Letter to the Editor

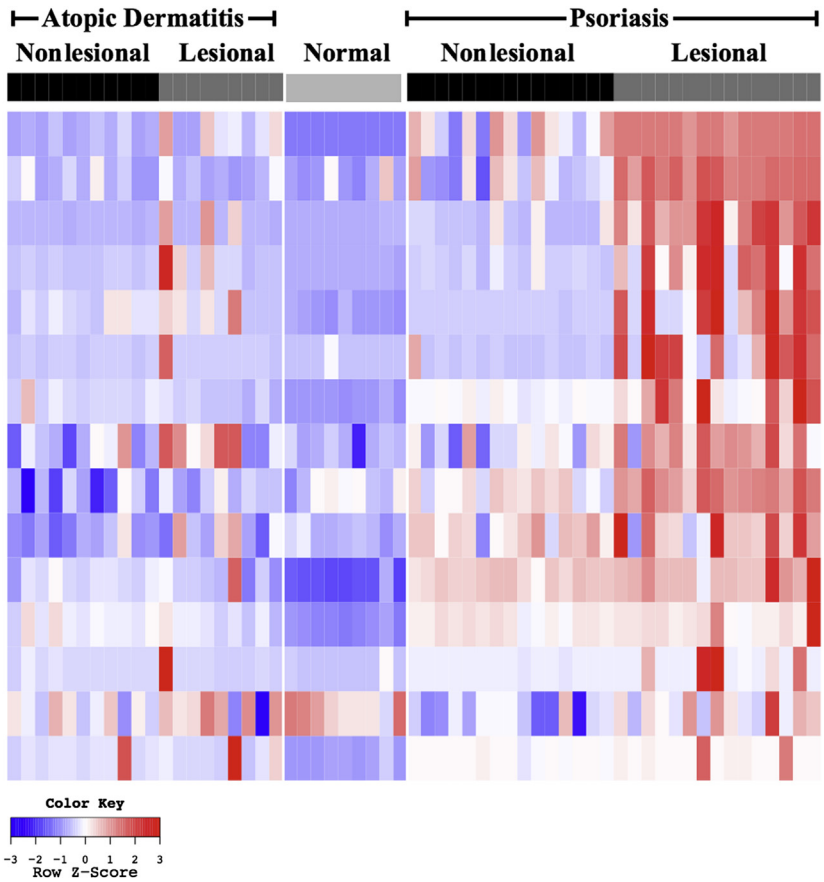
Attenuated neutrophil axis in atopic dermatitis compared to psoriasis reflects T_H17 pathway differences between these diseases

To the Editor:

Atopic dermatitis (AD) and psoriasis are the most common inflammatory skin diseases. While both are T-cell–associated and share common features such as epidermal hyperplasia and abundant infiltrates, these diseases have different immune and barrier phenotypes.[1] AD is T_H2/T_H22-polarized with an attenuated T_H17 axis and is associated with an impaired barrier, low levels of antimicrobial peptides (AMPs), and increased susceptibility to skin infections with fungi, viruses, and particularly, bacteria (ie, *Staphylococcus aureus*). In contrast, psoriasis is T_H1/T_H17-driven and does not have this increased infection rate.[1] The T_H17 cytokine IL-17 is a key inducer of AMPs and neutrophil chemoattractants.[2] This cytokine was found to be attenuated in AD compared with psoriasis and paralleled by highly decreased AMP levels, possibly explaining this increased susceptibility to pathogens. Importantly, a direct correlation between bacterial infections and disease exacerbation has been well established in AD.[3,4]

While psoriasis is established as a disease rich in neutrophils that contribute to cutaneous inflammation,[5] their role in AD immunity and inflammation is poorly understood. Recently, Choy et al[6] concluded on the basis of a transcriptome comparison of AD and psoriasis that genes encoding for neutrophil chemoattractants were comparably elevated in both diseases. Limited immunohistochemistry (hematoxylin and eosin, elastase, lipocalin-2 [LCN2]) in their study revealed neutrophilic infiltrates in lesional AD (AL) skin, though levels appear up to 2-fold lower than in lesional psoriasis (PL) skin per their quantification. However, as delineated in their patients' clinical characteristics, a majority demonstrated crusts and excoriations; superimposed infection was not thoroughly ruled out. In comparison, prior observations by our group suggested a relative paucity of neutrophils in AD versus psoriasis (unpublished data). Thus, we set to perform a direct comparison of neutrophils and neutrophil-related molecules in these diseases.

By using gene-array analysis of neutrophil-related genes, we compared nonlesional and lesional AD (ANL and AL; n = 11



	AD/N	Pso/N	AD/Pso
DEFB4A	5.36***	12.40***	−7.01***
LCN2	0.26	6.50***	−6.24***
IL8	1.76	7.24***	−5.47***
CXCL1	2.18*	4.88***	−2.70**
CXCL2	1.36*	3.09***	−1.73***
CCL20	0.49	3.63***	−3.15***
IL17A	0.24	1.15***	−0.91***
CCL2	1.73***	2.25***	−0.52
STAT3	−0.27	1.90***	−2.17***
CCR1	0.27	1.34***	−1.07***
CXCL3	0.43***	0.87***	−0.45***
CXCL5	0.15	0.32***	−0.17*
CXCL6	0.52	1.07*	−0.55
IL33	−0.49	−0.30	−0.19
IL6	0.31**	0.26**	0.05

FIG 1. Heatmap of the gene-array analysis of key neutrophil-related genes in normal, lesional, and nonlesional AD and psoriasis skin. Both AL and PL skin demonstrate elevated expression compared with normal skin. However, levels in lesional AD skin were markedly lower than in PL skin as demonstrated by significant fold-change (log_2) differences between groups (Table based on lesional expression values; *FDR < 0.05, **FDR < 0.01, ***FDR < 0.001). *Red*, Upregulated; *blue*, downregulated.

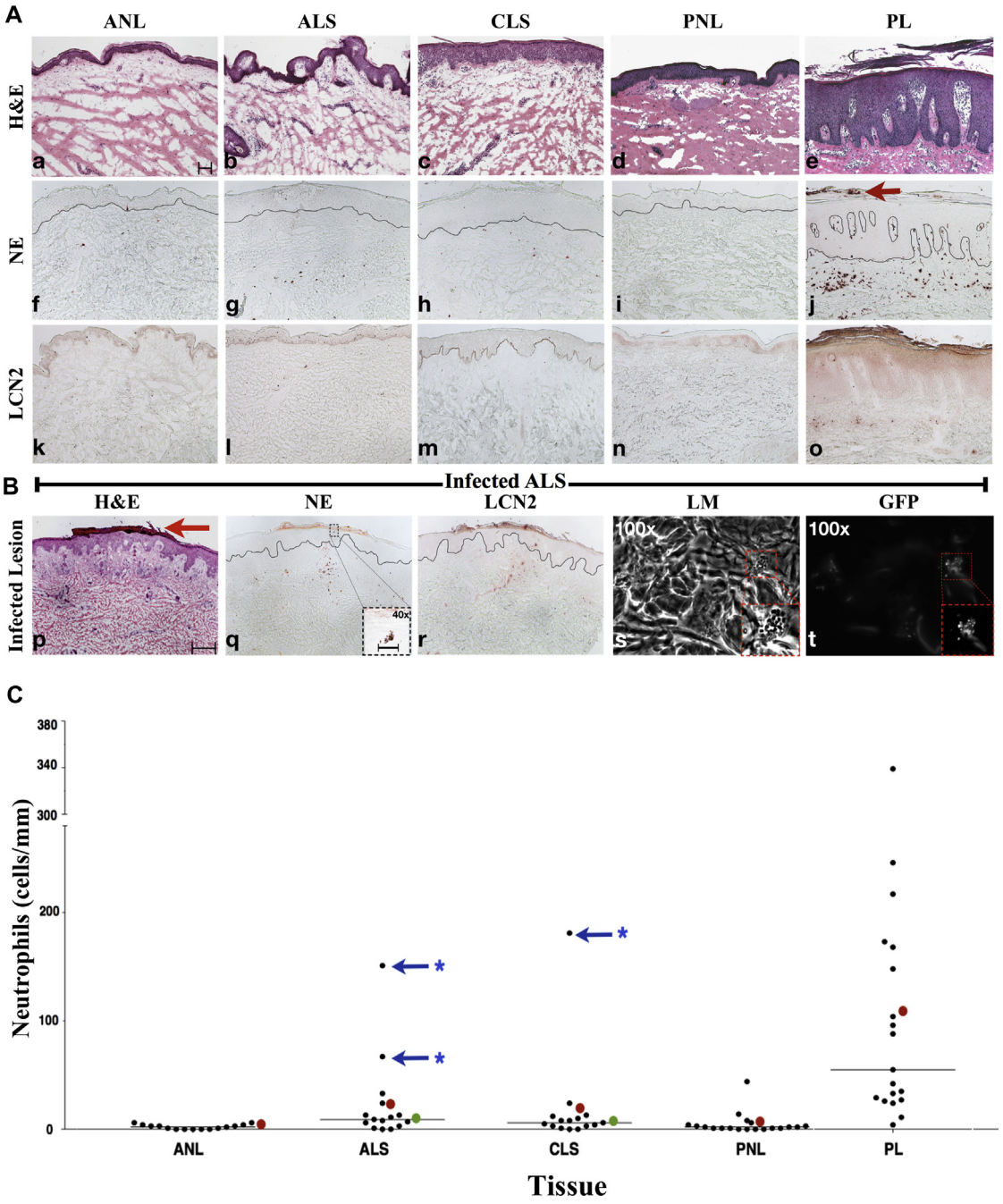


FIG 2. Immunohistochemistry of ANL, ALS, and CLS and PNL and PL skin. **A-a-e,** Representative hematoxylin and eosins *(H&Es)*. **A-f-j,** NE and **A-k-o,** LCN2 immunostainings demonstrate significantly increased neutrophils in PL compared with ALS and CLS skin (×10). **B-p,** H&E of ALS skin with elevated neutrophils illustrates a supraepidermal serum crust (*arrow*), with **B-q-r,** NE and LCN2 staining within the crust and dermis below (×4). Higher magnification (×40) shows a bacterial cluster (inset). **B-s,** A specimen with increased neutrophils reveals dermal clusters (×100, inset), confirmed as **B-t,** *Staphylococcus aureus* with a GFP-labeled bacteriophage lysin binding domain against *S aureus*. **C,** NE counts reveal significantly higher infiltrates in PL than in PNL, ALS, and CLS skin ($P < .01$) (*blue*, infected outliers; *red*, averages with outliers; *green*, averages excluding outliers; *bars*, median). *GFP*, Green-fluorescent protein; *LM*, light microscopy. *Scale bars* = 100 μm/×10, 500 μm/×4, 50 μm/×40.

and 9, respectively) and psoriasis (PNL and PL; n = 15 each, respectively) skin with normal skin (n = 9). Neutrophil infiltrates were compared by neutrophil elastase (NE) immunohistochemistry on nonlesional, acute/acute-on-chronic lesional, and chronic lesional AD (ANL, ALS, and CLS respectively; n = 15 each) and PL and PNL skin (n = 19 each).

Additional staining identified LCN2-positive neutrophils and differentiated epidermal keratinocyte expression levels of this potent AMP between the diseases (n = 9 ANL, 11 ALS, 9 CLS, 10 PNL, and 12 PL samples). All patients with AD and psoriasis exhibited moderate-to-severe disease as measured by Scoring of AD (mean, 55 ± 17) and Psoriasis Area Severity Index (≥12) (for details, see the Methods section in this article's Online Repository at www.jacionline.org).

Comparative gene-array analysis of neutrophil-signature genes revealed significantly decreased expression in AL skin compared with PL skin (but not compared with normal skin). For example, this difference was demonstrated in gene transcripts of IL-17 A (1.9-fold higher in PL, false-discovery rate [FDR] = .001) and neutrophil-inducing molecules such as defensin-β2/hBD2/DEFB4A (129-fold), LCN2 (76-fold), IL-8 (22-fold), CC family chemokine ligand 20 (8.9-fold), and CXC family cytokine ligand 1/CXCL1 (6.5-fold, FDR < 0.01) (FDR < 0.001 unless specified; Fig 1).

Using NE immunostaining, marked increases in neutrophils were observed in PL (mean, 98 cells/mm) compared with ALS (mean, 23/mm; $P = .007$), CLS (mean, 19/mm; $P = .005$), ANL (mean, 2/mm; $P = .0004$), and PNL skin (mean, 5/mm; $P = .0002$) (Fig 2, *A*-f-j-*C*; see Table E1 in this article's Online Repository at www.jacionline.org). In addition, characteristic psoriatic epidermal neutrophil clusters (Munro microabscesses) were seen only in PL tissues (Fig 2, *A*-j, red). In AD, only ALS samples demonstrated a notable increase in neutrophils compared with ANL skin ($P = .059$) (Fig 2, *C*). Staining for LCN2, a potent AMP expressed by keratinocytes[2] and neutrophils, also supported these findings, demonstrating abundant LCN2-positive neutrophils in PL compared with PNL, ALS, and CLS skin ($P = 4 \times 10^{-5}$, .002, and .0004, respectively) and greater keratinocyte expression in psoriasis (Fig 2, *A*-k-o).

Of note, 3 AD specimens demonstrated unusually elevated dermal neutrophil infiltrates (Fig 2, *C*, blue). On closer examination of these outliers, a supraepidermal serum crust was present on hematoxylin and eosin that was absent in other AD samples (Fig 2, *B*-p, red). This crust contained localized NE- and LCN2-positive neutrophil collections, with dermal infiltrates located directly below (Fig 2, *B*-q-r) and clusters suggestive of bacteria at higher magnification (Fig 2, *B*-q, inset). We hypothesized that these patients were secondarily infected, resulting in characteristic crust formation. To confirm infection, we used a green fluorescent protein–tagged binding domain of a bacteriophage lysin specific for *S aureus* on AD tissues with and without large neutrophil infiltrates (n = 3 each; see Methods in Online Repository). Dermal bacterial clusters, noted only in samples with large neutrophil infiltrates (Fig 2, *B*-s, 100×), fluoresced when exposed to this *S aureus*–specific construct (Fig 2, *B*-t). Interestingly, an uptrend in gene expression of some IL-17–modulated neutrophil chemoattractants (ie, CXCL1 and CXC family cytokine ligand 2/CXCL2) was noted in infected patients compared with other samples (not shown). Limiting mean calculations to uninfected AD samples resulted in marked decreases for ALS and CLS skin (means, from 23 and 19 cells/mm to 10 and 7/mm respectively; Fig 2, *C*, red to green), further enhancing significance compared with PL.

There are several explanations for the differences between our data and findings reported by Choy et al[6]: (1) Despite identifying crusts in 9 of 12 patients with AD and clearly depicting sample tissue similar to our infected patients' in Fig E1, *B* of their paper,[6] infection was not ruled out by staining or culture; (2) While the published findings focused on smaller regions of tissue consisting of 5 high-power fields at ×400, our histological samples were analyzed in a larger field of 1 mm at ×10, inclusive of the epidermis and dermis; (3) Alternatively, because disease severity measures were not included for psoriasis patients, inclusion of patients with mild psoriasis could skew neutrophil activity to levels comparable to AD.

Overall, we found that neutrophils and their associated molecules are significantly higher in psoriasis than in AD. The differences are particularly striking between uninfected AD lesions (with low neutrophil counts) and psoriasis lesions harboring large neutrophil numbers. In contrast, both diseases exhibit comparably significant $CD3^+$ T-cell and $CD11c^+$ dendritic-cell infiltrates.[7] Our data associate higher neutrophil levels in AD with *S aureus* infection and *Staphylococcal* coagulase–induced supraepidermal crust formation. These results suggest a much smaller contribution of the neutrophil axis to AD pathogenesis compared with psoriasis, paralleling similar differences observed in the T_H17/IL-17 pathway.[2] Interestingly, recent findings suggest that *S aureus* is capable of T_H17/IL-17 induction.[8,9] Together with our data, this potentially implicates an increasing role for the T_H17/IL-17 axis and its downstream neutrophil-associated molecules in cases of *S aureus* infection in AD, with possible therapeutic implications.

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METHODS

Patients' characteristics and skin samples

Cohorts of patients with AD (n = 30) and psoriasis (n = 33) previously published by our group[E1-E4] were used for this study. Lesional and nonlesional (≥10 cm from active lesions) skin biopsies were previously collected from these studies under protocols approved by the Institutional Review Board. Skin biopsies were taken from clinically uninfected areas. For one cohort of patients with AD, biopsies were taken of both acute and chronic lesions. Acute AD was previously defined in an earlier study by our group[E4] as less than 72-hour duration and without lichenification. No topical or systemic treatments were allowed within 2 and 4 weeks prior to biopsies, respectively. Tissue samples were frozen in Optimal Cutting Temperature (OCT) medium after collection. Because of limited tissue availability, some analyses were performed on a limited number of patients (n values specified in the text and figure legend for each analysis). Venous blood samples were collected from patients with AD for measurement of serum IgE levels (reference range, 0-200 kU/L), eosinophil counts (0%-7%), and DNA sequencing (see below). In addition, some analysis involved skin samples from 9 deidentified normal volunteers.

For this study, 2 cohorts of 15 patients with AD each were analyzed (n = 30; 17 males, 13 females; mean age, 41 years).[E1,E4] Serum IgE levels were increased in 21 of the 30 patients (range, 26-70,364 kU/L; mean, 3,930 ± 13,097 kU/L). Serum eosinophil counts were increased in 6 of the 30 patients (range, 0.30%-13.6%; mean, 4.8% ± 3.2%). Two patients out of the 30 exhibited FLG mutations (1 each heterozygous for the 2282del4 or R501x allele). The severity of AD was evaluated in all patients by using the Scoring of AD (SCORAD) index, as previously described,[E5] and the SCORAD score was used to classify AD in each patient as mild (<25), moderate (25-50), or severe (>50) (maximum SCORAD score, 103), as previously defined.[E5] The SCORAD index formula is as follows: $A/5 + 7B/2 + C$. In this formula, A is defined as the extent of disease (percentage of body surface area involvement, arrived at by application of the "rule of nines" applied to a front/back drawing of the patient), B is defined as the intensity (erythema, edema/papulation, excoriations, lichenification, oozing/crusts, and dryness), and C is defined as the subjective symptoms (daily pruritus and sleeplessness).[E5] SCORAD index scores ranged from 28 to 97.5 (mean, 55 ± 17).

Psoriatic lesional and nonlesional tissues were also collected as described above (total n = 33). A subset of this cohort (n = 19) consisted of deidentified residual samples of plaque-type psoriasis vulgaris from previous studies for whom no clinical characteristics are available; a Psoriasis Area Severity Index of more than 12 (moderate-to-severe psoriasis vulgaris with >10% body surface area involvement) was required for entry into these trials. The remainder of the cohort (15 total; 11 men, 4 women; age, 28-59 years; median, 48 years) exhibited moderate-to-severe psoriasis (with >10% body surface area involvement) and was free of therapy for more than 4 weeks prior to biopsy.[E2,E3]

Immunohistochemistry

Cryostat tissue sections of all patients were stained with hematoxylin (Fisher, Fair Lawn, NJ) and eosin (Shandon, Pittsburgh, Pa). Immunohistochemistry was performed by using purified mouse anti-human mABs against NE (Dako NP57 IgG_1 1:200; 15 ANL, ALS, and CLS AD samples each; 19 PNL and PL psoriatic samples each) and LCN2 (abcam ab23477 IgG_1 1:50; 9 ANL, 11 ALS, 9 CLS, 10 PNL, and 12 PL samples).

Biotin-labeled horse anti-mouse antibodies (Vector Laboratories, Burlingame, Calif) were used to detect the mouse mAbs. The staining signal was amplified with avidin-biotin complex (Vector Laboratories) and developed with chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich, St Louis, Mo). All immunostained sections were photographed and analyzed with ImageJ software to measure cell counts per millimeter (ImageJ 1.42; National Institutes of Health, Bethesda, Md). For NE staining, cell counts for 10 patients with AD (ANL, ALS, and CLS tissue) previously published by our group[E4] were combined with counts from 5 unpublished patients newly stained for this study. For LCN2 staining, individual quantification of LCN2 expression by epidermal keratinocytes was not possible given imaging resolution limits, which precluded the ability to identify individual positive cells within the epidermis.

Genotyping of filaggrin variants

Genomic DNA samples were isolated from whole blood from all patients included in the study and genotyped for 2 of the most common filaggrin null mutations, R501X and 2282del4, using TaqMan allelic discrimination assays as previously described,[E1-E4] with minor modifications that were described and referenced in supplementary methods for these studies. Briefly, TaqMan assays using Universal PCR Master Mix in 10-mL reaction volumes were run under standard TaqMan conditions on a ViiA7 Real-Time PCR System (Life Technologies, Grand Island, NY). For R501X, primers were used at 300 nM, probes at 200 nM, and template DNA at 0.625 ng. For 2282del4, the forward-1 and reverse primers were used at 1000 nM, the forward-2 primer at 200 nM, probes at 300 nM, and template DNA at 5 ng.

Detection of *S aureus* using green fluorescent protein-chimeric lysin binding domain for *Staphylococci*

Lysins are a class of antimicrobial bacteriolytic enzymes expressed by bacteriophages. They have 2 components: a catalytic domain preserved among many lysins and a species-specific binding domain that binds to bacterial cell wall moieties.[E6] Chimeric lysin for *Staphylococci* (ClyS) is a lysin binding domain specific for *Staphylococcus aureus* that was paired with green fluorescent protein (GFP) by using a specialized construct, pAR160-GFP. This construct was provided by the Laboratory of Bacterial Pathogenesis and Immunology at The Rockefeller University, New York, NY.

A fusion between GFP and the binding domain of the phage lysin ClyS[E7] was created by amplifying the cell wall–binding domain of ClyS by using primers 5_ClyS/BD_XbaI (5′-ccgtctagaatgaataagatcacaaataaagttaaaccacc-3′) and 3_ClyS/BD_PstI (5′-gcgctgcagttaaaacacttctttcacaatcaatctctc-3′), and inserting the resulting PCR product into the XbaI and PstI sites of pAR159,[E8] yielding pAR160_ClyS/BD-GFP.

Methodology for the detection of S aureus uses an approach similar to that described in a prior publication.[E9] Embedded samples of ALS, CLS, and ANL tissue with and without elevated neutrophil counts (n = 3 each) were fixed in acetone for 15 minutes, rinsed 3 times in PBS, blocked in 1% BSA (in PBS), incubated for a period of 1 hour with the GFP-ClyS/BD at a concentration of 1:100 in 1% BSA/PBS or with control (1% BSA/PBS alone), and rinsed again with PBS. Specificity of the construct was determined by using a slide containing only fixed *S aureus* cells. *S aureus* cells exposed to the construct, but not control cells, were fluorescent in the green channel (not shown). Slides were mounted for visualization with PBS. Images were captured by using a Nikon Eclipse E400 microscope, equipped with a Nikon 100/1.25 oil immersion lens, and a Retiga EXi fast 1394 camera (QImaging, www.qimaging.com). QCapture Pro version 5.1.1.14 software (QImaging) was used for image capture.

Sample preparation for gene chip analysis

Samples were previously prepared as described in supplementary materials and methods for an earlier study by our group.[E1] Briefly, we used human HGU133Plus2.0 GeneChip probe arrays (Affymetrix, Inc, Santa Clara, Calif) containing approximately 54,000 probe sets to assess the expression of more than 47,000 transcripts, including approximately 38,500 genes and unigenes. Total RNA was extracted from tissues frozen in liquid nitrogen by using the Qiagen RNeasy Mini Kit (Valencia, Calif). DNA was removed with on-column DNAse digestion by the Qiagen RNAse-free DNAse Set. Total RNA (50 ng) was reverse transcribed and amplified with Ovation Whole Blood Solution from NuGen (San Carlos, Calif). The labeled target was fragmented and hybridized to probe arrays, using Encore Biotin Module from NuGen (San Carlos, Calif). The chips were washed, stained with streptavidin-phycoerythrin, and scanned (HP GeneArray Scanner; Hewlett-Packard Company, Palo Alto, Calif). On each chip, the human housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase served as controls. Suite 5.0 software normalized the expression level values using these controls.

Statistical methods and heat maps

Two-tailed unpaired *t* tests and 1-way ANOVA analyses were used for statistical comparisons of NE and LCN2 cell counts. Statistical methodology for

analysis of gene array data was previously described. Briefly, for each of the comparisons of interest, the *P* values were obtained and adjusted for multiplicity by using the Benjamin Hochberg approach, which controls for false-discovery rate.[E1] Neutrophil-specific genes were extracted from this published analysis and relevant data, including fold change differences between normal (n = 9), lesional and nonlesional AD (n = 11 and 9, respectively), and lesional and nonlesional psoriasis (n = 15 each) skin were used to generate the table and heatmap of Fig 1. Heatmaps for these genes were generated by using Euclidian distance and McQuitty agglomeration strategy.

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TABLE E1. Neutrophil elastase cell counts for AD and psoriasis samples

Tissue type	Epidermis*	Dermis	Total
ALS	3	21	24
ALS	0	0	0
ALS	0	0	0
ALS†	15	136	151
ALS	0	1	1
ALS	0	6	6
ALS	0	7	7
ALS†	0	67	67
ALS	5	28	33
ALS	0	11	11
ALS	0	13	13
ALS	0	3	3
ALS	0	9	9
ALS	2	11	13
ALS	0	8	8
ANL	0	6	6
ANL	0	0	0
ANL	0	4	4
ANL	0	2	2
ANL	3	3	6
ANL	0	1	1
ANL	1	2	3
ANL	0	0	0
ANL	2	1	3
ANL	0	3	3
ANL	0	0	0
ANL	0	1	1
ANL	0	0	0
ANL	0	4	4
ANL	0	0	0
CLS	0	8	8
CLS	0	0	0
CLS	0	24	24
CLS	0	3	3
CLS	1	7	8
CLS†	1	180	181
CLS	4	2	6
CLS	0	0	0
CLS	3	2	5
CLS	0	13	13
CLS	0	10	10
CLS	0	12	12
CLS	0	4	4
CLS	0	1	1
CLS	0	3	3
PL	3	214	217
PL	177	162	339
PL	39	16	55
PL	2	31	33
PL	22	4	26
PL	9	2	11
PL	32	64	96
PL	45	43	88
PL	50	196	246
PL	9	26	35
PL	0	4	4
PL	41	63	104
PL	2	27	29
PL	4	23	27
PL	113	55	168
PL	54	94	148
PL	3	39	42
PL	1	23	24
PL	87	86	173
PNL	5	3	8
PNL	1	3	4
PNL	0	3	3
PNL	0	2	2
PNL	0	1	1
PNL	1	1	2
PNL	1	5	6
PNL	17	27	44
PNL	0	14	14
PNL	0	0	0
PNL	0	0	0
PNL	0	3	3
PNL	0	1	1
PNL	0	1	1
PNL	0	0	0
PNL	0	1	1
PNL	0	0	0
PNL	0	2	2
PNL	0	1	1

Counts are cells per millimeter.
*Epidermal counts inclusive of crust if present.
†Infected skin lesion.