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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

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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

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 publication¹⁸ 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 package²¹ 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 log₂-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

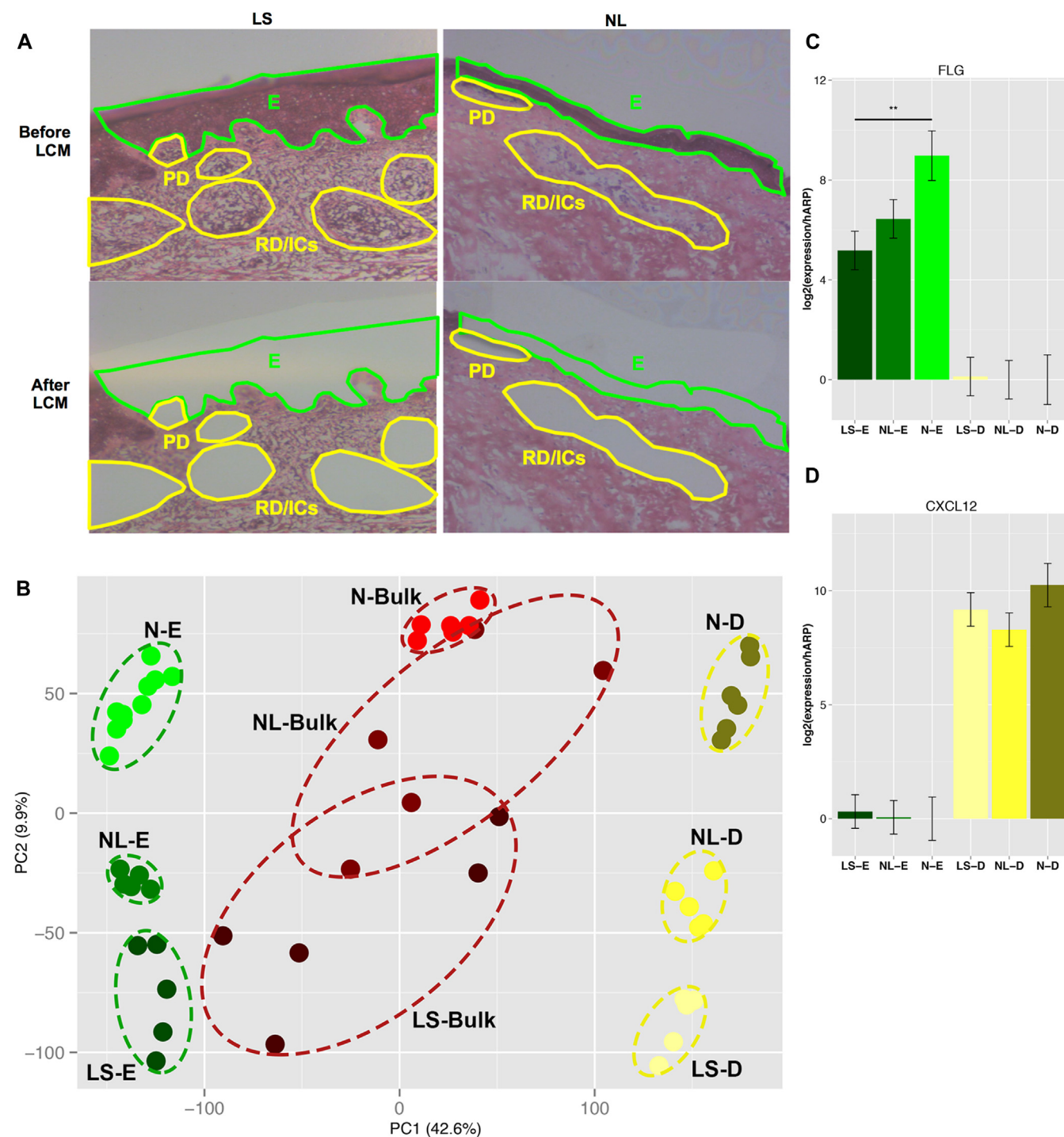


FIG 1. **A**, Representative hematoxylin and eosin staining of lesional (LS) and nonlesional (NL) AD skin. LCM was performed in the indicated zones (green: epidermis [E]; yellow: papillary dermis [PD], reticular dermis [RD], and inflammatory aggregates [ICs]). **B**, Principal component [PC] analysis plot showing clear separation of groups. **C** and **D**, mRNA expression of *FLG* (Fig 1, C) and *CXCL12* (Fig 1, D) in the epidermis (E) and dermis (D) of lesional (LS) and nonlesional (NL) AD and normal (N) skin. Values are presented as means \pm SDs. ** $P < .01$.

Online Repository at www.jacionline.org). A principal component analysis of expression values illustrates the lack of outliers and that samples cluster in accordance with tissue type (Fig 1, B). Markers primarily considered dermal (*CXCL12*, *CD93*, and collagens [*COL1A2* and *COL6A3*]) were highly

represented in the dermis (see Tables E4, A, and E5, A, in this article's Online Repository at www.jacionline.org), and epidermal markers (filaggrin [FLG], loricrin [LOR], late cornified envelope [LCE], and small proline-rich proteins [SPRRs]) were localized to the epidermis, which is consistent with the clear

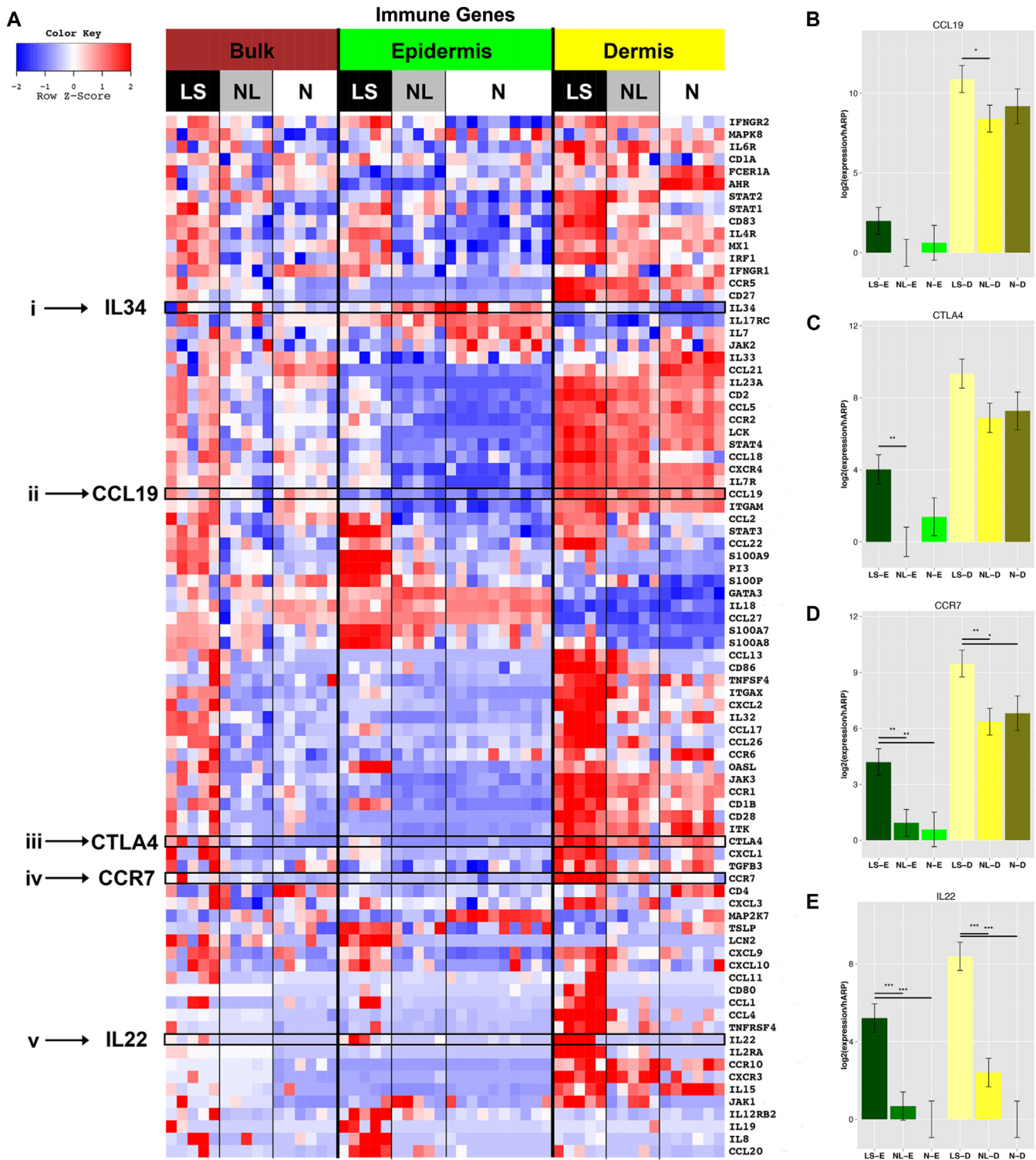


FIG 2. A, Heat map of immune-related genes organized by compartment. FCHs comparing lesional (LS), nonlesional (NL), and normal (N) skin in bulk samples (B) and the epidermis (E) dermis (D) are shown (n = 5 AD specimens and n = 6, 10, and 6 normal bulk, epidermal, dermal specimens, respectively). B-E, mRNA expression, as determined by using RT-PCR, of CCL19 (Fig 2, B), CTLA4 (Fig 2, C), CCR7 (Fig 2, D), and IL22 (Fig 2, E) in the epidermis (E) and dermis (D) of lesional (LS) and nonlesional (NL) AD skin and normal (N) skin. Values are presented as means ± SDs in Fig 2, B through E. *P < .05, **P < .01, and ***P < .001.

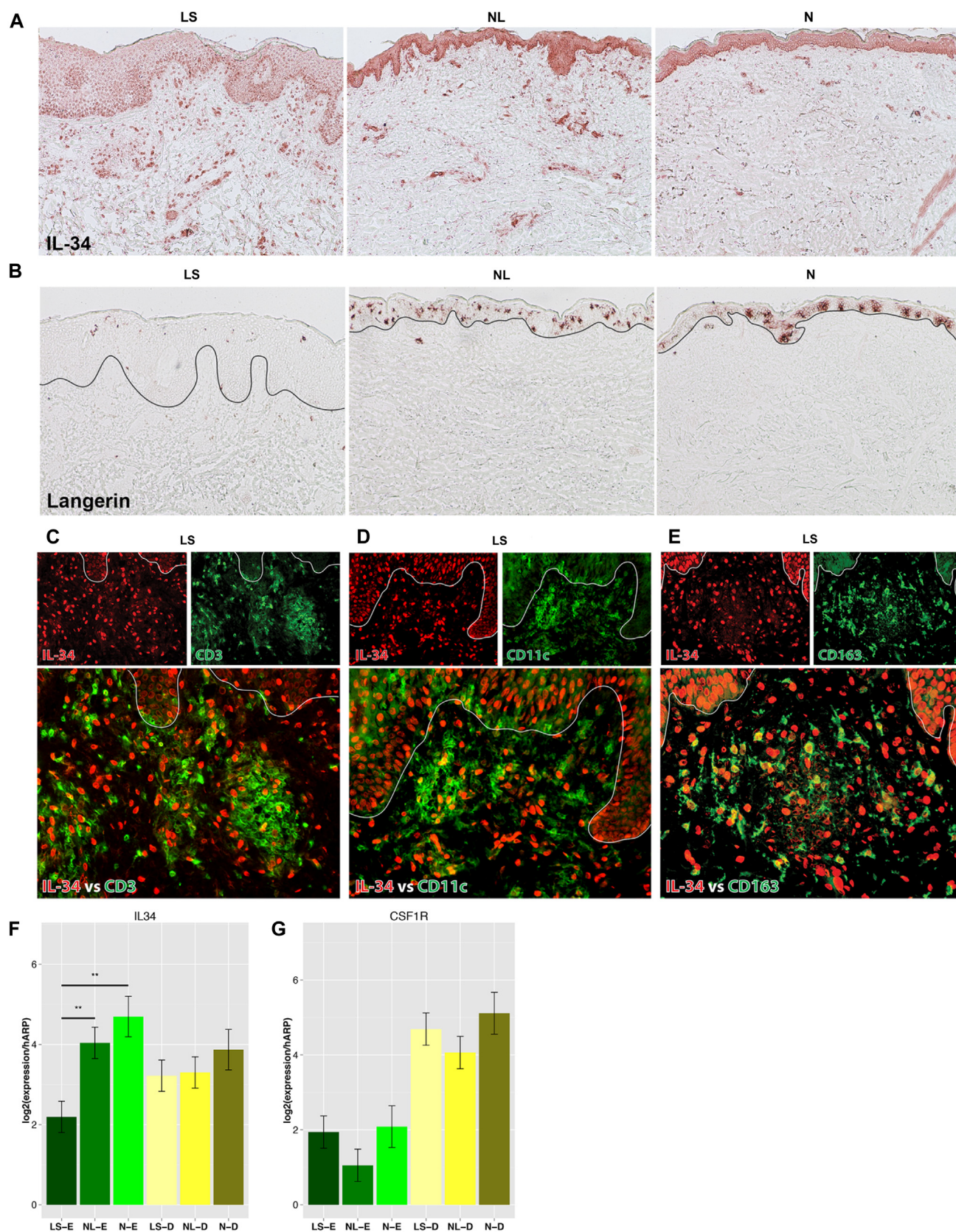


FIG 3. **A** and **B**, Representative staining of IL-34 (Fig 3, **A**) and langerin (Fig 3, **B**) in lesional (LS) and nonlesional (NL) AD and normal (N) skin. **C-E**, Representative double immunofluorescence for coexpression of IL-34 (red) versus CD3 (green; Fig 3, **C**), CD11c (green; Fig 3, **D**), and CD163 (green; Fig 3, **E**) in lesional (LS) skin. **F** and **G**, mRNA expression of IL-34 (Fig 3, **F**) and its receptor, CSF1R (Fig 3, **G**), in the epidermis (E) and dermis (D) of lesional (LS), nonlesional (NL), and normal (N) skin. Values are presented as means \pm SDs. ** $P < .01$.

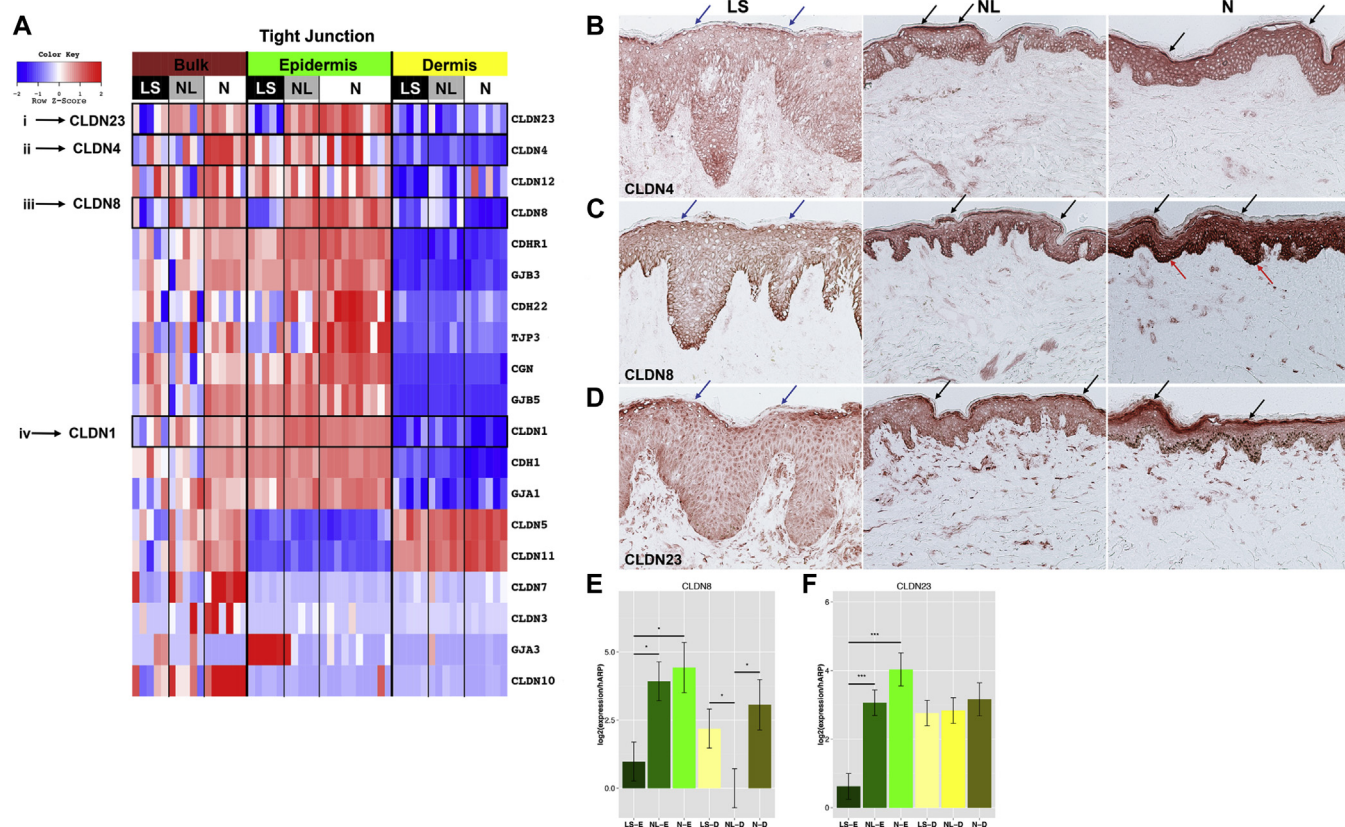


FIG 4. A, Heat map of barrier-related gene FCHs comparing lesional (LS), nonlesional (NL), and normal (N) skin in bulk, epidermal, and dermal specimens (n = 5 AD specimens and n = 6, 10, and 6 normal bulk, epidermis, and dermis specimens, respectively). B–D, Representative CLDN4 (Fig 4, B), CLDN8 (Fig 4, C), and CLDN23 (Fig 4, D) staining in lesional (LS), nonlesional (NL), and normal (N) skin. Arrows, Increased stratum corneum (black), decreased lesional stratum corneum (blue), and increased normal skin basal layer (red) staining. E and F, Expression of CLDN8 (Fig 4, E) and CLDN23 (Fig 4, F) in the epidermis (E) and dermis (D) of lesional (LS), nonlesional (NL), and normal (N) skin. Values are presented as means \pm SDs. *P < .05 and ***P < .001.

epidermal-dermal separation (see Tables E4, B, and E5, B). The accuracy of the LCM separation was validated by using RT-PCR, with greater than 69.7-fold and greater than 846.2-fold enrichment of the mRNA expressions of *FLG* and *CXCL12* in the epidermis versus dermis, respectively, in normal tissues (Fig 1, C and D).

LCM enlarges the AD transcriptome and increases detection of low-abundance immune genes on microarrays

To characterize the AD phenotype within each compartment, we defined the lesional epidermal and dermal transcriptomes as the set of DEGs between lesional and nonlesional tissue within each compartment, respectively, by using the classical criteria of an FCH of greater than 2.0 and an FDR of less than 0.05. Combining the epidermal and dermal transcriptomes, many more compartment-specific DEGs were identified (860 upregulated and 495 downregulated, see Fig E2 in this article's Online Repository at www.jacionline.org), adding 674 upregulated and 405 downregulated DEGs to the recently defined AD transcriptome.^{11,12,26} The bulk transcriptome consists of 710 upregulated and 487 downregulated DEGs, whereas the LCM epidermal transcriptome

contains 566 upregulated and 268 downregulated DEGs, and the LCM dermal transcriptome contains 330 upregulated and 244 downregulated DEGs. Little overlap was observed between the epidermal and dermal transcriptomes (only 36 upregulated and 17 downregulated DEGs were detected in both).

Overall, the top 25 upregulated and downregulated genes in each of the epidermal and dermal lesional transcriptomes included many genes that have been shown to contribute to the AD phenotype (see Tables E6 and E7 in this article's Online Repository at www.jacionline.org).^{11,12} The top 25 upregulated genes in the lesional epidermal transcriptome consisted of proliferation-related (*KRT6A*, *KRT6B*, and *KRT16*), epidermal differentiation complex (EDC; S100 genes), inflammatory (matrix metalloproteinase 12 [*MMP12*]), and antimicrobial peptide-derived (defensin, beta 4A [*DEFB4A*] and peptidase inhibitor 3, skin-derived [*PI3*]/Elafin) genes (see Table E6, A). The top 25 upregulated genes in the lesional dermal transcriptome included T-cell activation (granzyme B [*GZMB*] and IL-2 receptor α [*IL2RA*]), T_H2-related (*CCL17*, *CCL22*, and *CCL26*), T_H22/T_H17-related (*IL22*, *S100A8*, *CXCL1*, and *CXCL2*), and collagen (*COL6A6*, *COL6A5*, and *COL4A3*) genes (see Table E6, B). *IL22* expression was significantly increased in the lesional dermal transcriptome (12.46 FCHs and FDR < 0.001), whereas it was present

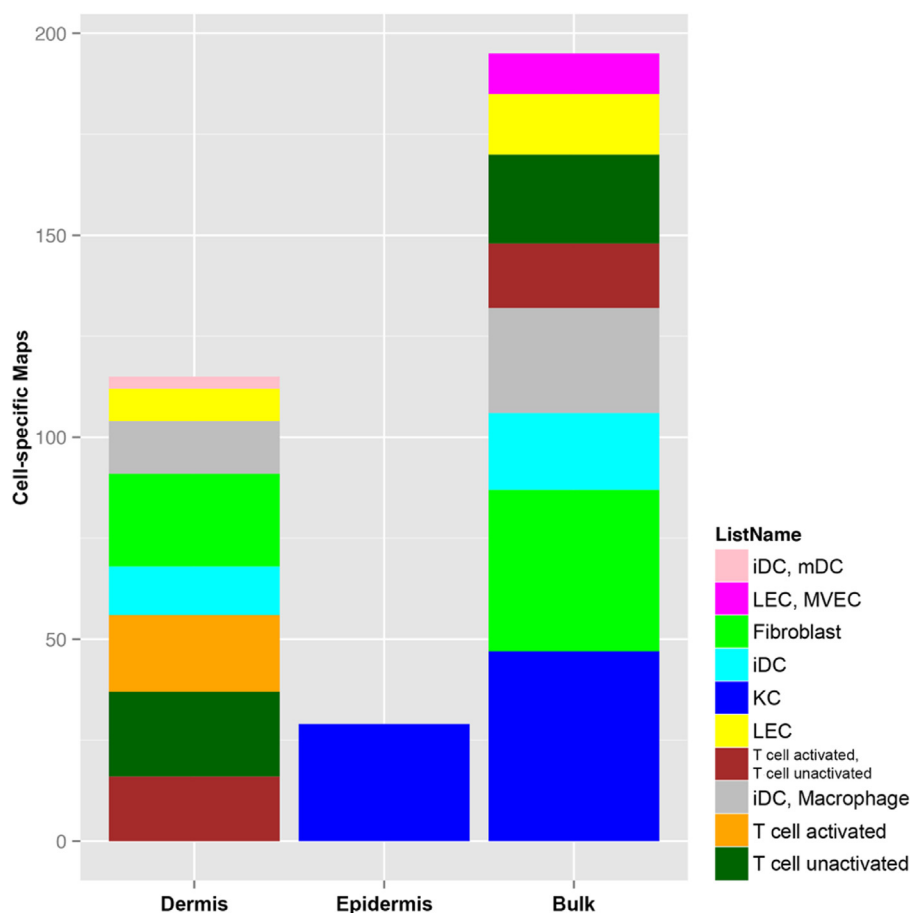


FIG 5. The cell-specific map of the LCM epidermal and dermal transcriptomes and bulk transcriptome. The cellular source of the epidermal transcriptome was mainly keratinocytes, whereas activated T cells were only significantly enriched in the LCM dermal transcriptome. *iDC*, Immature dendritic cell; *KC*, keratinocyte; *LEC*, lymphatic endothelial cell; *mDC*, mature dendritic cell; *MVEC*, microvascular endothelial cell.

at less than detection levels in previous reports,^{11,12,26} indicating that LCM is a useful method to increase the sensitivity of detecting low-abundance genes.

Key inflammatory and barrier genes were uniquely detected in the LCM epidermal (see Table E8, A, in this article's Online Repository at www.jacionline.org) and dermal (see Table E8, B) transcriptomes. The unique lesional epidermal transcriptome included IL-17-related (*DEFB4A* and *CCL20*) and inflammatory (*CXCR4*, signal transducer and activator of transcription 3 [*STAT3*], and *IL8*) genes (see Table E8, A). Key T_H2 (*CCL17*, *CCL22*, and *CCL26*) and T_H22 (*IL22*) markers and T-cell migration/activation markers (*CCR7* and inducible T-cell costimulator [*ICOS*]) were found within the unique lesional dermal transcriptome (see Table E8, B). Although the LCM approach detected an appreciably larger number of genes within bulk tissues, there was a subset of DEGs that were not identified in the corresponding epidermal and dermal transcriptomes (429 upregulated and 381 downregulated genes; Fig 2 and see Table E8, C). A deeper analysis of these data suggests that most of this discrepancy is created by increased or decreased gene abundance because of differing contributions of the epidermis and dermis in lesional versus nonlesional skin (see the Results section and Fig E3 in this article's Online Repository at www.jacionline.org).

We further explored the compartmental distribution of immune DEGs in lesional, nonlesional, and normal skin using our previously curated and reported immune gene subset,^{19,26,27} as shown in a heat map (Fig 2, A, and see Table E9 in this article's Online Repository at www.jacionline.org). Among significantly upregulated genes in the lesional dermal transcriptome were T-cell (cytotoxic T-lymphocyte antigen 4 [*CTLA4*] and *CD28*), DC (integrin α X, complement component 3 receptor 4 subunit [*ITGAX*]/*CD11c* and *CD1A*), lymphoid-organizing chemokine (*CCR7* and its ligands [*CCL19* and *CCL21*]), and T_H2-related (*CCL11*, *CCL13*, *CCL17*, *CCL22*, *CCL26*, TNF receptor superfamily, member 4 [*TNFRSF4*]/*OX40*), and *IL4R*) genes. Significant increases in T_H2-related (*IL7R*), T_H17-related (*PI3*, lipocalin 2 [*LCN2*], *CCL20*, and *STAT3*), T_H22/T_H17-related (*IL22*, *S100A8*, and *S100A9*), and T_H1-related (2'-5'-oligoadenylate synthetase-like [*OASL*], myxovirus [influenza virus] resistance 1, interferon-inducible protein p78 [mouse] [*MX1*], *IL12RB2*, IFN- γ receptor 2 [*IFNGR2*], and interferon regulatory factor 1 [*IRF1*]) products were found. Using RT-PCR, we validated the primarily dermal mRNA expression of selected markers, including *IL22*, *CTLA4*, *CCR7*, and *CCL19*. High *IL22* mRNA levels were characteristic of only lesional skin (Fig 2, B-E). A list of DEGs in all comparisons is presented in Table E8.

IL-34: A novel cytokine identified by means of LCM

IL-34 was significantly downregulated in lesional compared with both nonlesional and normal epidermis (Fig 2, A, and see Table E9). IL-34 is a recently identified cytokine in mice and normal human skin²⁸ and is suggested to regulate LC differentiation in steady states. It has been identified as an alternative ligand to the colony-stimulating factor 1 receptor (CSF-1R), which has been shown to be expressed in epidermal LCs, dermal monocytes, and DCs.²⁹ IL-34 has not been previously reported in human skin diseases. We performed immunohistochemical staining of IL-34, which showed stronger epidermal staining in normal and nonlesional skin compared with lesional skin and stronger dermal staining in lesional skin (Fig 3, A).

Because IL-34 was reported to induce LC differentiation only in steady states,²⁹ langerin (CD207) staining for LCs was performed. Many fewer LCs were detected in lesional compared with both nonlesional and normal skin (Fig 3, B). To identify the cellular distribution of IL-34 expression in the dermis, we performed double immunofluorescence for IL-34 (red) with CD3⁺/T cells (green; see Fig E4, A, in this article's Online Repository at www.jacionline.org), CD11c⁺/DCs (green; see Fig E4, B), and CD163⁺/macrophages (green; see Fig E4, C) in normal, nonlesional, and lesional skin. Few double-positive IL-34⁺/CD3⁺ cells were found. Many IL-34⁺ cells colocalized with CD11c⁺ and CD163⁺ cells (Fig 3, C-E). Thus IL-34 in the dermis is preferentially expressed by myeloid DCs and macrophages.

We validated the lower expression of IL-34 in lesional epidermis using RT-PCR, with significantly lower *IL34* mRNA expression observed in lesional versus both nonlesional and normal epidermis and with slightly lower dermal mRNA expression (Fig 3, F). We also measured *CSF1R* mRNA expression, which was significantly higher in the dermis, regardless of tissue (Fig 3, G).

LCM highlights tight junction defects in patients with AD

Because defective barrier function is a hallmark of AD,¹ we evaluated for EDC and cornified envelope (CE) markers. A heat map of EDC and CE genes (including *S100A* genes) is shown in Fig E5 in this article's Online Repository at www.jacionline.org. The majority of *S100* genes (*S100A8/S100A9/S100A12/S100A13*), IL-17-induced *PI3/Elafin*, and *SPRRs* (*SPRR1A*) showed a primarily epidermal expression and higher expression in lesional versus both nonlesional and normal skin (FDR < 0.001 for most markers), whereas a few *S100* genes (*S100A4/S100A6/S100A10*) showed predominantly dermal lesional expression. As previously reported in bulk tissues,²⁶ the differentiation genes (*LCE*, *LOR*, *FLG*, and periplakin [*PPL*]) showed decreased expression in lesional versus nonlesional and normal epidermis (see Fig E5 and Table E10 in this article's Online Repository at www.jacionline.org). Claudins, another crucial barrier component, are essential for tight junction (TJ) formation. Twenty-three claudins have been identified in human subjects; however, their contribution to AD is not fully defined.^{30,31} To gain insight into which claudins are expressed in patients with AD, we evaluated a subset of TJ genes, the expression profiles of which are visualized in a heat map (Fig 4, A, and see Table E11 in this article's Online Repository at

www.jacionline.org). Downregulation of claudins 1 and 23 was observed in lesional versus nonlesional and normal epidermis, as well as downregulation of claudins 4 and 8, which were previously unreported in patients with AD (FDR < 0.001).³² We also detected dermal claudins (5 and 11), expression of which was significantly downregulated in the lesional versus normal skin comparison, which was previously not reported in patients with AD.

The differential expression of claudin genes in the epidermal and dermal compartments was validated by using immunohistochemistry and RT-PCR. CLDN4, CLDN8, and CLDN23 stained all epidermal layers, except the stratum corneum (Fig 4, B-D), with highly attenuated staining in lesional versus both nonlesional and normal epidermis. The reduced staining was particularly evident in the granular layer. CLDN8 showed increased intensity of basal layer staining in normal epidermis (Fig 4, C). The mRNA expression of *CLDN8* and *CLDN23* was also significantly reduced in lesional epidermis ($P < .05$; Fig 4, E and F).

Markers associated with activated T cells and other inflammatory cells are enriched by using LCM

Using individual cell-culture expression data (keratinocytes, fibroblasts, activated and unactivated T cells, DCs, and LCs),³³⁻³⁵ we explored the distribution of DEGs identified in epidermal, dermal, and bulk AD transcriptomes²⁷ using a Gene Set Enrichment Analysis. Whereas only the keratinocyte gene subset was enriched in the epidermal transcriptome (Fig 5 and see Table E12, A, in this article's Online Repository at www.jacionline.org), the dermal transcriptome showed enrichment of key inflammatory subsets, including activated and unactivated T cells, various DC subsets (immature and mature DCs), macrophages, fibroblasts, and lymphatic endothelial cells (Fig 5 and see Table E12, B). The bulk transcriptome showed a significant enrichment for keratinocytes, fibroblasts, and inflammatory subsets, with no enrichment for activated T cells and mature DCs (Fig 5 and see Table E12, C).

The Gene Set Enrichment Analysis also linked key mediators (*IL22*, TNF superfamily, member 4 [*TNFSF4/OX40L*], and *CTLA4*) detected in the dermal transcriptome to the gene signature of activated T cells (see Table E12, B). Among the genes found to be associated with both activated and unactivated T cells in the dermal transcriptome were *GZMB*, *ICOS*, and *CD27*.

CCR7, which is associated with T-cell trafficking and cutaneous lymphoid aggregates,^{36,37} was shown to be expressed by various cells types, including T cells and DCs. To investigate the predominant cellular expression of CCR7, we performed double immunofluorescence staining for CD3⁺ T cells (green) or CD11c⁺ myeloid DCs (green) with CCR7 (red) in lesional, nonlesional, and normal skin. CCR7 colocalized mainly with CD11c, showing increased expression in lesional versus nonlesional and normal dermis and some colocalization with CD3, primarily in lesional dermis (see Fig E6 in this article's Online Repository at www.jacionline.org). Thus CCR7 is preferentially expressed by myeloid DCs rather than T cells.

DISCUSSION

Evolving disease concepts associate the AD phenotype with barrier and immune abnormalities.^{1,2} In this model altered proliferation and differentiation of keratinocytes result from

cytokines derived from distinct T-cell subsets.^{2,12} It is important to understand the relative contributions of the epidermal and dermal compartments in creating the abnormal lesional phenotype to fully understand the pathogenic mechanisms driving AD.²⁶

This is the first report that establishes robust epidermal and dermal genomic signatures of lesional and nonlesional AD and normal skin compared with corresponding whole-tissue fingerprinting. Using bulk skin, we have associated the AD phenotype with T_H2/T_H22 immune activation and abnormal epidermal differentiation.¹¹⁻¹⁴ However, in bulk genomic profiling we could not determine which compartments are responsible for individual gene expression or locate their cellular sources.^{4,12,27} Furthermore, because of a dilution effect in bulk tissues, which are composed of functionally heterogeneous cells, many genes linked to AD pathogenesis are present at less than the detection level on microarrays.^{11,12,26,27} Additionally, because a large subset of genes are expressed in either the epidermis or dermis only, some of the measured differential gene expression between lesional and nonlesional skin appears to be an artifact of the unequal contribution of epidermal expansion in lesional skin rather than because of true changes in gene expression at the cellular level. The previous disease model for AD, which has relied on bulk tissue genomic profiling, is limited by the inability to distinguish between these differences. A large proportion of DEGs found only in bulk tissue seem to derive from unequal contributions of lesional epidermis and dermis compared with nonlesional epidermis and dermis. Overall, we believe that the differences in cellular physiology created by AD are best detected in the LCM-generated DEGs.

Using the LCM method followed by genomic and cellular studies, we have identified a largely increased AD transcriptome, with an additional 674 upregulated and 405 downregulated genes compared with prior reports.^{11-14,26,27} By separating the 2 compartments, we have also identified key immune and barrier markers that are usually undetectable on arrays (ie, IL-22 and TSLP) and obtained more robust genomic differences for most immune genes when comparing lesional, nonlesional, and normal tissues. Through this approach and previously acquired pathways and cell-specific genomic maps,^{11-14,26,27} we have localized many immune and barrier genes to the epidermis or dermis (or both), leading to a deeper understanding of inflammatory circuits and the cellular subset involved in creating the AD phenotype. For example, dermal expression of CCL19 and CCL21 likely attracts $CCR7^+/CD11c^+$ DCs and might organize lymphoid tissue in the dermis. Our LCM and genomic approach also led to enrichment of gene products associated with activated T cells and inflammatory DCs that play a role in effector responses in patients with AD.³⁷

Our data are the first to identify IL-34, the newly identified cytokine in mice models, and its receptor, CSF-1R, in a human skin disease. The mouse studies demonstrated a critical role for IL-34 in differentiation and proliferation of LCs in the epidermis during steady states, whereas repopulation of LCs in inflammatory states was independent of IL-34.²⁸ Although IL-34 is mainly produced by keratinocytes, its receptor, CSF-1R, originates from dermal macrophages and mononuclear cells.²⁹ Our data show decreased epidermal expression of IL-34 in lesional epidermis compared with that seen in both nonlesional AD and normal epidermis (Fig 3, B and C). This confirms the role of IL-34 in the maintenance phase, corresponding to prior mouse data.²⁸

Thus the IL-34 cytokine might function as a negative regulator, and its induction in nonlesional skin might inhibit the propagation of the inflammatory cascade toward development of active skin lesions. Additionally, we found that IL-34 expression in lesional dermis colocalizes most commonly with myeloid DCs and macrophages. Future studies are needed to evaluate the possible functions of the IL-34/CSF-1R cytokine-receptor complex in background and diseased AD skin and whether strategies of increasing levels of this cytokine might be able to prevent development of skin lesions.

Separating the 2 compartments, we also identified 2 novel barrier genes, *CLDN4* and *CLDN8*, for the first time in the AD transcriptome. Claudins are pivotal for TJ formation.³⁸ Prior mouse and human skin equivalent models demonstrated that *CLDN4* colocalized with *CLDN1* to the epidermal granular layer,^{39,40} whereas *CLDN8* was previously shown in human kidneys and intestines.^{41,42} Nevertheless, our knowledge of claudins and their involvement in the barrier alterations seen in patients with AD remains incomplete.²⁹ De Benedetto et al³² reported that *CLDN1* and *CLDN23* show significantly reduced expression in nonlesional AD skin compared with that seen in healthy skin. In our study *CLDN8* and *CLDN23* showed significantly reduced expression in lesional compared with nonlesional and normal epidermis by using microarrays (Fig 4, A, and see Table E11), with respective fainter epidermal staining of *CLDN4*, *CLDN8*, and *CLDN23* in lesional versus nonlesional AD and normal epidermis (Fig 4, B and D). Claudin 11 was identified as a novel dermal claudin, which was previously shown in other tissues (brain, testis, and cochlea).⁴³⁻⁴⁵ Future experiments are needed to investigate the function of the newly identified *CLDN4* and *CLDN8*, but together with *CLDN1* and *CLDN23*, these might contribute to the barrier defect associated with the AD phenotype.

Our study also had some limitations. First, LCM is a labor-intensive method, usually allowing analyses of a small number of samples, as in our study ($n = 5$ lesional and nonlesional specimens each and $n = 3$ normal samples separated into epidermis and dermis categories), possibly resulting in weaker statistical power, particularly for bulk tissue comparisons. Second, although our LCM approach detected novel barrier genes, the downregulation of key differentiation markers (ie, *FLG*, *LOR*, and involucrin [*IVL*]) in the lesional and nonlesional (vs normal) epidermal transcriptomes was not as impressive as in prior bulk data,^{13,26} which is contrary to the enrichment of *FLG* and *LOR* in a prior normal skin epidermis LCM study,^{17,46} perhaps because of lower recovery of granular layer products using the epidermal-dermal separation approach.⁴⁷ Third, even though the combined LCM epidermal and dermal transcriptomes were larger than the bulk tissue transcriptome, many genes were only detected in the bulk transcriptome (see Fig E2 and Table E8, C). This might be explained by (1) the unequal contribution of lesional epidermis and dermis compared with their counterparts; (2) possible inclusion of subcutaneous tissue in bulk biopsy specimens; (3) platform/technical issues, as described in a previous LCM article separating the epidermis and dermis from only 3 psoriatic (lesional and nonlesional) and 3 normal tissues¹⁶; and (4) restriction of this analysis to patients with severe AD, in whom nonlesional skin has a more abnormal phenotype.²⁶ Thus many genes might not pass defined thresholds of FCH and significance when comparing lesional and nonlesional skin.

Our study establishes the utility of LCM in patients with AD to separate different skin compartments and cellular infiltrates. It provides complementary information to bulk analysis, allowing regional and/or cellular localization of key barrier or immune molecules, and enables detection of genes that are not usually detected on arrays because of the mixture of transcripts within the heterogeneous bulk tissue.^{48,49} LCM is particularly beneficial for AD, in which a complex network of immune and barrier abnormalities results in the global phenotypes of active and “normal-appearing” skin, and dissecting the individual skin components and cells is crucial to unraveling their respective contributions to pathogenesis. Our combined LCM and genomic approach can be useful in future studies aimed at dissecting the relative roles of barrier versus immune activation of different AD phenotypes (ie, intrinsic and extrinsic AD)¹¹ and for dissecting therapeutic responses to various agents that are now in clinical trials for patients with AD.⁵⁰

Clinical implications: Our approach can be useful to differentiate the ability of targeted treatments to reverse epidermal and immune alterations in AD skin.

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