

Age-specific changes in the molecular phenotype of patients with moderate-to-severe atopic dermatitis



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Background: Atopic dermatitis (AD) shows differential clinical presentation in older compared with younger patients. Nevertheless, changes in the AD molecular profile with age are unknown.

Objective: We sought to characterize age-related changes in the AD profile.

Methods: We evaluated age-specific changes in lesional and nonlesional tissues and blood from patients with moderate-to-severe AD (n = 246) and age-matched control subjects (n = 71) using immunohistochemistry, quantitative real-time PCR, and Singulex in a cross-sectional study. Patients were analyzed by age group (18-40, 41-60, and ≥61 years).

Results: Although disease severity/SCORAD scores were similar across AD age groups (mean, approximately 60 years; $P = .873$), dendritic cell infiltrates (CD1b⁺ and FcεRI⁺, $P < .05$) decreased with age. T_H2 measures (IL5, IL13, CCL13, CCL18, and CCL26) significantly decreased with age in patients with AD, despite increasing with age in control subjects. Consistent with T_H2 axis

decreases, serum IgE levels and eosinophil counts negatively correlated with age in patients with AD ($r = -0.24$ and $r = -0.23$, respectively; $P < .05$). T_H22-secreted IL22 expression levels also decreased with age uniquely in patients with AD ($P < .05$). Expression of T_H1-related (IFNG, IL12/23p40, STAT1, and CXCL9; $P < .05$ for CXCL9) and T_H17-related (IL17A and IL20; $P < .05$ for IL20) markers increased with age in both patients with AD and control subjects. Expression of terminal differentiation measures significantly increased in older patients with AD (loricrin [LOR] and filaggrin [FLG], $P < .05$), whereas expression of S100As (S100A8, $P < .01$) and hyperplasia markers (epidermal thickness, keratin 16, and Ki67; $P < .05$ for keratin 16) decreased. Serum trends in AD mimicked skin findings, with T_H2 downregulation (CCL26; $r = -0.32$, $P < .1$) and T_H1 upregulation (IFN-γ; $r = 0.48$, $P < .01$) with age.

Conclusion: The adult AD profile varies with age. Although T_H1/T_H17 skewing increases in both patients with AD and control subjects, patients with AD show unique decreases in T_H2/T_H22 polarization and normalization of epithelial abnormalities. Thus age-specific treatment approaches might be beneficial for AD. (J Allergy Clin Immunol 2019;144:144-56.)

Key words: Atopic dermatitis, biomarker, T_H1, T_H2, T_H17, T_H22, hyperplasia, loricrin, filaggrin, aging, skin

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Atopic dermatitis (AD) is the most common inflammatory skin disease, with an increasing prevalence of up to 10% in adults.¹⁻⁴ Although AD had been largely considered a pediatric disease with resolution by adolescence, this paradigm has recently changed. Newer studies demonstrate that persistence of disease into adulthood is more common than previously appreciated.⁵

Novel targeted therapeutics for AD are being developed, and dupilumab, a recently US Food and Drug Administration–approved T_H2 antagonist, shows efficacy in patients with AD.^{6,7} However, treatment response rates in patients with AD still lag behind those in patients with psoriasis.⁸ This discrepancy might be attributed to the heterogeneous nature of AD, which is driven by multiple polar immune pathways (T_H2/T_H22 centered with some T_H1/T_H17), as opposed to psoriasis, which is primarily T_H17 centered.⁸ Although T_H2 axis activation is common across all AD phenotypes, intrinsic, Asian, and pediatric AD subsets show greater increases in T_H17/T_H22 activation.⁹⁻¹¹

Abbreviations used

AD:	Atopic dermatitis
DC:	Dendritic cell
ET:	Epidermal thickness
FCH:	Fold change
FLG:	Filaggrin
FOXP3:	Forkhead box P3
IHC:	Immunohistochemistry
K16:	Keratin 16
LOR:	Loricrin
MMP12:	Matrix metalloproteinase 12
qRT-PCR:	Quantitative real-time PCR
STAT:	Signal transducer and activator of transcription 1
Treg:	Regulatory T

An emerging AD phenotype is “elderly AD” (age >60 years), which demonstrates a different clinical presentation.¹ There is a male predilection that exists only among older patients, and the skin manifestations demonstrate a “reverse sign” of lichenification surrounding clear flexural folds in contrast to the classic lichenification located within the flexures.¹ However, tissue analyses of this new category have not yet been performed. Differential immune activation with age has been observed in patients with other atopic diseases; for example, elderly asthmatic patients demonstrate significantly greater neutrophil and eosinophil values and increased levels of IL-1 β , IL-6, IL-8, eotaxin-1, GM-CSF, and T_H17-related cytokines.¹² Among healthy control subjects, aging has also been shown to alter the immune system, which is referred to as immunosenescence.^{13,14} Although some aspects of immune function in the elderly are impaired, such as vaccination responses,¹⁵ other features become hyperresponsive, leading to increased autoimmune and inflammatory disease.^{14,16}

Given the changes in normal immunity with aging, the unique clinical presentation of AD in the elderly, and the existence of age-dependent differential immune activation in patients with other atopic diseases, we sought to assess age-specific phenotypic changes in the AD disease mechanism.

METHODS

Study subjects and skin samples

For this cross-sectional study, we combined gene expression, immunohistochemistry (IHC), and serum data sets of patients with chronic moderate-to-severe AD (n = 246) and control subjects (n = 71) with skin biopsy specimens and serum samples collected under institutional review board–approved protocols.^{6,11,17–21} We separated patients and control subjects into age groups, with 130 patients with AD (85 male and 45 female patients) and 19 control subjects (12 male and 7 female control subjects) between the ages of 18 and 40 years, 89 patients with AD (42 male and 47 female patients) and 25 control subjects (18 male and 7 female control subjects) between the ages of 41 and 60 years, and 27 patients with AD (22 male and 5 female patients) and 27 control subjects (15 male and 12 female control subjects) older than age 60 years. All patients had chronic AD, which presented during early childhood (<5 years of age). No patients with AD had serious disease comorbidities, and no healthy control subjects had atopic or other comorbidities.

Recruitment occurred in New York at Mount Sinai and Rockefeller University. Demographic data of the patients are listed in Table 1. There were no significant differences among age groups in disease severity (evaluated by using SCORAD scores). There were significant differences in racial composition across the age groups.

Two hundred forty-four lesional and 194 nonlesional (≥ 10 cm from active lesions) skin biopsy specimens and 69 serum samples from patients with AD and 63 skin biopsy specimens from control subjects were evaluated. Similar affected areas underwent biopsy for lesional, nonlesional, and normal skin from all age groups. The majority of biopsy specimens were taken from the extremities, and if this was not possible, others were taken from the trunk.

Patients were stratified into extrinsic and intrinsic AD categories, with IgE levels of greater than 200 kU/L defining extrinsic AD and levels of less than 200 kU/L defining intrinsic AD. Filaggrin (FLG) mutations were evaluated in 52 of 246 patients, of which 13 mutations were found (see the Methods section in this article’s Online Repository at www.jacionline.org for more information).

IHC

IHC was performed on cryostat tissue sections by using purified mouse anti-human mAbs (see Table E1 in this article’s Online Repository at www.jacionline.org). Epidermal thickness (ET) and positive cells per millimeter of basement membrane were quantified for IHC by using ImageJ software (version 1.42; National Institutes of Health, Bethesda, Md).

Quantitative gene expression analyses

RNA was extracted, followed by quantitative gene expression analyses with quantitative real-time PCR (qRT-PCR), as previously described.^{21,22} The primers used are listed in Table E2 in this article’s Online Repository at www.jacionline.org.

Statistical analysis

Statistical analysis for gene expression was performed by using R software (www.R-project.org) and packages available through Bioconductor (www.bioconductor.org).

Human acid ribosomal protein–normalized qRT-PCR expression values of less than the limit of detection were imputed as 20% of the minimal value over the limit of detection. No other missing value imputation method was performed. Data were scaled based on data set origin by using the R function “scale.” Data were log₂-transformed before analysis with a linear mixed-effect model, with age group and tissue (normal, lesional, and nonlesional skin) as fixed factors and a random effect for each patient. Fold changes (FCHs) for comparisons of interest against age-controlled healthy subjects were estimated, and hypothesis testing was conducted with contrasts under the general framework for linear models in the R “nlme” package. IHC data and cell counts were similarly evaluated by using a linear mixed-effects model. Serum data comparisons were evaluated by using unpaired Student *t* tests. The association of skin samples and sera with age was evaluated by using Spearman correlation coefficients. Word clouds were created with the R “wordcloud” package, with word sizes proportional to FCHs. AD-associated immune pathways were quantified by using Gene Set Variation Analysis with the R “gsva” package.

RESULTS

To determine the presence of age-specific differences in healthy control subjects and adults with chronic moderate-to-severe AD, we evaluated a total of 246 patients with AD (18–40 years old, n = 130; 41–60 years old, n = 89; and ≥ 61 years old, n = 27) and 71 healthy control subjects (18–40 years old, n = 19; 41–60 years old, n = 25; and ≥ 61 years old, n = 27; Table 1). There were no significant differences in disease severity/SCORAD scores between the AD age groups.

Changes in normal skin as a function of age

To understand and provide context for AD-specific changes, we first evaluated changes in healthy control subjects as a function of

TABLE I. Baseline characteristics of patients and healthy control subjects

Age group (y): Characteristic	Patients with AD			P value	Healthy control subjects			P value
	18-40 (n = 130)	41-60 (n = 89)	≥61 (n = 27)		18-40 (n = 19)	41-60 (n = 25)	≥61 (n = 27)	
Age (y), mean (SD)	23.3 (0.4)	48.9 (0.4)	69.3 (0.8)	<.001	29.0 (1.4)	50.8 (1.0)	68.0 (2.7)	<.001
Sex (%)				.1564				.4693
Male	85 (65.4)	42 (47.2)	22 (81.5)		12 (63.2)	18 (72.0)	15 (55.6)	
Female	45 (34.6)	47 (52.8)	5 (18.5)		7 (36.8)	7 (28.0)	12 (44.4)	
Race (%)				<.001				<.001
African American	19 (14.6)	19 (21.3)	2 (7.4)		5 (26.3)	11 (44.0)	6 (22.2)	
Asian	48 (36.9)	8 (9.0)	3 (11.1)		12 (63.2)	3 (12.0)	0	
European American	63 (48.5)	61 (68.5)	21 (77.8)		2 (10.5)	11 (44.0)	21 (77.8)	
NA	0	1 (1.1)	1 (3.7)		0	0	0	
SCORAD score, mean (SD)	60.0 (17.1)	60.5 (15.4)	60.2 (16.9)	.873	—	—	—	—
IgE (kU/L), mean (SD)	6767.6 (9724.3)	5103.7 (7586.5)	5269.3 (15679.0)	.308	—	—	—	—

NA, Not available.

age. Measures of epidermal hyperplasia generally decreased with age (ET and Ki67, $P < .05$; see Fig E1, A and B, in this article's Online Repository at www.jacionline.org), with the exception of keratin 16 (K16), which showed an opposite trend ($P < .05$; see Fig E1, G). These findings were corroborated by significant negative correlations of ET and Ki67 values with age ($r = -0.52$, $P < .001$ and $r = -0.52$, $P < .01$, respectively; see Table E3 in this article's Online Repository at www.jacionline.org); however, there was no significant correlation of K16 values with age (see Table E3). Markers of dendritic cells (DCs; CD1a⁺, FcεRI⁺) increased in the older population ($P < .01$ for both; see Fig E1, C and E), with corresponding significant positive correlations with age ($r = 0.67$, $P < .001$ and $r = 0.47$, $P < .01$, respectively; see Table E3). CD1b⁺ cells showed no clear trend with age (see Fig E1, D).

mRNA expression of immune and barrier measures in control subjects was also found to vary with age. Expression of matrix metalloproteinase 12 (*MMP12*), a marker of general inflammation, increased as a function of age ($P < .01$; see Fig E1, F). Overall, normal skin appeared more inflamed in older control subjects across multiple T_H axes. Expression of T_H2 markers (*IL5*, *IL13*, *CCL13*, *CCL18*, and thymic stromal lymphopoietin receptor [*TSLPR*]) all increased stepwise as a function of age ($P < .05$ for all; see Fig E1, H-L), with positive correlations with age ($r = 0.41$, $P < .01$ for *IL13*; see Table E3). Expression of T_H22-secreted *IL22* and associated *S100A8* increased significantly in older control subjects ($P < .05$; see Fig E1, M and N) and positively correlated with age ($r = 0.29$, $P < .05$ for *IL22*; see Table E3). Expression of *IFNG* and other associated T_H1 measures (*IL12/23p40*, signal transducer and activator of transcription 1 [*STAT1*], and *CXCL9*; $P < .05$ for *CXCL9*) was upregulated with age (see Fig E1, O-R). Expression of *IL17A* also uptrended ($P < .1$; see Fig E1, S) and strongly correlated with age ($r = 0.63$, $P < .001$; see Table E3), and expression of T_H17-associated *IL20* significantly increased ($P < .05$; see Fig E1, T).

Although inflammation markers of T_H2, T_H22, T_H1, and T_H17 axes increased in older control subjects, expression of terminal differentiation markers (loricrin [*LOR*] and *FLG*) decreased ($P < .05$ for *FLG*; see Fig E1, U and V). Additionally, expression of the negative regulator *IL37* decreased in older control subjects ($P < .05$; see Fig E1, W) and negatively correlated with age ($r = -0.59$, $P < .001$; see Table E3).

Changes in AD profile with age

We next evaluated for AD-specific changes with age through normalization to age-matched control subjects, as well as direct comparisons of values between different AD age groups without normalization (see Fig E2 and Table E4 in this article's Online Repository at www.jacionline.org).

Changes in epidermal hyperplasia and cellular infiltrates. We observed age-specific changes in measures of epidermal hyperplasia in AD skin (ET, K16, and Ki67; Fig 1, A-F). We found significant decreases in levels of ET and K16 in lesional and nonlesional skin ($P < .05$; Fig 1, A-D), with a similar trend in nonlesional skin for Ki67 (Fig 1, E and F). Additionally, K16 level demonstrated significant negative correlation with age in nonlesional skin ($r = -0.35$, $P < .001$; Fig 1, K, and see Table E5 in this article's Online Repository at www.jacionline.org). Overall, epidermal hyperplasia attenuated with age, particularly in nonlesional skin. These findings were consistent in the direct age comparison as well, which demonstrated a decrease in levels of hyperplasia markers with age in both patients with AD and control subjects (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org).

We next evaluated for inflammatory infiltrates. In all age groups, there were high levels of cellular infiltrates, including T cells (CD3⁺ and CD8⁺) and DCs (CD1b⁺, FcεRI⁺, and CD206⁺) in lesional skin compared with respective nonlesional and normal skin (Fig 1, G-J, and see Fig E3, A-F, in this article's Online Repository at www.jacionline.org). CD3⁺ infiltrates showed inconsistent changes in skin of patients with AD with age, whereas levels of lesional CD8⁺ infiltrates significantly increased, which is consistent with reports of increased accumulation of memory CD8⁺ T cells with normal aging, particularly in the setting of chronic inflammation ($P < .05$; see Fig E3, A-D).^{13,23-25} Numbers of CD1b⁺ and FcεRI⁺ cells, both marking inflammatory dendritic epidermal cells, were reduced in lesional and nonlesional skin with age ($P < .05$ for both; Fig 1, G-J), which is in contrast to the increase observed in older control subjects. This change was also evidenced by using significant negative correlations with age for CD1b⁺ cells in both lesional and nonlesional skin ($r = -0.51$ and $r = -0.54$, $P < .001$ for both; Fig 1, L and N) and FcεRI⁺ cells in nonlesional skin ($r = -0.33$, $P < .001$; Fig 1, M). Numbers of CD1a⁺ cells, another marker of DCs, also demonstrated a significant decrease with increasing age groups ($P < .05$; see Fig E3, H). Numbers of CD206⁺ (the

mannose receptor) cells, marking both DCs and macrophages, showed a nonsignificant increase with age in both lesional and nonlesional skin (see Fig E3, F). No significant trends were seen with age for CD1c⁺ and CD11c⁺ cells (see Fig E3, I-L). Representative IHC panels are shown in Figs 1 and E3. We additionally conducted a subanalysis of cellular counts in the epidermis and dermis and found similar trends (see Table E6 in this article's Online Repository at www.jacionline.org).

Gene expression changes determined by using qRT-PCR. To evaluate the role of aging in key AD immune and epidermal barrier markers, we performed qRT-PCR on a robust panel of 34 mRNA measures. Overall, significant differences were found in lesional and nonlesional AD skin with age, both when normalizing first to respective control subjects and when performing direct comparisons between age groups (Fig 2 and see Figs E2 and E4 and Table E4 in this article's Online Repository at www.jacionline.org). Expression of the general inflammation marker *MMP12* significantly decreased with age in lesional and nonlesional skin ($P < .05$; Fig 2, A) and was negatively correlated with age in lesional and nonlesional skin ($r = -0.26$ and $r = -0.35$, $P < .001$ for both; see Table E5). Expression of *IL1B*, a marker of innate immunity, showed inconsistent changes across age groups (Fig 2, B).

The T_H2 axis was strongly activated in all AD age groups compared with control subjects, particularly in lesional skin (Fig 2 and see Table E4). When comparing between age groups, significant decreases were observed in expression of multiple T_H2 cytokines (*IL5*, *IL13*, *IL31*, *CCL13*, *CCL17*, *CCL18*, *CCL22*, *CCL26*, and *TSLPR*; $P < .05$ for all) in both lesional and nonlesional skin (Fig 2, C-J, and see Fig E4, A). Additionally, we observed significant negative correlations with age in expression of T_H2 cytokines (*IL5*, *IL13*, *CCL18*, and *CCL26*) in lesional and/or nonlesional skin ($P < .001$ for all; Fig 3, A-C and J-M). T_H2-related *IL22* expression decreased with age significantly for lesional and nonlesional skin ($P < .01$; Fig 2, K). This reduction was also evidenced by significant negative correlations of *IL22* expression with age in lesional and nonlesional skin ($r = -0.37$ and $r = -0.33$, $P < .001$ for both; Fig 3, D and N). We also observed significant reductions in expression of IL-22-induced S100As in lesional and/or nonlesional skin (Fig 2, L, and see Fig E4, B-D; $P < .05$ for all), with congruent negative correlations with age ($r = -0.44$, $P < .001$ for nonlesional *S100A8*; Fig 3, E and O, and see Fig E5, C, I, and J, in this article's Online Repository at www.jacionline.org).

IFNG and other associated T_H1 measures (*IL-12/23p40* and *CXCL9*) did not demonstrate a strong cumulative trend but rather remained consistent or slightly downtrended across age groups (Fig 2, M-O). Expression of *IL17A* and IL-17-related *CCL20* and *IL20* downtrended with increasing age (Fig 2, P and R, and see Fig E4, H); however, expression of other T_H17-related markers (*IL19*, *IL23p19*, and peptidase inhibitor 3 [*PI3*]) either remained constant or increased in older age groups (Fig 2, Q, S, and T). In the direct age group comparison, T_H1 measures (*IFNG*, *IL12/23p40*, *STAT1*, and *CXCL9*) increased with age in nonlesional and/or lesional AD skin at the same rate as in control subjects ($P < .05$ for *CXCL9*; see Fig E2, N-Q). Similarly, expression of *IL17A* and *IL20* uptrended across control, nonlesional, and lesional skin (see Fig E2, R and S). Thus the absence of age-specific trends in T_H1 and T_H17 axes in patients with AD after normalization is likely attributable to simultaneous increases in both control subjects and patients with AD with age. T_H9/*IL9*

mRNA expression decreased with age, which was significant in nonlesional skin ($P < .01$; Fig E4, J).

Expression of *IL37*, a negative regulator of inflammation, significantly increased with age in nonlesional skin ($P < .05$; Fig 2, U). Expression of forkhead box P3 (*FOXP3*), a marker of regulatory T (Treg) cells, did not change in lesional skin but decreased across age groups in nonlesional skin (see Fig E4, I). Because levels of the T_H2/*IL13* and T_H22/*IL22* driver cytokines decreased with age, we also evaluated age-associated changes in the ratio of *FOXP3/IL13* and *FOXP3/IL22* and found increasing *FOXP3* expression over driver cytokines in lesional skin with age ($P < .05$ for both; see Fig E4, K and L).

Expression of the terminal differentiation markers *FLG* and *LOR* significantly increased with age ($P < .05$; Fig 2, V and W), with accompanying positive correlations with age in lesional and/or nonlesional skin ($r = 0.38$, $P < .001$ for nonlesional *FLG*; Fig 3, S and T, and see Table E5). These changes opposed the decreases in expression of terminal differentiation markers observed in control subjects. A summary heat map of all mRNA changes with age is shown in Fig 4. Overall, levels of T_H2- and T_H22-related markers uniquely decreased in patients with AD, whereas T_H1- and T_H17-related markers increased simultaneously in control subjects and patients with AD with age. These changes were accompanied by AD-specific increases in terminal differentiation products.

We also evaluated which markers correlated closest with disease severity/SCORAD scores in each age group (Table II and see Table E7 in this article's Online Repository at www.jacionline.org). In the youngest age group (18-40 years), T_H2-associated (*TSLPR*; $r = 0.58$, $P < .01$), T_H17-associated (*IL20* and *IL19*; $r = 0.49$ and $r = 0.45$, $P < .05$ for both), and T_H1-associated (*CXCL9*; $r = 0.32$, $P < .05$) markers in nonlesional skin were all significantly positively correlated with SCORAD scores (Table II). The stronger SCORAD score correlations in nonlesional skin compared with lesional skin were consistent with previous findings.²⁶ Additionally, the negative regulator *IL37* ($r = -0.38$, $P < .1$) and terminal differentiation markers ($r = -0.31$, $P < .01$ for nonlesional *LOR*) were negatively correlated with SCORAD scores (Table II and see Table E7). In the 41- to 60-year-old age group, T_H17 markers (*IL20*, *IL19*, and *IL8*; $r = 0.74$, $r = 0.66$, and $r = 0.62$, respectively; $P < .05$ for all) demonstrated larger and significant positive correlations with SCORAD scores. T_H2-associated (*CCL22*; $r = 0.37$, $P < .01$) and T_H1-associated (*CXCL9*; $r = 0.36$, $P < .05$) measures were also significantly correlated with SCORAD scores (Table II). Terminal differentiation markers were again negatively correlated with SCORAD scores ($r = -0.27$, $P < .05$ for lesional *LOR*). In the oldest age group (≥ 61 years) markers overall had weaker correlations with SCORAD scores. The largest positive correlations with SCORAD scores were seen in T_H2 markers (*CCL26*, *IL5*, and *IL31*; $r = 0.52$, $P < .05$ for lesional *CCL26*; Table II and see Table E7). Interestingly, T_H1 markers were negatively correlated with SCORAD scores in this age group (*STAT1* and *CXCL9*; $r = -0.59$, $P < .1$ for lesional *STAT1*; Table II and see Table E7).

Serum correlations with age. Age-specific differences in serum levels of AD inflammatory markers followed similar trends to mRNA expression changes (Fig 5 and see Table E4). IgE levels negatively correlated with age ($r = -0.24$, $P < .01$), leading to a decreasing proportion of patients with extrinsic AD in older age groups (Fig 5, A and B). Eosinophil counts similarly negatively correlated with age ($r = -0.23$, $P < .05$; Fig 5, C). Serum

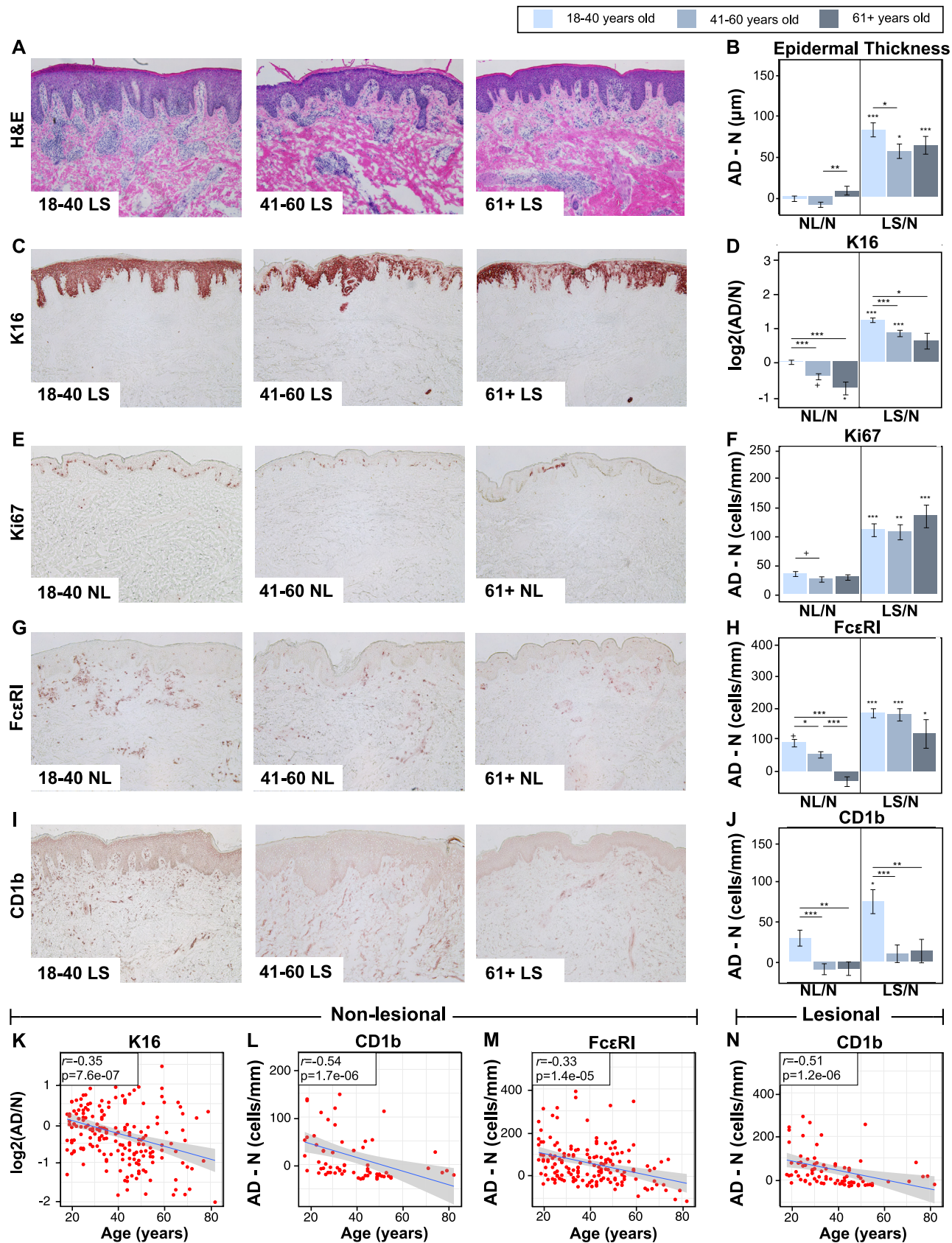


FIG 1. A-C and E-J, Representative IHC images and quantification of cellular markers. Measurements are conducted by counting cells per millimeter of basement membrane. Data are normalized to age-matched control subjects (cells from patients with AD/millimeter subtracted by cells from control subjects/

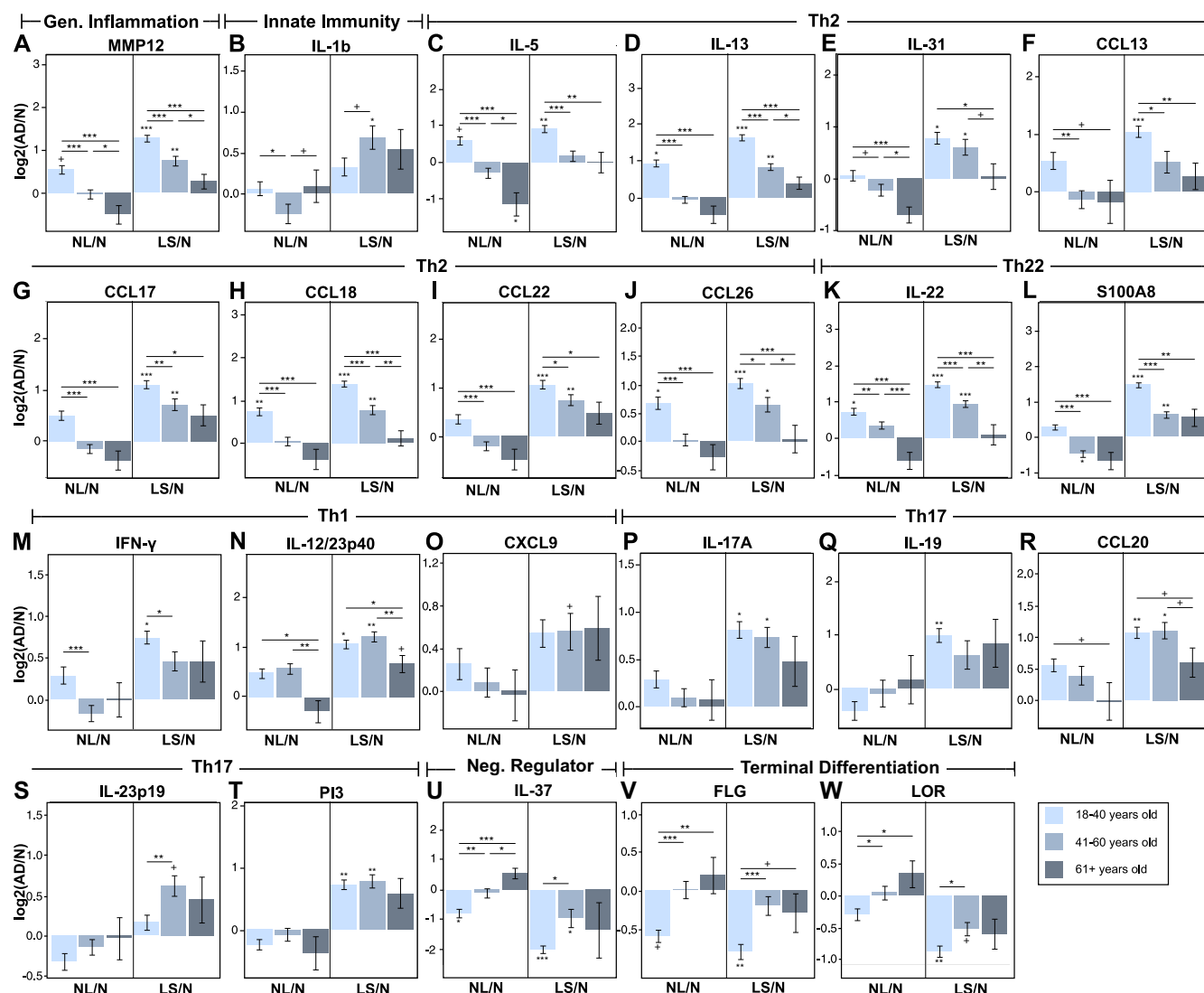


FIG 2. A-W, Bar plots denote $\log_2$ FCH (patients with AD vs control subjects) of mRNA expression. Presented data are normalized to age-matched control subjects. Light blue indicates age of 18 to 40 years, medium blue indicates age of 41 to 60 years, and dark blue indicates age 61 years or greater. Asterisks on top of each bar indicate significance of comparison between patients with AD and healthy control subjects. Asterisks that traverse bars indicate significance of comparison between age groups. LS, Lesional; N, normal; NL, nonlesional. + $P < .1$, * $P < .05$, ** $P < .01$, and *** $P < .001$.

IFN- γ levels increased somewhat ($P < .05$; Fig 5, D) and positively correlated with age ($r = 0.48$, $P < .01$; Fig 5, G). Levels of CCL2, another T_H1 marker, also increased with age ($P < .05$; Fig 5, E). Levels of the eosinophil-attracting chemokine CCL26 decreased across age groups ($P < .1$; Fig 5, F) and downtrended with age ($r = -0.32$, $P < .1$; Fig 5, H).

Because phenotypic differences have been previously observed between intrinsic and extrinsic AD⁹ and we found a greater proportion of intrinsic AD among our older patients, we also performed a separate analysis of age-related changes exclusively in patients with extrinsic AD (see Table E8 in this article's Online Repository at www.jacionline.org). As shown in Fig E6 in this

millimeter). D, Bar plot denotes $\log_2$ FCH (patients with AD vs control subjects) of mRNA expression of K16. Presented data are normalized to age-matched control subjects. Light blue indicates age of 18 to 40 years, medium blue indicates age of 41 to 60 years, and dark blue indicates age of 61 years or greater. Asterisks on top of each bar indicate significance of comparison between patients with AD and healthy control subjects. Asterisks that traverse bars indicate significance of comparison between age groups. LS, Lesional; N, normal; NL, nonlesional. + $P < .1$, * $P < .05$, ** $P < .01$, and *** $P < .001$. K, Spearman correlation of $\log_2$ FCH (patients with AD vs control subjects) K16 mRNA expression with age. L-N, Spearman correlations of cells from patients with AD/millimeter subtracted by cells from control subjects/millimeter with age for various cellular markers.

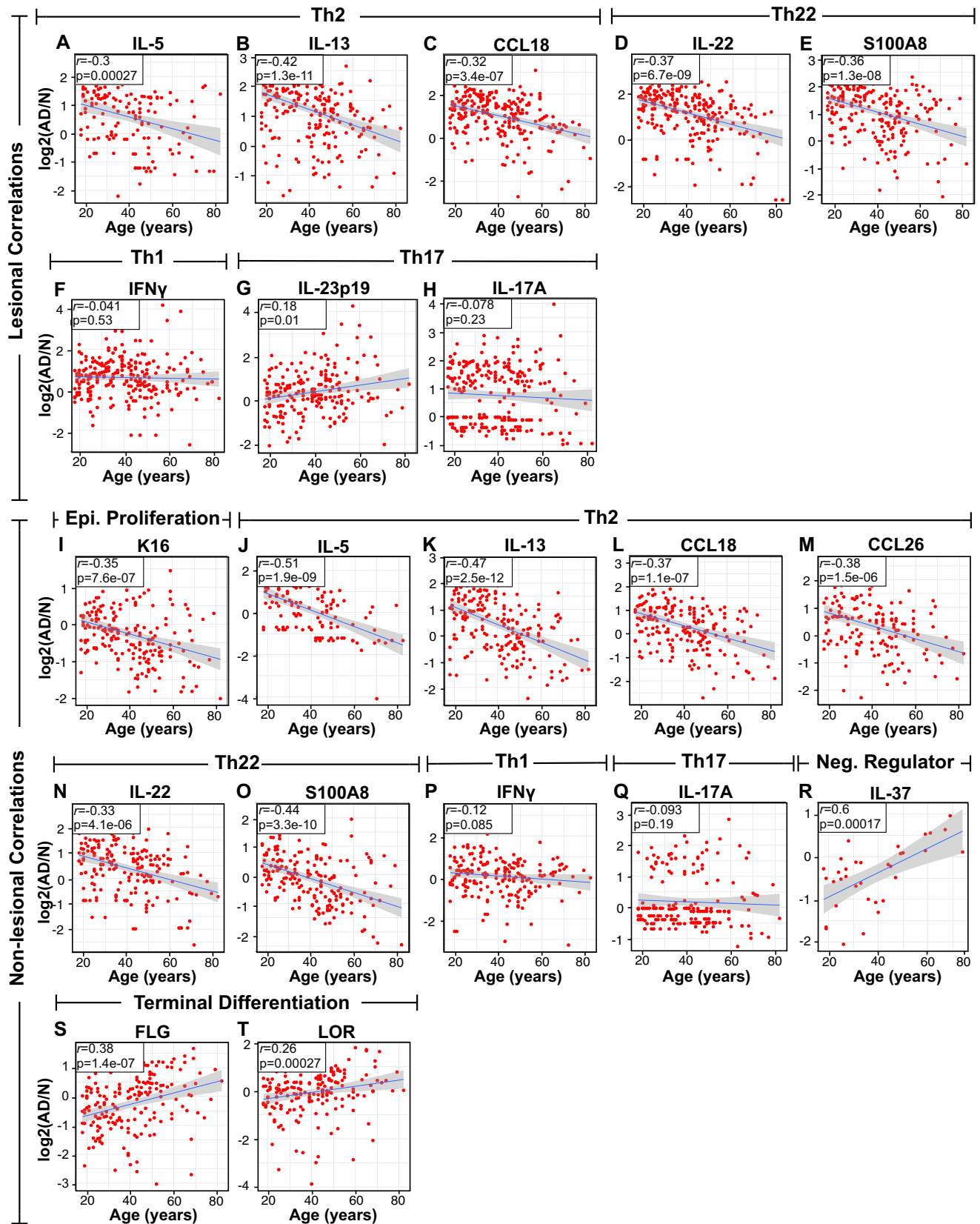


FIG 3. A-T, Spearman correlations of $\log_2$ FCH (patients with AD vs control subjects) of mRNA expression with age. Presented data are normalized to age-matched control subjects. *N*, Normal.

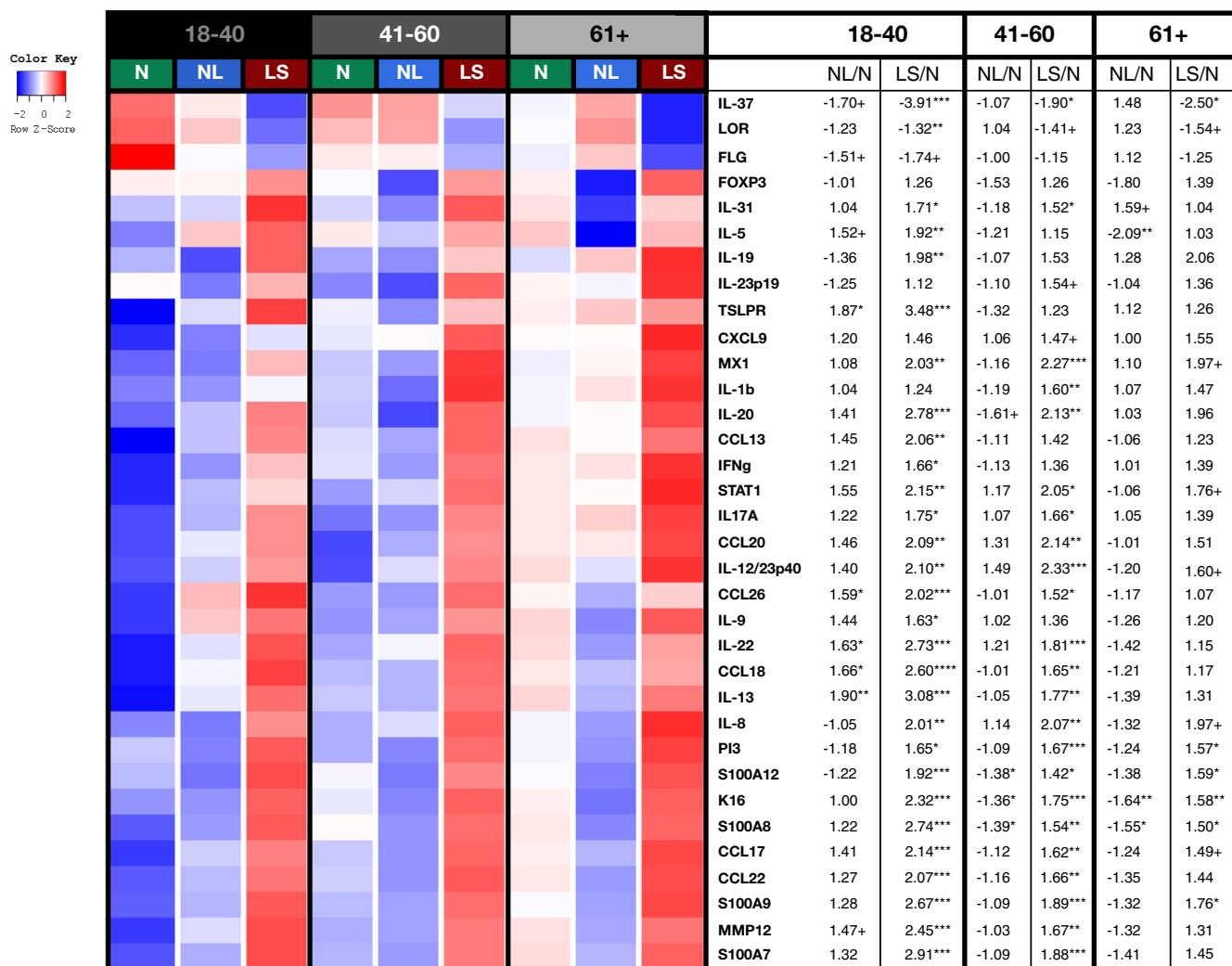


FIG 4. Summary heat map of qRT-PCR data. Red indicates gene upregulation, and blue indicates gene downregulation. Values represent FCH (patients with AD vs control subjects) of mRNA expression. Colors in the heat map correspond to expression values. LS, Lesional; N, normal; NL, nonlesional. + $P < .1$, * $P < .05$, ** $P < .01$, and *** $P < .001$.

article's Online Repository at www.jacionline.org, our findings in the extrinsic population are consistent with those of the entire cohort. These data suggest that the observed changes with age are independent of intrinsic/extrinsic status.

Word cloud analyses illustrate age-specific changes in T-cell polarization. To better visualize age-specific changes in AD across individual markers and pathways, we created word clouds based on mRNA data (Fig 6, A). The font size is scaled proportionally to FCH values (patients with AD vs age-matched control subjects), with reference numbers in black to allow quantification. The word colors are assigned based on T_H cell association (red, T_H2; blue, T_H22; green, T_H1; and purple, T_H17). Similar trends were noted across lesional and nonlesional skin, although greater immune activation was present in lesional compared with nonlesional skin across all age groups, as visualized by larger fonts. Overall, T_H2 and T_H22 markers (red and blue, respectively) dominate the 18- to 40-year-old age group, whereas T_H17 and T_H1 (purple and green, respectively) become increasingly predominant in the 61-year and older group. Gene

Set Variation Analysis plots of age-controlled mRNA data of key markers characterizing T_H pathways also serve to summarize the findings. T_H2 and T_H22 axes decreased in both lesional and nonlesional skin with increasing age groups ($P < .05$; Fig 6, B and C). There was a significant decrease in T_H1 activity isolated in nonlesional skin with increased age ($P < .05$; Fig 6, D); otherwise, there did not appear to be any age-specific changes in the T_H1 axis. The T_H17 axis also did not show any significant changes across age groups (Fig 6, E).

Although the majority of our participants were European Americans, we had some patients and control subjects of other ethnicities. To evaluate for race as a confounder, a sensitivity subanalysis was conducted by using only the European American cohort (our largest racial cohort), which demonstrated consistent findings with the presented analysis of the entire cohort (data not shown). Additionally, a sensitivity subanalysis on male and female subjects was conducted to evaluate for sex as a confounder, which also produced similar findings to the main analysis (data not shown).

TABLE II. Correlations of PCR markers with SCORAD scores

18-40 y			41-60 y			≥61 y		
Marker	r Value	P value	Marker	r Value	P value	Marker	r Value	P value
TSLPR NL	0.576	.006	IL20 NL	0.743	.009	CCL26 LS	0.521	.041
IL20 NL	0.492	.013	IL19 NL	0.662	.026			
IL19 NL	0.446	.025	IL8 LS	0.624	.040			
TSLPR LS	0.413	.036	FOXP3 LS	0.503	.005			
CXCL9 NL	0.318	.017	K16 LS	0.367	.001			
CCL13 NL	0.290	.045	CCL22 LS	0.365	.002			
IL1B LS	0.288	.007	CXCL9 NL	0.357	.041			
PI3 LS	0.253	.008	IL17A LS	0.338	.002			
S100A9 LS	0.245	.033	MMP12 LS	0.319	.008			
K16 LS	0.234	.011	IL13 LS	0.318	.004			
IL17A LS	0.228	.013	PI3 LS	0.309	.007			
S100A8 LS	0.206	.028	CCL18 NL	0.290	.013			
IL13 NL	0.203	.042	CCL18 LS	0.285	.009			
S100A12 LS	0.197	.039	K16 NL	0.281	.016			
FLG LS	−0.196	.040	IL9 LS	0.267	.045			
LOR LS	−0.272	.004	CCL17 LS	0.243	.028			
LOR NL	−0.308	.002	CCL17 NL	0.240	.041			
			LOR LS	−0.270	.015			

LS, Lesional; NL, nonlesional; PI3, peptidase inhibitor 3; TSLPR, thymic stromal lymphopoietin receptor.

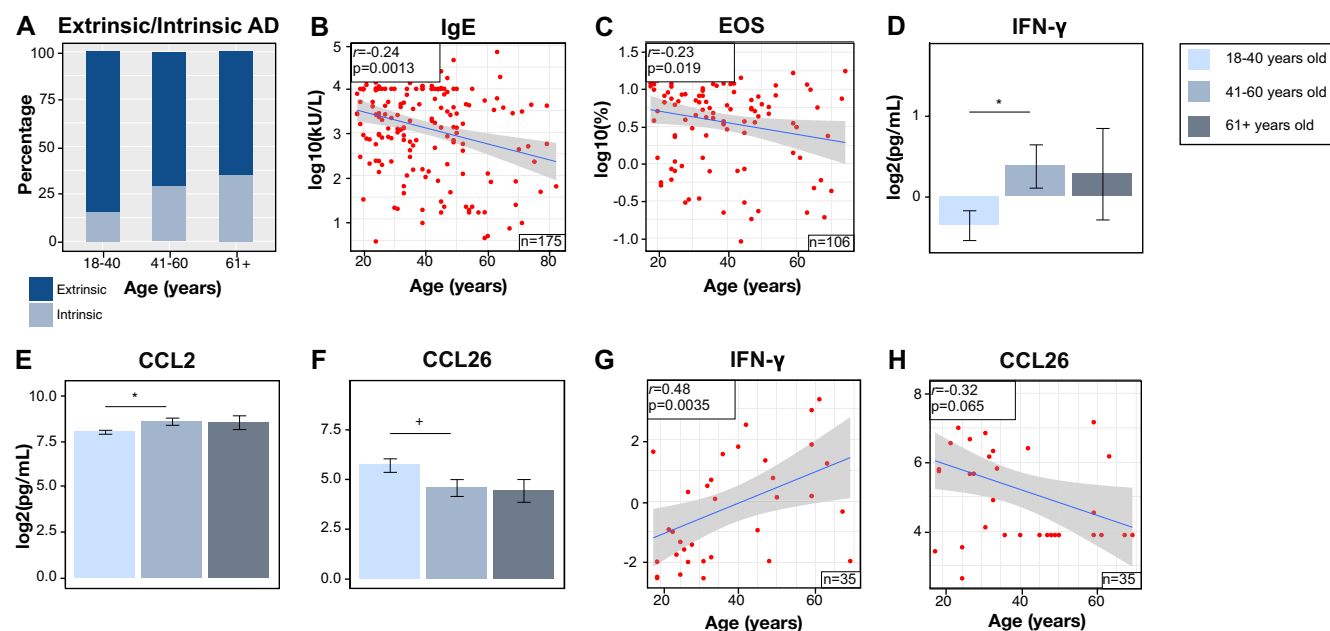


FIG 5. A, Bar plot demonstrating percentage of extrinsic and intrinsic AD present in each age group. Dark blue indicates extrinsic AD, and light blue indicates intrinsic AD. B, Spearman correlation of $\log_{10}(\text{IgE})$ with age. C, Spearman correlation of $\log_{10}(\text{eosinophil [EOS] \%})$ with age. D-F, Bar plots denote values of AD serum measures. Light blue indicates age of 18 to 40 years, medium blue indicates age of 41 to 60 years, and dark blue indicates age of 61 years or greater. Asterisks that traverse bars indicate significance of comparison between age groups. + $P < .1$, * $P < .05$, ** $P < .01$, and *** $P < .001$. G and H, Spearman correlations of AD serum measures with age.

DISCUSSION

This is the first study of molecular and cellular changes as a function of age in patients with AD. To understand our findings in the context of normal aging, we evaluated age-related changes in the skin of healthy control subjects, which have yet to be studied. Then, to capture the systemic nature of AD,²⁷ we evaluated lesional and nonlesional skin, as well as serum, of patients with moderate-to-severe AD. Our analyses included comparisons

between different age groups of patients with AD (18-40, 41-60, and ≥61 years), as well as normalization to appropriate control subjects. This allowed for evaluation of changes in AD fingerprinting with age while simultaneously accounting for potential changes attributable to the normal aging process.

Because the normal aging process is known to alter immunity (termed “immunosenescence”) and the skin barrier, we evaluated changes in normal skin with age to provide context for the

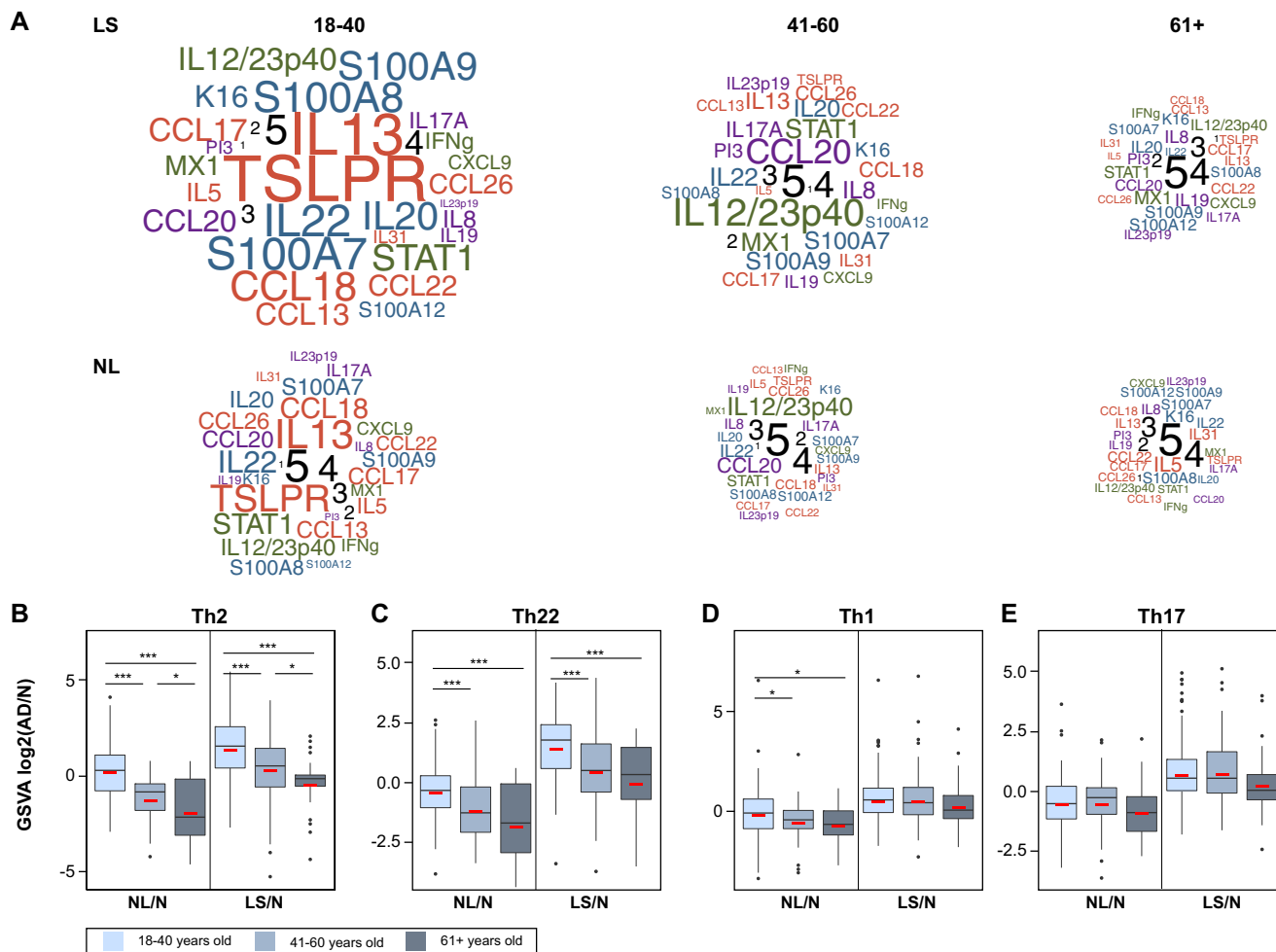


FIG 6. A, Word clouds summarize changes in qRT-PCR markers across age groups. Font size is scaled proportional to FCH values (patients with AD vs control subjects). Presented data are normalized to age-matched control subjects. Word colors are assigned based on T_H cell/pathway association (red, T_H2; blue, T_H22; green, T_H1; and purple, T_H17). Reference numbers are in black to allow quantification. LS, Lesional; N, normal; NL, nonlesional. B-E, Gene Set Variation Analysis of mRNA expression along major immune axis pathways. Presented data are normalized to age-matched control subjects. Asterisks that traverse bars indicate significance of comparison between age groups. The red bar indicates the mean. LS, Lesional; N, normal; NL, nonlesional. +P < .1, *P < .05, **P < .01, and ***P < .001.

AD-specific trends with age. In the skin of healthy control subjects, we observed significant changes in immunity with increasing age. These skin findings were consistent with prior studies on healthy subjects, which only evaluated the blood compartment. Overall, we found increased markers of general inflammation and T_H2, T_H22, T_H1, and T_H17 immune axes in older control subjects. Additionally, we found increased markers of DCs as a function of age.

Studies have previously shown that normal aging involves upregulation of innate and proinflammatory cytokines (IL-1, IL-6, IL-8, TNF- α , and C-reactive protein) in the absence of underlying infection or a systemic inflammatory disorder, a process termed “inflamm-aging.”^{23,28-33} Although this increase in levels of proinflammatory cytokines is associated with increased activation of DCs, these DCs have defective antigen-presenting and phagocytic capacity.³⁴⁻³⁶ Our findings of increased general inflammation and DCs in older control subjects parallel these previous studies.³⁷ Consistent with our findings of increased T_H1 and T_H2 activity in the skin of older control subjects, both

numbers of T_H1 and T_H2 T cells and levels of associated cytokines (IFN- γ /T_H1; IL-4 and IL-13/T_H2) have been found to increase in peripheral blood with normal aging^{14,38-41}; however, there is a T_H1-to-T_H2 shift, particularly within CD8⁺ T cells.^{33,39,42} This phenomenon might be a compensatory mechanism to counteract the increased proinflammatory cytokines associated with inflamm-aging.

Interestingly, despite the more T_H2-dominant cytokine profile in normal aging, a reduced serum IgE level is observed. A longitudinal study evaluated healthy adults over a 20-year period and found a decrease with aging in serum IgE levels and IgE sensitization to various allergens (ie, house dust mite).⁴³ This IgE reduction with aging might be attributable to the reduced B-cell repertoire available to respond to antigenic stimuli in the elderly with reduced isotype switching and lower antibody responses.⁴³⁻⁴⁶

Additionally, our findings of increased T_H17 and T_H22 activity in older control subjects follow a previously observed trend for increased levels of T_H17/IL-17 and T_H22/IL-22 cytokines in

peripheral blood.^{47,48} The frequency and function of FOXP3⁺ Treg cells were shown to significantly increase with aging,⁴⁹⁻⁵¹ potentially contributing to the age-related immune dysregulation. Although we did not observe a difference in FOXP3⁺ T cells in the skin, we did see significant decrease in expression of *IL-37*, a negative regulator, which suggests loss of immune regulation with age. With normal aging, restructuring of the T-cell compartment occurs as the number of naive T cells emerging from the thymus progressively decreases secondary to thymic involution.⁵² In both skin and peripheral blood, the memory/memory effector T-cell pool expands with aging, with a progressive increase in numbers of CD28⁻CD8⁺ T-cells,²³⁻²⁵ which have enhanced cytotoxicity and suppressor functions, potentially contributing to age-related immune dysregulation.

In addition to changes in skin immunity, we also observed decreases in measures of epidermal hyperplasia (ET and Ki67) and terminal differentiation (*LOR* and *FLG*) as a function of age. This is consistent with studies demonstrating reductions in epidermal hyperplasia and lipid content in aged skin,⁵³⁻⁵⁶ which is more easily disrupted by tape-stripping.⁵⁶ This suggests some degree of intrinsic barrier compromise in normal aged skin in addition to the presence of thinner skin.

In the AD cohort we observed significant changes with age in both skin and blood. T_H2-related measures (eg, *IL13*, *IL31*, and *CCL26*) decreased in lesional and nonlesional skin and serum in older patients with AD, as accompanied by concurrent reductions in levels of atopic DC/inflammatory dendritic epidermal cell markers (CD1a⁺, CD1b⁺, and FcεRI⁺), serum IgE levels, and eosinophil counts.^{57,58} *IL-22* and *IL-22*-induced S100As⁵⁹ showed attenuation in both lesional and nonlesional skin with age. These findings were apparent in both our direct comparison of AD values and our comparison of age-controlled normalized AD data. On the other hand, T_H1- and T_H17-related markers, when comparing expression values, incrementally increased with age in lesional and nonlesional AD skin at a rate consistent with that of normal skin but showed minimal changes when normalized to age-matched control subjects. We also found age-specific differences in markers that strongly correlated with disease severity. In the youngest group multiple axes (T_H2, T_H17, and T_H1) correlated with SCORAD scores, whereas markers became increasingly T_H17 dominated with age. In the oldest group the disease remained T_H2 driven because T_H2 markers highly correlated with SCORAD scores; however, T_H1 markers inversely correlated with SCORAD scores.

There are multiple mechanisms that could explain this age-related immune skewing with decreased T_H2/T_H22 and increased T_H1/T_H17 activation in older patients with AD. One explanation might be a T_H1/T_H2 counterregulatory effect because IFN-γ has been found to inhibit T_H2 proliferation and differentiation.⁶⁰ It is possible that increased T_H1 activation and subsequently greater IFN-γ production lead to reduced T_H2 skewing in older patients with AD. This is further supported by the negative correlation between T_H1-associated markers and disease severity in the oldest age group. Additionally, T_H2 cytokines have been shown to inhibit *IL-17A* production.⁶¹ Thus the reduction in the T_H2 axis might allow for increased T_H17 activity. Additionally, we observed increased negative regulators (*IL-37* and *FOXP3* vs T_H2/T_H22-driver cytokine ratio) in skin of patients with AD. This increase might also partially account for the reduced T_H2/T_H22 activation with age, particularly because T_H2/T_H22

cytokines are established drivers of AD.^{19,57,58} Although the changes in T_H1/T_H17 observed in patients with AD are in line with those observed in aged control subjects, the decreases in T_H2 and T_H22 activity were uniquely seen in patients with AD and were opposed to normal aging trends.

We also observed significant age-specific changes in measures of the AD-associated epidermal barrier defect, which is characterized by increased hyperplasia, upregulation of *IL-22*-induced S100As, and attenuated terminal differentiation. Epidermal hyperplasia measures (ET, K16, and Ki67) were diminished, whereas levels of terminal differentiation markers (*LOR* and *FLG*) were significantly increased with age, suggesting an amelioration of the epidermal pathology of AD in older patients. Our patients with AD did not demonstrate increased ET in nonlesional skin compared with control subjects, which has been observed in other smaller AD cohorts in previous reports.²⁶ Because T_H2 and T_H22 cytokines have been shown to suppress terminal differentiation,^{59,62-64} the improvement in skin barrier measures with age in patients with AD might be attributable to attenuation in the T_H2/T_H22 cytokine axes.

Some of the age-specific findings in our AD cohort can be viewed as part of the normal aging process, including increases in numbers of CD8⁺ T cells, innate immunity, T_H1 and T_H17 activity, and negative regulators, as well as reduced serum IgE levels.²⁶ However, the decreased T_H2/T_H22 measures observed with age in our AD cohort run contrary to the T_H1-to-T_H2 shift and increased T_H22 activity previously described in normal aging.⁴⁷ In our data set, older control subjects also demonstrated increased T_H2/T_H22 activity, whereas older patients with AD showed decreased T_H2/T_H22 polarization. Thus these particular age-related changes in the AD phenotype might not reflect the normal aging process but can be attributed to the complex interplay between the multi-cytokine activation involved in long-standing chronic AD that characterizes older patients with AD, which involves T_H2, T_H22, T_H1, and T_H17 cytokines,^{8,27,65,66} as well as Treg cells. The reduced T_H2 and T_H22 activity with age in patients with AD could be due to counterregulation by both T_H1 and T_H17, as well as by an increased regulatory tone, perhaps ultimately promoting disease attenuation or tolerance.^{61,67} The intrinsic skin barrier compromise associated with normal aging, marked by reduced key physiologic lipids and decreased resilience to tape-stripping,⁵⁶ might further explain the persistence of AD in elderly patients, despite amelioration in terminal differentiation markers.

To the best of our knowledge, there are no studies to date that evaluate age-specific changes in skin and blood biomarkers of patients with AD. A single prior study in patients with AD only assessed changes with aging in IgE sensitization and found that older patients with AD have lower rates of IgE sensitization compared with younger patients with AD (although greater than in age-matched healthy control subjects).⁶⁸

Our findings are largely consistent with age-related changes reported in other atopic diseases. Decreased serum IgE levels with aging have also been observed in patients with allergic rhinitis and asthma.⁶⁹⁻⁷¹ Another study evaluated change in levels of sputum cytokines of asthmatic patients with aging and, similar to our AD findings, observed significantly increased innate immunity (*IL-1β*, *IL-6*, and *IL-8*) and T_H17 activation (*CCL20* and *IL-23*) in older compared with younger asthmatic patients.¹² T_H1/IFN-γ showed a trend of upregulation. Neutrophil counts were also increased in older patients, likely because of greater

$T_H17/IL-8$ induction.⁷² Both IL-6 and IL-23 have been linked to T_H17 differentiation,⁷³ which is increased in aged asthmatic patients. This study also found significantly lower allergen sensitization in aged asthmatic patients. Interestingly, eosinophil counts and protein expression of IL-5 and eotaxin-1/CCL11 were significantly increased in older versus younger asthmatic patients, perhaps reflecting the greater severity and lack of asthma control characterizing the older versus younger group.¹²

This study had some limitations. This was not a prospective study; however, the cross-sectional trial design in this study allows for inclusion of greater numbers of patients and can inform future prospective studies. Patients from several ethnicities were included; however, when performing the same analyses in our largest ethnic population, European American patients with AD, our results showed similar trends, although with reduced significance likely caused by the decreased study size (data not shown). We also conducted a sex sensitivity subanalysis, with similar findings (data not shown). Future larger studies with appropriate age- and race-matched control subjects could further shed light on age-specific changes while taking into consideration different demographic factors.

Additional possible confounders include disease comorbidities; however, we recruited patients with no serious comorbidities. Variation of disease duration and chronic use of AD medications, especially among the elderly, can also serve as confounders. Nevertheless, all the patients had initiation of AD in early childhood. Therapeutic targeting with T_H2 -, T_H22 -, and T_H17/T_H1 -specific antagonists is needed to understand the varying pathogenic roles of these axes in patients with AD. Elucidating the differential patterns of immune skewing and barrier abnormalities among different AD phenotypes might be useful for developing personalized treatment approaches, especially in patients with recalcitrant AD.

Key messages

- As a function of age, although T_H1/T_H17 axis activation increases in both patients with AD and control subjects, T_H2/T_H22 activation decreases uniquely in patients with AD.
- Despite similar disease severity, older patients with AD demonstrate attenuated epithelial abnormalities through decreased hyperplasia and S100A expression and increased expression of terminal differentiation markers.

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SUPPLEMENTARY METHODS

Study subjects and skin samples

For this cross-sectional study, we combined gene expression, immunohistochemistry, and serum data sets of moderate-to-severe patients with chronic AD (n=246) and controls (n=71), with skin biopsies and serum samples collected under Institutional Review Board-approved protocols.¹⁻⁶ We separated patients and controls into age-groups, with 130 AD patients (85 males, 45 females) and 19 controls (12 males, 7 females) between ages 18-40, 89 AD patients (42 males, 47 females) and 25 controls (18 males and 7 females) between ages 41-60, and 27 AD patients (22 males, 5 females) and 27 controls (15 males and 12 females) older than age 60. All patients had chronic AD, which presented during early childhood (<5 years of age). All recruited patients were healthy with no serious disease comorbidities, and all recruited healthy controls did not have atopic or other comorbidities. Recruitment occurred in New York at Mount Sinai and Rockefeller University. Demographic data of the patients are listed in Table 1. There were no significant differences among age-groups in disease severity (evaluated by Scoring of AD/SCORAD). There were significant differences in racial composition across the age-groups. 244 lesional and 194 non-lesional (≥ 10 cm from active lesions) skin biopsies and 69 serum samples from AD patients and 63 skin biopsies from controls were evaluated. Similar affected areas were biopsied for lesional, non-lesional, and normal skin from all age groups. The majority of biopsies were taken from the extremities, and only if not possible, from the trunk. Patients were stratified into extrinsic and intrinsic AD categories, with IgE levels greater than 200 kU/L defining extrinsic AD and values less than 200 kU/L defining intrinsic AD.

FLG mutations were evaluated in 52 of 246 patients, of which 13 mutations were found. Mutations found were p.R501X, p.Q3569X, c.3321delA, p.S3671X, c.2282del4, p.R2447X, p.R826X, p.S3640X, 11783delA, and p.S2706X. The FLG mutation status was unavailable for the rest of the patients and healthy control subjects.

Immunohistochemistry

Immunohistochemistry/IHC was performed using purified anti-human monoclonal antibodies (see Table E1). For IHC, biotin-labeled horse anti-mouse secondary antibodies (Vector Laboratories) were used to detect the primary antibodies, and the staining signal was developed with chromogen 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). For cell counts, positive cells per millimeter of basement membrane were manually quantified by using computer-assisted image analysis software (ImageJ 1.42; National Institutes of Health, Bethesda, MD).

Quantitative gene expression analyses

RNA was extracted, followed by quantitative gene expression analyses with quantitative real-time PCR/qRT-PCR as previously described.^{7, 8} The TaqMan RT-PCR reaction was performed with EZ-PCR Core Reagents (Life Technologies), according to the manufacturer's directions. Human acidic ribosomal protein/hARP, a housekeeping gene, was used to normalize each sample and each gene. The primer sequences used were generated with the Primer Express algorithm (Applied Biosystems, Foster City, Calif), version 1.0, by using published genetic sequences (National Center for Biotechnology Information–PubMed), as follows: hARP forward CGCTGCTGAACATGCTCAA, hARP reverse TGTCGAACACCTGCTGGATG, and hARP probe 6-FAM- TCCCCCTTCTCCTTTGGGCTGG-TAMRA (Gene Bank accession

no. NM-001002). Primers and probes are listed in Table E2. The data were analyzed and quantified by using the software provided with the Applied Biosystems PRISM 7700 (Sequence Detection Systems, version 1.7).

Statistical analysis

Statistical analysis for gene expression was performed using R-language (www.R-project.org) and packages available through Bioconductor (www.bioconductor.org). hARP-normalized qRT-PCR expression values under the limit of detection/LOD were imputed as 20% of the minimal value over the LOD. No other missing value imputation method was performed. Data was scaled based on study of origin using R function *scale*. Data was log2-transformed prior to analysis with a linear mixed-effect model, with age-group and tissue (lesional and non-lesional skin) as fixed factor, and a random effect for each patient. Fold changes/FCHs for comparisons of interest against age-controlled normals were estimated, and hypothesis testing was conducted with contrasts under the general framework for linear models in the R *nlme* package. IHC data, measured as cell counts were also analyzed with the linear-mixed effect model. Serum data comparisons were evaluated using unpaired Student's t-tests. The association of skin and serum with age was evaluated using Spearman correlation coefficients. Word clouds were created using the R *wordcloud* package with word sizes proportional to FCHs. AD-associated immune pathways were quantified using Gene Set Variation Analysis (GSVA) with the R *gsva* package.

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SUPPLEMENTARY FIGURE LEGENDS

Figure E1. A-E, Bar plots denote quantification of epidermal thickness and cellular markers in skin of healthy controls. Measurements are conducted by counting cells per mm of basement membrane. **F-W,** Barplots denote hARP-normalized RT-PCR expression values in skin of healthy controls. Light blue indicates ages 18-40 years old, medium blue indicates ages 41-60 years old, and dark blue indicates ages 61+ years old. Stars that traverse bars indicate significance of comparison between age-groups.

*IHC: immunohistochemistry; PCR: polymerase chain reaction; +p<0.1, *p<0.05, **p<0.01, ***p<0.001.*

Figure E2. A-D, Bar plots denote quantification of epidermal thickness and cellular markers in skin of healthy controls and lesional and non-lesional skin of AD patients. Measurements are conducted by counting cells per mm of basement membrane. **E-V**, Barplots denote hARP-normalized RT-PCR expression values in skin of healthy controls and lesional and non-lesional skin of AD patients. Light blue indicates ages 18-40 years old, medium blue indicates ages 41-60 years old, and dark blue indicates ages 61+ years old. Stars that traverse bars indicate significance of comparison between age-groups. *IHC: immunohistochemistry; PCR: polymerase chain reaction; N: normal; NL: non-lesional; LS: lesional; +p<0.1, *p<0.05, **p<0.01, ***p<0.001.*

Figure E3. A-L, Representative immunohistochemistry images and quantification of cellular markers. Measurements are conducted by counting cells per mm of basement membrane. Data is normalized to age-matched controls (AD cells/mm subtracted by control cells/mm). Light blue indicates ages 18-40 years old, medium blue indicates ages 41-60 years old, and dark blue indicates ages 61+ years old. Stars on top of each bar indicate significance of comparison between AD and healthy controls. Stars that traverse bars indicate significance of comparison between age-groups. *AD: atopic dermatitis; N: normal; NL: non-lesional; LS: lesional; +p<0.1, *p<0.05, **p<0.01, ***p<0.001.*

Figure E4. A-J, Bar plots denote log2 FCH (AD versus control) of mRNA expression. Presented data is normalized to age-matched controls. **K-L**, Bar plots denote log2 FOXP3 expression over IL-13 or IL-22 expression in lesional skin. Presented data is normalized to age-matched controls. Light blue indicates ages 18-40 years old, medium blue indicates ages 41-60 years old, and dark blue indicates ages 61+ years old. Stars on top of each bar indicate significance of comparison between AD and healthy controls. Stars that traverse bars indicate significance of comparison between age-groups. *AD: atopic dermatitis; N: normal; NL: non-lesional; LS: lesional*; + $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig E5. A-L, Spearman correlations of log2 FCH (AD versus control) of mRNA expression with age. Presented data is normalized to age-matched controls. *AD: atopic dermatitis; N: normal*.

Fig E6. Barplots represent changes with age in the extrinsic AD subset. **A-D**, Barplots denote quantification of thickness and cellular markers in extrinsic AD skin. Measurements are conducted by counting cells per mm of basement membrane. Data is normalized to age-matched controls (AD cells/mm subtracted by control cells/mm). **E-P**, Barplots denote log2 FCH (AD versus control) of mRNA expression. Presented data is normalized to age-matched controls. **Q-S**, Barplots denote values of AD serum measures. Light blue indicates ages 18-40 years old, medium blue indicates ages 41-60 years old, and dark blue indicates ages 61+ years old. Stars on top of each bar indicate significance of comparison between AD and healthy controls. Stars that traverse bars

154 indicate significance of comparison between age-groups. *IHC: immunohistochemistry;*
 155 *PCR: polymerase chain reaction; AD: atopic dermatitis; N: normal; NL: non-lesional; LS:*
 156 *lesional; +p<0.1, *p<0.05, **p<0.01, ***p<0.001.*

Table E1. Antibodies used for IHC and immunofluorescence analyses.

Antibody	Vendor	Clone	Isotype	Concentration
CD3	BD Biosciences	SK7	IgG1	20 ug/ml
CD11c	BD Biosciences	B-ly6	IgG1	20 ug/ml
CD8	Abcam	144B	IgG1	25 ug/ml
FceRI	Thermo Scientific	CRA1 (AER37)	IgG2b	1 ug/ml
CD1a	Abcam	CD1a007	IgG1	20 ug/ml
CD1b	BD Biosciences	M-T101	IgG1	25 ug/ml
CD1c	Miltenyi Biotec	AD5-8E7	IgG2a	10 ug/ml
CD206	GenWay Biotech	15-2	IgG1	50 ug/ml
Ki67	Santa Cruz	MIB-1	IgG1	20 ug/ml

Table E2. Primers and probes used for qRT-PCR.

Gene Symbol		Sequence
RPLP0/hARP	Forward	CGCTGCTGAACATGCTCAA
	Reverse	TGTCGAACACCTGCTGGATG
	Probe	6-FAM-TCCCCCTTCTCCTTTGGGCTGG-2TAMRA
		Assay ID
KRT16		Hs00373910_g1
LOR		Hs01894962_s1
FLG		Hs00856927_g1
FOXP3		Hs01085834_m1
MMP12		Hs00159178_m1
IL-5		Hs00174200_m1
IL-13		Hs00174379_m1
IL-31		Hs01098710_m1
CCL13		Hs00234646_m1
CCL17		Hs00171074_m1
CCL18		Hs00268113_m1
CCL22		Hs01574247_m1
CCL26		Hs00171146_m1
TSLPR		Hs00845692_m1
IL-17A		Hs00174383_m1

IL-8	Hs00174103_m1
IL-19	Hs00604657_m1
PI3	Hs00160066_m1
IL-23p19	Hs00900828_g1
CCL20	Hs00355476_m1
IFNg	Hs00989291_m1
STAT1	Hs01013996_m1
MX1	Hs00895608_m1
CXCL9	Hs00171065_m1
IL-12/23p40	Hs01011518_m1
IL-20	Hs00218888_m1
IL-22	Hs01574154_m1
S100A7	Hs01923188_u1
S100A8	Hs01923188_u1
S100A9	Hs00610058_m1
S100A12	Hs00942835_g1
IL-1b	Hs01555410_m1
IL-9	Hs00174125_m1
IL-37	Hs00367201_m1

Table E3. Correlations of IHC and RT-PCR data of normal skin with age

IHC			PCR		
marker	R Value	P Value	marker	R Value	P Value
CD1a N	0.673	5.01E-06	IL17A N	0.629	8.06E-06
CD11c N	0.605	7.30E-05	IL37 N	-0.589	1.98E-04
thickness N	-0.515	9.35E-04	IL20 N	0.484	0.002
Ki67 N	-0.519	0.001	IL13 N	0.413	0.007
FCER1 N	0.471	0.003	TSLPR N	0.344	0.008
CD206 N	0.440	0.007	S100A7 N	0.381	0.013
CD1c N	0.418	0.010	STAT1 N	0.414	0.013
CD3 N	0.357	0.026	CCL20 N	0.410	0.015
CD1b N	-0.197	0.243	IL12.23p40 N	0.381	0.024
CD8 N	-0.155	0.361	IL22 N	0.290	0.033
			CCL18 N	0.264	0.034
			S100A9 N	0.254	0.055
			MX1 N	0.254	0.055
			MMP12 N	0.230	0.065
			K16 N	0.229	0.066
			CCL13 N	0.224	0.073
			CCL22 N	0.212	0.090
			CCL17 N	0.210	0.093
			IL5 N	0.221	0.095
			CXCL9 N	0.206	0.120
			FOXP3 N	-0.228	0.146
			LOR N	-0.181	0.150
			S100A8 N	0.171	0.173
			FLG N	-0.170	0.176
			CCL26 N	0.165	0.189
			IL9 N	0.136	0.309
			IL31 N	0.125	0.322
			IFNg N	0.105	0.406
			IL8 N	0.109	0.414
			S100A12 N	0.096	0.449
			IL1b N	0.079	0.529
			IL19 N	-0.067	0.687
			IL23p19 N	-0.032	0.842
			PI3 N	-0.019	0.883

N: normal

Table E4. Excel file of IHC, qRT-PCR, and serum comparisons in age analysis.**Table E5.** Correlations of IHC cell counts, mRNA expression by qRT-PCR, and serum measures in lesional and non-lesional AD skin with age.

IHC			PCR			Serum		
marker	R Value	P Value	marker	R Value	P Value	marker	R Value	P Value
CD1a NL	-0.573	7.80E-12	IL13 NL	-0.474	2.532E-12	log10(IgE)	-0.240	0.001
CD1a LS	-0.476	2.17E-09	IL13 LS	-0.420	1.304E-11	CCL2	0.512	0.002
CD1b LS	-0.506	1.21E-06	S100A8 NL	-0.437	3.313E-10	IFNg	0.480	0.003
CD1b NL	-0.540	1.66E-06	IL5 NL	-0.514	1.864E-09	log10(EOS)	-0.230	0.019
FCERI NL	-0.332	1.37E-05	IL22 LS	-0.370	6.731E-09	IL22	-0.351	0.038
CD11c NL	-0.150	0.041	S100A8 LS	-0.363	1.332E-08	CCL3	0.322	0.059
CD3 NL	-0.126	0.086	CCL18 NL	-0.369	1.128E-07	CCL26	-0.316	0.065
FCERI LS	-0.120	0.100	FLG NL	0.380	1.399E-07	CCL11	0.316	0.065
CD8 NL	0.144	0.181	S100A7 LS	-0.386	1.746E-07	CCL13	0.114	0.516
CD206 LS	0.101	0.203	CCL18 LS	-0.323	3.449E-07	IL13	-0.111	0.525
CD8 LS	0.107	0.285	CCL17 NL	-0.352	4.551E-07	IL17	-0.101	0.663
CD1c LS	0.083	0.338	K16 NL	-0.345	7.550E-07	CCL22	-0.056	0.748
Ki67 LS	0.055	0.447	CCL26 NL	-0.380	1.463E-06			
CD1c NL	-0.071	0.460	IL22 NL	-0.328	4.108E-06			
CD3 LS	0.049	0.469	MMP12 NL	-0.345	7.776E-06			
CD11c LS	0.048	0.480	S100A7 NL	-0.360	1.641E-05			
Ki67 NL	-0.053	0.492	CCL22 NL	-0.299	6.359E-05			
thickness NL	0.046	0.522	MMP12 LS	-0.264	1.431E-04			
thickness LS	-0.009	0.899	IL37 NL	0.601	1.724E-04			
CD206 NL	0.009	0.912	IL5 LS	-0.296	2.732E-04			
			LOR NL	0.262	2.737E-04			
			IL31 NL	-0.317	4.185E-04			
			S100A9 NL	-0.323	4.320E-04			
			CCL26 LS	-0.250	6.163E-04			
			FLG LS	0.222	8.558E-04			
			IL9 NL	-0.275	9.856E-04			
			TSLPR LS	-0.434	1.036E-03			
			IL1b LS	0.238	1.796E-03			
			IL20 NL	-0.407	9.125E-03			
			IL23p19 LS	0.184	0.010			
			S100A9 LS	-0.199	0.021			
			IL9 LS	-0.168	0.034			
			TSLPR NL	-0.346	0.036			

CCL17 LS	-0.134	0.038
K16 LS	-0.133	0.040
CCL22 LS	-0.141	0.044
FOXP3 NL	-0.263	0.048
CCL20 NL	-0.158	0.061
CXCL9 LS	0.174	0.068
LOR LS	0.115	0.081
IFNg NL	-0.124	0.085
S100A12 NL	-0.128	0.086
CCL13 LS	-0.153	0.099
CCL13 NL	-0.172	0.103
IL8 LS	0.201	0.157
IL8 NL	0.204	0.175
MX1 LS	0.177	0.176
S100A12 LS	-0.090	0.182
IL17A NL	-0.093	0.194
MX1 NL	0.172	0.209
IL23p19 NL	0.096	0.220
IL17A LS	-0.078	0.233
IL37 LS	0.193	0.246
IL12.23p40 NL	-0.081	0.264
IL31 LS	-0.091	0.276
STAT1 NL	-0.110	0.294
IL19 NL	0.148	0.362
FOXP3 LS	0.108	0.390
PI3 LS	0.058	0.391
PI3 NL	0.064	0.400
IFNg LS	-0.041	0.525
STAT1 LS	0.052	0.602
IL1b NL	-0.045	0.609
IL19 LS	0.078	0.616
CXCL9 NL	0.048	0.634
CCL20 LS	-0.024	0.767
IL12.23p40 LS	-0.013	0.847
IL20 LS	0.002	0.988

LS: lesional; NL: non-lesional

Table E6. Excel file of IHC sub-analysis with separate analysis of cell counts in epidermis and dermis.

Table E7. Correlations of PCR markers with SCORAD

18-40 years old			41-60 years old			61+ years old		
marker	R value	P value	marker	R value	P value	marker	R value	P value
LOR NL	-0.308	0.002	K16 LS	0.367	0.001	CCL26 LS	0.521	0.041
LOR LS	-0.272	0.004	IL17A LS	0.338	0.002	CCL22 NL	-0.455	0.059
TSLPR NL	0.576	0.006	CCL22 LS	0.365	0.002	STAT1 LS	-0.591	0.061
IL1b LS	0.288	0.007	IL13 LS	0.318	0.004	IL5 LS	0.510	0.062
PI3 LS	0.253	0.008	FOXP3 LS	0.503	0.005	IL31 LS	0.495	0.072
K16 LS	0.234	0.011	PI3 LS	0.309	0.007	CXCL9 LS	-0.500	0.121
IL20 NL	0.492	0.013	MMP12 LS	0.319	0.008	IL5 NL	-0.447	0.145
IL17A LS	0.228	0.013	IL20 NL	0.743	0.009	IL17A LS	-0.298	0.178
CXCL9 NL	0.318	0.017	CCL18 LS	0.285	0.009	S100A9 LS	-0.364	0.246
IL19 NL	0.446	0.025	CCL18 NL	0.290	0.013	IL31 NL	-0.356	0.257
S100A8 LS	0.206	0.028	LOR LS	-0.270	0.015	CCL17 NL	-0.265	0.272
S100A9 LS	0.245	0.033	K16 NL	0.281	0.016	LOR NL	-0.261	0.278
TSLPR LS	0.413	0.036	IL19 NL	0.662	0.026	IFNg NL	-0.261	0.278
S100A12 LS	0.197	0.039	CCL17 LS	0.243	0.028	FLG LS	0.230	0.315
FLG LS	-0.196	0.040	IL8 LS	0.624	0.040	S100A7 LS	0.257	0.354
IL13 NL	0.203	0.042	CCL17 NL	0.240	0.041	K16 LS	-0.206	0.356
CCL13 NL	0.290	0.045	CXCL9 NL	0.357	0.041	IL22 LS	-0.181	0.432
IL9 LS	0.205	0.056	IL9 LS	0.267	0.045	CCL18 NL	-0.189	0.436
MX1 LS	0.312	0.056	IL13 NL	0.220	0.062	S100A7 NL	0.236	0.437
IL22 LS	0.168	0.075	IL22 LS	0.208	0.062	CCL20 NL	-0.222	0.445
CCL18 NL	0.177	0.078	IL5 NL	0.285	0.064	CCL20 LS	-0.203	0.450
IL37 NL	-0.377	0.092	S100A8 LS	0.206	0.065	IL9 LS	-0.196	0.466
IL13 LS	0.155	0.094	IL12/23p40 LS	0.190	0.086	PI3 LS	-0.179	0.477
IL22 NL	0.171	0.097	IFNg LS	0.190	0.088	PI3 NL	0.196	0.482
S100A9 NL	0.185	0.146	CXCL9 LS	0.292	0.089	IL12/23p40 LS	-0.152	0.498
CCL26 NL	0.167	0.147	IL1b LS	0.238	0.089	CCL18 LS	-0.137	0.541
IL8 LS	0.245	0.156	MX1 LS	0.424	0.089	IL23p19 NL	-0.164	0.558
IL12/23p40 NL	0.140	0.164	S100A12 LS	0.196	0.092	S100A12 NL	-0.139	0.570
IL37 LS	-0.266	0.189	CCL13 LS	0.288	0.104	S100A8 NL	0.121	0.621
MX1 NL	0.230	0.191	FLG LS	-0.189	0.104	S100A12 LS	-0.101	0.661
IL5 NL	0.163	0.205	S100A9 LS	0.240	0.105	IL17A NL	-0.104	0.673
STAT1 LS	0.165	0.208	STAT1 LS	0.270	0.128	CCL26 NL	0.121	0.682
IFNg LS	0.116	0.211	CCL20 NL	0.210	0.132	IL1b NL	0.126	0.683

IL8 NL	0.216	0.252	STAT1 NL	0.275	0.135	IL13 NL	-0.093	0.705
CCL17 NL	0.115	0.254	CCL22 NL	0.170	0.180	S100A9 NL	0.127	0.714
IL20 LS	0.206	0.275	S100A8 NL	0.159	0.182	IFNg LS	-0.075	0.739
CCL22 NL	0.115	0.287	FOXP3 NL	0.263	0.185	IL12/23p40 NL	-0.075	0.759
CCL13 LS	-0.141	0.288	IL23p19 NL	-0.144	0.248	MMP12 LS	0.066	0.802
IL23p19 LS	0.105	0.292	S100A12 NL	0.141	0.255	IL1b LS	0.053	0.848
CCL18 LS	0.092	0.321	CCL26 NL	0.150	0.265	IL9 NL	0.047	0.872
IL19 LS	0.168	0.375	IL9 NL	0.140	0.319	S100A8 LS	-0.032	0.890
IL17A NL	-0.090	0.376	IL17A NL	0.118	0.319	IL13 LS	0.031	0.892
K16 NL	0.088	0.384	CCL20 LS	0.134	0.322	IL22 NL	-0.025	0.920
MMP12 NL	0.096	0.392	MMP12 NL	0.119	0.360	MMP12 NL	0.029	0.923
IL23p19 NL	-0.094	0.393	IL5 LS	0.124	0.418	CCL22 LS	-0.023	0.927
S100A8 NL	0.087	0.400	S100A9 NL	0.123	0.444	FLG NL	-0.019	0.940
IL31 NL	0.107	0.407	IL12/23p40 NL	-0.086	0.471	CCL17 LS	-0.018	0.940
IL12/23p40 LS	0.069	0.460	IL31 LS	0.107	0.486	IL23p19 LS	-0.017	0.951
IL31 LS	0.082	0.493	IFNg NL	0.080	0.499	K16 NL	0.014	0.957
IL9 NL	0.079	0.501	IL23p19 LS	0.066	0.571	LOR LS	0.000	1.000
STAT1 NL	0.087	0.540	CCL26 LS	0.067	0.602			
CCL26 LS	-0.063	0.557	IL8 NL	0.134	0.678			
FOXP3 NL	0.105	0.609	IL22 NL	0.042	0.727			
FOXP3 LS	-0.083	0.658	FLG NL	0.036	0.769			
PI3 NL	0.040	0.709	IL1b NL	-0.041	0.786			
MMP12 LS	0.036	0.719	S100A7 LS	0.034	0.802			
S100A12 NL	0.037	0.730	LOR NL	-0.028	0.816			
FLG NL	0.023	0.830	CCL13 NL	0.034	0.851			
CXCL9 LS	0.027	0.832	PI3 NL	-0.017	0.891			
CCL17 LS	0.019	0.837	MX1 NL	0.036	0.892			
CCL20 NL	-0.023	0.846	IL31 NL	-0.015	0.924			
CCL20 LS	0.020	0.857	S100A7 NL	0.010	0.943			
IL1b NL	0.021	0.862						
S100A7 LS	-0.019	0.866						
IFNg NL	0.017	0.868						
IL5 LS	-0.019	0.872						
S100A7 NL	0.016	0.898						
CCL22 LS	0.009	0.929						

LS: lesional; NL: non-lesional

Table E8. Excel file of IHC, qRT-PCR, and serum comparisons in extrinsic AD subset analysis.

Figure E1

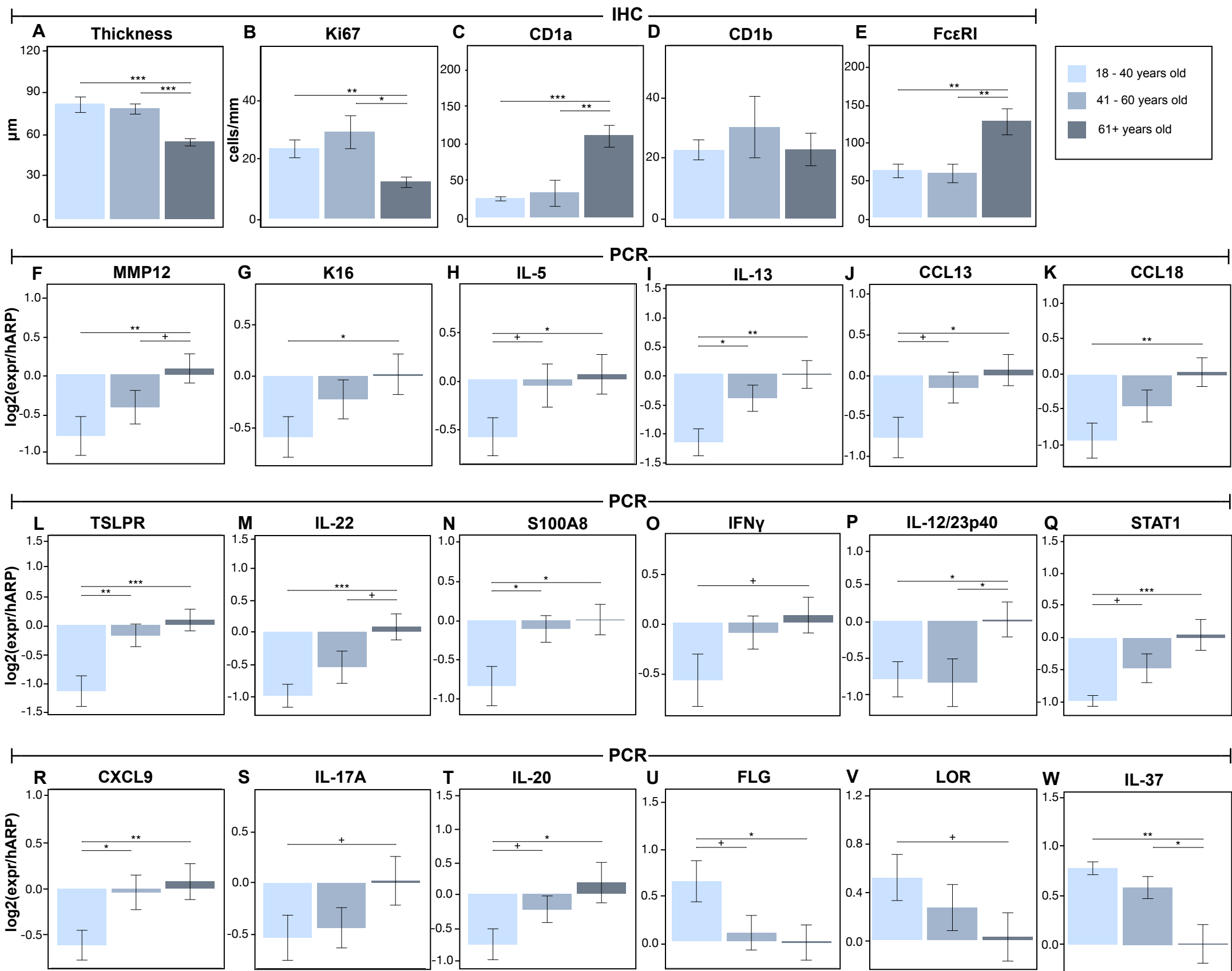
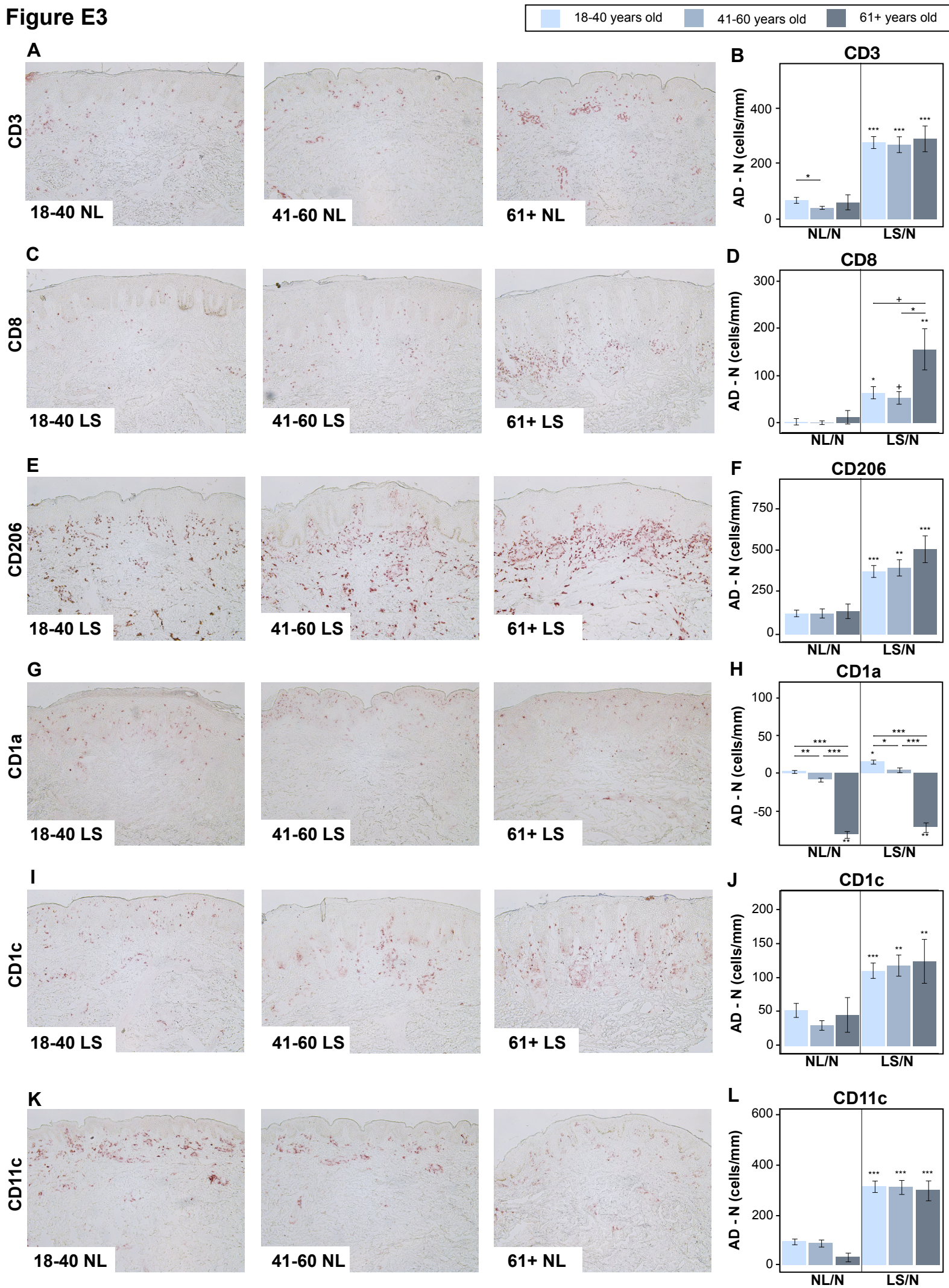


Figure E3



- 18-40 years old
- 41-60 years old
- 61+ years old

Figure E5

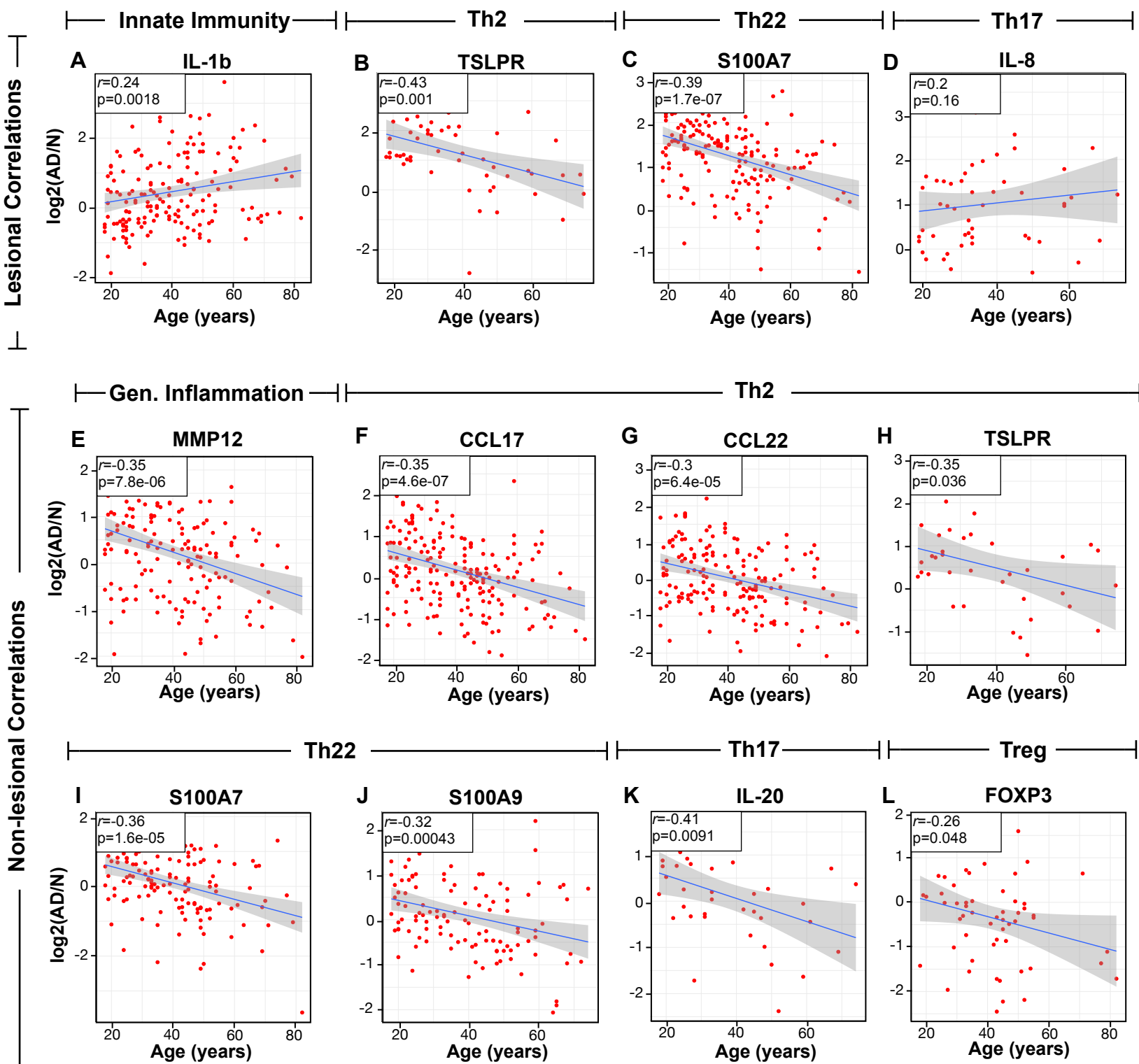


Figure E6

