

Letter to the Editor

Reductions in claudin-1 may enhance susceptibility to herpes simplex virus 1 infections in atopic dermatitis

To the Editor:

Atopic dermatitis (AD) is the most common inflammatory skin disease, affecting up to 20% of children in the United States, and is characterized by an increased susceptibility to cutaneous infections.^{1,2} One in 10 subjects with AD has difficulty clearing cutaneous infections with a host of viruses including herpes simplex, vaccinia, human papilloma, and/or molluscum contagiosum.¹ This typically manifests as more extensive cutaneous and sometimes systemic disease and/or resistance to standard therapies. One of the rarer but more severe viral complications is eczema herpeticum (EH), caused by herpes simplex virus (HSV)-1 that results in a widespread skin infection.¹ EH can be complicated by viremia and multiorgan involvement including keratoconjunctivitis, meningitis, and encephalitis.¹ Although we have shown that subjects with AD with more severe disease, greater allergen sensitization, and enhanced T_H2 polarity are at greatest risk for this viral complication, the molecular mechanisms responsible for this remain unclear.¹

Disruption of the epidermal barrier is now recognized as a cardinal feature of AD pathogenesis. The skin has 2 barrier structures: the stratum corneum (SC) and tight junctions (TJs). It is widely accepted that the SC in subjects with AD is dysfunctional. The loss-of-function mutations in filaggrin, a structural protein important for its hygroscopic properties, have been reproducibly and robustly associated with AD³ and more recently with EH.⁴ TJs are found on opposing membranes of stratum granulosum keratinocytes, directly below the SC. TJs consist of a complex of adhesive and scaffolding proteins that control the passage of water, ions, and solutes through the paracellular pathway.⁵ We have recently demonstrated that the nonlesional epithelium of subjects with AD has remarkable bioelectric abnormalities indicative of a TJ defect that may be the consequence of reduced levels of claudin-1 (CLDN1), a key TJ adhesive protein.⁶ This is in line with the landmark work of Furuse and Tsukita,⁷ whose CLDN1 knockout mouse established the importance of epidermal TJ and CLDN1. These mice died within 24 hours of birth with wrinkled skin, severe dehydration, and increased epidermal permeability as measured by dye studies and transepidermal water loss.⁷ In our AD studies, we also observed an inverse correlation between CLDN1 expression and markers of T_H2 polarity (total eosinophil counts and serum total IgE).⁶ This has led us to speculate that TJ defects may promote greater T_H2 polarity, which is a characteristic of subjects with EH.¹ We hypothesized that TJ defects such as those we observed in subjects with AD may promote HSV-1 infections on that basis.

In addition, viruses often commandeer intercellular junction proteins as a means of viral entry.⁵ For example, wild-type HSV-1 infects keratinocytes through a complex interaction involving the viral envelope glycoprotein gD with either nectin-1 (*PVRL1*) or HSV entry mediator.^{8,9} Nectin-1, a Ca²⁺-independent cell adhesion protein of the immunoglobulin superfamily, colocalizes with E-cadherin and β -catenin to form another intercellular junctional complex called the adherens junction. Previous studies have shown that the susceptibility of human keratinocytes to HSV-1

infection is inversely related to the degree of cell-cell contact and confluency.^{9,10} This suggests that healthy junctional complexes may be a key deterrent to the spread of HSV-1 from one keratinocyte to another.

In these studies, we tested the hypothesis that the silencing of epidermal CLDN1 would enhance HSV-1 infectivity of human keratinocytes. We had previously demonstrated that reductions in CLDN1 adversely affected measures of TJ integrity.⁶ Primary human keratinocytes (PHKs) were isolated from foreskin as previously described⁶ and transiently transfected with scrambled (control) or CLDN1 (100 nmol/L) small interfering RNA (siRNA; Santa Cruz Biotechnology, Santa Cruz, Calif) by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, Calif; see this article's complete [Methods](#) in the Online Repository at www.jacionline.org). We have previously demonstrated that this knockdown approach led to a >50% reduction in claudin-1 transcripts and adversely affected TJ function as demonstrated by a 50% reduction in transepithelial electrical resistance and doubling of the 50% permeability.⁶ Importantly, this level of CLDN1 reduction mirrored what we had observed in AD samples. Twenty-four to 48 hours posttransfection, PHKs were differentiated in Dulbecco modified Eagle medium (DMEM) with 10% heat-inactivated FBS (HI-FBS; Invitrogen/Gibco) for 24 hours and subsequently infected with the highly virulent HSV-1 strain F (provided by Dr D. C. Johnson, Oregon Health and Science University) at a multiplicity of infection of 0.1 (see this article's complete [Methods](#) in the Online Repository). The multiplicity of infection was chosen based on findings from viral titration (0.01-10 multiplicity of infection) studies on differentiated and undifferentiated PHKs, respectively, used as negative and positive controls (data not shown). After 2 hours, the viral inoculum was removed, and PHKs were cultured for 24 hours in DMEM containing 5% HI-FBS and 0.4% human- γ -globulin (0.5 mg/mL; Sigma, St Louis, Mo) to neutralize extracellular virus. HSV-infected PHKs were fixed in 4% formaldehyde and stained with a polyclonal rabbit anti-HSV-1 (1:500; Dako, Carpinteria, Calif) antibody followed by Alexa Fluor 488 donkey-antirabbit IgG H+L (1:1000; Invitrogen) and 4',6-diamidino-2-phenyl-indole, dihydrochloride (DAPI; 1:10,000; Invitrogen). For each sample, 6 random fields were captured at identical acquisition settings and analyzed computationally to quantify differences objectively in focal forming units (FFUs; see complete [Methods](#) in the Online Repository). A FFU was defined as a cluster of 3 or more adjacent HSV-1-positive cells. CLDN1 depletion significantly increased the number and size of HSV-1 FFUs ([Fig 1, A](#)) compared with scrambled siRNA-transfected PHK ([Fig 1, B](#)). An average of 4.8 ± 0.7 FFUs/field were counted in CLDN1 knockdown PHKs, whereas only 2.5 ± 0.5 FFUs/field were detected in the control cells ($P = .05$; $n = 6$; [Fig 1, C](#)). The diameters of FFUs were significantly greater in keratinocytes whose CLDN1 expression was reduced ($207 \pm 22 \mu\text{m}$) compared with controls ($152 \pm 40 \mu\text{m}$; $P = .03$; $n = 6$; [Fig 1, D](#)). Similar findings were observed when evaluating FFU area ($P = .04$; $n = 6$; [Fig 1, E](#)) in PHKs after CLDN1 knockdown ($15,649 \pm 4367$ pixels) compared with control transfection (9902 ± 4943 pixels). The increased frequency of HSV-1-infected cells in CLDN1 knockdown PHKs was confirmed by an infectious

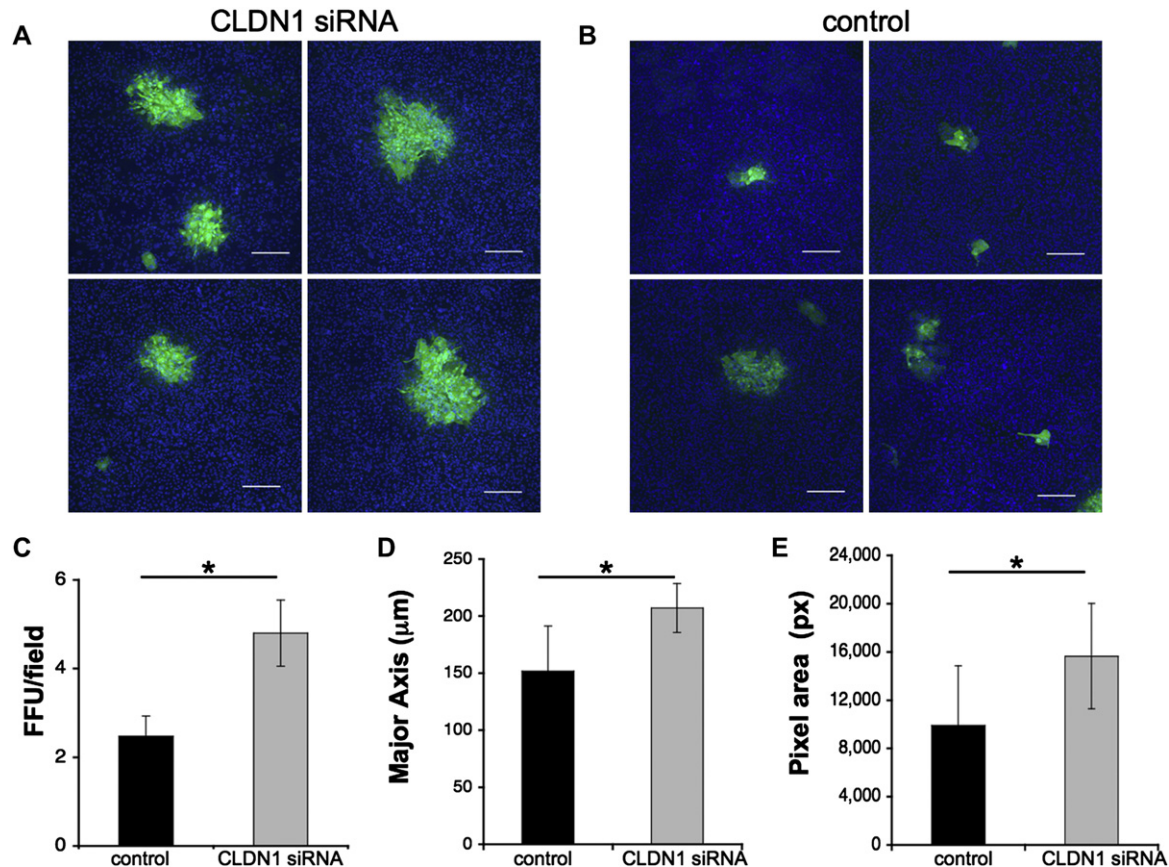


FIG 1. Knockdown of claudin-1 (CLDN1) in human keratinocytes increases HSV-1 infectivity. Green indicates HSV-1 staining. DAPI (blue) nuclear stain provides an assessment of cell density (bars = 200 μ m). Larger and more numerous FFUs were detected in PHKs treated with CLDN1 siRNA (A) compared with the control siRNA (B; representative images of $n = 6$ experiments). Cells stained with a rabbit IgG isotype control were all negative (data not shown). C, An average of 4.8 ± 0.7 FFUs/field were counted in CLDN1 knockdown PHKs and 2.5 ± 0.5 FFUs/field in the control samples ($*P = .05$; $n = 6$). D, The diameter of the major axis of FFUs was significantly greater in PHKs whose CLDN1 expression was reduced (207 ± 21 μ m) compared with controls (152 ± 40 μ m; $*P = .03$; $n = 6$). E, Similar observations were noted in FFU area ($*P = .04$; $n = 6$) in CLDN1 knockdown ($15,649 \pm 4367$ pixels [px]) compared with control transfection (9902 ± 4943 pixels).

center assay (see this article's complete [Methods](#) in the Online Repository). More CLDN1 siRNA-transfected PHKs were infected with HSV-1 ($38 \pm 6\%$; $n = 3$) than control PHKs ($28 \pm 7\%$; $n = 3$; $P = .003$; see this article's [Fig E1](#) in the Online Repository at www.jacionline.org). Importantly, the observed increase in HSV-1 infectivity in CLDN1 knockdown PHKs could not be explained by reduced expression of nectin-1, the HSV-1 receptor. Indeed, nectin-1 mRNA expression was unaffected by CLDN1 silencing ($n = 5$; see this article's [Fig E2](#) in the Online Repository at www.jacionline.org). However, we cannot rule out the possibility that reductions in CLDN1 and the effects on TJ function might reduce nectin-1 membrane localization. It is also important to highlight that our *in vitro* model is sufficient to induce functional TJ as demonstrated by enhanced transepithelial resistance and reduced permeability.⁶ However, it is not enough to induce terminal differentiation (eg, filaggrin expression and cornified envelope). Although this might be a limitation of the methodology, we believe this condition well reflects the *in vivo* situation. In AD subjects with history of EH (ADEH⁺), often the virus spreading is a result of reactivation of a silent infection (inside-out), suggesting that

the first barrier structure the virus will encounter is indeed the TJ (located in the stratum granulosum, just below the SC).

As part of the National Institute of Allergy and Infectious Diseases-funded Atopic Dermatitis and Vaccinia Network (ADVNI; reviewed in a previous publication by Beck et al¹), we performed a preliminary study to evaluate whether *CLDN1* polymorphisms were associated with EH in AD subjects. To do this, we used a haplotype-tagging SNP approach with genetic markers available in the public arena ($n = 132$ in single nucleotide polymorphism database) in 414 European Americans and 328 African Americans (see this article's complete [Methods](#) and [Table E1](#) in the Online Repository at www.jacionline.org). We previously reported modest associations ($P = .003-.05$) between SNP variants throughout the *CLDN1* gene and AD that were more significant among African Americans.⁶ In this study, we addressed whether genetic variations in *CLDN1* contribute to risk of EH in both North American AD populations. The EH subphenotype was defined as subjects with AD who had at least 1 EH episode documented by an ADVNI investigator (or a physician affiliated with the same academic center) or diagnosed clinically by an outside physician where the HSV infection was confirmed by PCR, tissue immunofluorescence,

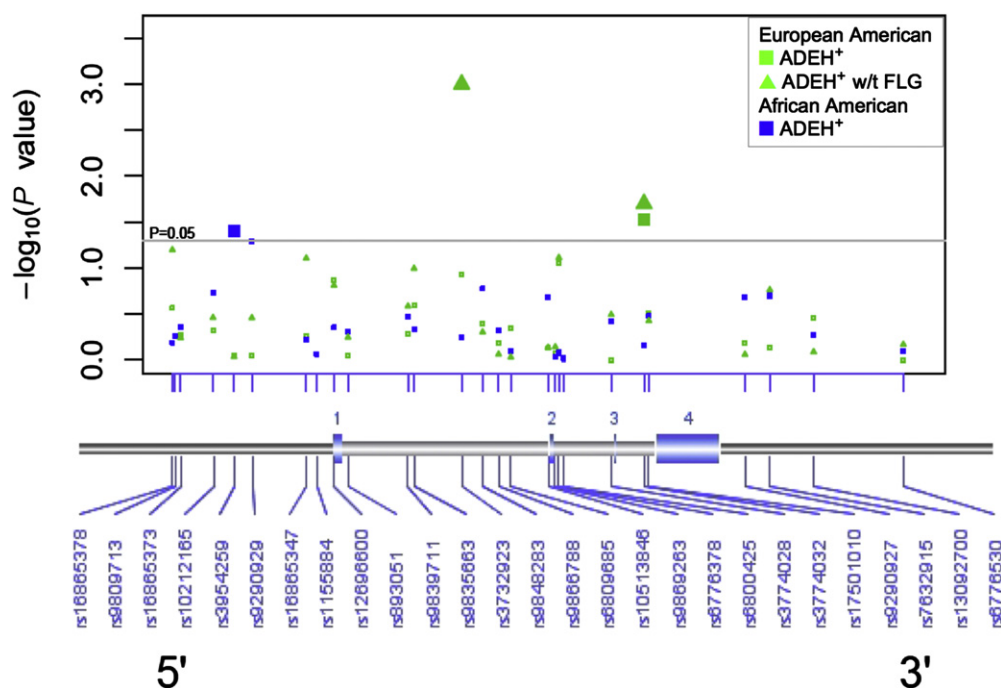


FIG 2. Excluding subjects with the 2 most common *FLG* mutations significantly enhanced *CLDN1* associations with EH. An SNP (rs3774032; $P = .03$) was significantly associated with EH in the European American population. When subjects were stratified for *FLG* (eg, without [w/o] *FLG* mutations), the association was slightly more significant ($P = .02$). In this group, we observed 1 additional intronic SNP that was significantly associated with EH (rs3732923; $P = .001$). In the African American population, an SNP (rs3954259; $P = .040$) in the promoter region was associated with EH. w/, With.

TABLE I. ADVN registry participant characteristics

Characteristic	European American		African American	
	ADEH ⁺	ADEH ⁻	ADEH ⁺	ADEH ⁻
Sample size	93	165	21	155
Males, n (%)	49 (52.7)	47 (28.5)	8 (38.1)	35 (22.6)
Age (y), mean (SD)	22.9 (21.5)	38.0 (14.5)	22.8 (19.0)	36.4 (11.0)
AD onset <5 y, n (%)	77 (97.5)	101 (78.3)	14 (93.3)	77 (68.1)
IgE level, median (interquartile range)*	2225 (611-9034)	252 (85-1275)	3505 (571-9429)	425 (129-1194)
EASI,† median (interquartile range)‡	10.0 (4.4-15.8)	3.2 (1.3-8.4)	10.9 (6.8-15.8)	3.0 (1.5-7.4)
FLG null allele carrier, n (%)	29 (36.3)	36 (22.1)	2 (12.5)	9 (6.0)

ADEH⁺, AD with a history of eczema herpeticum; ADEH⁻, AD without a history of EH; EASI, Eczema Area and Severity Index.

*Total serum IgE levels were significantly higher in patients with ADEH⁺ compared with both patients with ADEH⁻ and nonatopic, healthy controls even after adjusting for age ($P < .001$).

†EASI was determined by the percentage of eczema area on a 7-point ordinal scale: 0, no eruption; 1, <10%; 2, 10% to 29%; 3, 30% to 49%; 4, 50% to 69%; 5, 70% to 89%; and 6, 90% to 100%.

‡EASI was significantly higher among patients with ADEH⁺ compared with patients with ADEH⁻ in both racial groups even after adjusting for age ($P < .001$).

Tzanck smear, and/or culture. The study was approved by the institutional review boards at National Jewish Health, Johns Hopkins University, Oregon Health and Science University, the University of California San Diego, Children's Hospital of Boston, and the University of Rochester Medical Center. All subjects gave written informed consent before participation.

To determine whether SNPs in *CLDN1* were associated with EH, the Cochran-Armitage trend test using the PLINK software (<http://pengu.mgh.harford.edu/~purcell/plink/>) was performed within each AD population and confirmed with the adaptive permutation test. We observed only modest associations between *CLDN1* SNPs and EH in our North American AD populations.

We wondered whether the strong association we observed between both filaggrin (*FLG*) null mutations R501X and 2282del4 and EH (odds ratio [OR], 10.1 [value range: 4.7-22.1]; $P = 1.99 \times 10^{-11}$) was masking a lesser effect from *CLDN1* variants.⁴ To address this, we excluded subjects who had 1 or both of these *FLG* mutations and reanalyzed the associations with all *CLDN1* SNPs. In the European American group, an intronic SNP (rs3774032; OR, 0.59 [0.35-0.98]; $P = .037$) was marginally associated with EH (Fig 2) but not significantly after adaptive permutations adjustment ($P = .10$). When excluding subjects with a *FLG* mutation ($n = 29$ [36.3%]; Table I), the association became slightly more significant (OR, 0.44 [0.22-0.85]; $P = .0225$) and

still significant after adaptive permutations adjustment ($P = .035$). Interestingly, 1 additional intronic SNP emerged (rs3732923; OR, 1.93 [1.21-3.07]; $P = .0010$; adaptive permutation, $P = .0005$; Fig 2). Although the EH sample size in the African American population was too small ($n = 21$) for meaningful analyses, a SNP in the promoter region was associated with EH (rs3954259; OR, 2.16 [1.02-4.59]; $P = .040$; adaptive permutation, $P = .033$). Limitations of these studies are the small sample size and the superficial coverage of *CLDN1* loci. Nevertheless, these findings implicate *CLDN1* mutations in susceptibility to widespread HSV skin infections in subjects with AD, especially those who do not have a *FLG* mutation.

In conclusion, this study and previously reported work implicate both SC and TJ epidermal barrier defects as a mechanism to explain the susceptibility of subjects with AD to widespread cutaneous infections with HSV and possibly other viral pathogens.⁴ In support of this notion, our patients with EH more commonly experienced infections with molluscum contagiosum than subjects with AD with no history of EH (29% vs 2%, respectively).¹ More of our subjects with EH (16%) had recurrent HSV keratitis compared with only 1% of subjects with AD with no history of EH.¹ It is possible that the same mechanism may be responsible for their ocular disease because corneal epithelium possesses TJs and these structures express claudin-1.^{11,12} In this study, we show both mechanistic and genetic results that implicate TJs as a critical barrier structure important in containing the spread of epidermal HSV-1 infections. Defects in TJ compared with the SC may be particularly relevant in viral infections in which the virus enters from a basolateral direction, as is the case with HSV reactivation. Collectively, this work highlights a previously underappreciated role for TJ proteins in cutaneous host defense and may lead to new therapeutic strategies.

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METHODS

Culture of primary human keratinocytes

Human keratinocytes (PHKs) were isolated from neonatal foreskin.^{E1} PHKs were cultured in keratinocyte-SFM (Invitrogen/Gibco, Carlsbad, Calif) with 1% Pen/Strep, 0.2% amphotericin B (Invitrogen/Gibco). To differentiate PHK, cells were grown in DMEM (Invitrogen/Gibco) with 10% HI-FBS (Invitrogen/Gibco) and 1% Pen/Strep, 0.2% amphotericin B (Invitrogen/Gibco).

RNA interference

Primary human keratinocytes were plated on glass coverslips at 2 to 3×10^5 cells/well in a 6-well plate or at 2 to 3×10^4 cells/filter in Transwell inserts (Costar; Corning Inc, Corning, New York; Polyester Membrane, 0.4 μ m pore size, 6.5 mm insert) in keratinocyte-SFM without antibiotics. The day after plating, cells were transfected with claudin-1 specific or control (scrambled) siRNAs (Santa Cruz Biotechnology, Santa Cruz, Calif) by using Lipofectamine 2000 Transfection Reagent (Invitrogen).

HSV-1 infection of PHKs

Infection studies were performed by using the highly virulent HSV-1 strain F (provided by Dr D. C. Johnson). Twenty-four to 48 hours posttransfection with 100 nmol/L claudin-1 or control siRNA, the cells were differentiated in DMEM with 10% heat-inactivated FBS for 24 hours. Cells were washed twice with HBSS and infected with HSV-1 strain F at a multiplicity of infection of 0.1 in DMEM containing 1% heat-inactivated FBS at 37°C, with rocking every 15 minutes. After 2 hours, the viral inoculum was removed, and the cells were washed 2 times with HBSS and incubated in DMEM containing 5% HI-FBS and 0.4% human- γ -globulin (Sigma; final concentration, 0.5 mg/mL) for 24 hours to neutralize any extracellular virus. It is important to note that our PHKs are grown under differentiating conditions that induce functional TJs as demonstrated by enhanced transepithelial resistance and reduced permeability,^{E1} but they do not fully differentiate, as evidenced by the lack of filaggrin expression.

HSV-1 fluorescent-focus assay

Herpes simplex virus-infected PHKs were washed 2 times with PBS and fixed in 4% formaldehyde/PBS for 20 minutes at room temperature. A polyclonal rabbit anti-HSV-1 (Dako) antibody diluted 1:500 in PBS/1% BSA was placed on the PHK for 1 hour at 37°C followed by Alexa Fluor 488 donkey-antirabbit IgG H+L (1:1000; Invitrogen) and DAPI (1:10,000; Invitrogen). Coverslips were mounted onto slides with SlowFade (Invitrogen). For each sample, 6 random fields were captured at identical acquisition settings. Images were stored in Portable Network Graphics format and analyzed computationally to quantify differences objectively in FFUs. HSV-1 infected cells were assigned to the green channel. Cell density was calculated by counting the number of DAPI-labeled nuclei, assigned to the blue channel. Images were analyzed by using MATLAB (MathWorks, Inc, Natick, MA) to enumerate FFUs, and total cell number. The following FFU measurements were also taken: major axis length (μ m), area (μ m²), and pixel area (px).

The infected cell channel (green) was converted to a binary image using the Otsu method for threshold determination.^{E2} A morphologic closing (dilation followed by erosion) was performed to reduce noise and fuse individual infected cells into colonies. A distance transform was computed from the binary image, followed by a watershed transform. The colony binary image was then multiplied by its watershed image, the result was dilated slightly, and its perimeter was drawn. Only cells with sizes greater than a predefined threshold were included for further analysis. The infected cell channel (green) was filtered to reduce effects of noise. The resulting image was inverted, followed by a watershed transform. The infected cell watershed image was then multiplied by the colony watershed image to identify cells within previously identified colonies. Finally, a perimeter was drawn around cell borders.

Infectious center assay

Twenty-four hours postinfection, PHKs were treated with 0.05% trypsin-EDTA (Invitrogen/Gibco) for 5 minutes at 37°C followed by gently scraping to

lift the cells, washed 3 times in DMEM containing 1% HI-FBS, and vortexed for 30 seconds to disrupt cell clumps. Live cells were counted by using Trypan blue exclusion, and scalar PHK dilutions (1000 to 1 cells in 1 mL media) were plated onto pregrown monolayers of Vero cells in 6-well plates. After 2 hours, 1 mL DMEM containing 5% HI-FBS and 0.4% human- γ -globulin (Sigma; final concentration, 0.5 mg/mL) was added to each well. After 3 days of incubation at 37°C, cells were fixed (75% methanol/25% acetic acid) and stained with 0.1% crystal violet. Plaques were counted by 2 independent investigators, and results were expressed as a percentage of infected PHKs.

Quantitative PCR

Reverse transcription was performed from total RNA by using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol. Quantitative PCR (eg, real-time PCR) was performed by using the iQ SYBER Green Supermix assay system from Bio-Rad Laboratories. All PCR amplifications were carried out in triplicate on an iQ5 Multicolor real-time PCR detection system (Bio-Rad). Primers were designed and synthesized by Integrated DNA Technologies. Primers used in the study were as follows: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: GAA GGT GAA GGT CGG AGT C, and reverse: GAA GAT GGT GAT GGG ATT TC), CLDN1 (forward: CGA TGA GGT GCA GAA GAT GA, and reverse: CCA GTG AAG AGA GCC TGA CC), and PVRL1 (nectin-1; forward: AGC CAT TAA GGA GAA ACG A, and reverse: TTC CCA ATT TCT CTG CTC T).

Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches detection threshold.^{E3} The normalized Ct value of each sample was calculated by using GAPDH as an endogenous control gene.

Genetic study participants

DNA was isolated by using standard protocols from 258 unrelated European American patients with AD and 156 nonatopic healthy controls participating in the ADVN. The same set of markers was genotyped on 176 African American patients with AD.^{E4} AD was diagnosed by using the US consensus conference criteria.^{E5} ADEH⁺ was defined as AD patients with at least 1 EH episode documented by an ADVN investigator (or a physician affiliated with the same academic center) or diagnosed by another physician and confirmed by PCR, tissue immunofluorescence, Tzanck smear, and/or culture. AD severity was defined according to the Eczema Area and Severity Index, a standardized grading system,^{E6} and total serum IgE was measured. The study was approved by the institutional review boards at National Jewish Health, Johns Hopkins University, Oregon Health and Science University, the University of California San Diego, Children's Hospital of Boston, and the University of Rochester Medical Center. All subjects gave written informed consent before participation.

Genotyping and quality control

We performed genotyping on genomic DNA extracted from blood samples by using MagAttract DNA blood Mini M48 kit (QIAGEN) on a Biorobot M48, according to the manufacturer's instructions. DNA quantification was performed by using Pico-Green (Molecular Probes). Genotyping in these samples was determined for each of the selected tag single nucleotide polymorphism (SNP) with the Illumina GoldenGate custom panel containing 384-plex assays according to the manufacturer's protocol (Illumina Inc, San Diego, Calif).

Tagging SNPs were selected to represent the *CLDN1* gene in both the European American and African American groups. The SNP selection approach was to examine 10 kb upstream and 10 kb downstream in accordance with design score validations based on Illumina in-house measurements and the 60-bp limitation (a SNP cannot be closer than 60 bp to another SNP on this oligonucleotide pool assay). We initially selected all available *CLDN1* SNPs from the HapMap (<http://www.hapmap.org/>) to tag the linkage disequilibrium (LD) blocks in each of the racial groups (European American and African American). Tagging was based on the LDselect algorithm,^{E7,E8} with a minor allele frequency $\geq 10\%$ and an r^2 threshold of 0.80 (as reported in HapMap) to ensure

nearly perfect LD to infer information on all SNPs captured by the tag set. A final selection included 27 SNPs (Table E1) chosen for the Illumina oligonucleotide pool assay. Of the 27 tagging SNPs selected, 24 qualified as tagging SNPs from both the HapMap CEPH (Centre d'Etude du Polymorphisme Humain) Utah (CEU, with European ancestry) and the HapMap Yoruba (YRI, with African ancestry) samples; an additional 3 tagging SNPs (rs6800425, rs1155884, and rs9809713) were genotyped only in the African Americans. Two LD blocks were observed among the European American group (block 1, rs10212165, rs3954259, and rs9290929 [$D' = 0.982$ -1.0]; block 2, rs9835663 and rs3732923 [$D' = 0.976$]), and 3 LD blocks were observed among the African American group (block 1, rs3954259 and rs9290929 [$D' = 1.0$]; block 2, rs893051, rs9839711, and rs9835663 [$D' = 0.957$ -1.0]; block 3, rs6800425 and rs3774028 [$D' = 1$]) by using the criteria of Gabriel et al.^{E9}

The 27 SNPs were genotyped by using the custom-designed Illumina oligonucleotide pool assay for the BeadXpress Reader System and the GoldenGate Assay with VeraCode Bead technology (Illumina, Inc, San Diego, Calif) according to the manufacturer's protocol.^{E10} Briefly, the GoldenGate assay uses 3 primers designed for each locus. Two are specific to each allele at the SNP site, and a third hybridizes at a downstream locus from the site. All 3 primers have regions complementary to both genome and universal PCR primer sites. A total of 250 ng of high-quality genomic gDNA was plated and then activated. The activated DNA, paramagnetic particles, assay oligos, and hybridization buffer are combined in a hybridization step to allow DNA to bind to the particles. After hybridization of primers, plates were washed to reduce noise, and allele-specific oligos were extended and ligated to the downstream locus specific primer. This mix then served as a PCR template by using the universal primers, P1, P2, and P3. P1 and P2 are Cy3 and Cy5-labeled. After downstream processing, the single-stranded dye-labeled PCR products were hybridized to their complement VeraCode bead types. Plates were then scanned in the BeadXpress Reader for fluorescence and code identification. Scanned data and oligo assignments were uploaded into Illumina's BeadStudio software for downstream genotype cluster analysis. Genotyping quality was high, with an average completion rate of 97.2% to 98.2% for the BeadXpress genotyping.

The Cochran-Armitage trend test was used to test for association between each individual marker (under an additive model) and disease status with PLINK software (<http://pngu.mgh.harford.edu/~purcell/plink/>) and

confirmed with the adaptive permutation test. Analyses were performed for subjects of European and African ancestry separately to minimize confounding caused by racial differences in polymorphism frequency. We tested for association between genetic markers and total serum levels of log-adjusted IgE by using recessive logistic regression models adjusted for confounding variables including age and sex. Departures from Hardy-Weinberg equilibrium at each locus were tested by means of the χ^2 test separately for cases and controls using PLINK. Haplotype analyses were performed with PLINK by using sliding windows of 2 to 4 SNPs and the P values for haplotype frequency differences tested by 10,000 permutations.

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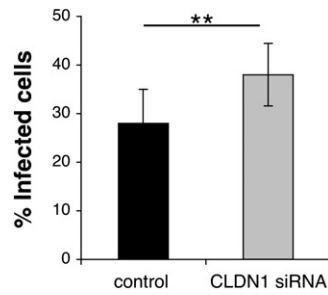


FIG E1. Knockdown of CLDN1 in human keratinocytes increases HSV-1 infectivity. Infectious center assay demonstrates that CLDN1 knockdown PHKs were more infected with HSV-1 ($38 \pm 6.43\%$; $n = 3$) than control transfected cells ($28 \pm 7\%$; $n = 3$; $**P = .003$).

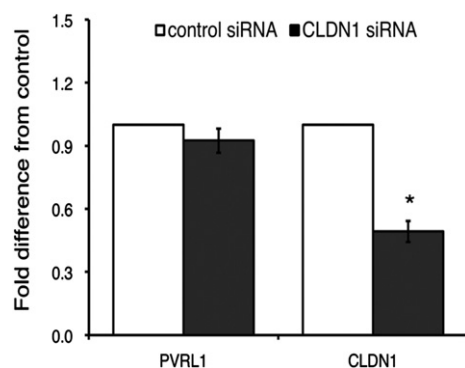


FIG E2. Silencing of CLDN1 does not affect expression of nectin-1 (PVRL1). CLDN1 siRNA (100 nmol/L) resulted in a 50% reduction in CLDN1 transcripts compared to control transfected cells (0.5 ± 0.06 fold; $*P = .5 \times 10^{-6}$; $n = 5$ /group). There was no effect on mRNA expression of nectin-1 (PVRL1; cldn-1 siRNA, 0.99 ± 0.16 ; control, 1.1 ± 0.14 ; $n = 5$ /group). Relative gene expressions were calculated by using the $2^{-\Delta\Delta C_t}$ method, in which C_t indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches detection threshold. The normalized C_t value of each sample was calculated by using GAPDH.

TABLE E1. *CLDN1* polymorphisms and minor allele frequencies

Gene	dSNP ID (build 129)	Location	Inter-SNP distance (bp)	Position	Type of variant	Minor allele frequency			
						CEU*	YRI*	European American† (n = 156)	African American† (n = 152)
CLDN1	rs6776530	191498394	0	C/A	Downstream	0.409	0.103	0.384	0.431
3q28	rs13092700	191502254	3860	A/G	Downstream	0.060	0.000	0.071	0.007
	rs7632915	191504142	1888	C/A	Downstream	0.525	0.192	0.394	0.224
	rs9290927	191505210	1068	A/T	Downstream	0.883	0.958	0.115	0.056
	rs17501010	191509348	4138	C/A	Intron	0.158	0.317	0.147	0.252
	rs3774032	191509554	206	G/A	Intron	0.142	0.042	0.210	0.059
	rs3774028	191510966	1412	T/A	Intron	0.415	0.034	0.332	0.082
	rs6800425	191513018	2052	G/A	Intron	0.542	0.108	NA	0.168
	rs6776378	191513220	202	G/A	Intron	0.198	0.263	0.276	0.311
	rs9869263	191513374	154	G/A	Coding exon	0.150	0.280	0.128	0.240
	rs10513846	191513683	309	G/A	Intron	0.158	0.108	0.136	0.056
	rs6809685	191515288	1605	G/A	Intron	0.833	0.703	0.424	0.482
	rs9866788	191515814	526	A/G	Intron	0.483	0.208	0.490	0.273
	rs9848283	191516502	688	A/G	Intron	0.491	0.096	0.442	0.194
	rs3732923	191517403	901	A/T	Intron	0.408	0.575	0.410	0.493
	rs9835663	191519452	2049	G/A	Intron	0.280	0.250	0.263	0.263
	rs9839711	191519713	261	C/G	Intron	0.100	0.158	0.126	0.178
	rs893051	191522295	2582	C/G	Intron	0.417	0.492	0.455	0.535
	rs12696600	191522901	606	A/C	5' UTR	0.446	0.198	0.457	0.287
	rs1155884	191523648	747	A/C	Promoter	0.534	0.458	NA	0.431
	rs16865347	191524091	443	A/G	Promoter	0.108	0.085	0.106	0.097
	rs9290929	191526433	2342	A/G	Promoter	0.433	0.133	0.410	0.240
	rs3954259	191527201	768	G/A	Promoter	0.533	0.167	0.513	0.293
	rs10212165	191528104	903	A/G	Promoter	0.150	0.250	0.173	0.237
	rs16865373	191529502	1398	G/A	Promoter	0.050	0.033	0.062	0.051
	rs9809713	191529746	244	G/A	Promoter	0.542	0.492	NA	0.496
	rs16865378	191529856	110	A/G	Promoter	0.050	0.000	0.045	0.017

NA, Not applicable.

*From HapMap (<http://www.hapmap.org>) for 60 Yoruba (YRI) and 60 Utah European American (CEU) founders.

†Minor allele frequencies from nonatopic healthy controls participating in the ADVN study.