

# Tight junction defects in patients with atopic dermatitis

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**Background:** Atopic dermatitis (AD) is characterized by dry skin and a hyperactive immune response to allergens, 2 cardinal features that are caused in part by epidermal barrier defects. Tight junctions (TJs) reside immediately below the stratum corneum and regulate the selective permeability of the paracellular pathway.

**Objective:** We evaluated the expression/function of the TJ protein claudin-1 in epithelium from AD and nonatopic subjects and screened 2 American populations for single nucleotide polymorphisms in the claudin-1 gene (*CLDN1*).

**Methods:** Expression profiles of nonlesional epithelium from patients with extrinsic AD, nonatopic subjects, and patients with

psoriasis were generated using Illumina's BeadChips.

Dysregulated intercellular proteins were validated by means of tissue staining and quantitative PCR. Bioelectric properties of epithelium were measured in Ussing chambers. Functional relevance of claudin-1 was assessed by using a knockdown approach in primary human keratinocytes. Twenty-seven haplotype-tagging SNPs in *CLDN1* were screened in 2 independent populations with AD.

**Results:** We observed strikingly reduced expression of the TJ proteins claudin-1 and claudin-23 only in patients with AD, which were validated at the mRNA and protein levels. Claudin-1 expression inversely correlated with T<sub>H</sub>2 biomarkers. We observed a remarkable impairment of the bioelectric barrier function in AD epidermis. *In vitro* we confirmed that silencing claudin-1 expression in human keratinocytes diminishes TJ function while enhancing keratinocyte proliferation. Finally, *CLDN1* haplotype-tagging SNPs revealed associations with AD in 2 North American populations.

**Conclusion:** Collectively, these data suggest that an impairment in tight junctions contributes to the barrier dysfunction and immune dysregulation observed in AD subjects and that this may be mediated in part by reductions in claudin-1. (J Allergy Clin Immunol 2010;■■■■:■■■■-■■■■.)

**Key words:** Atopic dermatitis, claudin-1, tight junctions

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Atopic dermatitis (AD) is the most common inflammatory skin disease, affecting up to 17% of children and about 6% of adults in the United States.<sup>1,2</sup> Its cardinal features include dry eczematous skin lesions, a relapsing and often chronic course, and an intense intractable pruritus. Many of these features have been attributed in part to an acquired and/or genetic epidermal barrier defects. Disturbance of the epithelial barrier is now recognized as a common feature in many inflammatory diseases, including inflammatory bowel disease, celiac disease, sinusitis, food allergy, asthma, and AD.<sup>3,4</sup> A defect in barrier has been argued to favor the penetration or reactivity to microbes, allergens/antigens, and irritants into the dermis and possibly contribute to the T<sub>H</sub>2 immune response observed in early AD lesions.<sup>5-7</sup>

The skin has 2 barrier structures: the stratum corneum (SC) and tight junctions (TJs).<sup>8</sup> It is widely accepted in patients with AD that the SC is dysfunctional as the result of 1 or more of the following defects: (1) reduced levels of SC lipids<sup>9-11</sup>; (2) acquired or genetic defects in filaggrin (FLG)<sup>12-14</sup> or other epidermal differentiation proteins; (3) acquired or genetic defects in proteases, antiproteases, or both<sup>13,15</sup>; and/or (4) simply the consequence of the physical

**Abbreviations used**

|                                    |                                                              |
|------------------------------------|--------------------------------------------------------------|
| AA:                                | African American                                             |
| AD:                                | Atopic dermatitis                                            |
| ADVN:                              | Atopic Dermatitis and Vaccinia Network                       |
| CLDN1:                             | Claudin-1 gene                                               |
| EA:                                | European American                                            |
| EASI:                              | Eczema Area and Severity Index                               |
| FITC:                              | Fluorescein isothiocyanate                                   |
| FLG:                               | Filaggrin gene                                               |
| GAPDH:                             | Glyceraldehyde-3-phosphate dehydrogenase                     |
| LD:                                | Linkage disequilibrium                                       |
| OPA:                               | Oligonucleotide pool assay                                   |
| OR:                                | Odds ratio                                                   |
| P <sub>Cl</sub> /P <sub>Na</sub> : | Relative permeability of Cl <sup>-</sup> and Na <sup>+</sup> |
| PHK:                               | Primary human foreskin keratinocyte                          |
| qPCR:                              | Quantitative PCR (eg, real-time PCR)                         |
| SNP:                               | Single nucleotide polymorphism                               |
| SC:                                | Stratum corneum                                              |
| siRNA:                             | Small interfering RNA                                        |
| TEER:                              | Transepithelial electrical resistance                        |
| TEWL:                              | Transepidermal water loss                                    |
| TJ:                                | Tight junction                                               |
| ZO:                                | Zonulae occludens                                            |

trauma from widespread scratching that predates the development of all skin lesions.

TJs function as the “gate” for passage of water, ions, and solutes through the paracellular pathway.<sup>16</sup> TJs also regulate the localization of apical and basolateral membrane components. Whether epidermal TJs have this cell polarity function is still debated.<sup>17</sup> Some investigators have speculated that TJs might regulate the lipid components found in the SC.<sup>18,19</sup> This has promoted the notion that these 2 epidermal barrier structures interact in a dynamic way to ensure that the skin is in fact a formidable barrier (Fig 1). The structure and function of keratinocyte TJs remains an area of active investigation. TJs are composed of a number of transmembrane proteins, such as the claudin family, junctional adhesion molecule A, occludin, and tricellulin. In addition, several scaffolding proteins, such as zonulae occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein 1, membrane-associated guanylate kinase, and cingulin, have been identified in the TJ cytosolic plaque.<sup>18</sup> Claudins are 4-transmembrane-spanning proteins that determine TJ resistance and permeability and include more than 24 members.<sup>20-22</sup> Based on *in vitro* experiments, claudins have been divided into those that increase transepithelial electrical resistance (TEER) or enhance barrier, including claudin-1 and claudin-4, and claudins that reduce TEER and therefore disrupt barrier function, such as claudin-2 and claudin-6.<sup>23</sup>

Although the existence of TJ-like structures in the epidermis has been suggested for some time,<sup>24</sup> the functional relevance of these structures has been addressed only recently.<sup>21,25,26</sup> A major breakthrough came in 2002, when Furuse et al<sup>27</sup> reported that claudin-1-deficient mice died within 24 hours of birth with wrinkled skin, severe dehydration, and increased epidermal permeability, as assessed by dye studies and transepidermal water loss (TEWL).<sup>27</sup> Importantly, these mice had no abnormalities in the expression of SC proteins (eg, loricrin, involucrin, transglutaminase-1, or Krueppel-like factor 4) or lipids that might explain the severe skin phenotype. Although this murine model

clearly established the importance of claudin-1 in skin barrier function, very little is currently known about the role of claudin-1 (or TJs) in human skin diseases.

Dysfunction of keratinocyte TJs could explain many of the consequences of a defective skin barrier. For example, increased TEWL, which is a well-established measure of skin barrier integrity and is notably increased in both lesional and nonlesional skin of patients with AD, is not attributable to *FLG* mutations (Fig 1).<sup>28-31</sup> Thus other genetic or acquired defects in the skin barrier likely explain increased TEWL. Defective structure and function of TJs could also have immunologic consequences (Fig 1). For example, Kubo et al<sup>32</sup> recently demonstrated that activated Langerhans cells, the resident antigen-presenting cell in the epidermis, gain access to foreign antigens by sending dendrites out through epidermal TJs.<sup>32</sup> It seems likely that Langerhans cells will be more likely to sample surface antigens and allergens when epidermal TJs are compromised. This, coupled with the recent evidence that Langerhans cells may be specialized to induce the differentiation of naive CD4<sup>+</sup> T cells to T<sub>H</sub>2 cells, strongly supports the notion that a breach in TJs is likely a critical feature in the initiation of AD.<sup>33</sup>

In the current study we report for the first time the reduced expression of a key TJ protein, claudin-1, in AD epidermis, which was not observed in patients with psoriasis, a T<sub>H</sub>17-polarized inflammatory skin disease also associated with barrier defects.<sup>34,35</sup> *In vitro* findings confirm that reductions in claudin-1 expression, comparable with those observed in human skin samples, significantly affect TJ function by using 2 different measures of TJ integrity. Our *ex vivo* studies with full-thickness epidermal samples demonstrate remarkable bioelectric defects in AD nonlesional skin and provide more insight into the mechanism of epidermal barrier dysfunction characteristic of AD. Preliminary single nucleotide polymorphism (SNP) analysis suggests that the claudin-1 gene (*CLDN1*) might be a novel susceptibility gene for AD. Collectively, our studies strongly suggest that claudin-1 might be a key determinant of skin barrier dysfunction in patients with AD and may play a role in the T<sub>H</sub>2 polarization characteristically observed in most subjects.

## METHODS

### Study participants: Expression profiling and validation experiments

The diagnosis of AD was made using the US consensus conference criteria.<sup>36</sup> All patients with AD had extrinsic disease, as defined by a serum total IgE level 2 SDs or more of age-dependent norms and a positive multiallergen RAST result (ImmunoCap Phadiatop). Nonatopic healthy subjects were defined as having no personal or family history of atopic diseases, no personal history of chronic skin or systemic diseases, and a serum total IgE level that was 2 SDs or less of age-dependent norms and a negative Phadiatop. The diagnosis of psoriasis was based on characteristic clinical features of plaque-type lesions by a board-certified dermatologist. Exclusion criteria were as follows: age of 18 years or less or 60 years or greater, use of systemic immunosuppressive therapy, use of leukotriene inhibitors within the last 6 weeks, use of topical steroids or calcineurin inhibitors within the last 6 weeks at the site of blister formation or biopsy, and presence of a recent systemic infection or course of oral antibiotics within the last 2 weeks. All subjects either underwent an epidermal procurement procedure (see below) or a 4-mm punch biopsy of their non-sun-exposed forearm. These studies were approved by the Research Subject Review Boards at the Johns Hopkins Medical Institution, the University of Rochester Medical Center, or both. All subjects provided written informed consent.

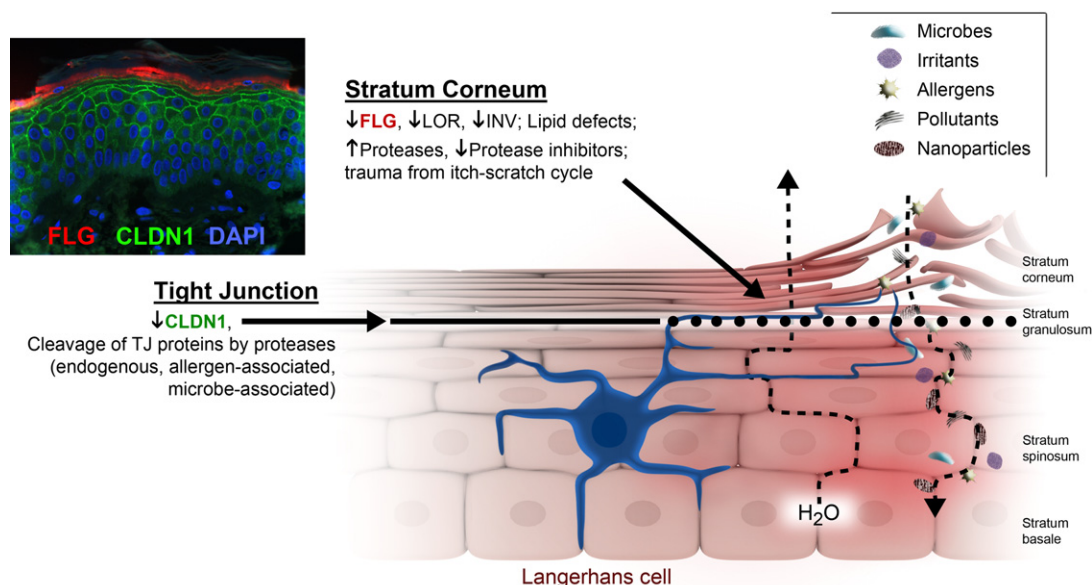


**FIG 1.** Skin epithelium is uniquely armed with 2 barrier structures: the SC and TJs. The SC is the outermost structure, consisting of multiple layers of enucleated keratinocytes called corneocytes. The SC barrier is maintained by the complex interaction of the cornified envelope, intracytoplasmic moisturizing factors, and a complex lipid mixture in the extracellular space.<sup>8</sup> TJs are located just below the SC at the level of the stratum granulosum. In this article we present evidence that this structure might be compromised in patients with AD, and this may be due to reductions in claudin-1 levels. These 2 barrier structures interact in a dynamic way to ensure that the skin maintains a formidable barrier. It is our premise that SC defects alone would not lead to the development or exacerbation of AD. We propose that a breach in both the SC and TJs would be required for sensitization to antigens/allergens, irritants, microbes, pollutants, or possibly even nanoparticles. A recent study demonstrates that the transient disruption of TJs results in the incorporation of Langerhans cell dendrites within a temporary TJ.<sup>32</sup> This extension of the Langerhans cell dendrite would enable antigen uptake and processing and might confer activation signals that would determine the immunologic fate of the antigen-presenting cell–T-cell interaction that follows. A fluorescence image of healthy human epidermis (*left*) demonstrates the expression patterns of 2 key players in AD pathogenesis, namely *FLG* (red) and *CLDN1* (green), and pictorially highlights the orientation of the 2 skin barrier components. *DAPI*, 4',6-Diamidino-2-phenylindole; *INV*, involucrin; *LOR*, loricrin.

## Epidermal procurement and processing

An NP-2 negative pressure vacuum apparatus (Electronic Diversities, Finksburg, Md) was applied to the volar forearm (*Fig 2, B-D*). The blister roof, which consists of full-thickness epidermis, was removed by using a sterile technique and placed in Hank's balanced salt solution (Invitrogen, Carlsbad, Calif). Total RNA was extracted from epidermis by using the QIAshredder spin column and RNeasy RNA isolation kits (Qiagen, Hilden, Germany). The quality of total RNA samples (RNA integrity number) was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif). Samples were selected for microarray analysis if they had a good RNA integrity number (range, 8-10).

## Gene expression profiling

Biotin-labeled complementary RNA was prepared from total RNA according to the manufacturer's protocol (Illumina, San Diego, Calif). Complementary RNA was hybridized to Illumina Sentrix HumanRef-8 Expression BeadChips (Illumina), which contain 24,350 probes corresponding to 21,429 unique genes. Signal intensity quantification was performed with an Illumina BeadStation 500GX Genetic Analysis Systems scanner.

Preliminary analysis of the scanned data was performed with Illumina BeadStudio software, which returns single-intensity data values for each probe after the computation of a trimmed mean average for each probe represented by a variable number of bead probes on the array. *Z* transformation for normalization was performed on each Illumina sample/array on a stand-alone basis, and *z* ratios were calculated by taking the difference between the averages of the observed gene *z* scores and dividing by the SD of all the differences for that particular comparison.<sup>37</sup> Significant changes in gene expression between class pairs were calculated by using the *z* test,<sup>38</sup> and significant genes were

defined as those that satisfy a *z* test *P* value of  $\pm 1.5E-3$ . The BeadStudio expression values for each sample/array were scaled to have a median of 256 and then  $\log_2$  transformed.

## Quantitative PCR

Quantitative PCR (qPCR) was performed with the iScript cDNA Synthesis kit and the iQ SYBER Green Supermix assay system (Bio-Rad Laboratories, Hercules, Calif). All PCR amplifications were carried out in triplicate on an iQ5 Multicolor real-time PCR detection system (Bio-Rad Laboratories). Primers were designed and synthesized by Integrated DNA Technologies (see *Table E1* in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Relative gene expression was calculated by using the  $2^{-\Delta\Delta C_t}$  method.<sup>39</sup> The normalized threshold cycle value of each sample was calculated by using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous control gene.

## Imaging of TJ proteins

For immunohistochemical staining, the following antibodies were used: claudin-1 (0.2  $\mu$ g/mL, JAY.8; Zymed, South San Francisco, Calif), occludin (2.5  $\mu$ g/mL, OC-3F10; Zymed), ZO-1 (2.5  $\mu$ g/mL, ZO1-1A12; Zymed), or isotype control. Five-micrometer sections from formalin-fixed skin biopsy specimens were deparaffinized and rehydrated. Slides were incubated in 1× EDTA solution, pH 8.0, at 95°C for 10 minutes. Samples were incubated overnight at 4°C, with primary antibodies titrated to the lowest concentration that produced immunoreactivity in control samples. The secondary antibodies and detection system used were reported previously.<sup>40</sup> For immunofluorescence labeling, skin samples were incubated in blocking solution (5% BSA, 0.1% saponin, and 1 mmol/L calcium in PBS) for 20 minutes, followed by

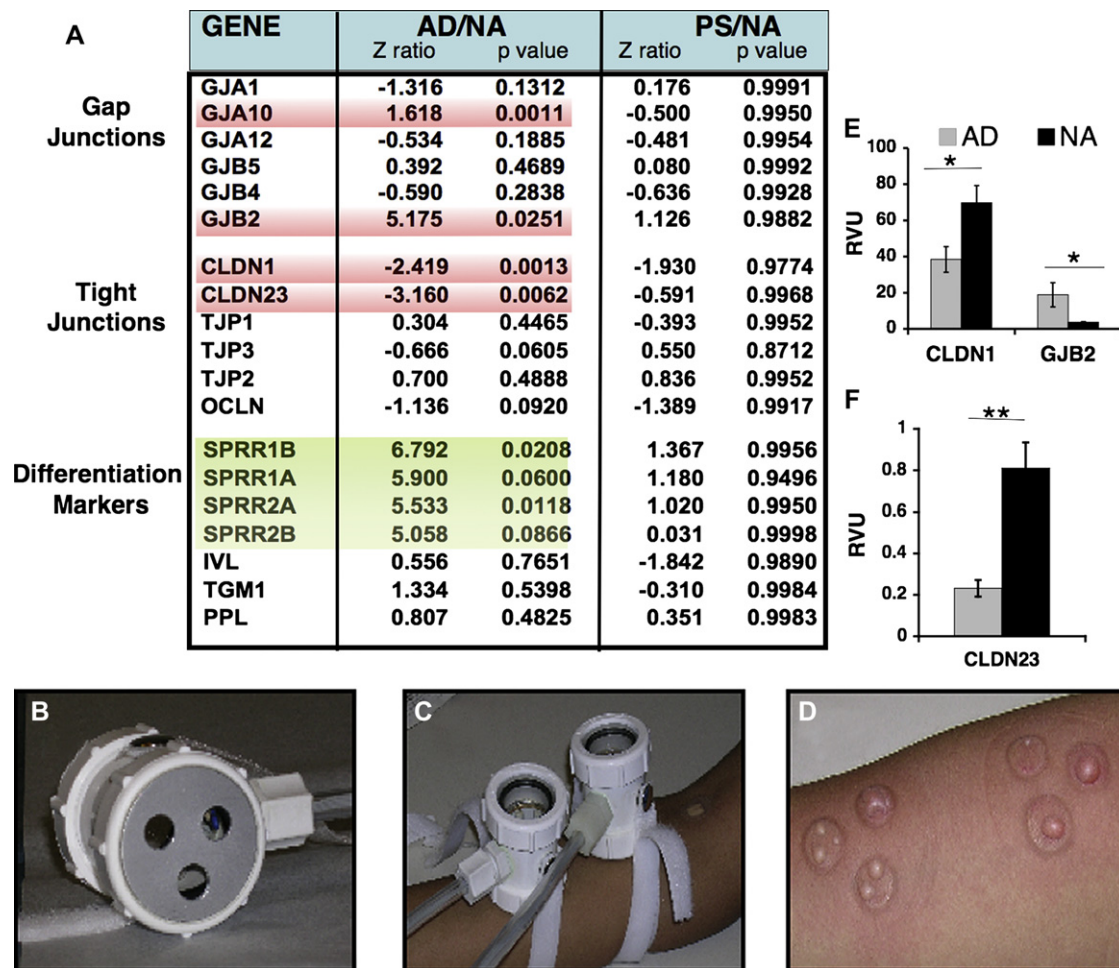


**FIG 2.** Interacellular junction proteins are dysregulated in patients with AD. **A-D**, z ratios from gene arrays performed on nonlesional epithelium (blister roofs; **B-D**). Claudin-1 and claudin-23 levels were reduced, whereas the gap junction proteins connexin-26 (*GJB2*) and connexin-62 (*GJA10*) were upregulated (**A**, red). Expression of genes indicative of dedifferentiation were either unaffected or increased (**A**, yellow). **E** and **F**, Validation of the key genes in newly recruited AD/nonatopic (NA) control samples. \**P* = .03 and \*\**P* = .001. *GJA* and *B*, gap junction; *IVL*, involucrin; *OCLN*, occludin; *PPL*, periplakin; *SPRR*, small proline rich proteins; *TGM1*, transglutaminase 1; *TJP1-3*, ZO-1 to 3.

90 minutes of incubation with primary antibodies diluted in blocking solution. This was followed by a 1-hour incubation with secondary antibodies: 1:1,000 AlexaFluor 488 donkey-anti-rabbit IgG H+L (Molecular Probes, Eugene, Ore), 1:1,000 Alexa Fluor 568 donkey-anti-mouse IgG H+L (Molecular Probes), and 1:10,000 4',6-diamidino-2-phenylindole (Molecular Probes). Primary human keratinocytes (PHKs) grown on transwell filters or a glass cover slip were fixed in methanol at  $-20^{\circ}\text{C}$  for 15 minutes, followed by blocking in PBS with 1% BSA and immunolabeled with the above TJ antibodies.

Fluorescent images were obtained with an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) using the FV10-ASW 1.7 software (Olympus). The Alexa Fluor 488 and 568 signals were imaged sequentially in frame interlace mode to eliminate crosstalk between channels. The saturation level of fluorescence intensity was set for the controls by using the Hi-Lo feature of the Fluoview software. For each experiment, the images were captured at identical settings during image acquisition, and no manipulations of the images occurred before importing into the FV1000 software at the workstation.

### Ex vivo electrophysiologic measurements in Ussing chambers

Isolated blister roofs (1 cm in diameter; Fig 2, **B-D**) were placed in the Ussing chamber in 2-mm-diameter sliders for Snapwell chambers (area,

$0.031\text{ cm}^2$ ; Physiologic Instruments, San Diego, Calif), bathed in Ringer lactate, and gassed with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The tissues were continuously voltage clamped to zero by using VCC MC8 (Physiologic Instruments). Transepithelial short-circuit current (expressed as micro-Angstroms per square centimeter of tissue surface area) was measured, and total tissue conductance (expressed as milliSiemens [mS] per square centimeter of tissue surface area) was calculated with Ohm's law by applying a 5-mV transepithelial pulse every 20 seconds and measuring the resulting current deflections.

Dilution potential measurements were performed to determine the changes in the permeability ratio between  $\text{Na}^+$  and  $\text{Cl}^-$  by using the Nernst equation.<sup>41-43</sup> Briefly, the tissues were allowed to equilibrate in the Ussing chamber for 45 minutes. Ten nanomoles per liter of bumetanide was added to both the apical/corneum and basolateral/basal bathing solutions to block  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransport, which feeds transepithelial  $\text{Cl}^-$  secretion through apical channels. Dilution potentials were induced by means of apical perfusion, basolateral perfusion, or both with Ringer solutions containing 2 different concentrations of  $\text{Na}^+$  (140 and 70 mmol/L) and  $\text{Cl}^-$  (119.8 and 50 mmol/L), and the remainder was replaced with mannitol to maintain equal osmolality between experiments. Dilution potentials were corrected for changes in junction potential (usually  $<1\text{ mV}$ ). Changes in membrane voltage ( $E_m$ ), along with known concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in the respective solutions, were

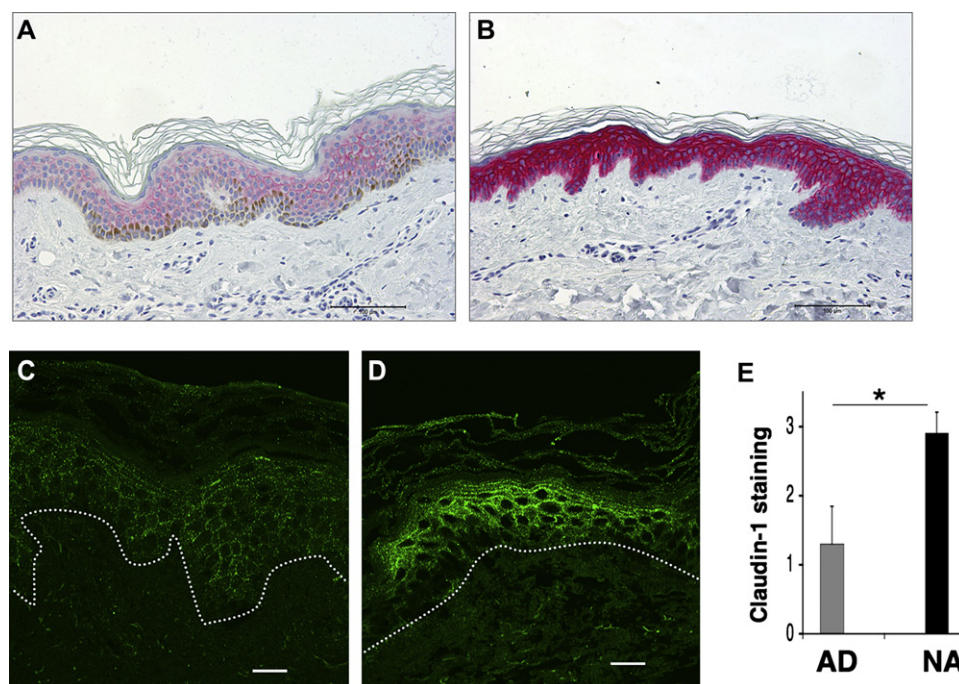


**FIG 3.** Claudin-1 expression is markedly reduced in AD epidermis. **A** and **B**, Reduced claudin-1 immunoreactivity was noted in nonlesional AD skin ( $n = 11$ ; **A**) compared with skin from nonatopic subjects ( $n = 12$ ; **B**). Red indicates positive staining ( $\text{bar} = 100 \mu\text{m}$ ). **C** and **D**, By using an FITC-conjugated secondary antibody, claudin-1 had a membranous pattern in both patients with AD (**C**) and nonatopic control subjects (**D**). The signal intensity was significantly reduced in AD epidermis (**C**). Positive staining is indicated in green ( $\text{bar} = 20 \mu\text{m}$ ). The dotted line denotes the epidermal-dermal junction. **E**, Semiquantitative scoring confirmed reduced epidermal expression of claudin-1 in patients with AD ( $1.3 \pm 0.3$ ) compared with that seen in nonatopic (NA) subjects ( $2.9 \pm 0.1$ ,  $*P < .0004$ ).

substituted in the modified Nernst equation to determine the change in permeability ratio between  $\text{Cl}^-$  and  $\text{Na}^+$  ( $P_{\text{Cl}}[\text{Cl}]_i/P_{\text{Na}}[\text{Na}]_i$ ).

$$E_m = RT/F^* 2.303 \log_{10} \{ P_{\text{Na}}[\text{Na}]_o + P_{\text{Cl}}[\text{Cl}]_i / P_{\text{Na}}[\text{Na}]_i + P_{\text{Cl}}[\text{Cl}]_o \} R = 8.314472 (\text{J/K/mol});$$

$$F = 96.48531 (\text{KJ/mol}); \text{ Permeability ratio } (\beta) = P_{\text{Cl}}/P_{\text{Na}};$$

$$T = 310 (\text{Kelvin}).$$

### Permeability studies in Ussing chambers

For the permeability assay, 0.02% fluorescein isothiocyanate (FITC)–albumin (Sigma, St Louis, Mo) was added to the apical side of the inserts, and buffer samples were collected at several time points (0.5–3 hours) from the other side of the membrane. Permeability was assessed by using a spectrophotometer (Multiskan EX; Thermo Electron Corporation, Vantaa, Finland) at 490 nm. Permeability data were expressed as FITC fluorescence intensity normalized to sample area and time (OD per square centimeter per hour).

### Culture of foreskin PHKs

Human keratinocytes were isolated from neonatal foreskin.<sup>44</sup> PHKs were cultured in Keratinocyte-SFM (Invitrogen/Gibco) with 1% Pen/Strep and 0.2% Amphotericin B (Invitrogen/Gibco). Cells were grown in Dulbecco modified Eagle medium (Invitrogen/Gibco) with 10% heat-inactivated FBS (Invitrogen/Gibco) and 1% Pen/Strep and 0.2% Amphotericin B (Invitrogen/Gibco) to differentiate PHKs. For TJ modulation experiments, the following cytokines were added alone or in combination to the differentiation media: human IL-4 (50 ng/mL; R&D Systems, Minneapolis, Minn) and IL-13 (50 ng/mL, R&D Systems).

### TEER and paracellular flux

PHKs were plated at a subconfluent density of  $2.5$  to  $3 \times 10^4$  cells per filter in Transwell insert and cultured in Keratinocyte-SFM media until confluent. TEER was measured with an EVOMX volttohmmeter (World Precision Instruments, Sarasota, Fla). The resistance of cell-free filters was subtracted from each experimental value.

To evaluate the paracellular flux of PHKs, 0.02% fluorescein (FITC) sodium (Sigma) in Hanks buffered saline solution was added to the upper chamber. Samples were collected from the lower chamber at different times (0.5–3 hours). The amount of FITC that had diffused from the apical to the basal side of the filter was measured with a spectrofluorimeter (Multiskan EX, Thermo Scientific, Milford, Mass), with excitation/emission wavelengths of 490/514 nm. Paracellular permeability was presented as follows:

$$\text{Relative fluorescence flux} = \text{Experimental condition/Filter alone} \times 100.$$

### Immunoblotting

PHKs were lysed on ice in RIPA lysis buffer (20 mmol/L Tris; 50 mmol/L NaCl; 2 mmol/L EDTA; 2 mmol/L ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid; 1% sodium deoxycholate; 1% TX-100; and 2% SDS, pH 7.4), with 1:100 Protease Inhibitor Cocktail (Sigma) and 1:100 Phosphatase Inhibitor Cocktail (SigmaCells) for 30 minutes at 4°C. The samples were heated at 95°C for 10 minutes and then centrifuged at 14,000 rpm for 15 minutes. Forty micrograms of protein, as determined by means of BCA (Pierce, Cheshire, United Kingdom) in NuPage LDS Sample Buffer (Invitrogen), was applied to 4–12% NuPage Bis-Tris gels (Invitrogen). Electrophoresis was performed under reducing conditions with MES SDS Buffer (Invitrogen). Membranes were incubated in blotting solution (5% nonfat dry milk in PBS plus 0.05% Tween 20) at room temperature for 1 hour and then incubated with primary antibodies: claudin-1 (JAY.8; Zymed), occludin (OC/3F10; Zymed), and GAPDH (FL-355; Santa Cruz Biotechnology, Santa Cruz, Calif). A horseradish peroxidase-linked secondary antibody

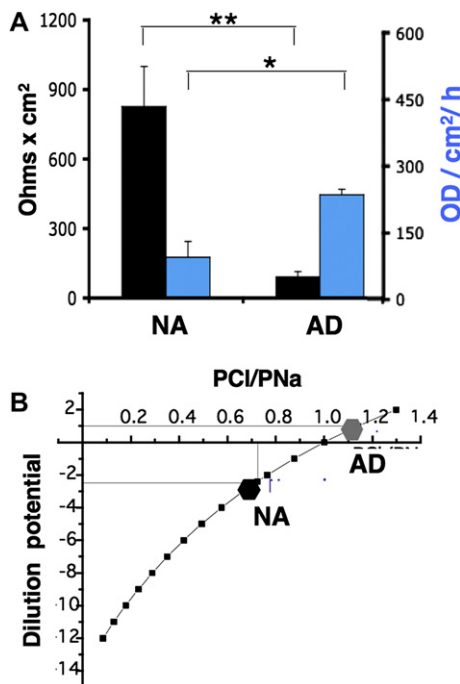


**FIG 4.** AD epidermis has altered bioelectric properties compared with epidermis from nonatopic (NA) control subjects. **A**, Ussing chamber measurements reveal a markedly reduced resistance ( $92 \pm 22 \Omega \times \text{cm}^2$ ,  $n = 4$ ) in AD epithelium compared with that from control subjects ( $827 \pm 173 \Omega \times \text{cm}^2$ ,  $P = .01$ ,  $n = 4$ ). This was reflected in an increased permeability to FITC-conjugated albumin in patients with AD ( $445 \pm 24 \text{ OD}/\text{cm}^2/\text{h}$ ,  $n = 4$ ) compared with that seen in nonatopic control subjects ( $175 \pm 68 \text{ OD}/\text{cm}^2/\text{h}$ ,  $P = .02$ ,  $n = 4$ ). **B**, Dilution potential studies noted the preservation of membrane selectivity in nonatopic subjects, with  $\text{Na}^+$  ions relatively more permeable than  $\text{Cl}^-$  ions ( $0.77 \pm 0.03$ -fold  $P_{\text{Cl}}/P_{\text{Na}}$ ). In contrast, the selectivity was completely lost in AD epidermis ( $1.10 \pm 0.02$ -fold  $P_{\text{Cl}}/P_{\text{Na}}$ ,  $P = .001$ ,  $n = 3$  per group), and both ions were equally permeable.

(GE Healthcare, Fairfield, Conn) was used with ECL (GE Healthcare) to visualize bands by means of autoradiography with Kodak BioMax MR film (Kodak, Rochester, NY). The pixel intensity of each band was estimated with Image J software.

## RNA interference

PHKs were plated on glass cover slips at  $2$  to  $3 \times 10^5$  cells per well in a 6-well plate or at  $2$  to  $3 \times 10^4$  cells per filter in a Transwell insert (Costar; PET membrane,  $0.4\text{-}\mu\text{m}$  pore size,  $6.5\text{-mm}$  insert) in Keratinocyte-SFM without antibiotics. The next day after plating, cells were transfected with claudin-1-specific or control (scrambled) small interfering RNAs (siRNAs; Santa Cruz Biotechnology) by using Lipofectamine 2000 transfection Reagent (Invitrogen). TEER, permeability, and proliferation experiments were conducted 48 hours after PHKs were switched to differentiation media.

## Proliferation assay

To evaluate cell proliferation we used Click-iT EdU (5-ethynyl-2'-deoxyuridine) kit following manufacturer's instructions (Molecular Probes, Eugene, Ore). Cells were stained with DAPI to identify nuclei and assess cell density. For each sample, 6 random fields (magnification  $\times 200$ ) were captured. EdU positive cells were counted by 2 investigators blinded to the conditions, and results expressed as mean EdU positive cells/hpf.

## Genetic study participants

DNA was isolated by using standard protocols from 258 unrelated European American (EA) patients with AD and 156 healthy control subjects

participating in the Atopic Dermatitis and Vaccinia Network (ADVNet). The same set of markers was genotyped in 176 African American (AA) patients with AD and 152 healthy control subjects. Baseline characteristics are presented in Table I, and further details can be found in previous publications.<sup>45</sup> AD was diagnosed by using the US consensus conference criteria.<sup>36</sup> Nonatopic healthy control subjects were defined as having no personal history of chronic disease, including atopy. AD severity was defined according to the Eczema Area and Severity Index (EASI), a standardized grading system,<sup>46</sup> and total serum IgE levels were 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 provided written informed consent before participation.

## Genotyping and quality control

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

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 (an SNP cannot be closer than 60 bp to another SNP on this oligonucleotide pool assay [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 ethnic groups (EA and AA subjects). Tagging was based on the LDSelect algorithm,<sup>47,48</sup> with a minor allele frequency of 10% or greater and an  $r^2$  threshold of 0.80 (as reported in HapMap) to ensure nearly perfect LD and infer information on all SNPs captured by the tag set. A final selection included 27 SNPs 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 3 tagging SNPs (rs6800425, rs1155884, and rs9809713) were genotyped only in the AA subjects. Two LD blocks were observed among the EA 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 AA 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.<sup>49</sup>

The 27 SNPs were genotyped by using the custom-designed Illumina OPA for the BeadXpress Reader System and the GoldenGate Assay with VeraCode Bead technology (San Diego, Calif), according to the manufacturer's protocol.<sup>50</sup> Genotyping quality was high, with an average completion rate of 97.2% to 98.2% for the BeadXpress genotyping (see the complete *Methods* section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

All samples were also genotyped for the 2 *FLG* mutations most commonly associated with AD in EA subjects (R501X and 2282del4), plus 9 additional polymorphisms (rs12730241, rs2065956, rs11582620, rs3126082, rs6587665, rs11204980, rs1933063, rs1933064, and rs3126091), as previously described.<sup>51</sup> Interaction among *FLG* mutations previously associated with AD and haplotype-tagging SNPs in *FLG* and *CLDN1* SNPs were investigated by using PLINK epistasis. DNA samples from subjects enrolled for the immunostaining study were also genotyped for the same 2 *FLG* null mutations.

## Statistical analysis

Data were expressed as means  $\pm$  SEMs of 3 or more experiments. Differences between groups were evaluated by using an appropriate  $t$  test. A  $P$  value of .05 or less was considered statistically significant. The statistical analyses performed as part of the expression profiling or SNP analyses are

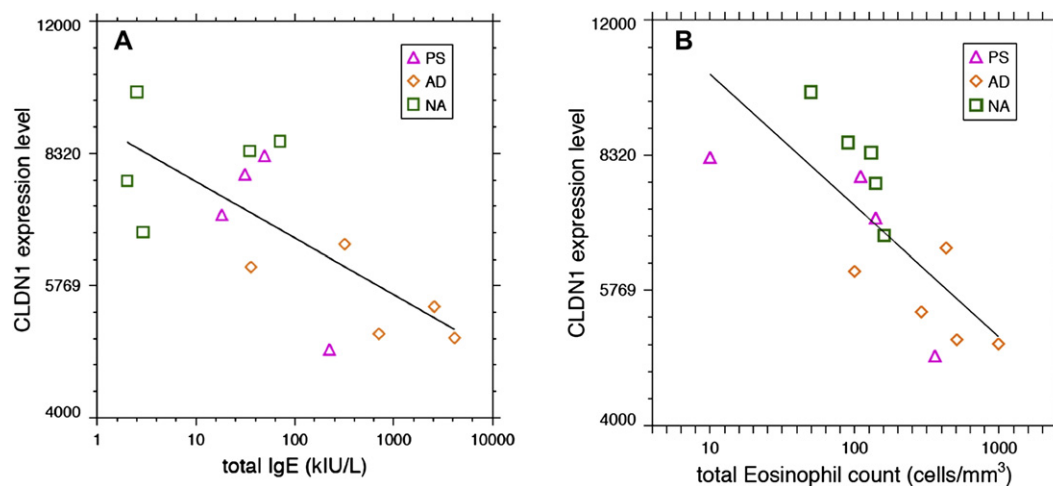


**FIG 5.** Claudin-1 expression from the gene arrays correlates with  $T_H2$  biomarkers. **A**, The line represents the linear least square fit for  $\log_2$  *CLDN1* expression level versus  $\log_{10}$  total serum IgE level (Pearson product-moment correlation coefficient [ $n = 14$ ]:  $r = -0.718$ ,  $P = .0038$ ). **B**, The plot of  $\log_2$  *CLDN1* expression level versus  $\log_{10}$  total eosinophil count ( $n = 14$ ;  $r = -0.761$ ,  $P = .0016$ ). The disease phenotypes (psoriasis [PS], AD, and nonatopic control subjects [NA]) are denoted by unique symbols.

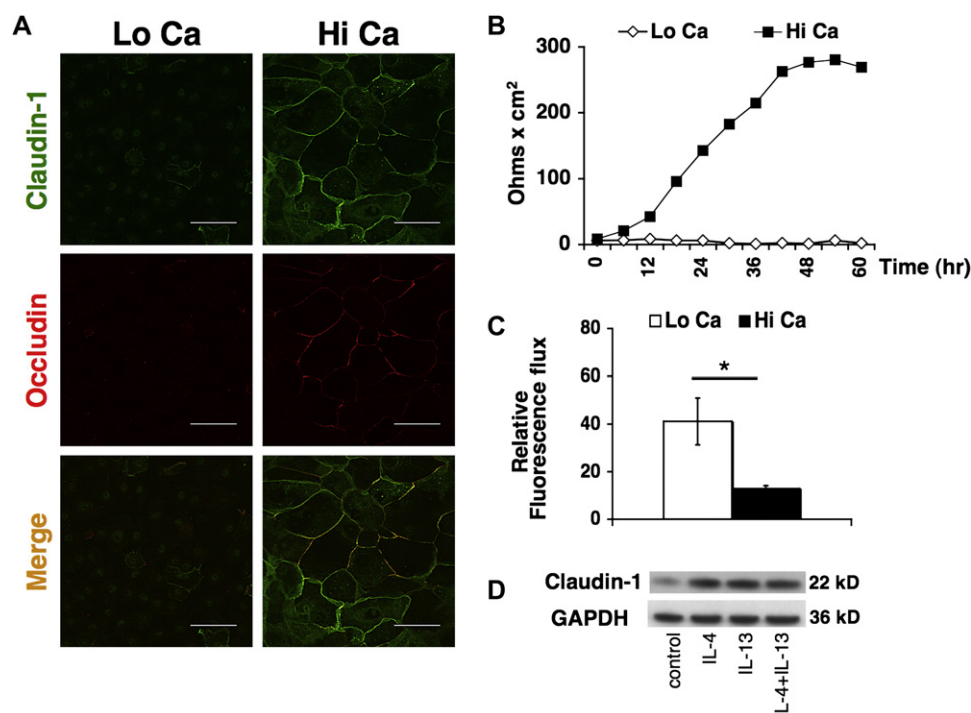


**FIG 6.** Claudin-1 colocalizes with other TJ proteins at the cell membrane in differentiated keratinocytes, and this coincides with TJ function. **A**, In  $Ca^{2+}$ -differentiated PHKs (*Hi Ca*) claudin-1 (green) colocalizes with occludin (red) at the cell membrane (bars = 50  $\mu m$ ). **B**, TEER is only observed in differentiated PHKs and peaks about 40 hours after the increase in  $Ca^{2+}$  level (representative of  $n = 5$ ). **C**, Paracellular diffusion of 0.02% sodium fluorescein is markedly reduced in differentiated PHKs ( $3.1 \pm 0.6$ -fold,  $*P = .046$ ,  $n = 3$ ). **D**, Western blots show enhanced expression of claudin-1 when PHKs were differentiated for 48 hours in the presence of stimulation with IL-4 (50 ng/mL), IL-13 (50 ng/mL), or both cytokines (representative of  $n = 3$ ).

described in that section. 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/to>). 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 the quantitative measure of severity, EASI, by using recessive logistic regression models. Departures from Hardy-Weinberg equilibrium at each locus were tested by means of the  $\chi^2$  test separately for cases and control subjects by using PLINK; all SNPs were in Hardy-Weinberg equilibrium. Tests for

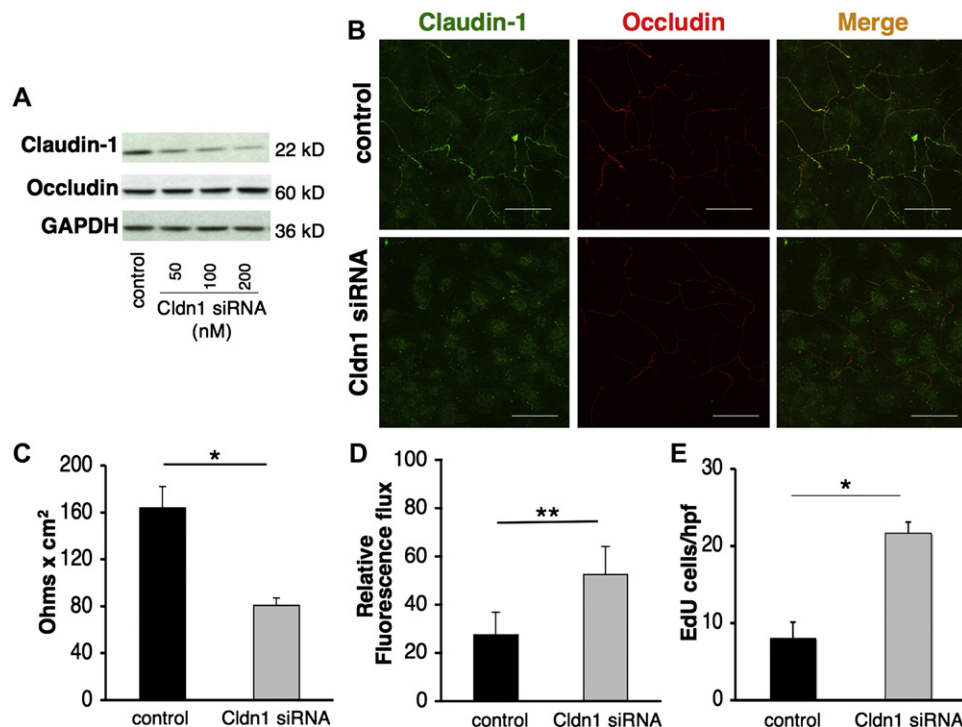


**FIG 7.** Claudin-1 silencing reduces TEER, increases paracellular permeability, and enhances cell proliferation. **A**, Western blots demonstrated a dose-dependent reduction of claudin-1 with *CLDN1* siRNA (48 hours). No change was observed for occludin. **B**, Immunostaining confirmed that claudin-1, but not occludin, expression was reduced in differentiated PHKs after transfection with *CLDN1* siRNA (bars = 50  $\mu$ m). **C**, Claudin-1 knockdown significantly reduced TEER (control:  $164.0 \pm 18.2$  and *CLDN1* siRNA:  $80.6 \pm 6.4$ ,  $*P = .007$ ,  $n = 4$ ). **D**, Increased sodium fluorescein permeability (control:  $27.6 \pm 11.7$  and *CLDN1* siRNA:  $52.5 \pm 9.2$ ,  $**P = .026$ ,  $n = 4$ ). **E**, Enhanced cell proliferation, as assessed by using the Click-iT EdU assay ( $**P = 0.002$ ,  $n = 3$ ).

association with a  $P$  value of less than .05 were then confirmed with the PLINK max(T) test by using 10,000 permutations.

## RESULTS

### Claudin-1 expression is markedly reduced in nonlesional AD epidermis

To characterize and quantify the expression of human epidermal proteins important for barrier function, we performed gene expression profiling of nonlesional or clinically unaffected epidermis by using blister roofs from patients with AD, patients with psoriasis, and nonatopic subjects (Fig 2, B-D). The Illumina Sentrix HumanRef-8 Chip contained 43 TJ genes (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), 8 gap junction genes, and 41 epidermal differentiation complex genes. A number of the epidermal differentiation complex genes were differentially expressed in epidermis from patients with AD versus nonatopic nonlesional epidermis, and several (eg, *S100A8*, *S100A7*, *FLG*, and *LOR*; see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) corroborate the findings observed by others in lesional skin biopsy specimens.<sup>52,53</sup> Of note, *FLG* was downregulated in patients with AD versus nonatopic subjects ( $z$  ratio,  $-5.29$ ); however, the  $P$  value of .01 did not reach the significance threshold. Using the expression profiling array data from nonatopic control subjects, we verified that human keratinocytes express claudin-1, claudin-4, claudin-8, claudin-12, and claudin-14<sup>54-56</sup> and demonstrated for the first time that they

also express claudin-15 and claudin-23 (Illumina detection values  $\geq 0.99$ , data not shown). Interestingly, of the intracellular junction genes, claudin-1 met our criteria for significance, demonstrating reduced expression in patients with AD with a  $z$  ratio of  $-2.4$  and a  $P$  value of .0013 compared with nonatopic subjects (Fig 2, A, red highlights). We observed no difference in claudin-1 expression in patients with psoriasis compared with that seen in nonatopic control subjects ( $P = .98$ ; Fig 2, A, red highlights). We also noted reduced expression of claudin-23 ( $z$  ratio,  $-3.2$ ;  $P = .0062$ ) in AD epithelium compared with that from nonatopic subjects, but the difference was only marginally significant (Fig 2, A, red highlights). Furthermore, patients with AD displayed enhanced expression of the gap junction proteins connexin-26 (*GJB2*;  $z$  ratio,  $+5.2$ ;  $P = .025$ ) and connexin-62 (*GJA10*;  $z$  ratio,  $+1.6$ ;  $P = .001$ ; Fig 2, A, red highlights). Patients with AD did not show decreased expression of proteins relevant for adherens junctions or desmosomes (data not shown). Importantly, the expression of a number of differentiation genes was either not affected or increased in patients with AD compared with that seen in control subjects (Fig 2, A, yellow-highlighted genes), indicating that the observed changes in TJ genes did not simply reflect dedifferentiation of the epidermis. We confirmed the reduced expression of claudin-1 in blister roofs obtained from newly recruited patients with AD and nonatopic subjects ( $n = 5$  per group) by means of qPCR (patients with AD:  $38.4 \pm 7.1$  relative value units vs nonatopic subjects:  $69.7 \pm 9.5$ ;  $P = .03$ ; Fig 2, E). We also confirmed the reduced expression of claudin-23 (patients

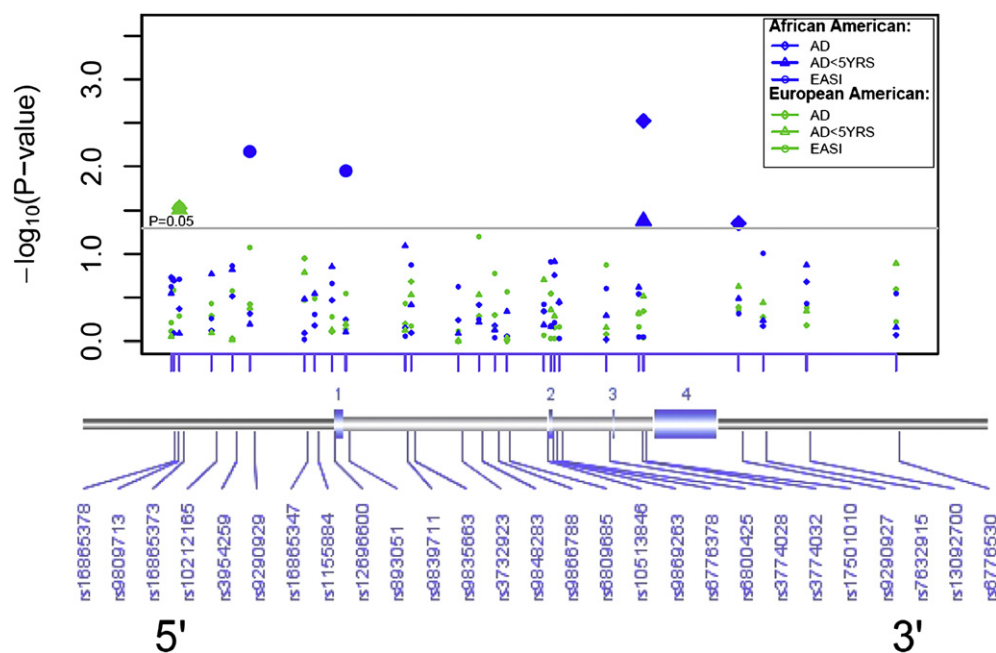


**FIG 8.** Evidence for *CLDN1* association with risk of AD, early-onset AD, and disease severity in 2 North American populations. The x-axis represents the physical position for each of 27 *CLDN1* SNPs shown in relationship to the exonic structure of the *CLDN1* gene on chromosome 3q.28-q29. The y-axis denotes the association test result as  $-\log(P\text{-value})$  corresponding to representative symbols for each of the phenotypes. The standard cutoff for significance ( $P = .05$ ) is shown as a horizontal solid line. Outcomes included risk of AD (diamonds), early age of onset (<5 years; triangle), and the clinical scoring system called EASI (circles) in AA (blue) and EA (green) populations.

with AD:  $0.23 \pm 0.04$  relative value units vs nonatopic subjects:  $0.81 \pm 0.12$ ;  $P = .001$ ) and the enhanced expression of connexin-26 (*GBJ2*; patients with AD:  $18.87 \pm 6.7$  vs nonatopic subjects:  $3.73 \pm 0.26$ ;  $P = .03$ ) in patients with AD versus nonatopic control subjects by means of qPCR (Fig 2, F). Although we considered the reduced expression of claudin-23 in AD samples very interesting, we were limited in our ability to pursue this finding by the lack of reliable reagents. Additional studies are clearly needed to clarify the role played by claudin-23 in epidermal barrier function.

To investigate claudin-1 expression in intact skin, we compared skin biopsy specimens from nonlesional AD skin with those from nonatopic subjects by using both immunohistochemistry (Fig 3, A and B) and immunofluorescent/confocal microscopy (Fig 3, C and D). Claudin-1 immunoreactivity was detectable in bright-field images in all suprabasal layers, whereas occludin and ZO-1 were detected only in the upper granulosum layer where TJs form (data not shown). This pattern has been noted by other investigators.<sup>16,57</sup> Importantly, the expression of claudin-1 was markedly reduced in nonlesional skin from patients with AD compared with that seen in nonatopic control subjects (Fig 3, A-D). Semiquantitative claudin-1 scoring confirmed the markedly reduced staining ( $\geq 50\%$ ) in the epidermis of patients with AD ( $1.3 \pm 0.3$ ) compared with that seen in nonatopic subjects ( $2.9 \pm 0.1$ ,  $P < .0004$ ), and the highly significant  $P$  value demonstrates the remarkable uniformity of staining intensity within each group (Fig 3, E). Immunofluorescent staining more clearly demonstrated claudin-1 immunoreactivity on the cell membranes. The signal intensity was again significantly less in AD samples (Fig 3, C and D). These findings provide the first indication, to

our knowledge, that the skin barrier defect in patients with AD might extend beyond the SC and include TJs.

### AD epidermis shows defective bioelectric properties

We next investigated the functional impairment of TJ barrier in AD epidermis by measuring bioelectric characteristics in Ussing chambers. This approach has been used in human and murine models to evaluate the TJ bioelectric property of mucosal epithelia.<sup>58,59</sup> The technique is based on the principle that an intact semipermeable membrane will maintain the electrochemical potential gradient generated artificially by bathing each side of the epidermal sheets with solutions of different ionic strength.<sup>60,61</sup> A leaky membrane will allow easy diffusion across the membrane and thus loss of electrochemical potential. Thus the higher the permeability across a membrane, the lower the potential gradient.<sup>59,62</sup> TEERs were stable and typically quite high, indicating intact paracellular barrier function of the electrically active epithelium. Using this approach, AD epidermis showed a dramatically lower resistance ( $92.0 \pm 22.0 \Omega \times \text{cm}^2$ ,  $n = 3$ ) compared with the nonatopic subjects ( $827.0 \pm 173.3 \Omega \times \text{cm}^2$ ,  $P = .01$ ,  $n = 4$ ; Fig 4, A). The lower resistance observed in the Ussing chambers was consistent with the increased permeability of FITC-conjugated albumin in AD samples ( $445 \pm 24 \text{ OD}/\text{cm}^2/\text{h}$ ,  $n = 3$ ) compared with nonatopic samples ( $175 \pm 68 \text{ OD}/\text{cm}^2/\text{h}$ ,  $P = .02$ ,  $n = 4$ ; Fig 4, A).

To better investigate the paracellular permeability properties, we measured the relative permeability of  $\text{Cl}^-$  and  $\text{Na}^+$  ( $P_{\text{Cl}}/P_{\text{Na}}$ ).<sup>42</sup> If  $P_{\text{Cl}}/P_{\text{Na}}$  is equal to 1, then there is no selectivity and

TABLE I. ADVN genetics study demographics

| Characteristic                     | EA subjects      |                    | AA subjects      |                    |
|------------------------------------|------------------|--------------------|------------------|--------------------|
|                                    | Patients with AD | Nonatopic subjects | Patients with AD | Nonatopic subjects |
| Sample size                        | 258              | 156                | 176              | 152                |
| Male subjects, no. (%)             | 96 (37.2)        | 63 (40.4)          | 43 (24.4)        | 77 (50.7)          |
| Age (y), mean (SD)                 | 33.1 (18.5)      | 36.6 (13.2)        | 35.3 (12.5)      | 41.1 (10.3)        |
| AD onset <5 y, no. (%)             | 178 (68.9)       | NA                 | 91 (51.7)        | NA                 |
| Geometric mean IgE levels (95% CI) | 670.0 (502-895)  | 59.1 (48-111)      | 556.8 (425-729)  | 141.2 (113-299)    |
| Geometric mean EASI* (95% CI)      | 4.6 (3.9-5.4)    | NA                 | 4.0 (3.3-4.9)    | NA                 |
| FLG null allele carrier, no. (%)   | 65 (25.2)        | 9 (5.8)            | 11 (6.2)         | 2 (1.3)            |

NA, Not applicable.

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

the membrane is freely permeable. Dilution potential studies on epidermal sheets showed that membrane selectivity is preserved in skin from nonatopic subjects, with  $\text{Na}^+$  ions relatively more permeable than  $\text{Cl}^-$  ions ( $0.77 \pm 0.03$ -fold  $\text{P}_{\text{Cl}}/\text{P}_{\text{Na}}$ ). However, in samples from patients with AD, the selectivity was completely lost ( $1.10 \pm 0.02$ -fold  $\text{P}_{\text{Cl}}/\text{P}_{\text{Na}}$ ,  $P = .001$ ,  $n = 3/\text{group}$ ) because both ions were equally permeable (Fig 4, B).

### Claudin-1 expression inversely correlated with $\text{T}_\text{H}2$ biomarkers

To address whether expression levels of epidermal claudin-1 might modulate adaptive immune responses, we looked at the relationship between claudin-1 mRNA expression in our epidermal samples from patients with AD, patients with psoriasis, and nonatopic control subjects and biomarkers of  $\text{T}_\text{H}2$  polarity, namely serum total IgE levels and peripheral blood eosinophilia (Fig 5, A and B). Claudin-1 levels were inversely correlated with both total IgE levels ( $r = -0.718$ ,  $P = .0038$ ) and eosinophil numbers ( $r = -0.761$ ,  $P = .0016$ ), suggesting that reductions in this key TJ barrier protein might affect the character of the immune response to environmental allergens or *vice versa*.

### Claudin-1 localizes to TJs only in differentiated human keratinocytes

Claudin-1 protein is expressed in PHKs *in vitro* but colocalizes with other TJ proteins at the cell membrane only after  $\text{Ca}^{2+}$ -induced differentiation (Fig 6, A). Confocal microscopy demonstrated that claudin-1 colocalizes with occludin and ZO-1 at the areas of cell-cell contact only in PHKs grown in media with a high concentration of  $\text{Ca}^{2+}$  (Hi Ca, 1.9 mmol/L) for at least 24 hours after confluency. Interestingly, in undifferentiated PHKs grown in 0.3 mmol/L  $\text{Ca}^{2+}$  (Lo Ca)-containing media, claudin-1 immunoreactivity was faint and largely nuclear or perinuclear, whereas occludin was undetectable (Fig 6, A), and ZO-1 was localized on the membrane with a discontinuous pattern (data not shown).

Next we investigated the functional relevance of these  $\text{Ca}^{2+}$ -induced changes in TJ protein expression and localization. We observed during  $\text{Ca}^{2+}$ -induced PHK differentiation a dramatic (approximately 300-fold) increase in TEER, which peaked between 30 and 60 hours after exposure to high extracellular  $\text{Ca}^{2+}$  (Fig 6, B) and confirmed previously published studies.<sup>27,63-67</sup> Additionally, we showed that sodium fluorescein flux was markedly reduced in differentiated PHKs ( $3.1 \pm 0.6$ -fold,  $P = .046$ ; Fig 6, C).

### $\text{T}_\text{H}2$ cytokines enhance claudin-1 expression and TJ barrier function

Previous studies have shown that claudin-1 expression in noncutaneous epithelial cells can be modulated by cytokines and growth factors.<sup>68-70</sup> Recent findings from murine models support the hypothesis that IL-4 could delay skin barrier recovery.<sup>71,72</sup> We therefore wondered whether claudin-1 downregulation observed in AD nonlesional skin biopsy specimens could be secondary to  $\text{T}_\text{H}2$  cytokines. A corollary to this hypothesis is that even nonlesional skin has been shown to express  $\text{T}_\text{H}2$  cytokines.<sup>73</sup> To test this possibility, PHKs were differentiated in the presence or absence of  $\text{T}_\text{H}2$  cytokines, IL-4, and IL-13, and the expression of claudin-1, occludin, and ZO-1 was evaluated. We observed a significant enhancement of claudin-1 expression after IL-4 (48 hours, 50 ng/mL;  $2.1 \pm 0.4$ -fold over control subjects;  $P = .05$ ;  $n = 3$ ) and IL-13 (48 hours, 50 ng/mL;  $1.6 \pm 0.3$ -fold over control subjects;  $P = .1$ ;  $n = 3$ ) stimulation (Fig 6, D). No synergism was noted with either cytokine, and interestingly, we observed no effect on the expression of occludin and ZO-1 (data not shown). We then investigated the effect of IL-4 and IL-13 on PHK barrier function. A significant increase in TEER peak was observed in IL-4 (50 ng/mL)-treated PHKs ( $166 \pm 24 \Omega \times \text{cm}^2$  vs  $109 \pm 11 \Omega \times \text{cm}^2$  for medium alone;  $P = .03$ ;  $n = 4$ ). Dose-response experiments confirmed IL-4's effect on TEER (dose range, 0.5-100 ng/mL; data not shown), whereas IL-13 (dose range, 0.5-100 ng/mL) induced only a slight increase in TEER (data not shown). The mechanisms by which  $\text{T}_\text{H}2$  cytokines paradoxically enhance barrier function in our PHKs is currently unknown, but these data suggest that the reduced claudin-1 expression and TJ dysfunction observed in patients with AD are not due to the actions of  $\text{T}_\text{H}2$  cytokines. Alternatively, we cannot rule out the possibility that AD epithelium would respond differently to  $\text{T}_\text{H}2$  cytokines, and therefore studies are planned to address this possibility. Therefore at this point we cannot exclude the possibility that the impaired barrier observed in AD epidermis is a consequence of the  $\text{T}_\text{H}2$  cytokines present in the underlying dermis.

### Claudin-1 knockdown disrupts TJ function and increased PHK proliferation

We used an RNA interference approach to evaluate the effect that claudin-1 reduction has on measures of TJ function (TEER and permeability) and proliferation. Fig 7, A, shows that claudin-1-specific siRNA induced a dose-dependent and significant

( $\leq 60\%$ ) decrease in protein expression compared with scrambled siRNA-transfected cells. Because the claudin-1 knockout mouse dies shortly after birth and our tissue staining demonstrated approximately 50% reduction in claudin-1 immunoreactivity in AD compared with nonatopic epithelium (Fig 3), we chose to perform our functional assessments with a dose of siRNA (100 nmol/L) that reduced claudin-1 transcripts by a similar amount ( $P = .01$ ). With only a 50% reduction in claudin-1, TEER decreased by 50% or more (Fig 7, C; control:  $164 \pm 18 \Omega \times \text{cm}^2$  and *CLDN1* siRNA:  $81 \pm 6 \Omega \times \text{cm}^2$ ;  $P = .007$ ;  $n = 4$ ), and permeability to sodium fluorescein increased by a similar extent (Fig 7, D; control:  $28 \pm 12$  and *CLDN1* siRNA:  $52 \pm 9$ ;  $P = .026$ ;  $n = 4$ ) when compared with that seen in the scrambled siRNA-transfected PHKs. Yamamoto et al<sup>63</sup> used a similar knockdown strategy but observed a more modest effect (14% reduction) on TEER and did not assess TJ function by using a permeability assay. We also investigated the effect of claudin-1 knockdown on connexin-26 (*GJB2*) because this was upregulated in AD array samples. We observed enhanced expression of connexin-26 in our knockdowns ( $1.7 \pm 0.8$ -fold over control;  $P = .05$ ;  $n = 6$ ; see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Using a proliferation assay based on EdU incorporation, we noted that claudin-1-depleted cells have more proliferating cells ( $21.6 \pm 1.4$  EdU-positive cells per high-powered field) compared with control ( $8.1 \pm 2.0$  EdU-positive cells per high-powered field;  $P = .002$ ;  $n = 3$ ; Fig 7, E). These studies confirm and extend (with permeability and proliferation assays) previously published studies,<sup>27,63-67</sup> which have implicated claudin-1 as a critical protein for the establishment of a functional paracellular epidermal barrier. The proliferation we observed in claudin-1 knockdowns suggests that this might be responsible for the enhanced epithelial thickness or acanthosis observed in nonlesional AD skin.

### ***CLDN1* variants are associated with risk of AD**

Because our findings strongly implicated claudin-1 as a critical element in the risk of AD, we took advantage of the ongoing National Institute of Allergy and Infectious Diseases-funded ADVN, which is currently enrolling patients with AD and nonatopic subjects (reviewed in a previous publication<sup>45</sup>) to examine whether common variants in the human *CLDN1* gene might be associated with susceptibility to AD and disease severity. Two independent groups (EA and AA subjects) of patients with AD and healthy control subjects were enrolled (Table I). In brief, AD was diagnosed by using the US consensus conference criteria,<sup>36</sup> and AD severity was defined according to the EASI, a standardized grading system.<sup>46</sup> Twenty-seven *CLDN1* SNPs spanning a 31.5-kb region on chromosome 3q28-q29 were selected by using a haplotype-tagging approach, of which 24 were common to both ethnic groups (see Table E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Adjusting for 10,000 permutations, we observed the most significant associations for a lower risk of AD in the AA group for an intronic SNP (rs17501010) between the third and fourth exons of *CLDN1* (odds ratio [OR], 0.5; 95% CI, 0.3-0.8;  $P = .003$ ) and an adjacent SNP (rs9290927) downstream of rs17501010 that was associated with a higher risk of AD (OR, 1.8; 95% CI, 1.0-3.3;  $P = .004$ ; Fig 8 and see Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). SNP rs17501010 also showed a modest

association with early-onset AD ( $< 5$  years of age:  $P = .04$ ). In addition, 2 SNPs (rs893051 in intron 1 and rs9290929 in the promoter region) were associated with greater disease severity (OR, 1.5; 95% CI, 1.1-2.1;  $P = .010$ ; and OR, 1.6, 95% CI, 1.1-2.3;  $P = .007$ , respectively; Fig 8 and see Table E4). Modest associations were observed in the EA group, including a promoter SNP (rs16865373) and lower risk of AD (OR, 0.5; 95% CI, 0.2-0.9;  $P = .034$ ) and lower risk of early-onset AD ( $< 5$  years of age: OR, 0.4; 95% CI, 0.2-1.0;  $P = .034$ ; Fig 8 and see Table E4). Haplotype analyses were performed but did not alter the evidence for association for any of the outcomes, including the *FLG* null mutation (eg, R501X and 2282del4) phenotype (data not shown). Considering the importance of *FLG* mutations in patients with AD and its subphenotypes, we also evaluated whether there was an interaction between haplotype-tagging SNPs in *FLG* and *CLDN1*. No significant interaction effect was found. Despite the limitations of relatively small sample sizes, these findings suggest that *CLDN1* genetic variations might determine the risk for and severity of AD in ethnically diverse populations and that this appears to be independent of *FLG*.

### **DISCUSSION**

This is the first report, to our knowledge, implicating a TJ defect in patients with AD, a human skin disease that affects up to 15 million Americans. We demonstrated reduced expression of epidermal claudin-1 in AD nonlesional epidermis (Figs 2 and 3). This was specific for AD and not observed in patients with psoriasis, a  $T_H17$ -driven inflammatory skin disorder (Fig 2). Although previous psoriasis publications have suggested that TJs might be altered in lesional epidermis, this has not been consistently observed by other groups.<sup>56,74-76</sup> Adapting the Using chamber to measure bioelectric properties of stratified squamous epithelium enabled us to characterize skin barrier properties from human subjects. Using this approach, we observed a remarkable alteration in the bioelectric characteristics of AD epidermis with markedly lower electrical resistance and higher albumin permeability that was associated with the loss of ion selectivity permeability (Fig 4). These defects are the signature of a TJ defect.

Our findings are highly consistent with observations made from genetically altered mice. For example, the claudin-1 knockout mouse dies within 24 hours of birth with wrinkled skin, severe dehydration, and increased epidermal permeability, as measured by dye studies and TEWL.<sup>27</sup> Importantly, these mice have no abnormalities in the expression of SC proteins or lipids that might explain their severe skin phenotype. Another recent study reported disruption of the epidermal barrier and severe dermatitis in transgenic mice overexpressing an adhesion-deficient mutant of claudin-6 in the suprabasal compartment of the skin.<sup>77</sup> Interestingly, a marked downregulation of claudin-1 expression was noted in these transgenic mice. It is tempting to speculate that the reduction in claudin-1 rather than the adhesion-defective claudin-6 *per se* was responsible for the barrier disruption observed in this mouse. Lopardo et al<sup>66</sup> also observed reduced claudin-1 expression in the epidermis of p63 mutant mice. These mice have a severe skin phenotype and die of dehydration within 1 day of birth, similar to the claudin-1 knockout mice. In summary, these murine models have highlighted the critical importance of claudin-1 for a functional epidermal TJ.

Recently, a human syndrome caused by an exonic mutation in *CLDN1* has been described.<sup>78-80</sup> Patients with this syndrome, called neonatal ichthyosis-sclerosing cholangitis, have features in common with AD, namely erythema, dry flaky skin, and patchy alopecia, in addition to unique features, such as severe liver and gallbladder abnormalities that likely arise because of the importance of claudin-1 in the barrier integrity of bile canaliculi.<sup>78,79</sup>

Our *in vitro* studies with PHK monolayers demonstrating a clear association between claudin-1 levels and TEER confirmed previous work.<sup>63,81,82</sup> We extended these findings by demonstrating that claudin-1 knockdown also enhanced TJ permeability and proliferation (Fig 7). In our study the claudin-1 siRNA was target specific, with no changes observed in the expression or localization, of other critical TJ proteins (occludin and ZO-1), adherens junction components (E-cadherin and nectin-1) or SC proteins (filaggrin; see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Additionally, we were able to reduce claudin-1 expression by approximately 50%, which is similar to the reductions we observed in claudin-1 immunoreactivity of AD epidermis, and this resulted in a remarkable reduction (approximately 50%) in TJ function (based on both electrical resistance and permeability assays, Fig 7). Although immunolabeling data allow only a correlative link between claudin-1 expression and the development of the epidermal barrier, our RNA interference result demonstrated a causal connection between these 2 events (Fig 7).

Recent studies with different types of mammalian epithelia indicated that the epithelial barrier is determined by 2 different components: charge-selective small pores that are permeable to molecules with up to a 4- $\mu$ m radius and large, poorly-defined breaks of the barrier without size or charge selectivity (reviewed in Anderson and Van Itallie<sup>83</sup>). TEER reflects small-pore permeability, whereas flux of FITC with a reported Stokes' radius of approximately 5.5 Å measures the permeability of large barrier breaks. Knockdown of claudin-1 in PHK monolayers decreased TEER and increased FITC flux, which implicates claudin-1 in regulation of both paracellular pores and breaks in the epidermal barrier. These findings strongly suggest that the skin of patients with AD would also be more permissive to a number of relevant environmental allergens, such as house dust mite. Purified allergens from the house dust mite *Dermatophagoides pteronyssinus* have a diameter of approximately 1.6 Å, as determined by means of radiographic crystallography, and therefore could penetrate even small paracellular pores formed by epithelial TJs.<sup>84</sup> Thus defective expression and function of claudin-1 in patients with AD provides a plausible molecular mechanism for increased sensitization to environmental antigens, allergens, irritants, or pollutants (Fig 1). This is in keeping with the distinction that AD is the allergic disorder with the greatest and most diverse allergen reactivity, as reflected in high serum total IgE values.

AD is also recognized for a reduced cutaneous irritancy threshold that could simply reflect greater epidermal penetration of irritants (Fig 1). Importantly, TJ disruption is a leading hypothesis to explain allergen reactivity in the airways, which manifests as asthma and allergic rhinitis or, in the intestinal tract, as food allergy.<sup>3,85,86</sup>

Although we observed an inverse correlation between claudin-1 expression and markers of T<sub>H</sub>2 polarity, we did not find that T<sub>H</sub>2 cytokines (IL-4 and IL-13 alone or together) reduced claudin-1 expression but in fact observed the opposite. This induction

of claudin-1 was observed in conjunction with enhanced TJ function (eg, TEER) and suggests that T<sub>H</sub>2 cytokines have a reparative effect on TJs in normal keratinocytes. Whether the actions of these T<sub>H</sub>2 cytokines would be different in AD epidermis will require further study. Interestingly, T<sub>H</sub>2 cytokines have been shown to reduce the expression of several SC components important for skin barrier function.<sup>72</sup> At this point, we conclude that TJ dysfunction observed in AD epidermis is not likely caused by T<sub>H</sub>2 milieu, which is present even at nonlesional sites. Instead, we would hypothesize that the connection we observed between TJ function and biomarkers of T<sub>H</sub>2 polarity suggest that AD TJ defects enable or promote T<sub>H</sub>2 responses, possibly by enhancing the trafficking of nonself antigens that are responsible for triggering the T<sub>H</sub>2 response in genetically predisposed persons. Alternatively, the upregulation of claudin-1 in response to IL-4 and IL-13 might represent a compensatory immune response to "protect" against further antigen uptake through the skin.

In preliminary studies we evaluated genetic associations between *CLDN1* polymorphisms and AD. We undertook a haplotype-tagging SNP approach using genetic markers available in the public arena ( $n = 132$  in dbSNP) in 414 EA subjects and 328 AA subjects. Interestingly, *CLDN1* is localized on chromosome 3q28-q29, very close to the *ATOD1* locus for AD.<sup>87</sup> Adjusting for 10,000 permutations to reduce a type I error caused by multiple comparisons, we observed several modest associations ( $P = .003-.05$ ) between variants throughout the *CLDN1* gene and the outcomes associated with AD, especially among AA subjects. In separate studies we tested for associations between several of the same *CLDN1* SNPs and risk of disease among a more robustly powered German dataset of patients with AD and control subjects; however, the full set of SNPs genotyped in the ADVN study were not available for direct comparisons, and phenotyping approaches differed (data not shown). Further studies are underway for comprehensive joint analyses between ADVN and the German study. Interestingly, *CLDN1* variants were also associated with asthma and its related traits, total serum IgE levels and FEV<sub>1</sub>, in 2 independent populations of African descent (K. Barnes, unpublished data). In addition, the rs893051 SNP associated with AD severity in our AA population was also associated with asthma and disease severity in a population of African descent. As part of the ADVN study, our population was also screened for the 2 most frequent *FLG* mutations (R501X and 2282del4, Table I) and 9 haplotype-tagging SNPs throughout the *FLG* gene.<sup>51</sup> There were no significant interaction effects between haplotype-tagging SNPs in *FLG* and *CLDN1* SNPs. Moreover, in the AA population, in which we had the strongest association with *CLDN1* SNPs, the 2 *FLG* mutations were considerably less common than in our EA population.<sup>51</sup> Our findings provide suggestive evidence for a role for *CLDN1* variants in AD and its associated phenotypes, further supporting the importance of *CLDN1* in patients with AD.

In conclusion, this study provides the first evidence that epidermal TJs are defective in patients with AD, the most common human skin disease. We observed that claudin-1 is selectively reduced in the epidermis of patients with AD and that *CLDN1* might be a novel AD susceptibility gene. Epidermal samples from patients with AD had remarkable defects in resistance and ion transport compared with those from healthy control subjects. Using the model of human keratinocyte monolayers, we observed enhanced claudin-1 expression and recruitment to intercellular junctions on cell differentiation, which coincided with the

development of a paracellular barrier. Selective downregulation of claudin-1 expression markedly increased paracellular permeability, decreased resistance, and enhanced proliferation indicative of a wound repair response. We hypothesize that the reduced expression of claudin-1 in AD epidermis might enhance the penetration of many relevant environmental antigens, leading to greater allergen sensitization, as well as greater susceptibility to irritants/pollutants and possibly even altered microbial flora (Fig 1). The inverse relationship between claudin-1 and serum total IgE values also suggests that this defect might promote T<sub>H</sub>2 responses. Collectively, these data suggest that barrier dysfunction in patients with AD extends beyond the SC to TJs, the second barrier structure, and that barrier regulation provides a novel therapeutic opportunity in patients with AD and possibly other atopic disorders.

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### Key messages

- Claudin-1 plays a critical role in human epidermal TJ function and keratinocyte proliferation.
- Expression of claudin-1 is significantly reduced in nonlesional skin of patients with AD compared with nonatopic subjects and patients with psoriasis.
- Claudin-1 levels are inversely correlated with T<sub>H</sub>2 biomarkers, suggesting that reductions in this key TJ barrier protein might affect the character of the immune response to environmental allergens.
- Analysis of *CLDN1* haplotype-tagging SNPs in 2 North American populations revealed associations with AD.

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## METHODS

## Genotyping and quality control

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

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 (an 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 LD blocks in each of the ethnic groups (EA and AA subjects). Tagging was based on the LDSelect algorithm, with a minor allele frequency of 10% or greater 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 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 HapMap Yoruba (YRI, with African ancestry) samples; an additional 3 tagging SNPs (rs6800425, rs1155884, and rs9809713) were genotyped only in the AA subjects. Two LD blocks were observed among the EA 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 AA 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.<sup>E1</sup>

The 27 SNPs were genotyped by using the custom-designed Illumina OPA for the BeadXpress Reader System and the GoldenGate Assay with VeraCode Bead technology (San Diego, Calif), according to the manufacturer's protocol.<sup>E2</sup> 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 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 with 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 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% to 98.2% for the BeadXpress genotyping.

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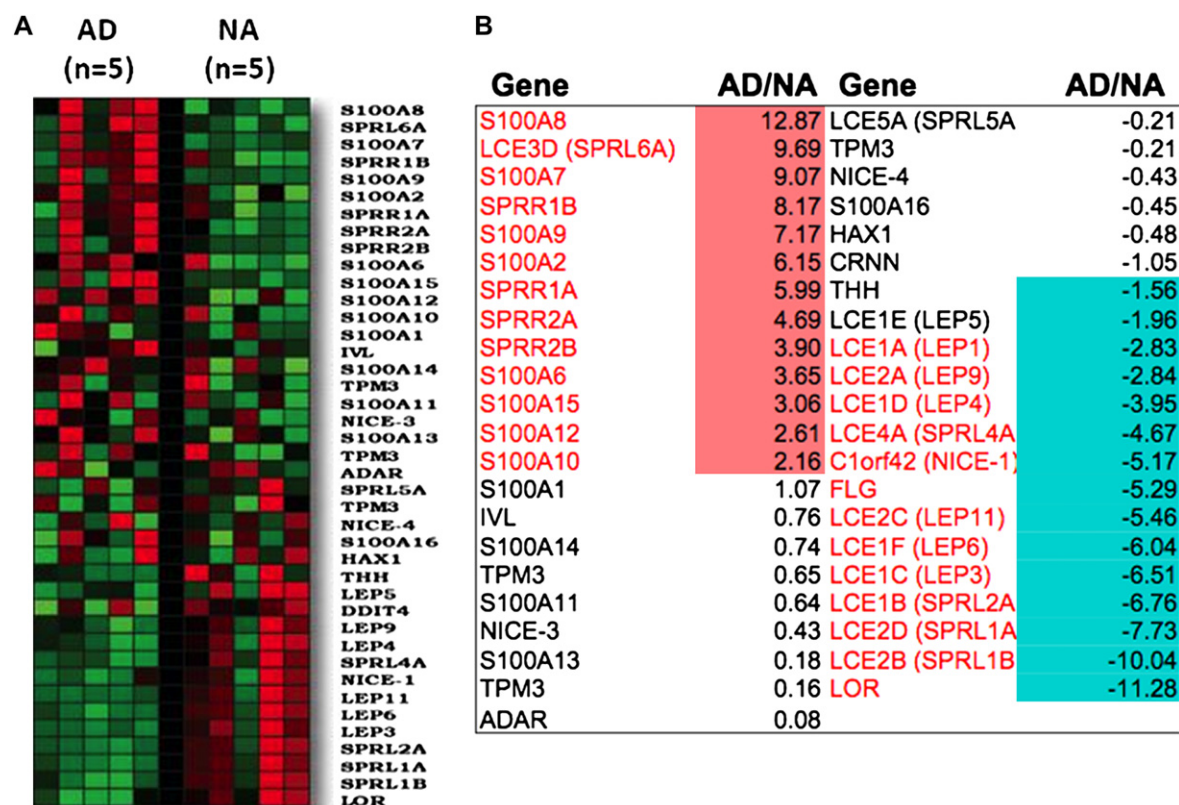


**FIG E1.** Epidermal differentiation complex genes (EDC) are differentially expressed in patients with AD. **A**, Gene arrays were performed on nonlesional epithelium (blister roofs) from patients with AD with extrinsic disease ( $n = 5$ ) and nonatopic control subjects (NA;  $n = 5$ ). Each column represents a single-array experiment on a single epidermal sample. A heat map of EDC genes was generated, where *red* represents upregulation and *green* represents downregulation. **B**, The data are presented with z ratios comparing patients with AD with nonatopic control subjects (NA).

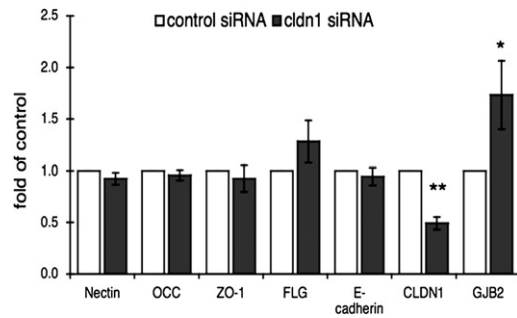


**FIG E2.** Claudin-1 silencing does not affect expression of other proteins relevant for barrier function. *CLDN1* siRNA (100 nmol/L) resulted in a 50% reduction in claudin-1 expression compared with that seen in control transfected cells ( $0.5 \pm 0.06$ -fold,  $**P = 0.5 \times 10^{-6}$ ,  $n = 5$  per group). Connexin-26 expression (*GJB2*) was significantly upregulated in *CLDN1* siRNA transfected PHKs ( $1.7 \pm 0.8$ -fold,  $*P = .05$ ,  $n = 6$ ).

**TABLE E1.** Real-time PCR primers

| Target                    | Forward                    | Reverse                    |
|---------------------------|----------------------------|----------------------------|
| <i>GAPDH</i>              | GAA GGT GAA GGT CGG AGT C  | GAA GAT GGT GAT GGG ATT TC |
| <i>CLDN1</i>              | CGA TGA GGT GCA GAA GAT GA | CCA GTG AAG AGA GCC TGA CC |
| <i>CLDN23</i>             | CTT GCC ATG CAA ACT CTC AA | TTC TCC TCT TGG CTT CTG GA |
| Occludin                  | CTG GCC TAC AGG AAT ACA AG | CTT GAT GTG TGA CAA TTT GC |
| <i>ZO1</i>                | CGG AAA ACA TGC TAC ACA C  | CCC ATT TAC TGG CTG GTA T  |
| <i>GJB2</i> (connexin-26) | GTT TAA CGC ATT GCC CAG TT | GGC CTA CAG GGG TTT CAA AT |
| <i>PVRL1</i> (nectin-1)   | AGC CAT TAA GGA GAA ACG A  | TTC CCA ATT TCT CTG CTC T  |
| <i>CDH</i> (E-cadherin)   | CAGAAAGTTTCCACCAAAG        | AAATGTGAGCAATTCTGCTT       |
| <i>FLG</i>                | GAGCTGAAGGAAGTTCTGG        | GATCCATGAAGACATCAACCA      |

**TABLE E2.** Modulation of TJ pathway genes (n = 43) in epidermal samples taken from patients with AD compared with healthy nonatopic control subjects

| Gene                | z ratio       | P value      | Fold change  |
|---------------------|---------------|--------------|--------------|
| <i>ACTN1</i>        | 2.796         | .0121        | 1.47         |
| <i>ACTN4</i>        | 0.798         | .3742        | 1.14         |
| <i>CGN</i>          | 0.063         | .9532        | 1.04         |
| <i>CIP98</i>        | 0.712         | .1986        | 1.1          |
| <b><i>CLDN1</i></b> | <b>−2.419</b> | <b>.0013</b> | <b>−1.42</b> |
| <i>CLDN10</i>       | 1.620         | .0641        | 1.27         |
| <i>CLDN12</i>       | −0.798        | .2518        | −1.09        |
| <i>CLDN14</i>       | −1.056        | .0896        | −1.13        |
| <i>CLDN15</i>       | 0.553         | .4967        | 1.12         |
| <i>CLDN16</i>       | −0.007        | .9881        | −1.02        |
| <i>CLDN18</i>       | −0.915        | .2421        | −1.08        |
| <i>CLDN23</i>       | −3.155        | .0062        | −1.6         |
| <i>CLDN4</i>        | −0.977        | .4279        | −1.07        |
| <i>CLDN6</i>        | −1.067        | .1561        | −1.02        |
| <i>CLDN7</i>        | −0.298        | .6320        | −1.03        |
| <i>CLDN8</i>        | −1.265        | .1616        | −1.26        |
| <i>CRB3</i>         | 0.755         | .3508        | 1.14         |
| <i>CSDA</i>         | −0.426        | .4062        | −1.02        |
| <i>CTNNA1</i>       | 0.139         | .8766        | 1.02         |
| <i>CTNNB1</i>       | −0.879        | .1319        | −1.1         |
| <i>INADL</i>        | 0.627         | .3357        | 1.12         |
| <i>LLGL2</i>        | −0.455        | .4178        | −1.06        |
| <i>MAGI1</i>        | 0.694         | .2086        | 1.12         |
| <i>MLLT4</i>        | 0.374         | .4000        | 1.04         |
| <i>MPDZ</i>         | 0.616         | .1559        | 1.06         |
| <i>MPP5</i>         | −0.136        | .8746        | 1.11         |
| <i>NR3C1</i>        | −1.299        | .0178        | −1.16        |
| Occludin            | −1.136        | .0920        | −1.17        |
| <i>PARD3</i>        | −0.621        | .3375        | −1.06        |
| <i>PPP2CA</i>       | 0.170         | .7934        | 1.03         |
| <i>PRKCA</i>        | 0.523         | .3959        | 1.02         |
| <i>PRKCI</i>        | 0.329         | .6671        | 1.04         |
| <i>RAB13</i>        | 0.532         | .4387        | 1.13         |
| <i>SEC6L1</i>       | 0.959         | .2246        | 1.19         |
| <i>SEC8L1</i>       | 0.494         | .5482        | 1.07         |
| <i>SPTAN1</i>       | −1.965        | .0017        | −1.21        |
| <i>SRC</i>          | −0.656        | .1983        | −1.05        |
| <i>TJP1</i>         | 0.304         | .4465        | 1.04         |
| <i>TJP2</i>         | 0.700         | .4888        | 1.14         |
| <i>TJP3</i>         | −0.666        | .0605        | −1.07        |
| <i>TJP4</i>         | 0.081         | .8330        | −1.02        |
| <i>YES1</i>         | 0.734         | .0692        | 1.08         |
| <i>ZAK</i>          | −0.287        | .6614        | 1.05         |

**TABLE E3.** *CLDN1* polymorphisms and minor allele frequencies

| Gene  | dSNP ID<br>(build 129) | Location  | Inter-SNP<br>distance (bp) | Position | Type of<br>variant | Minor allele frequency |       |                           |                           |
|-------|------------------------|-----------|----------------------------|----------|--------------------|------------------------|-------|---------------------------|---------------------------|
|       |                        |           |                            |          |                    | CEU*                   | YRI*  | EA subjects†<br>(n = 156) | AA subjects†<br>(n = 152) |
| CLDN1 | rs6776530              | 191498394 | 0                          | C/A      | Downstream         | 0.409                  | 0.103 | 0.384                     | 0.431                     |
| 3q28  | rs13092700             | 191502254 | 3,860                      | A/G      | Downstream         | 0.060                  | 0.000 | 0.071                     | 0.007                     |
|       | rs7632915              | 191504142 | 1,888                      | C/A      | Downstream         | 0.525                  | 0.192 | 0.394                     | 0.224                     |
|       | rs9290927              | 191505210 | 1,068                      | A/T      | Downstream         | 0.883                  | 0.958 | 0.115                     | 0.056                     |
|       | rs17501010             | 191509348 | 4,138                      | 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 | 1,412                      | T/A      | Intron             | 0.415                  | 0.034 | 0.332                     | 0.082                     |
|       | rs6800425              | 191513018 | 2,052                      | 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 | 1,605                      | 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 | 2,049                      | 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 | 2,582                      | 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 | 2,342                      | 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 | 1,398                      | 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                     |

UTR, Untranslated region.

\*From HapMap (<http://www.hapmap.org>) for 60 Yoruban (YRI) and 60 Utah EA (CEU) founders.

†Minor allele frequencies from healthy control subjects participating in the ADVN study.

**TABLE E4.** *CLDN1* SNP association test results

| dbSNP ID (build 129) | EA subjects |             |      | AA subjects  |             |              |
|----------------------|-------------|-------------|------|--------------|-------------|--------------|
|                      | AD          | AD at <5 y  | EASI | AD           | AD at <5 y  | EASI         |
| rs6776530            | .25*        | .13         | .61  | .86          | .7          | .28          |
| rs13092700           | .66         | .45         | .42  | .21          | .13         | .37          |
| rs7632915            | .53         | .36         | .54  | .67          | .58         | .1           |
| rs9290927            | .41         | .24         | .44  | .04 (.039†)  | .33         | .49          |
| rs17501010           | .46         | .31         | .92  | .003 (.005†) | .04 (.065†) | .91          |
| rs3774032            | .48         | .48         | .7   | .29          | .24         | .9           |
| rs3774028            | .84         | .7          | .13  | .96          | .51         | .25          |
| rs6800425            | NA          | NA          | .69  | .36          | .36         | .94          |
| rs6776378            | .7          | .52         | .94  | .18          | .12         | .62          |
| rs9869263            | .29         | .44         | .94  | .68          | .69         | .12          |
| rs10513846           | .46         | .2          | .86  | .46          | .65         | .38          |
| rs6809685            | .99         | .93         | .27  | .89          | .46         | NA           |
| rs9866788            | .5          | .74         | .17  | .67          | .75         | .93          |
| rs9848283            | .52         | .29         | .06  | .38          | .61         | .57          |
| rs3732923            | .99         | .99         | .77  | .58          | .82         | .24          |
| rs9835663            | .21         | .29         | .68  | .81          | .38         | .13          |
| rs9839711            | .64         | .75         | .37  | .7           | .08         | .89          |
| rs893051             | .73         | .64         | .29  | .57          | .79         | .01 (.010†)  |
| rs12696600           | .78         | .75         | .53  | .34          | .14         | .22          |
| rs1155884            | NA          | NA          | .32  | .66          | .29         | .49          |
| rs16865347           | .11         | .16         | .34  | .81          | .33         | .96          |
| rs9290929            | .38         | .42         | .08  | .49          | .64         | .007 (.006†) |
| rs3954259            | .94         | .97         | .27  | .31          | .15         | .14          |
| rs10212165           | .52         | .81         | .38  | .76          | .17         | .55          |
| rs16865373           | .03 (.034†) | .03 (.034†) | .52  | .43          | .82         | .2           |
| rs9809713            | NA          | NA          | .26  | .82          | .2          | .2           |
| rs16865378           | .77         | .89         | .62  | .19          | .28         | .24          |

NA, Not applicable.

\*Unadjusted *P* value based on Cochran-Armitage trend test.†Empiric *P* value based on 10,000 permutations.