

Specificity protein 1 is pivotal in the skin's antiviral response

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Background: Previous studies have found specificity protein (Sp) 1 transcription factor in the viral replication machinery and postulated that Sp1 was required for viral replication in host cells.

Objectives: We investigated the role of Sp1 in the skin's antiviral responses from the perspective of host defense and its biological relevance in patients with atopic dermatitis and a history of eczema herpeticum (ADEH⁺).

Methods: Small interfering RNA duplexes were used to knock down Sp1 in keratinocytes. The expression of vaccinia virus (VV), herpes simplex virus 1, and other genes were evaluated by real-time PCR, or combined with Western blot and immunohistochemistry staining. A total of 106 human subjects participated in this study.

Results: Both VV and herpes simplex virus 1 replication were enhanced in Sp1 knocked-down keratinocytes. Sp1 gene expression was significantly decreased in ADEH⁺ subjects compared with patients with atopic dermatitis without a history of eczema herpeticum and nonatopic subjects ($P < .0001$) and inversely correlated with VV DNA copy number in human skin explants incubated with VV *in vitro* (partial correlation $r = -0.256$; $P = .009$). Gene profiling revealed that the antiviral genes, double-stranded RNA-dependent protein kinase (PKR) and 2'5'-oligoadenylate synthetase 2 (OAS2), were significantly downregulated in Sp1-silenced keratinocytes. Gene expression of PKR and OAS2 was also significantly decreased in skin biopsies from ADEH⁺ subjects compared with patients with atopic dermatitis without a history of eczema herpeticum and nonatopic subjects. IFN- γ augmented the antiviral capacity of Sp1-silenced keratinocytes.

Conclusion: Specificity protein 1 knockdown enhances viral replication in keratinocytes by downregulating gene expression of PKR and OAS2. Sp1 deficiency in ADEH⁺ patients may

contribute to their increased propensity to disseminated skin viral infections. IFN- γ augmentation may be a potential treatment for ADEH⁺ patients. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Specificity protein 1, vaccinia virus, herpes simplex virus 1, double-stranded RNA-dependent protein kinase, 2'5'-oligoadenylate synthetase 2, atopic dermatitis, eczema herpeticum, IFN- γ

Atopic dermatitis (AD) is the most common chronic skin disease worldwide, affecting up to 30% of children in certain countries.^{1,2} A small subset of patients with AD have disseminated viral skin infections—that is, eczema herpeticum (EH) after herpes simplex virus infection³ or eczema vaccinatum (EV) after smallpox vaccination with vaccinia virus (VV) vaccine.⁴ As a result, patients with AD are generally excluded from smallpox vaccination. This is a major impediment to mass vaccination with the conventional smallpox vaccine.⁵⁻⁷ To address this major health care problem, the National Institutes of Health/National Institute of Allergy and Infectious Diseases funded the Atopic Dermatitis Vaccinia Network to identify mechanisms that predispose this subset of patients with AD to disseminated viral infections.

Specificity protein (Sp)-1 is a cellular transcription factor involved in diverse cellular functions.⁸⁻¹⁰ The recognition elements of Sp1 are frequently found in the promoters of various viral genes, and several lines of evidence have suggested the contribution of Sp1 in transactivation of viral genes.¹¹⁻¹⁴ Although no report has identified Sp1 recognition elements in the genome of VV, Sp1 has been reported to be co-localized with the DNA replication machinery of VV in host cell cytoplasm.¹⁵

To study further the relationship of Sp1 to VV replication, our current experiments examined the effect of Sp1 gene silencing on VV replication in normal human keratinocytes (NHKs). To our surprise, we found that Sp1 gene knockdown enhanced VV replication, and Sp1 gene expression was downregulated in the skin of patients with AD and a history of eczema herpeticum (ADEH⁺). We therefore explored, in further detail, the role of Sp1 in the skin's host response against VV. Our findings indicate that enhanced VV replication in Sp1-silenced keratinocytes is associated with attenuation of the double-stranded RNA-dependent protein kinase (PKR)/eukaryotic initiation factor 2 α (eIF2 α) signal pathway and decreased gene expression of 2'5'-oligoadenylate synthetase (OAS)-2. Our current findings suggest that Sp1 deficiency predisposes a subset of patients with AD to disseminated viral skin infections.

METHODS

Subjects

Human subjects included 31 nonatopic healthy individuals, 55 patients with AD without a history of eczema herpeticum (ADEH⁻), and 20 ADEH⁺ subjects.

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Abbreviations used

AD:	Atopic dermatitis
ADEH ⁻ :	Atopic dermatitis without a history of eczema herpeticum
ADEH ⁺ :	Atopic dermatitis and a history of eczema herpeticum
EH:	Eczema herpeticum
EV:	Eczema vaccinatum
eIF2 α :	Eukaryotic initiation factor 2 α
GO:	Gene ontology
HSV-1:	Herpes simplex virus 1
KLK:	Kallikrein
MFI:	Mean fluorescence intensity
MOI:	Multiplicity of infection
NHK:	Normal human keratinocyte
OAS:	2'5'-Oligoadenylate synthetase
PKR:	Double-stranded RNA-dependent protein kinase
siRNA:	Small interfering RNA
Sp:	Specificity protein
VV:	Vaccinia virus

The detailed demographics information of human subjects is included in this article's [Table E1](#) in the Online Repository at www.jacionline.org. The institutional review board at National Jewish Health approved this study. All subjects provided written informed consent to participate in the study.

Punch biopsies (2 mm) were collected from the uninvolved skin and immediately placed in a 96-well plate with RPMI 1640 (Cellgro, Herndon, Va) supplemented with 10% FCS (Gemini Bio Products, Calabasas, Calif) and penicillin (100 U/mL)/streptomycin (100 μ g/mL). Biopsies were cultured in the presence of medium alone or 2×10^5 plaque-forming unit vaccinia virus for 24 hours. After the exposure period, medium was removed and biopsies were submerged in 10% buffered formalin for immunohistochemical staining or Tri-Reagent (Molecular Research Center Inc, Cincinnati, Ohio) for RNA and DNA isolation.

Cells and virus source

Normal human keratinocytes were purchased from Cascade Biologics (Portland, Ore) and maintained in serum-free EpiLife Medium containing 0.06 mM CaCl₂ (Cascade Biologics). The Wyeth/ACAM2000 strain of VV was obtained from the Centers for Disease Control and Prevention (Atlanta, Ga). Herpes simplex virus 1 (HSV-1; VR-733) was purchased from American Type Culture Collection (Manassas, Va).

Small interfering RNAs silencing experiment and virus infection

Specificity protein 1 and silencer negative control 1 Small interfering RNA (siRNA) duplexes were purchased from Ambion (Austin, Tex). Sequences for targeting *Sp1* were as follows: *Sp1* #1 siRNA: 5'-GCAACAUGGGAAUUAU GAA-3'; *Sp1* #2 siRNA: 5'-GGCAGACCUUACAACUCA-3'; *Sp1* #3 siRNA: 5'-CCACAAGCCCAACAAUCA-3'. PKR and OAS2 siRNA duplexes were purchased from Dharmacon (Lafayette, Colo). Sequences for targeting OAS2 were as follows: 5'-AGAGGCAACUCCGAUGGUA-3'; 5'-AAGAGAAGCCAACGUGACA-3'; 5'-GGGAUA GCUGAAGUUCUG-3'; 5'-GUUGGUUAUCCAGGAUA-3'. Sequences for targeting PKR were as follows: 5'-GUAAGGGAACUUUGCGAUA-3'; 5'-GCGA GAAACUAGACAAAGU-3'; 5'-CGACCUAACAAUCUGAAA-3'; 5'-CC ACAUGAUAGGAGGUUA-3'.

NHK cells were plated in 24-well plates at 1×10^5 per well the day before transfection. Cells were transfected with 5 pmol siRNA duplexes per well by using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif). To knock down *Sp1* at different levels, 0.05 pmol, 0.5 pmol, and 5 pmol of *Sp1* #1 siRNA duplexes per well were transfected into NHK cells. Forty-eight hours after transfection, cells were infected with VV at various multiplicities of infection (MOIs) from 0.01 to 0.1 and

HSV-1 at MOI 0.05. We also used different concentrations of PKR and OAS2 siRNA duplexes to knock down PKR and OAS2 at different levels.

Plaque assays

To assess the production of infectious viral particles, cells and culture supernatants were harvested together after 24 hours of VV incubation. Cells were disrupted by 3 freeze-thaw cycles. Viral yields were determined by titration plaque assay as previously described.¹⁶

RNA isolation and PCR analysis

Total RNA was isolated from skin biopsies and cells by using Tri-Reagent (Molecular Research Center Inc, Cincinnati, Ohio) and RNeasy Mini Kits (Qiagen, Valencia, Calif) according to the manufacturer's guidelines. DNA was extracted from skin biopsies after RNA extraction according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA and analyzed by real-time RT-PCR using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, Calif) as previously described.¹⁷ Primers and probes for human *Sp1*, *OAS1*, *OAS2*, *OAS3*, *PKR*, and 18s ribosomal RNA were purchased from Applied Biosystems. The primer sequences for VV transcripts were prepared as previously described.¹⁸ The primer sequences for HSV-1 transcripts were prepared as previously described.¹⁹ Quantities of all target genes in test samples were normalized to the corresponding 18s ribosomal RNA levels.

Western blot analysis

Whole-cell extracts were prepared in the presence of 1% (vol/vol) of protease inhibitor cocktail and 1% (vol/vol) of phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, Mo). The cell nuclear compartment was extracted from cells by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, Ill) according to the manufacturer's instructions. Protein was then separated using SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, Calif). The blots were then blocked and incubated with primary and secondary antibodies. Rabbit antihuman *Sp1* and rabbit antihuman eIF2 α were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Monoclonal anti- β -actin (Sigma Chemical Co, St Louis, Mo) and monoclonal antihuman TATA box binding protein (Abcam Inc, Cambridge, Mass) were used as loading controls for whole-cell lysates and nuclear protein, respectively. Rabbit anti-phospho-eIF2 α mAb was purchased from Epitomics Inc (Burlingame, Calif). Blots were developed with ECL Detection Reagents (GE Healthcare Bio-Sciences, Piscataway, NJ). We analyzed the density of protein bands by using software Alpha Imager 2200 (Cell Biosciences, Inc, Santa Clara, Calif).

Immunofluorescence staining for human *Sp1*

Immunofluorescence staining for human *Sp1* with paraffin-embedded tissues followed the conventional staining protocol as previously described.²⁰ Paraffin-embedded tissues were cut at 5- μ m thickness on frosted microscope slides. By using toluene and a series of ethanol washes, the slides were deparaffinized and then rehydrated. Immunofluorescence staining was visualized with confocal microscopy (Leica, Wetzlar, Germany). Images were collected at $\times 630$, and levels of mean fluorescence intensity (MFI) were measured with Slidebook 4.1 (Intelligent Imaging Innovations, Denver, Colo). The MFI was determined for each exposure group and was reported as mean MFI $\pm$ SEM.

Statistical analysis

All statistical analysis was conducted by using Graph Pad prism, version 4.03 (San Diego, Calif) or SAS version 9.13 or greater (Cary, NC). Comparisons of expression levels were performed by using ANOVA techniques and independent sample *t* tests as appropriate. Log₁₀ transformations were applied when necessary to satisfy statistical assumptions. Pearson correlations and partial correlations were computed by using log₁₀-transformed values for *Sp1* mRNA expression and VV copies. Partial correlations were computed to describe the association between VV copies and *Sp1* mRNA expression

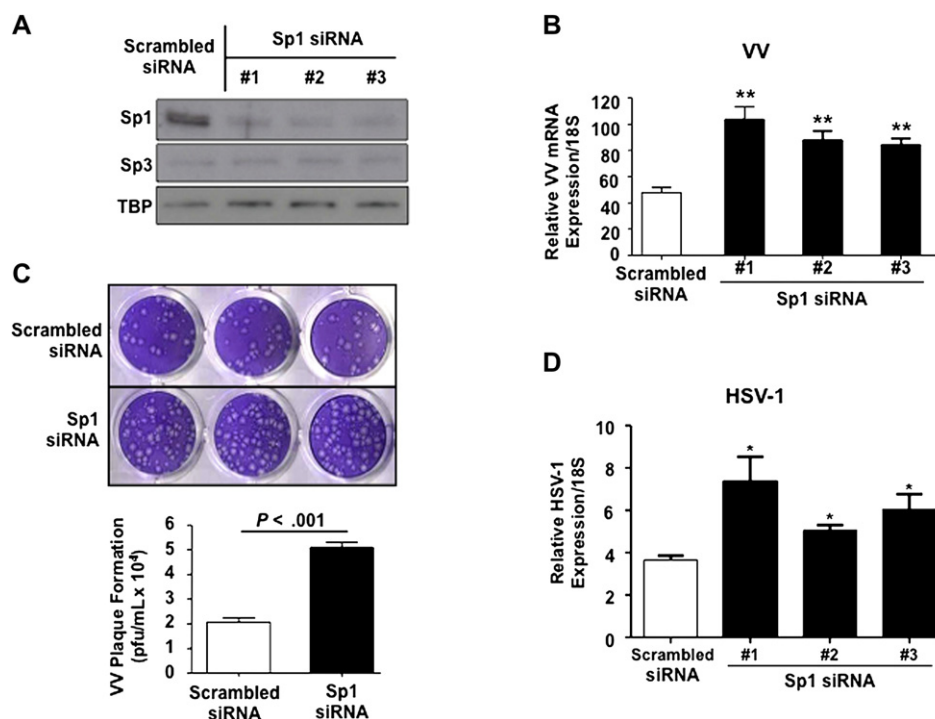


FIG 1. Inhibition of Sp1 enhances viral replication in NHK cells. **A**, Western blot detection of Sp1 and Sp3 protein expression in NHK cells after transfection with 3 independent siRNA duplexes targeting the *Sp1* gene for 48 hours. TATA box binding protein (TBP) is used as a loading control for nuclear protein. **B**, Relative VV mRNA expression was evaluated by real-time quantitative PCR assay. NHK cells were transfected with 3 different Sp1 siRNA duplexes and scrambled siRNA for 48 hours and then inoculated with VV (MOI, 0.1) for 24 hours. **C**, Twenty-four-hour viral yield of VV was evaluated by a plaque assay. **D**, Relative HSV-1 mRNA expression was evaluated by real-time quantitative PCR assay. NHK cells were transfected with 3 different Sp1 siRNA duplexes and scrambled siRNA for 48 hours and then inoculated with HSV-1 (MOI, 0.05) for an additional 24 hours. All data are presented as mean $\pm$ SEM; * P < .05 and ** P < .01 compared with scrambled siRNA. The data shown here are representative of 3 or 4 experiments.

while adjusting for the influence of diagnostic groups. Partial correlations were computed with multiple linear regression models by using the SAS REG procedure (Cary, NC). Dose-response inferences were made by using linear regression models with $\log_{10}$ of dose (either *Sp1* siRNA or IFN- γ doses) as the independent variable. Differences were considered significant at P < .05.

RESULTS

Gene silencing of Sp1 enhances VV replication in keratinocytes

To investigate the importance of Sp1 in skin keratinocyte responses against VV, we inhibited Sp1 expression in cultured NHK cells using an siRNA technique. Sp1 protein expression was significantly inhibited after transfection with 3 different specific siRNA duplexes targeting 3 independent mRNA sequences of Sp1 (Fig 1, A). Expression of Sp3, a protein from the same family of transcription factors as Sp1,⁹ did not change under the same gene silencing conditions, suggesting that the knockdown of Sp1 was specific. We did not find any morphologic changes of NHK cells after *Sp1* silencing. By using a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay to determine cell proliferation, we found that the cell growth of *Sp1*-silenced NHK cells did not change significantly compared with scrambled siRNA-silenced NHK cells (data not shown). After gene silencing, we inoculated the cells with VV at an MOI of 0.1 and incubated for 24 hours. The infected cells were then harvested for RNA

isolation and reverse-transcribed into cDNA. VV replication was evaluated by real-time quantitative PCR. We found that VV replication was significantly enhanced in NHK cells silenced by 3 independent *Sp1* siRNA duplexes compared with cells transfected with scrambled siRNA control duplexes (Fig 1, B; P < .01), indicating a specific effect of *Sp1* silencing and not an off-target effect. We used a functional approach to evaluate further whether *Sp1*-silenced NHK cells support increased production of infectious VV particles by using a viral plaque assay. As shown in Fig 1, C, after 24 hours, the yield of infectious virus was significantly increased in *Sp1*-silenced NHK cells compared with cells transfected with scrambled siRNA duplexes. We also inoculated *Sp1* and scrambled siRNA-silenced NHK cells with various MOIs, and we found that the enhancement of VV replication in *Sp1*-silenced NHK cells was consistent with different MOIs (data not shown). Extending the observation to another virus, we found that *Sp1* silencing also led to significantly increased HSV-1 replication in keratinocytes compared with transfection with scrambled siRNA (Fig 1, D). These data demonstrate that Sp1 plays a critical role in antiviral cellular responses.

Sp1 gene expression is significantly decreased in ADEH⁺ patients

Next, we determined the potential clinical relevance of our *in vitro* observation that inhibiting Sp1 gene expression enhances

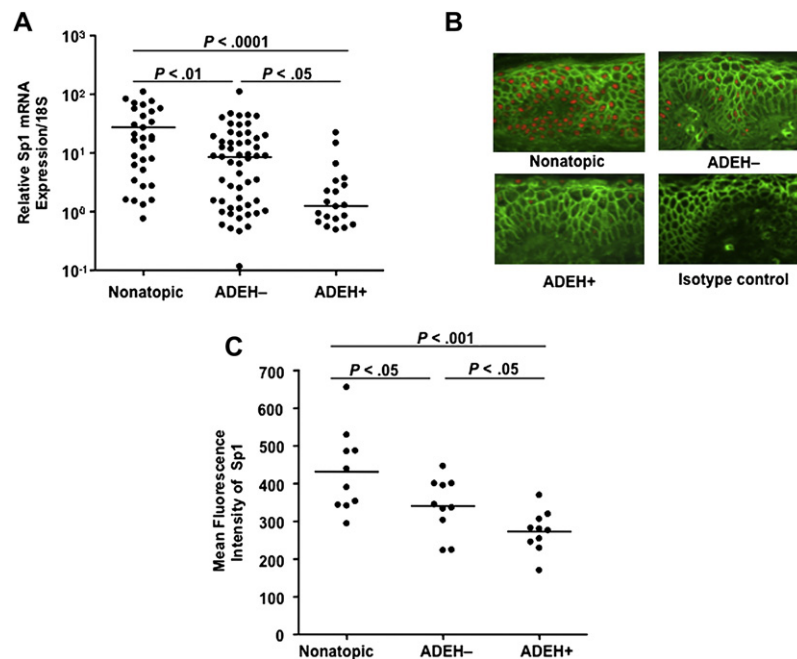


FIG 2. Sp1 gene expression is decreased in skin biopsies from ADEH⁺ patients. **A**, Relative Sp1 mRNA expression in skin biopsies from nonatopic subjects ($n = 31$), ADEH⁻ subjects ($n = 55$), and ADEH⁺ subjects ($n = 20$) evaluated by real-time quantitative PCR. **B**, Representative fluorescence images of Sp1 in skin biopsies from nonatopic, ADEH⁻, and ADEH⁺ subjects. Sp1 (red) presents as nuclear staining, and wheat germ agglutinin-conjugated fluorescein isothiocyanate (green) stains the cytoskeleton. Magnification $\times 630$. **C**, MFI of Sp1 in skin biopsies from nonatopic, ADEH⁻, and ADEH⁺ subjects. 18S, 18s ribosomal RNA.

VV replication in keratinocytes. Eczema herpeticum has been reported to resemble EV clinically,²¹ suggesting that ADEH⁺ patients would be good clinical models for mechanistic investigation of both EV and EH. We therefore examined gene expression of Sp1 in skin biopsies from ADEH⁺ subjects, comparing them with ADEH⁻ and nonatopic healthy subjects. As shown in Fig 2, A, Sp1 gene expression was decreased in skin biopsies from ADEH⁻ subjects (mean, 13.58 ± 2.50 ; $n = 55$) compared with nonatopic subjects (mean, 27.48 ± 5.26 ; $n = 31$; $P < .01$). However, Sp1 expression was significantly more decreased in ADEH⁺ (mean, 3.39 ± 1.23 ; $n = 20$; $P < .0001$) compared with nonatopic subjects. Most importantly, Sp1 gene expression was significantly lower in ADEH⁺ skin compared with ADEH⁻ skin ($P < .05$).

To determine which cell types in the skin express reduced Sp1 protein levels, we performed immunofluorescence staining of skin explants from 10 subjects in each group. Sp1 staining intensity was significantly decreased in the keratinocytes of the epidermis from ADEH⁺ subjects compared with keratinocytes of ADEH⁻ (Fig 2, B and C; $P < .05$) and nonatopic subjects (Fig 2, C; $P < .001$).

VV replication is inversely correlated with Sp1 expression level

Although Sp1 gene expression was reduced in both ADEH⁻ and ADEH⁺ patients compared with nonatopic subjects, the reduction of Sp1 gene expression was significantly greater in ADEH⁺ patients compared with ADEH⁻ patients. This suggested that the level of Sp1 expression might determine the ability of keratinocytes to control VV replication. We therefore inhibited Sp1 gene expression to various levels in NHK cells by

transfection with various concentrations of siRNA duplexes. Cells were then inoculated with VV at a MOI of 0.01 for 24 hours. We found that Sp1 protein levels kept reducing in NHK cells after transfection with increasing concentrations of Sp1 siRNA duplexes (Fig 3, A, upper panel), and VV replication in NHK cells increased in a dose-dependent manner with increasing concentrations of transfected Sp1 siRNA duplexes (Fig 3, A, lower panel; $P < .01$ from linear model relating $\log_{10}$ VV expression to $\log_{10}$ Sp1 siRNA dose).

We then investigated whether Sp1 gene expression level correlated with viral replication in skin explants from human subjects. Skin explants from ADEH⁺, ADEH⁻, and nonatopic healthy subjects were incubated with VV for 24 hours; RNA and DNA were then isolated from the skin explants for evaluation of Sp1 gene expression and VV viral copies, respectively. As shown in Fig 3, B, the number of VV viral copies in skin explants was inversely correlated with Sp1 gene expression levels (subject group adjusted; partial correlation $r = -0.256$; $P = .009$).

PKR/eIF2 α antiviral pathway is impaired in Sp1-silenced NHK and ADEH⁺ skin

We searched for mechanisms that could lead to enhanced viral replication in Sp1-silenced NHK. The mechanism of the PKR/eIF2 α pathway in host antiviral responses is well known to act by inhibition of viral translation.²²⁻²⁴ A previous study has shown that Sp1 binds to the gene promoter of PKR, and overexpression of Sp1 in mammalian cells induces activation of the PKR promoter.²⁵ Thus, we further investigated gene expression and protein phosphorylation of gene components in this signaling

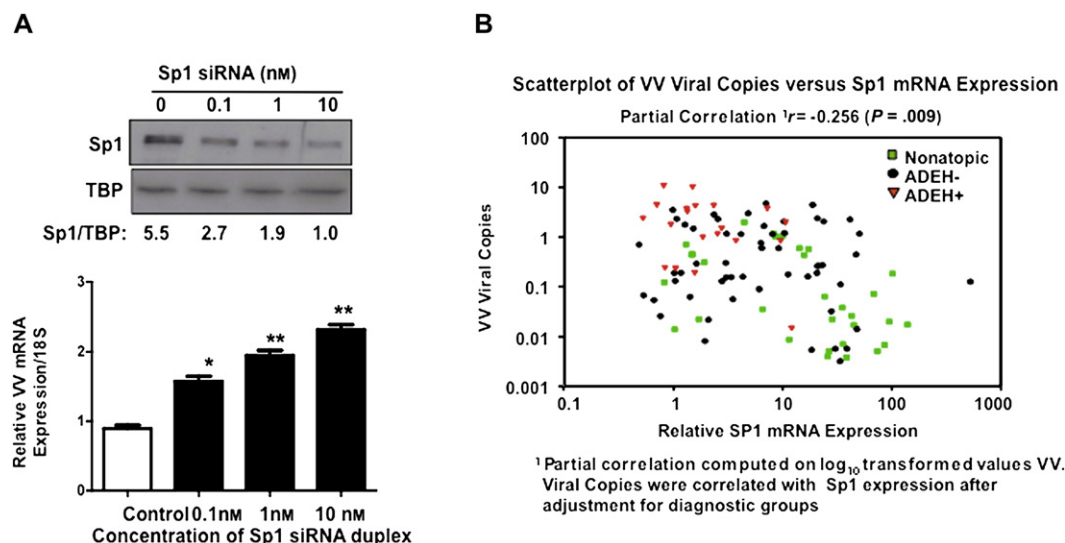


FIG 3. Sp1 expression level is inversely correlated with VV replication. **A**, Upper panel, Western blot detection of Sp1 expression in NHK cells after transfection with different concentrations of Sp1 siRNA duplexes for 48 hours. The concentration of Sp1 siRNA duplexes is indicated above each lane. Lower panel, Relative VV mRNA expression was evaluated with different levels of Sp1 silencing in NHK cells with VV inoculation (MOI, 0.01) for 24 hours (corresponding to upper panel) by real-time quantitative PCR. All data are presented as mean $\pm$ SEM; * $P < .05$ and ** $P < .01$ compared with scrambled siRNA control. **B**, Sp1 mRNA expression was inversely correlated with VV viral copies in skin explants (subject group adjusted; partial correlation $r = -0.256$; $P = .009$). 18S, 18s ribosomal RNA; TBP, TATA box binding protein.

pathway. We found that PKR was significantly downregulated in Sp1-silenced NHK cells compared with cells transfected with scrambled siRNA duplexes (Fig 4, A; $P < .01$).

After viral infection, PKR was activated by a viral replication intermediate of double-stranded RNA. Activated PKR then catalyzes the phosphorylation of eIF2 α . Phosphorylation of eIF2 α increases its affinity for guanine nucleotide exchange factor eukaryotic initiation factor 2B, blocking the ability of eukaryotic initiation factor 2B to catalyze nucleotide exchange, and subsequently inhibiting protein synthesis.²⁶ We therefore determined the protein expression and phosphorylation of eIF2 α in Sp1-silenced keratinocytes. We found that eIF2 α was constitutively phosphorylated in NHK cells (Fig 4, B). Protein phosphorylation of eIF2 α was slightly enhanced after VV inoculation of NHK cells transfected with scrambled siRNA duplexes 2 hours postinfection and persisted to 8 hours postinfection. However, eIF2 α phosphorylation was decreased in Sp1-silenced NHK cells during this same time course of VV infection compared with scrambled siRNA-silenced control cells. These data demonstrate that the PKR/eIF2 α signaling pathway is impaired in Sp1-silenced NHK cells.

We knocked down PKR in NHK cells to a similar expression level as in Sp1-silenced NHK cells. We then inoculated VV (MOI, 0.01) to those cells, and VV replication was evaluated. As shown in Fig 4, C, VV replication is significantly enhanced in PKR-silenced NHK cells, but the enhancement is not as great as in Sp1-silenced NHK cells, suggesting that the decrease in PKR expression is only a part of the mechanism resulting in enhanced VV replication in Sp1-silenced keratinocytes, and that other mechanisms may contribute to the viral replication enhancement as well.

Because Sp1 is significantly lower in skin biopsies from ADEH⁺ patients, we searched for evidence of attenuation of

the PKR/eIF2 α signal pathway in human subjects. Indeed, we found that PKR gene expression was also significantly decreased in the skin biopsies from ADEH⁺ patients compared with ADEH⁻ patients (Fig 4, D; $P < .001$) and nonatopic subjects (Fig 4, D; $P < .0001$). These data support the concept that the PKR/eIF2 α signal pathway is functionally decreased in the skin of ADEH⁺ patients with Sp1 deficiency.

OAS2 is significantly decreased in Sp1-silenced keratinocyte and ADEH⁺ skin

To search for additional mechanisms independent of PKR, which might account for increased propensity to disseminated viral skin infections, we used a gene microarray technique to identify candidate genes associated with impaired antiviral responses in Sp1-silenced NHK. We searched for genes that had greater than a 1.5-fold change in expression and also belonged to the gene ontology (GO) group designated "immune system process" (GO accession number, 0002376). Out of approximately 972 genes, 143 probes representing 67 genes were identified to have a greater than 1.5-fold change of gene expression in Sp1-silenced keratinocytes compared with cells transfected with scrambled siRNA (see this article's Fig E1 in the Online Repository at www.jacionline.org). Among these genes, we found that OAS2, a member of the OAS family involved in host innate immune responses, had a 2-fold reduction in gene expression.²⁷⁻³⁰ The effect of OAS2 on VV replication has not been previously reported. We therefore further explored the role of OAS2 in controlling VV replication.

In 3 independent experiments, we examined OAS2 gene expression levels in Sp1-silenced keratinocytes by using NHK cells from 3 different donors. We confirmed that OAS2 gene expression was significantly downregulated in Sp1-silenced

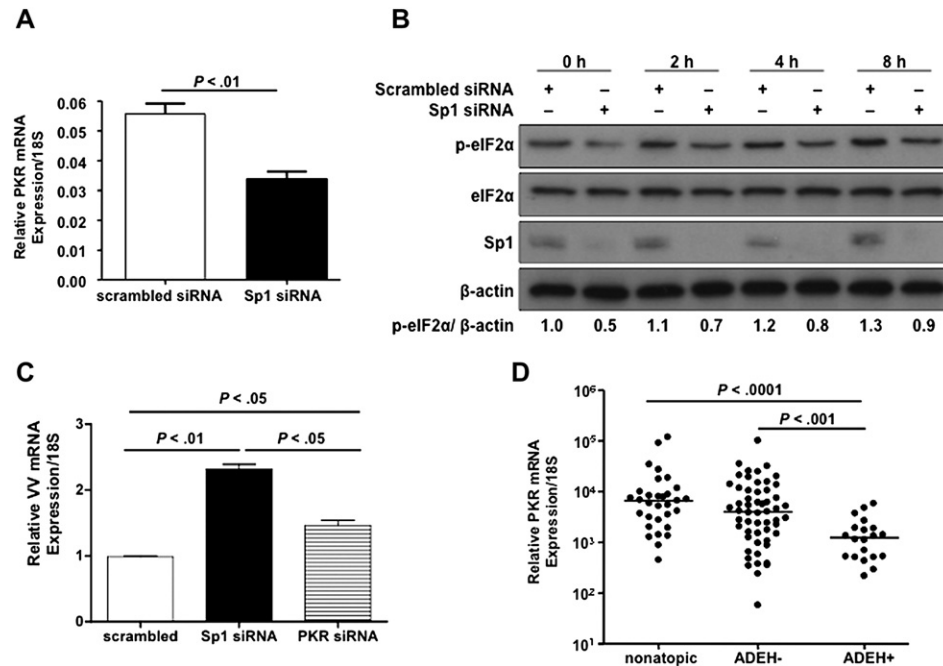


FIG 4. *PKR/elf2 α* pathway is impaired in *Sp1*-deficient NHK cells and skin of *ADEH*⁺ patients. **A**, Relative *PKR* mRNA expression in *Sp1*-silenced NHK cells evaluated by real-time quantitative PCR. Data are presented as mean $\pm$ SEM, representative 1 of 3 independent experiments. **B**, Western blot detection of phosphorylation of *elf2 α* (p-*elf2 α*), total *elf2 α* , *Sp1*, and β -actin in *Sp1*-silenced NHK cells and cells transfected with scrambled siRNA duplexes for 48 hours and then treated with VV (MOI, 0.1) and harvested at various time points. Time after treatment is indicated above each pair of lanes. **C**, VV mRNA expression in *Sp1* and *PKR*-silenced NHK cells compared with cells transfected with scrambled siRNA as evaluated by real-time PCR. **D**, Relative *PKR* mRNA expression in skin explants from *ADEH*⁺ subjects ($n = 20$), *ADEH*⁻ subjects ($n = 55$), and nonatopic subjects ($n = 31$). Data are presented as mean $\pm$ SEM.

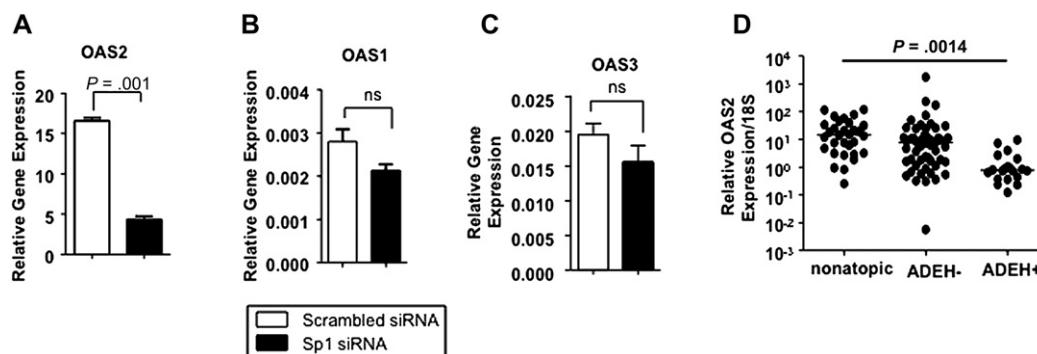


FIG 5. *OAS2* is decreased in *Sp1*-silenced keratinocytes and *ADEH*⁺ patients. **A-C**, Real-time PCR results show that *OAS2* is significantly downregulated, but *OAS1* and *OAS3* did not change significantly in *Sp1*-silenced NHK. Data are presented as mean $\pm$ SEM. **D**, *OAS2* gene expression is significantly downregulated in skin biopsies from *ADEH*⁺ subjects compared with *ADEH*⁻ and nonatopic subjects. ns, No significance.

keratinocytes, but not *OAS1* or *OAS3* (Fig 5, A-C). These results suggested that *Sp1* silencing regulated the gene expression of *OAS2*, but did not affect the other 2 *OAS* family members, although they are all closely located as a gene cluster.

To confirm the biological relevance of *OAS2* deficiency, we investigated *OAS2* gene expression in skin biopsies from *ADEH*⁻, *ADEH*⁺, and nonatopic subjects by real-time PCR. We found that *OAS2* gene expression is indeed significantly downregulated in the skin of patients with *ADEH*⁺ compared with *ADEH*⁻ and nonatopic subjects (Fig 5, D).

***OAS2* is a part of mechanism resulting in enhanced VV replication in *Sp1*-silenced keratinocytes**

To demonstrate the functional significance of *OAS2* deficiency, we also selectively silenced *OAS2* gene expression in NHK cells, then inoculated them with VV at an MOI of 0.01 for 24 hours. We found that VV replication was indeed enhanced in *OAS2*-silenced NHK cells compared with cells transfected with scrambled siRNA. However, enhancement of VV replication in *OAS2*-silenced NHK cells was not as great as in *Sp1*-silenced cells, although the *OAS2* expression level was lower in *OAS2*-silenced

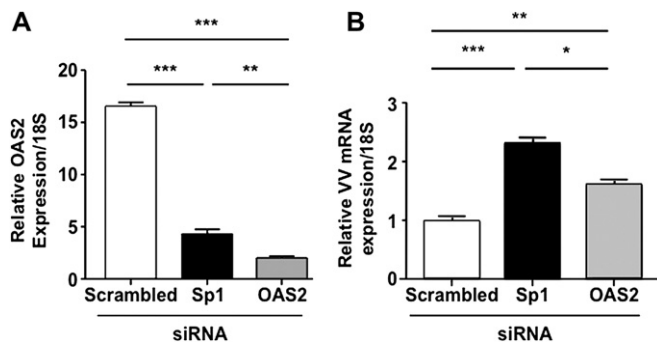


FIG 6. OAS2 reduction is part of the mechanism resulting in enhanced VV replication in Sp1-silenced keratinocytes. **A**, OAS2 gene expression in Sp1 and OAS2-silenced NHK cells compared with cells transfected with scrambled siRNA as evaluated by real-time PCR. **B**, VV mRNA expression in Sp1 and OAS2-silenced NHK cells compared with cells transfected with scrambled siRNA as evaluated by real-time PCR. All data are presented as mean $\pm$ SEM; * $P < .05$; ** $P < .01$; *** $P < .001$. The data shown are representative of 3 experiments.

cells than Sp1-silenced cells ($P < .01$; Fig 6, A and B). These results suggest that downregulation of OAS2 is a part of the mechanism involved in the enhancement of VV replication in Sp1-deficient cells other than downregulation of PKR.

IFN- γ treatment significantly augments the antiviral capacity of Sp1-silenced keratinocytes

Because IFN- γ treatment can induce OAS2 gene expression and enhance phosphorylation of eIF2 α protein, we tested whether IFN- γ treatment could bypass the effect of Sp1 deficiency in keratinocytes. We treated Sp1-silenced NHK and scrambled siRNA-transfected cells with different concentrations of IFN- γ for 8 hours, then inoculated the cells with VV at MOI of 0.01 for 24 hours. We found that OAS2 was significantly induced by IFN- γ in both Sp1-silenced NHK cells and control cells transfected with scrambled siRNA in a dose-dependent manner (from linear models relating $\log_{10}$ of relative OAS2 expression to $\log_{10}$ dose of IFN- γ , for both cell types $P < .01$). The induction of OAS2, however, in Sp1-deficient NHK was not as great as in cells transfected with scrambled siRNA (Fig 7, A). This result suggests that Sp1 only regulates the constitutive gene expression of OAS2 but does not affect its induction by IFN- γ . We also evaluated protein phosphorylation of eIF2 α in Sp1-silenced NHK cells and control cells transfected with scrambled siRNA after IFN- γ treatment. We found that IFN- γ treatment enhanced the phosphorylation level of eIF2 α protein in both Sp1-silenced NHK and scrambled siRNA-transfected control cells; however, the eIF2 α protein phosphorylation level was lower in Sp1-silenced keratinocytes than cells transfected with scrambled siRNA under both stimulated and unstimulated conditions (Fig 7, B). Accordingly, VV replication evaluated by real-time PCR was significantly inhibited in IFN- γ -treated Sp1-deficient NHK and control cells transfected with scrambled siRNA in a dose-dependent manner (Fig 7, C; $P < .01$ from linear models relating $\log_{10}$ relative VV expression to $\log_{10}$ IFN- γ dose). However, the inhibition of VV replication in Sp1-deficient cells was not as great as in cells transfected with scrambled siRNA (Fig 7, C). For example, when treated with 1 ng/mL IFN- γ , VV replication decreased almost 90-fold in scrambled siRNA-silenced NHK cells, whereas there was only about 8.5-fold reduction of VV

replication in Sp1-silenced NHK cells (Fig 7, C). Nevertheless, IFN- γ treatment did significantly augment the antiviral response of Sp1-deficient cells.

DISCUSSION

Because Sp1-binding sites are frequently found in various viral gene promoters, the potential role of Sp1 in promoting virulence has been suggested by numerous studies using modified virus with deletion of Sp1-binding sites of certain viral genes. To the best of our knowledge, the current study is the first report that demonstrates, from the perspective of immune host defense, that Sp1 is critical to antiviral responses in skin keratinocytes. Furthermore, this report is the first to demonstrate that OAS2 is a target gene of the Sp1 transcription factor, and downregulation of OAS2 is part of the mechanism that results in enhanced viral replication in Sp1-deficient keratinocytes. Sp1 target genes encompass many key players involved in cell growth, angiogenesis, and genomic stability. Indeed, overexpression of Sp1 has been found in several cancers, and its expression level correlates with tumor grade/stage and poor prognosis.³¹⁻³⁵ Aside from overexpression in patients with various types of cancer, promoter mutations in Sp1 target genes have been implicated in various human diseases.^{36,37} The current study, however, is the first to identify a human disease, ADEH, associated with decreased gene expression of Sp1. The *in vivo* relevance of Sp1 deficiency is supported by our observation that downstream effects of Sp1 (ie, activation of OAS2 and PKR) are both affected in the skin of patients with ADEH⁺.

To understand better the impact of Sp1 expression deficiency in keratinocytes, we conducted a gene array assay to study the gene expression profiling of Sp1-silenced NHK cells compared with scrambled siRNA-silenced NHK cells. Aside from looking for dysregulated genes associated with the immune process, we also searched for genes that were dysregulated after Sp1 silencing and involved in skin barrier integrity because ADEH⁺ patients have skin barrier defects. We found that multiple genes from the kallikrein (KLK) family, including KLK6, KLK12, KLK10, KLK8, KLK5, and KLK7, were upregulated after Sp1 silencing in NHK cells. Kallikrein genes encode proteins with serine protease activities, which are important for the desquamation process in epidermis.³⁸ Elevated KLK5 activity was found to induce AD in a mouse model of Netherton syndrome.³⁹ Three keratins, KRT13, KRT19, and KRT15, are significantly downregulated after Sp1 silencing by 8.2-fold, 7.5-fold, and 3.8-fold, respectively. We believe the altered expression of these genes could profoundly change skin barrier integrity. The functional study of Sp1 in skin barrier integrity is currently underway in our laboratory.

In the presence of IFN or viral infection, OAS1, OAS2, and OAS3, 3 functional genes from the human OAS gene family, can be activated to catalyze the oligomerization of ATP into 2',5'-linked oligoadenylate. The 2',5'-linked oligoadenylate binds to and activates the latent RNase, which in turn degrades viral RNA to inhibit viral replication in host cells.⁴⁰ Previous studies demonstrated that various human OAS proteins can be differentially induced in different types of cells and appear in different subcellular locations and enzymatic parameters, suggesting that these proteins might have distinct biological functions.^{27,28} Searching upstream of 2-kb DNA sequences from the starting code for the Sp1-binding site, we found 3 putative Sp1-binding

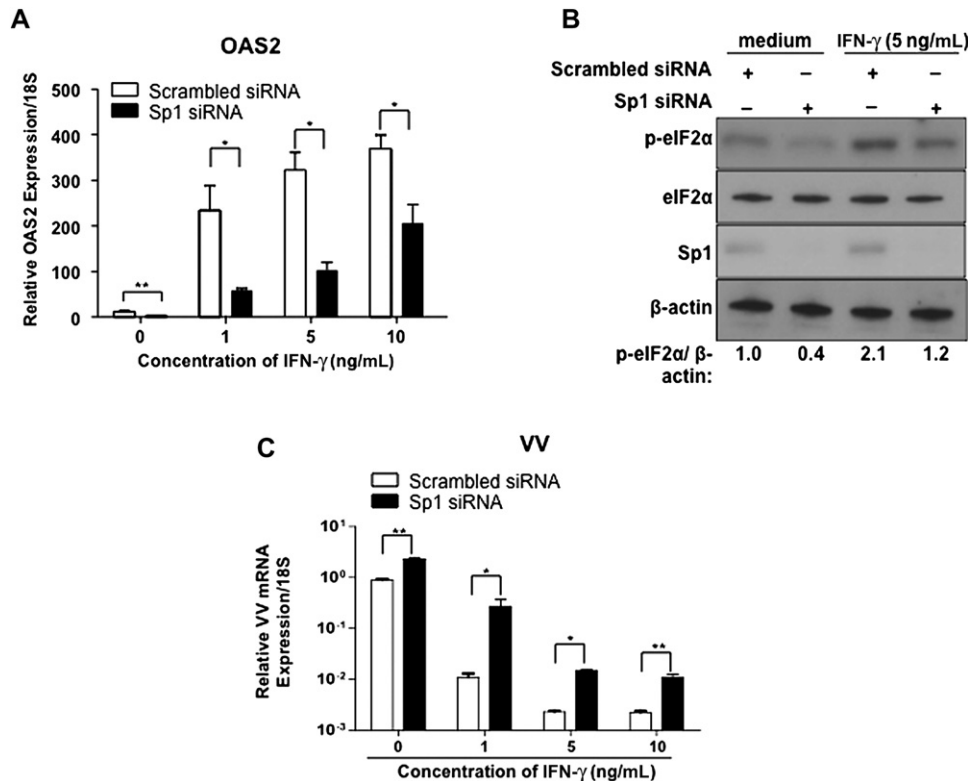


FIG 7. IFN- γ treatment significantly augments antiviral responses in Sp1-deficient keratinocytes. **A**, OAS2 gene expression in Sp1-silenced NHK and control cells transfected with scrambled siRNA in the presence of different doses of IFN- γ as evaluated by real-time PCR. **B**, Protein phosphorylation of eIF2 α in Sp1-silenced NHK and control cells transfected with scrambled siRNA in the absence and presence of IFN- γ (5 ng/mL) as evaluated by Western blot. **C**, VV mRNA expression in Sp1-silenced NHK and control cells transfected with scrambled siRNA in the presence of different doses of IFN- γ as evaluated by real-time PCR. All data are presented as mean $\pm$ SEM; * $P < .05$; ** $P < .01$. The data shown here are representative of 3 experiments. 18S, 18s ribosomal RNA.

sites in the *OAS2* gene promoter within 300 bp upstream of its starting code, suggesting that Sp1 may directly bind to *OAS2* promoter to maintain its constitutive expression. In contrast, we did not find Sp1-binding sites in the promoter of *OAS1* and *OAS3*, suggesting that Sp1 may only be involved in regulation of *OAS2*. This notion is supported by our data that the constitutive gene expression of *OAS2*, but not *OAS1* and *OAS3*, is significantly decreased in Sp1-deficient NHK cells.

Our laboratory previously demonstrated that production of the antimicrobial peptide cathelicidin was deficient in skin biopsies from patients with AD and demonstrated that this deficiency contributed to the susceptibility of patients with AD to viral infection.²⁰ Although cathelicidin-deficient mice had increased VV replication at the site of viral inoculation, they did not develop disseminated skin VV infection.¹⁸ This suggested that mechanisms other than cathelicidin deficiency were involved in the etiology of disseminated viral infections in ADEH⁺ patients. In the current study, we did not find downregulation of cathelicidin in Sp1-silenced keratinocytes (data not shown). This suggests that cathelicidin is not directly regulated by Sp1 in keratinocytes. Cathelicidin appeared to act extracellularly by damaging the viral envelope.¹⁸ This was a different but complementary mechanism to IFN-inducible genes that acted intracellularly to inhibit viral transcription and translation. Evidence collected by our laboratory and others strongly suggests that both intracellular and extracellular defects in antiviral host response are involved in

mechanisms leading to the increased propensity of viral infections in patients with ADEH⁺.

Our study demonstrated treatment with exogenous IFN- γ can significantly enhance both *OAS2* gene expression and protein phosphorylation of eIF2 α in Sp1-silenced keratinocytes. Although the enhancement is less than that in scrambled siRNA-transfected cells, this result suggests that the effect of deficiency in Sp1 can still be substantially corrected by IFN- γ treatment. Of interest, a previous study has demonstrated that IFN- γ production is deficient in ADEH⁺ patients compared with ADEH⁻ patients.⁴¹ Our study therefore suggests that the subset of patients with ADEH⁺ may benefit from IFN- γ treatment or an immune adjuvant that augments IFN- γ expression.

Taken together, we have demonstrated in this study that Sp1 silencing leads to enhanced VV and HSV-1 replication. The mechanisms resulting in this enhancement are associated with reduced gene expression of *OAS2* and an attenuated *PKR/eIF2 α* pathway in human keratinocytes. Furthermore, we report the novel finding that the Sp1, *OAS2*, and *PKR* antiviral pathways are decreased in the skin of ADEH⁺ patients, highlighting the clinical significance of the current study. Molecules identified by this study may be potential biomarkers for identification of the subpopulation of AD at risk for EV and EH. More importantly, the current report also identifies new molecular targets that may make smallpox vaccination safer for patients with AD at risk for life-threatening viral infections.

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Key messages

- Sp1 promotes host antiviral response by regulating gene expression of PKR and OAS2.
- Sp1, PKR, and OAS2 are significantly decreased in the skin of ADEH⁺ patients.
- IFN- γ treatment augments antiviral response in Sp1-deficient cells.

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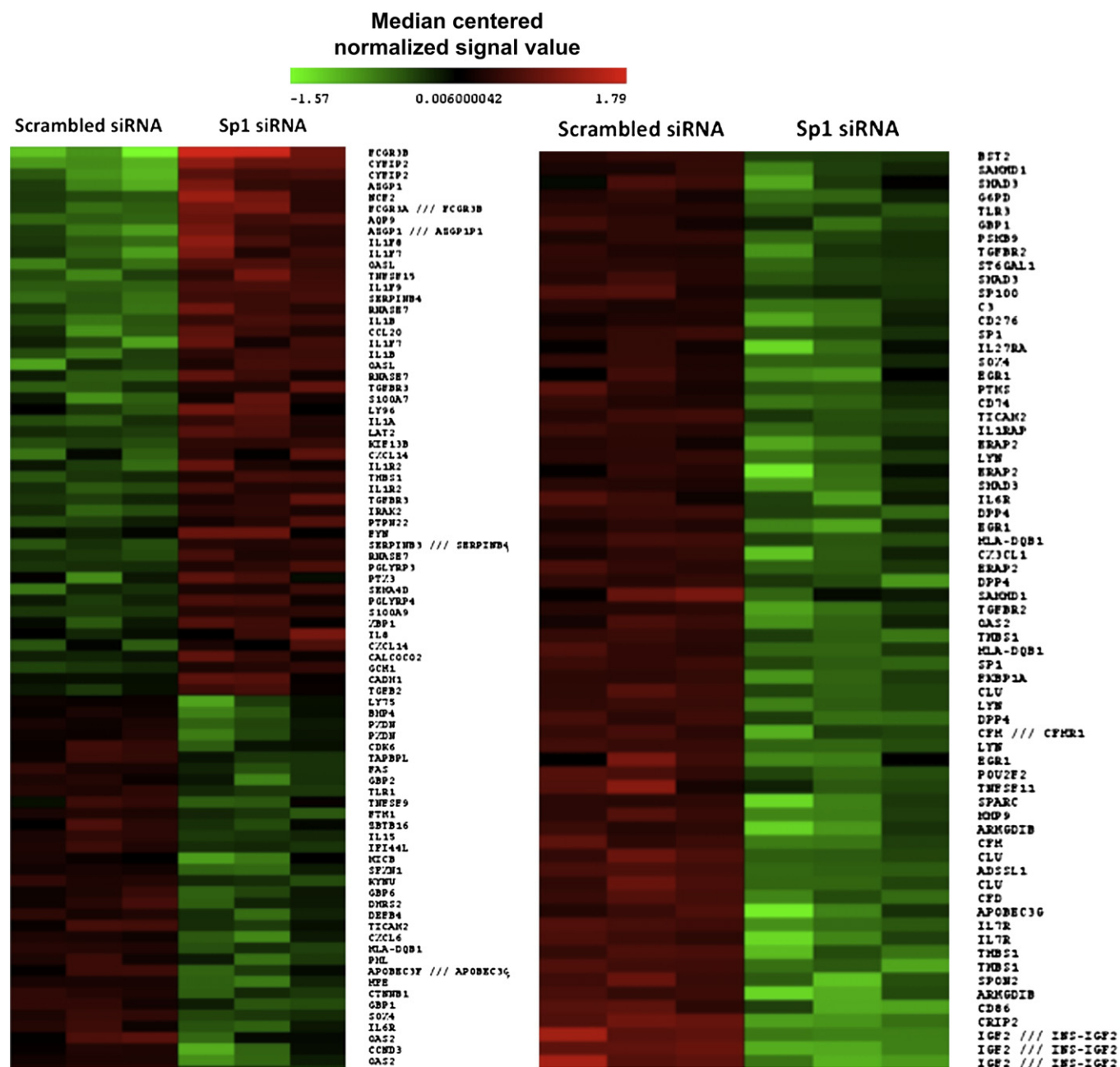


FIG E1. Heatmap presentation of genes in GO group of 0002376 that have greater than 1.5-fold change between *Sp1* siRNA and scrambled siRNA.

TABLE E1. Characteristics of human subjects

Demographic parameter	Diagnostic group		
	Normal (N = 31)	ADEH ⁻ (N = 55)	ADEH ⁺ (N = 20)
Age (y), mean (SD)	30.1 (8.9)	33.2 (12.3)	22.0 (18.9)
Female, n (%)	18 (58)	34 (62)	12 (60)
African American, n (%)	7 (23)	16 (29)	3 (15)
White, n (%)	19 (61)	33 (60)	17 (85)
Hispanic, n (%)	5 (16)	9 (16)	1 (5)
Rajka-Langeland, mean (SD)	—	6.2 (1.4)	6.9 (1.5)
Eczema area and severity index, mean (SD)	—	13.7 (12.5)	18.0 (14.3)