Identification of novel immune and barrier genes in atopic dermatitis by means of laser capture microdissection

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Background: The molecular signature of atopic dermatitis (AD) lesions is associated with T_H2 and T_H22 activation and epidermal alterations. However, the epidermal and dermal AD transcriptomes and their respective contributions to abnormalities in respective immune and barrier phenotypes are unknown.
Objective: We sought to establish the genomic profile of the epidermal and dermal compartments of lesional and nonlesional AD skin compared with normal skin.
Methods: Laser capture microdissection was performed to separate the epidermis and dermis of lesional and nonlesional skin from patients with AD and normal skin from healthy volunteers, followed by gene expression (microarrays and real-time PCR) and immunostaining studies.
Results: Our study identified novel immune and barrier genes, including the IL-34 cytokine and claudins 4 and 8, and showed increased detection of key AD genes usually undetectable on arrays (ie, *IL22*, thymic stromal lymphopoietin *[TSLP]*, *CCL22*, and *CCL26*). Overall, the combined epidermal and dermal transcriptomes enlarged the AD transcriptome, adding 674 upregulated and 405 downregulated differentially expressed genes between lesional and nonlesional skin to the AD transcriptome. We were also able to localize individual transcripts as primarily epidermal (defensin, beta 4A *[DEFB4A]*) or dermal (*IL22*, cytotoxic T-lymphocyte antigen 4 *[CTLA4]*, and *CCR7*) and link their expressions to possible cellular sources.
Conclusions: This is the first report that establishes robust epidermal and dermal genomic signatures of lesional and nonlesional AD skin and normal skin compared with whole tissues. These data establish the utility of laser capture microdissection to separate different compartments and cellular subsets in patients with AD, allowing localization of key barrier or immune molecules and enabling detection of gene products usually not detected on arrays. (J Allergy Clin Immunol 2015;135:153-63.)

Key words: *Atopic dermatitis, laser capture microdissection, IL-34, claudins 8 and 4, immune, barrier*

Atopic dermatitis (AD) is the most common inflammatory skin disease.[1,2] Although its pathogenesis is not fully understood, both barrier and immune components have been suggested to play key roles in AD, as indicated by the "outside-in" and "inside-out" hypotheses.[3-10] Whereas barrier-related molecules are largely epidermal, inflammatory responses are derived from both the epidermal (ie, keratinocytes and Langerhans cells [LCs]) and dermal (ie, T cells and dendritic cells [DCs]) compartments.

Using genomic analyses on whole tissue/bulk samples, we have previously shown that the AD phenotype/transcriptome is associated with polar immune activation of T_H2/T_H22, as well as T_H1 and T_H17, pathways and corresponding epidermal alterations (epidermal hyperplasia and abnormal differentiation).[11-14] However, bulk sample genomic analysis (by using microarrays and real-time PCR [RT-PCR]) presents some limitations. First, it is difficult to determine whether altered gene expression is due to expansion (hypertrophy) of one tissue compartment versus altered gene expression at the cellular level. Second, it cannot localize a particular gene/transcript to an epidermal/dermal compartment. Finally, low-abundance genes are often present at less than the detection level of conventional microarrays because of dilution of mRNA within full-thickness samples with more dominant products.

Laser capture microdissection (LCM) is an established technique for procuring subpopulations of tissues/cells of interest

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Abbreviations used
AD: Atopic dermatitis
CE: Cornified envelope
CLDN: Claudin
CSF-1R: Colony-stimulating factor 1 receptor
CTLA4: Cytotoxic T-lymphocyte antigen 4
DC: Dendritic cell
DEFB4A: Defensin, beta 4A
DEG: Differentially expressed gene
EDC: Epidermal differentiation complex
FCH: Fold change
FDR: False discovery rate
FLG: Filaggrin
GZMB: Granzyme B
ICOS: Inducible T-cell costimulator
LC: Langerhans cell
LCM: Laser capture microdissection
LOR: Loricrin
MX1: Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
PI3: Peptidase inhibitor 3, skin-derived
RT-PCR: Real-time PCR
SPRR: Small proline-rich protein
TJ: Tight junction

under direct microscopic visualization to study alterations in disease states.[15] Our group has previously demonstrated that epidermal and dermal separation of lesional and nonlesional samples from patients with psoriasis and normal samples by using LCM complemented by microarrays largely increases the detection of low-abundance genes compared with whole-tissue analyses.[16,17] Despite the pathogenic relevance of separating the epidermal and dermal compartments, such studies are unavailable in patients with AD.

In this study we sought to determine the molecular phenotypes of the epidermal and dermal compartments of lesional and nonlesional AD skin (compared with skin from healthy subjects). Overall, our results (1) enlarged the AD transcriptome; (2) detected low-abundance genes (which are usually present at less than detection levels on whole-tissue microarrays [eg, *IL22* and thymic stromal lymphopoietin *[TSLP]*); and (3) identified novel immune and barrier genes (ie, *IL34*, claudin 4 *[CLDN4]*, and *CLDN8*) and suggested possible cellular sources of immune markers (ie, CCR7).

METHODS

Skin samples

Paired nonlesional and lesional AD skin biopsy specimens were collected from 5 patients with moderate-to-severe chronic AD (3 male and 2 female patients; age, 27-59 years; mean age, 39.4 years; see Table E1 in this article's Online Repository at www.jacionline.org) under institutional review board–approved protocols. Normal skin samples from healthy volunteers (n = 10) that had been collected for a prior LCM publication[18] were also included. Paired epidermal, dermal, and full-thickness lesional and nonlesional samples were used for RT-PCR and microarray analysis (n = 5 in each group). Lesional and nonlesional expression values were compared with 10 epidermal, 6 dermal, and 6 bulk corresponding normal samples. For RT-PCR confirmation, 3 normal paired epidermal and dermal samples were used because of the limited available mRNA.

Slide preparation and LCM

LCM was performed according to the manufacturer's protocol for the CellCut system (Molecular Machines & Industries, Haslett, Mich; see the Methods section in this article's Online Repository at www.jacionline.org).

RNA extraction

Total RNA was extracted with the RNeasy Micro Kit (Qiagen, Valencia, Calif), according to the manufacturer's protocol, with on-column DNase digestion.

Sample preparation for gene chip analysis

Target amplification and labeling was performed according to the Affymetrix protocols for 2-cycle cDNA synthesis by using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, Calif), as previously reported.[16]

Sample preparation for quantitative RT-PCR

Reverse transcription to cDNA from RNA of LCM samples was carried out by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Calif), cDNA was amplified with TaqMan PreAmp Master Mix (Applied Biosystems), and the preamplified cDNA product was analyzed with TaqMan Gene Expression Master Mix, according to the manufacturer's instructions. The RT-PCRs for each assay were run in triplicate, and all data were normalized to human acidic ribosomal protein P0. The primers and probes used in this study are listed in Table E2 in this article's Online Repository at www.jacionline.org.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed on frozen skin sections, as previously described.[19] The antibodies used in this study are listed in Table E3 in this article's Online Repository at www.jacionline.org.

Statistical analysis

Preprocessing and statistical analysis of microarray data were conducted with R (R-project.org) and Bioconductor software packages.[20] Full details of the pipeline and downstream analysis are described in the Methods section in this article's Online Repository. Succinctly, the Harshlight package[21] was used to quality control the images, and expression values were obtained by using the GCRMA algorithm.[22] Batch adjustments were carried out with the ComBat algorithm, and mixed-effect models in the limma package were used to model differential expression.[23-25] Genes with a false discovery rate (FDR) of less than 0.05 and a fold change of greater than 2 were considered significantly differentially expressed. Similar models were used to analyze $\log_2$-transformed values of normalized RT-PCR data.

RESULTS

LCM localizes genes selectively expressed in the dermis and epidermis

We performed LCM to collect epidermal and dermal (papillary, reticular, and inflammatory aggregate) cells in frozen sections of lesional, nonlesional, and normal skin samples, as shown in Fig 1, *A*. Microarray profiling of lesional, nonlesional, and normal epidermal and dermal tissues was performed with Affymetrix HGU133Plus2.0 microarrays to define the epidermal and dermal transcriptomes. A heat map of epidermis- and dermis-specific genes shows clear separation of differentially expressed genes (DEGs) localized to the epidermis and dermis across lesional, nonlesional, and normal samples (see Fig E1 in this article's