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Sphingosine 1-phosphate receptor 2 is central to maintaining epidermal barrier homeostasis

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Abstract

The outer layer of the epidermis composes the skin barrier, a sophisticated filter constituted by layers of corneocytes in a lipid matrix. The matrix lipids, especially the ceramide-generated sphingosine 1-phosphate (S1P), are the messengers that the skin barrier uses to communicate with the basal layer of epidermis where replicating keratinocytes are located. S1P is a bioactive sphingolipid mediator involved in various cellular functions via S1P receptor (S1PR) 1–5, expressed by keratinocytes. We discovered that the *S1pr2* absence is linked to an impairment in skin barrier function. Although *S1pr2*$^{-/-}$ mouse skin have no difference in its phenotype and barrier function compared to wildtype (wt), after tape stripping, *S1pr2*$^{-/-}$ mice showed significantly higher transepidermal water loss (TEWL) and required another 24 more hours to normalize their TEWL levels. Moreover, after epicutaneous *S. aureus* application, impaired *S1pr2*$^{-/-}$ mouse epidermal barrier function allowed deeper bacterial penetration and denser neutrophil infiltration in the dermis. Microarray and RNA sequence of *S1pr2*$^{-/-}$ mouse epidermis linked the barrier dysfunction with a decrease in filaggrin 2 and tight junction components. In conclusion, *S1pr2*$^{-/-}$ mice have compromised skin barrier function and increased bacteria permeability, making them a suitable model for diseases that present similar characteristics, such as atopic dermatitis.

INTRODUCTION

The outer layer of human epidermis gives rise to a very specialized physical barrier constituted of corneocytes (the outermost layer of skin cells) in a lipid matrix. The lipid

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AUTHOR CONTRIBUTIONS

Conceptualization, resources, supervision and funding acquisition, AD; investigation, SI, AO, TN, TD and PP; formal analysis, SI and AD; visualization, SI; validation, ZW; writing the original draft, SI, AO, PP and AD; writing review and editing, SI, AD and PP.

CONFLICT OF INTEREST

The authors state no conflict of interest.

matrix is composed of ceramides, cholesterol and fatty acids (Elias, 1983, Li et al., 2020). This barrier is not just a physical protection to the inner layer, but it functions like a rheostat to convey signals from the surface to the base membrane using bioactive sphingolipids as messengers (Coant et al., 2017). Sphingosine 1-phosphate (S1P), one of those bioactive lipid mediators, is generated from ceramide by the consecutive actions of ceramidase and sphingosine kinase. S1P can potently regulate a variety of cell activities including cell proliferation via binding to and activating high-affinity G protein-coupled receptors which are named S1P receptor (S1PR) 1–5 (Coant et al., 2017, Obinata and Hla, 2019). S1PRs, including S1PR2, have diverse functions and have been implicated in many organ-system pathologies such as cell proliferation, differentiation, migration, and degranulation in endothelial, epithelial, nervous and immune systems including some kinds of cancer cells (Adada et al., 2013, Jolly et al., 2004, Nema et al., 2016, Wang et al., 2012). On the other hand, in the intestinal epithelium, S1P controls its barrier function by up-regulation of occludin (OCLN) expression (Paszti-Gere et al., 2016) and zonula occludens 1 (ZO1) expression via S1PR2 mediated PI3K/AKT signaling pathway (Chen et al., 2018), and it has already been shown that S1P induces keratinocyte differentiation by induction of the intracellular calcium concentration elevation (Japtok et al., 2014, Lichte et al., 2008), or inhibition of insulin-mediated keratinocyte proliferation through S1PR2 (Schuppel et al., 2008). However, the specific activity of S1P on S1PR2 in respect to epidermal barrier formation has not been investigated. Previously, we have reported that activation of S1PR1 and 2 during bacterial invasion controls proinflammatory cytokine synthesis in human keratinocytes (Igawa et al., 2019). We hypothesized that S1P and S1PR2 are involved in not only proinflammatory cytokine production but also barrier function in the epidermis as a response to various external stimuli, such as mechanical stress or bacterial invasion.

In this study, we have shown how S1P and S1PR2 contribute to maintaining epidermal barrier homeostasis both *in vivo* and *in vitro*. Our study started with the observation that *S1pr2*$^{-/-}$ mouse skin is fragile and characterized by a compromised skin barrier function. We demonstrated that while *S1pr2*$^{-/-}$ mouse skin phenotype and epidermal barrier function appeared normal, their various epidermal barrier-related protein expressions were significantly decreased, especially under bacterial invasion and mechanical stress. Here, we address how S1P stimulation increases these barrier-related protein mRNA expressions via S1PR2 in normal human epidermal keratinocytes (NHEKs), while the loss of cellular capacity to sense S1P through their S1PR2 brings alterations similar to those observed in skin disorders related to epidermal barrier dysfunction such as atopic dermatitis (AD).

RESULTS

S1pr2$^{-/-}$ mouse epidermal barrier function is impaired by mechanical stresses.

To investigate whether *S1pr2*$^{-/-}$ is involved in the epidermal barrier function, we decided to induce a mechanical stress using a sequential tape stripping followed by time-dependent transepidermal water loss (TEWL) evaluations. Before tape stripping, shaved back skin of *S1pr2*$^{-/-}$ mice showed slightly higher TEWL than wt mice with no significance (Figure 1a). After tape stripping, *S1pr2*$^{-/-}$ mice displayed significantly higher TEWL and required another 24 more hours to recover their TEWL levels than wt mice (Figure 1a). To rule out

the possible damaging effects of hair removal, we measured TEWL on the ear skin of wt and *S1pr2*$^{-/-}$ mice. We found that *S1pr2*$^{-/-}$ mice has significantly higher TEWL than wt mice even without tape stripping or hair removal (Figure 1b). We also investigated their phenotype with regards to histology and ultrastructure. *S1pr2*$^{-/-}$ mice showed no apparent skin phenotypical, histological or ultrastructural difference from wt mice (Supplementary Figure S1). This result suggests that *S1pr2*$^{-/-}$ mouse epidermal barrier function is slightly impaired at baseline when there are no barrier disruptions, but once the barrier has been broken by a mechanical stress, they have a more severe reaction to the stress and an impaired ability to recover their barrier function.

Reduced multiple junctional protein gene expression underlies impaired *S1pr2*$^{-/-}$ mouse epidermal barrier function

Since previous reports on S1P and S1PR2 function on the intestinal epithelia focused on tight junctions (TJs) (Chen et al., 2018, Chen et al., 2017), we proceed to verify whether the correlation between S1P signaling and TJs was also true in skin epithelia. To determine if junction proteins were responsible for the impaired barrier function of *S1pr2*$^{-/-}$ mice, we used RT^2 Profiler PCR Arrays® to analyze the epidermis of wt and *S1pr2*$^{-/-}$ mice before and after tape stripping. Based on the RT^2 Profiler PCR Arrays® data, we found that the *S1pr2*$^{-/-}$ mouse epidermis showed lower expression of multiple junctional protein genes including TJ protein genes (Supplementary Figure S2). Already at baseline, without tape stripping, *S1pr2*$^{-/-}$ mouse epidermis showed lower expression of the TJ protein *Zo1*, *Ocln*, claudin 1 (*Cldn1*) and of a corneodesmosome related protein, corneodesmosin (*Cdsn*), than wt mice (Figure 1c). After tape stripping, *S1pr2*$^{-/-}$ mice showed further downregulated *Zo1, Cldn1,* and *Cdsn* expressions than wt mice (Figure 1c and d). We also analyzed *S1pr1–5* mRNA expressions of wt and *S1pr2*$^{-/-}$ mouse epidermis before and three hours after tape stripping. We could not detect *S1pr3* mRNA expression in either wt or *S1pr2*$^{-/-}$ mouse epidermis and confirmed that after tape stripping, *S1pr1* and *4* mRNA expressions were increased, but *S1pr2* deficiency does not alter their transcription levels (Supplementary Figure S3). These data suggest that *S1pr2*$^{-/-}$ mice have a subclinical epidermal barrier dysfunction that becomes clinically evident after mechanical disruption.

S1pr2$^{-/-}$ mice show deeper *S. aureus* penetration and more massive neutrophil infiltration in the dermis after epicutaneous bacterial application.

To explore whether epidermal barrier dysfunction will compromise skin resistance against bacterial invasion, *Staphylococcus aureus* (*S. aureus*) was applied on the back skin of wt and *S1pr2*$^{-/-}$ mice. After epicutaneous *S. aureus* application, *S1pr2*$^{-/-}$ mice showed more pustular and erosive skin lesions than wt mice (Figure 2a). Histological analysis revealed that *S1pr2*$^{-/-}$ mice showed more massive neutrophil infiltration in the dermis (Supplementary Figure S4). Consistent with histological data, immunofluorescent staining also confirmed deeper *S. aureus* penetration and more massive neutrophil marker, Ly-6G, staining in the dermis of the *S1pr2*$^{-/-}$ mice compared to the wt (Figure 2b).

To confirm the role of S1PR2 in controlling the expression of TJ and corneodesmosome proteins ZO1 and CDSN, respectively, we performed Western blot analysis using whole skin extracts derived from wt or *S1pr2*$^{-/-}$ mice with or without application of *S. aureus*. Without

S. aureus application, *S1pr2*$^{-/-}$ mouse skin expressed less ZO1 compared to wt mouse skin (Figure 2c). After *S. aureus* application, ZO1 expression was substantially reduced in both wt and *S1pr2*$^{-/-}$ mouse skin; notably, in the *S1pr2*$^{-/-}$ mouse skin, we could not obtain any visible bands (Figure 2c). These data suggest that *S1pr2*$^{-/-}$ mouse skin have a decreased ZO1 expression compared to wt mice without bacterial infection. Furthermore, *S1pr2*$^{-/-}$ mouse skin also showed reduced CDSN expression after *S. aureus* application (Figure 2d). Consistent with the protein data, *S1pr2*$^{-/-}$ mice showed significantly lower *Zo1* (Figure 2e) and *Cdsn* (Figure 2f) mRNA levels compared to wt mouse skin without *S. aureus* application. After *S. aureus* application, *S1pr2*$^{-/-}$ mice showed the lowest *Zo1* and *Cdsn* expression (Figure 2e, f); however, there was no significance in the degree of mRNA reduction due to bacterial treatment in *Zo1* and *Cdsn* compared to TSB control treatment (Supplementary Figure S5). Taken together, these findings also suggest an association between *S1pr2* deficiency and impaired skin barrier functions due to decreased TJ and corneodesmosome expressions.

Filaggrin 2 expression was decreased in *S1pr2−/−* mice

In AD, the metabolism of ceramides is disturbed (Holleran et al., 2006) and reduced S1P level was found in canine AD (Baumer et al., 2011); however, S1P expression in human AD is still controversial (Japtok et al., 2014). At the same time, expression of filaggrin, a gene of great significance for the homeostasis of the skin, is also altered (Cabanillas and Novak, 2016, Palmer et al., 2006). Therefore, to investigate whether other skin barrier components were affected by *S1pr2*$^{-/-}$, we performed RNA sequencing using the epidermis of wt and *S1pr2*$^{-/-}$ mice. According to the RNA sequencing data, we actually found that the *S1pr2*$^{-/-}$ mouse epidermis showed lower gene expression of filaggrin 2 (*Flg2*) (Figure 3). FLG2 behaves similarly to filaggrin which undergoes proteolytic modification, suggesting that they may also have a function of providing natural moisturizing factors and its expression is also decreased in AD (Makino et al., 2014, Margolis et al., 2014, Pellerin et al., 2013). To assess the relationship between FLG2 and S1P *in vitro*, we incubated NHEKs with 1 μM S1P for 30 minutes. S1P treatment increased keratinocyte *FLG2* expression significantly (Figure 4a). To evaluate this result *in vivo*, we investigated the *Flg2* expression in the whole skin of wt and *S1pr2*$^{-/-}$ mice and confirmed that the *S1pr2*$^{-/-}$ mouse skin showed significantly lower *Flg2* expression than wt mice (Figure 4b). Moreover, immunofluorescent staining revealed that FLG2 staining was lost in *S1pr2*$^{-/-}$ epidermis while wt mouse epidermis showed granular and linear FLG2 staining in the granular layer (Figure 4c). These data suggest that S1P regulates FLG2 expression via S1PR2 and may be involved in the function to provide natural moisturizing factor derived from FLG2.

S1P stimulation increases barrier-related protein mRNA expression via S1PR2 in NHEKs.

To further analyze the effect of S1P-S1PR2 axis on skin barrier functions *in vitro*, we measured barrier-related protein mRNA expressions in NHEKs with or without S1P stimulation. We confirmed that 10μM S1P treatment significantly increased keratinocyte *ZO1*, *CDSN*, *FLG2* and keratin10 (*KRT10*) expression, whereas pre-treatment of 10μM S1PR2 antagonist, JTE013, in NHEKs prevented the S1P induced *ZO1*, *CDSN* and *FLG2* expressions but not *KRT10* (Figure 5a–d). These data suggest that S1P directly increases

the expression of epidermal barrier-related proteins in keratinocytes via S1PR2. Our results further highlight the role of S1PR2 in the regulation of the skin barrier function.

DISCUSSION

Maintenance of epidermal barrier homeostasis is important not only to prevent our body from external stimuli but also to avoid water loss from our body (Eyerich et al., 2018, Jonca et al., 2011). A variety of epidermal barrier gene-related genetic disorders, such as peeling skin syndromes, SAM syndrome, and Netherton syndrome, indicate how dysfunction of epidermal barrier affects body homeostasis (Chavanas et al., 2000, Ishida-Yamamoto and Igawa, 2014, Mohamad et al., 2018, Oji et al., 2010, Samuelov et al., 2013). These diseases are also known to have AD-like symptoms such as multiple allergies and increased serum IgE levels (Ishida-Yamamoto and Igawa, 2014). Since *FLG* gene mutation has been associated with AD symptoms (Palmer et al., 2006), a compromised epidermal barrier function is now recognized to be a key factor in AD development (Weidinger et al., 2018). The discovery of the association between AD, *FLG* and abnormal barrier function has led to the discovery of many other gene mutation and/or malformations as a genetic background of AD especially in TJ-related genes (Fortugno et al., 2012, Margolis et al., 2014, Saunders et al., 2013, Yuki et al., 2016).

S1P is widely known as a powerful modulator of homeostasis and pathogenesis in multiple organ systems (Obinata and Hla, 2019). The S1P-S1PR signaling system has been repeatedly shown to play a crucial role in regulating cell survival, proliferation, migration, phenotype and various inflammatory processes (Nema et al., 2016). S1P also induce keratinocyte differentiation by increasing its intracellular calcium concentration (Allende et al., 2013, Lichte et al., 2008) and is produced from both sphingomyelin and ceramides when the stratum corneum and/or epidermal keratinocytes are affected by external stimuli (Coant et al., 2017). These previous data made us consider the hypothesis that S1P-S1PR signals also control epidermal barrier functions. In this study, we demonstrated the crucial role of S1P-S1PR2 signals in regulating epidermal barrier-related protein expression and its function.

Using two *in vivo* mouse models, we have demonstrated that *S1pr2*$^{-/-}$ mouse skin has subclinical barrier impairment. After barrier disturbance via tape stripping, *S1pr2*$^{-/-}$ mice displayed significantly higher TEWL and required a longer time to normalize their TEWL compared to wt (Figure 1a), which indicates barrier dysfunction. After *S. aureus* epicutaneous application, *S1pr2*$^{-/-}$ mice also experienced more pustular and erosive lesions as well as increased inflammation and bacterial penetration in the dermis compared to wt. These data suggest that S1PR2 is central to maintaining epidermal homeostasis and integrity. Furthermore, since *S1pr2*$^{-/-}$ mice have reduced expression levels of TJ and barrier-related proteins such as ZO1, FLG2 and CDSN, S1PR2 deficiency may contribute to a dysregulation of vital TJs, corneodesmosomes and cornified envelopes needed for front-line innate defense against infection (Alberola et al., 2019, Ishida-Yamamoto and Igawa, 2015). Consistent with this observation, in endothelial and intestinal epithelial cells S1PR1 and 2 control TJ formation. (Chen et al., 2018, Lee et al., 2006, Li et al., 2015, Paszti-Gere et al., 2016). Epidermal TJs are dynamic structures formed by adhesive and

scaffolding proteins that play a role in maintaining cell polarity, regulating paracellular movement between different cell layers of the stratum granulosum as well as forming and maintaining epithelial and endothelial barriers (Aijaz et al., 2006, Kuo et al., 2013, Niessen, 2007, Shin et al., 2006). In addition, TJ proteins are involved in cell differentiation and proliferation, cell signaling and vesicle transport (Matter et al., 2005, Schneeberger and Lynch, 2004) and permeability barrier in epidermal keratinocytes (Yuki et al., 2007). When TJ proteins are impaired, the uptake of antigens is considerably increased, thus may result in increased levels of exogenous pathogens or particles entering the skin and triggering inflammatory response like in AD (Kezic et al., 2014, Yoshida et al., 2013). This explains why *S1pr2*$^{-/-}$ mouse skin displays increased inflammation and deeper *S. aureus* penetration consistent with dysregulation of junctional and barrier proteins. However, *S1pr2*$^{-/-}$ mice show no clinical or histological manifestations at baseline, possibly due to compensation by upregulation of other small proline rich proteins (*Sppr1b*), gap junction beta-2 protein (*Gjb2*), gap junction beta-6 protein (*Gjb6*) shown in RNA sequence and RT^2 Profiler PCR Arrays® (Figure 3 and Supplementary Figure S3). In addition, higher TEWL observed in *S1pr2*$^{-/-}$ mice could be a result of impaired stratum corneum (SC) integrity/cohesion and altered lipid composition related to CDSN and FLG2. FLG2 is protein of the filaggrin family expressed in the granular layer of the epidermis and localized in keratohyalin granules (Wu et al., 2009), and is diffusely present in the stratum corneum (Makino et al., 2014). Like filaggrin, FLG2 was suggested to also have hydration and photoprotection properties in the epidermis (Pendaries et al., 2015). Previous studies have shown that FLG2 levels were significantly decreased in tissue samples from patients with skin diseases such as ichthyosis vulgaris, AD and psoriasis vulgaris compared to normal skin samples (Makino et al., 2014). Our immunofluorescent staining confirmed that FLG2 staining was lost in *S1pr2*$^{-/-}$ epidermis while wt mouse epidermis showed FLG2 staining in the granular layer. Interestingly, filaggrin deficiencies were shown to influence TJ proteins (Nakai et al., 2012) and *FLG2* deficiency reduce CDSN expression and cause peeling skin syndrome type A (Bolling et al., 2018, Mohamad et al., 2018). A recent paper discovered that filaggrin mutations are responsible for an altered phase-separation dynamic between keratohyalin granules and cytoplasm, showing how associated skin barrier disorders can be exacerbated by environmental extremes (Quiroz et al., 2020). Hence, *FLG2* deficiency could result in decreased SC hydration and barrier dysfunction, leading to an increase in TEWL as previously discussed.

Taken together, we have demonstrated that *S1pr2*$^{-/-}$ skin displays barrier impairments associated with a downregulation of FLG2, CDSN and TJ-related proteins (Figure 1–4). Consistently, our *in vitro* data indicated that treatment of S1P directly increased barrier-related mRNA expression levels in keratinocytes, while chemical block with S1PR2 antagonist, JTE013, significantly reduced them (Figure 5). In the present study, we did not confirm that S1P directly increases FLG2 expression in keratinocytes after differentiation but only on undifferentiated keratinocytes. This is a limitation to our study that in the future could possibly be answered by studying keratinocytes' FLG2 response to S1P in a 3D skin model. Our data implicate S1P and S1PR2 as a modulator of skin barrier function and a possible pharmaceutical target for skin disorders based on impaired epidermal barrier function, such as AD. Since S1P regulates FLG2 via S1PR2 and may be involved in

providing natural moisturizing factors derived from FLG2, treatment with S1P might be able to alleviate dry skin and proinflammatory responses in AD. According to our RNA sequencing data, *S1pr2*$^{-/-}$ mouse epidermis showed increased IL19, 24 and 33 expressions (Figure 3), so we can also speculate that a *S1PR2* deficiency in the epidermis will affect Th2 response in the dermis, as observed in AD patients. Moreover, increased proinflammatory cytokines will further downregulate filaggrin and filaggrin 2 (Pellerin et al., 2013), creating a vicious cycle.

Since S1PR2 is present in other immune cell types such as macrophages and Th cells, to be able to assess the repercussion of epithelial deficiency of S1PR2 and to rule out the effects of system-wide S1PR2 deficiency, we plan to assess skin barrier function using Cre-loxP mice with conditional *S1pr2* deletion in the epidermis. We also should consider the possible compensation mechanism of *S1pr1, 3–5* in the absence of *S1pr2*. Moreover, to further establish the relationship between S1PR2 deficiency and barrier impairment, a thorough look at the morphology of tight junction network using confocal microscopy is needed.

In conclusion, our study indicates that S1P-S1PR2 signaling is critical in epidermal barrier function, suggesting their involvement in barrier dysfunctional phenotype of *S1pr2*$^{-/-}$ mice. To detect whether *S1pr2*$^{-/-}$ mice may have a weakened cytotoxic response against *S. aureus* infection and inability to lyse invasive pathogens and a propensity to develop a Th2 phenotype, further investigation into their dermal cytokine and antimicrobial profiles is needed. This will be the focus of our future investigation.

MATERIALS AND METHODS

Mice

BALB/c wildtype and *S1pr2*$^{-/-}$ mice were provided by Dr. Jerold Chun (Herr et al., 2016, Ishii et al., 2002). S1PR2 genotyping were performed by PCR using tail genomic DNA (Ishii et al., 2002). Three *S1pr2*$^{-/-}$ mice and three of their littermate controls were used for each experiment. We confirmed that in the epidermis, *S1pr2* deficiency does not affect *S1pr1, 3–5* expressions before and after tape stripping (Supplementary Figure S3). All animal protocols were reviewed and approved by the University of California San Diego (approval number: S10288).

Tape stripping and transepidermal water loss evaluation—To evaluate response to skin barrier disruption *in vivo*, transepidermal water loss (TEWL) was measured before and after tape stripping on wt and *S1pr2*$^{-/-}$ mouse back skin. After 24 hours from hair removal, we tape stripped mouse back skin ten times using D-squame® D100 (Clinical & Derm, Dallas, TX). TEWL was measured at 2, 6, 24, 48 and 72 hours after tape stripping using Tewameter TM300 Courage+ (Khazaka, Clologne, Germany). We also measured TEWL on ear skin without any treatment in both wt and *S1pr2*$^{-/-}$ mice. To investigate epidermal barrier function-related gene expressions before and after tape stripping, we isolated mouse epidermis by treating wt and *S1pr2*$^{-/-}$ mouse back skin samples with Dispase II (Sigma-Aldrich, St. Louis, MO) at 4°C overnight and these samples were analyzed by real-time quantitative RT-PCR (RT-qPCR).

Epicutaneous *S. aureus* application on the mouse back skin—According to the previous report (Nakatsuji et al., 2016), we applied *S. aureus* (sa113, ATCC35556, ATCC, Manassas, VA) on wt and *S1pr2*$^{-/-}$ mouse back skin. After their hair removal, we applied 10 mm-sized agar discs containing TSB as a control or 1×10^7 CFU *S. aureus* on their tape stripped back skin and the entire dorsal skin was then covered with Tegaderm (3M, Maplewood, MN) for 48 hrs. After disc removal, each whole skin samples were analyzed histologically, RNA was extracted for RT-qPCR, and protein was extracted for western blot analysis from each sample.

Primary normal human epidermal keratinocytes—Undifferentiated NHEKs (Thermo Fisher, Waltham, MA) were cultured in EpiLife™ Medium with 60 μM calcium (Thermo Fisher), seeded sufficiently for cell treatments. Subconfluently conditioned NHEKs were used for the experiments according to the previous report (Igawa et al., 2019).

S1P treatment and chemical S1PR2 block in NHEKs—To block S1PR2, NHEKs were incubated with 10μmol/L JTE013 (Cayman, Ann Arbor, MI) at 37°C for 2 hours prior to S1P treatment. After that, NHEKs were incubated with PBS and 1 or 10μmol/L S1P (TOCRIS, Minneapolis, MN) at 37°C for 30 minutes.

Histology and Immunofluorescence staining—Formalin fixed and paraffin embedded mouse skin samples were sectioned and hematoxylin-eosin stained at UCSD Comprehensive Moores Cancer Center Biorepository and Tissue and Technology Shared Resource. Skin sections were immunostained, as described previously (Wang et al., 2017), with the primary and secondary antibodies listed in Supplementary Table S1. Fluorescence images were obtained with a fluorescence microscope (Olympus BX51).

Quantitative real time reverse transcriptase-PCR—Total mouse RNA from whole mouse skin samples were isolated using Direct-zol Miniprep kit (Zymo Research, Irvine, CA). Total RNA from mouse epidermis and NHEKs were isolated by Quick RNA Miniprep kit (Zymo Research). cDNA conversion from RNA and real time RT-qPCR were performed according to the previous report (Igawa et al., 2019). We listed the probes used for real time RT-qPCR in Supplementary Tables S2 and S3 (Gandy et al., 2013, Li et al., 2012, Pendaries et al., 2014, Ryu et al., 2018, Vivinus-Nebot et al., 2014) online. The expression of target genes was normalized to GAPDH expression and analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis—According to the previous report (Takahashi et al., 2018), the mouse skin samples were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher) containing a protease inhibitor cocktail (Complete ultra mini, Sigma-Aldrich). Protein concentrations were determined with the BCA protein assay kit (Thermo Fisher). Proteins (10–20 μg/lane) were separated on a 4–20% precast polyacrylamide gel (Bio-Rad, Hercules, CA), transferred to PVDF membrane (Bio-Rad), followed by immunoblotting using indicated primary antibodies followed by fluorescent secondary antibodies (Supplementary Table S1) (LICOR Biosciences, Lincoln, NE) and imaging using fluorescent Odyssey System (LICOR Biosciences). Experiments were performed in triplicates and all results showed the same trend.

RNA sequencing—Purified RNA samples from wt and *S1pr2*$^{-/-}$ mice were submitted to the UCSD Institute for Genomic Medicine core facility for library preparation and high-throughput next-generation sequencing, according to the previous report (Liggins et al., 2019). Subsequent analysis was conducted by Center for Computational Biology and Bioinformatics at UCSD.

Statistical Analysis—In all *in vitro* experiments, all samples were performed in triplicates, and values are expressed as mean ± standard deviation. Mann-Whitney test were applied to analyze the differences between two groups. One- or two-way analysis of variance (ANOVA) and Tukey tests were applied to analyze the differences among more than two groups. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

Datasets related to this article can be found at https://data.mendeley.com/datasets/csrs6vw8ps/1, hosted at Mendeley. The RNA sequence data is uploaded to the Sequence Read Archive, hosted by NCBI (accession number PRJNA658157).

1 Abbreviations:

AD	atopic dermatitis
CDSN	corneodesmosin
CLDN1	claudin 1
FLG2	filaggrin 2
KRT10	keratin10
NHEK	normal human epidermal keratinocyte
OCLN	occluding
PBS	phosphate buffered saline
RT-PCR	Reverse transcriptase-PCR
S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
TEM	transmission electron microscope

TEWL	transepidermal water loss
TJ	tight junction
wt	wild type
TSB	3% tryptic soy broth, ZO1, zona occludens 1

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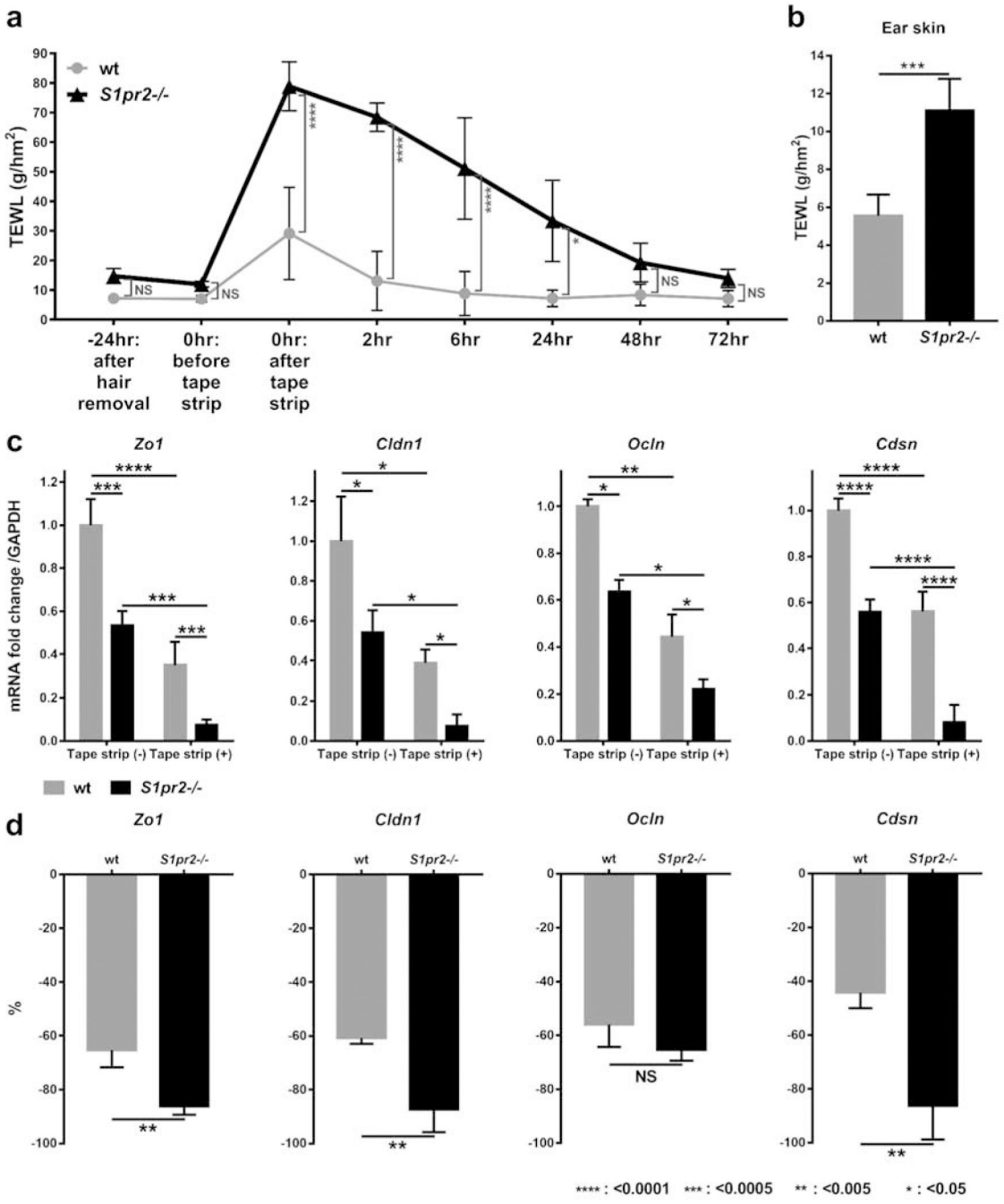

Figure 1. *S1pr2*$^{-/-}$ mouse epidermal barrier function was more severely impaired than wt after tape stripping and reduced multiple junctional protein gene expression underlay this impaired barrier function.

(a) The time-dependent change of TEWL measured from wt and *S1pr2*$^{-/-}$ mice before and after sequential tape stripping. (b) TEWL of wt and *S1pr2*$^{-/-}$ mouse ear skin without any treatments. (c, d) *Zo1, Cldn1, Ocln* and *Cdsn* transcriptions in wt and *S1pr2*$^{-/-}$ mouse epidermis before and three hours after tape stripping (c) and the ratio of downregulation in these mRNA expressions after tape stripping (d). Each data is shown by a comparison with the wt mRNA expression before tape stripping. TEWL: transepidermal water loss; *Zo1*:

Zonula occludens-1; *Cldn1*: Claudin-1, *Ocln*: occludin; *Cdsn*: corneodesmosine; wt: wild type.

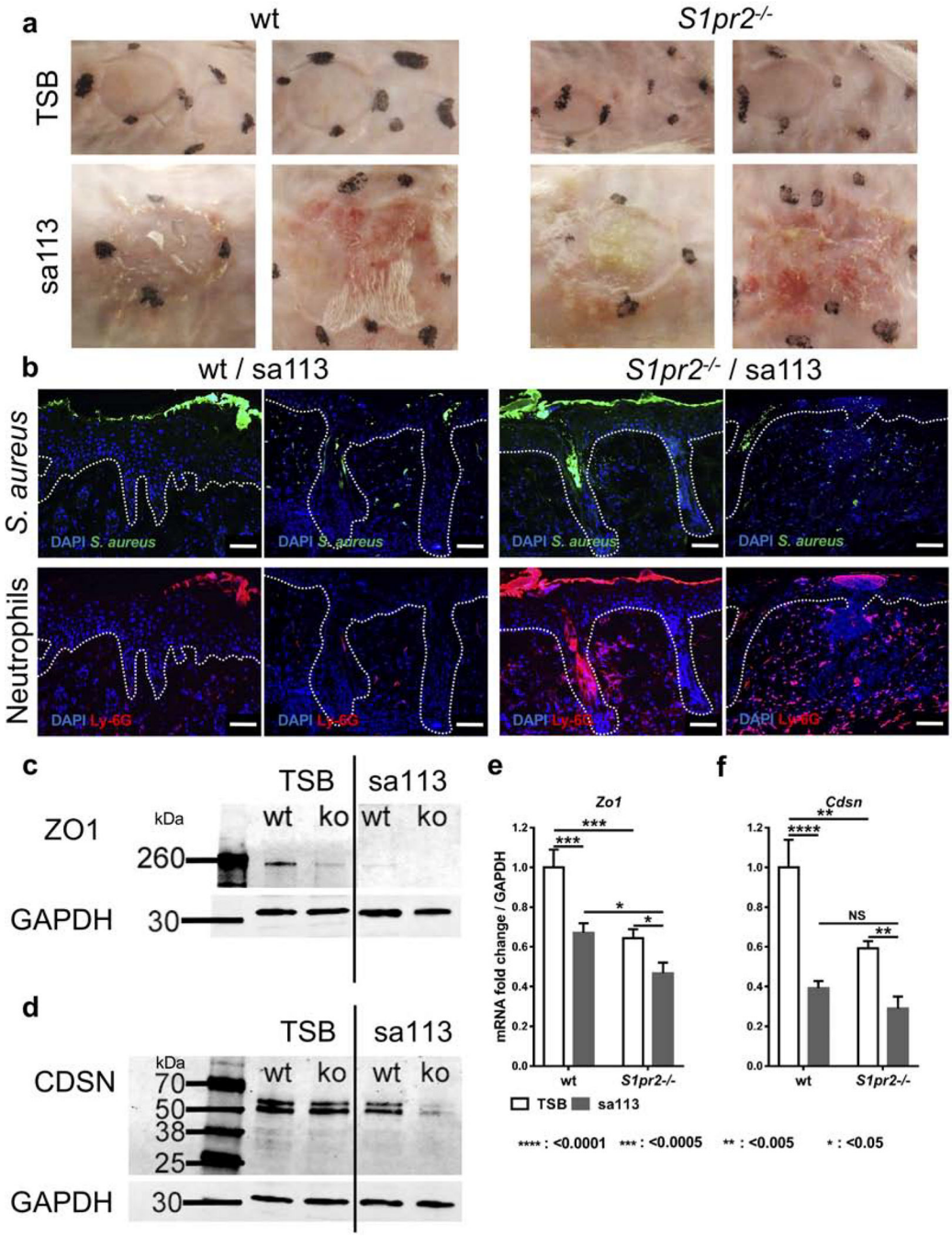

Figure 2. Decreased ZO1 and CDSN expression in *S1pr2*$^{-/-}$ mouse skin allowed deeper *S. aureus* penetration and more massive neutrophil infiltration in the dermis after epicutaneous bacterial application.

(a, b) wt and *S1pr2*$^{-/-}$ mouse clinical manifestations (a) and *S. aureus* (green) and Ly-6G (red) immunofluorescent images (b) 48hr-after TSB (a) or sa113 (a, b) application. (c-f) Western blot analysis (c, d) and mRNA expression (e, f) of ZO1 (c, e) and CDSN (d, f) of wt and *S1pr2*$^{-/-}$ mouse whole skin 48 hr after TSB or sa113 application. Each RT-qPCR data is shown by a comparison with the wt mRNA expression incubated with TSB. Scale

bars: (b) 20 μm, CDSN: corneodesmosin; ko: *S1pr2*$^{-/-}$ mice; TSB: 3% tryptic soy broth; wt: wild-type; ZO1: Zonula occludens-1.

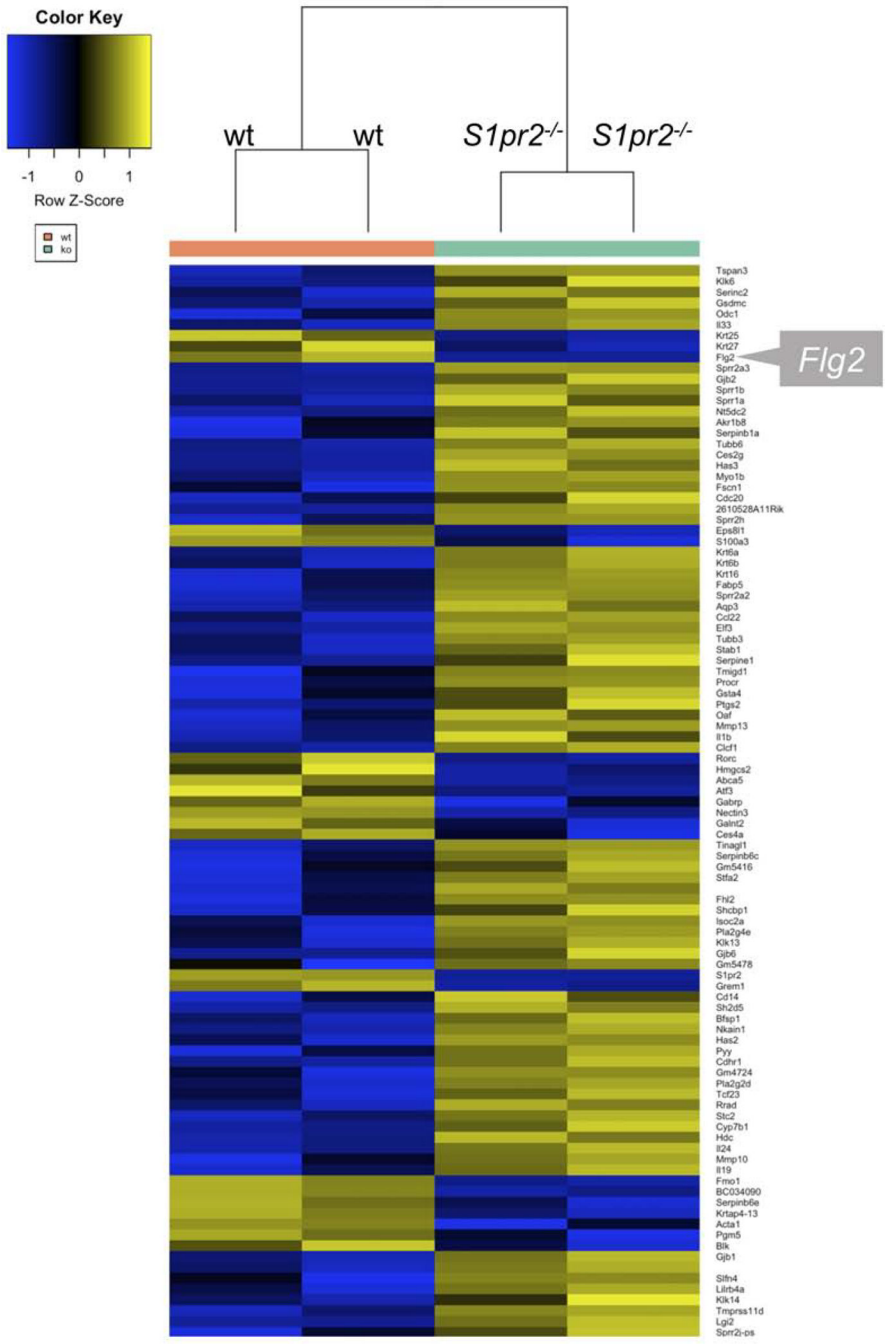

Figure 3. Filaggrin 2 expression was decreased in *S1pr2*$^{-/-}$ mice.

Heatmap clustering showing the difference of epidermal gene expressions in wt and *S1pr2*$^{-/-}$ mouse epidermis. Flg2: filaggrin 2; wt: wild type.

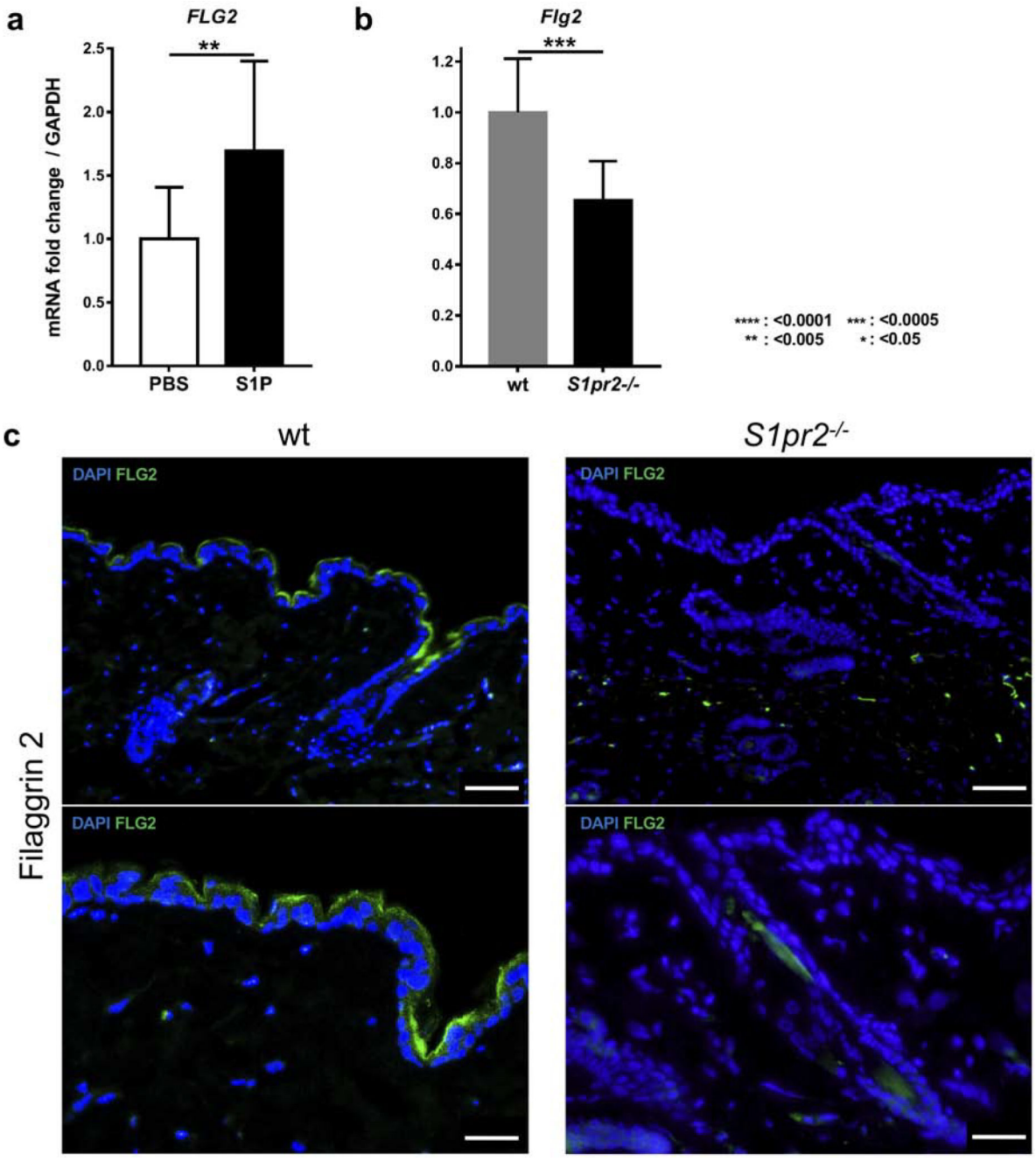

Figure 4. Filaggrin 2 expression is regulated via S1P-S1PR2 signaling.

(a) FLG2 mRNA expression levels in NHEKs incubated with PBS or 1μM S1P for 30 minutes. (b) *Flg2* mRNA expression levels in wt and *S1pr2*$^{-/-}$ mouse whole skin without any barrier disruption. Each RT-qPCR data is shown by a comparison with the PBS control (a) or wt skin (b) mRNA expression. (c) Flg2 immunofluorescence images of wt and *S1pr2*$^{-/-}$ mouse skin. Lower panels are magnified images of the different fields from upper panels. Scale bars: (c) upper panels 100 μm, lower panels 50 μm. *Flg2*: filaggrin 2; NHEK: normal human epidermal keratinocyte; PBS: phosphate-buffered saline; S1P: sphingosine 1-phosphate; S1PR: S1P receptor; wt: wildtype.

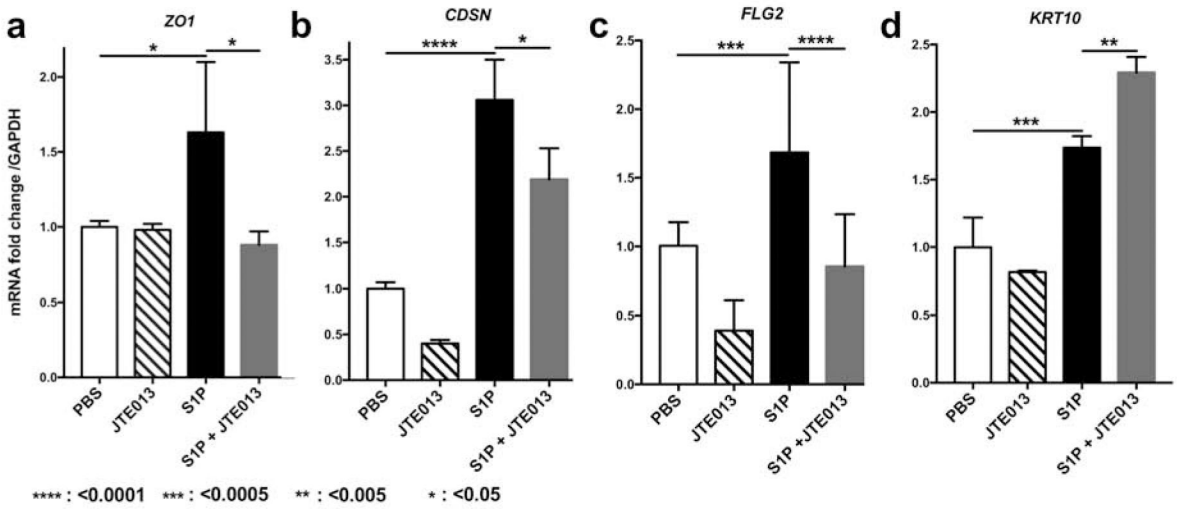

Figure 5. The S1P stimulation significantly increases epidermal barrier related protein expressions via S1PR2.

mRNA expression levels of (a) *Zo1* (b) *Cdsn* (c) *Flg2* and (d) *Krt10* measured in NHEKs incubated with PBS or 10μM S1P for 30 minutes. Before treatment, S1PR2 was inhibited by incubating NHEKs with 10μM JTE013 for 2 hours. Each data is shown by a comparison with mRNA expression of NHEK treated with PBS control. *Cdsn*: corneodesmosin; *Flg2*: filaggrin 2; *Krt10*: keratin 10; NHEK: normal human epidermal keratinocyte; PBS: phosphate-buffered saline; S1P: sphingosine-1-phosphate; S1PR: S1P receptor; *Zo1*: Zonula occludens 1.