

Comparative transcriptomic analyses of atopic dermatitis and psoriasis reveal shared neutrophilic inflammation

David F. Choy, BS,^{a,*} Daniel K. Hsu, PhD,^{d,e,*} Dhaya Seshasayee, PhD,^b Maxwell A. Fung, MD,^d Zora Modrusan, PhD,^c Flavius Martin, MD,^b Fu-Tong Liu, MD, PhD,^{d,e,*} and Joseph R. Arron, MD, PhD^{a,†} South San Francisco and Davis, Calif, and Taipei, Taiwan

Background: Atopic dermatitis (AD) and psoriasis are common inflammatory diseases canonically described as involving distinct T_H polarization and granulocytic infiltration. Acute AD lesions are associated with T_H2 and eosinophilic inflammation, whereas psoriatic lesions are associated with T_H1/T_H17 and neutrophilic inflammation. Despite intensive investigation, these pathways remain incompletely understood *in vivo* in human subjects.

Objective: Using AD and psoriatic lesional skin as exemplar T_H2 and T_H1/T_H17 diseased tissue, we sought to clarify common and unique molecular and pathophysiologic features in inflamed skin with different types of inflammatory polarization.

Methods: We conducted gene expression microarray analyses to identify distinct and commonly dysregulated expression in AD (based on Hanifin and Rajka criteria) and psoriatic lesions. We defined gene sets (GSs) as comprising genes encoding cytokines, chemokines, and growth factors that were uniquely or jointly dysregulated in patients with AD and those with psoriasis and calculated aggregate GS expression scores for lesional skin of patients with these dermatoses and healthy control skin.

Results: The atopic dermatitis gene set (AD-GS) score correlated with systemic and local measures of allergic inflammation, including serum IgE levels, blood eosinophil counts, and tissue eosinophil counts. Unexpectedly, genes encoding neutrophil chemoattractants among the common GS were highly expressed in AD lesional skin. Hematoxylin and eosin and immunohistochemical analyses showed the numbers

of neutrophils in AD lesional skin were comparable with those in psoriatic lesional skin, and both were correlated with the extent of expression of neutrophil chemoattractant genes.

Conclusion: These data are evidence that neutrophilic inflammation is a feature of lesional AD pathology comorbid with allergic inflammation. (J Allergy Clin Immunol 2012;■■■■:■■■-■■■.)

Key words: Atopic dermatitis, psoriasis, T_H2, T_H17, gene expression microarray, neutrophil, eosinophil

Atopic dermatitis (AD) and psoriasis are common and clinically distinct human diseases characterized by inflammatory skin lesions.¹⁻³ These diseases have been associated with contrasting polarization of the adaptive immune system and distinct granulocytic infiltration in lesional tissue. AD is canonically described as featuring excessive T_H2 and eosinophilic infiltration in acute lesions and a mixed T_H1 and T_H2 pattern in chronic lesions,⁴ whereas psoriasis features excessive T_H1/T_H17 inflammation and neutrophilic infiltration.^{2,5,6} Investigative studies of therapies that specifically target canonical T_H2 cytokines in patients with AD⁷ and T_H1/T_H17 cytokines in patients with psoriasis⁸⁻¹¹ have provided direct evidence of the causal roles of these T_H pathways in disease morbidity. However, variable therapeutic efficacy and the lack of sustained clinical response in the absence of continuous therapy underscore the need to better understand these types of inflammation. This concerns both the relationship between the activity of targeted pathways and specific pathologic measures and underlying bases of disease heterogeneity, which might directly influence clinical outcomes. Addressing this need might lead to the discovery of new therapeutic targets and facilitate the development of selection strategies to appropriately pair patients with molecularly targeted therapies.

Molecular phenotyping of diseased tissues by using high-dimensional gene expression technologies (eg, microarrays) comprehensively surveys the transcriptome. However, the high dimensionality of gene expression microarrays requires appropriate considerations for the manifold nature of using the gene-by-gene hypothesis testing that might consequently reduce statistical power.¹² Independent filtering, which aims to remove variables unlikely to be informative before testing, is a 2-stage approach that filters variables by criteria independent of the test statistic (eg, overall variance and gene annotation) and can increase the detection rate without loss of type I error control.¹³ Cytokines, chemokines, and growth factors (CCGFs) are potent molecular mediators involved in both homeostatic and aberrant processes of inflammation and tissue development. Given the well-characterized roles of CCGFs as mediators of inflammation and intercellular communication in patients with inflammatory diseases,¹⁴ we have focused our analyses on CCGFs based on both

From the Departments of ^aImmunology, Tissue Growth, and Repair Biomarker Discovery, ^bImmunology, and ^cMolecular Biology, Genentech, South San Francisco; ^dthe Department of Dermatology, School of Medicine, University of California–Davis; and ^ethe Institute of Biomedical Sciences, Academia Sinica, Taipei.

*These authors contributed equally to this work.

†These authors codirected this project.

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Corresponding author: Fu-Tong Liu, MD, PhD, Department of Dermatology, 3301 C St, Suite 1400, Sacramento, CA 95816. E-mail: fliu@ucdavis.edu. OR Joseph R. Arron, MD, PhD, 1 DNA Way, MS 231C, South San Francisco, CA 94080. E-mail: arron.joseph@gene.com.

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Abbreviations used

AD:	Atopic dermatitis
AD-GS:	Lesional atopic dermatitis gene set
CCGf:	Cytokines, chemokines, and growth factors
C-GS:	Common lesional gene set
DE:	Differential expression
FDR:	False discovery rate
GS:	Gene set
H&E:	Hematoxylin and eosin
MPO:	Myeloperoxidase
PC:	Principal component
PCA:	Principal component analysis
Ps-GS:	Lesional psoriasis gene set
SPCA:	Supervised principal component analysis

biological relevance and attractive statistical properties. We have previously derived a quantitative gene expression signature of T_H2 inflammation in bronchial biopsy specimens from asthmatic patients that correlated with physiologic measures of allergic inflammation. We then evaluated this signature against a gene set (GS) comprising CCGfs to better understand phenotypic heterogeneity in asthmatic patients and the involvement of key mediators of inflammation, cellular migration, and tissue remodeling.¹⁵ We anticipated that CCGf expression analyses in inflamed tissues from patients with AD and those with psoriasis would be particularly informative in understanding the pathophysiologic features of these inflammatory skin diseases.

In this study we investigated tissues from patients with AD and those with psoriasis using gene expression microarrays in which we aimed to (1) clarify T_H -associated inflammatory expression patterns in the context of human skin disease, (2) gain insight into the degree to which T_H type-associated inflammation and other pathways are involved in individual cases of disease, and (3) relate pathway activity to specific pathologic measures. Leveraging the distinct inflammatory causes associated with AD and psoriasis, we performed comparative analyses to delineate common and unique expression patterns associated with each disease. We derived a lesional AD gene set (AD-GS), a lesional psoriasis gene set (Ps-GS), and a common lesional gene set (C-GS) and observed dramatic T_H2 - and T_H1/T_H17 -associated gene expression patterns in patients with AD and those with psoriasis, respectively. We summarized these skin biopsy GSs into quantitative expression signature scores and observed correlations with related physiologic measures of inflammation, suggesting that transcriptional perturbations in lesional skin are involved in broader related systemic inflammatory manifestations. Unexpectedly, we found that patterns of lesional gene expression common to both AD and psoriasis are enriched for genes encoding neutrophil chemoattractants, the expression levels of which correlate with peripheral and immunohistochemical measures of neutrophilia. Collectively, these data suggest that neutrophilic inflammation is a pathologic feature of chronic AD coexisting with hallmarks of allergic inflammation.

METHODS**Subjects**

The subject population consisted of 12 patients with AD, 14 patients with psoriasis, and 5 healthy control subjects. Hanifin and Rajka criteria¹⁶ were used to identify adults with AD. Subjects had active lesions and were not

receiving systemic therapy. Patients' demographics, along with disease severity and the distribution of the affected skin, are summarized in Table E1 in this article's Online Repository at www.jacionline.org. All subjects had at least 3 of the 4 major features and at least 3 of the minor features defined in the Hanifin and Rajka criteria. All but 1 subject (MB19) met the revised criteria of the American Academy of Dermatology.¹⁷ However, this subject fulfills the criteria developed by Williams.¹⁸ The median total serum IgE level of these subjects was 303 kU/L (interquartile range, 527 kU/L).

The biopsy specimens were taken from established lesions in affected areas that exhibited characteristic eczematous lesions with signs of epithelial disruption, excoriations, and crusts but without clinical evidence of superinfection. Fig E1 in this article's Online Repository at www.jacionline.org depicts a representative clinical appearance of the AD lesions in this study.

Participants with psoriasis consisted of adults with chronic disease and active skin lesions with characteristic morphology and histologic presentation. Patients' demographics are tabulated in Table E1. See the Methods section in this article's Online Repository at www.jacionline.org for additional details.

RNA processing and microarray hybridization

Agilent 2-color Whole Human Genome expression microarray analysis (Agilent, Santa Clara, Calif) was conducted as described previously¹⁵ on RNA isolated from biopsy specimens preserved in RNALater (Invitrogen, Carlsbad, Calif; see the Methods section in this article's Online Repository).

Microarray data analysis and statistics

All statistical calculations were performed with the R Project software package, version 2.14.0 (<http://www.R-project.org>). R package hgu4112a.db, version 2.6.3, was used for microarray annotation. A moderated paired *t* test was used to assess differential expression (DE) by using the limma package of Bioconductor.¹⁹

Correlation coefficients between gene expression and physiologic parameters were performed by using the Spearman method. Continuous versus categorical variable testing was implemented with Kruskal-Wallis ANOVA. Multiple testing for pairwise tests was addressed through the calculation of the false discovery rate (FDR).

For detailed statistics, see the Methods section in this article's Online Repository.

Histochemistry and immunohistochemistry

Detection of inflammatory cells in paraffin-embedded skin biopsy specimens was performed as previously described (see the Methods section in this article's Online Repository).²⁰ Detection of neutrophils was accomplished by using optimized neutrophil lipocalin (lipocalin-2) and neutrophil elastase (elastase-2) staining supplemented with hematoxylin and eosin (H&E), both in combination with observation of polymorphic nuclei. Initial neutrophilic assessment involved myeloperoxidase (MPO) staining.

RESULTS**AD-GS, Ps-GS, and C-GS are defined by using comparative DE analyses of CCGf genes**

Leveraging the distinct inflammatory causes associated with AD and psoriasis, we performed comparative expression analyses to define disease-specific GSs that were composed of distinguishing T_H -associated genes and those that were dysregulated in common. We compared within-disease lesion versus nonlesion moderated paired *t* tests (see the Methods section in this article's Online Repository) and defined AD-GS, Ps-GS, or C-GS as being associated with lesional AD (red and purple genes in figures) or psoriasis (blue and purple genes in figures) or being commonly dysregulated in both diseases (green genes in figures, Fig 1 and see Table E2 in this article's Online Repository at www.jacionline.org). The *t*-statistic scatter plot of CCGf genes

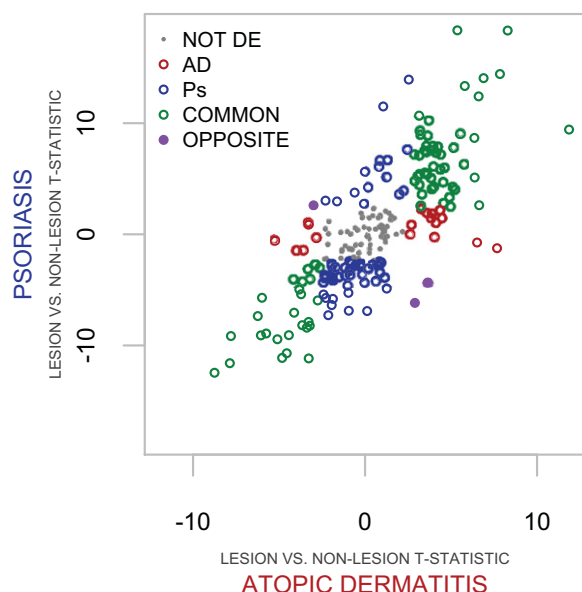


FIG 1. Comparative expression analysis of CCGfs defines GSs. T-statistics of within-disease lesional versus nonlesion comparisons are depicted by using scatter plots. The AD-GS (red symbols) and Ps-GS (blue symbols) comprise unique and opposing differentially expressed genes (purple symbols). Commonly and not differentially expressed (NOT DE) genes are represented by green and gray symbols, respectively.

comparing diseased lesions versus nonlesions depicts how the intersection of magnitude and direction of dysregulation define the GSs (Fig 1). DE analysis revealed a substantial number of genes that are significantly altered (FDR < 0.05) commonly (C-GS) in the 2 diseases (75/217 [34.6%] CCGfs) compared with the AD-GS (21/217 [9.7%] CCGfs) and the Ps-GS (59/217 [27.2%] CCGfs).

AD-GS comprises T_H2 -associated genes and signature expression intensity correlates with serum IgE levels and blood and tissue eosinophil counts

The AD-GS (see Table E2 and Fig 2, A) includes *IL13*, *CCL13*, *CCL17*, *CCL26*, and *TNFSF4*, which are established mediators of T_H2 inflammation.^{5,15,21} *IL13* has been under intense investigation as a therapeutic target in T_H2 -driven inflammatory diseases.²² We have observed *CCL13* and *CCL26* expression in bronchial biopsy specimens to be associated with a “ T_H2 -high” subphenotype of asthma.¹⁵ *CCL17* is a chemoattractant for T_H2 cells through *CCR4*²² and is potently upregulated in T_H2 central memory T cells stimulated by thymic stromal lymphopoietin–dendritic cells.²³ *TNFSF4* (also known as OX40 ligand) has been demonstrated to be a key component in the initiation of thymic stromal lymphopoietin–dependent allergic inflammation.^{21,24}

Given the strong representation of dysregulated T_H2 -associated genes in patients with AD, we performed supervised principal component analysis (SPCA) on the 21 AD-GS genes. This allowed us to derive a quantitative signature^{15,25} to link pathway gene expression in lesional skin and skin from healthy control subjects to systemic clinical covariates related to allergy and inflammation. Expression intensity of these genes is represented by heat map and organized by SPCA factors (Fig 2, A). We found that

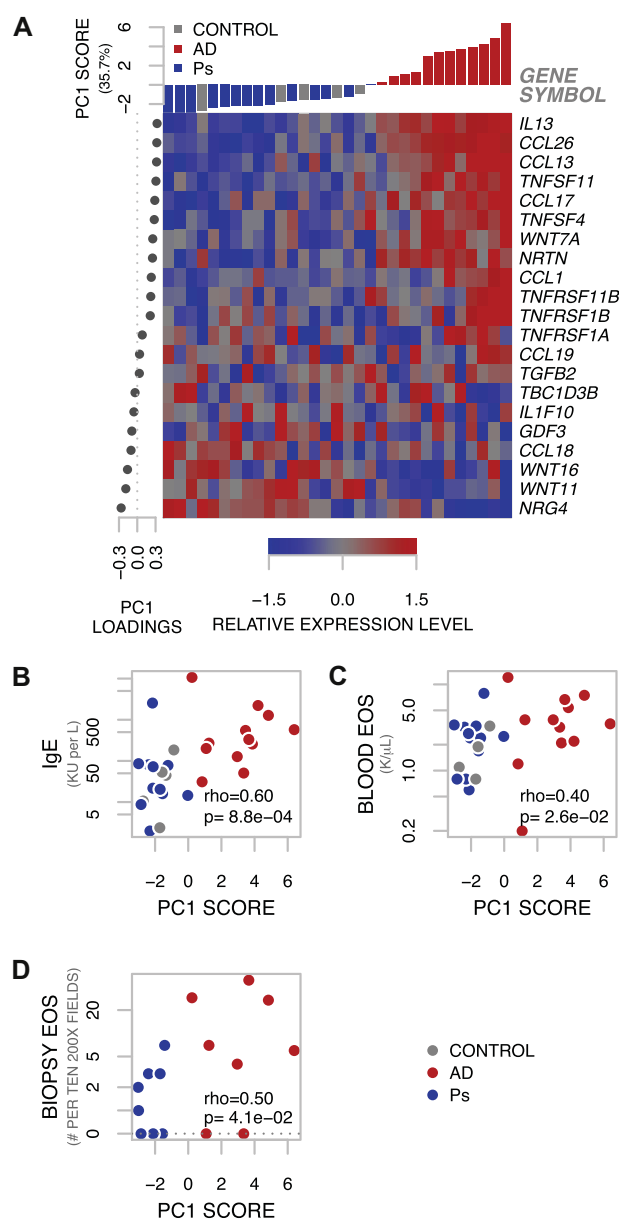


FIG 2. AD-GS. **A**, Genes comprising AD-GS are represented by an expression heat map and organized by PCA factors. Columns (samples) are arranged by PC1 score. Rows (genes) are organized by PC1 loadings. **B–D**, PC1 significantly correlates with serum IgE levels (Fig 2, B), blood eosinophil counts (Fig 2, C), and biopsy specimen eosinophil counts (Fig 2, D).

principal component (PC) 1, representing 35.7% of the variance of these 21 genes, correlated (Spearman rank correlation) strongly with serum IgE levels ($\rho = 0.60$, $P = 8.8 \times 10^{-4}$; Fig 2, B). Statistically significant correlations with PC1 were also observed with blood eosinophil counts ($\rho = 0.40$, $P = .026$; Fig 2, C) and tissue eosinophil counts ($\rho = 0.50$, $P = .041$; Fig 2, D).

Ps-GS comprises upregulated T_H17 -associated and downregulated T_H2 -associated CCGf genes

The Ps-GS (see Table E2 and Fig 3) comprises 59 CCGf genes. Upregulated Ps-GS genes included *IL17A*, *IL17F*, *IL1B*, and *CCL20*, all of which are associated with T_H17 inflammation. *IL17A* and *IL17F* are expressed by T_H17 cells with normal

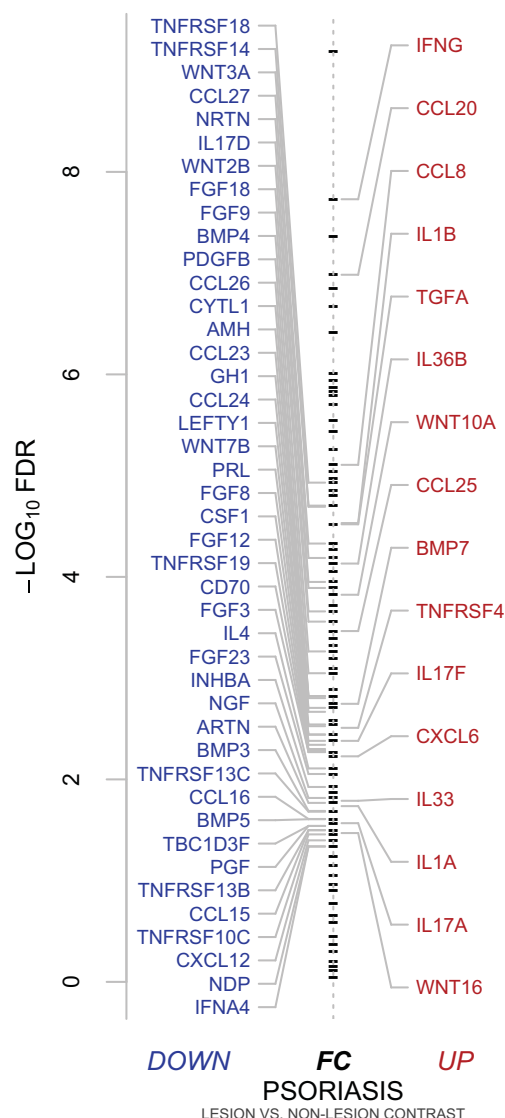


FIG 3. Ps-GS expression. Summary statistics ($-\log_{10}$ FDRs and fold-change direction) of moderated paired t tests of lesional psoriasis versus nonlesional psoriasis is plotted by using an annotated strip chart for all 217 CCGs. Genes of the Ps-GS, which are all differentially expressed with FDRs of less than 0.05, are annotated.

functions that are associated with defense against extracellular pathogens.²⁶ *IL1B* and *CCL20* expression has been reported to be significantly upregulated in lesional skin in patients with psoriasis *in vivo* and in IL-17A-stimulated keratinocytes *in vitro* and synergistically upregulated by IL-17A and TNF stimulation.²⁷ These are expected and reassuring observations because these genes, the expression of which are the result of T_H17 inflammation, have been previously implicated in the pathophysiology of psoriasis.²⁷

Among downregulated Ps-GS genes were T_H2 inflammation-associated *CCL16*, *CCL23*, *CCL26*, *CCL24*, and *IL4*. *CCL16* potently mediates eosinophil migration *in vitro*.²⁸ Microarray analysis of PBMCs stimulated by IL-13 identified *CCL23* (myeloid progenitor inhibitory factor 1) and *CCL26* (eotaxin-3) as significantly upregulated genes.²⁹ *CCL24* (eotaxin-2), like *CCL26*, is a CCR3 agonist and a specific eosinophil chemoattractant.³⁰ *IL4*

is produced by T_H2 cells and drives B-cell differentiation to IgE-producing plasma cells.⁵

C-GS comprises genes encoding neutrophil chemoattractants and signature expression intensity correlates with blood and tissue neutrophils

Among the C-GS genes similarly dysregulated in patients with AD and those with psoriasis compared with nonlesional skin are *CXCL1*, *CXCL2*, *IL8*, and *CSF2* (GM-CSF). These are all potent neutrophil chemoattractants.^{31,32} We hypothesized that the dysregulation of neutrophil chemoattractant genes would have pathologic relevance and sought to relate the expression of the C-GS genes with local and systemic measures of neutrophilic inflammation. We performed SPCA^{15,25} on the 75 C-GS genes to quantitatively relate pathway gene expression of lesional skin from patients with AD or those with psoriasis and skin from healthy control subjects with measures of blood and lesional neutrophils. The expression intensity of these genes is represented by using a heat map organized by SPCA factors (Fig 4, A). We found that PC1, representing 42.4% of the variance of these 75 genes, correlated (Spearman rank correlation) with blood neutrophil counts ($\rho = 0.49$, $P = 5.6 \times 10^{-3}$; Fig 4, B).

We detected neutrophil infiltration in AD and psoriatic lesional skin but not in control or perilesional AD or psoriatic skin using histologic and immunohistochemical analyses (representative AD lesional skin; Fig 5, A). MPO-positive, elastase-2-positive, and neutrophil cell counts determined by using H&E in the dermis are shown in Fig 5, B.

We initially observed a large number of MPO-positive cells (a neutrophil marker³³) in both AD and psoriatic skin, and the amounts of these cells were comparable between these 2 disease cases. These cells included intensely stained cells without distinct nuclear morphology typical of neutrophils and can include immature neutrophils, monocytes, or both.

We then used a more specific marker for neutrophils, elastase-2 (and, in a limited subset, lipocalin-2), and confirmed that the amounts of positively stained cells were also comparable between patients with AD and those with psoriasis. Other cell types are known to express elastase-2, although at lower levels relative to neutrophils.^{34,35} However, we established that most cells exhibiting intense positivity for neutrophil elastase-2 and lipocalin-2 in both AD and psoriatic lesions were neutrophils on the basis of polymorphic nuclear morphology. These cells were largely present in the papillary dermis.

Importantly, using H&E staining, we noted that extravascular neutrophil counts with the characteristic morphology of neutrophils (ie, multilobular nuclei and eosinophilic cytoplasm) were also comparable between patients with lesional AD and those with psoriasis, although they were substantially lower than elastase-positive cell counts (Fig 5, B).

Each of the 3 neutrophil indicators in lesional skin positively correlated (Spearman rank correlation) with PC1 of the C-GS (positive elastase-2 staining and cell morphologies consistent with neutrophils: $\rho = 0.46$, $P = 4.7 \times 10^{-2}$; positive MPO staining: $\rho = 0.51$, $P = 3.1 \times 10^{-2}$; and characteristic morphology by means of H&E analysis: $\rho = 0.52$, $P = 3.4 \times 10^{-2}$; Fig 5, C). Finally, we found that these 3 indicators of neutrophils were positively intercorrelated ($P < .05$, all pairwise correlations, Spearman).

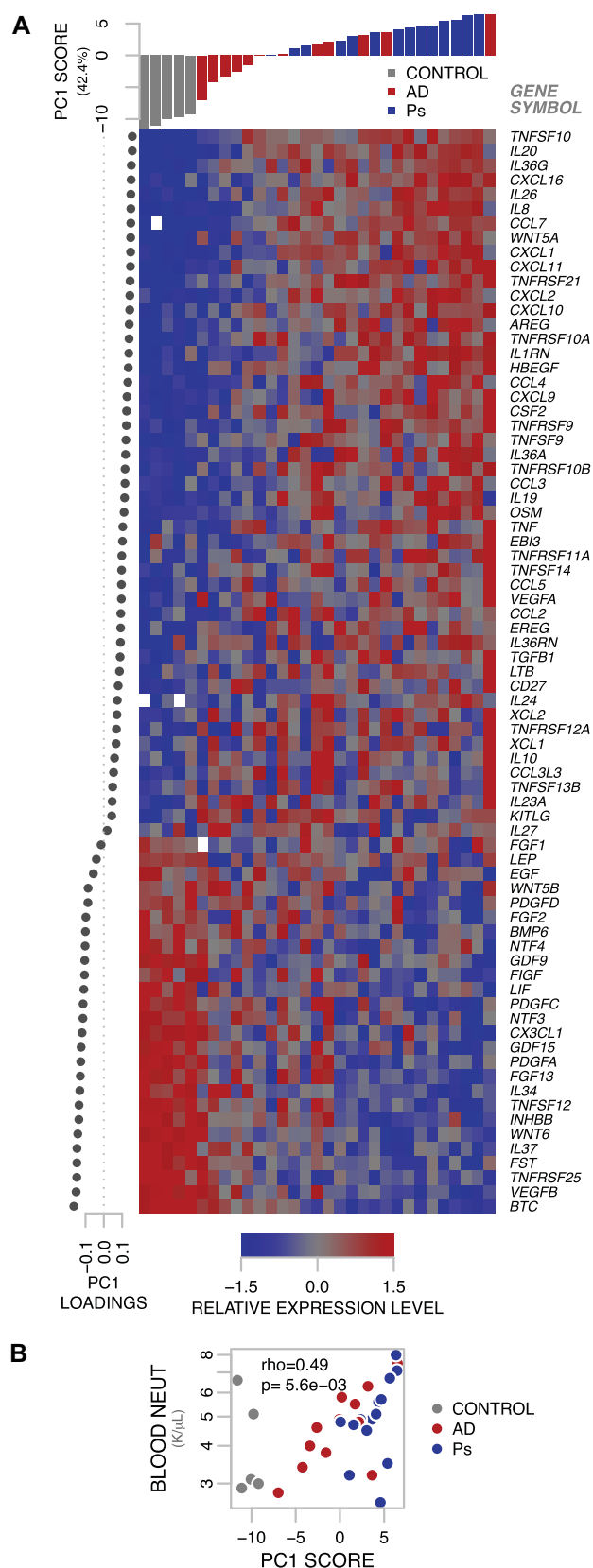


FIG 4. C-GS expression signature correlates with blood neutrophil counts. **A**, Genes comprising the C-GS are represented by using an expression heatmap and organized by PCA factors. Columns (samples) are arranged by PC1

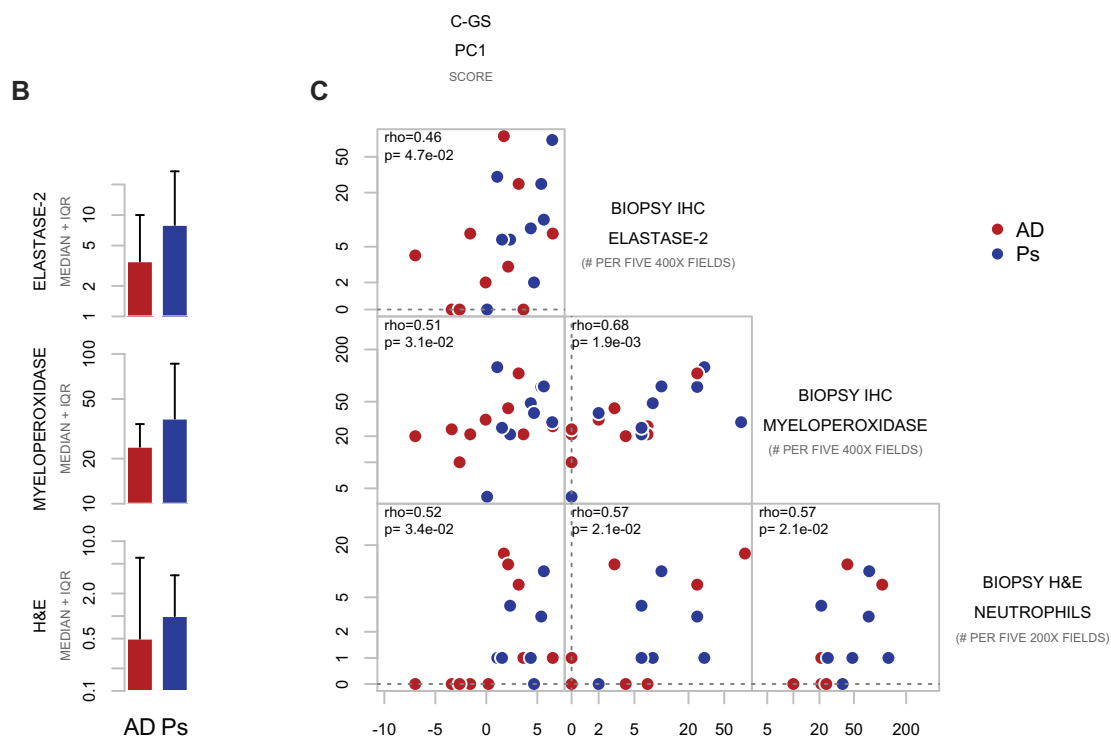
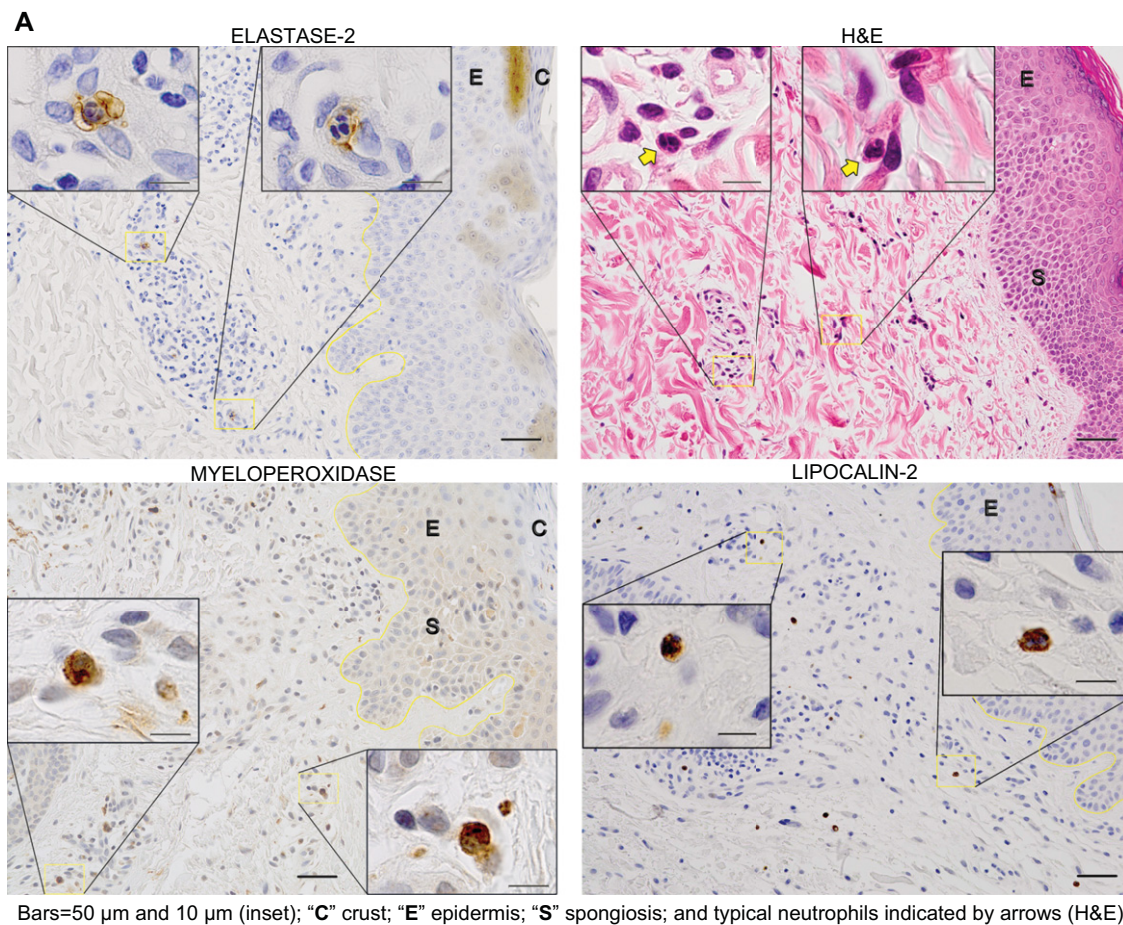
At the microscopic level, most biopsy specimens contained surface crust, which is consistent with a typical clinical feature of AD. Fig E1 in this article's Online Repository at www.jacionline.org depicts the maximum crust identified microscopically. The numbers of elastase-2-positive cells correlated with the presence of surface crust in the stratum corneum. None of the patients with AD exhibited prominent crust or microscopic evidence of bacterial colonization or clinical or microscopic evidence of secondary infection (impetiginization, see Table E1), and neutrophilic infiltration was clearly evident in the dermis deep to the crusts in all cases.

DISCUSSION

Genome-wide association studies have identified susceptibility loci implicating the T_H2 cytokines IL-4 and IL-13 for both AD and psoriasis but with opposing effects for the same alleles in each disease,^{2,36,37} suggesting that these diseases represent opposing extremes of T_H2 dysregulation. An investigational study of 20 patients with psoriasis treated with recombinant human IL-4 demonstrated clinical efficacy, further suggesting that psoriasis might involve a relative deficiency in T_H2 activity.³⁸ *CCL26* (eotaxin-3) was one of 3 CCGf genes to be differentially expressed (FDR < 0.05) in opposite directions in patients with AD and those with psoriasis (upregulated in patients with lesional AD and downregulated in patients with lesional psoriasis, see Fig E2 and Table E2 in this article's Online Repository at www.jacionline.org). *CCL26* expression is induced by IL-13 or IL-4 *in vitro*, and because of its function as a CCR3 agonist, *CCL26* is a potent eosinophil chemoattractant.^{29,39} Its expression has been observed to be upregulated *in vivo* in patients with other T_H2 -associated human diseases, including bronchial biopsy specimens of asthmatic patients and esophageal biopsy specimens from patients with eosinophilic esophagitis.^{15,40} *CCL26* expression can be induced through activation of signal transducer and activator of transcription 6, which is the predominant signal transducer downstream of IL-4 and IL-13 receptor complexes.^{15,29,39,40} Therefore if *CCL26* expression is taken as an indicator of IL-4 activity, IL-13 activity, or both, we found that the expression patterns of *CCL26* in patients with AD and those with psoriasis were consistent with relatively increased IL-4/IL-13 activity in patients with AD and relatively decreased IL-4/IL-13 activity in patients with psoriasis.

Supporting the relevance of CCGfs in this study, principal component analysis (PCA, see Fig E3 in this article's Online Repository at www.jacionline.org) revealed a prominent relationship between the study factors of lesion status and diagnosis with respect to CCGf expression. PC1 accounted for 24% of all gene expression variance. As illustrated by arrows connecting individual subjects' nonlesional and lesional skin samples, PC1 clearly separates sample lesion status, regardless of diagnosis. A smaller proportion of gene expression variance was explained by disease diagnosis. PC2 (accounting for 10.1% of all gene expression variance) effectively separates lesional samples from patients with AD and those with psoriasis. These data suggest that

score. Rows (genes) are organized by PC1 loading. Missing expression values are represented in white. **B**, PC1 significantly correlates with blood neutrophil counts. Ps, Psoriasis.



CCGf expression is highly enriched with informative gene expression variance for the assessment of lesional AD and psoriasis.

The findings that AD-GS expression in AD and psoriatic lesional skin is correlated with serum IgE levels and biopsy specimen eosinophil counts is analogous to our previous description of a correlation between a quantitative T_H2 gene signature in bronchial biopsy specimens from asthmatic patients and local and systemic pathophysiologic features of allergic inflammation: serum IgE levels, bronchial alveolar lavage eosinophil counts, and peripheral blood eosinophil counts.¹⁵ The findings that the magnitude of T_H2-associated gene expression in asthmatic airways, as well as lesional skin from patients with AD and those with psoriasis, is mirrored by relevant local and systemic physiologic measures of allergic inflammation suggest a direct relationship between gene expression patterns and disease phenotype and that the pattern of expression is useful in understanding the heterogeneity of pathway involvement. These findings are reconcilable with the expected function of genes implicated in these analyses. The T_H2-associated cytokines IL-4 and IL-13 drive immunoglobulin class-switching in B cells and the production of IgE.⁵ Clinically, atopy is an important criterion in the diagnosis of AD,⁴¹ and IgE is thought to play a key role in the atopic response.⁴² T_H2 cells also produce IL-5, a dominant eosinophilopoietic factor with activity that directly affects peripheral blood eosinophil counts.⁴³ These observations suggest that serum IgE levels, blood eosinophil counts, or both might be useful as biomarkers for assessing the extent of T_H2 inflammation in diseased tissues, which could be directly relevant when considering patient selection strategies for therapies targeting T_H2 inflammation in patients with allergic disorders. Accordingly, we have found in the context of bronchial allergen challenge that asthmatic patients with increased serum IgE levels, blood eosinophil counts, or both experienced enhanced clinical benefit from therapeutic IL-13 blockade compared with patients with lower IgE levels, blood eosinophil counts, or both.⁴⁴

As expected, the Ps-GS was enriched with upregulated T_H17-associated genes, such as *IL17A* and *IL17F*. Interestingly, we observed T_H2-associated genes among downregulated Ps-GS genes. The observation of downregulated *IL4* was notable with respect to the previously mentioned study in 20 patients with psoriasis effectively treated with recombinant IL-4.³⁸ Because allergic disease is characterized by excessive T_H2 inflammation,⁵ these findings further support previous genetic^{37,44} and therapeutic³⁸ studies that psoriasis might be characterized by a deficiency of T_H2 pathway involvement.

Our comparative transcriptomic analyses of patients with AD and those with psoriasis yielded the unexpected finding that commonly differentially expressed genes (the C-GS) are strongly enriched for those coding for neutrophil chemoattractants and that the extent of expression of the C-GS correlates with physiologic measures of neutrophilic inflammation. Highlighting an advantage of our study design (patient-matched lesional and nonlesional skin), these findings can be reconciled with the those of previous smaller studies, such as a genome-wide expression analysis of skin lesions that have described *IL8* and *CXCL2* to be

among other genes differentially expressed among 6 patients with AD versus 7 patients with psoriasis.⁶ As expected for the C-GS signature scores, which are derived from strongly differentially expressed lesional versus nonlesional skin comparisons common to both dermatoses, we found these values to be significantly increased in patients with AD versus control subjects ($FDR = 4.8 \times 10^{-4}$, see Fig E4 in this article's Online Repository at www.jacionline.org) and in patients with psoriasis versus control subjects ($FDR = 4.8 \times 10^{-4}$, see Fig E4). However, C-GS scores in patients with lesional psoriasis were also significantly increased versus those in patients with lesional AD ($FDR = 6.4 \times 10^{-3}$). Taken together, this suggests that a similar mechanism of neutrophilic inflammation operates in both diseases, although factors relating to the recruitment of neutrophils might be involved to a more variable extent in patients with AD than in patients with psoriasis.

In support of our transcriptomic observations is the remarkable and important finding that numbers of neutrophils present in the dermis of AD lesions are comparable with those found in psoriatic lesions. Although the presence of neutrophils in lesional skin is characteristic of psoriasis, this is not typically considered a feature associated with AD.^{2,45-47} The unexpected finding of the presence of neutrophils in AD lesions at levels comparable with those found in psoriatic lesions was prompted by our observation of commonly differentially expressed neutrophil chemoattractant genes in both patients with AD and those with psoriasis.

We noted that substantial numbers of MPO-positive cells possessed morphologic characteristics of neutrophils. In addition, we definitively identified neutrophils by means of positive lipocalin-2 and elastase-2 staining and the presence of characteristic neutrophil morphologies in those positively stained cells. Thus we believe that the numbers of neutrophils might be higher than is evident by using H&E staining alone. To our knowledge, immunodetection of neutrophils in human AD lesions has not been previously described. However, neutrophilia in the dermis has been described in antigen-specific dermatitis driven by a T_H2-polarized response in a murine model of AD,⁴⁸ although murine models by no means duplicate the human disease.

We found a correlation between the number of neutrophils and the presence of crusts in the AD lesions. The presence of crusts and excoriations is a characteristic and diagnostic feature of AD. They reflect epithelial disruption and, in the case of AD, are likely associated with scratching that results from itching, which is a prominent and essential clinical feature of AD. Thus a contributing factor to neutrophilia might be tissue damage and the resultant release of neutrophil chemotactic factors,⁴⁹ as has been observed in skin wounding when neutrophils are the initial dermal infiltrating cell type.⁵⁰ In fact, mRNAs for neutrophil chemoattractants, including *CXCL1*, *CXCL2*, *IL8*, and *CSF2* (GM-CSF), were detected by using our microarray analyses in AD lesions. These chemokines were also detected by using our microarray in psoriatic lesions, as expected. However, the psoriatic lesions are typically devoid of encrustation, and thus the neutrophil

FIG 5. C-GS expression signature correlates with neutrophil enumeration by histology. **A**, Detection of neutrophil elastase-2, lipocalin-2, and MPO by means of immunohistochemistry and H&E in representative AD lesional skin without excoriations from different subjects. **B**, Cell counts for lesions from patients with AD and those with psoriasis (Ps). **C**, Scatter plot matrix of biopsy neutrophil indicators and C-GS PC1 scores ($P < .05$, all pairwise comparisons).

infiltrations are likely not due to tissue damage but represent a part of the immune-mediated inflammatory response specific for this skin disease.^{51,52} It is noteworthy that AD lesions are characterized by *Staphylococcus aureus* colonization, which might be facilitated by reduced levels of β -defensin 2 as a consequence of attenuated IL-17 in the T_H2 milieu.⁵³ Although the lesions we studied did not have clinical evidence of frank infection and bacteria were not detected by using H&E staining of the biopsied tissues, we cannot rule out bacterial colonization as a contributing factor to tissue neutrophilia in patients with AD. Indeed, this feature of AD might provide another mechanism by which neutrophil infiltration in these lesions could occur. Specifically, scratching might introduce colonizing bacteria into the affected skin, leading to a neutrophilic response.

Although neutrophils were identified in the majority of cases, none exhibited zones of dermal neutrophilia (ie, dense neutrophilic infiltrates) or even areas in which neutrophils predominated over lymphocytes. Thus there were no dermal neutrophil "hot spots." In all cases the infiltrates were confined to the papillary and upper reticular dermis, as is typical of patients with AD and those with psoriasis, and were entirely sampled in 5 nonoverlapping fields. The fields that were not selected were consistently areas of the reticular dermis that contained minimal or absent inflammatory cells, as expected in patients with AD and those with psoriasis.

We believe that our comparative analyses in patients with AD and those with psoriasis provide a precise transcriptomic description in the context of primary human tissue, which might provide insights into understanding the bases of disease heterogeneity. However, there are limitations to the design and execution of this study that could be subjects of further investigation. Although focusing our analyses on CCGf genes offered greater statistical advantages for detecting DE, it precludes the discovery of genes outside of our search space that might have uncharacterized relevance.

The subjects in our cohorts discontinued systemic and topical anti-inflammatory therapy for at least 2 weeks before biopsy. Although this measure potentially reduces the confounding effects of treatment, it might also accentuate the magnitude of inflammatory patterns that are ameliorated by available topical therapies. Refractory AD is often associated with concomitant herpesvirus infections, staphylococcal infections, or both²; thus the extent of neutrophilic infiltration in patients with AD should be evaluated with respect to infection in future studies. Future studies in patients with AD and those with psoriasis refractory to topical anti-inflammatory therapies might build on the observations presented here to further pinpoint potential therapeutic targets most relevant to the residual unmet medical need in these dermatoses. Intriguingly, a subset of asthmatic patients refractory to inhaled corticosteroid therapy also exhibits mixed eosinophilic and neutrophilic airway inflammation,⁵⁴ which might reflect a common pathologic mechanism to steroid-refractory AD with mixed eosinophilic and neutrophilic skin inflammation.

Finally, the cross-sectional nature of this study does not elucidate the mechanism of neutrophil infiltration in AD, and although a neutrophil response might not be a primary event, whether these cells contribute to the augmentation and perpetuation of the primary inflammatory response is worthy of further investigation.

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Key messages

- AD lesions are enriched for transcripts encoding T_H2 -associated factors, which correlate with tissue and systemic eosinophilia and IgE levels.
- Psoriatic lesions exhibit upregulated transcripts encoding T_H17 -associated factors and downregulated T_H2 -associated factors.
- Expression of neutrophil chemoattractants is enriched in both patients with AD and those with psoriasis, and the magnitude of gene expression correlates with the degree of lesional neutrophilia.

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METHODS

Study methods and design

All subjects were recruited and processed according to the protocol approved by the Institutional Review Board at the University of California–Davis School of Medicine and guidelines established by the National Institutes of Health. Hanifin and Rajka criteria^{E1} were used to identify and recruit adults with AD. Subjects had active lesions and were not receiving systemic therapy. Participants with psoriasis consisted of adults with chronic disease and active skin lesions with characteristic morphology and histologic presentation. Subjects were instructed not to apply topical medications to selected sites for 2 weeks, and biopsies were performed on those sites. Six-millimeter biopsy specimens were obtained with informed consent after achievement of local anesthesia from diseased skin and uninvolved skin, with a minimum distance of 10 cm away from lesions. Subjects with no history of skin disease were also recruited, and 6-mm biopsy specimens were obtained from healthy-appearing skin.

RNA processing and microarray hybridization

RNA was isolated from biopsy specimens preserved in RNALater (Invitrogen), according to instructions provided in the RNeasy Fibrous Tissue extraction kit (Qiagen, Valencia, Calif). RNA was extracted from three fourths and one half of lesional and nonlesional skin biopsy specimens, respectively. Agilent 2-color Whole Human Genome expression microarray analysis was conducted as described previously.^{E2}

Microarray data analysis and statistics

All statistical calculations were performed with the R Project software package, version 2.14.0 (<http://www.R-project.org>). Array quality control was assessed by using the arrayQualityMetrics package for R. CCGf^{E2} array features were selected by using Entrez gene. If individual Entrez genes mapped to multiple probes, the probe with the greatest interquartile range was retained, and the others were removed. Linear model test statistics of gene expression microarray data were performed with the limma^{E3} package of Bioconductor.

Adjustment for multiple testing was implemented by means of calculation of FDRs with the Benjamini-Hochberg method. Significantly dysregulated genes are defined as those with an FDR of less than 0.05. Among the 238 CCGfs queried, DE testing was only possible for 217 genes for both AD and psoriasis. These 217 genes are tabulated in Table E2.

A moderated paired *t* test was used to assess DE.^{E3} Genes from the AD-GS and PS-GS were determined as genes within disease DE FDRs of less than 0.05 between nonlesional and lesional samples and DE FDRs of 0.05 or greater for the paired *t* test in the other disease if the direction of dysregulation was shared (Fig 1). Differentially expressed genes (FDR < 0.05) in both comparisons, although in different directions (Fig 1, purple genes; Table E2), exhibited disease-specific patterns of expression and were therefore included in both the AD-GS and PS-GS. We defined the C-GS as commonly dysregulated genes for AD and psoriasis: genes differentially expressed (FDR < 0.05) for both *t* tests and having the same direction of dysregulation.

PCA was performed on gene *z* score–transformed values. Because the computation of PCA requires complete data matrices,^{E4} we have implemented zero substitution (equivalent to mean substitution of *z* scores) of missing values as a means of missing data mitigation.^{E5} Herein, PCA was used in 3 instances:

1. Fig 2: AD-GS. Among 21 AD-GS genes and 31 samples, 0 (0%) of 651 data points were missing. Therefore missing data mitigation was unnecessary.

2. Fig 4: C-GS. The C-GS is comprised of 75 genes, and among 31 samples, there are only 4 missing microarray values (0.17% of 2325; Fig 4, A), which required substitution for PCA.
3. Fig E2. PCA was conducted on all 238 CCGf genes for 57 samples. Among the data matrix of 13,566 data points, 329 were missing (2.4%).

Histochemistry and immunohistochemistry

Detection of inflammatory cells in paraffin-embedded skin biopsy specimens was performed as previously described.^{E6} Sections were processed for multiplex detection with diaminobenzidine, SG, and VIP (Vector Laboratories, Burlingame, Calif) development to conserve tissue blocks. Visualization of MPO was performed with rabbit antibody (0.2–0.5 µg/mL; LabVision, Thermo Scientific, Uppsala, Sweden) after antigen retrieval (citrate buffer, 95°C, 15 minutes), followed by detection with the UltraVision LP HRP Detection System and diaminobenzidine substrate. Additional confirmation of neutrophil infiltrates was performed with neutrophil lipocalin (lipocalin-2, clone 220310; R&D Systems, Minneapolis, Minn) and neutrophil elastase (elastase-2, clone NP57; Dako-Cytomation, Carpinteria, Calif) preceded by antigen retrieval with citrate at 95°C for 15 minutes and 42°C for 1 hour, respectively. Staining for lipocalin-2 was performed with 0.1 to 0.5 µg/mL 220310 antibody, and staining for elastase-2 was performed with 5 to 10 µg/mL NP57 antibody, which were both incubated at 23°C for 1 hour. Detection was performed with the UltraVision LP Detection HRP System (Labvision, Thermo Scientific) and diaminobenzidine substrate.

The immunohistochemical staining was assessed by means of light microscopy and digital image analysis. Light microscopy was performed by one coinvestigator (M.A.F., a board-certified pathologist), who recorded positive-staining dermal extravascular cell counts in 5 high-power fields (×400 magnification) containing the densest infiltrates in each case. The amount of neutrophil infiltration was initially assessed by using MPO staining, and only intensely stained cells were considered neutrophils. Subsequently, staining for lipocalin-2 and elastase was performed, and positively stained cells were further assessed by cell morphologies characteristic of neutrophils. Eosinophil counts in H&E-stained sections were performed at ×200 magnification. Data are represented as absolute cell counts.

AD biopsy specimens were assessed, and results were found to be negative for bacterial infection by using microscopic analyses of H&E sections by a certified pathologist (M.A.F.). A representative clinical appearance of an AD lesion and typical crusts of AD and psoriatic lesions depicted microscopically are shown in Fig E1.

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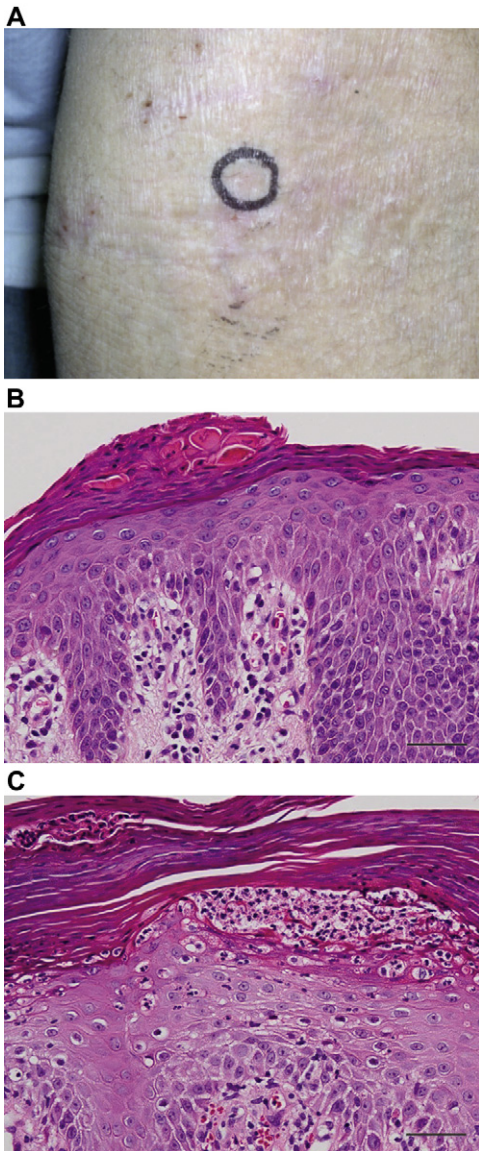


FIG E1. Representative lesion profiles. **A**, Representative AD skin lesion biopsied. **B**, Representative histology of AD. Most AD biopsy specimens in this study contained focal crust, with none more prominent than the degree depicted here. Scale bar = 50 μ m. **C**, Histology of psoriasis. Occasional cases exhibited prominent crust. Scale bar = 50 μ m.

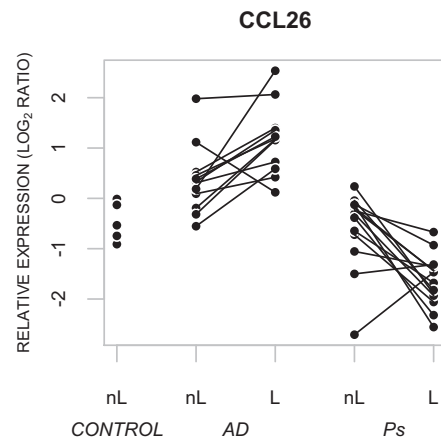


FIG E2. Chemokine (C-C motif) ligand 26 (*CCL26*) expression. *CCL26* is an opposing dysregulated (FDR < 0.05) CCGf gene in patients with lesional AD and those with psoriasis (*Ps*). Subject-matched nonlesional (*nL*) and lesional (*L*) sample expression values are linked by line segments.

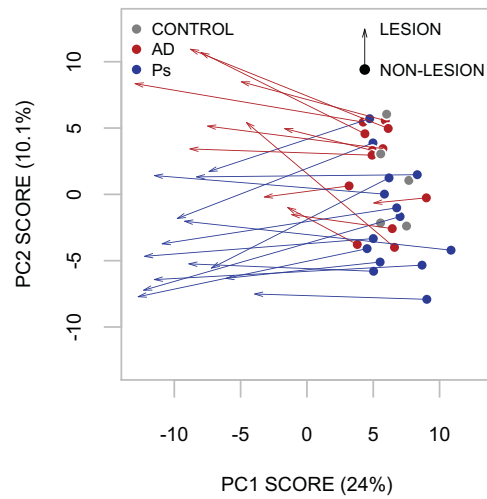


FIG E3. Lesion, diagnosis, and subject ID are important sources of CCGf expression variation. PCA demonstrates that lesion and diagnosis are informative sources of CCGf expression variation. Patient-matched non-lesional and lesional samples are connected by arrows. *Ps*, Psoriasis.

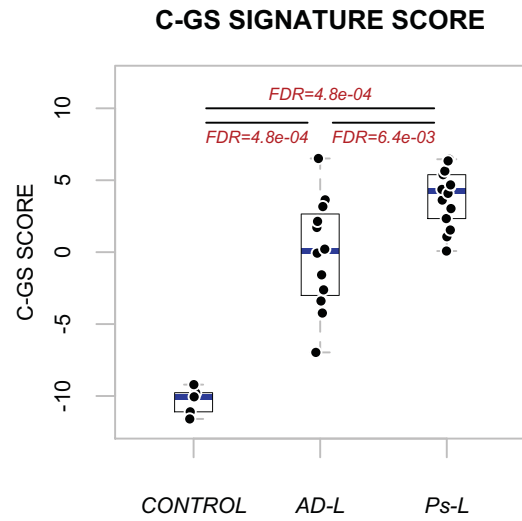


FIG E4. C-GS signature score. C-GS expression was summarized by using SPCA (see the [Methods](#) section). C-GS is represented by box plots and points. Statistics were calculated by using the pairwise Wilcoxon test, and significance is expressed as the FDR. AD-L, AD lesion; Ps-L, psoriatic lesion.