

Inhibition of S100A11 gene expression impairs keratinocyte response against vaccinia virus through downregulation of the IL-10 receptor 2 chain

Lianghua Bin, PhD,^a Michael D. Howell, PhD,^{a,b} Byung Eui Kim, MD,^a Clifton F. Hall, MS,^a Joanne E. Streib, BA,^a and Donald Y. M. Leung, MD, PhD^{a,b} *Denver and Aurora, Colo*

Background: The mechanism that predisposes patients with atopic dermatitis (AD) to disseminated vaccinia viral (VV) skin infection after smallpox vaccination is unknown. We have demonstrated that expression of S100A11, a calcium-binding protein involved in keratinocyte differentiation, is downregulated in AD.

Objective: We investigated whether inhibiting expression of S100A11 increased VV replication in human keratinocytes and the mechanism by which S100A11 affects the innate immune response of keratinocytes.

Methods: Small interfering RNA duplexes were used to reduce gene expression of S100A11 in keratinocytes. VV replication was evaluated by real-time PCR and viral plaque assay. VV cytopathic effect was assessed by crystal violet staining.

Affymetrix GeneChip assay was used to compare gene expression profiles. Real time PCR, Western blotting, and immunohistochemistry staining assay were used to evaluate gene expression in keratinocytes and AD skin biopsies.

Results: Keratinocytes with deficient S100A11 expression supported increased VV replication and manifested augmented VV cytopathic effects. Gene microarray analysis revealed that the IL-10 receptor 2 chain (IL-10R2), which binds IFN- λ s, was downregulated by 2.26-fold in S100A11-silenced keratinocytes. IL-10R2 expression was found to be decreased in skin biopsies from patients with acute AD (mean, 25.21 ± 5.25 ; $n = 20$) compared with skin from normal healthy subjects (mean, 137.1 ± 34.46 ; $n = 19$; $P < .01$). Furthermore, deficient S100A11 gene expression significantly impaired IL-29 (IFN- $\lambda 1$) responsiveness (2' 5'-oligoadenylate synthetase and Myxovirus [influenza virus] resistance induction) and its anti-VV effects in keratinocytes.

Conclusions: Inhibition of S100A11 gene expression impairs the ability of keratinocytes to control VV replication via downregulation of IFN- λ receptor IL-10R2. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: S100A11, atopic dermatitis, vaccinia virus, IL-10 receptor, IFN- λ , IL-29

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by overexpression of T_H2 cytokines, IL-4 and IL-13, in skin.^{1,2} Patients with AD are predisposed to *Staphylococcus aureus* infection, eczema herpeticum, and eczema vaccinatum. The increased susceptibility of these patients to recurrent bacterial and viral skin infection is thought to be related to impaired skin innate immune responses that protect the host from invading pathogens.³⁻⁵ Recent studies have demonstrated that T_H2 cytokines inhibit the skin innate immune response and allow enhanced vaccinia virus (VV) replication in keratinocytes. Treatment of atopic skin with *ex vivo* neutralizing antibodies to IL-4 and IL-13 inhibits replication of VV.⁴

Using a proteomics approach, we recently demonstrated that S100A11, a gene located in the epidermal differentiation complex gene cluster on chromosome 1q21, was downregulated by IL-4 and IL-13 in cultured human keratinocytes, and its expression was significantly suppressed in AD skin.⁶ This prompted the current study to examine the functional role of S100A11 in controlling VV replication in keratinocytes and the molecular mechanism by which this molecule modulates the skin innate immune response. In this study, we demonstrate that inhibition of S100A11 gene expression increases VV replication in keratinocytes. Moreover, we demonstrate for the first time that deficient expression of S100A11 leads to downregulation of the IL-10 receptor 2 chain (IL-10R2), required for the cellular action of the IFN- λ family known to have potent antiviral actions, and compromises IFN signaling pathways in keratinocytes.

METHODS

Subjects

Human subjects included 19 healthy individuals with no history of active or chronic disease (mean age, 29.8 ± 3.8 years) and 20 patients with acute AD (mean age, 34.7 ± 3.4 years). All human subjects' skin biopsy specimens were used for RT-PCR analysis. Twelve different skin biopsy specimens from each subject group were used for immunohistochemical staining. None of the subjects had received topical corticosteroids or calcineurin inhibitors for at least 1 week before enrollment. This study was approved by the institutional review board at National Jewish Health. All subjects provided written informed consent to participate in the study.

Two-millimeter punch biopsies were collected from the acute lesional skin of subjects with AD and healthy skin from normal subjects. Skin samples were submerged immediately in either Tri-Reagent (Molecular Research Center, Inc, Cincinnati, Ohio) or 10% buffered formalin for real-time RT-PCR and immunohistochemical studies, respectively.

From ^athe Department of Pediatrics, National Jewish Health, Denver; and ^bthe Department of Pediatrics, University of Colorado Denver, Aurora.

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Reprint requests: Donald Y. M. Leung, MD, PhD, National Jewish Health, 1400 Jackson Street, Room K926i, Denver, CO 80206. E-mail: leungd@njhealth.org.
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Abbreviations used

AD: Atopic dermatitis
 GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
 IL-10R2: IL-10 receptor 2 chain
 MOI: Multiplicity of infection
 NHK: Normal human keratinocyte
 OAS: 2' 5'-Oligoadenylate synthetase
 siRNA: Small interfering RNA
 VV: Vaccinia virus

Virus source

The Wyeth strain of VV was obtained from the Centers for Disease Control and Prevention (Atlanta, Ga). VV was propagated in Hela S3 cells (ATCC, Manassas, Va) as previously described.⁷

Keratinocyte cultures

Normal human keratinocytes (NHKs) were purchased from Cascade Biologics (Portland, Ore) and maintained in serum-free EpiLife Medium containing 0.06 mmol/L CaCl₂ as previously described.⁸ The HaCaT cell line was grown in Dulbecco modified Eagle medium (Cellgro, Herndon, Va) containing 1.5 mmol/L CaCl₂ and 10% FBS. In some experiments, cells were differentiated by increasing the concentration of CaCl₂ to 1.3 mmol/L in NHK or 10 mmol/L in HaCaT cells as previously described.⁹

Small interfering RNAs experiment

HaCaT and NHK cells were plated in 24-well plates at 60% confluence for transfection. Cells were transfected with small interfering RNA (siRNA) duplexes in low CaCl₂ media for 48 hours, then subjected to VV infection or human recombinant IL-29 (R&D Systems, Inc, Minneapolis, Minn) treatment. siRNA duplexes for S100A11 and Silencer Negative Control One were purchased from Dharmacon (Lafayette, Colo). Keratinocytes were transfected with siRNAs by using lipofectamine 2000 according to the manufacturer's guidelines (Invitrogen, Carlsbad, Calif).

Viral plaque assays

Vaccinia virus infection and replication were evaluated in BS-C-1 cells (African green monkey kidney fibroblast cells) by using a standard viral plaque assay as previously described.⁷ To assess 24-hour virions' yield, 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 for plaque assay.

RNA isolation and PCR analysis

Total RNA was isolated from skin biopsies and cells using Tri-Reagent and RNeasy Mini Kits (Qiagen, Valencia, Calif) according to the manufacturer's guidelines. 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.⁸ Primers and probes for human GAPDH, S100A11, IL-29, IL-28A, IL-10R2, and IL-28R1 were purchased from Applied Biosystems. The primer sequences for VV transcripts were prepared as previously described.⁷ Quantities of all target genes in test samples were normalized to the corresponding GAPDH levels.

Gene-microarray analysis

Total RNA was isolated from HaCaT cells that were transfected with either S100A11 siRNA or scrambled siRNA duplexes for 48 hours. A total of 5 µg of RNA from each sample was used for double-stranded cDNA conversion, biotin-labeling, and hybridization with the Affymetrix GeneChip Human Genome U133 Plus 2.0 (Affymetrix Inc, Santa Clara, Calif). The

experiments were carried out in the microarray core laboratory at the University of Colorado Denver and followed the procedure previously described.¹⁰

Western blot analysis

Cells were lysed in RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) to isolate total protein. Protein was separated by using SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, Calif). The blots were blocked and incubated with primary and secondary antibodies. Rabbit antihuman S100A11 (ProteintechGroup Inc, Chicago, Ill), mouse antihuman IL-10R2 (Santa Cruz Biotechnology, Santa Cruz, Calif), monoclonal anti-β-actin (Sigma Chemical Co, St Louis, Mo), and mouse anti-β-tubulin (ZYMED, San Francisco, Calif) were used in this study. After stringent wash, blots were developed with ECL Detection Reagents (GE Healthcare Bio-Sciences, Piscataway, NJ).

Immunohistochemical staining for human IL-10R2

Paraffin skin sections (5 µm) were stained using the Cell and Tissue Staining Kit (R&D Systems, Minneapolis, Minn). Tissue samples were deparaffinized through a series of ethanol washes and then incubated with a polyclonal rabbit antihuman IL-10R2 (Cell Sciences, Canton, Mass) at 4°C overnight. Slides were washed and incubated with a biotinylated secondary antibody. Positive signal was visualized by using diaminobenzidine. The sections were counterstained with hematoxylin (Sigma Chemical Co). All slides were read and analyzed blindly. The intensity of the immunostaining was graded under microscopy on a scale from 0 to 5, with 0 indicating no staining and 5 the most intense staining.

Statistical analysis

All statistical analysis was conducted by using Graph Pad prism, version 4.03 (San Diego, Calif). Data were analyzed by using an unpaired 2-tailed *t* test or a 1-way ANOVA for multiple comparisons. Differences were considered significant at *P* < .05.

RESULTS**Inhibition of S100A11 gene expression increases VV replication**

To examine the potential involvement of S100A11 in the control of VV replication, we used a siRNA technique to inhibit gene and protein expression of S100A11 in keratinocytes. HaCaT cells were harvested at 24 hours, 48 hours, and 72 hours after transfection with scrambled versus S100A11 siRNA duplexes to determine the proper time point for our experiment. The protein expression of S100A11 was significantly reduced in cells transfected with siRNA targeting S100A11 compared with cells transfected with scrambled siRNA (nontargeting control; Fig 1, A). The reduction of endogenous S100A11 protein expression did not become significant until 48 hours after transfection; therefore, we chose this time point for further investigation.

After 48 hours of transfection with siRNA, HaCaT keratinocytes were treated with VV for 24 hours under low calcium conditions (1.5 mmol/L CaCl₂). The cells were then harvested for assessment of viral mRNA. As shown in Fig 1, B, VV gene expression was increased in infected cells in a dose-dependent manner. Viral mRNA transcription was significantly increased in cells transfected with S100A11 siRNA compared with cells transfected with scrambled siRNA (mean, 4.22 ± 0.07 vs 1.36 ± 0.03 ng VV/ng GAPDH, at multiplicity of infection [MOI] 0.05; *P* < .01).

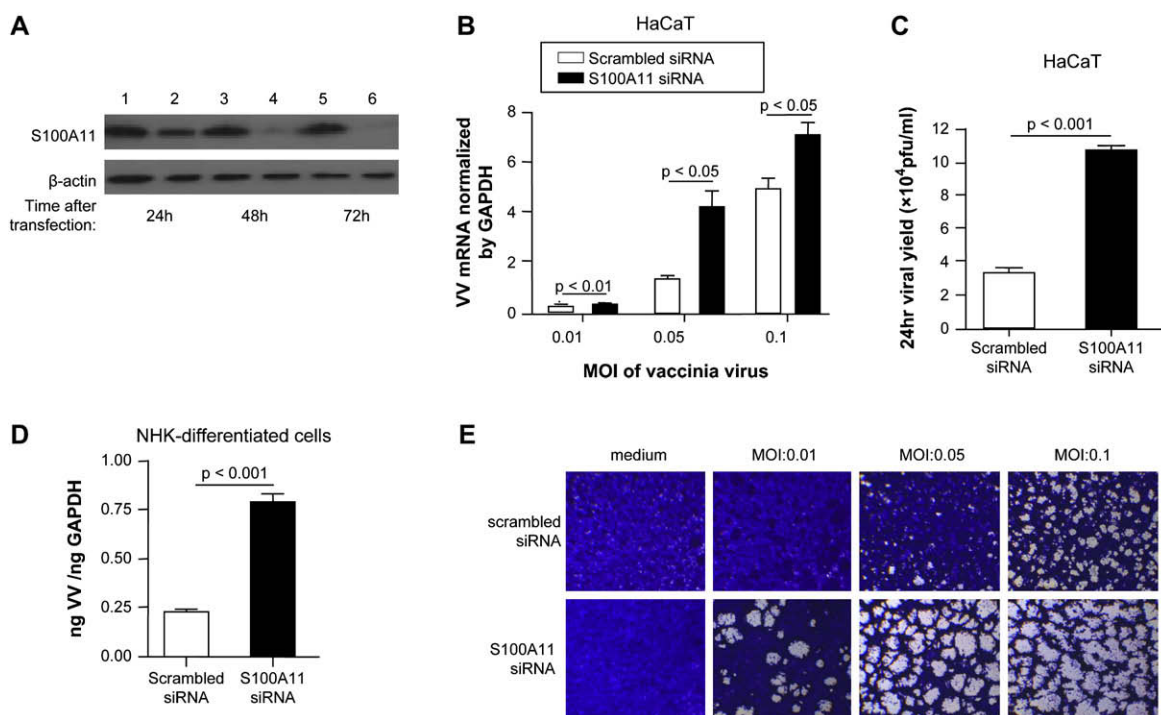


FIG 1. Inhibition of S100A11 expression enhances VV replication in keratinocytes. **A**, S100A11 protein was detected by Western blot. Lanes 1, 3, 5, Scrambled siRNA; lanes 2, 4, 6, S100A11 siRNA. **B**, VV mRNA in HaCaT cells was evaluated by RT-PCR. **C**, 24-Hour viral yield of VV in HaCaT cells was evaluated by plaque assay. **D**, VV mRNA in NHK cells was evaluated by real-time RT-PCR. **E**, VV cytopathic effects were observed in HaCaT cells after crystal violet staining. Original magnification $\times 13$. Data shown are representative of 3 independent experiments. Columns and error bars represent means $\pm$ SEMs.

TABLE I. Genes with more than 2-fold change in S100A11-silenced cells compared with control cells

Gene name	Probe set	Fold change
IL-1RL1	207526_s_at	(Up) 4.92
IL-1R2	205403_at	(Up) 3.89
IL-1R2	211372_s_at	(Up) 3.66
IL-13RA2	206172_at	(Up) 3.47
IL-1B	205067_at	(Up) 2.97
IL-1A	210118_s_at	(Up) 2.71
IL-1B	39402_at	(Up) 2.28
IL-18R1	206618_at	(Up) 2.06
IL-15	205992_s_at	(Down) 2.49
IL-15	217371_s_at	(Down) 2.41
IL-10R2	209575_at	(Down) 2.26
IL-6R	205945_at	(Down) 2.20
S100A11	200660_at	(Down) 3.52

Down, Downregulation compared with scrambled siRNA-transfected cells; up, upregulation compared with scrambled siRNA-transfected cells.

Ca^{2+} is an important regulator for keratinocyte differentiation.¹¹ Because S100A11 is a calcium-binding protein and can be activated by Ca^{2+} ,⁹ we investigated whether Ca^{2+} stimulation affected S100A11's ability to control VV replication. To study this, HaCaT cells were transfected with siRNA duplexes in low Ca^{2+} media for 48 hours, and cells were allowed to undergo differentiation in high Ca^{2+} media for 24 hours. VV was then added to the cells, which were propagated in high Ca^{2+} for the final 24 hours. As shown in Fig 1, C, the VV yield as assessed by viral

plaque-forming assay was increased about 3-fold more in S100A11-deficient cells (mean, $[10.73 \pm 0.17] \times 10^4$ plaque forming unit/mL) compared with scrambled siRNA knock-down cells (mean, $[3.33 \pm 0.26] \times 10^4$ pfu/mL; $P < .001$).

The HaCaT cell line is a transformed human keratinocyte cell line. To investigate our current observations in a more biologically relevant system, we also used cultured primary NHKs to study the involvement of S100A11 in the control of VV replication in normal cells. NHK cells were transfected separately with S100A11 siRNA and scrambled siRNA for 48 hours. The cells were then switched to media with a high concentration of calcium (1.3 mmol/L) for 24 hours; VV (MOI of 0.001) was added to the cells for the final 24 hours, and cells were then harvested for the assessment of VV mRNA by RT-PCR. As shown in Fig 1, D, VV mRNA was increased significantly in S100A11-silenced NHK cells compared with the scrambled siRNA-transfected cells ($P < .001$).

We also investigated the cytopathic effects of VV in S100A11-silenced HaCaT cells compared with the control cells transfected with scrambled siRNA. We observed that viral plaque formation was increased in S100A11-silenced keratinocyte monolayers in high Ca^{2+} media compared with the control cells transfected with scrambled siRNA (Fig 1, E).

IL-10R2 is decreased after inhibition of S100A11 gene expression in keratinocytes

We were interested in determining the potential mechanism by which the silencing of S100A11 expression leads to increased VV replication. To this end, we performed a screening gene

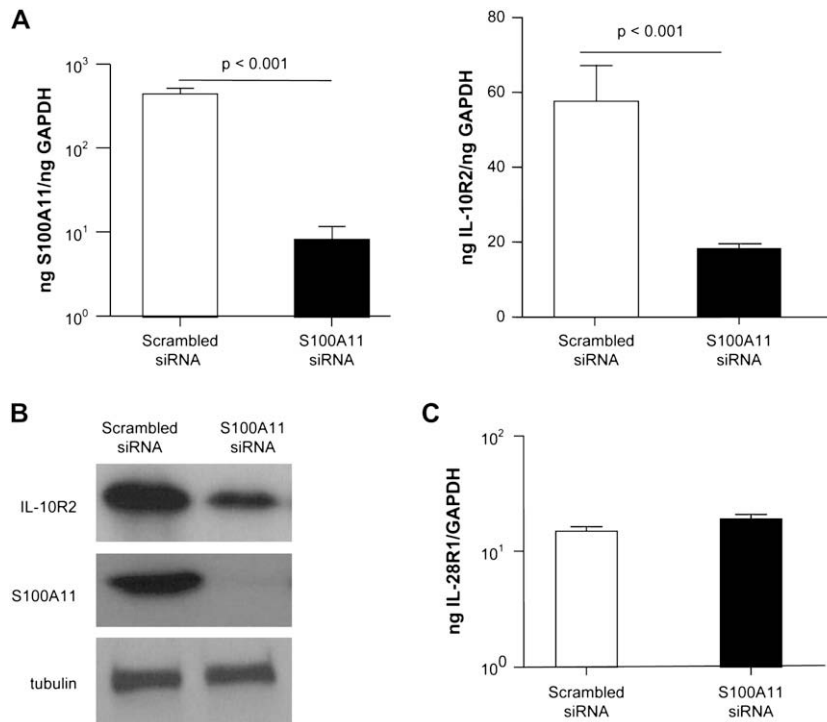


FIG 2. IFN- λ receptor IL-10R2 is downregulated in S100A11-silenced keratinocytes. NHK cells were transfected with S100A11 and control siRNA for 48 hours. S100A11 and IL-10R2 mRNA (**A**), S100A11 and IL-10R2 protein were evaluated by using RT-PCR and Western blot analysis, respectively (**B**), and IL-28R1 mRNA was evaluated by RT-PCR analysis (**C**). Columns and error bars represent means $\pm$ SEMs. Data are representative of 3 independent experiments.

microarray assay to investigate the gene expression profiles of HaCaT cells transfected with S100A11 siRNA compared with cells transfected with scrambled siRNA. We searched for genes that changed their expression levels greater than 2-fold in S100A11-silenced cells. We found a group of cytokines and cytokine receptors with more than 2-fold changes that were related to inflammation and immune responses (Table I). Among them, IL-10R2, which was downregulated by 2.26-fold in S100A11-silenced cells compared with the control cells transfected with scrambled siRNA, has important functions in the innate immune response. IL-10R2 is one of the receptor chains for IFN- λ s.^{12,13} IFN- λ , also called type III IFN, is a newly identified IFN family composed of 3 family members: IL-28A/B (IFN- λ 2/3) and IL-29 (IFN- λ 1). These 3 cytokines bind to the same receptor pair, IL-28R1 and IL-10R2, and activation of downstream signaling requires both subunits of the receptor. Recent studies have demonstrated that IFN- λ s are important mediators for epithelial cell antiviral innate immune responses.¹⁴ IFN- λ s protect host cells against a broad spectrum of viruses including influenza A,¹⁵ hepatitis C,¹⁶ and rhinovirus.¹⁷ Therefore, we chose IL-10R2 to study further in the current study. We performed real-time RT-PCR and Western blot analysis in 3 independent experiments to confirm independently our initial observations made in the gene microarray analysis. As shown in Fig 2, A and B, gene expression of IL-10R2 was significantly ($P < .001$) downregulated in NHK cells that were silenced for S100A11 gene expression. To rule out any potential off-target effect of siRNA duplexes, we transfected 3 independent S100A11 siRNA duplexes into NHK cells. All 3 S100A11

siRNA duplexes successfully knocked down S100A11 gene expression and also led to decreased IL-10R2 expression in NHK cells compared with the cells transfected with scrambled siRNA duplexes. These data suggest that the effect of S100A11 siRNA on reduction of IL-10R2 expression is specific (data not shown). We also evaluated the gene expression of IL-28R1, another receptor chain essential for IFN- λ signaling transduction, in the same experimental samples by using real-time PCR. In contrast, we found that IL-28R1 did not demonstrate any significant changes in S100A11-silenced NHK cells compared with the control cells transfected with scrambled siRNA (Fig 2, C).

IL-10R2 expression is decreased in AD skin

To determine the association of IL-10R2 expression with S100A11 in a clinically relevant disease, we examined IL-10R2 gene expression in acute AD skin biopsies known to be associated with increased T_H2 cytokine expression, in comparison with skin biopsies from healthy individuals. We found that IL-10R2 expression was significantly decreased in acute AD skin lesions (mean, 25.21 ± 5.25 ng IL-10R2/ng GAPDH; $n = 20$) compared with the skin of normal healthy subjects (mean, 137.1 ± 34.46 ng IL-10R2/ng GAPDH; $n = 19$; $P < .01$; Fig 3, A). We also performed immunohistochemical staining to assess IL-10R2 protein levels in acute AD skin biopsies compared with normal subjects. As shown in Fig 3, B and C, IL-10R2 protein was significantly decreased in acute AD skin lesions ($P < .001$) and uninvolved AD skin ($P < .05$) compared with normal subjects.

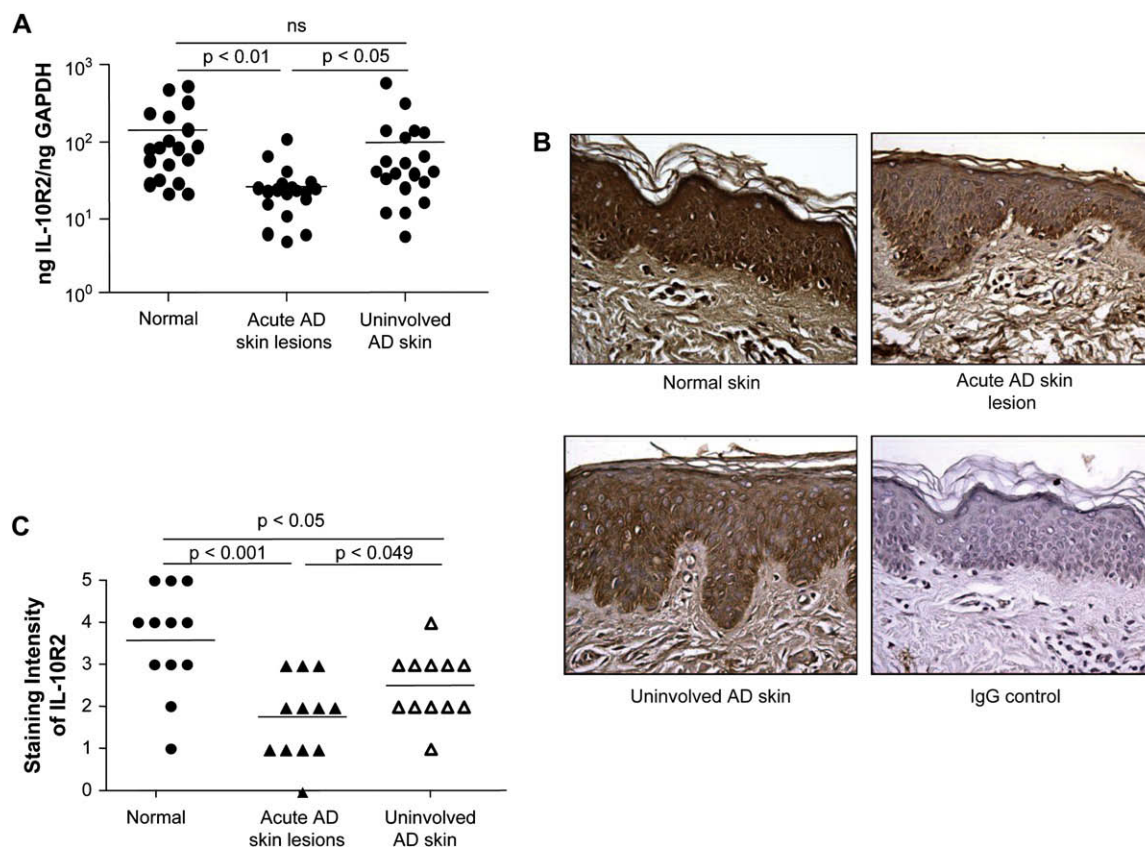


FIG 3. IL-10R2 expression is significantly decreased in AD skin. **A**, IL-10R2 gene expression was evaluated by using real-time PCR in skin biopsies. **B**, Representative immune-stained skin sections are shown. Original magnification, $\times 100$. **C**, The intensity of immunostaining was graded visually on a scale from 0 (no staining) to 5 (the most intense staining) for skin sections from 12 normal subjects, 12 acute AD skin lesions, and 12 unaffected AD skin samples.

The antiviral responsiveness of IFN- λ 1 (IL-29) is attenuated in S100A11-knocked-down keratinocytes

Next we investigated the responsiveness of IFN- λ s in S100A11-silenced NHK cells compared with cells transfected with scrambled siRNA. Because IL-29 is more effective than IL-28A/B (IFN- λ 2/3),¹⁸ we chose IL-29 for further investigation. IL-29 initially binds to IL-28R1 and causes a conformational change that subsequently allows IL-10R2 to bind IL-29. The receptor complex is then activated and induces the expression of many IFN-stimulated genes.¹² This action induces a so-called *antiviral state* in target cells. Mx-1 and 2' 5'-oligoadenylate synthetase (OAS) are well known to be induced by IL-29 stimulation. These proteins inhibit viral replication by interfering with viral RNA transcription and protein translation. To investigate whether IL-29 impairs the formation of an antiviral state after S100A11 gene silencing, we knocked down S100A11 gene expression in NHK cells. We then treated the cells with various concentrations of IL-29 for 24 hours under high Ca^{2+} conditions (1.3 mmol/L). As shown in Fig 4, *A and B*, we found that the induction of Mx-1 and OAS were significantly inhibited in S100A11-silenced cells compared with scrambled siRNA-transfected cells ($P < .001$).

After pretreatment with IL-29, NHK cells were also subjected to inoculation with VV for an additional 24 hours; VV mRNA transcription in these cells was then assessed by RT-PCR. As

shown in Table II, pretreatment of NHK cells with IL-29 significantly ($P < .001$) reduced VV replication in both scrambled siRNA and S100A11 siRNA-transfected cells. However, the anti-VV effect of IL-29 treatment was more efficient in NHK cells transfected with scrambled siRNA compared with cells treated with S100A11 siRNA. VV mRNA in the presence of IL-29-treated NHK cells with scrambled siRNA was only 9.01% of that found in cells without IL-29 treatment, whereas VV mRNA in S100A11-silenced NHK cells with IL-29 treatment was 32.55% of VV mRNA found in IL-29 nontreated cells ($P < .001$).

DISCUSSION

Human diseases that are mediated by $\text{T}_\text{H}2$ cytokines such as AD and asthma are exacerbated by bacterial and viral infections.^{1,19} It is well established that $\text{T}_\text{H}2$ cytokines suppress the secretion of $\text{T}_\text{H}1$ cytokines, thereby impairing cell-mediated immune response.^{20,21} Recent studies suggest that $\text{T}_\text{H}2$ cytokines also directly impair the innate immune response of epithelial cells.^{3,4} However, the underlying mechanisms for this phenomenon are not well understood. Using a proteomics approach, we recently found that S100A11 was selectively downregulated in keratinocytes by IL-4 and IL-13 treatment, and S100A11 is significantly decreased in AD skin.⁶ In addition, we have observed that S100A11 gene expression is significantly decreased only in

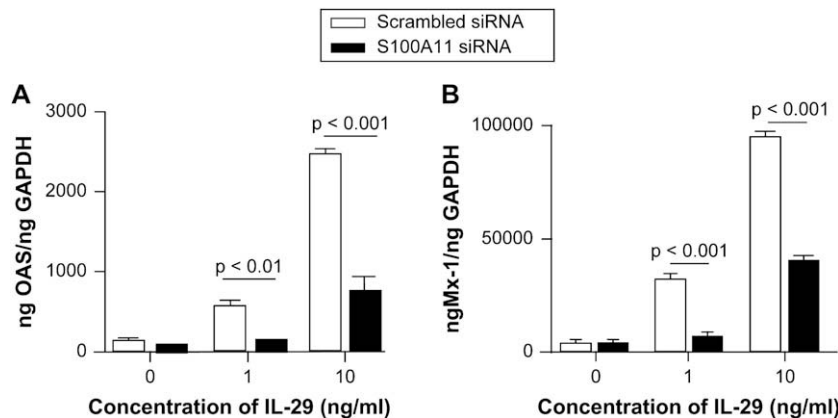


FIG 4. Antiviral responses of IFN- λ 1 (IL-29) were impaired in S100A11-silenced NHK cells. NHK cells were transfected with siRNA duplexes for 48 hours and then treated with IL-29 for 24 hours. OAS (**A**) and Mx-1 (**B**) were evaluated by real-time PCR. Columns and error bars represent means $\pm$ SEMs. Data shown are representative of 3 independent experiments.

TABLE II. VV mRNA expression in NHK cells with siRNA in the presence of IL-29 compared with media

Treatment	Scrambled siRNA	S100A11 siRNA	P value§
Media + VV*	2.33 $\pm$ 0.19†	6.39 $\pm$ 0.56†	$P < .01$
IL-29‡ + VV*	0.21 $\pm$ 0.05†	2.08 $\pm$ 0.13†	$P < .001$
P value	$P < .001$	$P < .01$	

*VV: MOI 0.01.

†VV mRNA normalized by GAPDH.

‡IL-29: 10 ng/mL.

§Scrambled vs S100A11 siRNA.

||Media + VV vs IL-29 + VV.

AD acute skin lesions, not in AD uninvolved skin, suggesting that reduction of S100A11 in AD is most likely acquired by the modulation of locally overexpressed IL-4/IL-13 and not by genetic polymorphisms.⁶ These previous data suggest that IL-4 and IL-13 may subvert the skin's innate immune response via downregulation of S100A11 gene expression. S100A11 has 2 calcium-binding EF-hand domains and belongs to the epidermal differentiation complex on chromosome 1q21. Its expression is abundant in human keratinocytes, but its biological function remains largely unknown.⁹ We have clearly demonstrated in this study that inhibition of S100A11 gene expression leads to significantly enhanced VV replication in keratinocytes and an augmented VV cytopathic effect (Fig 1, A). These observations suggest that S100A11 plays a role in the skin's innate immune response.

To understand better the mechanism by which S100A11 maintains the innate immune response of keratinocytes, we investigated the gene expression profiles of S100A11-silenced keratinocytes compared with the control cells by using a gene microarray approach. We focused our attention on genes that had greater than 2-fold change in expression and functions related to innate immune responses and regulation of inflammation. As shown in Table I, 5 members of the IL-1 and IL-1R families have greater than 2-fold changes in S100A11-silenced keratinocytes. Among them, IL-1RL1(ST2) was upregulated 4.92-fold in S100A11-silenced cells. IL-1RL1 and its ligand IL-33 are known to activate the signaling pathways that induce T_H2 cytokine production in several human cells.²² Interestingly, IL-1RL1 was

reported upregulated in atopic diseases including asthma and AD.^{23,24} In addition, Shimizu et al²⁴ found that genetic polymorphisms within the IL-1RL1 gene region had a strong association with AD. Expression of IL-1RL1 is also reported to reduce innate immune responses by sequestering the adaptor molecules Myeloid differentiation primary response gene (88) and MyD88-adaptor-like for IL-1 and Toll-like receptor 4 signaling transduction,²⁵ but IL-1 and Toll-like receptor 4 signaling pathway do not directly activate antiviral responses. Our gene microarray data also show that IL-1R2 is upregulated by 3.89-fold in S100A11-silenced keratinocytes. IL-1R2, which blocks the IL-1 β signaling pathway, is a decoy receptor for IL-1R1.²⁶ Consistent with our current observations, IL-4 and IL-13 are reported to antagonize the activity of IL-1 by inducing the expression of IL-1R2.^{26,27} Upregulation of IL-1RL1 and IL-1R2 in S100A11-silenced keratinocytes implies that S100A11 may be associated with T_H2 polarization in AD pathogenesis although these genes, as well as others like IL-1A, IL-1B, IL-15, and IL-6R1, are not directly involved in innate immune responses that inhibit viral replication. Association studies of S100A11 and inflammatory responses are underway in our laboratory; however, we chose not to pursue these genes as targets for antiviral innate immune response in the current study.

The ligands of IL-10R2 include the extended IL-10 superfamily members: IL-10, IL-22, IL-26, and IFN- λ s.^{28,29} IL-10R2 is distributed ubiquitously on human cells, but the selective receptor chains for its ligands are preferentially expressed on different cell types. The primary cellular targets for IL-10 are leukocytes, and nonhematopoietic cells do not express IL-10R1. Keratinocytes express the selective receptor chain IL-20R1 for IL-26, but previous studies have not shown that IL-26 functions in the innate immune response.³⁰ As with IFN- λ s, the selective receptor chain for IL-22, IL-22R1, is expressed predominantly by epithelial cells in skin, lung, and gut. Recent studies with IL-22 suggest that this cytokine is important in mediating innate immune responses against gram-negative bacterial infections; however, this cytokine has no direct effect against viruses,³¹⁻³³ and patients with AD are not predisposed to gram-negative bacterial infections. Thus, IL-10, IL-26, and IL-22 were not studied further because our current work focused on VV infection.

Recent studies have shown that IFN- λ s are important cytokines for antiviral immune responses.^{12,13} Even though IFN- λ induces the same set of IFN-stimulated genes as type I IFNs, they have different cell targets. The primary target cells for IFN- λ are epithelial cells because the selective receptor chains for IFN- λ , IL-28R1, are only expressed on epithelial cells and 1 subset of dendritic cells.^{14,18} Our results show that expression of IL-10R2 was significantly decreased in S100A11-silenced cells, and this was found to impair responsiveness of keratinocytes to IFN- λ 1 (Fig 3), manifested by impairment of their innate immune response to VV (Table II). Previous studies have shown that IFN- λ s are expressed by human peripheral blood monocytes and dendritic cells on stimulation with viruses.^{12,13,34} In our current study, we found that IFN- λ s were greatly induced by VV in NHK cells (Fig E1). The induction of IFN- λ s in S100A11-silenced cells is normal; however, the responsiveness of IFN- λ 1 in NHK cells with deficient expression of S100A11 was significantly attenuated compared with S100A11 normally expressed cells (Fig 4). Consequently, we observed that the protective effect of IFN- λ 1 (IL-29) against VV is impaired significantly in S100A11-silenced cells compared with the control cells (Table II). These data therefore indicate that the enhancement of VV replication in S100A11-silenced keratinocytes is caused by inadequate IFN- λ responsiveness via decreased expression of IL-10R2. We further examined IL-10R2 expression in skin biopsies from AD skin lesions compared with normal healthy subjects, and found that IL-10R2 expression was significantly decreased in AD skin lesions. This finding highlights the clinical significance of our current study. The deficient expression of IL-10R2 in AD skin would be expected to impair IFN- λ responsiveness in control of viral infection.

Our group has previously demonstrated that skin antimicrobial peptide LL37 can protect keratinocytes from VV infection by destruction of VV viral particles, and LL37 induction in AD skin is greatly impaired on VV stimulation.^{4,5,7} However, LL37 expression is not affected in S100A11-silenced keratinocytes compared with the control cells (data not shown). In addition, type I IFNs (IFN- α and IFN- β) have been reported to have protective effects against VV in cultured cells and *in vivo* animal models.³⁵ However, we did not find the responsiveness to type I IFNs in S100A11-silenced keratinocytes is impaired compared with scrambled siRNA transfected cells (data not shown), suggesting that S100A11 is not involved in type I IFN signaling pathway of keratinocytes. Taken together, a deficit in IFN- λ -IL-10R2 signaling in AD skin is at least partly responsible for the propensity of patients with AD to disseminated VV skin infection.

There are 13 S100 family members located in the epidermal differentiation complex gene cluster including S100A11, S100A7, S100A8, S100A9, S100A12, and so forth.³⁶ Interestingly, S100A7, S100A8, S100A9, and S100A12 are found to be activated by IL-22, another ligand for IL-10R2, suggesting that S100A11 can regulate other family members' expression by regulating IL-10R2 through an as yet unknown mechanism. Our current study is the first to demonstrate that S100A11 is involved in cytokine signaling pathways through regulation of IL-10R2 expression at the transcriptional level. This function of S100A11 is most likely related to its subcellular location in the nucleus. Sakaguchi et al⁹ found that S100A11 nuclear translocation releases the transcription factor, specificity protein 1, from nucleolin. This action leads to the activation of P21 transcription. There are 2 putative specificity protein 1 binding sites in the IL-10R2

promoter, suggesting that S100A11 may use the same model to regulate the transcription of IL-10R2.

The threat of bioterrorism with smallpox has led to advocacy for reinstatement of voluntary vaccinations against smallpox. Because patients with AD are susceptible to disseminated VV infection, the Centers for Disease Control and Prevention has developed guidelines excluding children with AD and those in close contact with them for smallpox vaccination.³⁷ Approximately 17% of children in the United States are diagnosed as having AD,¹ and these children would be exposed to life-threatening danger if a smallpox bio-weapon was used. Understanding the mechanisms that result in poor control of VV in AD skin is critical for the development of new approaches to prevent the adverse effects after smallpox vaccination and biomarkers to identify the population at risk for side effects from this vaccine. Our findings in this study not only provide an explanation for the potential mechanism by which T_H2 cytokines mediate deficiency of epithelial innate immune responses to VV, but also identify novel gene targets to enhance innate immune responses in AD via upregulation of the expression of S100A11 and/or IL-10R2.

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Clinical implications: Reduced S100A11 and IL-10R2 expression may increase the propensity of patients with AD toward disseminated viral skin infection.

REFERENCES

- Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. *J Clin Invest* 2004;113:651-7.
- Homey B, Steinhoff M, Ruzicka T, Leung DY. Cytokines and chemokines orchestrate atopic skin inflammation. *J Allergy Clin Immunol* 2006;118:178-89.
- Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 2002;347:1151-60.
- Howell MD, Gallo RL, Boguniewicz M, Jones JF, Wong C, Streib JE, et al. Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. *Immunity* 2006;24:341-8.
- Howell MD, Wollenberg A, Gallo RL, Flaig M, Streib JE, Wong C, et al. Cathelicidin deficiency predisposes to eczema herpeticum. *J Allergy Clin Immunol* 2006;117:836-41.
- Howell MD, Fairchild HR, Kim BE, Bin L, Boguniewicz M, Redzic JS, et al. Th2 cytokines act on S100A11 to downregulate keratinocyte differentiation. *J Invest Dermatol* 2008;128:2248-58.
- Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol* 2004;172:1763-7.
- Kim BE, Leung DY, Streib JE, Kisich K, Boguniewicz M, Hamid QA, et al. Macrophage inflammatory protein 3 α deficiency in atopic dermatitis skin and role in innate immune response to vaccinia virus. *J Allergy Clin Immunol* 2007;119:457-63.
- Sakaguchi M, Miyazaki M, Takaishi M, Sakaguchi Y, Makino E, Kataoka N, et al. S100C/A11 is a key mediator of Ca(2+)-induced growth inhibition of human epidermal keratinocytes. *J Cell Biol* 2003;163:825-35.
- Nomura I, Goleva E, Howell MD, Hamid QA, Ong PY, Hall CF, et al. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 2003;171:3262-9.
- Hennings H, Holbrook K, Steinert P, Yuspa S. Growth and differentiation of mouse epidermal cells in culture: effects of extracellular calcium. *Curr Probl Dermatol* 1980;10:3-25.
- Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003;4:69-77.
- Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003;4:63-8.

14. Sommereyns C, Paul S, Staeheli P, Michiels T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog* 2008;4:e1000017.
15. Mordstein M, Kochs G, Dumoutier L, Renauld JC, Paludan SR, Klucher K, et al. Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. *PLoS Pathog* 2008;4:e1000151.
16. Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 2005;79:3851-4.
17. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, et al. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12:1023-6.
18. Meager A, Visvalingam K, Dilger P, Bryan D, Wadhwa M. Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine* 2005;31:109-18.
19. Holgate ST. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 2007;28:248-51.
20. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol* 1994;12:635-73.
21. Stanford MM, McFadden G. The "supervirus"? lessons from IL-4-expressing poxviruses. *Trends Immunol* 2005;26:339-45.
22. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23:479-90.
23. Oshikawa K, Kuroiwa K, Tago K, Iwahana H, Yanagisawa K, Ohno S, et al. Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am J Respir Crit Care Med* 2001;164:277-81.
24. Shimizu M, Matsuda A, Yanagisawa K, Hirota T, Akahoshi M, Inomata N, et al. Functional SNPs in the distal promoter of the ST2 gene are associated with atopic dermatitis. *Hum Mol Genet* 2005;14:2919-27.
25. Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, et al. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol* 2004;5:373-9.
26. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, et al. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 1993;261:472-5.
27. Colotta F, Re F, Muzio M, Polentarutti N, Minty A, Caput D, et al. Interleukin-13 induces expression and release of interleukin-1 decoy receptor in human polymorphonuclear cells. *J Biol Chem* 1994;269:12403-6.
28. Donnelly RP, Sheikh F, Kotenko SV, Dickensheets H. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J Leukoc Biol* 2004;76:314-21.
29. Commins S, Steinke JW, Borish L. The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *J Allergy Clin Immunol* 2008;121:1108-11.
30. Hor S, Pirzer H, Dumoutier L, Bauer F, Wittmann S, Sticht H, et al. The T-cell lymphokine interleukin-26 targets epithelial cells through the interleukin-20 receptor 1 and interleukin-10 receptor 2 chains. *J Biol Chem* 2004;279:33343-51.
31. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* 2008;14:275-81.
32. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 2008;14:282-9.
33. Dambacher J, Beigel F, Zitzmann K, Heeg MH, Goke B, Diepolder HM, et al. The role of interleukin-22 in hepatitis C virus infection. *Cytokine* 2008;41:209-16.
34. Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, et al. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol* 2004;34:796-805.
35. Martinez J, Huang X, Yang Y. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. *J Immunol* 2008;180:1592-7.
36. Eckert RL, Broome AM, Ruse M, Robinson N, Ryan D, Lee K. S100 proteins in the epidermis. *J Invest Dermatol* 2004;123:23-33.
37. Rotz LD, Dotson DA, Damon IK, Becher JA. Vaccinia (smallpox) vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2001;50:1-25.

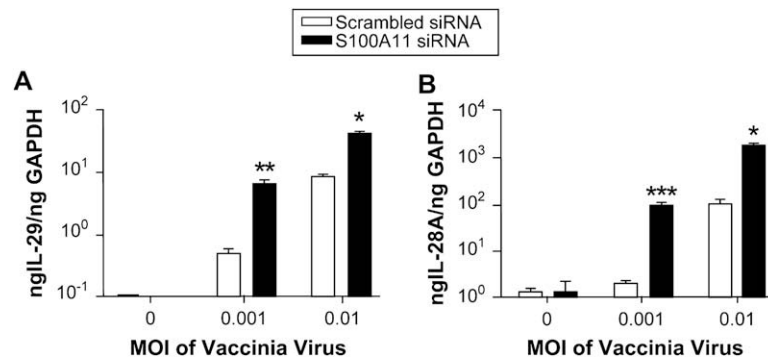


FIG E1. Type III IFNs are induced by VV. Primary NHK cells were transfected with siRNA for 48 hours and incubated with VV for 24 hours. Cells were then harvested for RNA extraction. IL-29 (A) and IL-28A (B) expression were evaluated by using real-time PCR. Columns and error bars represent means $\pm$ SEMs. Data shown are representative of 3 independent experiments. * $P < .05$; ** $P < .01$; *** $P < .001$.