Loss of ATP2A2 Allows Herpes Simplex Virus 1 Infection of a Human Epidermis Model by Disrupting Innate Immunity and Barrier Function

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Destruction of epidermal barrier function associated with atopic dermatitis or Darier’s disease often causes severe secondary skin infections. Patients with skin barrier disorders often repeatedly acquire Kaposi varicelliform eruption, which is caused by herpes simplex virus, but the underlying mechanisms and effective preventive methods have yet to be found. Viral infection through an impaired epidermal barrier can be prevented by enhancing innate immunity and/or inhibiting viral entry. In this study, we established a three-dimensional skin barrier dysfunction model by silencing ATP2A2, which is mutated in some Darier’s disease patients. We confirmed the loss of desmosomes and presence of histopathological clefts in the suprabasal layer. Herpes simplex virus 1 applied to the stratum corneum infected the deep epidermis. An innate immune reaction was assessed by evaluating the expression of IFNB1 and related genes. Pretreatment with polynosinic–polycytidylic acid alone or plus the antimicrobial peptide, LL37 enhanced IFN-β production and suppressed viral replication. Furthermore, topical application of a white petrolatum ointment containing heparin, which binds viral glycoproteins related to virus entry, strongly inhibited viral replication, probably by inhibiting invasion. Our human barrier-dysfunctional model will have future application for identifying the mechanism of Kaposi varicelliform eruption onset, preventive methods, and therapies.

INTRODUCTION

Epidermal barrier dysfunction often promotes bacterial and/or viral infection and prolongs or worsens a skin condition. Disseminative herpes simplex virus (HSV) infection of eczematous sites in atopic dermatitis or heritable skin diseases such as Darier disease (DD) and Hailey-Hailey disease is called eczema herpeticum or Kaposi varicelliform eruption (KVE) and is a serious complication for these patients (Beck et al., 2009; Gao et al., 2009; Molinelli et al., 2016). Although treatment with antiviral drugs is effective, KVE often develops repeatedly in the same patient and becomes more refractory than ordinary herpes simplex infection.

Dupilumab, a monoclonal antibody against the IL-4 and IL-13 receptors, has recently been reported to significantly prevent skin infections, including KVE, in moderate to severe atopic dermatitis patients (Fleming and Drucker, 2018). However, effective drugs that directly inhibit viral infection have not yet been found, and there is no known preventive method that has sufficient effect.

DD is an autosomal-dominantly inherited skin disorder in which desmosomal adhesion between keratinocytes is abnormal (Ohitavat et al., 2004; Ringpfeil et al., 2001; Takagi et al., 2016). We have successfully developed a three-dimensional (3D) in vitro model of DD in which loss of function mutations are replicated by silencing the ATP2A2 gene in keratinocytes (Sato et al., 2017a). ATP2A2 encodes a calcium pump found on the endoplasmic reticulum. The ATP2A2-silenced 3D epidermis formed from these keratinocytes has suprabasal clefts and irregular hyperkeratosis, which impairs skin barrier function. Using this barrier-dysfunctional 3D model, we established an experimental KVE model by infecting the keratinous layer with HSV1. We then assessed whether enhancing innate immunity reduces HSV replication in this 3D model and also evaluated topical reagents that may help compensate for barrier dysfunction and reduce HSV1 infection.

RESULTS

ATP2A2 silencing suppresses desmosome formation in intercellular spaces in a 3D epidermal model

First, we selected the most effective small interfering RNA (siRNA) for targeting ATP2A2 using the human keratinocyte...
ATP2A2 silencing promotes HSV1 invasion into the deep epidermal layers of 3D skin constructs

In Figure 1, we show that ATP2A2 silencing clearly suppressed desmosome formation and induced clefts at the intercellular spaces in 3D skin constructs. Rahn et al. (2017) reported that exogenous HSV1 could not invade into a normal human 3D epidermis model that had been air-exposed for more than 48 hours without wounding (Rahn et al., 2017). Following their report, we carefully applied HSV1 to the stratum corneum side of 3D skin constructs that had been air-exposed for more than 5 days. We harvested the constructs 24 hours after infection and then confirmed that HSV1-gB protein could not be detected in all epidermal layers of 3D skin constructs that were transfected with a negative control siRNA (Figure 2a, upper panels). However, ATP2A2 silencing allowed the invasion of HSV1 into the deep epidermal layers, including the basal and spinous layers (Figure 2a middle panels). We evaluated the expression of HSV1-gD (gD) mRNA (Figure 2b) and protein (data not shown) in these 3D skin constructs and found that mRNA expression was strongly up-regulated in the ATP2A2-silenced constructs.

HSV1 cannot survive in differentiated keratinocytes, but ATP2A2 silencing allows viral persistence after keratinization because of barrier dysfunction and reduced IFN-β expression

To clarify the reason why ATP2A2 silencing allowed HSV1 invasion into the deep layers of the epidermis without wounding, we investigated the underlying mechanism using two-dimensional (2D) keratinocyte cultures that were incubated in medium containing low (0.06-mmol/L) or high (1.0-mmol/L) concentrations of calcium. As previously reported (Rahn et al., 2017), HSV1 replication was time-dependently suppressed by calcium-induced differentiation in NHEKs (Figure 3a, and see Supplementary Figure S1b). Immunofluorescence staining showed that HSV1-gB protein was detected only in the keratinocytes that had been incubated in low-calcium medium (Figure 3b). Next, we infected HSV1 into siRNA-transfected 2D keratinocyte cultures. Surprisingly, ATP2A2 silencing suppressed HSV1 replication (see Supplementary Figure S1c) and reduced plaque numbers (Figure S1d) in low-calcium medium. Immunofluorescence staining showed that diffuse perinuclear HSV1-gB protein was detected in control keratinocytes around plaques (see Supplementary Figure S1f, HSV1-gB staining of siControl). On the other hand, HSV1-infected ATP2A2-silenced keratinocytes appeared apoptotic, and focal HSV1-gB expression was seen (see Supplementary Figure S1e, HSV1-gB staining of siATP2A2). We then evaluated expression of the apoptosis-related genes BAX and BCL2 (data not shown) and confirmed that BAX expression was significantly up-regulated in HSV1-infected ATP2A2-silenced keratinocytes in low-calcium medium (see Supplementary Figure S1d).

Taking these results together, we concluded that the loss of ATP2A2 facilitates the induction of apoptosis in low-calcium conditions, even though HSV1 inhibits endoplasmic reticulum stress and host cell apoptosis to enhance viral survival (Cheng et al., 2005; Galvan and Roizman, 1998; Jerome et al., 1999). Furthermore, previous studies reported that the inhibition of ATP2A2 induced endoplasmic reticulum stress in primary keratinocytes from DD patients (Savignac et al., 2014) Also, Celli et al. (2011) reported that short-term inhibition of ATP2A2 by thapsigargin activates XBP1 and caspase14, then induces terminal differentiation of keratinocytes incubated in low-calcium medium.

However, in the 3D skin constructs, we found that loss of ATP2A2 allows HSV1 to invade into the deep epidermal layers without wounding (Figure 2). This observation shows that, if HSV1 breaks through the stratum corneum barrier and then invades into the deep epidermal layers, then viral replication is amplified more in normal skin compared with DD skin.

Next, we transfected siRNA into the 2D keratinocyte cultures and then added 1.0 mmol/L CaCl₂ for keratinization (high-calcium medium). As previously reported (Celli et al., 2012), we observed the significantly suppressed expression of extracellular E-cadherin in the area where keratinocytes are detached, probably because of vulnerable intracellular adhesion by ATP2A2 silencing (Figure 3c). We infected HSV1 into the siRNA-transfected keratinocytes 72 hours after calcium-induced differentiation and then observed HSV1-gB protein by immunofluorescence staining (Figure 3d).
Figure 1. ATP2A2 silencing suppresses desmosome expression in intercellular spaces in 3D skin constructs. NHEKs were transfected with siRNA for 72 hours and then analyzed by (a) quantitative real-time reverse transcriptase–PCR and (b) Western blotting of ATP2A2 and β-actin (β-actin was used as a loading control).
HSV1-gB was not detected in control keratinocytes, which were differentiated for more than 72 hours (Figure 3d, left panel), but ATP2A2 silencing allowed HSV1 to survive after keratinization (Figure 3d, right panel).

To investigate why HSV1 can infect differentiated keratinocytes after the loss of ATP2A2, we evaluated the expression of genes related to apoptosis, type I IFNs, and IFN-stimulated genes in 3D skin constructs that had been differentiated for more than 6 days. We found that the expressions of IFNB1 and Isg15 were significantly down-regulated in ATP2A2 silenced constructs after HSV1 infection compared with controls (Figure 3d). Expression of the apoptosis marker BAX was not changed after epidermal differentiation by ATP2A2 silencing (Figure 3e), even though it was up-regulated before differentiation (see Supplementary Figure S1e). Furthermore, silencing of ATP2A2 did not increase the active form of caspase14 in the same condition (we evaluated Supplementary Figure S1f). These data suggest that ATP2A2 knockdown suppressed cell adhesion and terminal differentiation partially but may not be greatly involved in apoptosis and filaggrin processing (Nicotera and Melino, 2007) in high extracellular calcium condition. These results may suggest that enough extracellular calcium can overcome the dysfunction of calcium pumps on the endoplasmic reticulum to inhibit apoptosis. It is well known that the toll-like receptor 3 agonist polyinosinic-polycytidylic acid (Poly(I:C)) or a complex of Poly(I:C) plus the antimicrobial peptide LL37 (Poly(I:C)/LL37) is a strong inducer of IFN-β in keratinocytes (Zhang et al., 2016). To increase the production of IFN-β in ATP2A2 silenced 3D skin constructs, we applied a solution of Poly(I:C) or Poly(I:C)/LL37 for 4 hours (see Supplementary Figure S2b online). Although the effects of Poly(I:C)/LL37 were weaker in the ATP2A2 silenced constructs than in the controls, the expression of IFNB1 was significantly up-regulated by Poly(I:C) or Poly(I:C)/LL37 treatment compared with a phosphate-buffered saline (PBS) control (Figure 3f).

Based on these results, we stimulated the ATP2A2 silenced 3D skin constructs with PBS, Poly(I:C), or Poly(I:C)/LL37 for 3 hours and then infected them with HSV1 for 24 hours (see Supplementary Figure S2c). As expected, Poly(I:C) or Poly(I:C)/LL37 significantly suppressed viral replication compared with the control (Figure 3g).

**Pretreatment with glycosaminoglycans inhibits HSV-1 invasion into the epidermis**

In Figure 3, we show that the induction of IFN-β inhibited HSV1 replication in the barrier-dysfunctional epidermal model. However, large effects could not be obtained because

![Figure 2. Exogenous HSV1 invades into deep layers of ATP2A2-silenced epidermis. (a) Immunofluorescence staining of 3D skin constructs transfected with anti-ATP2A2 or control siRNAs. β-actin (red), HSV1-gB protein (green), DAPI (blue). Scale bar = 100 μm. (b) mRNA expression of HSV1-gD in 3D skin constructs 24 hours after infection (normalized to β-actin expression). Quantitative real-time reverse transcriptase PCR data are means ± standard error of the mean, n = 3. **P < 0.005 (Student’s t test). 3D, three dimensional; siControl, normal human epidermal keratinocytes that transfected siRNA negative control; siATP2A2, normal human epidermal keratinocytes that transfected anti-ATP2A2 siRNA; siRNA, small interfering RNA.**
Figure 3. Loss of ATP2A2 suppresses IFN-β production, and Poly(I: C) or Poly(I: C)/LL37 complex increases HSV1 infection. (a) mRNA expression time-course of HSV1-gD (gD) in 2D NHEKs cultured in 1.0 mmol/L CaCl₂ (normalized to β-actin expression). Data are mean ± standard error of the mean, n = 3. *P < 0.05 (Tukey multiple comparison test). (b) Immunofluorescence staining of 2D NHEKs cultured in 0.06 mmol/L or 1.0 mmol/L CaCl₂. β-actin (red), HSV1-gB protein (green), DAPI (blue). Scale bar = 100 μm. (c, d) Immunofluorescence staining of anti-ATP2A2 or control siRNA-transfected NHEKs cultured in 1.0 mmol/L CaCl₂; (c) For staining extracellular E-cadherin, NHEKs were fixed by 4% paraformaldehyde without permeabilization. (d) NHEKs were fixed by ice cold methanol and then stained by β-actin (red), HSV1-gB protein (green), and DAPI (blue). Scale bar = 100 μm. (e) Expression of BAX, IFNB1, and ISG15 in 3D skin E Sato et al. ATP2A2 Loss Allows Epidermal HSV1 Infection Journal of Investigative Dermatology (2018), Volume 138
the transcription of type 1 IFNs and IFN-stimulated genes was suppressed in epidermis in which ATP2A2 was lost (Figure 3g). We therefore attempted to pretreat host cells with glycosaminoglycans, which are very important for the entry phase of HSV1 infection. In particular, it is known that heparan sulfate (HS), or heparin, binds to the gB and gC glycoproteins of HSV1 and is important for the entry phase of virus into the host cells (Shieh et al., 1992; Shukla et al., 1999). Furthermore, pretreatment of primary keratinocytes or HaCaT cells with HS or heparin strongly suppresses HSV1 infection (MacLeod et al., 2013; Mader et al., 2016). There are a few reports that high concentrations of chondroitin sulfate (CS), especially CS-B, also adsorb HSV1 and inhibit viral entry to host cells (Banfield et al., 1995; Marchetti et al., 2004). Based on these results, we first observed the effect of CS-A, CS-B, HS, or heparin adsorption on HSV1 using 2D NHEKs that had been differentiated with 1.5 mmol/L CaCl2 for 24 hours (see Supplementary Figure S2a). As a result, although 10 µg CS-A could adsorb only 60% of the 5 x 10^2 plaque-forming units of HSV1 that was applied, the same amount of HS or heparin almost entirely adsorbed the virus (Figure 4a–d). The on the other hand, 10 µg CS-B had no effect at all, but 100 µg CS-B entirely adsorbed the HSV1 (Figure 4b). Immunofluorescence staining showed that 100 µg CS-A or CS-B, or 10 µg HS or heparin, significantly inhibited HSV1 infection into the 2D NHEKs (Figure 4e). Finally, we applied heparin in a white petrolatum (WP) vehicle to ATP2A2-silenced 3D skin constructs and then infected them with 5 x 10^2 plaque-forming units of HSV 1 (see Supplementary Figure S2d). WP with heparin strongly suppressed the invasion and replication of HSV1 (Figure 4f and g), but it did not affect the production of IFN-β and ISG15 (see Supplementary Figure S2e).

**DISCUSSION**

Skin diseases that involve epidermal barrier dysfunction, such as atopic dermatitis and DD, are associated with severe secondary infections, and elucidation of the underlying mechanisms and preventive strategies is required. In this study, we silenced the ATP2A2 gene, which encodes one of the calcium pumps on the endoplasmic reticulum, succeeded in creating a 3D epidermal model with DD-like barrier dysfunction, and observed the model’s epidermis by histopathology and transmission electron microscopy. We confirmed the presence of clefts between the keratinocytes, which seem to be due to the loss of intercellular desmosomes (Figure 1c and e). This barrier dysfunction not only permitted the invasion of HSV1 into the deep epidermal layers (Figure 2), but also suppressed the production of IFN-β and the antiviral factor, ISG15, that it induces (Figure 3d). A recently published article reported that the reduction of intracellular Ca2+ affects the phosphorylation of NF-kB and IRF3 (Schappe et al., 2018). Therefore, the poor elevation of intracellular Ca2+ caused by viral infection associated with the loss of ATP2A2 is likely to be related to insufficient phosphorylation of IRF3 and low production of IFN-β. Although solutions of the toll-like receptor 3 agonist Poly(I:C) and the mitochondrial antiviral signaling protein agonist LL37 complexed with Poly(I:C) both permeated into the 3D epidermis and strongly induced IFN-β, type 1 IFN production in the epidermis was suppressed by ATP2A2 silencing (Figure 3e). Thus, pretreatment with Poly(I:C) or Poly(I:C)/LL37 complex significantly suppressed HSV1 replication in epidermis lacking ATP2A2, but the effects were weaker than those seen in control epidermis (Figure 3f).

The HSV1 glycoproteins gB and gC, which play an important role in host cell invasion, are known to bind strongly to the glycosaminoglycans HS and heparin (MacLeod et al., 2013; Mader et al., 2016; Shieh et al., 1992; Shukla et al., 1999). We showed that pretreatment with WP ointment containing heparin strongly inhibited HSV1 invasion into the epidermis (Figure 4f and g). However, when we pretreated the 3D skin constructs with heparin dissolved in PBS, the heparin penetrated deeper into the epidermis, conversely resulting in the active invasion of virus into the epidermis (data not shown).

Based on these considerations, a schema of our experimental results is shown in Figure 5. In epidermis with barrier dysfunction, HSV1 easily infects from the outside (Figure 5, left). However, by infiltrating Poly(I:C) into the epidermis, IFN-β is induced, and HSV1 replication is inhibited (Figure 5, middle). Furthermore, intraepidermal invasion of HSV1 is strongly inhibited by pretreating the epidermis with heparin in WP, which strongly prevents heparin penetration into the epidermis (Figure 5, right).

Human beings are the only natural host of HSV, and it is arguable whether mouse infection models accurately represent human infection. However, our barrier-dysfunctional 3D skin model using primary NHEKs can reproduce KVE by permitting HSV infection from the surface. We hope that this human barrier-dysfunctional model will be useful for further elucidating the mechanisms, preventive methods, and treatment of external HSV infection.

**MATERIALS AND METHODS**

**Chemicals and antibodies**

CS-A, CS-B, HS, and heparin were obtained from Wako (Tokyo, Japan), LL37 peptides, WP, and Poly(I:C) were obtained, respectively, from AnaSpec (Fremont, CA), Maruishi Pharmaceutical (Osaka, Japan), and Enzo Life Science (Farmingdale, NY). Mouse anti-HSV1+HSV2 gB [10B7] (Abcam, Cambridge, MA), mouse anti-E-cadherin [HECD-1] (Abcam), rabbit anti-Jacalin [13E5] (CST, Danvers, MA) and rabbit anti-ATP2A2 (CST) were used as primary antibodies for immunoblotting or immunostaining. Alexa Fluor constructs at 24 hours after HSV1 infection. Data are means ± standard error of the mean, n = 3. *P < 0.05, **P < 0.01 (unpaired Student t test). (f, g) Expression of IFNB1, gD, and HSV1-VP16 (UL48) in siRNA-transfected 3D skin constructs. Data are means ± standard error of the mean, n = 3. *P < 0.05, **P < 0.005 (one-way analysis of variance with Tukey's multiple comparison test). (f) siRNA-transfected constructs treated with phosphate buffered saline, 10 µg Poly(I:C) or 10 µg Poly(I:C)/10 µg LL37 complex for 3 hours. (g) ATP2A2-silenced constructs pretreated as in f and then infected with HSV1 for 24 hours. 2D, two dimensional; 3D, three dimensional; h, hour; HSV, herpes simplex virus; M, mol/L; ND, no data; NHEK, normal human epidermal keratinocyte; Poly(I:C), polyinosinic-polycytidylic acid; siATP2A2, NHEKs that transfected anti-ATP2A2 siRNA; siControl, NHEKs that transfected siRNA negative control; siRNA, small interfering RNA.
Figure 4. Glycosaminoglycans protect host cells from HSV1 infection. (a–d) mRNA expression of HSV1-gD (gD) and HSV1-VP16 (UL48) in 2D NHEKs infected with $5 \times 10^3$ plaque-forming units of HSV1 (multiplicity of infection = 0.01) after pre-incubation with (a) CS-A, (b) CS-B, (c) HS or (d) heparin. Data are mean ± standard error of the mean, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001 (Tukey multiple comparison test). (e) Immunofluorescence staining of 2D NHEKs infected with HSV1 after pre-incubation with glycosaminoglycans. β-actin (red), HSV1-gB protein (green), DAPI (blue). Scale bar = 100 µm. (f, g) ATP2A2-silenced 3D skin constructs pretreated for 3 hours with WP alone or 10 µg heparin in WP and infected with $5 \times 10^3$ plaque-forming units of HSV1 for 24 hours. (f) Quantitative real-time reverse transcriptase–PCR and (g) immunofluorescence staining of HSV1-infected ATP2A2-silenced 3D skin constructs pretreated with WP or WP/heparin. β-actin (red), HSV1-gB protein (green), DAPI (blue). Scale bar = 100 µm. Data are mean ± standard error of the mean, n = 3. *P < 0.05 (Student t test). 2D, two dimensional; 3D, three dimensional; CS, chondroitin sulfate; HS, heparan sulfate; HSV, herpes simplex virus; NHEK, normal human epidermal keratinocyte; ns, not significant; siATP2A2, NHEKs that transfected anti-ATP2A2 siRNA; WP, white petrolatum.
were dissolved in distilled water. WP ointment was melted in a 20°C water bath before it was applied to 3D skin constructs. Ten microliters of melted WP was added to each 3D skin construct to cover the stratum corneum before HSV1 infection.

**Cells and reagents**

Adult NHEKs were obtained from Lonza (Basel, Switzerland) and cultured as previously described (Sato et al., 2016). 3D skin constructs were prepared as described (Li and Sen, 2015; Sato et al., 2017a). All glycosaminoglycans (CS-A, CS-B, HS, and heparin) were dissolved in distilled water. WP ointment was melted in a 60°C water bath before it was applied to 3D skin constructs. Ten microliters of melted WP was added to each 3D skin construct to cover the stratum corneum before HSV1 infection.

**Figure 5.** Pretreatment with Poly(I:C) or glycosaminoglycan ointment protects ATP2A2-silenced epidermis from exogenous HSV1 infection. A schema of the major results. The effects of pretreatment with vehicle control (left), Poly(I:C) (middle), and WP/heparin complex (right) are shown. Loss of ATP2A2 allowed exogenous HSV1 infection from outside (left). Pretreatment with Poly(I:C) protects host cells from HSV1 by inducing production of IFN-β (middle). WP/heparin inhibits HSV1 invasion into the epidermis because the heparin binds and retains HSV1-gB and gC glycoproteins in the ointment and thereby inhibits the entry phase of HSV1 infection (right). HSV, herpes simplex virus; Poly(I:C), polyinosinic-polycytidylic acid; WP, white petrolatum.

488- or 568-conjugated goat IgG (Thermo Fisher Scientific) were used as secondary antibodies for immunostaining.

**DNA extraction and reverse transcription—PCR**

Total RNA from 3D skin constructs and 2D keratinocyte cultures was extracted using ISOGEN II (NIPPON GENE, Toyama, Japan), and cDNA was synthesized as previously described (Sato et al., 2013), using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan). Quantitative real-time reverse transcriptase—PCR primer sequences for human ATP2A2, gD, UL48, human IFNB1, human ISG15, human BAX, and ACTB are presented in Supplementary Table S1 online. Expression of each mRNA was calculated relative to β-actin expression, and all data are presented as fold change compared with their respective control (the mean of nonstimulated cells).

**Protein extraction and immunoblotting**

Samples were lysed in RIPA buffer as previously described (Sato et al., 2017b). Protein concentrations were measured using a bicinchoninic acid assay kit (Thermo Fisher Scientific). For immunoblotting, 10 µg of protein was separated on a 5%–20% Tris-Glycine e-PAGE (ATTO Corporation, Tokyo, Japan), transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), followed by immunoblotting using the indicated primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse or anti-rabbit IgG—horseradish peroxidase conjugate; GE Healthcare, Little Chalfont, UK). The protein bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare), and images were captured with an LAS-3000 instrument (FUJIFILM, Tokyo, Japan).

**Immunofluorescence staining of cultured cells**

Cells were fixed in −20°C methanol or 4% paraformaldehyde for 10 minutes, then blocked with PBS containing 5% goat serum at room temperature for 30 minutes. Mouse anti-HSV1–hHSV2 gB, mouse anti-ε-cadherin, rabbit anti-β-actin, and secondary antibodies (Alexa Fluor 488- or 568-conjugated goat IgG) were used at 1 µg/ml. Cover slips were mounted using ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific). Images were captured using a BIORIGO BX-9000 instrument (KEYENCE, Osaka, Japan).

**Immunofluorescence staining of paraffin-embedded sections**

Samples of 3D skin constructs were fixed in 4% paraformaldehyde/ PBS (Wako) for 24 hours at room temperature. Paraffin embedding and sectioning were performed using a Leica ASP200 S, Leica EG1160, and JUNG RM2045 Multicut Rotary Microtome (Leica, Wetzlar, Germany). Next, 4-µm paraffin sections were deparaffinized and rehydrated before heat-induced antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes at 121°C. Immunostaining was performed as previously described (Sato et al., 2016). Mouse anti-HSV1–hHSV2 gB, rabbit anti-β-actin, and secondary antibodies (Alexa Fluor 488- or 568-conjugated goat
IgG were used at 1 µg/mL. Images were captured using a BIORÉVO BZ-9000 instrument (KEYENCE).

Skin permeability assay
Skin permeability was assayed as described previously (Hardman et al., 1998; Turksen and Troy, 2002). Briefly, unfixed and freshly isolated 3D skin constructs were rinsed in PBS and then immersed in X-gal solution at pH 4.6 (100 mmol/L Na3PO4, 1.3 mmol/L MgCl2, 3 mmol/L K3Fe(CN)6, 3 mmol/L K4Fe(CN)6 and 1 mg/ml X-gal) and incubated at room temperature for 5 hours. After reaction, 3D skin constructs were washed by PBS and then fixed by 4% paraformaldehyde at room temperature overnight.

Transmission electron microscopy
The Center for Electron Microscopy at Fukuoka University Faculty of Medicine performed all steps of the procedures after sample fixation. Samples were immersed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for at least 1 hour, postfixed in 2% osmium tetroxide in 0.1 mol/L cacodylate buffer for 1 hour, and stained en bloc in 2% uranyl acetate and 2.66% lead nitrate for 5 minutes. Samples were dehydrated in ethanol, embedded in epoxy resin (MilliporeSigma), sectioned at 60–90 nm on a Reichert Ultracut S (Leica), and picked up on Formvar- and carbon-coated copper grids (Niissin EM, Tokyo, Japan). Sections were stained with 2% uranyl acetate for 5 minutes and 2.66% lead nitrate for 5 minutes. Grids were viewed using a HITACHI H-7100 transmission electron microscope (HITACHI, Tokyo, Japan).

CONFLICT OF INTEREST
SI receives a fee from Maruho as a speaker and an advisor. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
ES designed and performed the majority of the experiments and wrote the manuscript. KM performed the transmission electron microscopy, and KH designed and performed the majority of the experiments and wrote the manuscript. All authors supervised and designed experiments and wrote and prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

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.2018.05.019.

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