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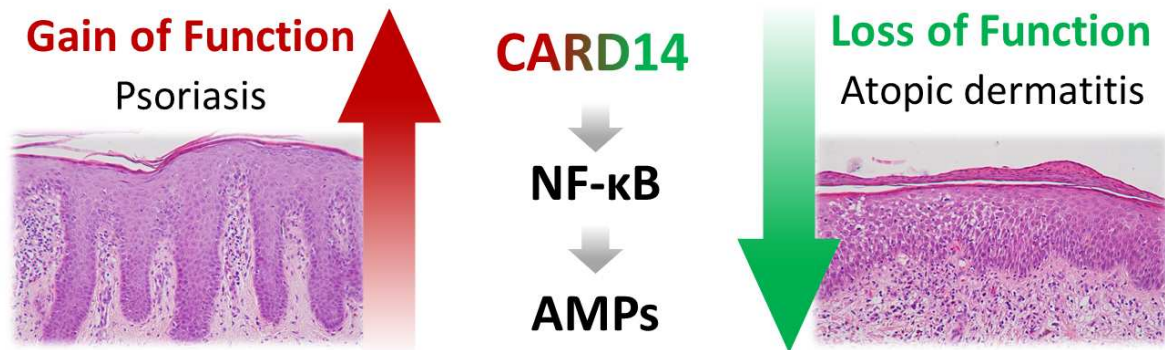
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NF- κ B - nuclear factor kappa-light-chain-enhancer of activated B cells; AMPs - Antimicrobial peptides

ORIGINAL ARTICLE

Loss-of-function mutations in *CARD14* are associated with a severe variant of atopic dermatitis

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ABBREVIATIONS USED

AD, Atopic dermatitis; WES, Whole exome sequencing; WT, Wild-Type; NF- κ B, Nuclear factor
 κ B; qRT-PCR, Quantitative RT-PCR; siRNA, Small interfering RNA; ELISA, Enzyme-linked
immunosorbent assay

CAPSULE SUMMARY

Dominant gain-of-function mutations in *CARD14*, encoding a known regulator of NF- κ B, cause
psoriasis and related disorders. Here, the authors show that dominant negative mutations in the
same gene result in severe atopic dermatitis and decreased NF- κ B signaling.

Clinical Implications

While up-regulation of *CARD14* leads to psoriasis, down-regulation of the same molecule
results in atopic dermatitis and decreased levels of antimicrobial peptides which not only protect
the skin against infections but also regulate cutaneous inflammatory circuits.

ABSTRACT

Background

Atopic dermatitis (AD) is a highly prevalent chronic inflammatory skin disease which is known to be, at least in part, genetically determined. Mutations in *CARD14* have been shown to result in various forms of psoriasis and related disorders.

Objective

We aimed to identify rare DNA variants conferring a significant risk for AD through genetic and functional studies in a cohort of patients affected with severe atopic dermatitis.

Methods

Whole exome and direct gene sequencing, immunohistochemistry, real-time PCR, ELISA and functional assays in human keratinocytes were used.

Results

In a cohort of individuals referred with severe atopic dermatitis, DNA sequencing revealed in 4 patients two rare heterozygous missense mutations in *CARD14* encoding the Caspase Recruitment Domain-Containing Protein 14, a major regulator of NF- κ B. A dual luciferase reporter assay demonstrated that both mutations exert a dominant loss-of-function effect and result in decreased NF- κ B signaling. Accordingly, immunohistochemistry staining showed decreased expression of *CARD14* in patient skin as well as decreased levels of activated p65, a surrogate marker for NF- κ B activity. *CARD14*-deficient or mutant-expressing keratinocytes displayed abnormal secretion of key mediators of innate immunity.

72

73 **Conclusions**

74 While dominant gain-of-function mutations in *CARD14* are associated with psoriasis and related
75 diseases, loss-of-function mutations in the same gene are associated with a severe variant of
76 atopic dermatitis.

77

78 **KEYWORDS**

79 Atopic dermatitis, psoriasis, *CARD14*, NF- κ B

80

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INTRODUCTION

Atopic dermatitis (AD) is an extremely prevalent disorder, very often manifesting initially in infancy and childhood, and persisting in a minority of affected individuals in adulthood.¹ AD is recognized as a prototypical multifactorial condition, resulting from a combination of genetically determined defects and environmental exposures, eventually leading to skin barrier disruption and both cutaneous and systemic immunologic dysfunction.^{2,3}

Extensive attempts at delineating the genetic causes of the disease through genome wide association studies have revealed a large number of susceptibility loci near genes affecting both barrier function and immune regulation, most of which contain variations conferring a slight to moderate risk for the disease only.^{4,5} A notable exception is *FLG*, encoding filaggrin, in which germline mutations have been shown to confer a remarkably high risk for AD.⁶ Nonetheless, although null mutations in *FLG* are considered as the strongest genetic risk factors for AD, they are found in less than half of the patients.⁶ In fact, currently available genetic data seem to barely explain 25% of AD heritability.⁷

As an alternative to genome wide association-based approaches, the study of rare instances of quasi-monogenic inheritance of conditions usually inherited as complex traits can often reveal genetic variations exerting a strong effect on the propensity to develop complex traits⁸ such as allergy and atopic dermatitis.⁹ The description of dominant negative mutations in *CARD11*, a structurally and functionally homologous gene to *CARD14*, leading to severe atopic dermatitis,¹⁰ following the description of *CARD11* variants identified as risk factors for common atopic dermatitis in genome-wide association studies,¹¹ remarkably illustrates the strengths of this approach. Similarly, the role of *CARD14* in the pathogenesis of several inflammatory conditions was initially revealed through the study of rare familial cases of psoriasis and pityriasis rubra

pilaris,^{12, 13} which later on led to the recognition of *CARD14* as a strong susceptibility gene in sporadic forms of these diseases.¹⁴⁻¹⁶ Psoriasis-causing mutations in *CARD14* were found to exert a gain-of-function effect and to result in heightened NF- κ B signaling^{12, 13, 17} leading to the production of pathogenic inflammatory mediators. Here, we demonstrate that dominant loss-of-function mutations in *CARD14* result in an unusually severe form of AD, decreased NF- κ B signaling and concomitant dysregulation of critical innate immunity-associated mediators previously implicated in AD pathogenesis.

METHODS

Patients

All affected and healthy family members or their legal guardian provided written and informed consent according to protocols approved by the institutional review board of the National Institute of Health (NCT00557895, NCT00852943) and of the Johns Hopkins All Children Hospital (IRB00097062). Genomic DNA was extracted from peripheral blood leukocytes of each participant using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Whole exome sequencing

DNA samples obtained from individuals belonging to families 1 and 3 were subjected to whole exome sequencing using the Ion Torrent AmpliSeq RDY Exome Kit (Life Technologies) and the Ion Chef and Proton instruments (Life Technologies). Briefly, 100 ng gDNA was used as the starting material for the AmpliSeq RDY Exome amplification step following the manufacturer's protocol. Library templates were clonally amplified and enriched using the Ion Chef and the Ion

PI Hi-Q Chef Kit (Chef package version IC.4.4.2, Life Technologies), following the manufacturer's protocol. Enriched, templated Ion Sphere Particles were sequenced on the Ion Proton sequencer using the Ion PI chip v3 (Life Technologies). Reads were aligned to the Genome Reference Consortium Human Build 37 (GRCh37/hg19) using Burrows-Wheeler.¹⁸ Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. Reads mapping to a region of known or detected insertions or deletions were re-aligned to minimize alignment errors. Single-nucleotide substitutions and small insertion deletions were identified and quality filtered using the Genome Analysis Tool Kit (GATK).¹⁹ Rare variants were identified by ANNOVAR²⁰ and filtered using data from dbSNP142, the 1000 Genomes Project, HGMD, gnomAD, Ensemble, Exome Variant Server, and an in-house database of individual exomes. Variants were classified by predicted protein effects using Polyphen2²¹ and SIFT.²² Validation and co-segregation of the disease phenotype with the mutation were verified using Sanger sequencing. For patient II-1, family 2, a targeted 207 gene immune-related disease clinical sequencing panel was performed by Invitae using Illumina next-gen sequencing technology.

Direct sequencing

Genomic DNA was PCR-amplified using oligonucleotide primer pairs spanning relevant exon sequences (**Table E1**) with Taq polymerase (Qiagen, Hilden, Germany). Cycling conditions were as follows: 94°C, 2 min; 94°C, 40 sec; 61°C, 40 sec; 72°C 50 sec, for 3 cycles, 94°C, 40 sec; 59°C, 40 sec; 72°C 50 sec, for 3 cycles, 94°C, 40 sec; 57°C, 40 sec; 72°C 50 sec, for 34 cycles. Gel-purified (QIAquick gel extraction kit, QIAGEN, Hilden, Germany) amplicons were subjected to bidirectional DNA sequencing with the BigDye terminator system on an ABI Prism

3100 sequencer (Applied Biosystems, Foster City, NY, USA) using oligonucleotides used for PCR.

Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR), cDNA was synthesized from 1000 ng of total RNA using qScript kit (Quanta Biosciences, Gaithersburg, MD, USA). cDNA PCR amplification was carried out with the PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA) on a StepOnePlus system (Applied Biosystems, Waltham, MA, USA) with gene-specific intron-crossing oligonucleotides (**Table E2**). Cycling conditions were as follows: 95°C, 20 sec and then 95°C, 3 sec; 60°C, 30 sec for 40 cycles. Each sample was analyzed in triplicates. For each set of primers, standard curves were obtained with serially diluted cDNAs. Results were normalized to *GAPDH* mRNA levels.

Immunohistochemical staining

Following antigen retrieval with 0.01M citrate buffer, pH 6.0 (Invitrogen, Carlsbad, CA) in a microwave for 25 min, blocking with hydrogen peroxide for 10 min and protein blocking for 40 min, 5 µm-thick paraffin-embedded sections fixed on ®Plus glass slides (MenzelGlazer, Braunschweig, Germany) were processed using an automated immunostainer (Benchmark-XT, Ventana Medical System, Tucson, AZ, USA) with primary antibodies directed against hBD-1 (ab14425), hBD-2 (ab63982), hCCL20 (ab9829) (Abcam, Cambridge, MA, USA), CARD14 (Novus Biologicals, Littleton, CO, USA) and the activated p65 subunit (Millipore, Billerica, MA) as previously described¹³. Negative controls consisted of slides processed while omitting the primary antibody. Visualization of the bound primary antibodies was performed using the HRP/AEC (ABC) Detection IHC Kit (Abcam, Cambridge, MA, USA). The sections were then

counterstained with Gill's hematoxylin, dehydrated and mounted for microscopic examination. Specimens were examined using a Nikon 50I microscope connected to a DS-RI1 digital camera. Immunohistochemistry staining intensity was quantified using ImageJ software.

Cell cultures

Primary keratinocytes were isolated from adult skin obtained from plastic surgery specimens after having received written informed consent from the donors according to a protocol reviewed and approved by the Tel Aviv Sourasky Medical center institutional review board as previously described.²³ Primary keratinocytes were maintained in Keratinocyte Growth Medium (KGM; Lonza, Walkersville, MD, USA).

HEK293 cells were cultured in high-glucose DMEM medium with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin (Biological Industries, Beit-Haemek, Israel).

siRNA transfection

Primary human keratinocytes were grown in triplicates in 6-well plates at 37°C in 5% CO₂ in a humidified incubator. After 5 days, keratinocytes at 60-70% confluency were transfected with a *CARD14*-specific siRNA (sc-60330) or control siRNA-Stealth RNAi Negative Control Duplex (Invitrogen, Carlsbad, CA, USA). Efficacy of siRNA-mediated *CARD14* down-regulation was ascertained using real-time PCR and Western blot (**Figure E1**).

NF-κB reporter assay

To examine the impact of the *CARD14* variants on NF-κB activation, we co-transfected HEK293 cells (15,000 cells per well were seeded in a white flat bottom 96 wells microplate) with a luciferase reporter under a NF-κB responsive element, a Renilla expression vector and

several *CARD14* cDNA constructs carrying either a wild-type sequence or the *CARD14* mutations (pCMV6-Entry Vector, Origene, Rockville, MD), using Lipofectamine2000, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The mutations were introduced into the *CARD14* sequence by mutagenesis service (Genscript, Piscataway, NJ) and validated by Sanger sequencing. As a positive control, we used a construct encoding the gain-of-function p.E138del variant¹³ as described previously.¹⁵ Twenty four hours after transfection, luciferase activity was read using a dual luciferase assay (Promega, MD, USA). Luciferase activity was normalized to Renilla luciferase activity.

Enzyme-linked immunosorbent assay (ELISA)

Primary keratinocytes were cultured in KGM (Lonza, Walkersville, MD, USA) containing 0.075 mM CaCl₂ supplemented with 0.4% bovine pituitary extract, 0.1% human epidermal growth factor (hEGF), 0.1% insulin, 0.1% hydrocortisone and 0.1% gentamicin/amphotericin B. Keratinocytes cells were seeded into 6-well or 12 plates, at 37°C in 5% CO₂ in a humidified incubator, adhered overnight, washed, and incubated with KGM up to 70% confluency. Before performing the experiment, cells were washed and fresh medium added without KGM supplements. Cells were then transfected with either human *CARD14* small interference RNAs (siRNA) (Santa Cruz; sc-60330) or control siRNA (Stealth™ RNAi Negative Control Duplex, Invitrogen) using Lipofectamine RNAiMax (Invitrogen) or with various *CARD14* constructs as indicated in the legends to figures. The transfection medium was replaced 6 hours after transfection with the same minimal medium and the cells were then maintained in growth medium (KGM) without supplements for 48 hours. For hCCL20 level measurements, the cells were maintained after transfection in KGM without hydrocortisone supplemented with 0.05%

inactivated fetal bovine serum for 24 hours . The cell lysates and media were collected, aliquoted and kept at -80°C until analyzed using qRT-PCR or ELISA, respectively.

Protein levels of hBD-1, hBD-2, hLL-37, hCCL20 and TSLP secreted into the cell medium or hIL-33 present in cell lysates (proteins were extracted from cells lysates using Lysis Buffer 17 (R&D systems, cat # 895943) according to manufacturer's instructions) were measured using the following ELISA kits: hBD-1 (PeproTech, Cat# 900-K202), hBD-2 (PeproTech, Cat# 900-K172), hLL-37 (Hycult Biotech, Cat# HK321), hCCL20 (R&D systems, Cat# DM3A00), hIL-33 (R&D systems, Cat# D3300B) and TSLP (R&D systems, Cat# DTSLP0) according to manufacturers' instruction. Each experiment was repeated at least 3 times and ELISA measurements were done in duplicates.

Western blotting

Cells were homogenized in Cellytic MT lysis/extraction reagent (Sigma-Aldrich) supplemented with protease inhibitor mix, including 1 mM phenylmethanesulphonylfluoride, and 1 mg/ml aprotinin and leupeptin (Sigma-Aldrich). Following centrifugation at $10,000 \times g$ for 10 minutes at 4 °C, proteins were electrophoresed through a 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane (BioTrace NT Nitrocellulose, Pall Corporation, Washington, NY, USA). After blocking for 1 hour using 1 × TBST (50 mM Tris, 150 mM NaCl, 0.01% Tween 20) with 3% BSA, blots were incubated over-night at 4 °C with a primary rabbit polyclonal anti-CARD14 antibody (diluted 1:500; Proteintech, Rosemont, IL, USA). The blots were washed 5 times for 5 min with 1 × TBST and 1.5% BSA. After incubation with a secondary horseradish peroxidase-conjugated anti-rabbit antibody (diluted 1:5000; Sigma-Aldrich) and subsequent washings (5 times 5 min each with 1 × TBST), proteins were detected using the EZ-ECL chemiluminescence detection kit (Biological Industries). To compare the amount of protein

loaded in the different samples, the blots were re-probed using a mouse monoclonal antibody against β -actin (Sigma-Aldrich).

RESULTS

Clinical findings

We studied three patients (the index patients from Families 1, 2, and 3; **Figure 1, A**) from a cohort of patients referred to tertiary care centers for severe atopic dermatitis. Clinical information on all families is summarized in **Table E3**.

Patient 1 (Family 1, individual II-1; **Figure 1, A**) is a 14 year-old male of Hispanic ancestry who developed very dry skin during the first year of life and was diagnosed with atopic dermatitis. His condition was controlled with topical agents until age 9 when he developed recurrent skin infections and bacterial abscesses requiring multiple hospitalizations. In addition, he had poorly controlled moderate persistent asthma, food allergy and allergic rhinitis. He had no significant systemic non-cutaneous infections. Short stature and precocious puberty were thought to be secondary to chronic steroid use. Aside for elevated IgE (>74,000 IU/mL) as well as marginally elevated IgG (2000 mg/dL) with slightly low IgM (28 mg/dL), laboratory values were unremarkable with normal lymphocyte subsets and specific antibody titers. His family history was remarkable for childhood atopic dermatitis with superinfection, asthma, and seasonal allergy in his mother and mild AD in his father. He tested negative for *STAT3* and *DOCK8* mutations (not shown). He failed oral cyclosporine, and multiple regimens of oral corticosteroids but improved with inpatient wet wrap therapy.

Patient 2 (Family 2, individual II-1; **Figure 1, A**) is a sporadic case in his family. Is a 17 year old Caucasian female with a lifelong history of atopic dermatitis beginning at 6 months of age and

worsening during the adolescent years, which led to multiple hospitalizations. Atopic dermatitis flares were complicated by skin infections. She had food allergies, and moderate persistent asthma which led to multiple hospitalizations during respiratory infection episodes, allergic rhinitis requiring immunotherapy, alopecia secondary to severe scalp involvement and vitiligo. Immunological evaluation demonstrated intermittently low B cell counts and highly elevated IgE levels ($>50,000$ IU/ml) which persisted despite improvement of her skin condition. Specific antibody responses to polysaccharide vaccine were normal, but titer persistence was short-lived. T cell studies were normal. She had no family history of atopy. Mycophenolate mofetil lead to partial improvement and intravenous immunoglobulins resulted in almost clear skin.

Patient 3 (Family 3, individual II-1; **Figure 1, A**) is a 13 year old African-American female born at term. Severe atopic dermatitis was diagnosed at 3 months of age. The patient had also poorly controlled moderate persistent asthma with multiple exacerbations requiring hospitalization, especially when secondary to upper respiratory infections. At least 4 asthma exacerbations were accompanied by symptoms and chest x-ray consistent with pneumonia, however, no infectious organism was isolated and subsequent chest CT was normal. She was noted to have food allergy, gastric esophageal reflux disease, non-traumatic fractures, retained primary teeth, viral skin infections and recurrent skin abscesses as well as extremity osteomyelitis likely secondary to chronic subungual infections. Her laboratory workup was largely unremarkable aside from markedly elevated IgE (5-13,000 IU/mL) and pneumococcal antibody titers which varied from low to normal without immunization. Her family history was significant for atopic dermatitis in her father associated with asthma, food allergy skin boils and respiratory tract infections. She has 3 maternal half-brothers with asthma, allergic rhinitis and mild atopic dermatitis. Her disease was resistant to multiple treatments including wet wraps, bleach baths, UVB therapy and multiple courses of systemic steroids which resulted in adrenal suppression.

Mutation analysis

DNA samples obtained from the patients were subjected to deep sequencing. Putative pathogenic changes were validated in the patients and relatives from whom material was available using Sanger sequencing (see **Table E4** for list of rare variations found in the WES analysis). Individual II-1 from family 1 and individual II-1 from family 2 were found to carry the same heterozygous missense mutation in *CARD14* (c.1778T>C, p.I593T), whereas individual II-1 from family 3 was found to carry a different heterozygous missense mutation (c.2209A>C, p.N737H) (**Figure 2, A**). The two mutations result in single amino acid substitutions (**Figure 2, B**), are extremely rare, affect highly conserved residues and are foreseen to be pathogenic by various prediction algorithms (**Table E5**). The mutations were found to segregate with the atopic phenotype in family 1 and 3 (**Figure 1, A**) although the phenotype of the mother of the proband in family 1 was less severe (**Figure 1, A; Table E3**).

Atopic dermatitis-associated mutations in *CARD14* exert a dominant-negative effect and impair NF- κ B activation

The two mutations are located in the structural PDZ-SH3-GUK module, also known as membrane-associated GUK (MAGUK) domain (**Figure 2, B**). This domain is essential for proper *CARD14* function,²⁴ which suggests that the mutations may affect *CARD14* capacity to regulate NF- κ B signaling.²⁴ We therefore used a NF- κ B luciferase reporter system as previously described.¹⁵ Briefly, we co-transfected HEK293 cells with a NF- κ B luciferase reporter plasmid and with a *CARD14*-wild-type expression vector, or the same vector carrying either of the two *CARD14* atopic dermatitis-associated mutations. Both mutations significantly attenuated the ability of *CARD14* to activate NF- κ B (**Figure 3, A**). Co-transfection of equal quantities of mutant and wild-type *CARD14* expression vectors led to impaired luciferase activity when

compared to that measured after transfection of the same quantity of wild-type vector alone, showing that the loss-of-function variants exert a dominant-negative effect (**Figure E2**) Gain-of-function mutations in *CARD14* result in increased expression of both *CARD14* itself and activated p65 (a surrogate marker for NF- κ B activity) in the skin of patients with psoriasis and pityriasis rubra pilaris.^{12, 13} We therefore hypothesized that loss-of-function mutations in the same gene should lead to the reverse phenotype in our patients with severe atopic dermatitis. Indeed, we found a significant decrease in the expression of both *CARD14* and the activated p65 subunit of NF- κ B, compared with healthy controls (**Figure 3, B, C**).

***CARD14* loss-of-function impairs epidermal secretion of antimicrobial peptides and hCCL20**

The pathogenesis of atopic disease caused by *CARD11* dominant negative mutations both in humans¹⁰ and in mice²⁵ is likely due to marked defects in the hematopoietic tissues where it is mostly expressed. Conversely, *CARD14* expression is largely confined to epidermal cells.¹³ *CARD14* is a major regulator of NF- κ B and NF- κ B signaling is known to regulate innate immunity which in turn has been implicated in the pathogenesis of inflammatory skin diseases.²⁶ We therefore used RNAi to down-regulate *CARD14* in keratinocytes or transfected wild type or mutant *CARD14* expression vectors in keratinocytes, and then examined the expression of pivotal mediators of innate immunity in human primary keratinocytes. Keratinocytes with reduced *CARD14* expression expressed significantly less hBD1, hBD2 and hCCL20 than keratinocytes transfected with control siRNA at the protein level (**Figure 4, A**) as well as at the RNA level (**Figure E3**). Moreover, keratinocytes expressing mutant *CARD14* also released significantly less hBD1, hBD2 and hCCL20 (**Figure 4, B**). LL37 levels remained unchanged in both sets of experiments (**Figure 4**). Accordingly, hBD1, hBD2 and hCCL20 expression was

markedly reduced in the epidermis of a patient carrying a loss-of-function *CARD14* mutation as compared with healthy controls or psoriasis patients (**Figure 5**). Of note, no significant changes were seen in the expression levels of IL-33 and TSLP in keratinocytes transfected with mutant *CARD14*, suggesting that the pathogenesis of the allergic inflammation is not primarily driven by these epithelium-produced, AD-related proteins (**Figure E4**).

DISCUSSION

In the present study, we demonstrate the presence of dominant-negative, loss-of-function mutations in *CARD14* in three patients with unusually severe AD. *CARD14* has recently emerged as a major player in psoriasis as well as the related disorder, pityriasis rubra pilaris.^{12, 13} In contrast with the dichotomist approach to the relationship between psoriasis and AD which was prevalent in the past,²⁷ more recent studies have highlighted overlapping pathomechanisms in those two conditions, which mostly involve elements traditionally associated with adaptive immune responses.^{28, 29} For example, the Th₁₇ pathway has been implicated in the pathogenesis of both diseases³⁰: the Th₁₇ axis plays a major role in the pathogenesis of psoriasis³¹, while its role has also been documented in a number of AD subsets including AD in patients of Asian origin, pediatric AD and intrinsic AD.^{32, 33} The fact that only some forms of AD are (at least in part) Th₁₇-driven may explain the lack of uniform response of AD to Th₁₇-targeting treatments.³⁴⁻³⁷ The combination of these common pathomechanisms with other and unique alterations in other signaling systems has been proposed to eventually result in different AD endotypes or disease phenotypes (e.g. AD and psoriasis).^{29, 32} Along with adaptive immunity defects, AD patients demonstrate a wide range of abnormalities related to the secretion of central epidermal inflammatory mediators. Although some studies

have demonstrated increased expression of some antimicrobial peptides (AMPs) in the skin of AD³⁸, most studies show that while AMPs are abundant in psoriasis, they seem to be present in contrast in very low amounts in AD, which may explain the susceptibility of individuals with AD to skin microbial infections,^{39, 40} as also seen in our patients. **(Table E3).**

Although Th₂-associated cytokines may modulate the expression of AMPs in AD,^{41, 42} the regulation of the secretion of these inflammatory mediators, has also been shown to be under the direct regulation of CARD14.^{13, 43} Indeed, CARD14 increased activity was found to be associated with elevated AMPs levels.⁴⁴ Here, we found that down-regulation of CARD14 led to decreased expression of two key AMPs that were also found to be less expressed in patient skin **(Figure 5).**

AD is considered to result from a combination of immunological abnormalities and epidermal defects, eventually leading to infections and allergic reactions.¹ AMP deficiency may not only underlie infectious complications displayed by AD patients, but could also contribute to other aspects of AD pathogenesis including impaired epidermal barrier function and mucosal surface immunity. Indeed, AMPs, in addition to their antibacterial and antiviral properties, seem to play an important role in connecting innate and adaptive immune responses as well as in regulating keratinocyte proliferation and differentiation.⁴⁵⁻⁴⁹ Whether the inflammatory disease seen in the respiratory tracts of our patients also reflects AMP impairment, as opposed to the product of the atopic march alone, is a matter for further studies. did not affect LL-37 expression in spite of the fact that NF- κ B has been shown to regulate LL-37. This may be due to the fact LL-37 expression can be induced through alternative pathways.⁵⁰

hCCL20 expression was also found to be decreased in CARD14-deficient keratinocytes as well as in keratinocytes cells expressing CARD14 loss-of-function mutations, which is consistent with the fact that hCCL20 expression is increased in keratinocytes exhibiting increased CARD14

expression¹³ Some reports have shown that hCCL20 expression is elevated in the serum and skin of AD patients⁵¹, but most other studies have shown hCCL20 to be deficient in AD patients. Since hCCL20 displays antiviral activity, hCCL20 deficiency may underlie the susceptibility of AD patients to herpes and pox viruses.^{52, 53} More importantly, hCCL20 seems to play an important role in triggering the recruitment of protective inflammatory cells to a compromised epidermis.⁵² The decreased expression of this and other inflammatory mediators in the context of severe atopic dermatitis seen in these patients provides a potential model to dissect the primary roles of global inflammatory mediators in psoriasis and some endotypes of AD. How do these heterozygous *CARD14* mutations interfere with normal signaling is an important topic for future research. The *CARD14* homolog, *CARD11*, is known to multimerize with activation⁵⁴; thus, *CARD14* deleterious mutations could render heteromultimers inefficient in inducing MALT1 protease activity. Alternatively, *CARD14* activity may be dependent upon different signaling mechanism in keratinocytes than those seen in lymphocytes for *CARD11*.

It is of interest to note that the *CARD14*^{-/-} mice do not develop spontaneous atopic dermatitis.⁵⁵ Besides inherent dissimilarities in keratinocyte biology in mice and in humans, the lack of triggering microbial exposures may underlie these observations— as was observed in the *Relb*^{-/-} or *Trim32*^{-/-} models which required viral or viral product exposure to induce AD.^{56, 57} These studies, as well as others pointing towards a direct driving effect of abnormal microbial colonization on AD-related inflammation⁵⁸⁻⁶⁰, provide a potential explanation for variable penetrance of *CARD14* mutations, and suggest that the lack of host defense genes alone can, in combination with microbial exposure, result in AD-like phenotypes.

In summary, we have identified germline mutations in *CARD14* in patients with severe atopic dermatitis, resulting in decreased NF- κ B activity and secretion of AMPs. These data expand the

spectrum of CARD14-associated phenotypes and shed new light on the partially overlapping but also distinct pathophysiologic mechanisms underlying AD and psoriasis.

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FIGURE LEGENDS

FIG 1. Clinical and histopathological features. (A) Pedigrees of the three families. Black symbols indicate severe AD; wild-type and mutant alleles are denoted as – and +, respectively. Individual II-1, family 1, presented with (B) erythroderma with fine scales involving more than 90% of his body surface area, (C) and prominent fissuring and accentuation of the skin folds; (D) Histopathological examination of a skin biopsy obtained from this individual's leg reveals parakeratosis, hypogranulosis, prominent spongiosis, acanthosis and psoriasiform epidermal hyperplasia, associated with occasional lymphocyte exocytosis and a perivascular mild lymphocytic infiltrate.

FIG 2. Mutation analysis. (A) Direct sequencing of *CARD14* revealed a heterozygous T>C transition (arrow) at position c.1778 of the cDNA sequence in individual II-1, family 1 (upper left panel) as well as a heterozygous A>C transversion (arrow) at position c.2209 of the cDNA sequence in individual II-1, family 3 (upper right panel). The wild type sequences (WT/WT) are given for comparison (lower panels); (B) The location of the two mutations is depicted along a schematic representation of the *CARD14* protein and its domains.

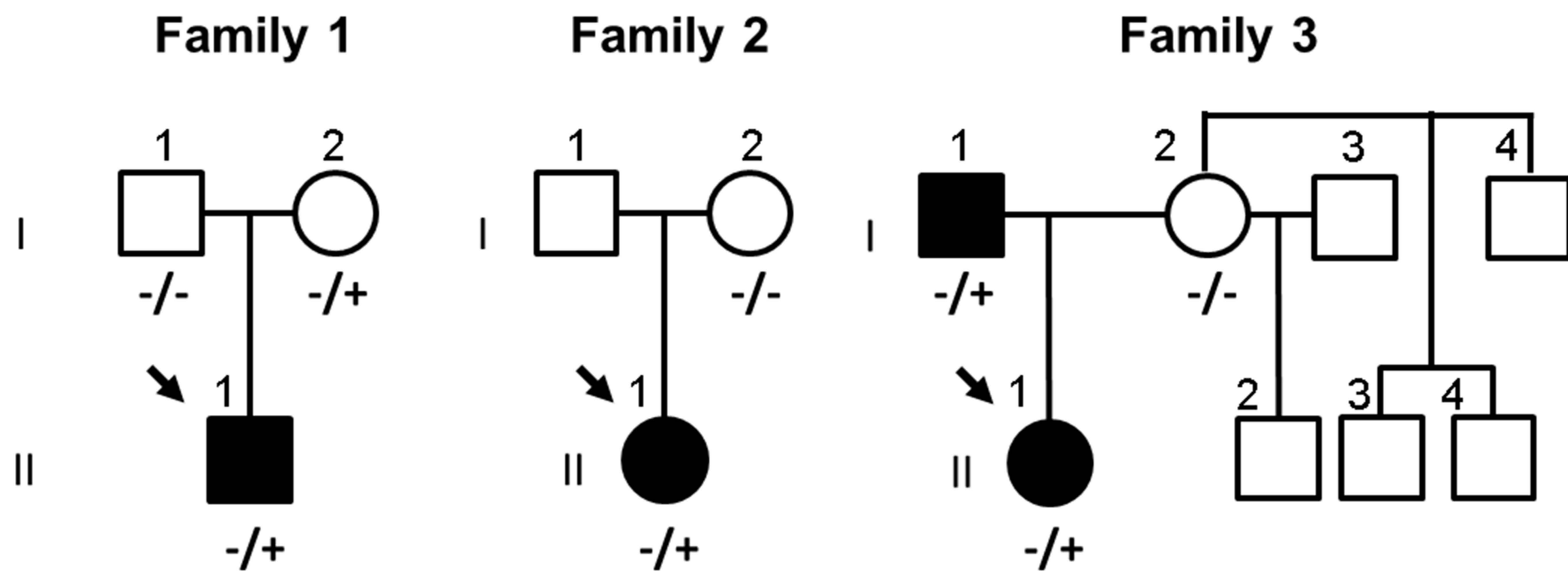
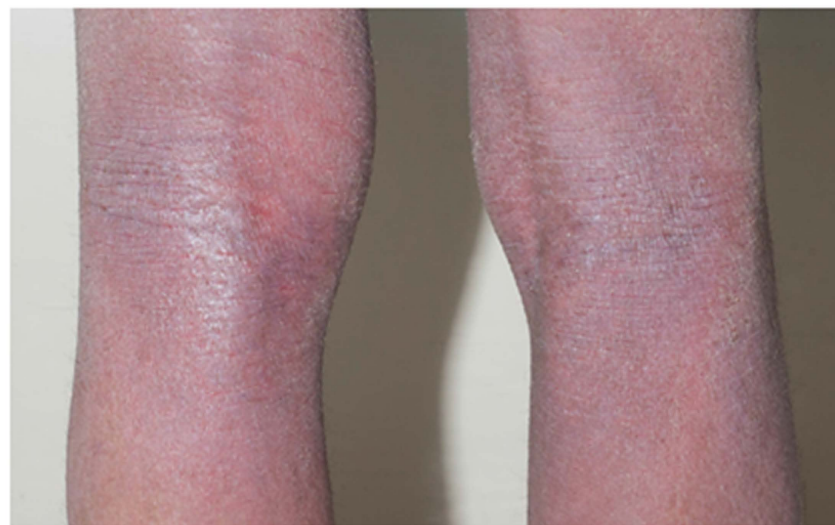
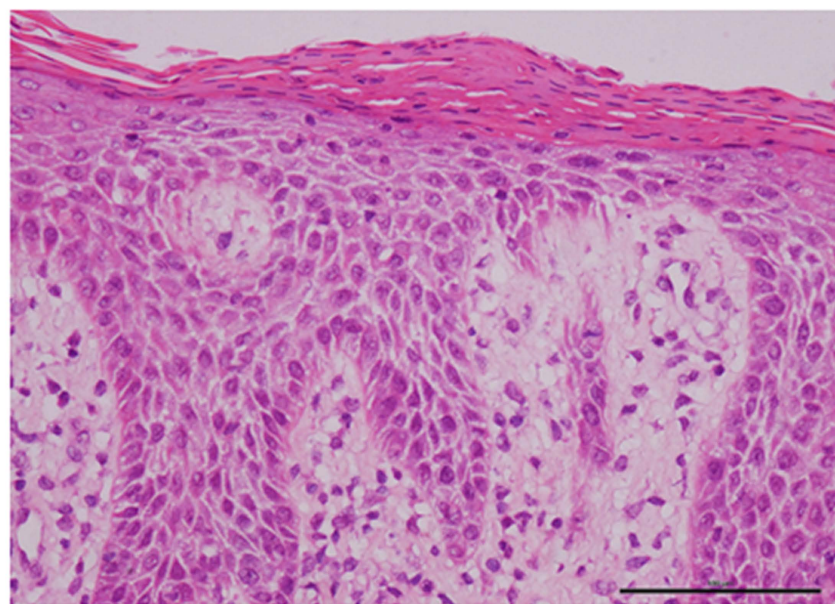
FIG 3. Consequences of AD-associated mutations in *CARD14*. (A) HEK293 cells were co-transfected with a NF- κ B-responsive luciferase reporter gene and plasmids expressing either wildtype *CARD14* cDNA or *CARD14* cDNA harboring p.I593T or p.N737H mutations. Luciferase activity was measured after 24 hours and normalized to Renilla luciferase. Results represent the mean of 3 independent experiments $\pm$ standard error (two sided t-test; *** $p < 0.001$); (B) Skin biopsies obtained from the thigh of individual II-1, family 1, and from a healthy control

were stained for CARD14 or for activated p65 (scale bars = 100 μ m, counterstaining with hematoxylin). (C) Immunohistochemistry staining intensity was quantified using ImageJ software (two sided t-test, *** p <0.001).

FIG 4. Inflammatory gene expression in CARD14-deficient keratinocytes and CARD14 loss

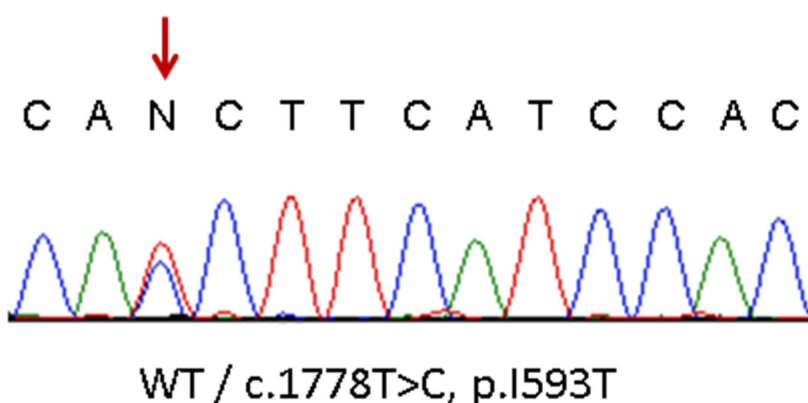
of function mutations. (A) Keratinocytes were transfected with *CARD14*-specific siRNA or with control siRNA. The protein levels of hBD-1, hBD-2, LL-37 and hCCL20 secreted into the culture medium were measured using ELISA assays according to manufacturer's instructions. Results are expressed as percentage of protein expression levels relative to expression in control siRNA-transfected samples $\pm$ standard error; (B) Keratinocytes were transfected with wild-type (WT) or mutant *CARD14* (p.I593T or p.N737H) complementary DNA (cDNA) constructs and then maintained in growth medium (KGM) without supplements for 48 hours or for 24 hour in the case of hCCL20. The protein levels of hBD-1, hBD-2, hLL-37, and hCCL20 secreted into the culture medium were measured using ELISA assays according to manufacturer's instructions. Results are expressed as percentage of protein expression levels relative to expression in wildtype *CARD14*-transfected samples $\pm$ standard error. Results represent the mean of at least three independent experiments (two sided t-test; * p <0.05, ** p <0.01, *** p <0.005).

FIG 5. hCCL20, hBD-1 and hBD-2 expression in skin biopsies. Skin biopsies obtained from the thigh of individual II-1, family 1, from a healthy control and a psoriasis patient were stained for hCCL20, hBD-1 and hBD-2 (scale bars = 100 μ m,). Immunohistochemistry staining intensity was quantified using ImageJ software (right panels) (two sided t-test: *** p <0.001).

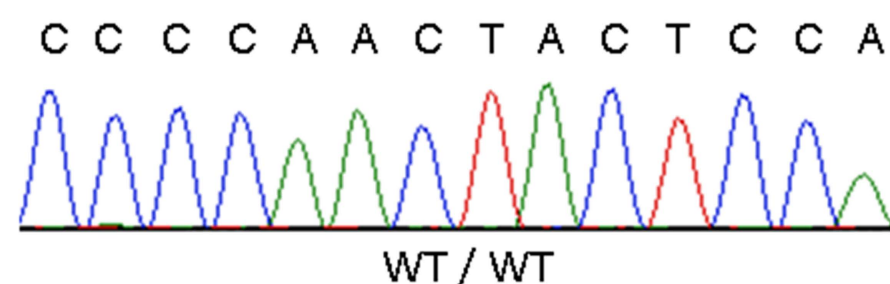
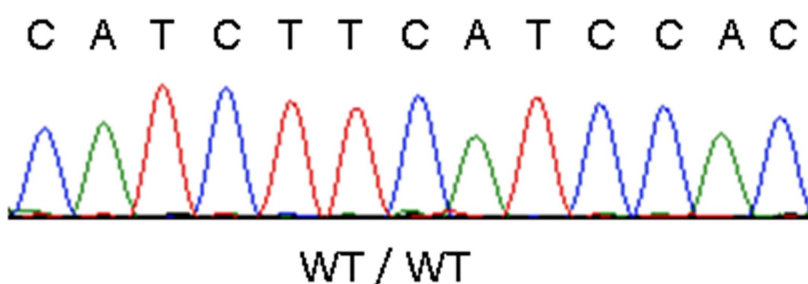
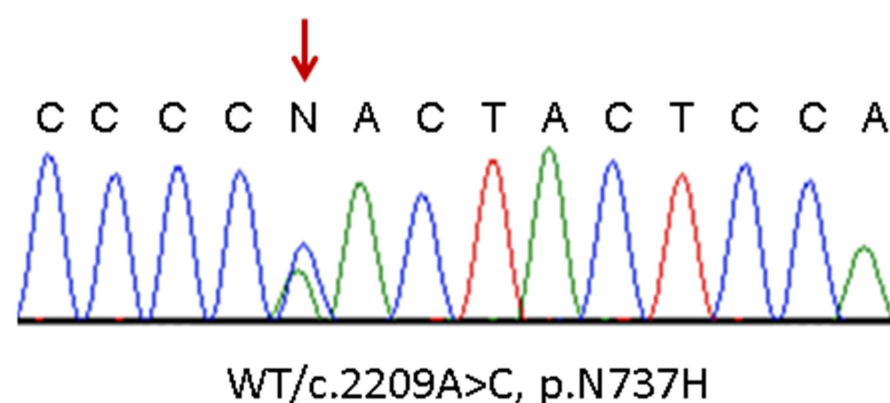
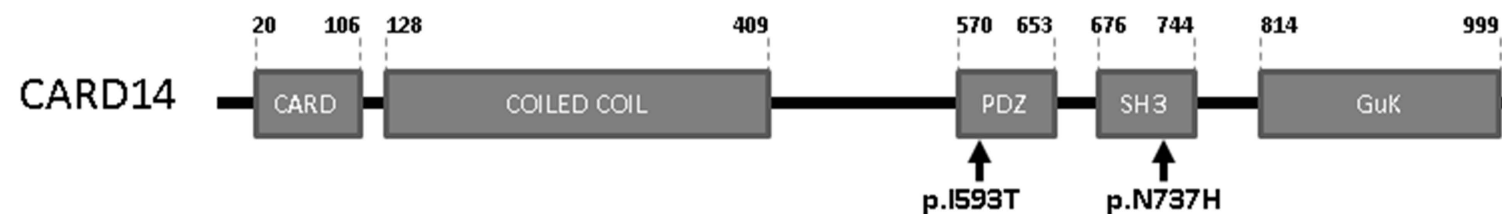
A**B****C****D**

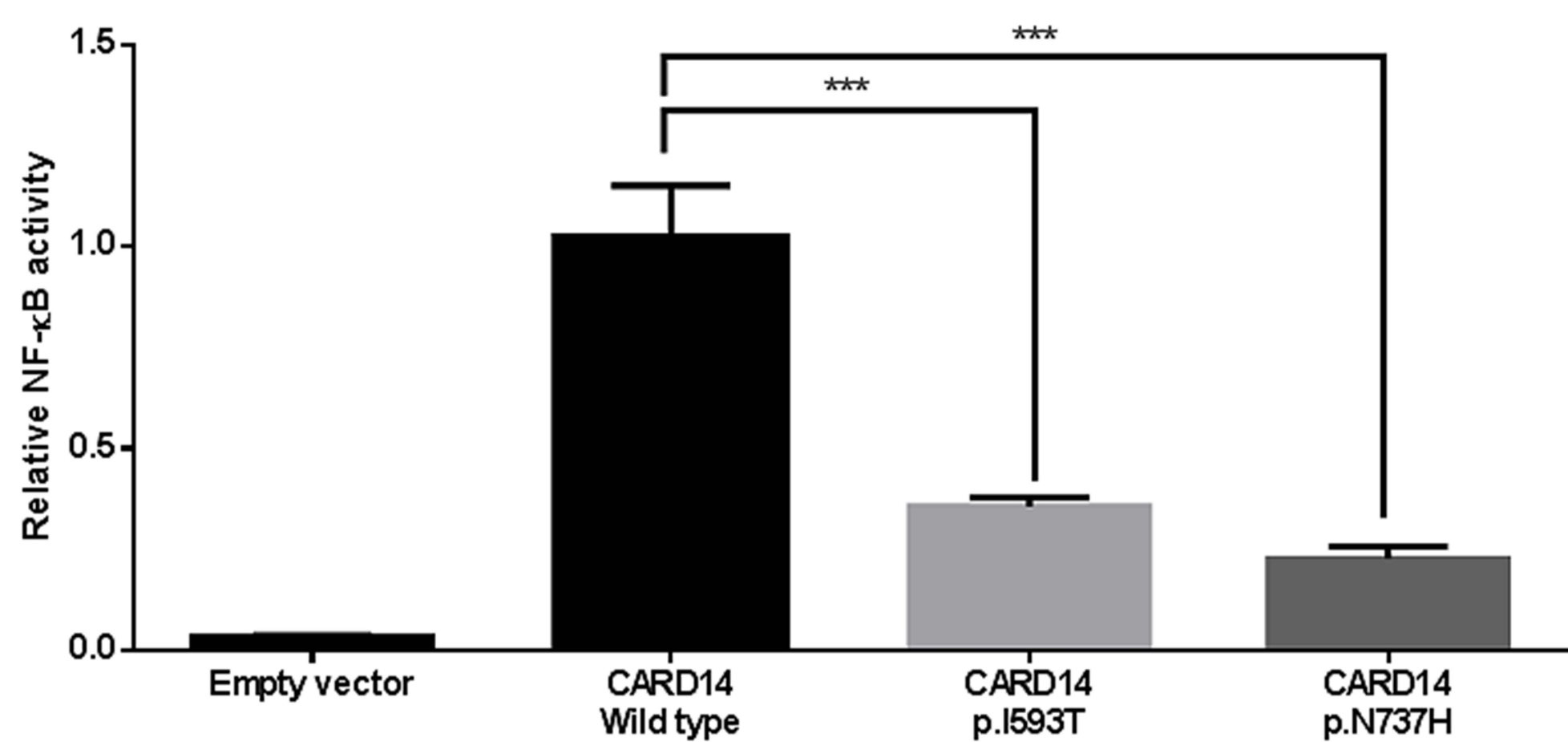
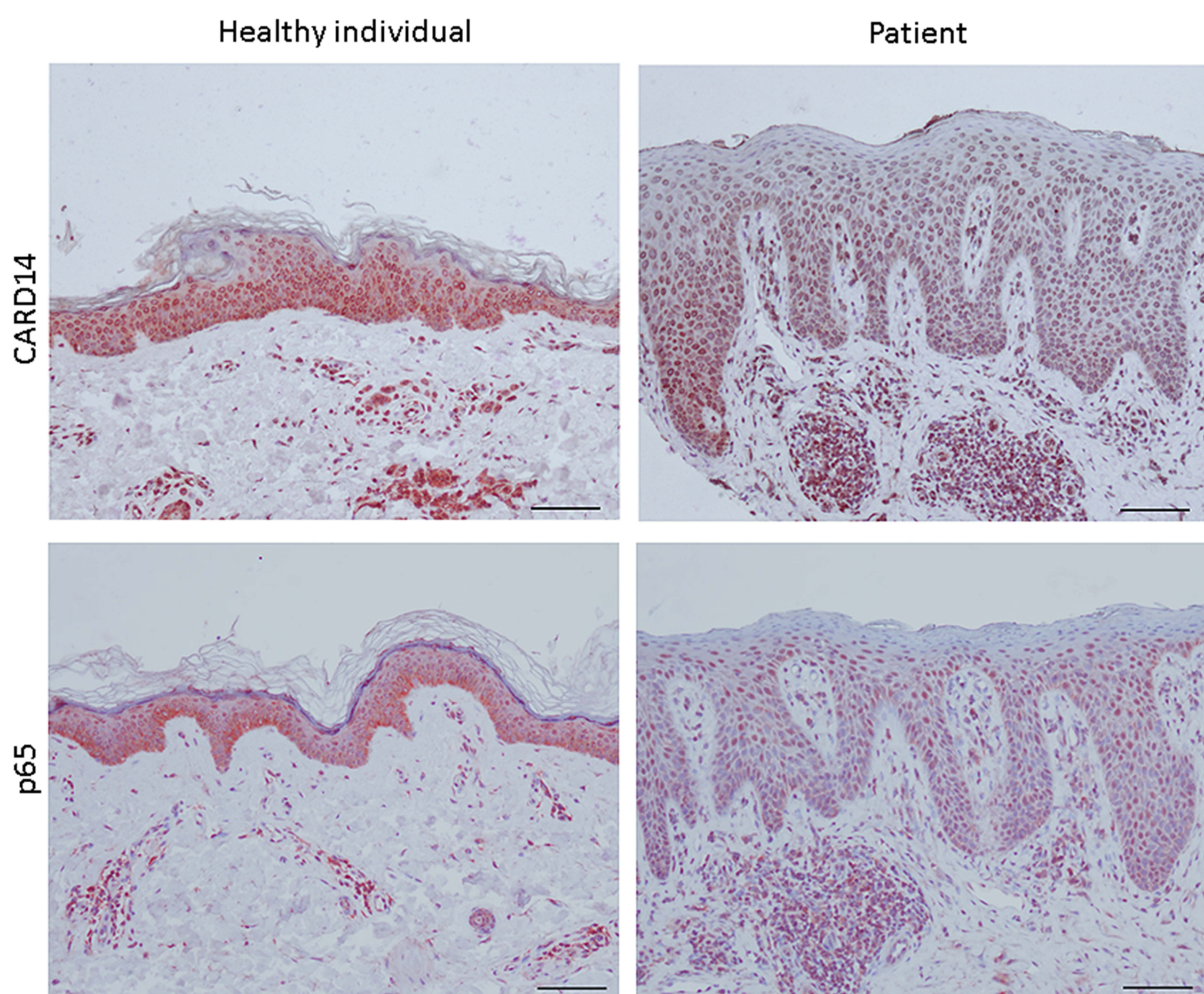
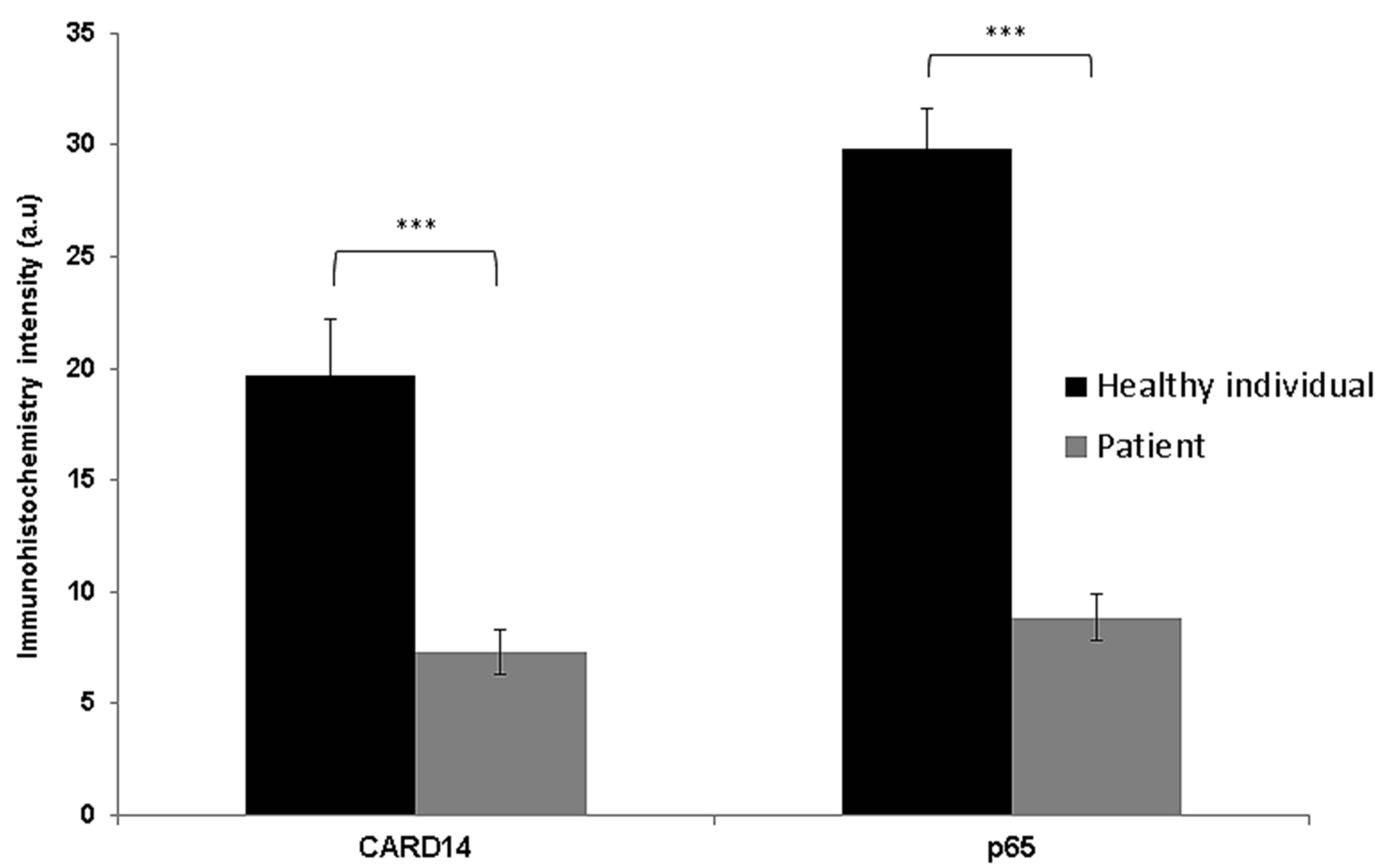
A

Patient 1

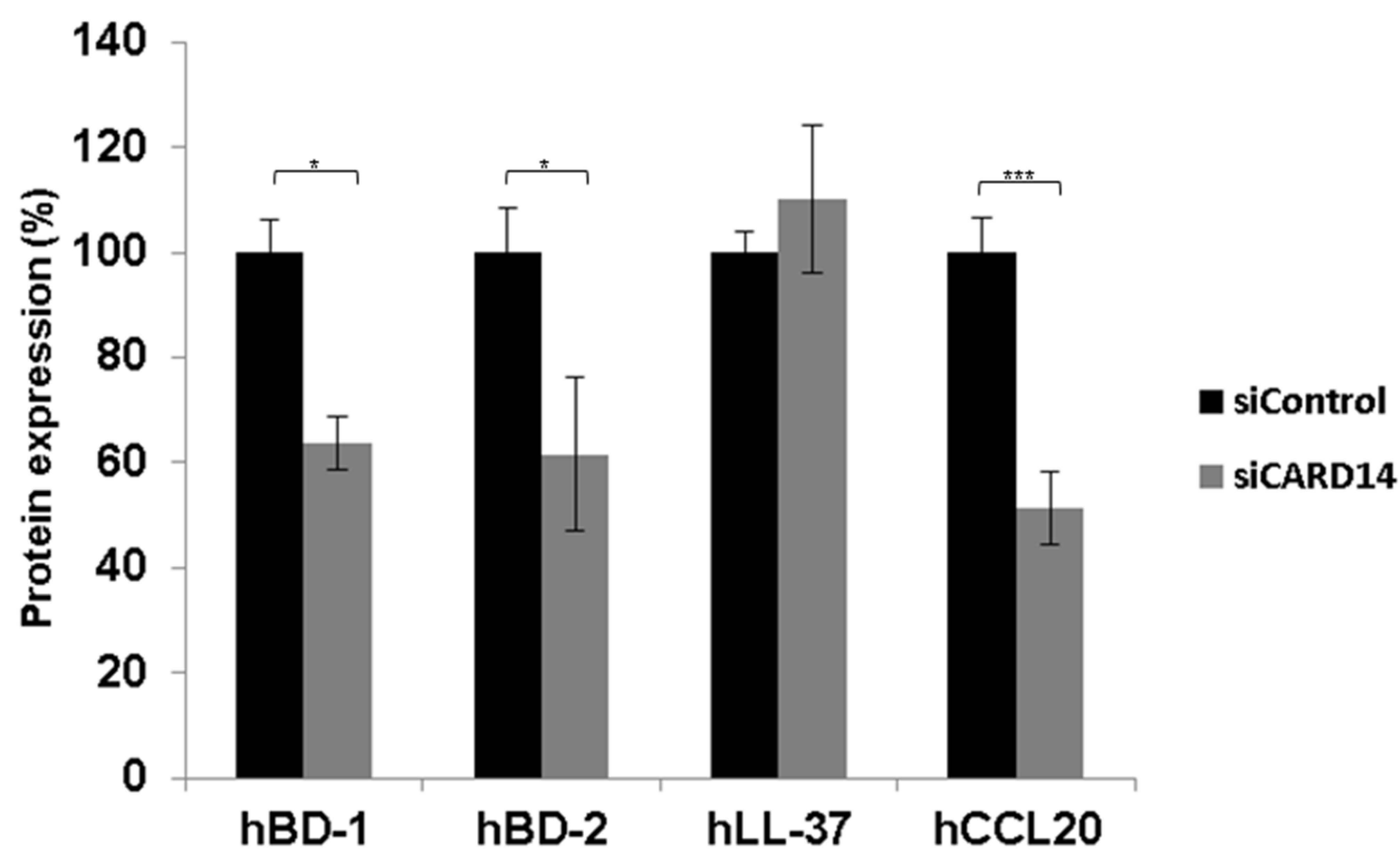


Patient 3

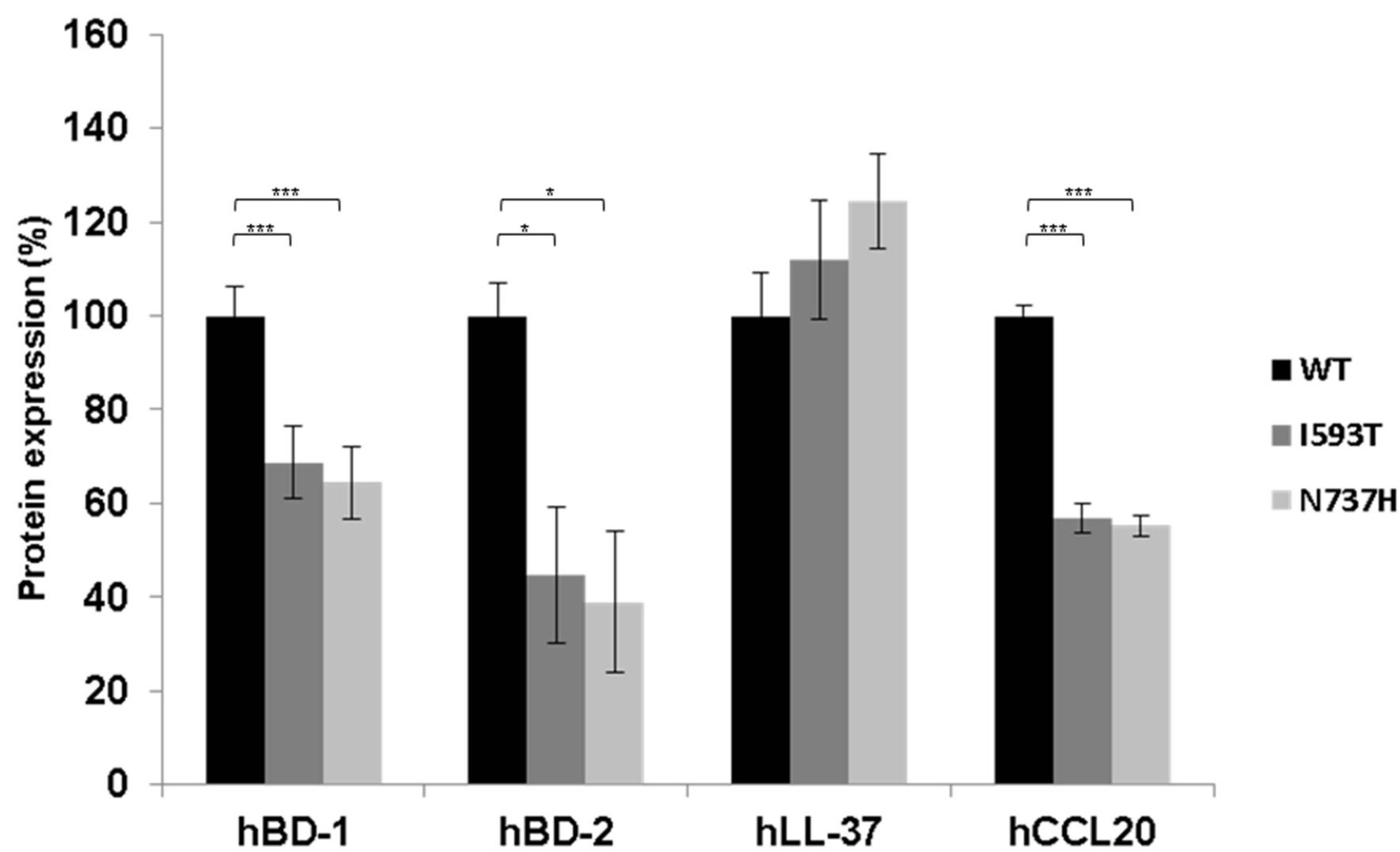
**B**

A**B****C**

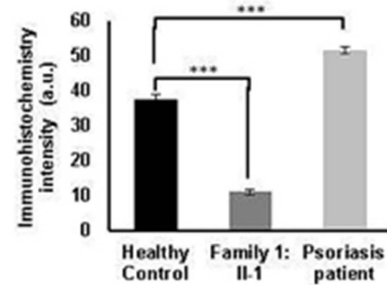
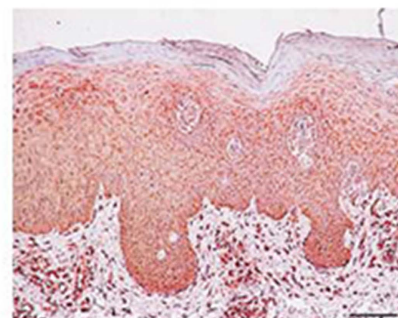
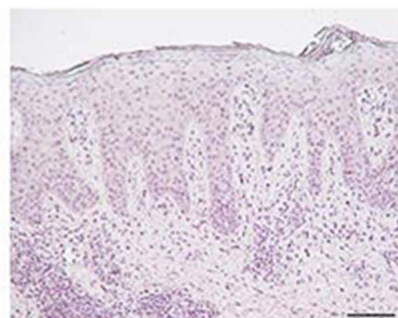
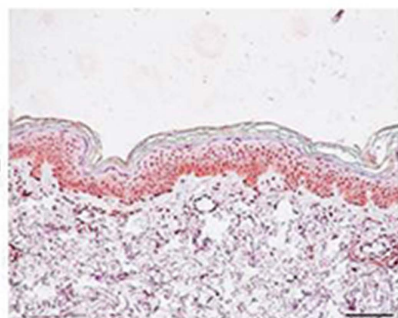
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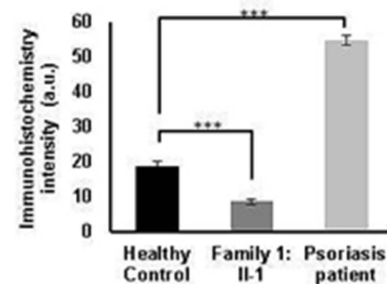
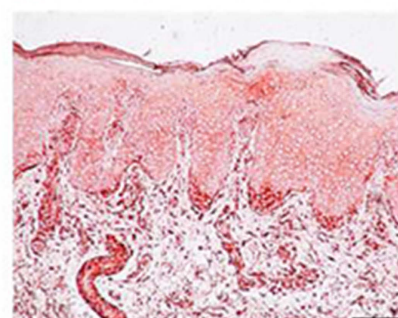
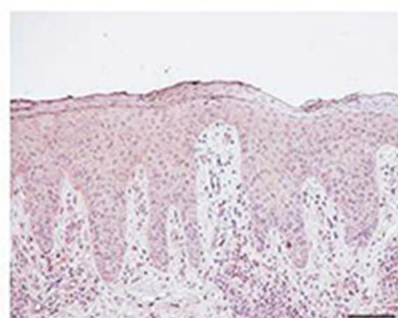
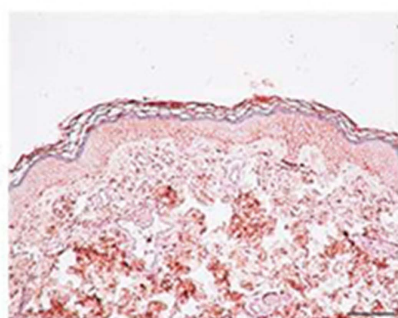
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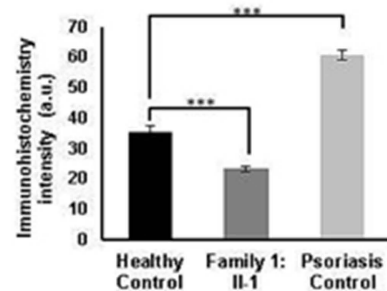
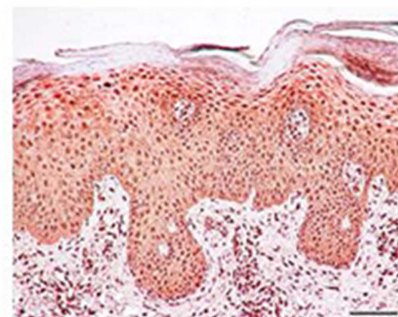
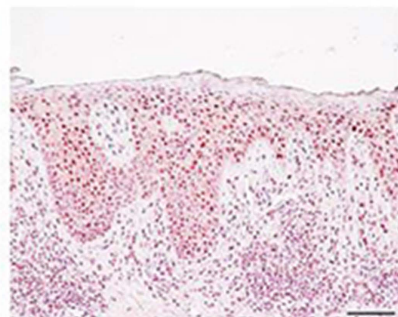
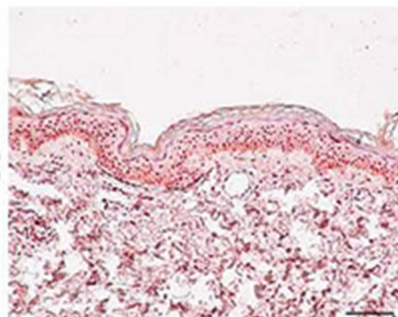
hCCL20



hBD-1



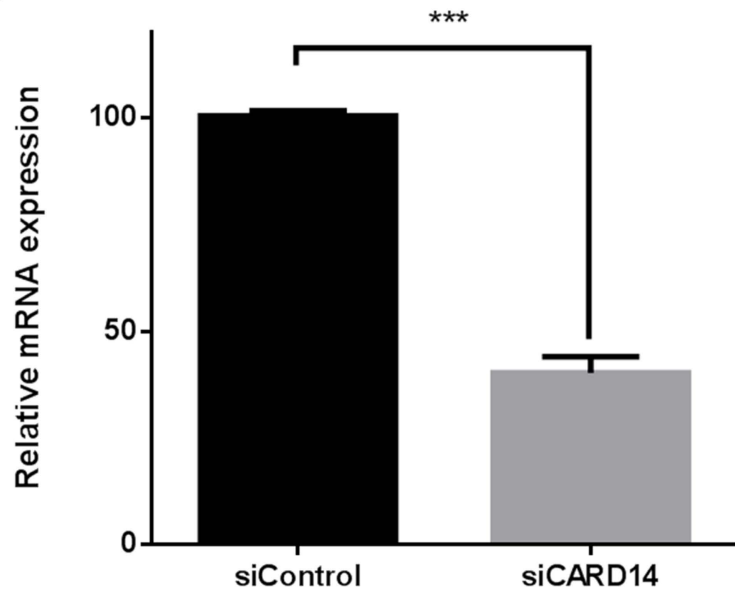
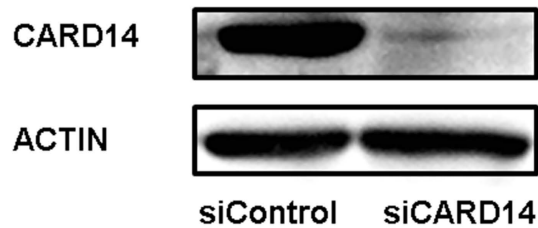
hBD-2

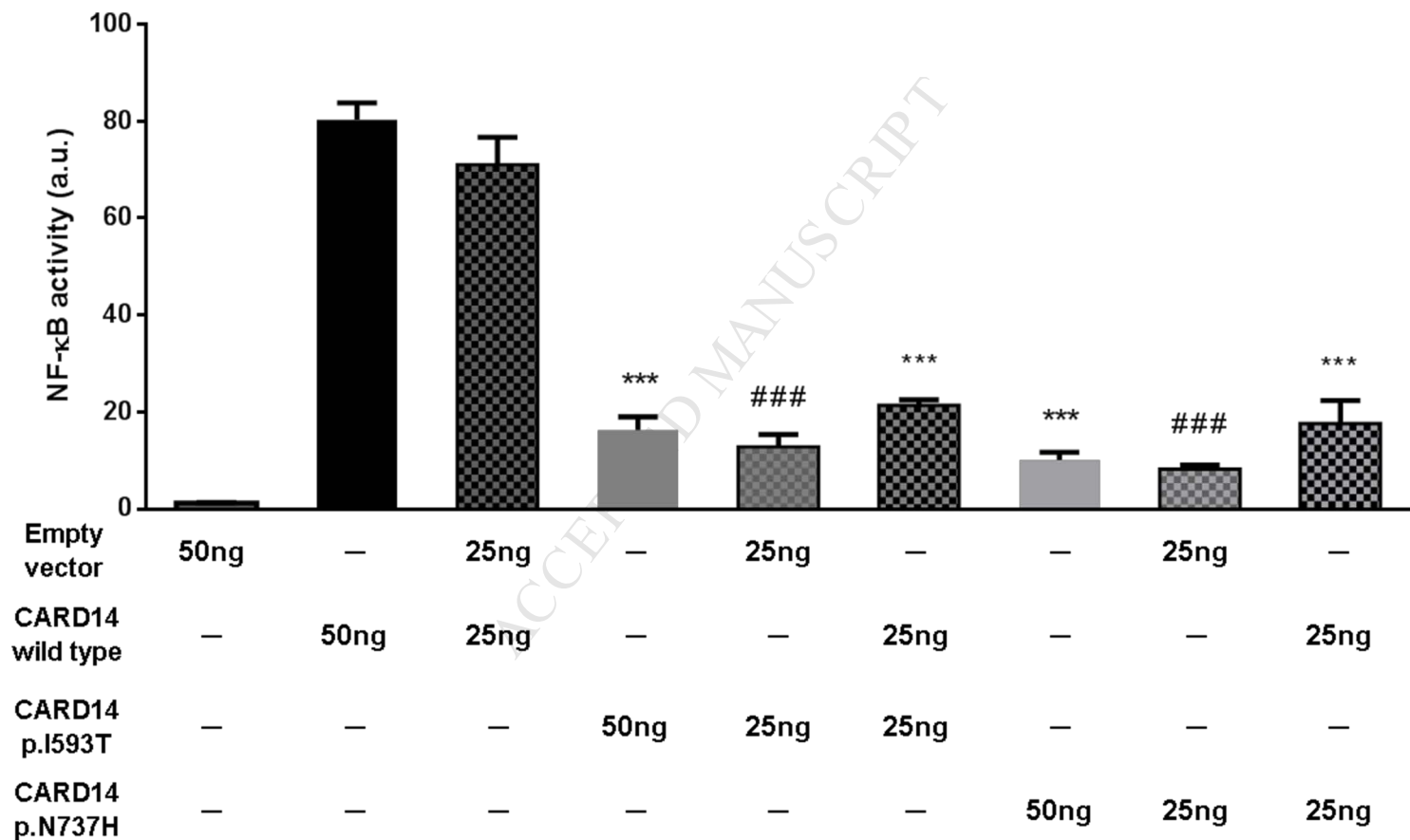


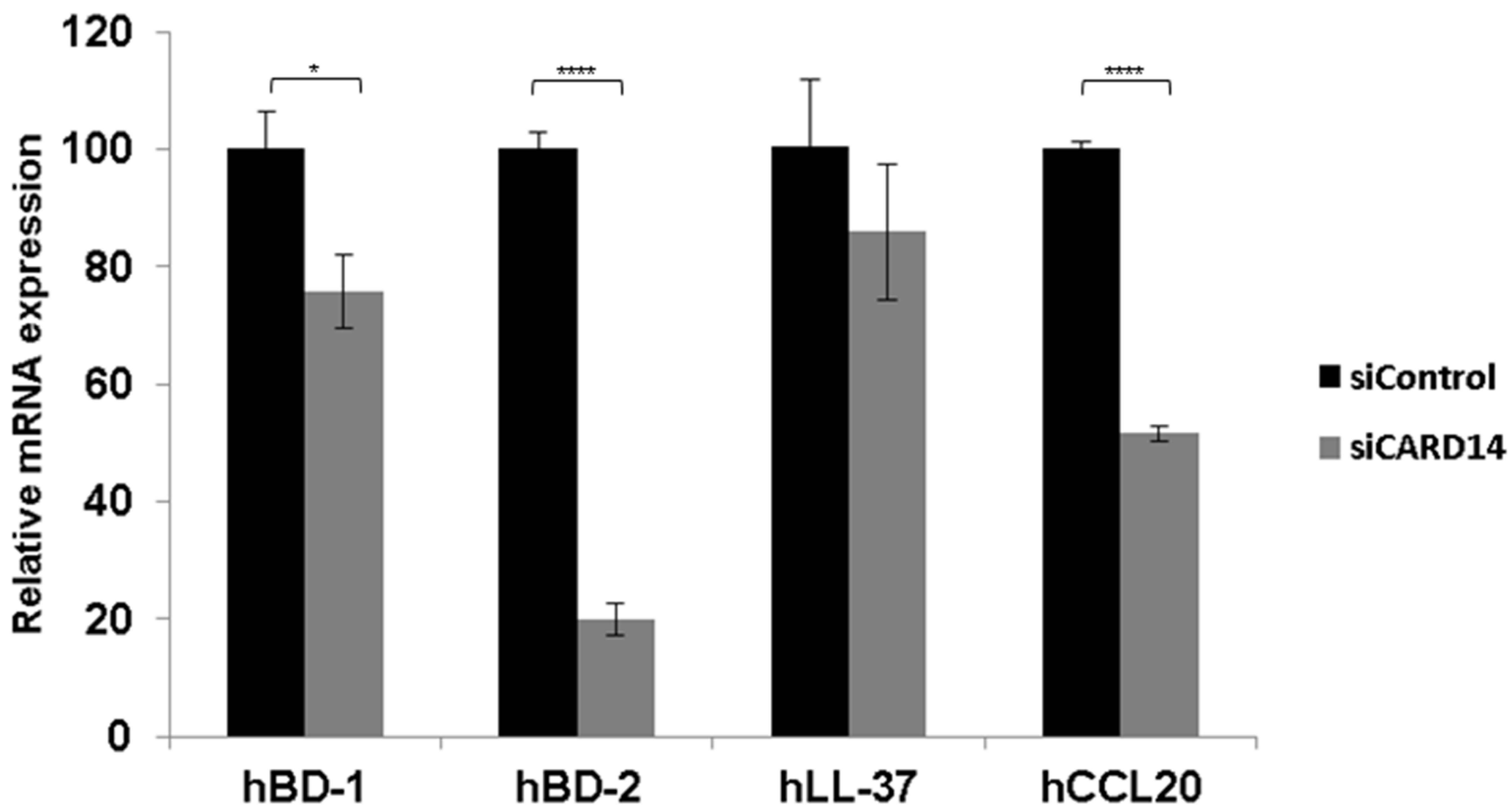
Healthy control

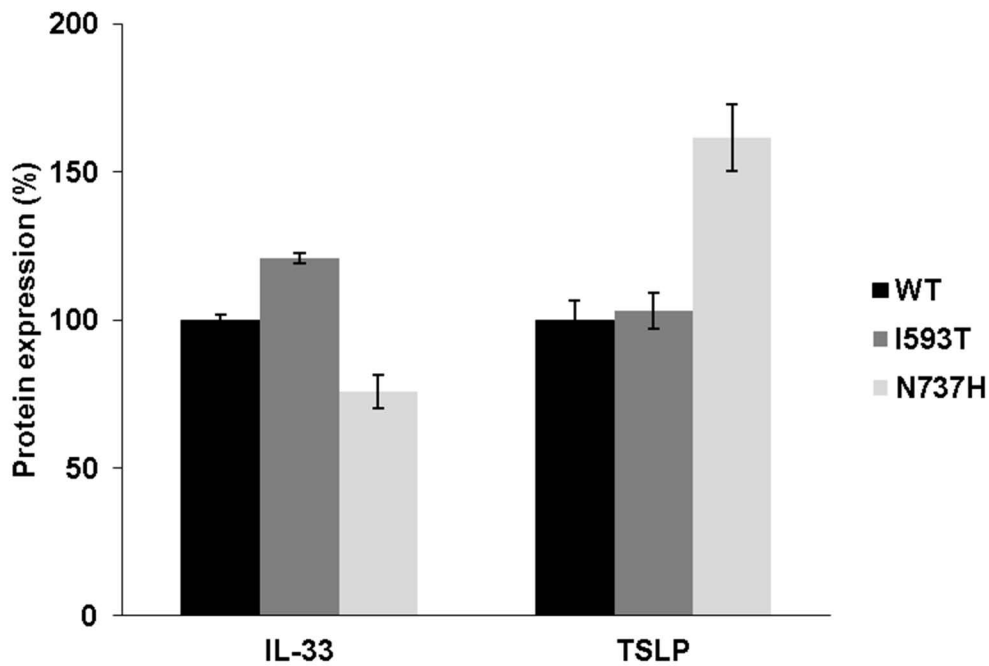
Family 1: II-1

Psoriasis patient

A**B**







Supplementary data

Loss-of-function mutations in *CARD14* are associated with a severe variant of atopic dermatitis

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Supplementary tables

ACCEPTED MANUSCRIPT

Supplementary Table E1. Oligonucleotides used to sequence *CARD14*

Exons	Forward oligonucleotide sequence	Reverse oligonucleotide sequence	Expected product size (bp)
2	TTAAAACGGTGTCACCCTG	ACAGGACGAGAAGAGACCCC	404
3	CGATTCTTACATGTGCGGG	GGCACCTGGGGTTACCAG	331
4	ACCTGCTCACCTACCCACC	GACAAGGAAGAGGGGAAAGG	506
5	TTAGGTGAACCCTTTTCGTGG	ACCTGTCAGAAACCCACAG	402
6	AAGACTGCATCCGTCCACAC	ATCTGGCTTCCCCACAGAC	320
7	AACTGTCTCCCTCCCTCCAC	GAGACTGTCCCCGGAACC	303
8	GGCTAGAAACAGGGCTCTCC	CTGGAGCCCAGCTCTGTC	308
9	ACCTGGTAGAAACTCCACGG	CAGGGAAGAGGTTGGTACGA	315
10-11	CTGTGGCTCTCTACACCG	TTCTATCTGCCCTTTCCCTG	659
12-13	GATCTGTGAAGAAGGGGCTG	GTGAAGTCTGCCTGGGTCAC	671
14	GTGCAGGCAGTGGTCCTAC	AACCACCAGGACTTAAGGG	323
15	ATTTTCTGCAACCTTCCTCG	CCACGCCACCCCTCTATTG	419
16	ACTCTCCCCTGCTCGGC	ACTCTCCACACAGTGCCTCC	237
17	ATCATCTCCCCTGAATTCCC	ACTAGCAGCAGCTCCCAAAG	263
18	AGCAAAGCAGACCCAGTCC	GGGGAGGGAAGGAGGAG	364
19	GGGGACAGGGGTGTTTACC	AGGTCACCCAGGTCTCAGG	315
20	CTTCTGACCTGGGCGTTG	CAAACCGCAGAGCACACTC	294
21	TGTTTAGGGGTGTTTGGGTG	CTGGGCTGAGGAACAGGAC	382

Supplementary Table E2. Oligonucleotides used to perform qRT-PCR.

Gene	Forward oligonucleotide sequence	Reverse oligonucleotide sequence	Expected product size (bp)
<i>CARD14</i>	AGGCAGGTGTTGAGCTG	GGTCCTGGCTTCCTGCTT	102
<i>DEFB1</i>	GGAGGGCAATGTCTCTATTCTG	TCATTTCACTTCTGCGTCATTTC	127
<i>DEFB2</i>	TTAAGGCAGGTAACAGGATCGC	TCCTCTTCTCGTTCCTCTTCATATTC	82
<i>CAMP</i>	TGTGCTTCGTGCTATAGATGG	GCACACTGTCTCCTTCACTG	145
<i>CCL20</i>	GGTGAAATATATTGTGCGTCTCC	ACTAAACCCTCCATGATGTGC	148
<i>GAPDH</i>	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAGCC	185

Supplementary Table E3. Clinical features of family 1-3 members

Patient	CARD14 Genotype	Atopic dermatitis	Allergic rhinitis	Asthma	Food allergy	Pyogenic skin infections	Viral skin infections	Respiratory tract infections	SCORAD	Others
Fam. 1: I-1	WT/WT	+++	+	+	-	+	-	-	NA*	-
Fam. 1: I-2	WT/I593T	++	++	++	++	++	-	+	NA	-
Fam. 1: II-1	WT/I593T	+++	++	++	++	+++	+	-	67	Short stature, precocious puberty (due to chronic oral steroid use ?)
Fam. 2: I-1	Unknown	-	-	-	-	-	-	-	-	-
Fam. 2: I-2	WT/WT	-	-	-	-	-	-	-	-	-
Fam. 2: II-1	WT/I593T	+++	-	++	+	+++	+	-	60-70	Vitiligo, alopecia
Fam. 3: I-1	WT/N737H	+++	+	+	+	++	-	-	NA	-
Fam. 3: I-2	WT/WT	-	-	-	-	-	-	-	-	-
Fam. 3: I-3	unknown	-	-	-	-	-	-	-	-	-
Fam. 3: I-4	unknown	-	-	-	-	-	-	-	-	-
Fam. 3: II-1	WT/N737H	+++	-	++	+++	+++	+++	+++	60-72	Extremity osteomyelitis, recurrent paronychia, non-traumatic fracture and retained primary teeth
Fam. 3: II-2	unknown	+	+	+	-	-	-	-	NA	-
Fam. 3: II-3	unknown	+	+	+	-	-	-	+	NA	-
Fam. 3: II-4	unknown	-	-	+	+	-	-	-	-	-

* NA, not available

** +, mild; ++, moderate; +++, severe

Supplementary Table E4. Rare immune-related variations found through WES analysis

Gene	Chromosome	Position	Reference allele	Alternative allele	MAF	Genotyping	
						Fam. 1: II-1	Fam. 3: II-1
JAK1	1	65321300	G	A	4.16E-05	G/A	G/G
BEND3	6	107391391	A	G	0	A/G	A/A
IKZF1	7	50467806	G	A	0	G/A	G/G
DOCK8	9	368288	GT	AC	0	GT/AC	GT/GT
SLC29A3	10	73115941	TG	CA	0	CA/CA	TG/TG
PSMA3	14	58737688	T	C	8.52E-06	T/C	T/T
CARD14	17	78172317	T	C	4.978E-06	T/C	T/T
SON	21	34922824	GC	G	0	GC/G	GC/GC
TLR8	X	12937536	A	C	0	A/C	A/A
NOD2	16	50733636	T	C	0	T/T	T/C
PLCG2	16	81973641	A	G	4.14E-05	A/A	A/G
CARD14	17	78176209	A	C	8.741E-05	A/A	A/C
G6PC3	17	42148520	A	C	0.0003	A/A	A/C
TREX2	X	152710624	G	A	0.0003	G/G	G/A

Supplementary Table E5. Bioinformatics analysis of *CARD14* mutations

Mutation	Polyphen2 (range 0-1)^a	SIFT (range 1-0)^b	Consurf (range 1-9)^c	Mutation Taster^d	SNAP^e	Allele frequency^f
p.I593T	0.999	0.5	9	Disease causing	Pathogenic	4.078e-6
p.N737H	0.993	0.01	8	Disease causing	Pathogenic	8.741e-5

^a <http://genetics.bwh.harvard.edu/pph2/index.shtml>¹^b http://sift.jcvi.org/www/SIFT_enst_submit.html²^c <http://consurf.tau.ac.il/2016/>³^d <http://www.mutationtaster.org/index.html>⁴^e <https://www.rostlab.org/services/SNAP/>⁵^f <http://gnomad.broadinstitute.org/gene/ENSG00000141527>⁶

Supplementary figures legends

Supplementary Figure E1: siRNA-mediated down-regulation of *CARD14*. Keratinocytes were transfected with *CARD14*-specific siRNA (siCARD14) or with control siRNA (siControl) and then maintained in growth medium (KGM) for 48 hours. (A) *CARD14* mRNA expression was ascertained using qRT-PCR. Results represent the mean of 3 independent experiments and are expressed as percentage of gene expression in primary keratinocytes cells transfected with *CARD14*-specific siRNA (siCARD14) relative to gene expression in siRNA control (siControl)-transfected cells $\pm$ standard error (two sided t-test: *** $p < 0.001$). Results are normalized to *GAPDH* RNA levels. (B) *CARD14* protein expression was ascertained using immunoblotting with an anti-CARD14 antibody (CARD14). β -actin (ACTIN) served as a loading control.

Supplementary Figure E2: Dominant negative effect of AD-associated mutations in *CARD14*.

HEK293 cells were co-transfected with a NF- κ B-responsive luciferase reporter and a combination of an empty vector or the same vector carrying either the wild type *CARD14* cDNA sequence or the *CARD14* cDNA sequence harboring mutations p.I593T or p.N737H. The amount of each expression vector used in each combination is given below the graph. The total amount of transfected DNA was kept at 50 ng. Luciferase activity was measured 24 hours after transfection and normalized to Renilla luciferase activity. Results represent the mean of 3 independent experiments $\pm$ standard deviation. Results were statistically tested against luciferase activity recorded upon transfection of 50 ng of wild type *CARD14* cDNA (asterisk) or against luciferase activity recorded upon transfection of 25 ng of wild type *CARD14* cDNA and 25 ng of non empty vector DNA (pound sign) (two-sided t-test; ### $p < 0.001$, *** $p < 0.001$).

Supplementary Figure E3: Inflammatory gene expression in CARD14-deficient

keratinocytes. Keratinocytes were transfected with *CARD14*-specific siRNA or with control siRNA and then maintained in growth medium (KGM) without supplements for 48 hours. Real-time PCR analysis was used to assess RNA expression of *DEFB1* encoding hBD-1, *DEFB2* encoding hBD-2, *CAMP* encoding LL-37 and *CCL20* encoding hCCL20. Results are expressed as percentage of RNA expression relative to expression in control siRNA-transfected samples $\pm$ standard error. Results represent the mean of three independent experiments (two sided t-test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

Supplementary Figure E4: IL-33 and TSLP expression in keratinocytes expressing

CARD14 loss of function mutations. Keratinocytes were transfected with wild-type (WT) or mutant *CARD14* (p.I593T or p.N737H) complementary DNA (cDNA) constructs and then maintained in growth medium (KGM) without supplements for 48 hours. The protein levels of TSLP secreted into the culture medium or IL-33 in cell lysate were measured using ELISA assays according to manufacturer's instructions. Results are expressed as percentage of protein expression levels relative to expression in wildtype *CARD14*-transfected samples $\pm$ standard error. Results represent the mean of three independent experiments.

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