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Reductions in Claudin-1 May Enhance Susceptibility to HSV-1 Infections in Atopic Dermatitis

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METHODS

Culture of Primary Human Foreskin Keratinocytes (PHK).

Human keratinocytes were isolated from neonatal foreskin[1]. PHK were cultured in Keratinocyte-SFM (Invitrogen/Gibco) with 1% Pen/Strep, 0.2% Amphotericin B (Invitrogen/Gibco). To differentiate PHK, cells were grown in DMEM (Invitrogen/Gibco) with 10% heat-inactivated fetal bovine serum (Invitrogen/Gibco) and 1% Pen/Strep, 0.2% Amphotericin B (Invitrogen/Gibco).

RNA Interference.

PHK were plated on glass coverslips at 2 - 3 x 10^5 cells/well in a 6-well plate or at 2 - 3 x 10^4 cells/filter in Transwell inserts (Costar; PET membrane, 0.4 µm pore size, 6.5 mm insert) in Keratinocyte-SFM without antibiotics. Next day after plating, cells were transfected with claudin-1 specific or control (scrambled) siRNAs (Santa Cruz) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen).

HSV-1 infection of PHK.

Infection studies were performed using the highly virulent HSV-1 strain F (provided by Dr. D.C. Johnson). Twenty-four to 48h post-transfection with 100 nM claudin-1 or control siRNA, the cells were differentiated in DMEM with 10% heat-inactivated FBS for 24h. Cells were washed twice with HBSS and infected with HSV-1 strain F at a multiplicity of infection (MOI) of 0.1 in DMEM containing 1% heat-inactivated FBS at 37°C, with rocking every 15 min. After 2h, the viral inoculum was removed, and the cells were washed 2x 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 hr to neutralize any extracellular virus. It is important to note that our PHK are grown under differentiating conditions that induce functional tight junctions as demonstrated by enhanced trans epithelial resistance and reduced permeability[1], but do not fully differentiate as evidenced by the lack of filaggrin expression.

HSV-1 Fluorescent-Focus Assay.

HSV-infected PHK were washed 2x with PBS and fixed in 4% formaldehyde/PBS for 20 min at room temperature. A polyclonal rabbit anti-HSV-1 (Dako) antibody diluted 1:500 in PBS/1% BSA was placed on the PHK for 1h at 37°C followed by Alexa Fluor 488 donkey-anti-rabbit IgG H+L (1:1000, Molecular Probes) and 4',6-diamidino-2-phenyl-indole, dihydrochoride (DAPI) (1:10,000, Molecular Probes). Coverslips were mounted onto slides with SlowFade (Molecular Probes). For each sample, six random fields were captured at identical acquisition settings. Images were stored in Portable Network Graphics (PNG) format and analyzed computationally to objectively quantify differences in focal forming units (FFU). 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 using MATLAB to enumerate FFU, and total cell number. The following FFU measurements were also taken: Major Axis Length (μm), Area (μm^2), and Pixel Area (px).

The infected cell channel (green) was converted to a binary image using the Otsu method for threshold determination. Then a morphological closing (dilation followed by erosion) was done to reduce noise and fuse individual infected cells into colonies. Then 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 post-infection, PHK were treated with 0.05% Trypsin - EDTA (Invitrogen/Gibco) for 5 min at 37°C followed by gently scraping to lift the cells, washed 3 times in DMEM containing 1% HI-FBS, and vortexed for 30 sec to disrupt cell clumps. Alive cells were counted using Trypan blue exclusion and scalar PHK dilutions (1000 to 1 cells in 1 ml of media) were plated onto pre-grown monolayers of Vero cells in six well plates. After 2h, 1 ml of 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 incubation at 37°C, cells were fixed (75% MeOH/25% acetic acid) and stained with 0.1% crystal violet. Plaques were counted by 2 independent investigators and results expressed as percent of infected PHK.

qPCR.

Reverse transcription was performed from total RNA using iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol. qPCR was performed 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: **GAPDH** (forward: GAA GGT GAA GGT CGG AGT C and reverse: GAA GAT GGT GAT GGG ATT TC); **Cldn-1** (forward**:** CGA TGA GGT GCA GAA GAT GA and reverse: CCA GTG AAG AGA GCC TGA CC); **PVRL1** (Nectin1; 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[2]. The normalized Ct value of each sample was calculated using GAPDH as an endogenous control gene.

Genetic Study Participants.

DNA was isolated using standard protocols from 258 unrelated European American AD patients and 156 non-atopic healthy controls participating in the ADVN. The same set of markers was genotyped on 176 African American AD patients[3]. AD was diagnosed using the US consensus conference criteria[4]. ADEH+ was defined as AD patients with at least one EH episode documented either 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' (EASI), a standardized grading system[5], and total serum IgE was measured. The study was approved by the institutional review boards at National Jewish Health, Johns Hopkins University, Oregon Health & Science University, University of California San Diego, Children's Hospital of Boston and University of Rochester Medical Center. All subjects gave written informed consent prior to participation.

Genotyping and Quality Control.

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

Tagging SNPs were selected to represent the *CLDN1* gene in both the EA and AA 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 OPA). 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 (EA and AA). Tagging was based on the LDSelect algorithm[6, 7], with a minor allele frequency (MAF)

≥10% and an r^2 threshold of 0.80 (as reported in HapMap) to ensure nearly perfect linkage disequilibrium (LD) in order to infer information on all SNPs captured by the tag set. A final selection included 27 SNPs (**Table E1**) chosen for the Illumina OPA. Of the 27 tagging SNPs selected, 24 qualified as tagging SNPs from both the HapMap CEPH Utah (CEU, with European ancestry) and the HapMap Yoruba (YRI, with African ancestry) samples; an additional three tagging SNPs (rs6800425, rs1155884, and rs9809713) were genotyped only in the AAs. 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 three 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)) using the criteria of Gabriel et al[8].

The 27 SNPs were genotyped using the custom-designed Illumina oligonucleotide pool assay (OPA) for the BeadXpress Reader System and the GoldenGate Assay with VeraCode Bead technology (San Diego, CA, USA) according to the manufacturer's protocol[9]. Briefly, the GoldenGate assay employs three 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 three primers have regions complementary to both genome and universal PCR primer sites. A total of 250 ng of high quality *g*DNA 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. Following 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 using the universal primers, P1, P2, and P3. P1 and P2 are Cy3 and Cy5 labeled. After down-stream processing, the single-stranded dye-labeled PCR products were hybridized to their complement VeraCode bead type. 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 97.2-98.2% 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 using PLINK software (http://pngu.mgh.harford.edu/~purcell/plink/to) and confirmed with the adaptive permutation test. Analyses were performed for subjects of European and African ancestry separately to minimize confounding due to racial differences in polymorphism frequency. We tested for association between genetic markers and total serum levels of log-adjusted IgE using recessive logistic regression models adjusted for confounding variables including age and gender.

Departures from Hardy-Weinberg equilibrium at each locus were tested by means of the Chi-squared test separately for cases and controls using PLINK.

Haplotype analyses were performed with PLINK using sliding windows of 2-4 SNPs and the P-values for haplotype frequency differences tested by 10,000 permutations.

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239 **Table E1**. *CLDN1* polymorphisms and minor allele frequencies (MAF)

						MINOR ALLELE FREQUENCY			
Gene	dSNP ID(build 129)	Location	Inter-SNP distance (bp)	Position	Type of Variant	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

240

241 [#]Minor allele frequencies (MAFs) from non-atopic healthy controls participating in the ADVN study.

242 *from HapMap (http://www.hapmap.org) for 60 Yoruba [YRI] and 60 Utah European American [CEU]

243 founders.

244

FIGURE LEGENDS

Figure E1. Knockdown of CLDN1 in human keratinocytes increases HSV-1 infectivity. Infectious center assay demonstrates that CLDN1 knockdown PHK were more infected with HSV-1 (38 ± 6.43%; n=3) than control transfected cells (28 ± 7%; n=3; ***P*=0.003).

Figure E2. **Silencing of CLDN1 does not affect expression of nectin-1 (PVRL1).** CLDN1 siRNA (100 nM) resulted in a 50% reduction in CLDN1 transcripts compared to control transfected cells (0.5% $\pm$ 0.6 fold; **P* = 0.5 x10^{-6}; *n* = 5/group). There was no effect on mRNA expression of nectin-1 (PVRL1; cldn-1 siRNA: 0.99 $\pm$ 0.16 and control: 1.1 $\pm$ 0.14; *n* = 5/group). Relative gene expressions were 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. The normalized Ct value of each sample was calculated using GAPDH.

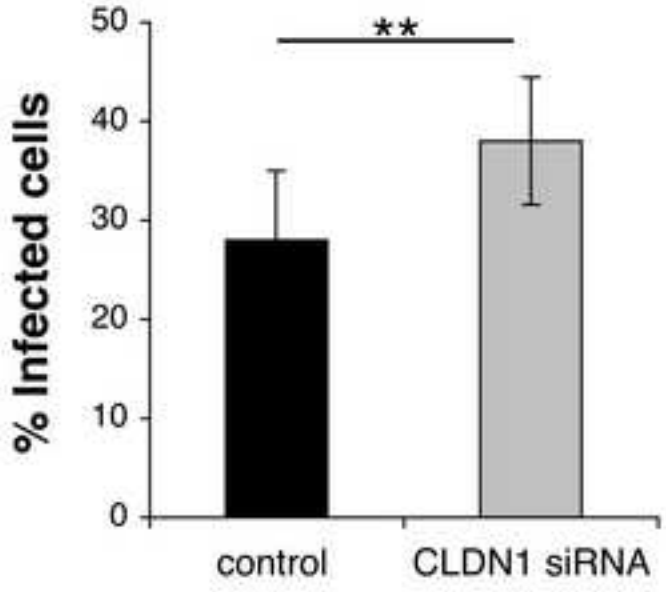

FIGURE E1

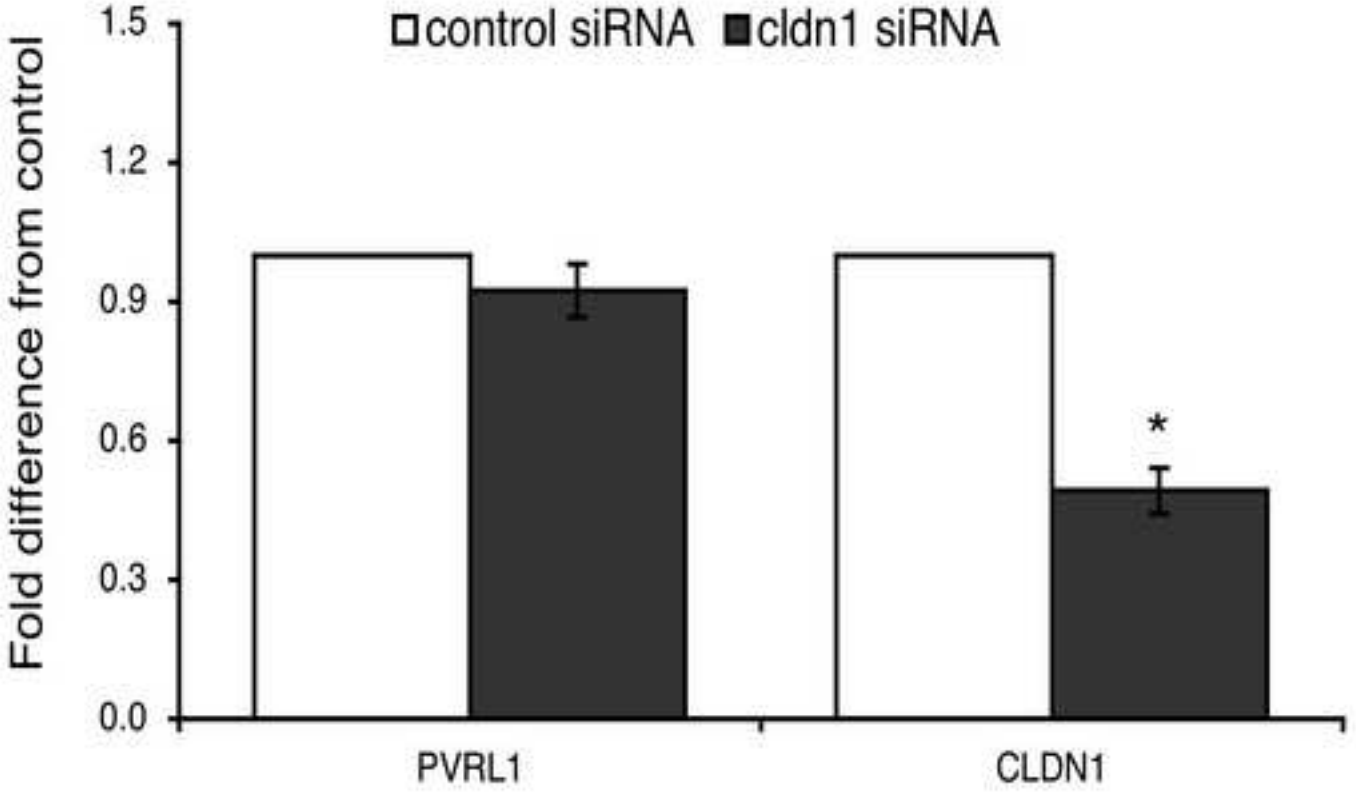
control siRNA
cldn1 siRNA
Fold difference from control
1.5
1.2
0.9
0.6
0.3
0.0
*
PVRL1
CLDN1

FIGURE E2