Early-onset pediatric atopic dermatitis is T_H2 but also T_H17 polarized in skin

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Background: Atopic dermatitis (AD) affects 15% to 25% of children and 4% to 7% of adults. Paradigm-shifting discoveries about AD have been based on adult biomarkers, reflecting decades of disease activity, although 85% of cases begin by 5 years. Blood phenotyping shows only T_H2 skewing in patients with early-onset pediatric AD, but alterations in early pediatric skin lesions are unknown, limiting advancement of targeted therapies.

Objective: We sought to characterize the early pediatric AD skin phenotype and its differences from pediatric control subjects and adults with AD.

Methods: Using immunohistochemistry and quantitative real-time PCR, we assessed biopsy specimens from 19 children with AD younger than 5 years within 6 months of disease onset in comparison with adults with AD or psoriasis and pediatric and adult control subjects.

Results: In lesional skin children showed comparable or greater epidermal hyperplasia (thickness and keratin 16) and cellular infiltration ($CD3^+$, $CD11c^+$, and $Fc\varepsilon RI^+$) than adults with AD. Similar to adults, strong activation of the T_H2 (IL-13, IL-31, and CCL17) and T_H22 (IL-22 and S100As) axes and some T_H1 skewing (IFN-γ and CXCL10) were present. Children showed significantly higher induction of T_H17-related cytokines and antimicrobials (IL-17A, IL-19, CCL20, LL37, and peptidase inhibitor 3/elafin), T_H9/IL-9, IL-33, and innate markers (IL-8) than adults ($P < .02$). Despite the characteristic downregulation in adult patients with AD, filaggrin expression was similar in children with AD and healthy children. Nonlesional skin in pediatric patients with AD showed higher levels of inflammation (particularly IL-17A and the related molecules IL-19 and LL37) and epidermal proliferation (keratin 16 and S100As) markers ($P < .001$).

Conclusion: The skin phenotype of new-onset pediatric AD is substantially different from that of adult AD. Although excess T_H2 activation characterizes both, T_H9 and T_H17 are highly activated at disease initiation. Increases in IL-19 levels might link T_H2 and T_H17 activation. (J Allergy Clin Immunol 2016;138:1639-51.)

Key words: *Atopic dermatitis, pediatric, adult, skin, T_H2, T_H9, T_H17, IL-17, IL-19, antimicrobials*

Atopic dermatitis (AD) is one of the most common pediatric disorders, affecting 15% to 25% of children and 4% to 7% of adults.[1,2] It usually begins within the first 5 years of life, and when encountered in adults, the disease has generally been present for decades.

In adults AD skin immune fingerprinting has been linked to more than 1 cytokine pathway, including possible roles for T_H2, T_H22, and even T_H17/IL-23 activation in creating the AD phenotype.[3-6] Furthermore, suppression of these pathways correlates with clinical disease resolution with both broad[7-9]

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Abbreviations used
AD: Atopic dermatitis
ADQ: Atopic Dermatitis Quickscore
AMP: Antimicrobial peptide
DC: Dendritic cell
EASI: Eczema Area and Severity Index
FOXP3: Forkhead box P3
hARP: Human acidic ribosomal protein
IRB: Institutional review board
K16: Keratin 16
OX40L: OX40 ligand
PI3: Peptidase inhibitor 3
PIQoL: Parent's Index of Quality of Life
TEWL: Transepidermal water loss
TSLPR: Thymic stromal lymphopoietin receptor

and specific T_H2-targeting treatments.[10,11] The increased understanding of the molecular circuits associated with chronic AD in adults has accelerated therapeutic development and testing of possible targets, largely targeting the adult population with AD.[6,12]

Despite its predominance in children, the factors that influence development of early onset of AD lesions in children have received minimal attention. The immune polarity and epidermal changes in patients with early-onset pediatric AD might differ from those in adult patients with AD, reflecting decades of disease activity and chronic use of immunosuppressants in adults. Understanding molecular circuits of evolving AD skin lesions in children and their differences and similarities from those of adults is critical for prioritizing pathogenesis-based AD therapies in children. Studies of early pediatric AD are largely limited to peripheral blood[13-26] and have shown that disease activity correlates with a few serum biomarkers (ie, IL-31, CCL17, CCL22, CCL27, eosinophils, and IgE) and limited mRNA expression of T_H2/T_H1 markers.[27-31] Increases in memory T-cell numbers have been observed with age,[32-35] and recently, we reported expanded T_H2 cells, but not other polar T-cell subsets, in blood.[36] One study investigated nonlesional skin in adolescents with AD, highlighting interferon responses similar to those seen in the setting of adult chronic AD.[26]

To evaluate immune and epidermal factors that contribute to early AD development, we investigated lesional and nonlesional biopsy specimens from infants with AD (<5 years old and within 6 months of diagnosis) and compared them with those from age-matched pediatric control subjects. Tissues from adults with well-characterized AD and patients with psoriasis and control tissues from prior cohorts[3,8,37] were also analyzed to contrast early pediatric AD across a different range of polar cytokines. We found that nonlesional skin of infants with AD is already characterized by overt immune activation with strong T_H2 skewing but even more impressive activation of innate and IL-17–associated mediators, which is further amplified in pediatric lesions.

METHODS

Patients' characteristics

Nineteen children aged 3 months to 5 years with AD onset in the previous 6 months and moderate-to-severe disease were enrolled. Parents signed institutional review board (IRB)–approved written consent forms. Use of systemic immunosuppressants in the past 4 weeks, topical steroids or immunomodulators within 1 week, or moisturizers within 12 hours from biopsy were restricted, and patients with skin infections were excluded. Demographic data, serum IgE levels, disease scoring with SCORAD and Eczema Area and Severity Index (EASI), and quality-of-life and itch assessments (Atopic Dermatitis Quickscore [ADQ] and the Parent's Index of Quality of Life in Atopic Dermatitis [PIQoL-AD]) were performed. Transepidermal water loss (TEWL) was measured at biopsy sites by using the Tewameter (Courage and Khazaka GmbH, Cologne, Germany). Lesional and nonlesional 4-mm biopsy specimens from affected popliteal and unaffected buttock skin, respectively, were obtained. Control skin was collected during routine surgical procedures from 14 age-matched subjects without personal/familial atopy after IRB-approved parental consent (see Table E1 and the Methods section in this article's Online Repository at www.jacionline.org).

Tissues from the extremities of adults with lesional and nonlesional moderate-to-severe AD (n = 15; age, 33-72 years) and patients with moderate-to-severe lesional psoriasis (n = 10; ages, 30-64 years) and control subjects (n = 8; age, 40-57 years) from previously reported IRB-approved cohorts[3,8,37] were included in the analyses. SCORAD scores were available for adults with AD, and Psoriasis Area and Severity Index scores were available for patients with psoriasis. Baseline characteristics were similar within groups, except for ethnicity in children (Table I), but a sensitivity analysis in the white children subgroup showed similar results to the entire population (data not shown). Demographics and laboratory data are summarized in Table I, Table E1, and the Methods section in this article's Online Repository.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence procedures were performed on frozen sections by using purified mouse anti-human mAbs (see Table E2 in this article's Online Repository at www.jacionline.org for details), as previously described[9,38,39] and as outlined in the Methods section in this article's Online Repository.

Quantitative real-time PCR

RNA was extracted, and reverse transcription to cDNA from RNA was carried out with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif). cDNA was amplified with TaqMan PreAmp Master Mix (Applied Biosystems), and the preamplified cDNA product was analyzed with TaqMan Gene Expression Master Mix, as previously described.[3] Primers and probes used in this study are listed in Table E3 in this article's Online Repository at www.jacionline.org. All expression values were normalized to human acidic ribosomal protein (hARP; see Table E4 in this article's Online Repository at www.jacionline.org).

Statistical analyses

hARP-normalized RT-PCR expression values of less than the limit of detection were imputed as 20% of the minimum observed values (over the limit of detection) and $\log_2$-transformed before analysis. No other missing value imputation method was performed, and all available observations from eligible subjects were included in analyses and carried out in R software (www.R-project.org) and its available packages. Differences in expression values (in $\log_2$-scale), cell counts, and clinical variables between conditions were assessed by using linear models.

In paired lesional and nonlesional analyses from single patients, a mixed model with random intercept for each patient was used instead. Once the model was fitted (using lm and lme functions), least-squares means were obtained, and group comparisons were assessed by using the 2-tailed t test with contrasts (by using functions lsmean and contrast in R package lsmeans). Significance levels were less than .05.

Unsupervised hierarchical clustering of variables or samples/patients was performed by using the correlation coefficient as a distance metric with an average agglomeration algorithm and represented as a heat map with a

TABLE I. Baseline characteristics

Patients	Healthy children n = 14	Children with AD n = 19	*P* value (children)	Healthy adults n = 8	Adults with AD n = 15	Adults with psoriasis n = 10	*P* value (adults)
Age (y), mean (range)	1.3 (0.6-3.0)	1.3 (0.3-5.0)	.923	50.9 (40-57)	51.4 (33-72)	51.3 (30-64)	.994
Sex, no. (%)			.364				.162
Female	9 (64.3)	8 (42.1)		1 (12.5)	8 (53.3)	4 (40.0)	
Male	5 (35.7)	11 (57.9)		7 (87.5)	7 (46.7)	6 (60.0)	
Ethnicity, no. (%)			.019				1
Asian/Pacific Islander	1 (7.1)	2 (10.5)		0 (0.0)	0 (0.0)	0 (0.0)	
African American	0 (0.0)	5 (26.3)		0 (0.0)	0 (0.0)	0 (0.0)	
Hispanic	0 (0.0)	4 (21.1)		0 (0.0)	0 (0.0)	0 (0.0)	
White	13 (92.9)	8 (42.1)		8 (100.0)	15 (100.0)	10 (100.0)	
SCORAD score,* mean (SD)	NA	57.8 (12.8)		NA	56.7 (11.1)	NA	
PASI score, mean (SD)	NA	NA		NA	NA	20.3 (15.4)	
Biopsies	n = 14 (HC)	n = 19 (LS and NL)		n = 8 (HC)	n = 15 (LS and NL)	n = 10 (LS)	
IgE (kU/L)† (log_{10}), mean (SEM)	ND	2.11 (0.35), n = 7		ND	2.15 (0.25), n = 15	ND	

HC, Healthy control; *LS*, lesional; *NA*, not applicable; *ND*, not done; *NL*, nonlesional; *PASI*, Psoriasis Area and Severity Index.
**P* value for SCORAD scores in children with AD versus adults with AD = .794.
†Reference range: adults = 0-200 kU/L; children = 0-100 kU/L; *P* value for children versus adults = .93.

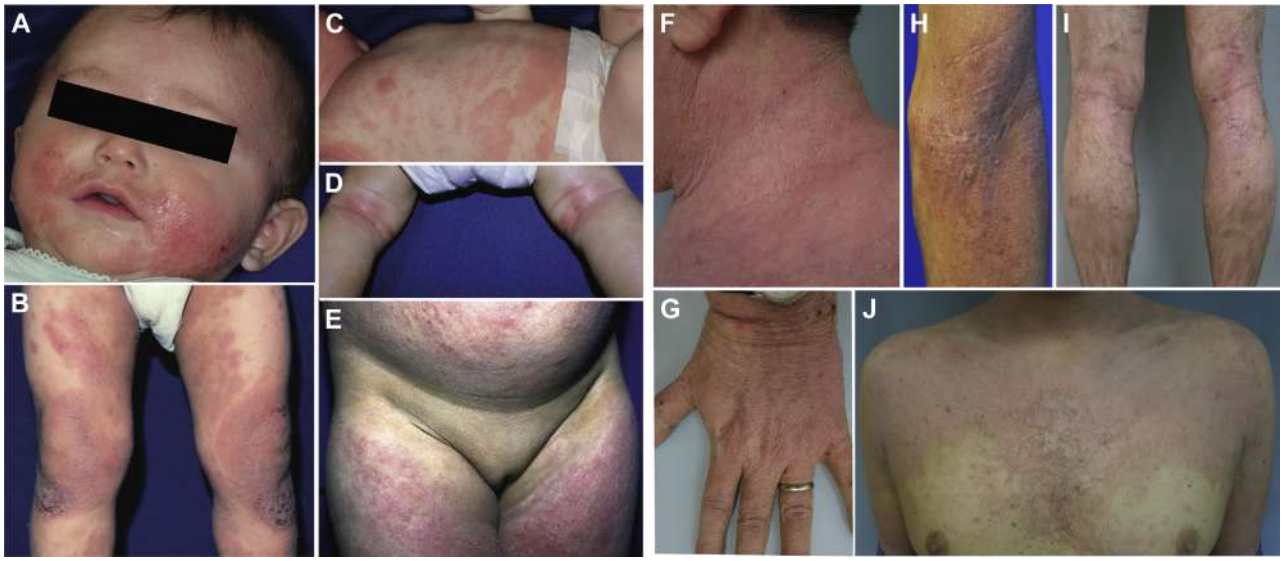

FIG 1. Clinical differences between pediatric and adult AD. Representative pictures of infants during the first 6 months of disease onset **(A-E)** and adults with years to decades of chronic disease **(F-J)** are shown.

dendrogram or PhyloTree (by using the R package ape; see extended statistics in this article's Online Repository).

RESULTS

Clinical distribution of AD differs in children and adults.[2] In infants lesions typically develop on the face (Fig 1, *A*), extensors (Fig 1, *B*), trunk (Fig 1, *C*), and folds (Fig 1, *D*), classically sparing the diaper areas (Fig 1, *E*). In adolescents and adults AD extends to the flexures, hands, neck, upper trunk, and shoulders (Fig 1, *F-J*).

To investigate early-onset AD in children, we analyzed lesional and nonlesional biopsy specimens from 19 children younger than 5 years with moderate-to-severe AD (mean age, 1.3 years) with a disease duration of less than 6 months and healthy skin from age-matched control subjects (n = 14). Adult patients with AD (n = 15), adult patients with psoriasis (n = 10), and adult control subjects (n = 8) were run concurrently to allow appropriate comparisons with all polar cytokine pathways displayed by the chronic phenotypes of these inflammatory skin diseases.

Early pediatric AD exhibits profound epidermal hyperplasia but lacks the filaggrin deficiency of adult AD

We first evaluated characteristics of the pathologic epidermal phenotype using disease biomarkers established for adult AD (Fig 2, *A-E*).[4,8,9,38] Despite the short disease duration in our pediatric cohort, remarkable epidermal hyperplasia (as measured by thickness, expression of keratin 16 [K16] mRNA and protein, Ki-67$^+$ counts, and S100A8/9 expression) was seen both in patients with lesional and those with nonlesional pediatric AD compared with that healthy skin, which was comparable or even higher than in adults with AD and psoriasis (Fig 2, *F-I*). K16 epidermal expression was noted in 14 (74%) of 19 nonlesional pediatric but only 3 (23%) of 13 nonlesional adult biopsy specimens (Fig 2, *B*). Healthy

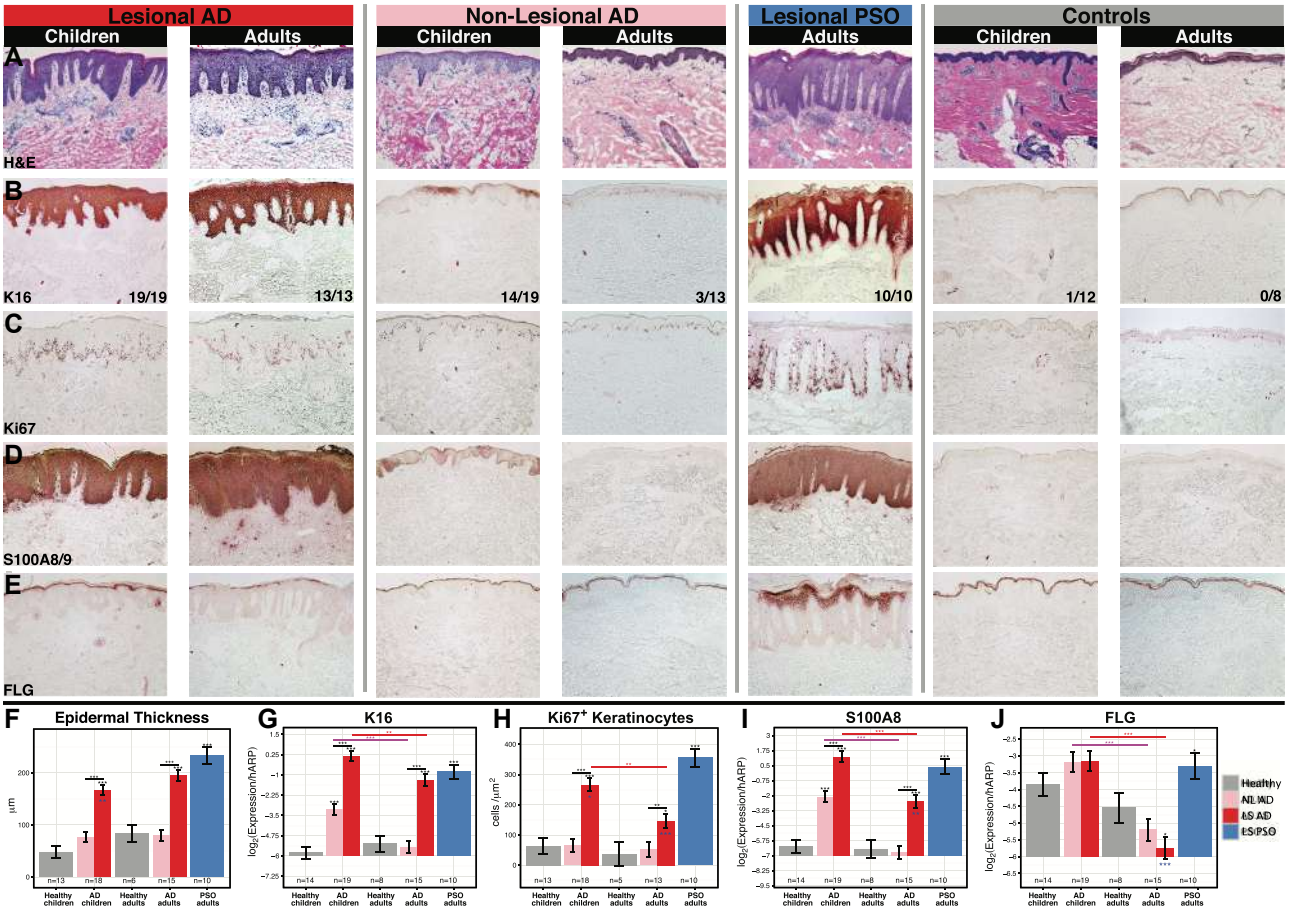

FIG 2. A-E, Representative staining in pediatric and adult patients with AD, patients with psoriasis *(PSO)*, and control subjects by using hematoxylin and eosin (Fig 2, *A*), K16 with fractions of immunohistochemistry-positive samples (Fig 2, *B*), Ki-67 (Fig 2, *C*), S100A8/A9 (Fig 2, *D*), and FLG (Fig 2, *E*). **F-J,** Quantification of epidermal thickness (Fig 2, *F*), K16 mRNA (Fig 2, *G*), Ki-67$^+$ keratinocytes (Fig 2, *H*), S100A8 mRNA (Fig 2, *I*), and FLG mRNA (Fig 2, *J*). mRNA $\log_2$ values were adjusted to hARP values. Values are means ± SEMs. *Stars above and below error bars* denote comparisons to matched healthy control skin *(black stars)* and psoriasis *(blue stars)*, respectively. *LS*, Lesional; *NL*, nonlesional. **P* < .05, ***P* < .01, and ****P* < .001.

control subjects were essentially negative for K16 both in pediatric and adult subjects (Fig 2, *B*). Higher Ki-67$^+$ counts and K16 and S100A8 mRNA levels were observed in pediatric compared with adult lesions ($P < .01$; Fig 2, *G-I*). *FLG* mRNA expression was higher in childhood versus adult AD, with an expression pattern more similar to that of adult psoriasis (Fig 2, *J*). Filaggrin protein expression in children with AD was similar to that in control subjects, showing a more continuous expression in the outer layers of the epidermis than in adult patients (Fig 2, *E*).

T-cell and dendritic cell infiltrates are similarly increased in pediatric and adult patients with AD

We next assessed cellular infiltrates, including CD3$^+$ T cells, CD11c$^+$ myeloid dendritic cells (DCs), and cells that express the high-affinity IgE receptor FcεRI$^+$, which is found, among others (see Fig E1 in this article's Online Repository at www.jacionline.org), on inflammatory epidermal dendritic cells.[40,41] Both pediatric and adult patients with AD showed large cellular infiltrates (Fig 3). Although counts of T cells and CD11c$^+$ DCs were comparable in patients with AD (regardless of age) and psoriasis, FcεRI$^+$ cell counts were significantly higher in children with AD compared with both adult patients with AD and those with psoriasis (Fig 3, *D-F*).

Pediatric AD is characterized by T_H17 activation and related antimicrobial peptides

To evaluate for mRNA expressions of innate and polar T_H cytokines, as well as regulatory and epidermal differentiation markers, which are usually less than detection levels on gene arrays,[42] we performed quantitative real-time PCR for a large array of mediators (Fig 4 and see Fig E2 in this article's Online Repository at www.jacionline.org). Mean expressions of all 53 assessed immune and epidermal markers are also depicted in an unsupervised hierarchical clustering heat map (Fig 5, *A*). Distinct differences were detected between pediatric and adult patients with AD, with several molecules being strongly upregulated in adult patients with but less so in children with AD (Fig 5, *A*, yellow box). Conversely, many markers were similarly upregulated in pediatric patients with AD and psoriasis and, to a lesser extent, in adult patients with AD (Fig 5, *A*, green box).

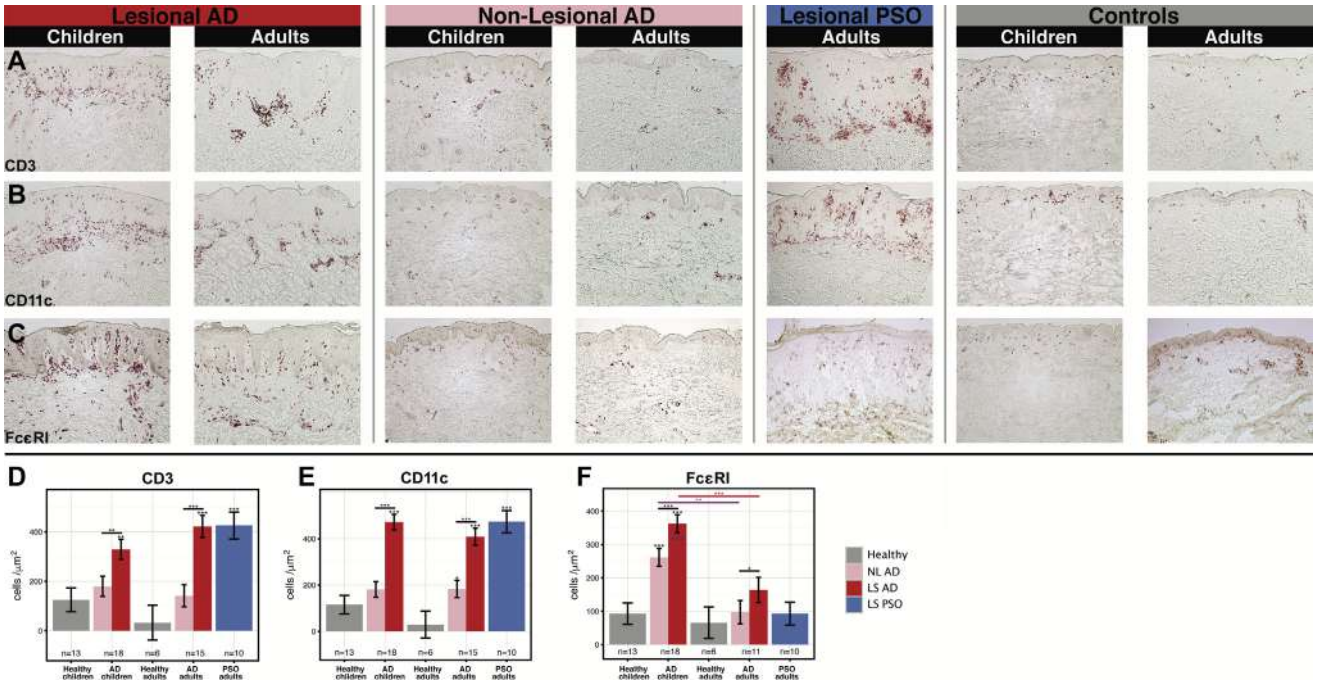

FIG 3. Representative immunohistochemistry staining in pediatric and adult patients with AD, patients with psoriasis *(PSO)*, and control subjects. $CD3^+$ T cells **(A)**, $CD11c^+$ DCs **(B)**, $Fc\varepsilon RI^+$ staining **(C)**, and respective cell counts **(D-F)** are shown. Values are presented as means ± SEMs. *Stars above and below error bars* denote comparisons with matched healthy control skin *(black stars)* and psoriasis *(blue stars)*, respectively. *LS*, Lesional; *NL*, nonlesional. $*P < .05$, $**P < .01$, and $***P < .001$.

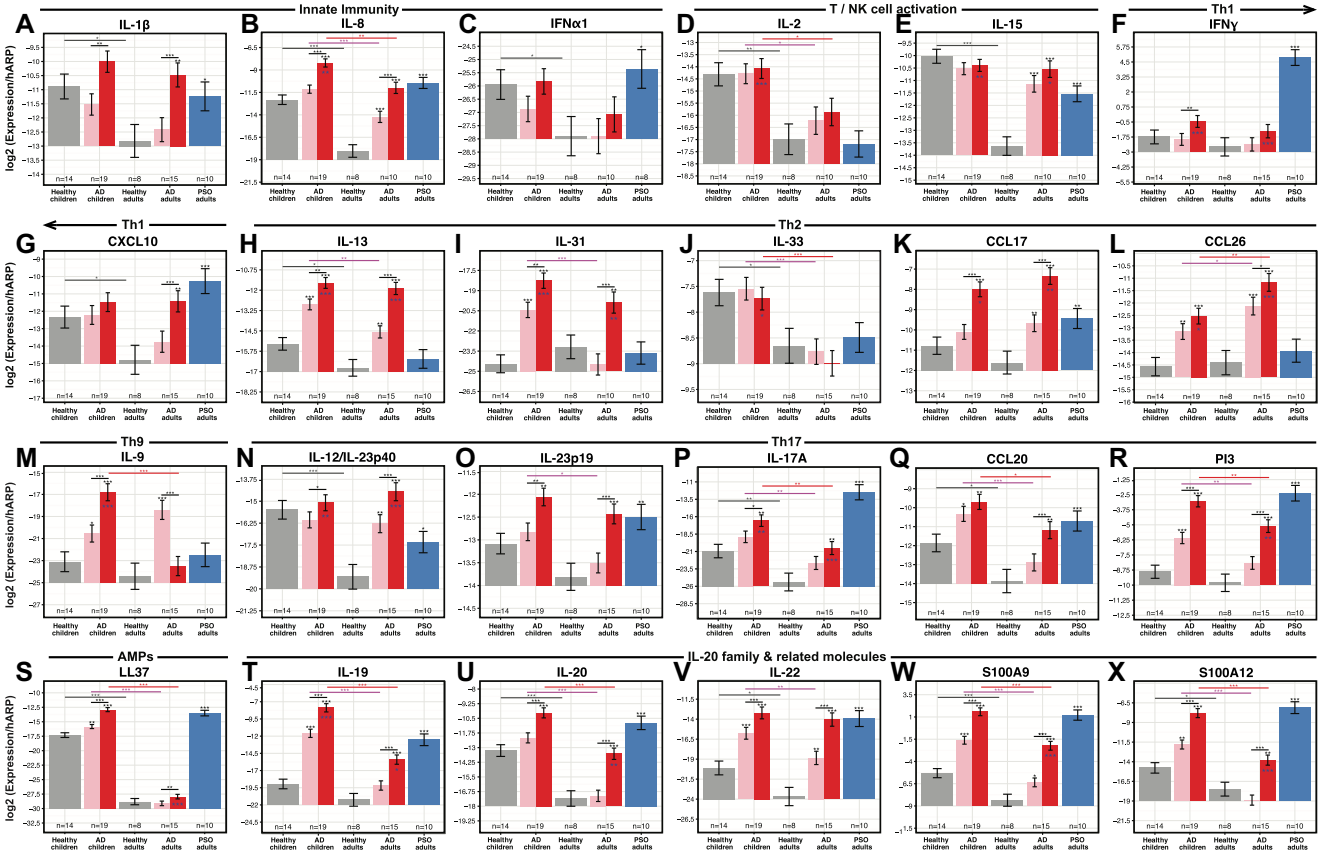

FIG 4. A-X, Quantitative real-time PCR comparisons of selected inflammatory and epidermal barrier markers are shown. Values show $\log_2$ expression/hARP and presented as means ± SEMs. *Stars above and below error bars* denote comparisons with matched healthy control skin *(black stars)* and psoriasis *(blue stars)*, respectively. $*P < .05$, $**P < .01$, and $***P < .001$.

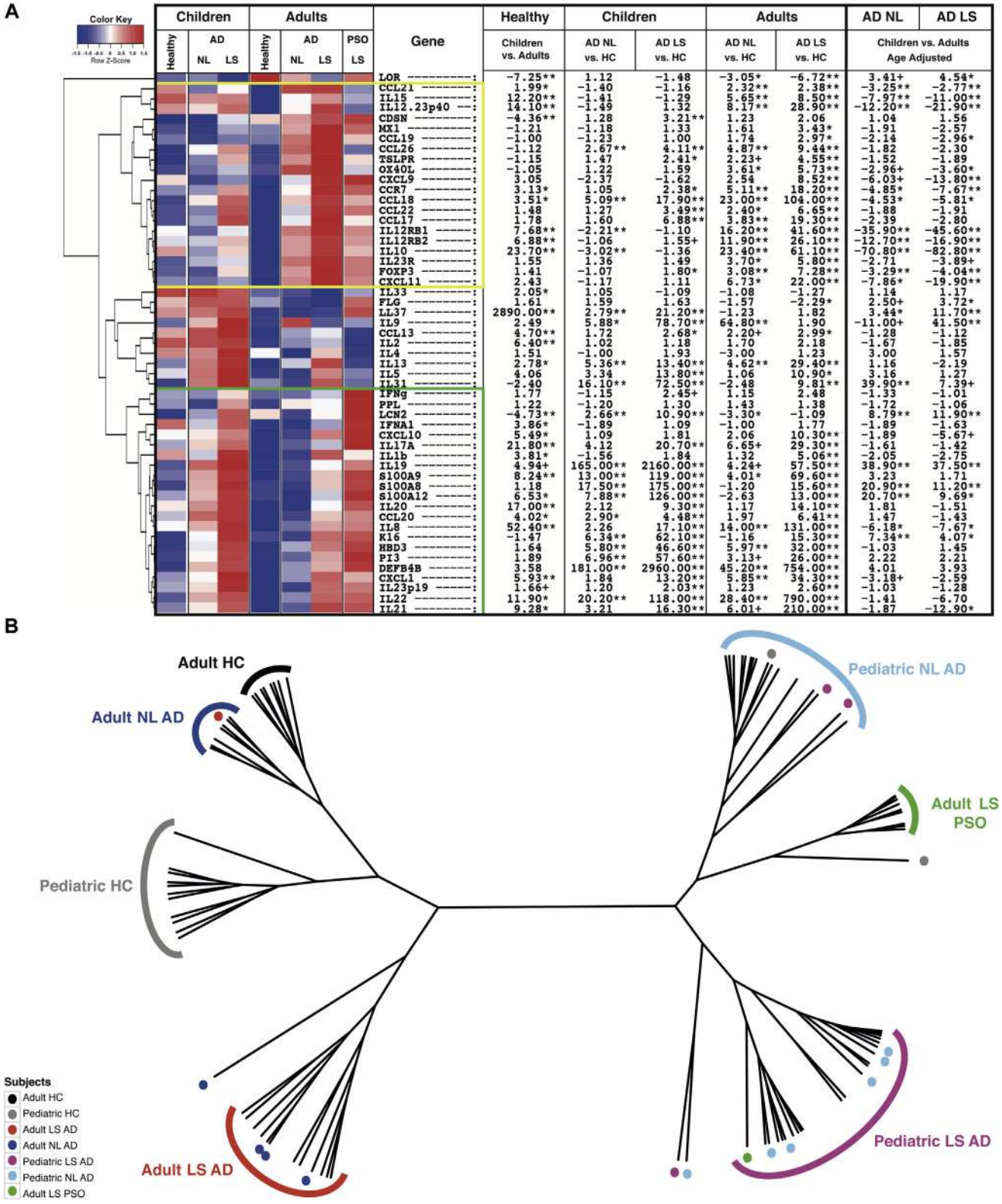

FIG 5. Unsupervised hierarchical clustering of mRNA/hARP expression. **A,** Heat map (mean) with fold changes. *Yellow box,* Cluster of upregulation in adult versus pediatric patients with AD; *green box,* upregulation in pediatric patients with AD/psoriasis versus adult patients with AD. $+P < .1$, $*P < .05$, and $**P < .01$. *Red,* Upregulation; *blue,* downregulation. **B,** Unsupervised clustering of samples (phylogenetic tree) based on expression profiles of 53 immune/barrier markers: distance, Pearson correlation; agglomeration, average. *Single dots* denote individual samples interspersed in otherwise homogeneous clusters. *LS,* Lesional; *NL,* nonlesional.

Much higher immune activation was seen in nonlesional pediatric versus adult AD skin, which might reflect AD initiation (Fig 5, *A*).

In comparison with adult healthy skin, pediatric control skin showed large increases in innate markers (IL-1β, IL-8, and IFN-α1), T/natural killer cell activation, and regulatory markers (IL-2, IL-15, and IL-10), with less evident increases in pediatric versus adult lesions compared with respective controls ($P < .05$; Fig 4, *A-E*, and Fig 5, *A*; and see Fig E2, *X*, and Table E4 in this article's Online Repository at www.jacionline.org). T_H17/T_H22-related genes (IL-17A, IL-12/23p40, CCL20, LL37, and IL-22) also showed significant increases in pediatric versus adult control subjects ($P < .05$; Figs 4, *N*, *P*, *Q*, *S*, and *V*, and 5, *A*, and see Table E4). Much smaller increases in IFN-γ–related mRNAs (CXCL9, CXCL10, CXCL11, and MX1) were seen in pediatric versus adult patients with AD (compared with respective control subjects), although IFN-γ levels were comparable (Figs 4, *G*, and 5, *A*, and see Fig E2, *A-C*, and Table E4). Expression of The IL-12/IL-23p40 and IL-12/23 receptor subunits *IL12RB1* and *IL12RB2* were significantly increased in pediatric versus adult control subjects yet were significantly decreased in pediatric versus adult patients with AD (Figs 4, *N*, and 5, *A*, and see Fig E2, *D* and *E*, and Table E4). As expected,[43] adult patients with psoriasis had significant increases in innate (IL-1β, IL-8, and IFN-α1), T_H1/IFN-γ–related (IFN-γ and CXCL10), T_H17/IL-23 and related antimicrobial peptides (AMPs; IL-17A, IL-23p19, CCL20, DEFB4, and LL37) and differentiation markers (FLG, loricrin, and PPL) compared with control subjects ($P < .05$; Figs 4, *A-C*, *F*, *G*, *O-Q*, and *S*, and 5, *A*, and see Fig E2, *Q*, and Table E4).

Levels of T_H2 cytokines (IL-13, CCL17, CCL18, CCL22, CCL26, OX40 ligand [OX40L], and thymic stromal lymphopoietin receptor [TSLPR]) were greatly increased in both pediatric and adult patients with AD; with exceptions of IL-5 and IL-31, increases were much higher in adults (Figs 4, *H*, *I*, *K*, and *L*, and 5, *A*, and see Fig E2, *H* and *J-M*, and Table E4). IL-33 levels were increased in children but were not increased in patients with AD (Figs 4, *J*, and 5, *A*, and see Table E4). IL-9/T_H9 levels were markedly increased in pediatric AD lesions (Figs 4, *M*, and 5, *A*, and see Table E4).

Although levels of IL-17–related mediators and AMPs were already increased in healthy children compared with adults (ie, LL37: 2890-fold changes in children/adults; $P < .01$), highly significant increases were still observed in many of these markers in pediatric patients with AD (IL-19, S100A8, S100A12, lipocalin 2, and LL37), even after adjusting for baseline levels ($P < .05$; Fig 5, *A*). Overall, expression of T_H17-related and some hyperplasia mediators (IL-19, S100As, and K16) in pediatric patients with AD were comparable with that seen in patients with psoriasis (Figs 2, *G* and *I*; 4, *T*, *W*, and *X*; and Fig 5, *A*, and see Table E4). IL-20, IL-21, and IL-22 levels showed significant increases in pediatric versus adult control subjects, as well as increased expression in patients with AD compared with control children but not compared with adult patients with AD after adjusting for respective healthy skin (Fig 5, *A*). In contrast, levels of regulatory markers (IL-10 and forkhead box P3 [FOXP3]) showed significant decreases in pediatric versus adult patients with AD, despite increases in respective control subjects (Fig 5, *A*, and see Fig E2, *X* and *Y*, and Table E4). Levels of differentiation markers (FLG and loricrin) were reduced only in adult patients with AD (Fig 5, *A*).

Early pediatric AD shows phenotypic similarities to psoriasis

In view of similar activation in many inflammatory genes in childhood AD and psoriasis (Fig 5, *A*, green box) and to better visualize the relations between pediatric and adult patients with AD, patients with psoriasis, and respective control subjects, we performed unsupervised clustering analyses using all expression values. Results are represented as a phylogenetic tree (Fig 5, *B*). As expected, lesional psoriasis (a highly inflammatory disease typically involving adults)[43] clustered far from adult AD. Surprisingly, lesional and nonlesional pediatric AD clustered closely with lesional adult psoriasis and far from healthy control subjects and adult AD. Lesional and nonlesional adult AD formed distinct clusters, which were much more distant from their pediatric counterparts, reflecting molecular differences between early AD in children and chronic adult disease (Fig 5, *B*).

Correlations of clinical, cellular, and molecular markers in pediatric patients with AD

We next evaluated associations of different clinical measures (SCORAD, EASI, ADQ/pruritus, PIQoL, and TEWL) with immune and barrier pediatric AD skin biomarkers. A graphic representation of the distance between variables for nonlesional and lesional pediatric AD are presented as phylogenetic trees (Fig 6 and see Fig E3 in this article's Online Repository at www.jacionline.org, respectively) and as heat maps showing positive (red) or negative (blue) correlations, with the color intensity reflecting strength of correlations. In patients with lesional AD, clinical scores clustered with hyperplasia markers (Ki-67 and thickness) and close to T_H2 cytokines (IL-13 and IL-31), IgE, and $FcεRI^+$ (see Fig E3, *A*). IL-17A (and its associated chemokine CXCL1) formed a single cluster with IL-20 family cytokines (IL-19, IL-20, and IL-22; see Fig E3, *A* and *B*, green box); AMPs (DEFB4B, LL37, HBD3, and S100As) clustered with IL-23p19 and IL-23 receptor (see Fig E3, *A* and *B*, black box).

Several positive correlations (see Table E5 in this article's Online Repository at www.jacionline.org) were found between clinical activity scores (EASI and SCORAD scores, see Table E5, *A* and *B*) and expression of inflammatory mediators in AD lesions, including innate (IFN-α1), T_H2-related (CCL18 and TSLPR), or T_H17/T_H22-related (IL-19 and S100A8) markers, as well as quality of life, pruritus (Pruritus ADQ and PIQoL), and barrier (TEWL) measures. Impressive correlations were found between TEWL, a functional barrier measure, and many immune markers, particularly T_H17/T_H22 (IL-21, S100As, IL-23R, CCL20, IL-12/23p40, IL-23p19, and IL-22) and T_H2/TSLPR markers (see Table E5, *C*). Pruritus scores showed correlations with disease activity and epidermal hyperplasia (see Table E5, *D* and *E*). Serum IgE levels positively correlated with cellular infiltrates (particularly $FcεRI^+$ cells) and IL-13 and negatively with IFN-γ– and IL-17–related (IL-17A, S100A9, and peptidase inhibitor 3 [PI3]/elafin) genes (see Table E5, *F*).

Many correlations were observed at the nonlesional skin level (Fig 6 and Tables II-X), with a clustering pattern similar to that seen in lesional skin (Fig 6, *A*). Clinical scores clustered in proximity to hyperplasia/Ki-67 and T_H2 markers (IL-4, IL-5, OX40L, and TSLPR). Strong correlations were observed between disease activity scores and T_H2 (TSLPR and OX40L)

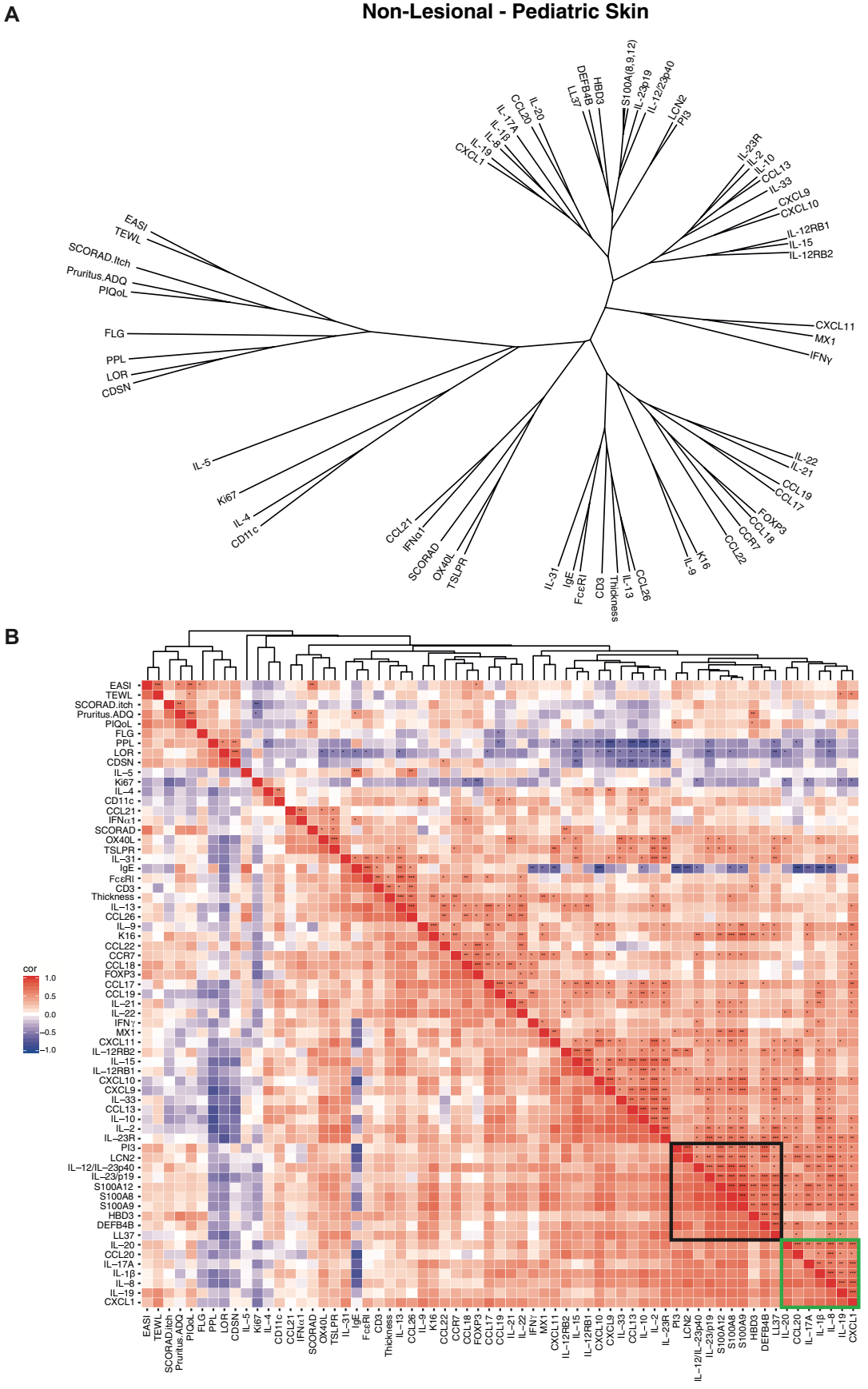

FIG 6. Correlation matrix of clinical, immune, and barrier measures in pediatric patients with nonlesional AD. **A,** Phylogenetic tree showing unsupervised hierarchic clustering using Spearman correlations as a similarity metric and average agglomeration algorithm. **B,** Correlation heat map and dendrogram. *Red/blue*, Positive/negative correlations. Intensity and *stars* reflect the strength and significance of pairwise correlations, respectively. *Green* and *black boxes* denote IL-17 and AMP-associated clusters. **P* < .05, ***P* < .01, and ****P* < .001.

TABLE II. Spearman correlations with EASI in nonlesional pediatric skin

Marker	ρ	*P* value
TEWL	0.733	.001
PIQoL	0.668	.002
SCORAD	0.648	.003
Pruritus ADQ	0.553	.016
FOXP3	0.47	.044
FLG	0.467	.046
IL19	0.435	.064
CCL18	0.414	.079
CDSN	0.409	.083

CDSN, Corneodesmosin.

TABLE III. Spearman correlations with SCORAD scores in nonlesional pediatric skin

Marker	ρ	*P* value
EASI	0.648	.003
IL12RB2	0.581	.009
TSLPR	0.561	.012
PIQoL	0.544	.016
Pruritus ADQ	0.497	.03
OX40L	0.478	.039
PI3	0.424	.07
IL12RB1	0.424	.07
FOXP3	0.418	.075
FcεRI	0.401	.099
CCL21	0.4	.09
HBD3	0.395	.094
CCL18	0.386	.103

HBD3, Human beta defensin 3.

TABLE IV. Spearman correlations with IgE in nonlesional pediatric skin

Marker	ρ	*P* value
FcεRI	0.786	.048
IL5	0.714	.088
IL13	0.679	.1
PI3	−0.786	.048
IL1B	−0.786	.048
CXCL10	−0.786	.048
CCL20	−0.821	.034

TABLE V. Spearman correlations with TEWL in nonlesional pediatric skin

Marker	ρ	*P* value
EASI	0.733	.001
CXCL1	0.554	.023
IL19	0.529	.031
PIQoL	0.456	.066
CCL18	0.424	.091
CDSN	0.409	.104

CDSN, Corneodesmosin.

and IL-17–associated (IL-19 and PI3/elafin) markers (Fig 6, *B*, and Tables II and III). FOXP3 levels were associated also with disease activity (Tables II and III). High correlations were found between serum IgE levels and $FcεRI^+$ cell counts in nonlesional skin, which approached significance with IL-5 and IL-13/T_H2 (Table IV). Similar to lesional skin, negative correlations were found between IgE levels and T_H1/T_H17 markers (CXCL10, CCL20, and PI3/elafin; see Table E2, *C*). TEWL in nonlesional skin was highly correlated with EASI scores, as well as with several T_H17-related (CXCL1 and IL-19) and to a lesser extent with T_H2-associated (CCL18) genes (Table V). Many correlations were found between hyperplasia measures (thickness and K16) and immune markers (Tables VI and VII). Epidermal thickness showed highest associations with T_H2 mediators (IL-13 and CCL26), followed by correlations with known hyperplasia mediators (IL-22, S100As, and IL-21; Table VI).[44] High correlations were also found between K16 levels and IL-22/IL-17–related (S100As, IL-12/23p40/IL-23p19, IL-22, IL-17A, LL37, and IL-19) and a few T_H2 (CCL17 and CCL18) markers (Table VII).

DISCUSSION

Recent data from adults with moderate-to-severe AD shed light on the immune and epidermal abnormalities that characterize nonlesional and acute and chronic AD lesional skin.[4,6,8,38,45] Acute lesions in adults are characterized by robust T_H2/T_H22 activation, with some IL-17 skewing. With disease chronicity, there is intensification of these axes and marked T_H1 activation.[4,46-48] Barrier defects and, to a lesser extent, immune abnormalities are apparent in nonlesional skin, with abnormalities in terminal differentiation proteins, tight junctions, and lipids.[26,38,45,49-55] Clinical severity in adult patients with AD has been positively associated with increases in T_H2 and T_H22/T_H17 markers and negatively correlated with differentiation markers. Furthermore, disease improvement with broad and specific treatments lead to decreases in $T_H2/T_H22/T_H17$ cytokine levels and some increases in barrier gene expression.[7-9,11] The increased pathogenic understanding in adult patients with AD is now translating into rapid development and testing of therapeutics for AD.[10,56]

Nevertheless, AD usually begins in children younger than 5 years, when it also shows the highest prevalence,[57-60] and the current concepts of pathogenesis largely rely on studies from adult patients in whom the disease is usually present for many years.[2,6,52] Little is known about alterations in skin immunity and barrier function that occur during early-onset AD in children or even about expression patterns in healthy skin of young children. This paucity of investigation of early pediatric AD skin has limited advancement of targeted treatments for children, with their large unmet need for safe and effective therapeutics.[2] The few US Food and Drug Administration–approved treatments for children with AD (some topical corticosteroids and calcineurin antagonists) were based on empirical evidence and are suboptimal for many pediatric patients with moderate-to-severe AD.[61,62] Comparing the cellular and molecular changes that characterize lesional and nonlesional skin from recent-onset AD in infants and young children with healthy pediatric skin, as well as with adult AD and psoriasis as a point of reference for polar inflammatory diseases, is critical for advancing current interventions into children and might suggest novel targets for pediatric AD.

This is the first study of the cellular and molecular changes that characterize lesional and nonlesional skin from infants with early

TABLE VI. Spearman correlations with epidermal thickness in nonlesional pediatric skin

Marker	ρ	*P* value
IL13	0.704	.001
CCL26	0.684	.002
CD3	0.654	.003
MX1	0.623	.006
CCR7	0.615	.007
K16	0.589	.0102
IL22	0.548	.0185
S100A8	0.528	.024
IL12RB1	0.527	.025
CXCL11	0.521	.026
IL31	0.504	.033
CCL22	0.482	.043
IL21	0.475	.046
FcεRI	0.471	.049
IL2	0.468	.05
FOXP3	0.449	.062
S100A9	0.444	.065
IL12RB2	0.431	.074
TSLPR	0.417	.085
CD11c	0.412	.089
CCL18	0.408	.093
IFN-γ	0.402	.099
LL37	0.394	.105

TABLE VII. Spearman correlations with K16 mRNA expression in nonlesional pediatric skin

Marker	ρ	*P* value
S100A9	0.717	.001
S100A8	0.698	.001
IL9	0.694	.001
IL12 23p40	0.644	.004
S100A12	0.635	.004
CCR7	0.623	.005
IL22	0.602	.008
HBD3	0.589	.009
Epidermal thickness	0.589	.01
MX1	0.54	.018
CCL17	0.537	.019
IL17A	0.525	.021
CCL22	0.525	.023
LL37	0.518	.025
CXCL1	0.514	.026
CXCL10	0.486	.037
DEFB4B	0.467	.046
IL19	0.465	.047
IL23p19	0.444	.058
SCORAD itch	0.441	.059
CD3	0.44	.0678
IL23R	0.427	.07
CCL18	0.423	.073
FOXP3	0.414	.079
CXCL9	0.414	.079
IL21	0.399	.091
CXCL11	0.398	.092
IL31	0.397	.093
IL13	0.391	.099
CCL26	0.381	.11

HBD3, Human beta defensin 3.

AD (<5 years old, within 6 months of disease onset). In healthy pediatric skin we found an array of increased innate and IL-17/IL-22–related markers and AMPs when compared with adult control subjects (IL-8, IL-17A, LL37, and defensins), which is consistent with previous reports from neonatal mice or human foreskin.[63,64] This pattern might reflect the response of young skin to infectious agents when adaptive immunity is not fully functional[65] but could also promote the development of AD, which peaks in infancy and early childhood.[2]

Nonlesional skin of infants with a new diagnosis of AD already harbors keratinocyte hyperactivation (K16 and S100A) accompanied by significant inflammation, as evidenced by infiltrates ($FcεR1^+$ cells)[40,41] and activated cytokines increased to levels often higher than those in adults.[38] Consistently, disease activity was correlated with nonlesional pediatric expression levels of several immune mediators that might play a role in disease initiation, including TSLPR and OX40L.[66-69]

Lesional pediatric AD skin showed marked epidermal hyperplasia comparable with that in adult patients chronically affected by AD and psoriasis. TEWL, a predictor of allergic sensitization in infancy,[70] closely correlated with T_H17/T_H22 and a few T_H2 biomarkers, suggesting an interplay between activation of these axes and barrier dysfunction. The negative correlation between IgE and T_H17 molecules supports the concept that intrinsic AD harbors stronger T_H17 responses.[5]

Despite remarkable hyperplasia and abnormal TEWL,[70,71] the FLG deficiency of adult AD[72,73] is missing in patients with early AD, which is consistent with a previous report in nonlesional adolescent skin from *FLG* wild-type patients.[26] *FLG* has been suggested to play a key role in early development of AD and other atopic disorders because of a breach in the epidermal barrier allowing for sensitization to environmental antigens.[44,74] Our findings might challenge the notion of filaggrin as central for disease elicitation and an instigator of the atopic march.[75] Another feature of adult AD is reduction in AMP levels, in contrast to psoriasis,[76] explaining why patients with AD but not those with psoriasis are predisposed to recurrent infections.[76,77] In our pediatric AD cohort, AMP levels (LL37, DEFB4B, and lipocalin 2) were highly increased, often to levels even higher than those in patients with psoriasis. These increased AMP levels could potentially trigger skin inflammation through DNA/RNA complexes binding Toll-like receptors 7 to 9, similar to what is seen in patients with psoriasis,[78,79] in which LL37 might function as an autoantigen, possibly initiating and perpetuating the disease.[80]

While T_H2 polarization is generally higher in adult patients with AD, IL-9 and IL-33, previously associated with peanut and mite sensitization and induction of food allergies,[81-85] which are frequent in children but rare in adults,[2] were increased in pediatric control subjects and/or lesional pediatric AD. T_H17-related mediators showed consistently higher levels in children with AD; this is reminiscent of what is seen in patients with psoriasis, which is considered a T_H17-centered disease.[43] Our findings are in line with the dominant role of the T_H17 immune axis in some murine models of AD,[86,87] with T_H17 being critically involved in the regulation of T_H2 responses.[46] Importantly, IL-17 can be produced by several cellular subsets that are best determined by using flow cytometry,[88,89] but this analysis was not possible with the limited available tissue. The profound epidermal hyperplasia seen in early pediatric AD is also reflected

TABLE VIII. Spearman correlations with PIQoL in nonlesional pediatric skin

Marker	ρ	*P* value
Pruritus ADQ	0.702	.001
EASI	0.668	.002
SCORAD	0.544	.016
HBD3	0.476	.039
PI3	0.469	.043
TEWL	0.456	.066
DEFB4B	0.449	.054
SCORAD itch	0.442	.058
CCL22	0.408	.083
FOXP3	0.382	.107

HBD3, Human beta defensin 3.

TABLE IX. Spearman correlations with SCORAD itch in nonlesional pediatric skin

Marker	ρ	*P* value
Pruritus ADQ	0.685	.001
PIQoL	0.442	.058
K16	0.441	.059
IL12.23p40	0.432	.065
PPL	0.405	.085

by significant increases in levels of IL-20 family cytokines (IL-19, IL-20, and IL-22) and associated S100As already in nonlesional skin. These cytokines induce hyperplasia,[90,91] and S100As are chemotactic mediators[92,93] that affect cell growth and differentiation[94,95] and might contribute to the recruitment of immune cells to trigger disease onset.[92,96,97] In particular, there were impressive upregulations of IL-19 levels in early-onset nonlesional and lesional pediatric skin. IL-19 is induced by both IL-17 and IL-4/IL-13[98,99] and amplifies the effects of IL-17 on keratinocytes.[98] Indeed, IL-19 levels and T_H17 activation are highly increased in Asian patients with AD, which bears some similarities with psoriasis.[3] Thus the marked hyperplasia and correlations between hyperplasia and T_H2 markers in skin of pediatric patients with AD might be attributable to highly increased IL-19 levels, possibly bridging T_H2 and T_H17 axes.

Although increased T_H2 and T_H17 responses are consistent with a bias toward these axes in blood from healthy infants,[100] our skin data differ from peripheral blood findings in children with AD, which showed primarily T_H2 skewing among skin-homing T cells.[36] Perhaps pathogenically relevant T-cell populations have already migrated to the skin and cannot be detected in blood during early AD, in contrast to longstanding adult disease, in which T_H2- and T_H22-skewed T cells are found in the periphery.[101]

Despite strong T_H2 induction, both pediatric lesional and nonlesional AD clustered around psoriasis but not adult AD. These data suggest profound differences between new-onset pediatric and longstanding adult disease, with important implications for future therapeutic approaches. In addition, nonlesional pediatric AD shows strong immune activation, clustering farther away from normal skin than adult lesional and nonlesional AD. Thus nonlesional pediatric AD might facilitate an understanding of mechanisms permissive for disease initiation, as reflected by decreased T_H1 and regulatory responses

TABLE X. Spearman correlations with pruritis ADQ in nonlesional pediatric skin

Marker	ρ	*P* value
PIQoL	0.702	.001
SCORAD itch	0.685	.001
HBD3	0.6	.008
EASI	0.553	.0156
SCORAD	0.497	.0303

HBD3, Human beta defensin 3.

but upregulated T_H17/T_H22-associated mediators when compared with adult disease. The profound immune activation in nonlesional pediatric skin might suggest the need for early systemic intervention at disease initiation, especially if future studies suggest that early intervention reduces the development of allergic disease of other organs (the atopic march).

In conclusion, AD begins as a multicytokine response in the skin, with marked T_H17, T_H9, T_H2, and T_H22 activation at disease onset in both lesional and nonlesional skin; differential immune skewing and barrier responses compared with adult patients with AD; and shared molecular features with psoriasis. These findings are likely to result in both a different understanding of AD onset and distinct treatment approaches for infants and children.

Clinical implications: Targeting of multiple cytokine axes might be needed to effectively treat early-onset AD in children.

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