Evolution of pathologic T-cell subsets in patients with atopic dermatitis from infancy to adulthood

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GRAPHICAL ABSTRACT

Evolution of polar T-cell subsets in healthy controls and atopic dermatitis patients from infancy to adulthood

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Background: The circulating immune phenotype was defined in adults and young children with early atopic dermatitis (AD), but chronicologic changes in the blood of infants and children with AD through adolescence have not been explored.

Objective: We sought to compare immune activation and cytokine polarization in the blood of 0- to 5-year-old (n = 39), 6- to 11-year-old (n = 26), 12- to 17-year-old (n = 21) and 18-year-old or older (n = 43) patients with AD versus age-matched control subjects.

Methods: Flow cytometry was used to measure IFN-γ, IL-9, IL-13, IL-17, and IL-22 cytokine levels in CD4+CD8- T cells, with inducible costimulator molecule and HLA-DR defining midterm and long-term T-cell activation, respectively, within skin-homing/cutaneous lymphocyte antigen (CLA)+ versus systemic/CLA- T cells. Unsupervised clustering differentiated patients based on their blood biomarker frequencies.

Results: Although CLA+ Tfh1 frequencies were significantly lower in infants with AD versus all older patients (P < .01), frequencies of CLA+ Tem2 T cells were similarly expanded across all AD age groups compared with control subjects (P < .05). After infancy, CLA+ Tfh1 frequencies were increased in patients with AD in all age groups, suggesting systemic immune activation with disease chronicity. IL-22 frequencies serially increased from normal levels in infants to highly significant levels in adolescents and adults compared with levels in respective control subjects (P < .01). Unsupervised clustering aligned the AD profiles along an age-related spectrum from infancy to adulthood (eg, inducible costimulator molecule and IL-22).

Conclusions: The adult AD phenotype is achieved only in adulthood. Future longitudinal studies, comparing the profile of patients with cleared versus persistent pediatric AD, might introduce safe, effective, and age-tailored targeted approaches.

The therapeutic arsenal available for 0- to 12-year-old patients with AD is limited to topical agents, broad systemic immune suppressors, or both. Active development of targeted therapeutics is ongoing for adults and adolescents with AD and will eventually move to children, further necessitating the elucidation of pediatric endotypes at successive preadult age groups to introduce safe, effective, and age-tailored targeted approaches.

We compared T-cell memory subset activation and polarized CD4/CD8 subset frequencies within the skin-homing/cutaneous lymphocyte antigen (CLA)+ and systemic/CLA- compartments in the blood of infants and toddlers (0-5 years old), young children (6 to 11 years old), adolescents (12 to 17 years old), and adults (≥18 years old) with moderate-to-severe AD. Age-matched healthy control subjects were included to differentiate pathologic from physiologic immune maturation. We found that decreased Tfh1/Tem2 ratios were shared across all AD ages, but unique fingerprinting characterizes individual AD age groups, differentiating them from their age-matched peers. Our intracellular and T-cell blood biomarker data grouped the AD cohort, but not the control subjects, into 3 unique age phenotypes aligned along a spectrum.

Abbreviations used

- AD: Atopic dermatitis
- CLA: Cutaneous lymphocyte antigen
- EASI: Eczema Area and Severity Index
- FCH: Fold change
- ICOS: Inducible costimulator molecule
- ILC: Innate lymphoid cell
- Tfh: Cytotoxic T
- Tcm: Central memory T
- Tem: Effector memory T
- TEWL: Transepidermal water loss
- Treg: Regulatory T

Key words: Atopic dermatitis, endotypes, T cell, cutaneous lymphocyte antigen, IL-13, IL-22, IFN-γ, inducible costimulator molecule, HLA-DR

Infancy, childhood, and adolescence are critical periods for immune system maturation. Early abnormal immune development can cause immune-related disorders. Indeed, 85% of patients with atopic dermatitis (AD) present before 5 years of age. Although young adults have a different AD phenotype from elderly patients, immune changes in patients with AD between early childhood and adulthood are unknown.

Blood studies that dissect developmental changes from infancy through adulthood are limited and primarily focused on Tfh1/Tem2 subsets. Some studies with healthy control subjects showed expansion of Tfh1/Tem2/cytotoxic T (Tc1) 1 subsets with age, whereas others reported no changes in Tfh2/Tc2 subsets over time. Immature IFN-γ response and low Tfh1/Tc1 cell frequencies were seen in early stages of normal development, and abnormal Tfh1/Tem2 ratios seen in cord blood and infants with AD were described. However, few studies compared pediatric and adult AD populations, and none directly compared consecutive age groups of patients with AD with age-matched control subjects, which is critical in understanding normal versus pathologic development of acquired immunity.

METHODS

Patients’ characteristics and blood samples

Blood was obtained (with signed institutional review board–approved informed consent from parents and patients ≥12 years of age) from 39 infants and toddlers (0-5 years old; mean, 23 months), 26 children (6-11 years old; mean, 7.5 years), 21 adolescents (12-17 years old; mean, 14.9 years), and 43 adults (≥18 years old; mean, 43 years) with moderate-to-severe AD, as well as healthy age-matched control subjects (24-30 in each group; demographic and laboratory data are shown in Table E1 in this article’s Online Repository at www.jacionline.org). Sensitivity analyses on patient subsets who were matched for all demographic parameters yielded similar results to analysis, including all subjects (see Table E2, A and B, in this article’s Online Repository at www.jacionline.org).

Disease severity was scored by using SCORAD scores for adults and SCORAD scores, Eczema Area and Severity Index (EASI) scores, and Atopic Dermatitis Quickscore in those less than 18 years old. The Atopic Dermatitis Quickscore is a parent-administered tool that assesses involvement and laboratory data are shown in Table E1 in this article’s Online Repository at www.jacionline.org).
allergies (allergic rhinitis/asthma/allergic conjunctivitis/environmental), food allergies, both, or neither. Control subjects had no personal history of AD. Only 1 child and 5 adolescent control subjects had histories of noncutaneous atopic manifestations; however, sensitivity analysis excluding these subjects did not alter the results (see Table E2, C).

Isolation of PBMCs
PBMCs were isolated from whole blood by using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Briefly, the blood placed under a Ficoll gradient, and after spinning, PBMCs were collected at the interface between the plasma and the Ficoll gradient (see the Methods section in this article’s Online Repository at www.jacionline.org).

Stimulation of blood cell populations for cytokine responses
*Ex vivo* cell activation is required to detect cytokine production because less than 1% of nonstimulated cells produce cytokines. Whole blood was incubated with phorbol 12-myristate 13-acetate (25 ng/mL) plus ionomycin (2 μg/mL) in the presence of brefeldin A (10 μg/mL) for 4 hours at 37°C to induce cytokine responses. After stimulation, red blood cells were lysed with FACS lysing solution to obtain leukocytes (see the Methods section in this article’s Online Repository).

Cell-surface and intracellular staining on PBMCs and stimulated and nonstimulated CD4/CD8 T cells
PBMCs were stained with fluorochrome-labeled antibodies to cell-surface markers (CD3, CD8, CD4, CD45RO, CCR7, inducible costimulator molecule [ICOS], HLA-DR, CLA, CCR4, CD25, and CD127). Stimulated and nonstimulated blood cells were also stained for cell-surface markers (CD3, CD4, and CLA [CD8+ T cells were gated thorough the CD3+CD4- T-cell subpopulation]) and permeabilized with FACS/perm to stain for cytokines, including IL-13, IL-22, IL-9, IFN-γ, and IL-17 (see the Methods section in this article’s Online Repository).

Statistical analysis
Statistical analyses were performed with R software (www.R-projets.org). Means and medians were compared by using the Welch *t* test and the Wilcoxon-Mann-Whitney test, respectively. Unsupervised hierarchical clustering of variables (T-cell subset frequencies, age, and clinical scores) was performed by using the R package “hclust,” with a McQuitty agglomeration algorithm and Spearman coefficient as a similarity metric and presented as a heat map and a dendrogram. Individual scatter plots were constructed that display Spearman coefficients, 95% CIs, and P values for samples from patients with AD and healthy control subjects. We performed k-means unsupervised clustering across principal components of the frequencies of all AD subsets and separately among all healthy control subsets. We found that 3 clusters separated patients with AD, but not control subjects, along a chronologic age spectrum. ANOVA, in conjunction with the Tukey test, was used to find markers that differentiated any 2 clusters.

RESULTS

Flow cytometry was used to measure frequencies of IFN-γ−, IL-9−, IL-13−, IL-17A−, and IL-22− polarized T cells, defining T<sub>H1</sub>/T<sub>C1</sub>, T<sub>H9</sub>/T<sub>C9</sub>, T<sub>H2</sub>/T<sub>C2</sub>, T<sub>H17</sub>/T<sub>C17</sub>, and T<sub>H22</sub>/T<sub>C22</sub> subsets in CD4<sup>+</sup>/CD8<sup>+</sup> T cells, respectively. Cell-surface staining was used to assess expression of midactivation (ICOS) and late activation (HLA-DR) markers in Tcm (CCR7<sup>+</sup>CD45RO<sup>+</sup>) and effector memory T (Tcm; CCR7<sup>+</sup>CD45RO<sup>+</sup>) cells in skin-homing/cutaneous/CLA<sup>+</sup> and systemic/CLA<sup>−</sup> compartments.

Patients and control subjects were divided into 4 consecutive age groups (infants and toddlers 0-5 years old, children 6-11 years old, adolescents 12-17 years old, and adults ≥18 years old). To display both healthy versus pathologic developmental changes and immune abnormalities within each age group versus control subjects, we present 2 types of comparison plots; both contain similar data but focus on either patients with AD versus control subjects for each age group or patients with AD versus control subjects across all ages.

The comparison plots presented below contain both the mean and median ± SE and their respective P values to better represent the effect of value distribution. Results discuss mean values, whereas median percentages for main comparisons discussed are presented in Table E3 in this article’s Online Repository at www.jacionline.org.

Skin-homing memory T-cell expansion and ICOS activation feature in early AD
Tcm and Tem cells are the main components of the adaptive immune system, harboring distinct homing capacities. Although both express the skin-homing marker CLA, only Tcm cells retain CCR7 positivity, which enables them to migrate into lymph nodes and function as an immunologic reserve. After gating on CD3<sup>+</sup> viable T cells using flow cytometry, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells were defined and analyzed separately. CCR7 and CD45RO were used to differentiate memory subsets within the CD4 and CD8 populations. CCR7<sup>+</sup>CD45RO<sup>−</sup> defined naive cells, CCR7<sup>+</sup>CD45RO<sup>+</sup> defined Tem cells, CCR7<sup>+</sup>CD45RO<sup>−</sup> defined Tcm cells, and CCR7<sup>+</sup>CD45RO<sup>−</sup> defined effector/Temra/terminally differentiated T cells (see Fig E1, A-C, in this article’s Online Repository at www.jacionline.org). We then further defined the activated ICOS/HLA-DR−activated Tcm/Tem cell subset using CLA to segregate skin-homing (CLA<sup>+</sup>) versus systemic (CLA<sup>−</sup>) subsets.

Normal development was characterized by a slight decrease in frequencies of CLA<sup>−</sup>CD4<sup>+</sup> Tem (but not Tcm) cells between infancy and childhood (infants: 26.7% vs children: 19%, P = .01; Fig 1, A and B, and see Fig E1, D and E), but this decrease was significantly more evident in patients with AD (P < .05; Fig 1, A and B, and see Fig E1, D and E). CD4<sup>+</sup>CLA<sup>−</sup> Tem/Tcm cell counts were significantly greater in infants with AD (Tcm cells: 20.4% vs 13.4%, P = .006; Tem cells: 38% vs 26.7%, P = .002; see Fig E1, D and E) and children with AD (Tcm cells: 18.8% vs 11.3%, P = .01; Tem cells: 27.7% vs 19%, P = .03; see Fig E1, D and E) than in control subjects, a difference that diminished with increasing age. T-cell memory subset fluctuations with age are presented in Fig E2 in this article’s Online Repository at www.jacionline.org.

Because of increased proportions of effector and naive cells, CD8<sup>+</sup> Tcm/Tem cell subset frequencies decreased between infancy and childhood exclusively in control subjects (Tcm cells: 12.8% [infants] vs 5.6% [children], P = .008; Tem cell: 15.4% [infants] vs 5% [children], P < .001; Fig 1, C and D), leading to higher frequencies in children with AD (P < .01), but otherwise, frequencies were overall similar between patients with AD and...
control subjects over time (see Fig E1, F and G). A significant increase in CD4+/CD8+ effector cells with age characterized patients with AD versus control subjects, who showed a decrease in this subset during the study (see Fig E2, J and L).

CD4+ Tcm/Tem ICOS levels at midactivation continuously increased in both control subjects and patients with AD; however, they decreased significantly between adolescence and adulthood exclusively in control subjects (Tcm CLA+ cells: 24% vs 9%, P = .003; Tcm CLA- cells: 9.2% vs 3.7%, P = .001; Fig 1, E-H). Both skin-homing/CLA+ and systemic/CLA- ICOS-activated CD4+ T-cell frequencies were significantly increased in infants and adults with AD versus those in respective control subjects (P < .05; Fig 1, E-H). Skin-homing CD8+ Tcm/Tem cell ICOS activation increased gradually, most notably in patients with AD (Fig 1, I-L), with frequency differences uniquely seen in adults with AD versus control subjects (Tem cells: 18.4% vs 12.5%, P = .01; Tem cells: 26% vs 13.4%, P < .001; Fig 1, I-L).

The HLA-DR antigen, indicating chronic activation,25 had similar (or even lower) expression in infants with and without AD (P > .1; see Fig E1, H-K) but started to increase in children with AD (Tcm+CD4+CLA+ AD: 15.8% vs 8.4%, P = .02; CLA-: 7.2% vs 2.8%, P = .06; see Fig E1, H and I), reaching consistently high levels across CD4+/CD8+/CLA+/CLA-/Tcm/Tem cells in adults with AD compared with healthy subjects (P < .05; CD8+ Tcm/Tem cell data are not shown; see Fig E1, H-K).

Decreased Th1/Th2 ratio characterizes AD across ages

Because T-cell activation leads to cytokine polarization, we next studied different polar T-cell subsets. Representative flow cytometric plots and the gating strategy are presented in Fig E3 in this article’s Online Repository at www.jacionline.org. Congruent with past publications,9,10 IFN-γ levels increased.

FIG 1. Frequency of CLA+ Tem (CD45RO+CCR7+) and Tcm (CD45RO+CCR7+) cells among CD4+/CD8+ T cells (A-D) and ICOS+ activation in CLA+/CLA- CD4+/CD8+ Tcm/Tem cells (E-L) in healthy control subjects and patients with AD across ages (ICOS+ CLA+/CLA-). Bar plots represent means (black)/medians (red) ± SEMs. *P < .05, **P < .01, ***P < .001, and ++P < .1.
with age in both control subjects and patients with AD, with the lowest frequencies seen in infants (Fig 2, A-D). Nevertheless, in patients with AD, IFN-γ did not reach control levels, particularly within the skin-homing compartment (CD4+CLA+: 12% [control infants] vs 7.7% [infants with AD], P = .04; 19.5% [control children] vs 13.6% [children with AD], P = .05; 18% [control adolescents] vs 13% [adolescents with AD], P = .07) until adulthood (P = .2; Fig 2, A-D, and see Fig E4, A-D, in this article’s Online Repository at www.jacionline.org). Even in adulthood, levels trended toward lower frequencies. Interestingly, the only population that showed lower systemic/CLA− CD4+ CD8+ IFN-γ levels was the 5- to 12-year-old age group (CD4+: 20% [control subjects] vs 14% [patients with AD], P = .04; CD8+: 39% [control subjects] vs 28% [patients with AD], P = .05; see Fig E4, B and D).

In control subjects IL-13+ CD4+ CLA+ levels were lowest in infants, reaching a plateau in childhood. Conversely, in patients with AD, levels were similarly increased across all ages (P > .1; Fig 2, E). Systemic/CLA− T\(\text{H2}^\text{+}\) cell counts were overall low in control subjects (Fig 2, F). Counts were significantly higher in patients with AD than in control subjects among children (2.8% vs 0.6%, P = .005; see Fig E4, F), adolescents (1.5% vs 0.7%, P = .006; see Fig E4, F), and adults (1.3% vs 0.7%, P = .005; see Fig E4, F). Although T\(\text{H2}^\text{−}\) cell counts were slightly higher in children with versus those without AD, differences in CD8+ subsets were more prominent in adulthood (Fig 2, G and H, and see Fig E4, G and H). Reflecting the T\(\text{H1}^\text{+}\) and T\(\text{H2}^\text{+}\) imbalances characterizing AD, the T\(\text{H1}^\text{+}\)/T\(\text{H2}^\text{+}\) ratio was decreased in patients with AD compared with that in control subjects in both CLA+ /CLA+ subsets across the ages. T\(\text{H1}^\text{+}/\text{T}_{\text{H2}}^\text{+}\) cell counts were significantly lower in children and adults with AD versus control subjects (Fig 2, I-L, and see Fig E4, I-L).

Both healthy control subjects and patients with AD had T\(\text{H9}^\text{+}\) cell count increases over time, peaking in adolescence and decreasing in adulthood (Fig 3, A and B, and see Fig E5, A and B, in this article’s Online Repository at www.jacionline.org).
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No TH9 level differences were observed between patients with AD and control subjects, with the exception of 5- to 12-year-old children with AD, who showed significantly increased CLA levels (0.76% vs 0.3%, \( P = .04 \); see Fig E5, B). This AD age group also showed increased CLA+/CLA− Tc9 cell counts (Fig 3, C and D, and see Fig E5, C and D). CLA+ TH17 cell counts were generally stable and similarly abundant across ages among control subjects and patients with AD (Fig 3, E, and see Fig E5, E) contrary to CLA− TH17 cell counts, which showed developmental expansion in both (Fig 3, F, and see Fig E5, F). Contrary to adolescents with AD, who had lower CLA+ Tc17 cell counts than control subjects (1% vs 4.7%, \( P = .04 \)), adults had significantly higher frequencies (2.1% vs 1.4%, \( P = .05 \); Fig 3, G and H, and see Fig E5, G and H).

Systemic/CLA− CD4+/CD8+ IL-22+ cell counts similarly increased with age in both control subjects and patients with AD (Fig 3, J and L, and see Fig E5, J and L), whereas skin-homing Tc22/Tc22 cell counts increased with age primarily in patients with AD (Fig 3, I and K, and see Fig E5, I and K). Starting in childhood, skin-homing TH17/Tc22 cell counts were significantly higher in patients with AD versus control subjects and incrementally increased with age (TH17 cell: children, 6.7% vs 4%, \( P = .07 \); adolescents, 7.9% vs 3.6%, \( P = .001 \); adults, 8.2% vs 4.4%, \( P < .0001 \); Fig 3, I and K, and see Fig E5, I and K). Polar T-cell subset development in control subjects and patients with AD is summarized in Table E4 in this article’s Online Repository at www.jacionline.org and shown in the graphical abstract.

The unsupervised hierarchical clustering heat map in Fig 4 summarizes the above, displaying all polarized T-cell subsets for control subjects and patients with AD across age groups (red, positive/increase; blue, negative/decrease). FCHs of the mean frequencies of patients with AD versus control subjects for each age group are presented. The green cluster includes subsets that were relatively low and stable among control subjects but incrementally increased with age in patients with AD. Most of these subsets were significantly increased in adults with AD versus control subjects (FCH > 1.57, \( P < .05 \)), whereas younger patients with AD showed lower or no significance. The pink
box shows increased IL-9 frequencies in childhood, which decrease in adulthood, particularly in patients with AD. The yellow cluster shows markers that increased in both control subjects and patients with AD, therefore minimizing the differences between groups.

**T-cell activation, clinical measures, and IFN-γ levels are associated with age and AD chronicity**

We also evaluated how clinical characteristics, including AD severity (SCORAD and EASI scores), patient age, disease duration, eosinophil counts, pruritus, and TEWL relate to different polar T-cell subsets. Unsupervised hierarchical clustering of all T-cell subset frequencies, clinical scores, AD duration, and age was performed by using Spearman correlations as a similarity metric, as displayed in the correlation heat map and dendrogram in Fig 5 (red, positive correlation; blue, negative correlation; stars and plus signs display significance). Congruent with recent AD data,22,26 which showed positive correlations between IL-13– and IL-22–producing T cells,22,26,27 these cytokines largely grouped together. IFN-γ–producing T-cells clustered together with AD duration and patient age. A tight cluster gathering multiple clinical measures was located adjacent to IL-9/IL-13–producing T cells. Significant correlations depicted in this heat map are listed in Table E5 in this article’s Online Repository at www.jacionline.org, with selected individual scatter plots presented in Figs 6 and 7 and Figs E6 to E8 in this article’s Online Repository at www.jacionline.org.

SCORAD and EASI scores were positively correlated \( (r = 0.74, P < .001; \text{Fig 6}, A) \). SCORAD scores also correlated with skin-homing CD4+ cell counts \( (r = 0.24, P = .0016; \text{Fig 6}, B) \), pruritus \( (r = 0.54, P < .0001; \text{Fig 6}, C) \), and eosinophil counts \( (r = 0.25, P = .001; \text{Fig 6}, D) \). To evaluate normal versus pathologic development with age, we comprehensively assessed age correlations in control subjects and patients with AD, presenting both on the same scatter plots for clarity, when applicable. Age correlated positively with severity based on SCORAD \( (r = 0.25, P = .0016; \text{Fig 6}, E) \) and EASI \( (r = 0.34, P < .001; \text{Fig 6}, F) \) scores and with all other clinical measures,
including pruritus (Fig 6, G), eosinophil counts (Fig 6, H), and lesional TEWL (Fig 6, I and J).

Although skin-homing TcM/Tem cell counts correlated negatively with age (Fig 6, K and L; red, patients with AD; blue, control subjects), the proportion of ICOS- and HLA-DR–activated skin-homing T cells increased exclusively in patients with AD (see Fig E6, A-D). AD duration, which highly correlated with patient age ($r = 0.98$, $P < .0001$; see Fig E6, E), also demonstrated significant positive correlations with EASI score and pruritus (see Fig E6, F-H). Among the cytokine subsets

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**FIG 5.** Unsupervised hierarchical clustering of polarized cytokine subset frequencies (percentages) with AD clinical measures using Spearman correlation as a similarity metric. CD4$^+$/CD8$^+$ IL-13$^+$, IL-17$^+$, and IL-22$^+$ subsets clustered together (turquoise box). IFN-$\gamma$–producing T-cell subsets grouped together with age and disease duration (yellow box). IL-9$^+$ and some IL-13$^+$ and IL-22$^+$ T cells (purple box) clustered adjacent to AD clinical measures (green box). The heat map shows positive (red) or negative (blue) correlations of all parameters, with color intensity reflecting the strength of the correlation ($-1$ to $+1$). Dendrograms are shown as a tree, representing the distance between variables. *$P < .05$, **$P < .01$, ***$P < .001$, and $+P < .1$. LS, Lesional; NL, nonlesional.
The most significant correlation was noted between AD duration and IFN-γ–producing cell counts (r = 0.58, P < .0001; see Fig E6, L).

Overall, IFN-γ levels increased with age in both patients with AD and control subjects (see Fig E7, A–D); however, although the TH1/TH2 (but not TC1/TC2) ratio, particularly its skin-homing component, increased with age in both groups, it remained significantly decreased in patients with AD across all ages versus control subjects (Fig 7, A–D). Conversely, differences between patients with AD and control subjects were observed in skin-homing TH2/TC2 cell counts throughout development, with control subjects never reaching the levels seen in patients with AD (Fig 7, E–H). CLA+TH22/TC22 cell counts showed significantly higher developmental increases in patients with AD (Fig 7, I and K), whereas systemic subsets generally overlapped (Fig 7, J and L). Similar negative trends of IL-9–producing cells (see Fig E7, E–H) and expansion of systemic TH17 cells were seen in control subjects and patients with AD (see Fig E7, J–L).

Positive correlations between SCORAD scores and IL-13–, IL-9–, and IL-22–producing cells were recorded (P < .03; see Fig E8, A–C, in this article’s Online Repository at www.jacionline.org). EASI scores correlated with TH9 and TH22 cell counts and pruritus (P < .03; see Fig E8, D–F), whereas pruritus was associated with TH22 cell and eosinophil counts (P < .032; see Fig E8, G and H). TEWL correlated with CD4+CLA+ cell counts only in nonlesional skin (Fig E8, I and J). TH22 cell counts positively correlated with TEWL in lesional tissues (see Fig E8, K and L), and AD severity correlated with TEWL in nonlesional skin (see Fig E8, M–P).

Because forkhead box P3 staining requires cell permeabilization, surface markers were used for regulatory T (Treg) cell identification. Ninety percent of CD25–CD127–CCR4+ cells coexpress forkhead box P3, and therefore the CD25+CD127–CCR4+ phenotype defined Treg cells.28 In analyzing Treg cells, both total Treg cell (CD25+CD127–) and skin-homing (CCR4+CLA+) Treg cell frequencies were captured. The only AD age group that showed significant increases in both total (see Fig E9, A, in this article’s Online Repository at www.jacionline.org) and skin-homing (see Fig E9, B) Treg cells versus control subjects was the 5 to 12-year-old group (P < .01). Developmentally, patients with AD showed similar total Treg cell trends as control subjects, whereas discrepancies were more evident in skin-homing subsets (see Fig E9, C and D).
Cytokine polarization and T-cell activation differentiate patients with AD into separate age clusters along a spectrum

We integrated T-cell and cytokine biomarkers to differentiate the entire AD cohort based on their blood phenotype. The principal components of all biomarker data for all subjects were analyzed by using unsupervised k-means clustering separately for patients with AD and control subjects. As shown in Fig 8, in the AD cohort the frequencies of different markers defined 3 meaningful clusters aligning along a spectrum. Although infants clustered on the far left and adults clustered on the right, children and adolescents generally clustered together between the infants and adults. The markers that best distinguished between each set of clusters appear in the boxes between the 2 cohorts and are summarized in Table E6 in this article's Online Repository at www.jacionline.org. The TH1/TH2 ratio, CD8+ activation, and IFN-γ–producing T-cell counts differentiated adults from younger groups. Treg cells, T-cell activation, and different cytokine subsets were able to differentiate distinctive AD age groups. Applying the same model to the control population did not distinguish between age groups (Fig 8).

DISCUSSION

This is the first comprehensive study that compares systemic immune profiles of different AD age groups (0-5, 6-11, 12-17, and ≥18 years old) with appropriate comparisons with control subjects. Because circulating CLA+ T cells have been suggested as peripheral biomarkers in patients with AD,29 we used CLA to segregate between skin-homing and systemic immune changes that accompany development in healthy subjects and patients with AD. Our data suggest unique endotypes in infants and toddlers, children, adolescents, and adults with AD, possibly advocating for personalized, endotype-driven approaches rather than “one-size-fits-all” therapeutic strategies. In addition, the results start to define age-specific differences between pediatric patients with AD and age-matched control subjects as a complement to our recent data showing differences between elderly versus young AD adult endotypes.3 Our data suggest
that even adolescents have a different profile from adults, possibly contributing to the somewhat lower response to the recently approved IL-4 receptor α antagonist dupilumab in adolescents versus adults.30,31

Immune responses at birth are immature,10 with decreases in frequencies of naive T cells in healthy children with increasing age paralleled by increases in Tcm/Tem cell counts.32 Skin-homing Tcm/Tem cell counts are higher in infants and children with AD compared with control subjects and decrease with age exclusively in patients with AD. Increased skin-homing memory cell counts cluster in infants with AD separately from other age groups, suggesting early relevance. Excessive naive-to-memory switch, which causes an influx of memory cells into the circulation in infancy and early childhood, might play a role in AD initiation, whereas exclusive decreases in the AD population over time might suggest increased migration of CLA⁺Tcm/Tem cells to the skin.

ICOS is involved in multiple adaptive immune responses, including induction of various T-cell polarization,33-35 Treg cell regulation,36 antibody responses (including IgE switching),37 and group 2 innate lymphoid cell (ILC)–mediated cytokine production.38,39 ICOS has also been implicated in the pathogenesis of asthma.40,41 Previously, we have shown that Tcm/Tem cell ICOS activation is higher in children with AD versus control children.17 Subsequent data showed accelerated systemic/CLA⁺ICOS T-cell activation in 0- to 3-year-old infants with AD.42 Our present data support ICOS expansion in development in both healthy subjects and patients with AD, particularly in CD4⁺T cells. However, positive correlations with age were exclusively seen in patients with AD and specifically in skin-homing memory subsets.

Also, infants and adults with AD have higher CD4⁺CLA⁺/CLA⁻ICOS activation than control subjects, a difference that is also significant for CD8⁺ cell subsets in children. The low CD8⁺ cell frequencies in infants, which increase with time,10 might explain the earlier activation of CD4⁺ subsets. Early involvement of ICOS in patients with AD indicates its possible role in disease induction through either stimulation of T-cell subset polarization, Treg cell modulation, IgE induction, or group 2 ILC activation. Furthermore, targeting ICOS/ICOS ligand in an asthma mouse model altered T-cell differentiation and ameliorated asthma.44 Because AD often precedes asthma,45 CLA⁻ICOS expansion in infants with AD might also be associated with noncutaneous atopic manifestations. These data suggest the potential for ICOS targeting as a preventive or therapeutic approach for AD and atopic associations, although this is speculative, and further studies are needed.

The chronic T-cell activation MHC class II antigen HLA-DR increases with age in healthy subjects.46,47 HLA-DR activation has been shown in adults with chronic AD.42 Although its level are not increased in infants with AD, its activation increases with age.

At birth, immune responses are TH2 biased, with low TH1/IFN-γ levels in both healthy newborns8,9,48,49 and those who eventually have AD.13 Our data show that TH1 frequencies increase with age in both infants with AD and control infants. However, congruent with recent blood data,50 patients with AD have significantly lower IFN-γ frequencies than control subjects, particularly among CD4⁺T cells. Conversely, CLA⁺TH2 cell counts are similarly increased across all AD age groups and are significantly higher than in control subjects, even in infants. Systemic/CLA⁻TH2 cell counts are significantly higher in patients with AD, frequencies of different markers defined 3 meaningful age clusters aligning along a spectrum. Although infants (pink ellipse) clustered on the far left and adults (green ellipse) clustered on the right, children and adolescents (blue ellipse) clustered together between the other age cohorts. Markers that best distinguished between clusters appear in the boxes between 2 cohorts (colors of markers parallel colors of the relative age group). Arrows designate increased frequencies of a given marker among the specific age group. In healthy control subjects clusters did not clearly align patients along an age spectrum.
patients with AD starting in childhood, which implies systemic Th17 activation with greater chronicity. Consistent with the less dominant role of CD8 suggested in children with early AD \(^{42}\) and low CD8 abundance in infants, \(^{10} \) Th2 cell increases are mostly prominent in adults with AD. CLA\(^+\) Th1/T12 ratio correlation with age is consistently decreased in patients with AD compared with control subjects, and the decreased CLA\(^+\)/CLA\(^-\) Th1/T12 ratio among all AD age groups best reflects the immune imbalance that accompanies AD from birth to adulthood.

Circulating Th19 cells have skin-homing predilection. \(^{31,52}\) IL-9 levels are increased in both the skin and serum of children with AD and were also correlated with disease severity. \(^{53,54}\) Although the mechanism by which IL-9 contributes to AD is unknown, IL-9 is linked to mast cell, eosinophil, and ILC function and has been thought to play a role in patients with AD, \(^{55}\) as well as in patients with other atopic disorders. \(^{56-59}\) Accordingly, IL-9 subsets cluster closest to all AD clinical measures (eosinophil count/pruritus/SCORAD score/EASI score/TEWL). Development in both healthy subjects and patients with AD are characterized by IL-9 level increases with age, which decrease in adulthood. We show considerably increased systemic/CLA\(^-\) T-cell counts exclusively among the 5- to 12-year-old group with AD versus control subjects, which is consistent with early allergy development in childhood and a subsequent decrease in adulthood. \(^{60,61}\) CLA\(^-\) systemic activation and cytokine polarization with age support the systemic nature of AD and the need for systemic therapeutic approaches in patients with moderate-to-severe disease, as well as the association of comorbidities and increased inflammatory and cardiovascular-associated proteins in patients with more chronic AD. \(^{62-65}\)

Black et al. \(^{66}\) showed strong Th17 skewing early in life. Although similar CLA\(^+\) Th17 frequencies are seen in infants and older subjects regardless of AD, systemic/CLA\(^-\) Th17 cell counts are relatively low in infants of both groups and mature with time. IL-17 signatures characterize the skin of pediatric patients with AD, \(^{64,66}\) but these changes are not mirrored in the circulation. This discrepancy might be due to increased skin residence of Th17 cells or caused by reciprocal peripheral regulation of the Th17 and Th12 axes. \(^{67,68}\)

Skin-homing Th12 levels are increased in children, adolescents, and adults with AD versus control subjects. Congruent with past studies showing that IL-22 is correlated with AD severity, \(^{69}\) we present positive correlations between IL-22-producing CD4\(^+\) cells, SCORAD scores, EASI scores, and pruritus. CD8\(^+\) T cells are major producers of IL-22 in the skin of adults with AD. \(^{69,70}\) Lower proportions of CD8\(^+\) versus CD4\(^+\) cells in early childhood \(^{46,70}\) and the fact that IL-22 marks AD chronicity \(^{71}\) might explain why skin-homing Th12/Th17 cell frequencies are highest in adolescents and adults. Increased IL-22 levels, which gave been shown to negatively regulate IFN-\(\gamma\), \(^{72}\) might also contribute to the low Th11 frequencies seen in patients with AD. Systemic/CLA\(^-\) Th12/Th12 subset development was similar in patients with AD and control subjects, potentially suggesting a greater pathogenic relevance of IL-22 in skin of patients with AD \(^{73}\) and the impaired skin barrier. \(^{74}\) Studies have shown positive correlations and cellular coproduction of IL-13 and IL-22/13 and of IL-17 and IL-22. \(^{27,75}\) Indeed, these markers cluster together in the correlation heat map. IFN-\(\gamma\) clusters with patient age and disease duration, differentiating infants from children and adolescents and adolescents from adults with AD. The association of IFN-\(\gamma\) with disease chronicity agrees with previous chronic adult AD data, suggesting that IFN-\(\gamma\) possibly plays a role in inflammatory disorder chronicity rather than in disease initiation. \(^{26,42,76}\) Expansion of CD8\(^+\) memory cells, increases in CLA\(^+\) IL-22–producing cells, and intensification of ICOS\(^+\)/HLA-DR\(^+\) activation with maturity might account for the positive correlations between age, disease duration, and severity measures.

Increasing severity with age was not seen in a recent study that followed infants with AD only between 0 and 11 months. \(^{77}\) TEWL increases with age in patients with AD and correlates with IL-22 (which also increases with age), likely again reflecting the contribution of IL-22 to the barrier impairment in patients with AD.

In addition to maintaining immune tolerance, \(^{78}\) Treg cells influence activation of effector cells. \(^{79,80}\) Only 5- to 12-year-old children with AD have significantly higher total and skin-homing Treg cells than control subjects. This age group is characterized by other unique features, including increased skin-homing Tcm/Tcm cell counts, decreased systemic IFN-\(\gamma\) levels, and increased systemic IL-9 frequencies. Additionally, skin-homing IFN-\(\gamma\) levels are almost doubled from infancy to childhood in patients with AD, although they are still lower than those in age-matched control subjects. Multiple immunologic changes occurring during these years might be involved in AD clearance or development of noncutaneous manifestations commonly occurring during these years. \(^{81,82}\)

Profiling AD across ages is imperative for targeted therapeutic development. Unlike psoriasis, in which targeted treatments lead to remarkable responses in most patients, AD responses to targeted therapeutics are much lower. \(^{83}\) This disparity can be attributed to the multicytokine activation seen in patients with AD, despite the shared Th12 activation, versus the Th17-centered responses in patients with psoriasis. \(^{83}\) Additionally, AD has a highly varied endotype repertoire, with different immune polarization. \(^{3,84,85}\) Despite common features, particularly increased Th12 expression, AD is endotypically different across ages, and treatments should be tailored to the unique age endotype. Although one could hypothesize that the immune changes merely reflect developmental phenomena that are age related, the lack of clear clustering in control subjects implicates AD as the driver of the distinct, progressive, age-related endotypic characteristics rather than age alone. The clustering model, based on flow cytometric biomarkers, splits the entire cohort into 3 separate age clusters only in patients with AD. Expectedly, IFN-\(\gamma\), IL-22, and HLA-DR levels increased chronologically, distinguishing older subjects from infants with AD. Skin-homing Tcm/Tcm cell counts and systemic ICOS activation, both of which were higher in infants with AD, separate infants from other groups. This model demonstrates that a limited group of blood biomarkers can distinguish among various AD endotypes based on patient age, with a spectrum of age-dependent versus only “infantile” or “adult” phenotypes. Because our data show that the “adult” or “stable” AD phenotype is only achieved in adulthood, it might be possible to intervene before adulthood and prevent establishment of the adult AD phenotype.

This study has a few limitations. Despite inclusion of different age groups, this study was not longitudinal and thus did not follow the same cohort with time. Additionally, studies in blood do not
allow one to measure T-cell subsets in skin, such as tissue-resident memory T cells. Furthermore, the study characterized polyclonal T-cell responses and not antigen-specific responses induced by culprit triggers. Finally, the pathogenicity of immune axes presented here cannot be further dissected without future targeted therapeutic studies to check mechanisms.

AD was initially considered an early-onset pediatric disease, with 75% “outgrowing” their disease by age 10 years. More recent studies have established AD as a disorder that often persists into adulthood. Comparing the profile of cleared versus persistent pediatric AD, ideally through longitudinal studies, will better define age-specific characteristics that predict AD clearance.

**REFERENCES**

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