Human Keratinocytes Use Sphingosine 1-Phosphate and its Receptors to Communicate *Staphylococcus aureus* Invasion and Activate Host Defense

Satomi Igawa[1], Jae Eun Choi[1], Zhenping Wang[1], Yu-Ling Chang[1], Chia-Chi Wu[1], Tyler Werbel[1], Akemi Ishida-Yamamoto[2] and Anna Di Nardo[1]

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator generated when a cell membrane or its components are damaged by various factors. S1P regulates diverse cell activities via S1P receptors (S1PRs). Keratinocytes express S1PR1−5. Although it is known that S1PRs control keratinocyte differentiation, apoptosis, and wound healing, S1PR functions in keratinocyte infections have not been fully elucidated. We propose that the S1P-S1PR axis in keratinocytes works as a biosensor for bacterial invasion. Indeed, in human impetigo infection, we found high epidermal expression of S1PR1 and S1PR2 in the skin. Furthermore, in normal human epidermal keratinocytes in vitro, treatment with *Staphylococcus aureus* bacterial supernatant not only induced S1P production but also increased the transcription of S1PR2, confirming our in vivo observation, as well as increased the levels of TNFA, IL36G, IL6, and IL8 mRNAs. However, direct treatment of normal human epidermal keratinocytes with S1P increased the expressions of IL36G, TNFA, and IL8, but not IL6. In both S1P- and *S. aureus* bacterial supernatant-treated normal human epidermal keratinocytes, S1PR1 knockdown reduced IL36G, TNFA, and IL8 transcription, and the S1PR2 antagonist JTE013 blocked the secretion of these cytokines. Overall, we have proven that during infections, keratinocytes communicate damage by using S1P release and tight control of S1PR1 and 2.

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INTRODUCTION

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator (Nema et al., 2016; Park et al., 2016) that regulates a variety of cell activities, including cell growth, differentiation, apoptosis, migration, inflammation, metabolism, and angiogenesis (Coant et al., 2017; Nema et al., 2016). As an extracellular signal, S1P can affect many processes through its G-protein coupled receptors, S1PRs (Rivera et al., 2008). In the epidermis, keratinocytes express S1PR1−5 (Allende et al., 2013; Vogler et al., 2003), with S1PR5 being the most abundant and S1PR1 and 2 being the second and third most expressed (see Supplementary Figure S1 online).

S1P is the product of the metabolism of sphingomyelin, a component of the cell wall. Sphingomyelinase mediates ceramide synthesis from sphingomyelin. Ceramide is then further metabolized by ceramidase to produce sphingosine, and finally, two sphingosine kinases (SPHK1 and 2) generate S1P (Rivera et al., 2008; Skoura and Hla, 2009; Thieme et al., 2017). Some bacteria, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have sphingomyelinase and ceramidase, respectively, as part of their toxins (Oizumi et al., 2014; Salgado-Pabon et al., 2014). Therefore, we hypothesized that during skin infection, the activity of bacterial toxins in the epidermis results in the generation of S1P, which, in this context, signals bacterial invasion and acts as an alarmin.

In the epidermis, it is known that SIP is involved in keratinocyte differentiation, growth arrest, apoptosis, and wound healing (Amano et al., 2004; Lichte et al., 2008; Schmitz et al., 2012; Schuppel et al., 2008). It has also been shown that S1P can play a role in the pathogenesis of some skin disorders such as allergic contact dermatitis, psoriasis, and systemic sclerosis (Castelino and Varga, 2014; Reines et al., 2009; Schaper et al., 2013; Thieme et al., 2017). However, even though S1P-mediated cathelicidin antimicrobial peptide production (Park et al., 2013, 2016), and increases in TNFA and IL8 mRNA expressions (Oizumi et al., 2014) have already been shown, the role of S1P and S1PRs in host defense against pathogens in keratinocytes has not been fully elucidated.

The aim of this study was to define the role of S1P and S1PRs in keratinocytes during bacterial skin infection. We have determined that the expression levels of S1PR1 and 2

[1]*Department of Dermatology, School of Medicine, University of California, San Diego, La Jolla, California, USA; and* [2]*Department of Dermatology, Asahikawa Medical University, Asahikawa, Japan*

Correspondence: Anna Di Nardo, Department of Dermatology, School of Medicine, University of California, San Diego, 9500 Gilman Drive #0869, La Jolla, California 92093, USA. E-mail: adinardo@ucsd.edu

Abbreviations: ATP, adenosine triphosphate; NHEK, normal human epidermal keratinocyte; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-PCR; S1P, sphingosine 1-phosphate; S1PR, sphingosine 1-phosphate receptor; si, small interfering; SPHK, sphingosine kinase; TSB, tryptic soy broth

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change in response to *S. aureus* invasion and that S1P induces keratinocyte proinflammatory cytokine expression and secretion that is precisely controlled by S1PR1 and 2.

RESULTS

S1PR2 is increased in impetigo lesional skin and *S. aureus* bacterial supernatant-treated normal human epidermal keratinocytes

To assess how *S. aureus* affects S1PR expression, we incubated primary normal human epidermal keratinocytes (NHEKs) with *S. aureus* sa113 bacterial supernatant or 3% tryptic soy broth (TSB), as a control, for 4 hours and measured the mRNA expression levels of the different S1PR genes. In NHEKs, S1PR2 expression was found to be significantly greater in cells incubated with sa113 supernatant than in control cells incubated with TSB. However, although the expression levels of the other S1PR isoforms were increased after incubation with sa113 supernatant, they did not reach significance (Figure 1a). Next, to investigate how *S. aureus* affects the expressions of S1PR1 and 2 in human skin in vivo, we performed immunofluorescent staining for these receptors in normal and impetigo lesional skin sections (Figure 1b and c). In normal epidermis, both S1PR1 and 2 showed diffuse cytoplasmic expression in the granular layer and stratum corneum (Figure 1b, and see Supplementary Figure S2a online), but in the basal and squamous layers, they were distributed in a perinuclear pattern (see Supplementary Figure S2a and b). In contrast, both S1PR1 and 2 showed increased diffuse cytoplasmic expression in the whole epidermis of impetigo lesional skin (Figure 1c). To confirm these findings, we performed immunofluorescent staining for S1PR1 and 2 in NHEKs stimulated with sa113 supernatant in vitro. NHEKs incubated with TSB for 24 hours showed perinuclear expression of S1PR1 and 2; however, cells incubated with sa113 supernatant for 24 hours showed diffuse cytoplasmic expression of these receptors (Figure 1d), similar to the in vivo findings (Figure 1c). $Ca2^{+}$-differentiated NHEKs also showed diffuse cytoplasmic expression (see Supplementary Figure S2c) of S1PR1 and 2, similar to the pattern seen in the granular layer and stratum corneum in normal epidermis. Taken together, these data suggest that the expression levels of S1PRs, particularly S1PR1 and 2, on keratinocytes are increased and mobilized to the surface in response to *S. aureus* invasion.

S. aureus bacterial supernatant increases SPHK activity and induces S1P production in NHEKs

To assess whether *S. aureus* induces keratinocyte production of S1P, we measured the expression levels and activities of SPHKs, the rate-limiting enzymes for S1P production (Rivera et al., 2008; Skoura and Hla, 2009). After a 4-hour incubation with sa113 supernatant, both SPHK1 and 2 mRNA expressions were increased in NHEKs, with only the level of SPHK1 mRNA being significantly greater than that in TSB-treated control cells (Figure 2a). In addition to SPHK mRNA levels, we also measured SPHK activity in sa113 supernatant-treated NHEKs. Our results showed that sa113 supernatant-treated NHEK lysates had significantly higher SPHK activity than lysates from control TSB-treated cells (Figure 2b). As expected from the increased SPHK activity, ELISA assays for S1P confirmed significantly higher levels of secreted S1P from NHEKs incubated with sa113 supernatant for 48 hours than TSB-treated cells (Figure 2c).

S. aureus induces NHEK production of TNF-α, IL-6, IL-8, and IL-36γ cytokines

To assess the inflammatory response of NHEKs to bacterial products, we measured the expression levels of the proinflammatory cytokines TNFA, IL6, and IL8 after treatment with sa113 supernatant. Our results showed that treatment of NHEKs with sa113 supernatant resulted in the increased expressions of these proinflammatory cytokines (see Supplementary Figure S3a online). The secretion of TNF-α and IL-8 from NHEKs was also increased after sa113 supernatant treatment, in accordance with the observed increases in mRNA expression (see Supplementary Figure S3b) and previous reports (Aufiero et al., 2007; Krishna and Miller, 2012). Because the IL-36 cytokine family was recently reported to be involved in the response to *S. aureus* (Liu et al., 2017; Nakagawa et al., 2017; Williams et al., 2017), we also investigated the expression levels of the IL-36 family of cytokines. These results showed that only IL36G mRNA expression was increased in NHEKs after incubation with sa113 supernatant (see Supplementary Figure S3a), with a corresponding increase in IL-36γ protein secretion (see Supplementary Figure S3b). Because Nakagawa et al. (2017) and Liu et al. (2017) proved the importance of mouse keratinocyte IL-36α production in signaling against *S. aureus* (USA300 LAC) invasion in the skin, we also tested whether two strains of *S. aureus* (sa113 and USA300 LAC) supernatant could increase IL36A mRNA levels in human keratinocytes. Unlike mouse keratinocytes, we could not find any detectable IL36A mRNA expression in NHEKs incubated with either TSB, sa113, or USA300 LAC supernatant (see Supplementary Figure S3a, and USA300 LAC negative data not shown). These data suggest that in NHEKs, IL-36γ is the most important member of the IL-36 family of cytokines for the anti-*S. aureus* response.

S1P-S1PR1 and 2 activations control the expression and secretion of proinflammatory cytokines in NHEKs

Since *S. aureus* supernatant induces proinflammatory cytokine synthesis in NHEKs, we examined whether S1P and its receptors are involved in this mechanism of cytokine production. First, we incubated NHEKs with PBS, 1 μmol/L S1P, or 10 μmol/L S1P at different time points and found that the expression levels of IL36G, TNFA, and IL8 but not IL6 mRNAs were significantly increased in S1P-treated NHEKs compared with cells treated with PBS (Figure 3a). We also observed increases in IL-36γ, TNF-α, and IL-8 secretion from NHEKs after 1 μmol/L S1P treatment (see Supplementary Figure S4 online), even though this lower concentration of S1P did not induce a significant increase in TNFA mRNA expression (Figure 3a).

Because our data indicate that keratinocytes S1PR1 and 2 are involved in the NHEK response to *S. aureus* (Figure 1a–d), we next investigated the function of NHEK S1PR2 on cytokine production in response to S1P using the S1PR2 antagonist JTE013. Our results showed that treating NHEKs with 10 μmol/L JTE013 blocked S1P-induced TNFA, but not IL36G or IL8 mRNA expression (Figure 3b). However, the inhibitor was still able to block the S1P-induced secretion of all three cytokines

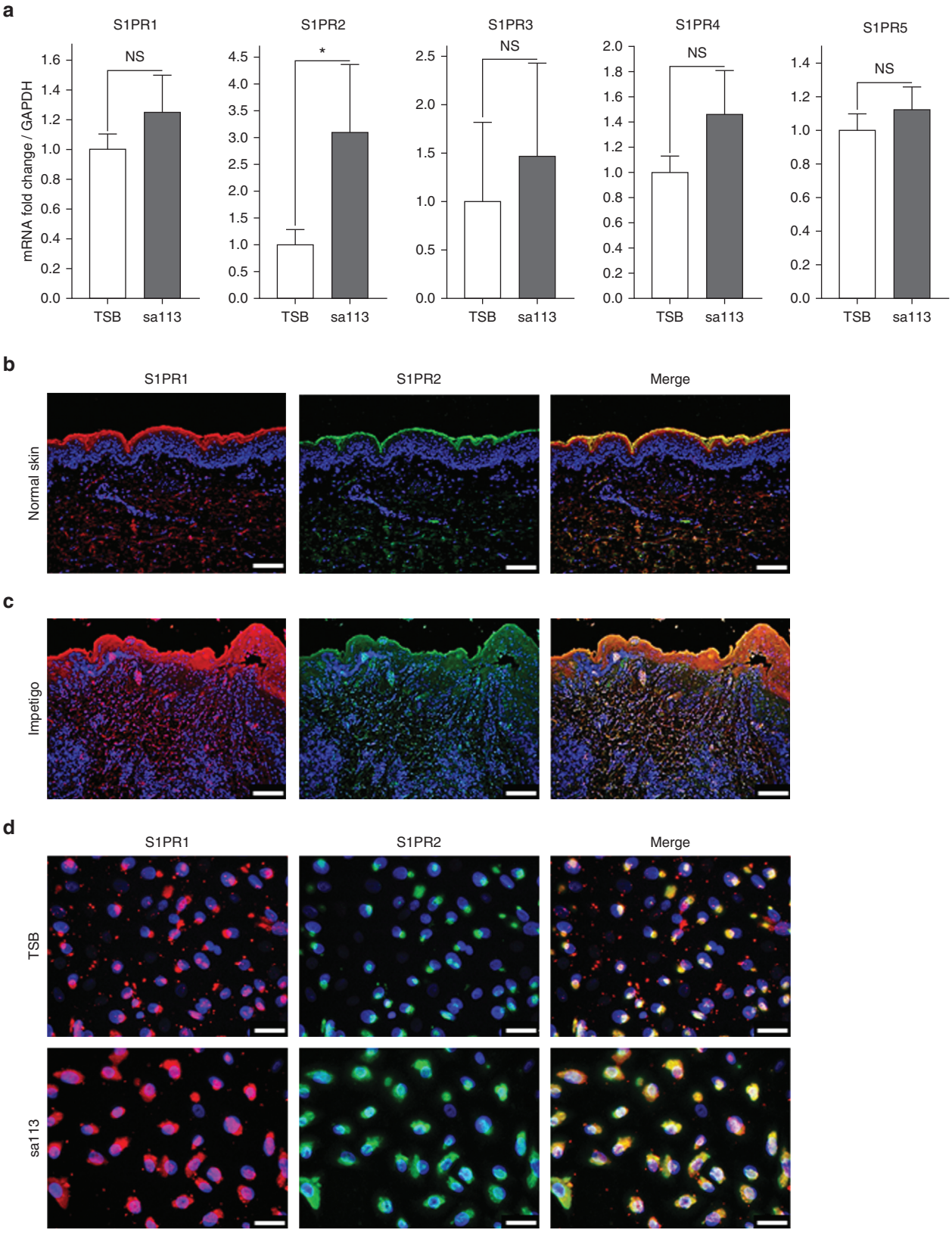

Figure 1. The expression and distribution of sphingosine 1-phosphate receptors (S1PRs) changes in impetigo skin and *Staphylococcus aureus* bacterial supernatant-treated normal human epidermal keratinocytes (NHEKs). (**a**) S1PR1−5 mRNA expression levels in NHEKs incubated with 50 μl/ml TSB or supernatant derived from *S. aureus* sa113 for 4 hours. Data are shown as the fold change versus TSB control. $*P < 0.05$. (**b**–**d**) S1PR1 and 2 immunostaining in (**b**) normal and (**c**) impetigo lesional skin sections, as well as (**d**) NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for 24 hours. Bar graphs indicate mean ± standard deviation. Scale bars = 50 μm in **b** and **c**, 10 μm in **d**. NS, not significant; TSB, tryptic soy broth.

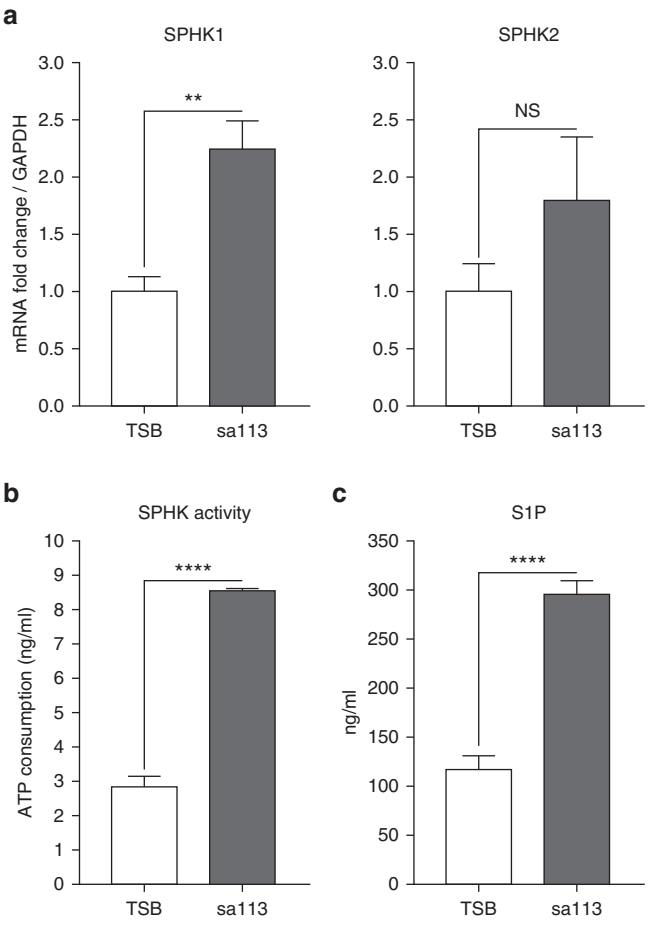

Figure 2. *Staphylococcus aureus* bacterial supernatant increases sphingosine kinase (SPHK) activity and induce S1P production in NHEKs. (**a**) mRNA expression levels of SPHK1 and 2 in NHEKs incubated with 50 μl/ml of TSB or sa113 supernatant for 4 hours. Data are shown as the fold change versus TSB control. (**b**) ATP consumptions indicating SPHK activity in NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for 24 hours. (**c**) S1P secreted into culture medium of NHEKs incubated with 50 μl/ml of TSB or sa113 supernatant for 48 hours. Bar graphs indicate mean ± standard deviation. $^{**}P < 0.005$; $^{****}P < 0.0001$. ATP, adenosine triphosphate; NHEK, normal human epidermal keratinocyte; NS, not significant; TSB, tryptic soy broth.

(Figure 3c). For better analysis of S1PR1 function in NHEKs, we transfected cells with small interfering (si) RNA for S1PR1 before S1P treatment. siS1PR1-transfected NHEKs showed reduced S1P-induced IL36G, TNFA, and IL8 mRNA expression levels (Figure 3d); however, when we analyzed cytokine secretion, we found that only TNF-α was affected by the loss of S1PR1 (Figure 3e). These data suggest that both S1PR1 and 2 are involved in TNF-α expression and secretion. However, for IL-36γ and IL-8 synthesis, S1PR1 and 2 fulfill different roles; namely, S1PR1 controls only IL36G and IL8 transcription, whereas S1PR2 controls only IL-36γ and IL-8 secretion, as summarized in Figure 3f.

S1P production is necessary for *S. aureus* bacterial supernatant-induced NHEK TNF-α, IL-8, and IL-36γ synthesis

To verify whether S1P production is actually involved in sa113 supernatant-induced cytokine synthesis in NHEKs, we transfected the cells with siRNA for SPHK1 and blocked SPHK2 with its inhibitor, ABC294640. SPHK1 knockdown combined with the SPHK2 block resulted in significantly reduced levels of TNF-α, IL-8, and IL-36γ transcription (Figure 4a) and protein secretion (Figure 4b) after sa113 supernatant treatment, whereas IL6 transcription was unaffected by the reduced S1P production (Figure 4a). These data suggest that for keratinocytes, SPHK1- and SPHK2-mediated S1P production contributes to TNF-α, IL-8, and IL-36γ synthesis in response to *S. aureus* stimulation.

S1PR1 and 2 inhibitions affect *S. aureus* bacterial supernatant-induced transcription and secretion, respectively, of IL-36γ, TNF-α, and IL-8 in NHEKs

Finally, to confirm that the different roles of keratinocyte S1PR1 and 2 in the cytokine response to S1P (Figure 3b–f) are consistent with those observed during the response to bacteria, we investigated how S1PR1 knockdown and S1PR2 block affect *S. aureus*-induced IL-36γ, TNF-α, and IL-8 synthesis. After sa113 supernatant stimulation, siS1PR1-transfected NHEKs showed significantly reduced levels of IL36G, TNFA, and IL8 mRNAs (Figure 5a). Furthermore, treatment with 10 μmol/L JTE013 before stimulation with sa113 supernatant resulted in significantly decreased amounts of secreted IL-36γ, TNF-α, and IL-8 (Figure 5b, and see Supplementary Figure S5 online). In siS1PR1-transfected NHEKs, IL6 mRNA expression tended to decrease, but not significantly (Figure 5a). These results confirm that for IL-36γ, TNF-α, and IL-8 synthesis in keratinocytes, S1PR1 mainly controls their transcription and S1PR2 mainly controls their release when stimulated with either S1P (Figure 3b–f) or *S. aureus* bacterial supernatant (Figure 5a and b).

DISCUSSION

Keratinocytes are known to synthesize diverse cytokines and chemokines in response to external stimuli. This includes activation by pathogens such as *S. aureus*, a major cause of skin and soft tissue infections such as impetigo, folliculitis, and cellulitis. During *S. aureus* infection, cytokines and chemokines produced by keratinocytes, particularly the chemoattractant IL-8 (Krishna and Miller, 2012; Ley et al., 2007), are essential for neutrophil recruitment from the circulation to lesional skin sites and are essential for mounting a proper immune response (Krishna and Miller, 2012). Keratinocytes also recognize *S. aureus* through their pattern recognition receptors, including toll-like receptor 2 (Miller and Cho, 2011), and can produce antimicrobial peptides, such as cathelicidin antimicrobial peptide, in response to bacterial infection (Ryu et al., 2014). Recently, it was shown that phenol soluble modulin (PSM)-α derived from *S. aureus* induces IL-1α and IL-36α production and that these cytokines orchestrate IL-17–dependent skin inflammation in mouse epidermis (Nakagawa et al., 2017). Our data confirm these previous reports that *S. aureus* supernatant-treated NHEKs produce TNF-α, IL-6, and IL-8; however, we could not detect any IL-36α production, and we detected IL-36γ only as an NHEK response to *S. aureus* supernatant stimulation (see Supplementary Figure S3). According to our data, although IL-36α is a very important mediator in mouse keratinocytes, IL-36γ is dominant in human keratinocytes in response to *S. aureus*. The role of IL-36γ in skin has been well

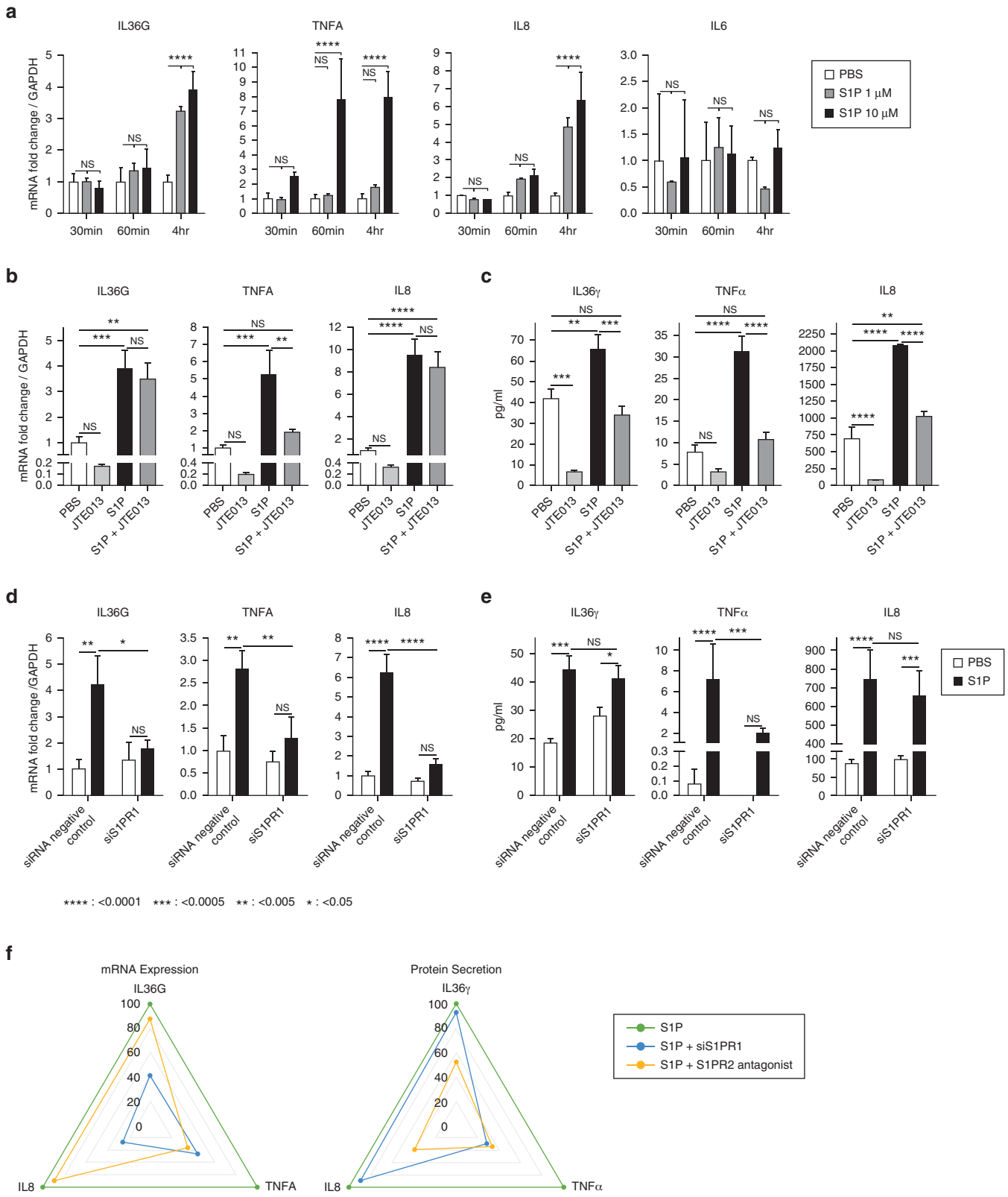

Figure 3. S1P-S1PR1 and 2 control NHEK cytokine synthesis. (**a**) IL36G, TNFA, IL6 and IL8 mRNA levels were measured in NHEKs treated with PBS or 1 or 10 μmol/L S1P. Data are shown as the fold change versus PBS control at each time point. (**b, d**) IL-36γ, TNF-α, and IL-8 mRNA levels and (**c, e**) protein secretion in NHEKs incubated with PBS or 10 μmol/L S1P for (**b, d**) 4 hours and (**c, e**) 24 hours. Before the treatment, NHEKs were treated with (**b, c**) 10 μmol/L JTE013, (**d, e**) negative control siRNA, or siS1PR1. (**b, d**) Transcription data are shown as the fold change versus PBS control without inhibitors. (**f**) Radar charts summarizing the percentage of each cytokine's mRNA expression and secretion with or without siS1PR1 or JTE013. Bar graphs indicate mean ± standard deviation. hr, hour; M, mol/L; min, minute; NHEK, normal human epidermal keratinocyte; NS, not significant; PBS, phosphate buffered saline; si, small interfering.

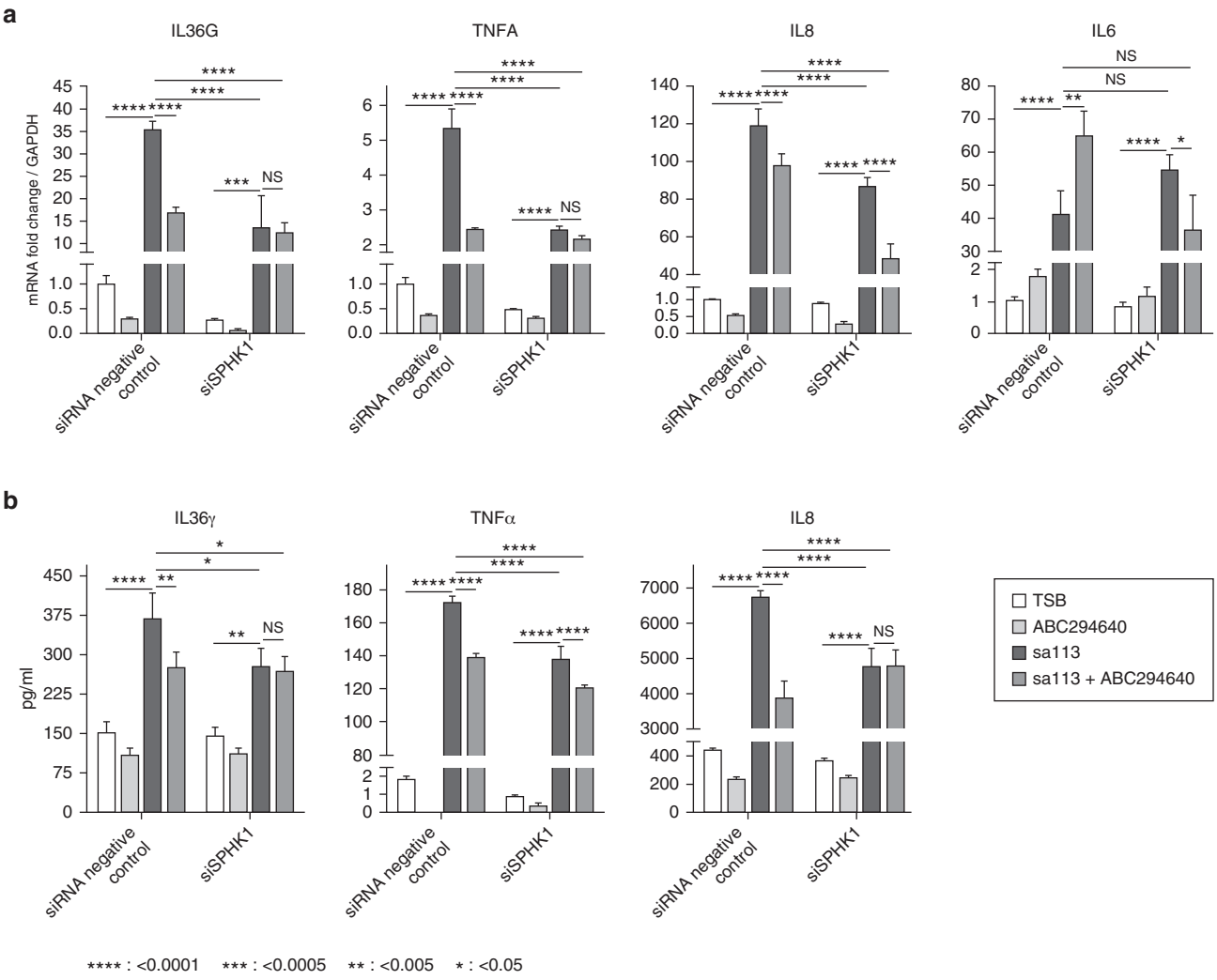

Figure 4. SPHK1 knockdown and SPHK2 inhibition reduces *Staphylococcus aureus* bacterial supernatant-induced TNF-α, IL-8, and IL-36γ cytokine synthesis in NHEKs. (**a**) IL36G, TNFA, IL8 and IL6 transcription and (**b**) IL-36γ, TNF-α, and IL-8 protein secretion was measured in NHEKs after incubation with 50 μl/ml TSB or sa113 supernatant for (**a**) 4 hours and (**b**) 24 hours. Before stimulation, cells were treated with control or SPHK1 siRNAs with or without 1 μmol/L ABC294640, an SPHK2 inhibitor, as indicated. mRNA expression data in **a** are shown as the fold change versus TSB control without inhibitors. Bar graphs indicate mean ± standard deviation. NHEK, normal human epidermal keratinocyte; NS, not significant; TSB, tryptic soy broth.

investigated in the pathophysiology of psoriasis to induce chronic skin inflammation (Bassoy et al., 2018; D'Erme et al., 2015; Li et al., 2014; Wang W et al., 2017). IL-36γ can induce human keratinocytes to produce cytokines, including IL-1β, IL-8, and IFN-γ (Li et al., 2014; Wang W et al., 2017). Furthermore, our data indicate that among the four IL-36 subtypes (IL-36α, -β, -γ, and RN), IL-36γ is the most responsive to *S. aureus* invasion and plays a major role in proinflammatory activity in human skin (Ganesan et al., 2017; Li et al., 2014).

Keratinocytes produce S1P as a response to various stimulations (Oizumi et al., 2014; Park et al., 2016). Here, we have shown that *S. aureus* supernatant induces S1P synthesis and release in keratinocytes to signal skin *S. aureus* invasion (Figure 2). Although we did not analyze the precise component of *S. aureus* supernatant that induces S1P production in NHEKs, *S. aureus* β-toxin, a specific sphingomyelinase, has the potential to initiate signaling through this pathway (Salgado-Pabon et al., 2014). Furthermore, our transcriptional data show that NHEK S1PR2 mRNA expression is significantly increased in response to *S. aureus* supernatant and that the levels of other S1PRs are also induced, although not significantly (Figure 1a). This observation is in line with our immunofluorescent staining data showing that both S1PR1 and 2 change their expression and distribution in the epidermis of impetigo skin and in NHEKs treated with *S. aureus* supernatant (Figure 1b–d). Moreover, a previous report on S1PR1 showed that this receptor is tightly associated with immune responses in many cell types (Blaho and Hla, 2014; Jiang et al., 2017). These data indicate that *S. aureus* not only induces S1P production but also influences the expression of S1PR1 and 2 in human keratinocytes.

Our results have shown that S1P induces keratinocyte TNF-α, IL-36γ, and IL-8 release (Figure 3, and see Supplementary Figure S4), which has been shown to result in subsequent neutrophil infiltration (Krishna and Miller, 2012; Li et al., 2014). Although direct S1P stimulation resulted in mRNA and secreted protein level inductions that were smaller than

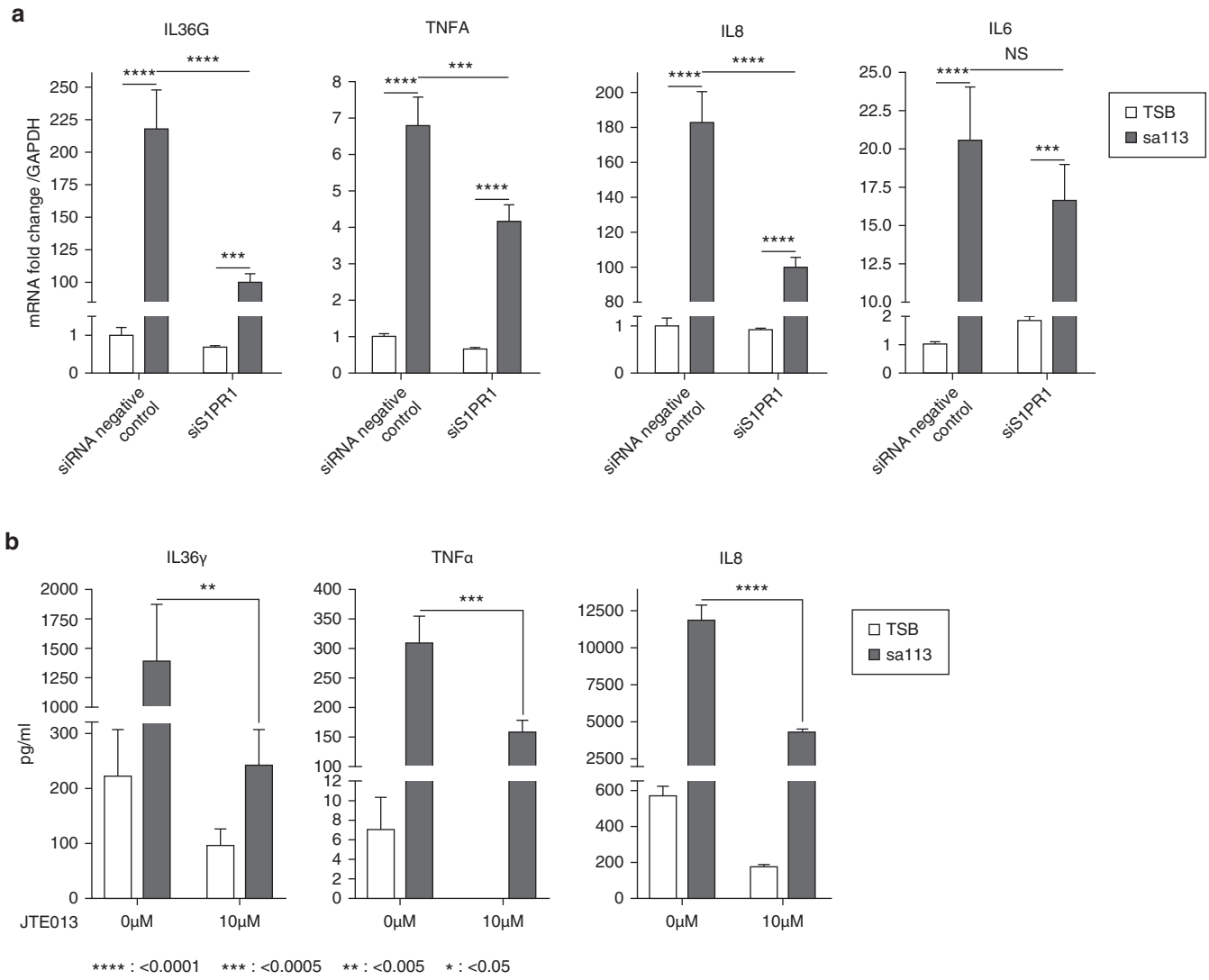

Figure 5. In NHEKs, S1PR1 knockdown affects *Staphylococcus aureus* bacterial supernatant-induced IL-36γ, TNF-α, and IL-8 transcription, and JTE013 blocks their secretion. (**a**) IL36G, TNFA, IL8 and IL6 transcription and (**b**) IL-36γ, TNF-α, and IL-8 protein secretion in NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for (**a**) 4 hours and (**b**) 24 hours. Before stimulation, cells were (**a**) transfected with control or S1PR1 siRNAs or (**b**) treated with or without 10 μmol/L JTE013. mRNA expression data in **a** are shown as the fold change versus control siRNA/TSB-treated cells. Bar graphs indicate mean ± standard deviation. M, mol/L; NHEK, normal human epidermal keratinocyte; si, small interfering; TSB, tryptic soy broth.

what was observed with sa113 supernatant stimulation (Figure 3, and see Supplementary Figures S3 and S4), we still saw that reduced S1P production caused by SPHK1 knockdown and SPHK2 inhibition significantly decreased TNF-α, IL-36γ, and IL-8 transcription (Figure 4a) and secretion (Figure 4b) in sa113 supernatant-treated NHEKs. This stronger response of NHEKs after sa113 supernatant treatment (see Supplementary Figure S3) than S1P stimulation (Figure 3a) could potentially be explained by the presence of different toxins in the supernatant that have the capacity to stimulate many different receptors at the same time. However, TNF-α, IL-36γ, and IL-8 transcriptions were reduced by S1PR1 knockdown (Figures 3d and 5a), and their protein secretion was decreased by blocking S1PR2 (Figures 3c and 5b, and see Supplementary Figure S5) similarly in S1P and sa113 supernatant-treated NHEKs. These data ensure that the S1P-S1PR axis plays a significant role in NHEK anti-*S. aureus* cytokine synthesis.

A previous report has shown that in keratinocytes, S1P-derived IL8 mRNA expression is mediated by S1PR1/3 and NF-κB (Oizumi et al., 2014). Our data are consistent with this report in that we have identified S1PR1 in NHEKs as the mediator of TNF-α, IL-36γ, and IL-8 transcription; however, the control of secretion of these cytokines is regulated by S1PR2. More specifically, both S1PR1 and 2 control TNF-α secretion, and S1PR2 controls IL-36γ and IL-8 secretion (Figures 3 and 5, and see Supplementary Figure S5). In different cell types, S1P binding to S1PRs activates different intracellular signaling pathways and differentially regulates cytokine production (Brunnert et al., 2015; Chandru and Boggaram, 2007; Hamidi et al., 2014; Oskeritzian et al., 2010; Oz-Arslan et al., 2006; Zhao et al., 2008). In endothelial cells, S1PR1 couples to inhibitory regulative G-protein (Gi) and activates the phosphatidylinositol-3-kinase (PI3K) pathway. In contrast, S1PR2 antagonizes S1PR1−G_i−PI3K signaling through activation

of the G_{12}–Rho pathway (Kim et al., 2015). In trophoblast-derived cells, S1PR1 and 2 have distinct functions for the transcription and secretion of IL-8 (Brunnert et al., 2015). A similar different activation of the intracellular pathway by S1PR1 or 2 is possible in NHEKs, and these findings support our data that TNF-α synthesis seems to be regulated by a different intracellular pathway from IL-36γ and IL-8 in keratinocytes (Figure 3b–f). Our findings also indicate that *S. aureus* is a trigger to activate the S1P-S1PR pathway in keratinocytes.

The epidermis uses diverse pathways to recruit neutrophils from the circulation to sites of inflammation (Krishna and Miller, 2012). We provide evidence that there is another pathway centered on the S1P-S1PR axis that contributes to neutrophil recruitment in the epidermis when it is exposed to *S. aureus* invasion. Although Park et al. (2013, 2016) reported that S1P induces cathelicidin antimicrobial peptide production through an S1PR-independent intracellular mechanism in keratinocytes, our findings show that S1P also has an extracellular antibacterial response in keratinocytes that induces proinflammatory cytokine synthesis through an S1PR-dependent mechanism.

In this study, we have proven that *S. aureus*-induced S1P production in keratinocytes (Figure 2) results in the release of proinflammatory cytokines from keratinocytes as an extracellular signal (Figure 4), but there are many different kinds of cells that express S1PRs on their surface in skin tissue, such as Langerhans cells, mast cells, innate and adaptive immune cells, fibroblasts, and endothelial cells (Bock et al., 2016; Hamidi et al., 2014; Reines et al., 2009; Rivera et al., 2008; Wilkerson and Argraves, 2014), which may have similar mechanisms of activation. We have not investigated the effects of *S. aureus*-induced, keratinocyte-secreted S1P on other types of S1PR-expressing cells in skin tissue. Our future plan includes investigating the role of keratinocyte-released S1P in orchestrating skin inflammation through S1P receptors on different cell types. To achieve this aim, more precise studies about the communication between keratinocytes and other cell types will be required.

S1P (molecular weight = 379.47 g/mol) is a low-molecular-weight lipid (Chun and Hartung, 2010), so S1P and/or its analogues have the capacity to cross the skin barrier and become topical therapeutic agents that may be used to fight *S. aureus* infections. Our data indicate the importance of controlling S1PR1 and 2 function in the skin, especially in the epidermis, where skin bacterial infection has occurred. Thus, S1PR modulators may contribute to the suppression of excessive inflammation-derived tissue damage and/or activate more selective inflammation to remove bacterial burdens. Although S1P and its receptors have multiple functions in many types of cells, we may be able to limit and control their effects in skin tissue through the use of topical applications of S1P and/or related therapeutics.

MATERIALS AND METHODS

Primary NHEKs

Primary normal human epidermal keratinocytes (Thermo Fisher Scientific, Waltham, MA) were cultured in EpiLife medium (60 μmol/L calcium) (Thermo Fisher Scientific) supplemented with 1% EpiLife Defined Growth Supplement and 1% Antibiotic-Antimycotic (Thermo Fisher Scientific). Subconfluent NHEKs were seeded in six-well plates, 12-well plates, or chamber slides and grown to sufficient confluence before treatment. For the induction of NHEK differentiation, confluent NHEKs were cultured in EpiLife medium (1.2 mmol/L calcium) for 48 hours.

NHEK treatment with bacterial supernatant or S1P

NHEKs were treated with PBS, 1 or 10 μmol/L S1P (Tocris Bioscience, Minneapolis, MN), 50 μl/ml 3% TSB (MilliporeSigma, Burlington, MA) or 50 μl/ml 0.22 μm-filtered (MilliporeSigma) flow of *S. aureus* (sa113, 35556; ATCC, Manassas, VA) bacterial supernatant in 3% TSB.

Chemical S1PR2 or SPHK2 block in NHEKs

To block S1PR2 or SPHK2, NHEKs were incubated with 10 μmol/L JTE013 (Cayman, Ann Arbor, MI) or 1 μmol/L ABC294640 (Cayman) at 37 °C for 2 hours or 4 hours, respectively, before S1P or *S. aureus* bacterial supernatant treatment, according to previous reports (Schaper et al., 2014; Terashita et al., 2016; Zhou et al., 2018).

RNA interference

Before S1P or *S. aureus* bacterial supernatant treatment, NHEKs were transfected with 10 nmol/L siRNA for SPHK1 (Silencer Select s16957, Thermo Fisher Scientific), S1PR1 (Silencer Select s4448, Thermo Fisher Scientific), or negative control siRNA (Silencer Select Negative control #1, Thermo Fisher Scientific) with Lipofectamine RNAiMAX (Thermo Fisher Scientific).

Histology and immunofluorescent staining

Human normal and impetigo de-identified skin samples were kindly provided by Asahikawa Medical University. All samples were collected under written informed consent, and the protocol was approved by the medical ethics committee of Asahikawa Medical University. The study was conducted according to the principles of the Declaration of Helsinki. Immunofluorescent staining of skin sections and NHEKs was performed as described previously (Wang Z et al., 2017), with the primary and secondary antibodies listed in Supplementary Table S1 online. Incubation with secondary antibodies only was served as a negative control (see Supplementary Figure S6 online). Fluorescence images were detected with an immunofluorescent microscope.

Quantitative real-time reverse transcriptase–PCR

Total RNA from NHEKs was isolated by RNeasy Mini Kit (Qiagen, Hilden, Germany). At least 1 μg RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. Quantitative real-time reverse transcriptase–PCR (real time RT-qPCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) or TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The hS1PR1-5 primers (Gandy et al., 2013) and the other probes used for real time RT-qPCR are listed in Supplementary Tables S2 and S3 online, respectively. The expression of target genes was normalized to GAPDH expression and analyzed by the $2^{-\Delta\Delta Ct}$ method.

ELISA

Supernatants from NHEKs were collected after 4, 24, or 48 hours of incubation with sa113 supernatant or S1P, and S1P and/or proinflammatory cytokine concentrations were detected with the ELISA kits listed in Supplementary Table S4 online, according to the

manufacturer's instructions. Optical density was determined with a DTX 880 multimode detector (Beckman Coulter, Brea, CA).

SPHK activity assay

To measure SPHK activity in NHEKs, we used the adenosine triphosphate (ATP) depletion assay. SPHK activity was measured with a sphingosine kinase activity assay kit (Echelon, Salt Lake City, UT), according to the manufacturer's instructions. Briefly, NHEKs were incubated with 50 μl/ml TSB or sa113 supernatant for 24 hours and lysed in reaction buffer. Cell lysates were incubated with 100 μmol/L sphingosine and 10 μmol/L ATP for 90 minutes at room temperature. Reaction buffer and 500 ng/ml recombinant SPHK1 and 2 (Cayman) were also incubated, instead of cell lysates, as negative and positive controls, respectively (see Supplementary Figure S7 online). ATP detector was added to stop the reaction, and luminescence was measured using the SpectraMax Gemini EM (Molecular Devices, San Jose, CA). ATP concentrations were calculated by linear regression analysis, and the data are represented as the amount of ATP consumed.

Radar charts

Radar charts were used to compare the percentages of cytokine mRNA expression and protein secretion from 10 μmol/L S1P-treated NHEKs with or without siS1PR1 or 10 μmol/L JTE013 treatment.

Statistical analysis

In all in vitro experiments, all samples were in triplicate, and values are expressed as mean ± standard deviation. Student's or Welch's *t* tests were applied to analyze the differences between two groups. One- or two-way analysis of variance and Tukey tests were applied to analyze the differences among more than two groups. $P < 0.05$ was considered significant.

Data availability statement

Data sets related to this article can be found at https://data.mendeley.com/datasets/cjhgx6wbxy/1, hosted at Mendeley.

ORCIDs

Anna DiNardo: https://orcid.org/0000-0002-5575-9968
Satomi Igawa: https://orcid.org/0000-0001-9483-2526
Jae Eun Choi: https://orcid.org/0000-0002-1215-7078
Zhenping Wang: https://orcid.org/0000-0003-3360-3775
Yu-Ling Chang: https://orcid.org/0000-0003-4650-7117
Chia-Chi Wu: https://orcid.org/0000-0002-2182-360X
Tyler Werbel: https://orcid.org/0000-0003-3441-6875
Akemi Ishida-Yamamoto: https://orcid.org/0000-0002-3104-102X

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization, resources, supervision and funding acquisition, AD and AY; investigation, SI, JEC, and TW; formal analysis, SI; visualization, SI and C-CW; validation, ZW and Y-LC; writing the original draft, SI; writing review and editing, AD.

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.2019.02.010.

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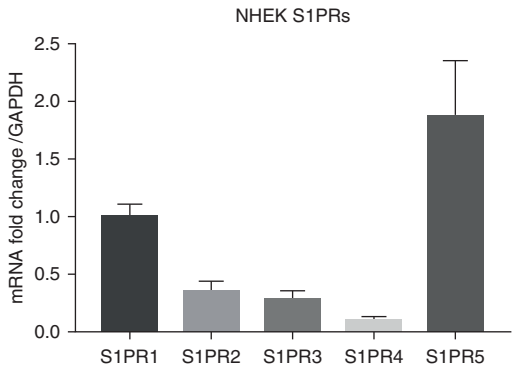
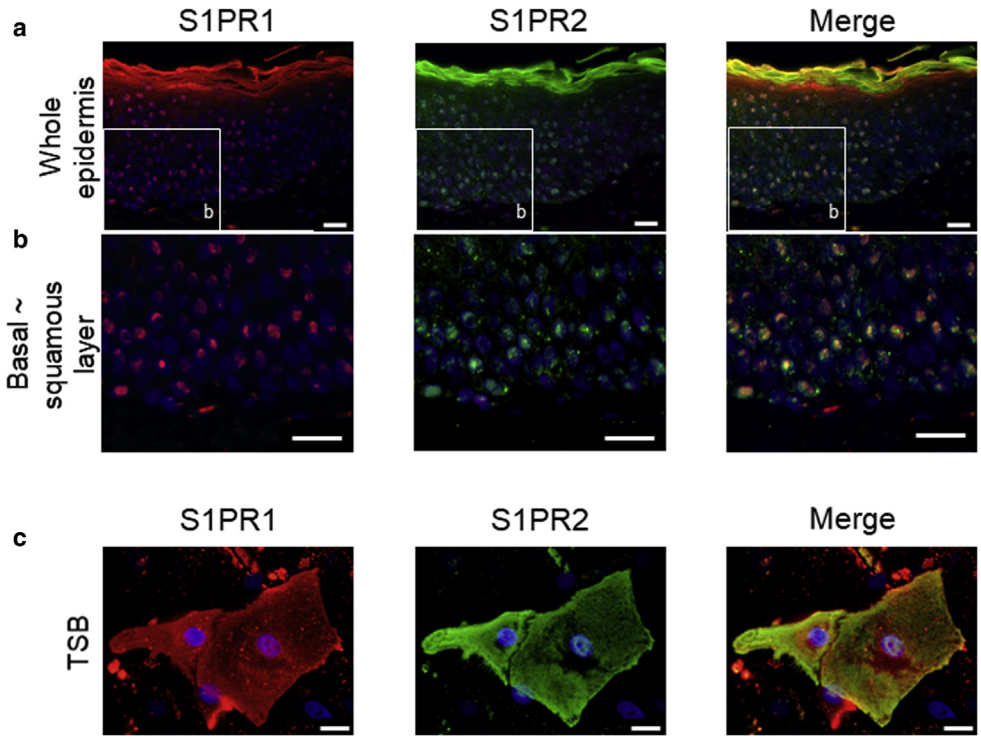
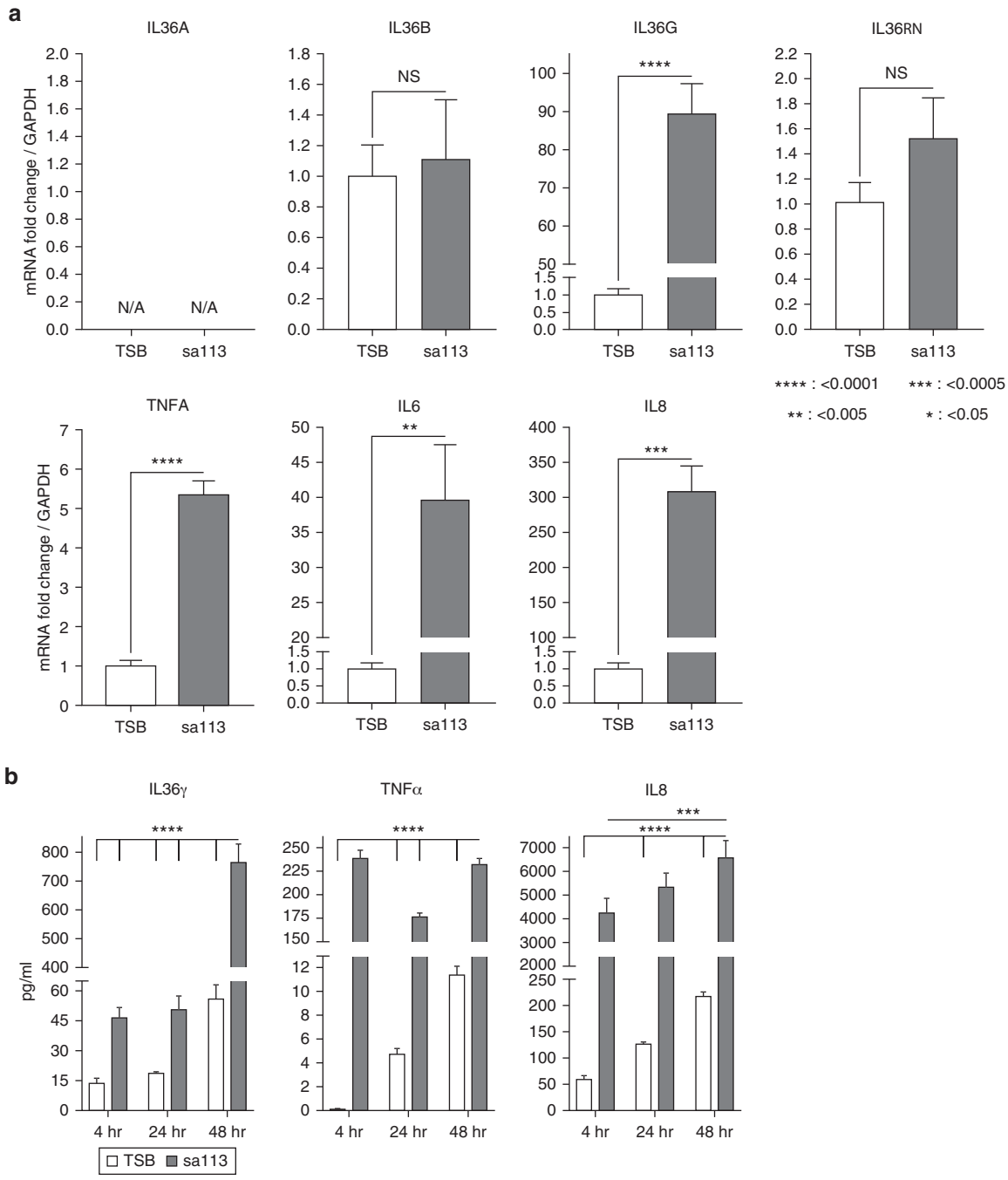


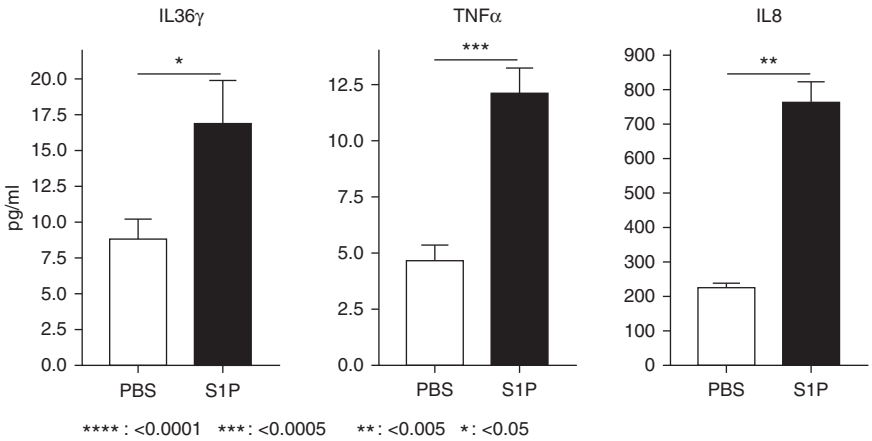
Supplementary Figure S1. mRNA expression levels of sphingosine 1-phosphate receptor (S1PR) isoforms in normal human epidermal keratinocytes (NHEKs). S1PR1-5 mRNA expressions in NHEKs without any treatment. Data is shown as the fold change versus S1PR1 mRNA expression.



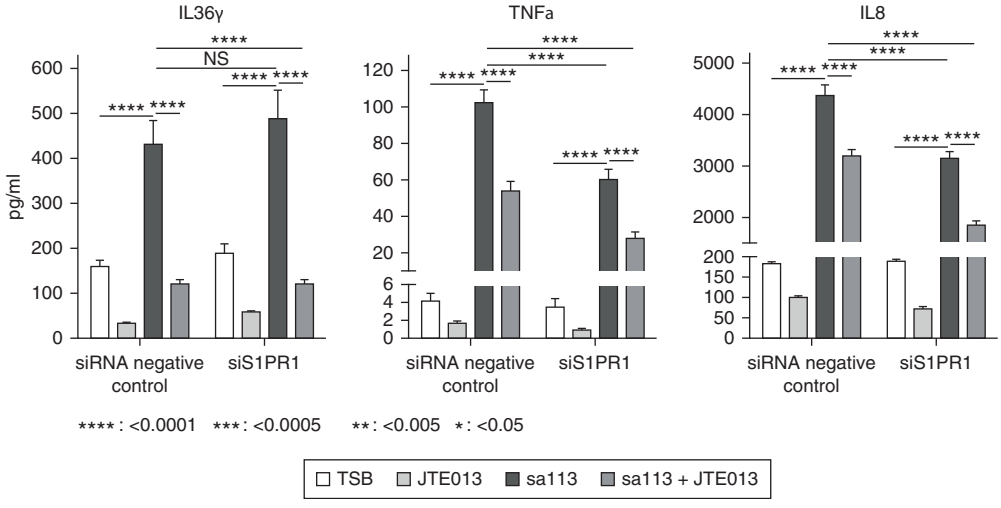
Supplementary Figure S2. Immunofluorescent staining of S1PR1 and 2 in normal skin sections and Ca^{2+}-differentiated NHEKs. (**a**) Magnified images of S1PR1 and 2 immunofluorescent staining in normal skin sections. (**b**) Further magnified images of the white rectangles shown in **a**. (**c**) S1PR1 and 2 immunostaining of Ca^{2+}-differentiated NHEKs incubated with 50 μl/ml TSB for 24 hrs. (**a-c**) Scale bars = 10 μm.



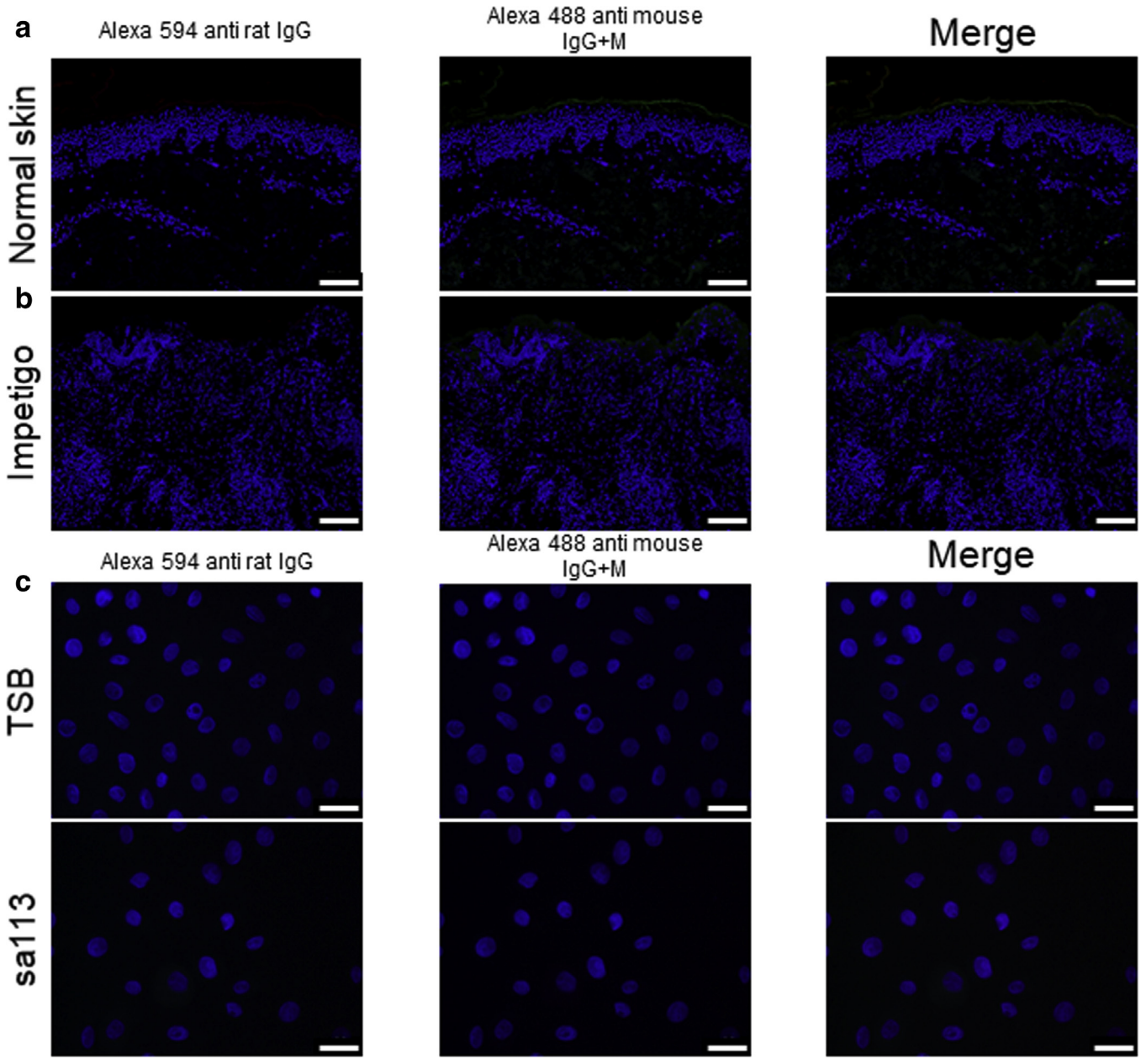
Supplementary Figure S3. *S. aureus* bacterial supernatant induces NHEKs to synthesize TNFα, IL6, IL8 and IL36γ cytokines. (**a**) mRNA expression of IL36 (A, B, G, RN), TNFA, IL6 and IL8 in NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for 4 hours. Data is shown as the fold change versus TSB control. (**b**) Secretion of IL36γ, TNFα, and IL8 from NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for 4–48 hours.



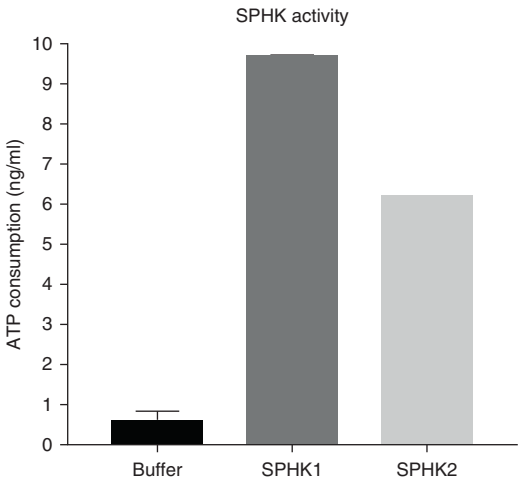
Supplementary Figure S4. 1 μmol/L S1P induces significant increases in TNFα, IL8, and IL36γ cytokine secretion in NHEKs. IL36γ, TNFα, and IL8 protein secretion was measured in NHEKs incubated with PBS or 1 μmol/L S1P for 24 hours.



Supplementary Figure S5. S1PR2 inhibitor dominantly reduces *S. aureus* bacterial supernatant-induced TNFα, IL8, and IL36γ cytokine secretion in NHEKs. Secretion of IL36γ, TNFα, and IL8 from NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for 24hrs. Before stimulation, cells were treated with control or S1PR1 siRNA, and S1PR2 was blocked with 10 μmol/L JTE013.



Supplementary Figure S6. Negative controls for immunofluorescent staining in skin sections and NHEKs. (**a**–**c**) Immunofluorescent images of (**a**) normal and (**b**) impetigo skin sections, as well as (**c**) NHEKs incubated with 50 μl/ml TSB or sa113 supernatant for 24 hours following incubation with secondary antibodies only, as negative controls. Scale bars: (**a, b**) 50 μm, (**c**) 10 μm



Supplementary Figure S7. Negative and positive controls for the sphingosine kinase (SPHK) activity assay. To confirm that the SPHK activity assay worked properly, ATP consumption was measured in the reaction buffer (black bar) as a negative control, and in reactions containing SPHK1 (gray bar) and 2 (pale gray bar) as positive controls.

Supplementary Table S1. Primary and secondary antibody list

Antibody	Product Description	Dilution	Source
Primary			
S1PR1 (clone # 713412)	Rat, Monoclonal	1 : 50	R&D Systems, Minneapolis, MN
S1PR2 (clone 6E8.1)	Mouse, Monoclonal	1 : 100	MilliporeSigma, Burlington, MA
Secondary			
Alexa Fluor 488 F(ab')2 IgG/M	Goat, anti-mouse	1 : 500	Thermo Fisher Scientific, Waltham, MA
Alexa Fluor 594 IgG	Goat, anti-rat	1 : 100	Thermo Fisher Scientific, Waltham, MA

Supplementary Table S2. Real-time RT-PCR primer list for SYBR green

Gene	Forward Primer Sequence	Reverse Primer Sequence
hS1PR1	TGCGGGAAGGGAGTATGTTTG	AGGAAGAGGCGGAAGTTATTGC
hS1PR2	CCCAACAAGGTCCCAGGAACAC	GCAACAGAGGATGACGATGAAGG
hS1PR3	AACAATAGCACGCACTCTCC	ATAAGAACACAGCCGGACAG
hS1PR4	TGGTGGTGCTGGAGAACTTG	TGATGTTCACCAGGCAATAGTAG
hS1PR5	AAATGTGCCCATGTGTTCTAAGAAATG	AACTACTCCTTCAGCTCC

Abbreviations: A, adenine; C, cytosine; G, guanine; RT-PCR, reverse transcriptase-PCR; T, thymine.

Supplementary Table S3. Real-time reverse transcriptase–PCR TaqMan (Thermo Fisher Scientific) probe list

Gene	TaqMan Assay ID
hSPHK1	Hs01116530_g1
hSPHK2	Hs01016543_g1
hIL36A	Hs00205367_m1
hIL36B	Hs00758166_m1
hIL36G	Hs00219742_m1
hIL36RN	Hs01104220_g1
hIL6	Hs00174131_m1
hIL8	Hs00174103_m1
hTNFA	Hs00174128_m1

Abbreviation: ID, identification.

Supplementary Table S4. ELISA kit list

ELISA Target	Cat#	Source
Human S1P	MBS2516132	MyBiosource, San Diego, CA
Human IL8	DY208	R&D Systems, Minneapolis, MN
Human TNFα	DY210	R&D Systems, Minneapolis, MN
Human IL36γ	DY2320	R&D Systems, Minneapolis, MN
Human IL36α	DY1078	R&D Systems, Minneapolis, MN

Abbreviations: Cat, catalog.