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ORIGINAL RESEARCH

IL-4, IL-13, TNF-α and IFN-γ Downregulate CLDN8 Expression Through Activating JAK Signaling Pathway in HaCaT Cells

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Background: Tight junctions (TJs) are important for skin barrier function. Claudin-8 (CLDN8), a member of TJs, was indicated decreased in several RNA sequencing studies in dermatitis conditions.

Methods: Bioinformatics analysis was performed to extract CLDN8 mRNA expression from atopic dermatitis (AD) related datasets in the Gene Expression Omnibus. CLDN8 protein expression was detected in AD lesions and healthy control skin tissues using immunohistochemistry staining (IHC). Cldn8 expression was detected in MC903-induced AD-like mouse model. AD-related cytokines with or without Janus kinase (JAK) inhibitor were added to HaCaT cells, and CLDN8 expression was detected by quantitative Polymerase Chain Reaction (qPCR).

Results: CLDN8 mRNA expression is decreased in AD lesions and MC903-induced AD-like mouse model. Downregulation of CLDN8 mRNA expression is alleviated after dupilumab or crisaborole treatment. CLDN8 protein was not detected by IHC in human or mouse skin tissues. Interleukin (IL)-4, IL-13, tumor necrosis factor (TNF)-α and interferon (IFN)-γ downregulated CLDN8 mRNA expression in HaCaT cells through activating JAK.

Conclusion: CLDN8 mRNA is decreased in AD lesions, and the decreased CLDN8 is alleviated along with therapy. Skin tissues might not express CLDN8 protein. AD-related cytokines including IL-4, IL-13, TNF-α and IFN-γ could downregulate CLDN8 mRNA expression through activating JAK.

Keywords: claudin-8, tight junction, atopic dermatitis, Janus kinase

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease clinically characterized by recurrent eczematous lesions and intense itching.[1] AD affects around 20% children and up to 10% of adult in high-income countries.[2] The etiology of AD is complex, mainly including genetic predisposition, skin barrier dysfunction and immune dysregulation.[3] Disturbance of skin barrier favors the penetration of pathogens, allergens and irritants into the dermis, which exacerbates the immune imbalance in AD lesions.[4]

Intact skin barrier function is maintained by several components: the microbiome, stratum corneum (SC), tight junctions (TJs), the chemical barrier and the immunological barrier.[5] TJs locate in the second layer of stratum granulosum, which seal the intercellular space to regulate the movement of water, ions and molecules. TJs also participate in regulation of differentiation, proliferation, cell polarity and signal transduction.[6] TJs are composed of transmembrane proteins, including claudins (CLDNs), occludin (OCLN) and junctional adhesion molecules (JAMs), as well as TJ plaque proteins, including zonula occludens (ZO) and cingulin.[7] TJ dysregulation has been involved in AD pathogenesis.[7–9] Cutaneous inflammation causes epidermal TJ barrier dysfunction, while TJ impairment results in SC

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damaging and increases the penetration of exogenous pathogens and allergens, thus leading to a vicious circle of skin inflammation.[10]

Previously, we found decreased CLDN1, CLDN4 and OCLN expression in HaCaT cells following treatment with AD-related cytokines through JAK signaling activation.[11] Interestingly, in this study, we found CLDN8, another member of CLDNs family, was among the common downregulated differentially expressed genes (DEGs) between lesional and non-lesional skin samples at baseline screened from four AD-related Gene Expression Omnibus (GEO) datasets. As a member of CLDN family, CLDN8 has been shown to be important for intestinal barrier integrity and lung epithelial barrier function.[12–14] CLDN8 also participates in ion permeability in the kidney.[15] Furthermore, many studies have revealed a promoting role of CLDN8 in cancers including colorectal cancer,[16] prostate cancer,[17] breast cancer[18] and so on. Meanwhile, several RNA sequencing studies have indicated decreased CLDN8 expression during skin inflammation.[19–23] However, the expression, regulation and function of CLDN8 in AD skin remains largely unknown.

In this study, we first used bioinformatics analysis to evaluate the CLDN8 mRNA expression in AD. Then human skin tissues and MC903-induced AD-like mouse model were used for detection of CLDN8 expression. HaCaT cells were treated with AD related cytokines to explore the mechanisms underlying the downregulation of CLDN8.

Material and Methods

Identification of Differentially Expressed Genes in Atopic Dermatitis Datasets

AD-related datasets GSE130588[24], GSE58558[25], GSE27887[26] and GSE32924[27] were searched and downloaded from the GEO (https://www.ncbi.nlm.nih.gov/geo/) database, and basic information about the four datasets is listed in Table S1. DEGs between lesional and non-lesional skin samples from AD patient before treatment in GSE130588, GSE58558, GSE27887 and GSE32924 were extracted using RStudio limma software package (version 1.4.1106; Boston, MA, USA). The screening criteria for DEGs was |log2 fold change| (|log2(FC)|>1.0 and P-value <0.05. The intersections of the downregulated DEGs from the four datasets were generated using the website Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/).

Relative mRNA Expression of CLDN8 in Atopic Dermatitis Datasets

Gene expression profiles of datasets GSE130588,[24] GSE58558,[25] GSE27887,[26] GSE32924[27] and GSE133477[28] were downloaded from GEO database. Gene expression levels were extracted using RStudio software, and the relative mRNA expressions of CLDN8 from the five datasets were selected for further analysis, and the basic information about the five datasets is listed in Table S1.

Clinical Samples

The formalin-fixed, paraffin embedded AD lesional tissues (n=6, 2 males, 4 females, mean age 47.5±17.59 years) and normal control skin tissues (n=6, 2 males, 4 females, mean age 50.33±11.66 years) were collected at Peking University People's Hospital. Patients enrolled had not used systemic treatments for at least 3 months and topical drugs for at least 1 week. The basic information about the patients and healthy controls is listed in Table S2. The study was approved by the Ethics Committee of Peking University People's Hospital, China, and all participants provided written informed consent.

Immunohistochemistry (IHC) Staining

Tissue sections were baked at 60°C for 50 min, followed by dewaxing and rehydrating. EDTA Antigen Retrieval Solution or Citrate Antigen Retrieval Solution (Beyotime, Shanghai, China) was used for antigen retrieval in 95°C for 15 min. Sections were then incubated with endogenous peroxidase blocking buffer (ZSGB-BIO, Beijing, China) for 10 min and blocked with normal goat serum (Solarbio, Beijing, China) for 1 h. Then, the sections were incubated with primary antibodies at 4°C overnight. Secondary antibody (ZSGB-BIO) was added and incubated for 20 min, after which diaminobenzidine (DAB) (ZSGB-BIO) was added for visualization. The sections were then stained with hematoxylin, fixed with neutral balsam and observed under a light microscope (Zeiss, Oberkochen, Germany). Primary antibodies used are listed in Table S3.

MC903-Induced AD-Like Mouse Model

Eight-week-old BALB/c female mice were purchased from SPF Biotechnology (Beijing, China), then were bred and maintained in the standard pathogen-free (SPF) environment in the Laboratory Animal Unit of Peking University People's Hospital. Mice were divided into four groups randomly (n=6 per group). About 20 μL of MC903 (Calcipotriol; Leo Pharma, Ballerup, Denmark) was painted to mice ears of AD, vaseline and crisaborole groups, whereas 20 μL normal saline was painted to mice ears of normal control (NC) group for 7 consecutive days. After 7 days, vaseline or crisaborole (Eucrisa; Pfizer Inc, USA) was applied to mice ear 30 min after painting MC903. After 14 days, the mice were euthanized. Ears were separated into two parts: one part was fixed in formalin for hematoxylin–eosin (HE) and IHC staining; another part was snap-frozen for RNA extraction. All animal experiments were approved by the Ethics Committee of Peking University People's Hospital and conducted according to the NIH Guide for the Care and Use of Laboratory Animals.

Cell Culture

The human keratinocyte HaCaT cell line was from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and was authenticated by short tandem repeat profiling. HaCaT cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA, USA) containing 4.5 g/L glucose, 2 mm L-glutamine, and 10% fetal bovine serum (Gibco). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Reagents

Recombinant human IL-4, IL-13, TNF-α and IFN-γ (PeproTech, Rocky Hill, NJ, USA) were dissolved and stored according to the manufacturer's protocol. The JAK1/2 inhibitor ruxolitinib was purchased from MCE (MedChemExpress, Monmouth Junction, NJ, USA). Concentration of cytokines used was as follows: IL-4, 100 ng/mL; IL-13, 100 ng/mL; TNF-α, 20 ng/mL; IFN-γ, 20 ng/mL.

RNA Isolation and Real-Time Quantitative PCR (RT-qPCR)

Total RNA of mice ears was extracted using the RNeasy Plus Universal Kit (Qiagen, Dusseldorf, German) according to the manufacturer's protocol. Total RNA from HaCaT cells was extracted using Trizol™ (Beyotime, Shanghai, China). RNA was reverse-transcribed into cDNA using EasyScript® All-in-One First-Strand cDNA synthesis SuperMix for qPCR (Transgen, Beijing, China). qPCR was performed using PerfectStart™ Green qPCR SuperMix (Transgen). All primers were synthesized by Sangon Biotech (Shanghai, China) and the sequences are listed in Table S4. RPLP0 was used as an endogenous reference gene for HaCaT cells and Gapdh for mouse samples. Measurements were conducted using an Applied Biosystems® 7500 Fast Real-Time PCR System (Waltham, MA, USA). Relative expression levels were calculated using the comparative threshold cycle (Ct) and $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Prism 8 (GraphPad Software, San Diego, CA, USA) was used for graph generation and statistical analyses. Data were analyzed using two-tailed one-way ANOVA (for 3 or more groups) or Student's *t*-test (for 2 groups). Data were expressed as mean ± standard deviation (SD) of at least 3 replicates unless otherwise mentioned. Differences with $P < 0.05$ were regarded as statistically significant.

Results

Relative mRNA Expression of CLDN8 Is Decreased in AD Lesions

Intersection calculation of downregulated DEGs between lesional and non-lesional skin samples at baseline screened from the AD-related GEO datasets GSE130588, GSE58558, GSE27887 and GSE32924 was showed in Figure 1A. In total, there were 37 common downregulated DEGs in datasets GSE130588, GSE58558, GSE27887 and GSE32924, among which, CLDN8 was included. The 37 common downregulated DEGs were listed in Table S5. We then extracted and analyzed the relative mRNA expression data from the four datasets and GSE133477. Results from skin tissues at

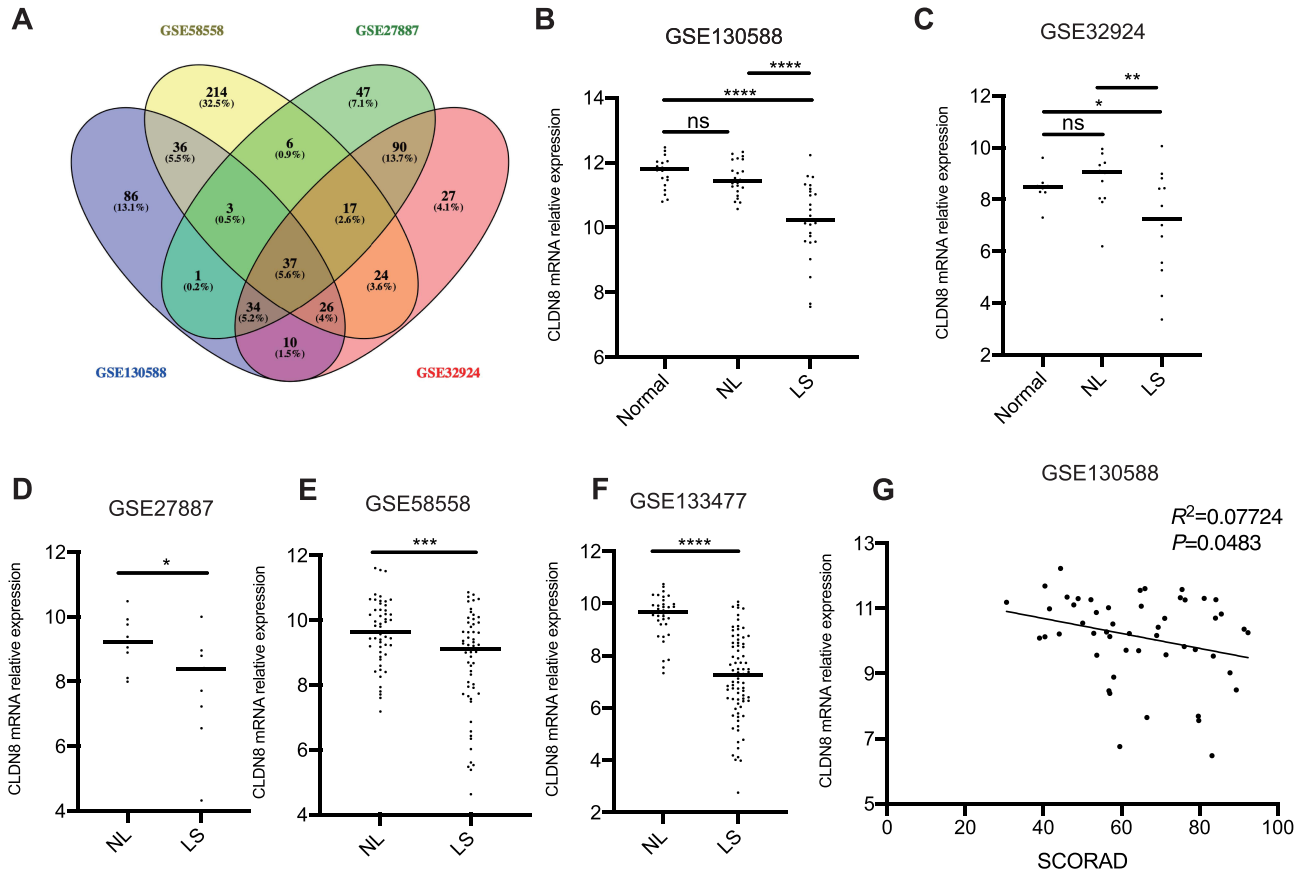

Figure 1 Relative mRNA Expression of CLDN8 is Decreased in AD Lesions. (**A**) Venn diagram of the downregulated DEGs from GEO datasets GSE130588, GSE58558, GSE27887 and GSE32924. (**B**) CLDN8 mRNA relative expression in GSE130588. (**C**) CLDN8 mRNA relative expression in GSE 32924. (**D**) CLDN8 mRNA relative expression in GSE27887. (**E**) CLDN8 mRNA relative expression in GSE58558. (**F**) CLDN8 mRNA relative expression in GSE133477. (**G**) Correlation between SCORAD and CLDN8 relative mRNA expression. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Abbreviation: NL, nonlesion; LS, lesion; SCORAD, SCORing Atopic Dermatitis; ns, not significant.

baseline (before starting treatment) showed that, in GSE130588 and GSE32924, CLDN8 relative mRNA expression was significantly decreased in AD lesions compared with normal and non-lesional skin tissues (Figure 1B and C). Also, significantly decreased CLDN8 relative mRNA expression was observed in AD lesions compared with non-lesions in all the five datasets (Figure 1B–F). Furthermore, in GSE130588, where the SCORing Atopic Dermatitis (SCORAD) index was supplied, a negative correlation between SCORAD and CLDN8 relative mRNA expression was observed (R^2=0.07724, P<0.05) (Figure 1G). These results indicated decreased CLDN8 mRNA expression in AD lesions.

Relative mRNA Expression of CLDN8 Is Increased After Treatment with Dupilumab or Crisaborole

In GSE130588, where gene expression data were provided during the treatment with dupilumab, we found that CLDN8 relative mRNA expression in AD lesions was significantly increased after treatment for 4 or 16 weeks compared with that at baseline (week 0, W0) (Figure 2A). Furthermore, after 16 weeks of therapy, CLDN8 relative mRNA expression was significantly increased in dupilumab group compared with placebo group (Figure 2B). In GSE133477, where gene expression data were provided during the treatment with crisaborole, we found that CLDN8 relative mRNA expression in AD lesions was significantly increased after treatment for 8 or 15 days compared with that at baseline (day 1, D1) (Figure 2C). Meanwhile, after 8 or 15 days of treatment, CLDN8 relative mRNA expression in AD lesions was also

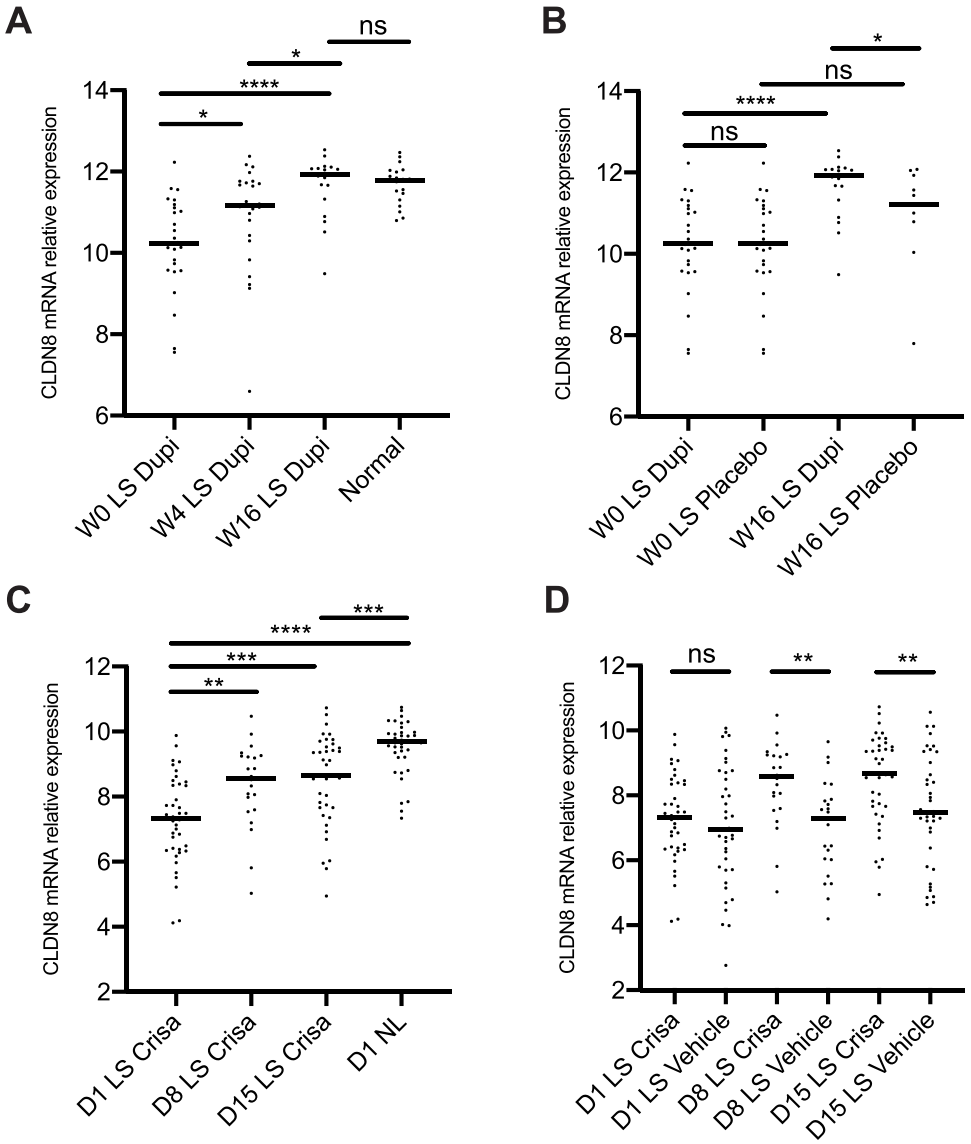

Figure 2 Relative mRNA Expression of CLDN8 is Increased After Treatment with Dupilumab or Crisaborole. (**A**) CLDN8 mRNA relative expression in GSE130588. (**B**) CLDN8 mRNA relative expression in GSE130588. (**C**) CLDN8 mRNA relative expression in GSE133477. (**D**) CLDN8 mRNA relative expression in GSE133477. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Abbreviations: W, week; LS, lesion; Dupi, dupilumab; D, day; NL, nonlesion; Crisa, crisaborole; ns, not significant.

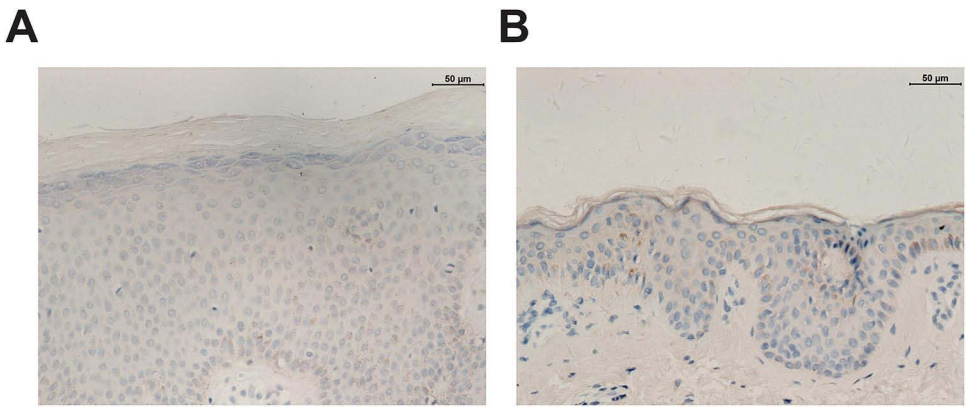

Figure 3 IHC Staining Showed no CLDN8 Protein Expression in Human Skin Tissues. Protein levels of CLDN8 measured with IHC in AD (**A**) and healthy control skin (**B**) tissues. Bar=50 μm.

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significantly increased in the crisaborole group compared with vehicle group (Figure 2D). These results indicated recovery of CLDN8 mRNA expression in AD lesions after therapy.

IHC Staining Showed No CLDN8 Protein Expression in Human Skin Tissues

Given to the decreased CLDN8 mRNA expression indicated by bioinformatic analyses above, we intended to detect CLDN8 in human normal skin tissues and AD lesions at protein level. Interestingly, we used two different antibodies against CLDN8 to detect its expression in AD lesions and healthy control skin tissues, neither showed positive stain (Figure 3A and B). Antibody against CLDN1, another member of CLDNs family, was used as positive control (Figure S1A). We then searched CLDN8 in

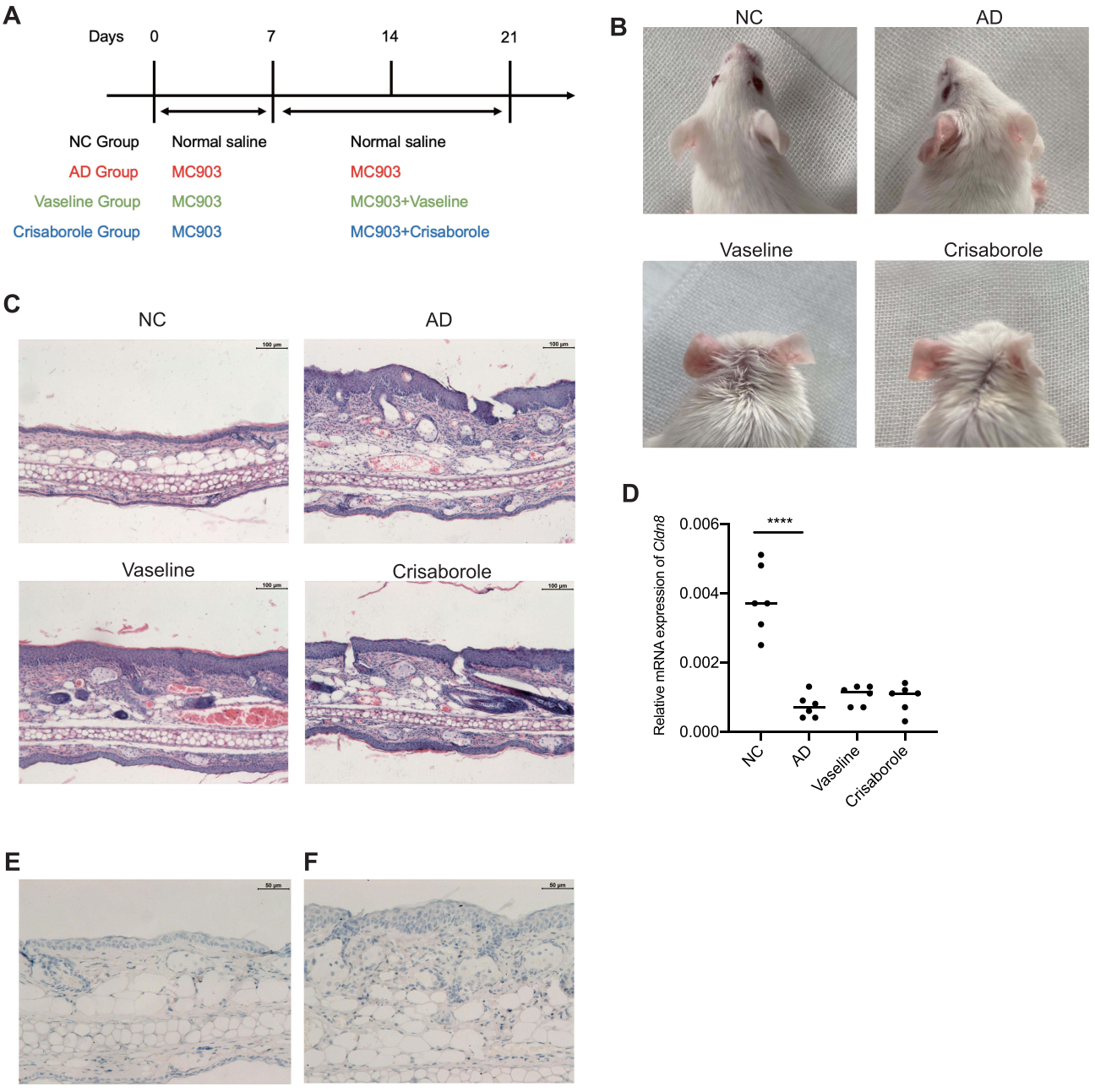

Figure 4 Cldn8 mRNA expression is Decreased in MC903-Induced AD-Like Mouse Model. (**A**) The schematic diagram of mouse experiment. Appearance (**B**) and HE staining (**C**) of mouse ears in the four groups. Bar=100 μm. (**D**) Relative mRNA expression of Cldn8. Protein levels of CLDN8 measured with IHC in NC (**E**) and AD (**F**) mouse ears. Bar=50 μm. ****P < 0.0001.

Abbreviations: NC, normal control; AD, atopic dermatitis; HE, hematoxylin-eosin.

the Human Protein Atlas (HPA, https://www.proteinatlas.org), a database that maps more than 26,000 kinds of human proteins in cells, tissues and organs using an integration of various omics technologies, which also shows positive RNA expression and negative IHC staining for CLDN8 in skin tissues. These results revealed that CLDN8 protein might not express or could not be detected with IHC in human skin tissues.

Cldn8 mRNA Expression is Decreased in MC903-Induced AD-Like Mouse Model

To detect the expression of *Cldn8* under dermatitis and therapy conditions, BALB/c mice were topically treated with MC903, vaseline or crisaborole (Figure 4A). General appearance of mouse ears showed thickening, redness and scaling after applying MC903, while vaseline and crisaborole could reduce thickening, redness and scaling in MC903 treated mouse ears (Figure 4B). Histological analysis of ear sections also showed decreased epidermal and dermal thickening by vaseline and crisaborole in MC903 treated mouse ears (Figure 4C). We also observed significantly decreased *Cldn8* mRNA expression in the AD group compared with NC group, while the expression was slightly increased in the vaseline and crisaborole group compared with AD group, although the difference was not significant (Figure 4D). We then used IHC method to detect CLDN8 protein expression and it showed negative staining in NC (Figure 4E) and AD (Figure 4F) mouse ears, whereas positive in the intestine (Figure S1B).

IL-4, IL-13, TNF-α and IFN-γ Downregulate CLDN8 mRNA Expression Through Activating JAK in HaCaT Cells

Considering the downregulation of CLDN8 mRNA expression in AD lesions and MC903-induced AD-like mouse model, we then explored the mechanisms underlying these phenomena. By treating HaCaT cells with Interleukin (IL)-4 for 24 h or 48 h, we observed significantly decreased CLDN8 mRNA expression (Figure 5A), and the same was also true for IL-13, tumor necrosis factor (TNF)-α and Interferon (IFN)-γ (Figure 5B–D). Treating HaCaT cells with IL-4 and IL-13 simultaneously resulted in lower CLDN8 mRNA expression (Figure 5E). Likewise, lower CLDN8 mRNA expression was also observed when treating HaCaT cells with TNF-α and IFN-γ simultaneously (Figure 5F). Whereas Janus kinase (JAK) 1/2 inhibitor ruxolitinib could dose dependently recover the downregulated CLDN8 mRNA expression caused by IL-4/IL-13 (Figure 5G) or TNF-α/IFN-γ (Figure 5H). These results indicated that IL-4, IL-13, TNF-α and IFN-γ could downregulate CLDN8 mRNA expression through activating JAK.

Discussion

Increasing evidence has shown the prominent role of epidermal barrier impairment in the pathogenesis of AD.[29,30] As the outermost organ of the body, skin has two physical barrier, the SC and TJs.[31] TJs seal the intercellular space between epithelial cells, thus forming a functional barrier to regulate the paracellular movement of water, ions and molecules.[32] TJs are also involved in many cellular functions, such as cell proliferation, differentiation, and signaling transduction.[5] TJ dysregulation could contribute to the dysfunction of skin barrier in AD. Disrupted TJ barrier can disturb the pH condition of SC, thus affecting the processing of polar lipids and profilaggrin.[33] Moreover, defects of TJs have been related to the penetration of irritants, toxins and allergens, leading to a vicious cycle of barrier dysfunction.[10]

Decreased CLDN1 and CLDN4 expression in AD lesions have been reported.[34] We also found decreased CLDN1, CLDN4 and OCLN expression following AD-related cytokines through JAK signaling pathway in HaCaT cells.[11] In the last few years, studies showed decreased CLDN8 mRNA expression in the skin of delayed type hypersensitivity reactions,[23] tape strips from AD,[21,22] and skin after paraphenylenediamine exposure[20] through RNA sequencing. In this study, using bioinformatics analyses, we identified 37 common downregulated DEGs in GSE130588, GSE58558, GSE27887 and GSE32924, among which, CLDN8 was included. Considering the lack of investigations on CLDN8 in the skin, we then focused our study on CLDN8. By extracting gene expression information from datasets, we also observed decreased CLDN8 mRNA expression in AD lesions, which had a correlation with increased SCORAD. Meanwhile, the decreased CLDN8 mRNA expression was alleviated after dupilumab or crisaborole treatment. Using a MC903-induced AD-like mouse model, we also found decreased CLDN8 mRNA expression after MC903 application and slight recovery after treatment with vaseline or crisaborole. However, CLDN8 protein could not be detected with IHC in both human and

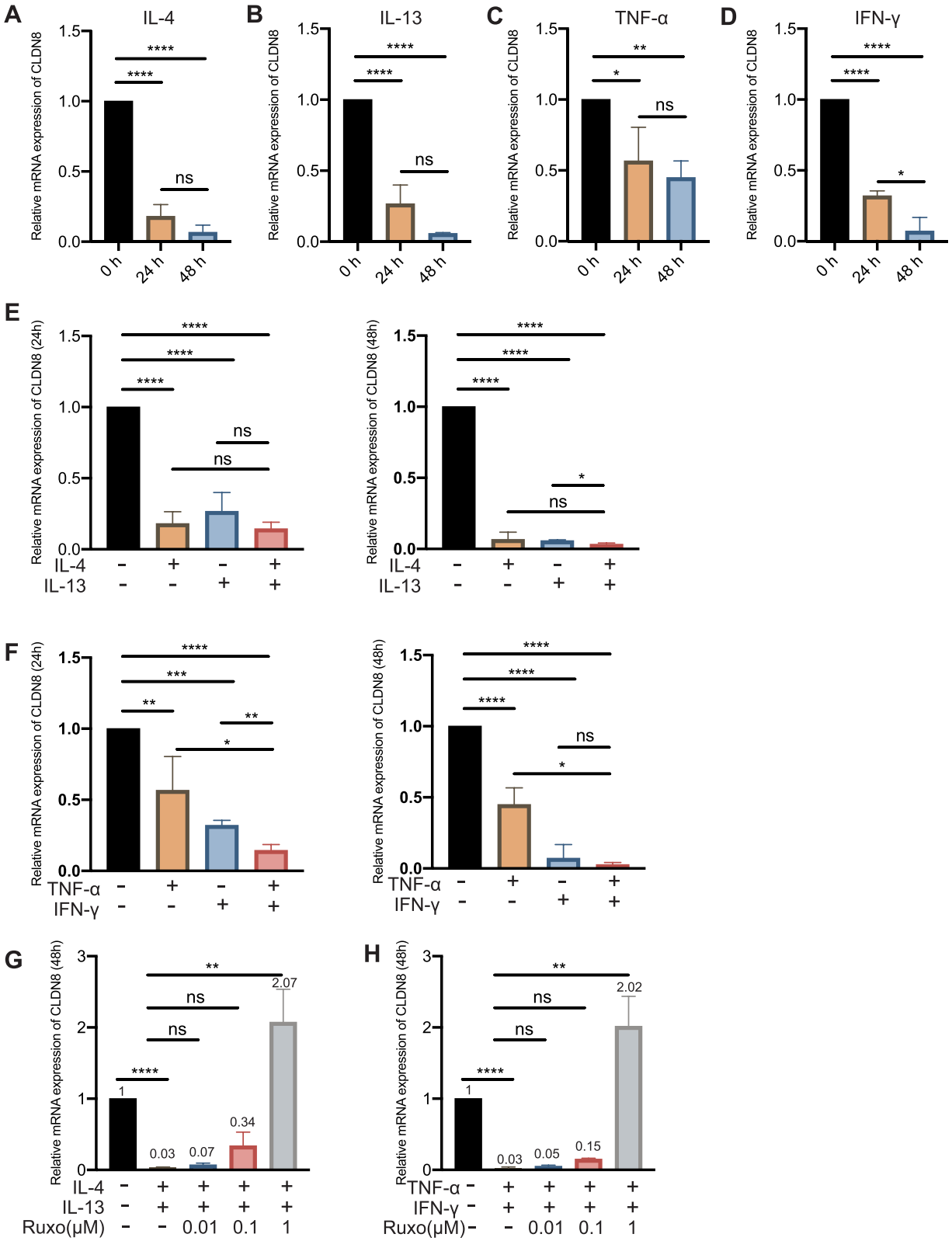

Figure 5 IL-4, IL-13, TNF-α and IFN-γ Downregulate CLDN8 mRNA Expression through Activating JAK in HaCaT Cells. Relative mRNA expression of CLDN8 after treatment with IL-4 (**A**), IL-13 (**B**), TNF-α (**C**), IFN-γ (**D**), IL-4 and IL-13 simultaneously (**E**) or TNF-α and IFN-γ simultaneously (**F**) in HaCaT cells. Relative mRNA expression of CLDN8 after treatment with ruxolitinib followed by IL-4 and IL-13 (**G**) or TNF-α and IFN-γ (**H**) in HaCaT cells. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Abbreviation: Ruxo, ruxolitinib; ns, not significant.

BALB/c mouse skins in our study; meanwhile, Human Protein Atlas also showed negative IHC staining for CLDN8 in human skin, which indicates that CLDN8 protein may not be detected using IHC or CLDN8 protein may not express in skin tissues. The expression and regulation of CLDN8 protein in the skin epidermis need further exploration.

Cutaneous inflammation, especially T helper (Th) 2 induced skin inflammation, plays a central role in AD pathogenesis. In acute AD lesions, Th2 and Th22 cell infiltration is dominant, and in chronic skin lesions, Th1 and Th17-mediated responses have also been reported.[1,35] Activated Th2 cells release IL-4 and IL-13, which activate downstream JAK pathways, thus promoting inflammation, pruritus and production of Immunoglobulin E.[36,37] It was reported that IL-4, IL-13 and IL-31 could downregulate CLDN1 expression in reconstructed human epidermis.[34] IL-33[38] and IFN-γ[39] could decrease CLDN1 expression in keratinocytes through extracellular regulated protein kinases (ERK)-signal transducer and activator of transcription (STAT) 3 and JAK-STAT1 signaling pathways, respectively. In this study, by treating HaCaT cells with IL-4, IL-13, TNF-α or IFN-γ, we found significantly decreased CLDN8 mRNA expression, which could be recovered by JAK inhibitor ruxolitinib. These results indicated that the downregulated CLDN8 mRNA expression in AD lesions may be caused by cytokines secreted from Th2 and Th1 cells infiltrated in AD lesions through activating the JAK signaling pathway.

Limitations

Our study showed negative IHC staining for CLDN8 in both human and mouse skins. The underlying mechanisms or reasons for the lack of detectable CLDN8 protein, like post-translational regulation or staining methods limitations, need further study. Furthermore, this study did not investigate the function of CLDN8 in the skin, which may also need further exploration.

Conclusion

Our study demonstrated that CLDN8 mRNA expression is downregulated in AD lesions and MC903-induced AD-like mouse model, while downregulation of CLDN8 mRNA expression is alleviated after treatment. CLDN8 protein might not express in human skin and mouse skin tissues, and further investigation is needed. IL-4, IL-13, TNF-α and IFN-γ could downregulate CLDN8 mRNA expression in HaCaT cells through activating JAK.

Abbreviations

SC, stratum corneum; TJ, tight junction; CLDN8, claudin-8; AD, atopic dermatitis; IHC, immunohistochemistry, JAK, Janus kinase; qPCR, quantitative Polymerase Chains Reaction; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; OCLN, occludin; JAMs, junctional adhesion molecules; ZO, zonula occludens; DEGs, differentially expressed genes.

Data Sharing Statement

The authors confirm that the data supporting the findings of this study are available in the article and its Supplementary Materials. Datasets referenced and analyzed in this study are available in the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/),[40] with the accession code numbers GSE130588, GSE133477, GSE58558, GSE27887 and GSE32924.

Ethics Approval

The study was approved by the Ethics Committee of Peking University People's Hospital. The study was conducted according to the Declaration of Helsinki principles. Animal studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animals. The ethical approval numbers are 2020PHB353-01 and 2022PHE126.

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Disclosure

The authors report no conflicts of interest in this work.

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