

Early pediatric atopic dermatitis shows only a cutaneous lymphocyte antigen (CLA)⁺ T_H2/T_H1 cell imbalance, whereas adults acquire CLA⁺ T_H22/T_C22 cell subsets

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Background: Identifying differences and similarities between cutaneous lymphocyte antigen (CLA)⁺ polarized T-cell subsets in children versus adults with atopic dermatitis (AD) is critical for directing new treatments toward children.

Objective: We sought to compare activation markers and frequencies of skin-homing (CLA⁺) versus systemic (CLA⁻) “polar” CD4 and CD8 T-cell subsets in patients with early pediatric AD, adults with AD, and control subjects.

Methods: Flow cytometry was used to measure CD69/inducible costimulator/HLA-DR frequency in memory cell subsets, as well as IFN- γ , IL-13, IL-9, IL-17, and IL-22 cytokines, defining T_H1/

cytotoxic T (T_C) 1, T_H2/T_C2, T_H9/T_C9, T_H17/T_C17, and T_H22/T_C22 populations in CD4 and CD8 cells, respectively. We compared peripheral blood from 19 children less than 5 years old and 42 adults with well-characterized moderate-to-severe AD, as well as age-matched control subjects (17 children and 25 adults). **Results:** Selective inducible costimulator activation ($P < .001$) was seen in children. CLA⁺ T_H2 T cells were markedly expanded in both children and adults with AD compared with those in control subjects, but decreases in CLA⁺ T_H1 T-cell numbers were greater in children with AD (17% vs 7.4%, $P = .007$). Unlike in adults, no imbalances were detected in CLA⁻ T cells from pediatric patients with AD nor were there altered frequencies of T_H22 T cells within the CLA⁺ or CLA⁻ compartments. Adults with AD had increased frequencies of IL-22-producing CD4 and CD8 T cells within the skin-homing population, compared with controls (9.5% vs 4.5% and 8.6% vs 2.4%, respectively; $P < .001$), as well as increased HLA-DR activation ($P < .01$).

Conclusions: These data suggest that T_H2 activation within skin-homing T cells might drive AD in children and that reduced counterregulation by T_H1 T cells might contribute to excess T_H2 activation. T_H22 “spreading” of AD is not seen in young children and might be influenced by immune development, disease chronicity, or recurrent skin infections. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Atopic dermatitis, T cell, cutaneous lymphocyte antigen, IL-13, IL-22, IFN- γ , inducible costimulator, CD69, HLA-DR

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Supported by a research grant from the LEO Foundation (research grant no. 0266-2572, GCO no. 13-1310 to E.-G.-Y. and A.S.P.). T.C. was cosponsored by the Center for Basic and Translational Research on Disorders of the Digestive System through the generosity of the Leona M. and Harry B. Helmsley Charitable Trust. J.G. was supported in part by grant no. UL1TR00000 from the National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH) Clinical and Translational Science Award (CTSA) program. D.M. received funding from the American Dermatological Association Medical Student Fellowship. J.G.K. was supported by grant no. 5UL1RR024143-02 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Blood acquisition was supported by the Northwestern University Skin Disease Research Center (NIAMS P30AR057216).

Disclosure of potential conflict of interest: J. G. Krueger reports that money was paid to his institution by and/or he has received personal fees from Pfizer, Amgen, Janssen, Lilly, Merck, Novartis, Kadmon, Dermira, Boehringer, Innovaderm, Kyowa, BMS, Serono, BiogenIdec, Delenex, AbbVie, Sanofi, Baxter, Parexel, Xenoport, and Kineta. A. S. Paller has received research support from the LEO Foundation; has received consultancy fees from AbbVie, Anacor, Celgene, Chugai Pharma, Galderma, GSK-Stiefel, Novartis, and Promius Pharma; and is an investigator for AbbVie. E. Guttman-Yassky is a board member for Sanofi Aventis, Regeneron, Stiefel/GSK, Medimmune, Celgene, Anacor, and Leo Pharma; has received consultancy fees from Regeneron, Sanofi Aventis, Medimmune, Celgene, Stiefel/GSK, Celgene, BMS, Amgen, and Draisi; and has received research support from Regeneron, Celgene, BMS, and Janssen. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication April 2, 2015; revised May 13, 2015; accepted for publication May 19, 2015.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2015.05.049>

Despite being one of the most common pediatric disorders,^{1,2} atopic dermatitis (AD) harbors immunologic alterations within the first 6 months of onset that are largely unexplored in children.^{3,4} Although 85% of AD cases present before 5 years of age and immune polarization in children can differ from that in adults with long-standing disease, studies have largely been limited to adults.⁵⁻⁷

The percentage of CD4 memory T cells (CD45RO⁺) in the circulation of newborns (5% to 10%) is significantly lower than that in 3- to 10-year-old children (approximately 15%) and adults (30% to 50%), whereas the percentage of naive T cells (CD45RA⁺) is highest in newborns (approximately 90%).⁸⁻¹¹ Most T cells in AD lesions are CD45RO⁺ memory cells that express the skin-homing receptor cutaneous lymphocyte antigen (CLA).¹²⁻¹⁴ Circulating CLA⁺ T cells are expanded in patients with AD, respond to skin-associated allergens, and play a role in disease pathogenesis.¹⁴⁻¹⁷ CLA⁺ T cells have been also demonstrated to be peripheral biomarkers in several inflammatory skin diseases.¹⁸ The need for large biopsy specimens to classify

Abbreviations used

AD:	Atopic dermatitis
CLA:	Cutaneous lymphocyte antigen
ICOS:	Inducible costimulator
PE:	Phycoerythrin
PMA:	Phorbol 12-myristate 13-acetate
T _C :	Cytotoxic T
T _{CM} :	Central memory T
T _{EM} :	Effector memory T

T-cell subsets limits the feasibility of intracellular cytokine staining of skin from adults and even more so in children. Therefore studying polarized, activated circulating CLA⁺ memory T-cell subsets might provide a surrogate to skin phenotyping.

Previous studies indicate low IFN- γ and high T_H2 signals in cord blood of newborns with AD.¹⁹⁻²¹ Subsequent infant and toddler AD studies mostly demonstrate increased T_H2 signals,²²⁻²⁴ with controversial data regarding T_H1 frequency,^{23,25-28} CD8 polar subsets,^{23,28} and levels of T-cell activation.²⁹⁻³¹ Several studies include cohorts of adults and children, but most do not provide direct comparison between these groups.^{22,23,26,32} Although we have recently reported a detailed blood phenotype of adult AD,³³ few pediatric studies differentiate cytokine expression in CLA subsets,³⁰ and virtually none compare adults and children.

Defining the similarities and differences between activated polarized T-cell subsets in adults and patients with early-onset pediatric AD is critical for understanding initial pathogenic immune mechanisms. Furthermore, linking IgE levels with T-cell polarization in patients with newly diagnosed AD might help define the sequence of events that leads to AD development.

In this study we compared differences between T-cell memory subset activation within the skin-homing/CLA⁺ and systemic/CLA⁻ compartments, as well as frequencies of polarized CD4 and CD8 subsets in blood of pediatric and adult patients with AD. We found that although AD onset in children is T_H2 dominated and characterized by short-term activation, adult AD extends to additional T_H subsets, particularly T_H22.

METHODS**Patients' characteristics and blood samples**

Blood was obtained (with patients' or parents' informed consent) from 19 children (11 female and 8 male children; age range, 5-70 months; mean age, 25 months) with moderate-to-severe AD, as well as from 17 pediatric control subjects (7 female and 10 male subjects; age range, 6-67 months; mean age, 32 months) and 42 adults (18 female and 24 male subjects; age range, 18-74 years; mean age, 42 years) with moderate-to-severe AD and 25 adult control subjects (12 female and 13 male subjects; age range, 19-66 years; mean age, 39 years) under an institutional review board-approved protocol. No age ($P = .3$), ethnicity ($P = .8/P = .055$, children/adults), or sex ($P = .8/P = .5$, children/adults) disparities were observed between the groups. Children with AD were within 6 months from AD diagnosis.

AD adult serum IgE levels (normal, <200 kU/L) ranged from 4 to 50,000 kU/L (mean, 6,238 kU/L), and levels in children with AD (normal, <100 kU/L) ranged from 23 to 1,772 kU/L (mean, 526 kU/L). Control children's levels ranged from 0 to 43 kU/L (mean, 26 kU/L).

SCORAD scores were used to evaluate disease severity (adults: range, 32-97; mean, 65; children: range, 30-73; mean, 54).

Control subjects had no personal history of inflammatory disease and no family history of atopy. Demographic and laboratory data are summarized in Table 1.

Isolation of PBMCs

PBMCs were isolated from whole blood by using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Briefly, the blood was laid under Ficoll gradient; 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

Whole blood was incubated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) plus ionomycin (2 μ g/mL) in the presence of brefeldin A (10 μ g/mL) for 4 hours at 37°C to induce T-cell cytokine responses. After stimulation, RBCs were lysed with FACS Lysing solution to obtain leukocytes (see the [Methods](#) section in this article's Online Repository).

Cell-surface staining 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 [ICOS], HLA-DR, and CLA). Stimulated and nonstimulated blood cells were also stained for cell-surface markers (CD3, CD4, CD69, and CLA) and then permeabilized with FACS/permeabilization solution 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

Data were analyzed by using the Student *t* test to compare variables. IgE values were log₁₀-transformed before analyses. Variables were correlated by using Pearson correlations. A *P* value of less than .05 was considered significant.

RESULTS

Surface staining was used to measure expression of early (CD69), middle (ICOS), and late (HLA-DR) activation markers in central memory (T_{CM}/CCR7⁺CD45RO⁺) and effector memory (T_{EM}/CCR7⁻CD45RO⁺) T cells in skin-homing/CLA⁺ and CLA⁻ subsets to examine immune activation in children versus adults with AD. The CD25⁺CD127⁻CCR4⁺ phenotype was used to exclude regulatory T cells from all PBMC analyses. Subsequently, intracellular cytokine staining was used to measure frequencies of IFN- γ , IL-13, IL-22, IL-17A, and IL-9-producing T cells after PMA/ionomycin activation, defining T_H1/cytotoxic T (T_C) 1, T_H2/T_C2, T_H22/T_C22, T_H17/T_C17, and T_H9/T_C9 subsets among CD4 and CD8 T cells, respectively, in CLA⁺ and CLA⁻ subsets. The gating strategy appears in [Fig E1](#) in this article's Online Repository at www.jacionline.org.

Effector T-cell numbers are uniquely increased in adults with AD

As previously described,¹¹ our data show that the proportion of naive T cells was higher in both control children versus control adults (CD4: 79% vs 60%, $P < .001$; CD8: 68% vs 56%, $P = .01$; [Fig 1](#)) and children versus adults with AD (CD4: 78% vs 59%, $P < .001$; CD8: 77% vs 55%, $P < .001$; [Fig 1](#)), whereas T_{CM}/T_{EM} cell subsets were lower in control children and children with AD compared with adults ($P = .001$, [Fig 1](#)). No significant differences in memory subsets were observed between patients with AD and control subjects ($P > .05$, [Fig 1](#)).

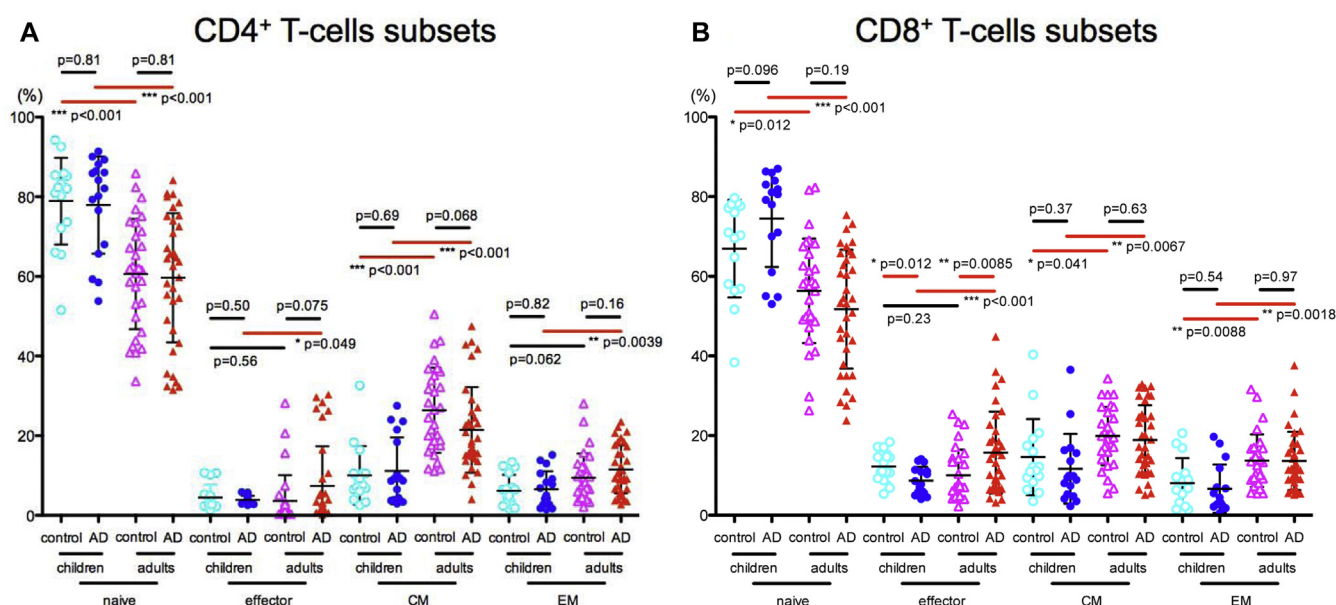
TABLE I. Epidemiologic, laboratory, and clinical data for patients with AD and control subjects

	Adults with AD	Adult control subjects	P value	Children with AD	Control children	P value
Age (y for adults; mo for children)	42 ± 2.1 (18-74)	39 ± 2.8 (19-66)	.3	25 ± 1.9 (5-70)	32 ± 1.8 (6-67)	.34
Sex, no.			.8			.51
Female	18	12		11	7	
Male	24	13		8	10	
Ethnicity, no.			.8			.055
Asian	3	3		5	0	
Black	6	2		2	1	
White	33	19		12	16	
SCORAD score	64.5 ± 2.45 (32-97)	NA		53.5 ± 14.4 (30-73)	NA	
IgE* (kU/L)	628.6 ± 114 (4-50,000)	NA		719.8 ± 318.3 (23-1,772)	28.8 ± 13.3 (0-141)	.082
Eosinophil count† (K/ μ L)	0.45 ± 0.05 (0-1.2)	NA		0.71 ± 0.26 (0.20-2.12)	0.27 ± 0.088 (0-1.12)	.14

Data are presented as means ± SEMs unless otherwise specified.

K/ μ L, Thousand cells per microliter.

*Reference range: 0 to 200 kU/L for adults and 0 to 100 kU/L for children.

†Reference range: 0 to 0.8 K/ μ L for absolute eosinophil counts.**FIG 1.** CD4 (A) and CD8 (B) T-cell subsets in children and adults with AD versus control subjects. Naive T-cell frequencies were higher in both control subjects and children with AD compared with those in adults, whereas T_{CM}/T_{EM} cell subsets were lower in control children and children with AD than adults. Adults with AD had significantly higher numbers of effector cells compared with those in children with AD. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Although no significant differences in effector cell frequencies were observed among pediatric versus adult control subjects (CD4: 4.5% vs 3.6%, $P = .5$; CD8: 12% vs 10%, $P = .2$; Fig 1), adults with AD had significantly greater effector cell numbers compared with those in pediatric patients with AD (CD4: 7.5% vs 3.8%, $P = .049$; CD8: 15% vs 8%, $P < .001$; Fig 1).

Memory subsets are positively correlated with IgE levels, but not with SCORAD scores, in children with AD

T_{CM} and T_{EM} cells are crucial components of the adaptive immune system and expand on antigenic stimulation.³⁴ T_{CM} and T_{EM} cell frequencies were correlated with IgE levels and disease severity/SCORAD scores to evaluate potential associations between disease severity and antigenic stimulation.

Significant correlations were only observed between T_{CM}/T_{EM} cell frequencies and IgE levels in children with AD (CD4: T_{CM} cells, $r = 0.88$, $P = .004$; T_{EM} cells, $r = 0.82$, $P = .01$; see Fig E2, B, in this article's Online Repository at www.jacionline.org). Memory subset frequencies were correlated with neither SCORAD scores in AD populations nor with IgE levels in control children (see Fig E2, A and C-E).

ICOS is highly expressed on CD4 T_{CM} and T_{EM} cell subsets of patients with AD

ICOS is a midactivation surface marker that is expressed up to 24 hours after T-cell receptor stimulation and is essential for effective T_H responses.^{35,36} Comparing ICOS expression on CD4 T cells among pediatric patients with AD, adults with AD, and control subjects showed that although patients with AD had

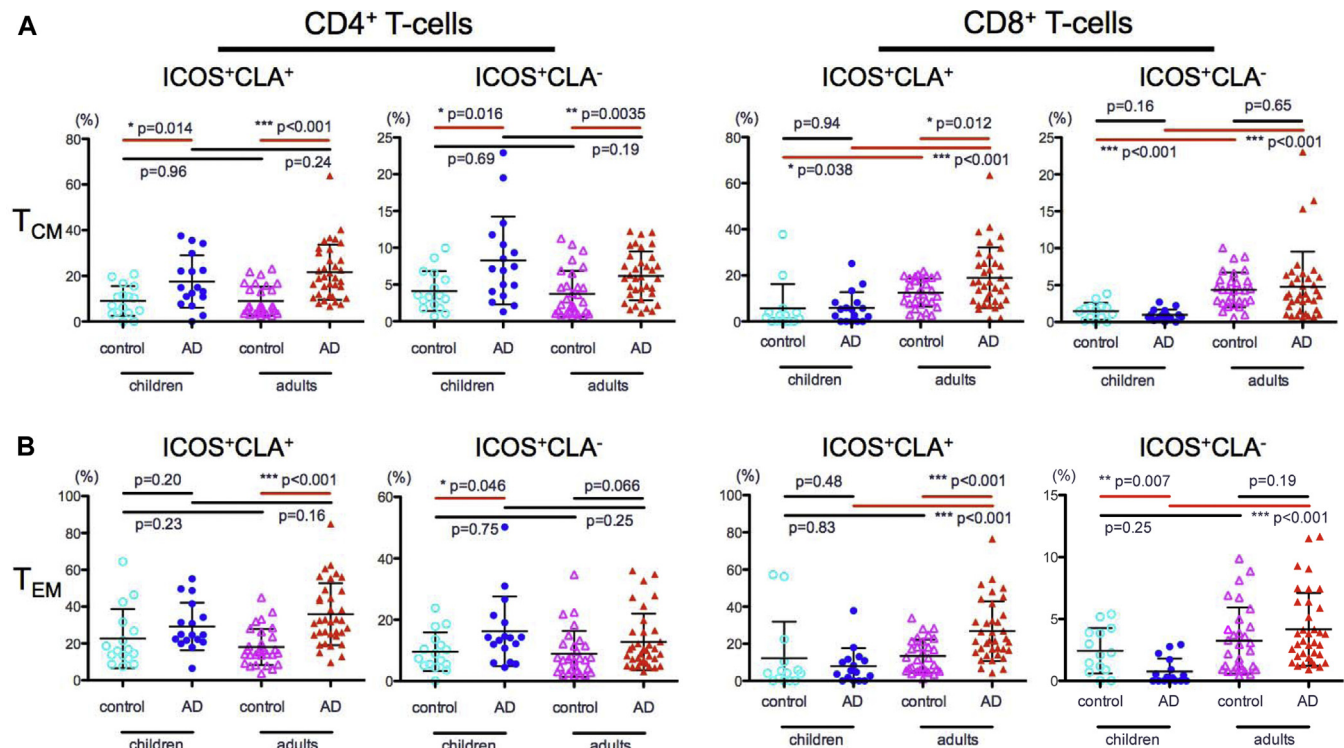


FIG 2. ICOS expression in CLA⁺ and CLA⁻ memory T-cell subsets in adults and children with AD. **A**, Patients with AD had increased ICOS expression in both CLA⁺ and CLA⁻ memory cell subsets compared with their relative control subjects. No significant CD4 ICOS expression differences were observed between the AD groups in both CLA⁺/CLA⁻ T_{EM}/T_{CM} cell subsets. **B**, Contrary to adults, in which higher CD8 ICOS expression was measured in CLA⁺ T_{CM}/T_{EM} cells compared with their relative control subjects, no differences were observed between children with AD and control subjects. **P* < .05, ***P* < .01, and ****P* < .001.

increased ICOS expression in both the CLA⁺ and CLA⁻ memory subsets compared with their relative controls (T_{CM} CLA⁺: children, 17% vs 9%, *P* = .01; adults, 22% vs 9%, *P* < .001; Fig 2, A), CLA⁺ ICOS levels were substantially higher (T_{CM} CLA⁻: children, 8% vs 4%, *P* = .01; adults, 6% vs 3.7%, *P* = .003; Fig 2, A). No significant CD4 ICOS expression differences were observed between AD groups in both CLA⁺ and CLA⁻ T_{EM}/T_{CM} cells (*P* > .16, Fig 2).

Contrary to adults, in whom higher CD8 ICOS expression was measured in CLA⁺ T_{CM}/T_{EM} cells compared with control subjects (T_{CM} cells: 19% vs 12%, *P* = .01; T_{EM} cells: 26.7% vs 13.5%, *P* < .001; Fig 2), no parallel differences were observed between children with AD and control subjects (*P* > .48, Fig 2). Among AD groups, adults had higher ICOS expression in CD8 T_{CM}/T_{EM} cell subsets compared with their pediatric counterparts (T_{CM} cells: CLA⁺ 19% vs 6%; CLA⁻ 4.7% vs 1%; T_{EM} cells: CLA⁺ 27% vs 10%; CLA⁻ 4% vs 1.8%, *P* < .001 for all; Fig 2).

HLA-DR is preferentially expressed on T_{CM}/T_{EM} cells in adults but not children with AD

HLA-DR is a human class II MHC antigen that indicates chronic activation.³⁷ Although comparable levels of T_{CM}/T_{EM} cell HLA-DR expression were observed in children regardless of AD status (*P* > .1; Fig 3, A and B), higher HLA-DR activation was found in adults with AD compared with control subjects, particularly among CLA⁺ populations (*P* < .001; Fig 3, A and

B). Adults with AD also showed higher HLA-DR activation than children with AD, mainly among CD8 subsets (T_{CM} cells: CLA⁺, 20% vs 6.5%; CLA⁻, 11.5% vs 3.3%; T_{EM} cells: CLA⁺ 19% vs 8%; CLA⁻ 8% vs 1.5%, *P* < .001 for all; Fig 3, A and B).

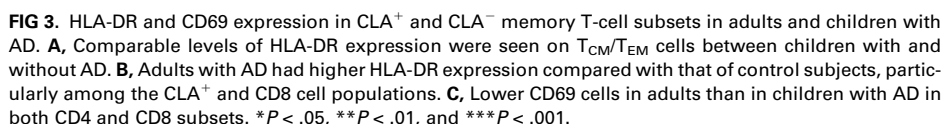
CD69 expression is higher in children compared with adults with AD

CD69 is the earliest activation marker expressed on T cells after CD4 and CD8 T-cell receptor activation.³⁸ CD69 was the only activation marker that was higher in children and particularly prominent in children versus adults with AD for both CD4 (CLA⁺: 40% vs 18%, *P* = .0013; CLA⁻: 70% vs 35%, *P* < .001; Fig 3, C) and CD8 subsets (CLA⁺: 66% vs 40%, *P* < 0.001; CLA⁻: 62% vs 36%, *P* < .001; Fig 3, C).

Skin-selective T_H1/T_H2 imbalance in pediatric AD

Because prior T-cell activation drives subset differentiation, we quantified polar T-cell subsets, including T_H1/T_C1, T_H2/T_C2, T_H22/T_C22, T_H17/T_C17, and T_H9/T_C9 frequencies, in CLA⁺/CLA⁻ subsets in all child and adult populations.

Adults with AD had normal to slightly lower IFN-γ⁺CLA⁺ levels compared with those in healthy subjects (CD4: 17% vs 20%, *P* = .18; Fig 4, A), and this difference is significant in patients with severe AD, as we recently described.³³ In contrast, children with AD had significantly lower IFN-γ⁺CLA⁺ levels



Because adult AD is characterized by T_H22/T_C22 cell expansion in skin^{39,40} and blood,³³ these subsets were measured in children. No significant differences in IL-22 levels were observed between children with AD and control subject ($P > .12$; Fig 5, A), whereas significantly higher IL-22 levels were observed in adults versus children with AD (CD4: CLA⁺, 9.5% vs 4.7%; CLA⁻, 3% vs 0.2%; CD8: CLA⁺, 8.6% vs 4.8%; CLA⁻, 0.6%

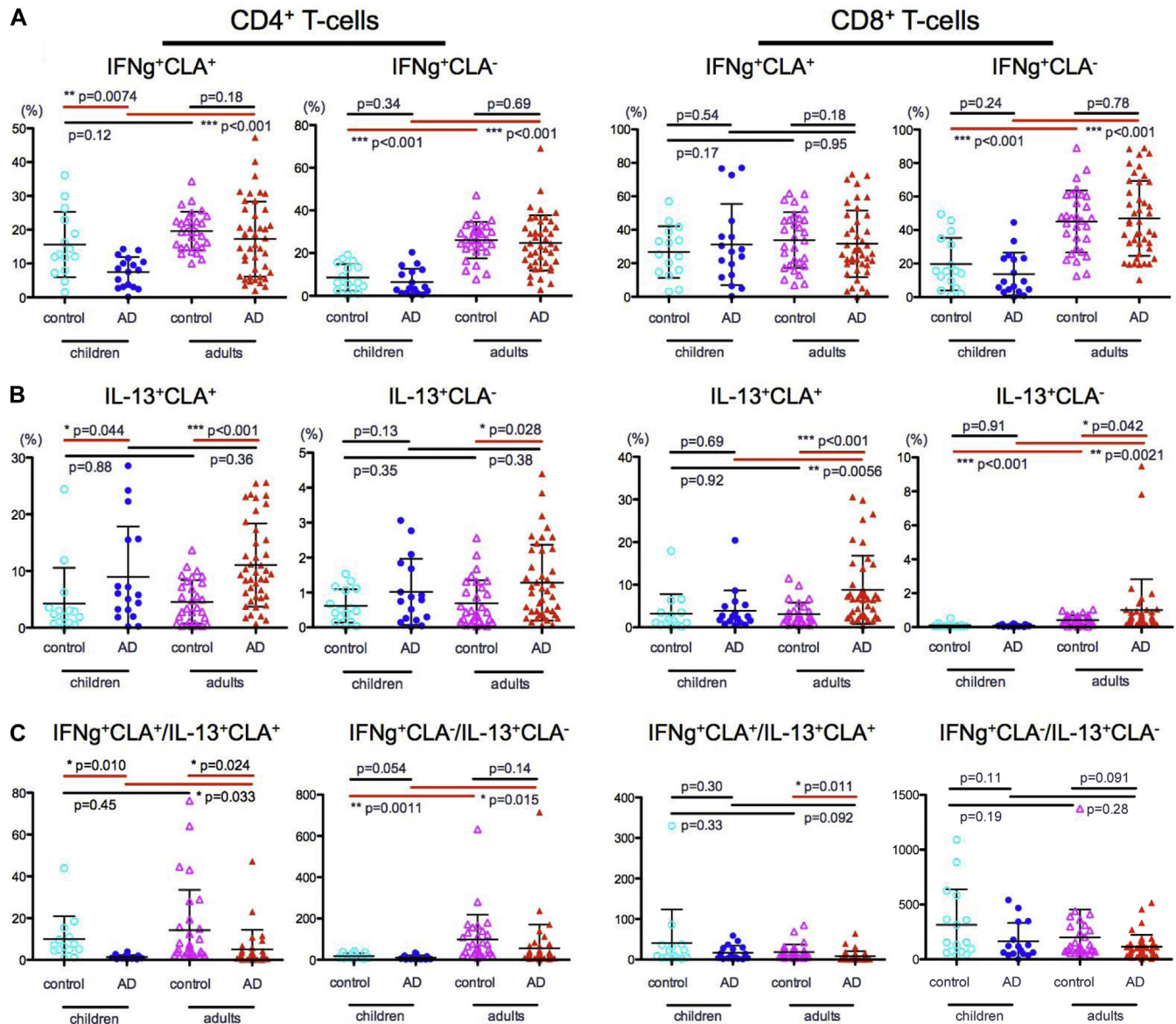


FIG 4. IFN- γ and IL-13 polarization in CLA⁺ and CLA⁻ CD4 and CD8 T cells. **A**, Compared with children, adults with AD had significantly higher IFN- γ levels, particularly in CD4 T cells. IFN- γ ⁺ CLA⁺ cell numbers in children with AD were significantly lower than in control children. **B**, In adults IL-13 levels were higher in the AD group compared with control values in both CLA⁺/CLA⁻ CD4/CD8 cell subsets. In children this difference was most prominent among CD4 CLA⁺ T cells. **C**, Children with AD had markedly lower T_H1/T_H2 ratios compared with control subjects, particularly in CD4 CLA⁺ T cells. **P* < .05, ***P* < .01, and ****P* < .001.

vs 0.1%; *P* < .001 for all; Fig 5, A). T_H17/T_C17 levels were higher in adults versus children with AD, specifically in the CLA⁻ subsets (CD4: 1.3% vs 0.3%, *P* < .001; CD8: 0.4% vs 0.1%, *P* < 0.001; Fig 5, B), with no difference between adults with and without AD. Finally, although no IL-9 differences were observed between patients with AD and control subjects, adults with AD had higher T_H9 levels in CLA⁺ cells compared with children with AD (2.3% vs 1.3, *P* = .01; Fig 5, C).

DISCUSSION

Although initial pathogenic events in AD development remain elusive, IgE switching, T-cell activation, and cytokine polarization have been suggested as key players.^{41,42} Our current

understanding of AD pathogenesis derives mostly from studies in adults with long-standing disease. However, only by studying young children with new-onset AD can we gain insight into primary pathogenic events. This is the first comprehensive report characterizing T-cell polarization and activation within skin-homing and systemic memory subsets in early-onset disease in young children with well-characterized disease severity in comparison with age-matched pediatric control subjects and severity-matched adults with AD.

At birth, the T-cell repertoire consists mostly of naive cells. With antigenic exposure, memory cells comprise approximately 35% of circulating T cells by the second decade of life.⁴³ Our data on memory T-cell subsets corroborate this naive memory CD4/CD8 T-cell shift. Furthermore, it shows that CD4/CD8

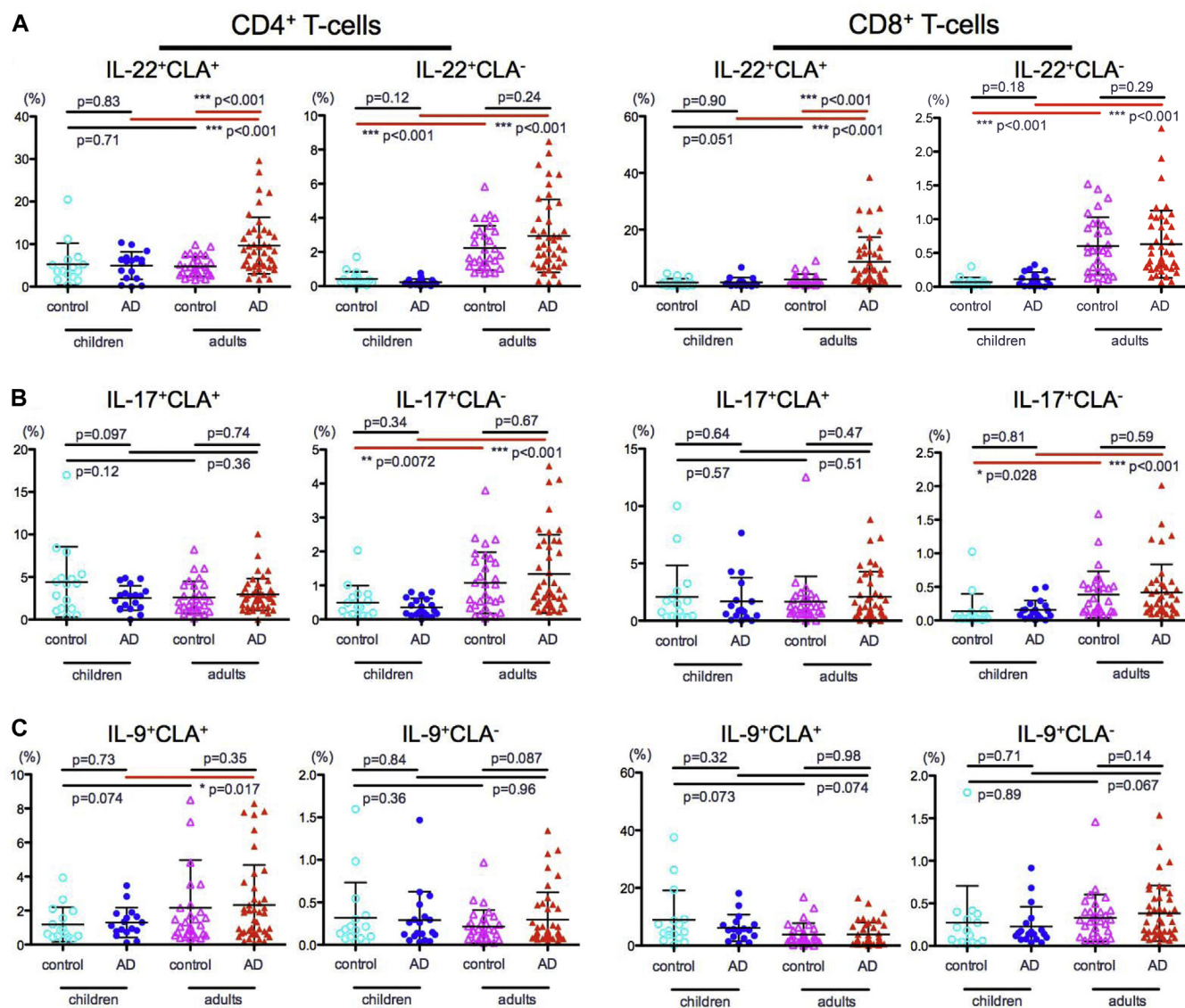


FIG 5. IL-22, IL-17, and IL-9 frequencies in CLA⁺ and CLA⁻ CD4 and CD8 T cells. **A**, No significant IL-22 differences were measured between children with and without AD. **B**, T_H17/T_C17 levels were higher in adults versus children with AD, specifically in the CLA⁻ CD4/CD8 cell subsets. **C**, Adults with AD had higher T_H9 levels in CLA⁺ cells than children with AD. **P* < .05, ***P* < .01, and ****P* < .001.

effector cells are the only consistently higher subset in adults versus children with AD, with no parallel differences in their control subjects. Normally, effector cell proliferation is part of the “expansion phase” of the immune reaction, which is replaced shortly after by a “death phase” of greater than 90% of these effectors, with remaining cells entering a “memory phase.”⁴⁴ Because antigenic exposure and microbial phenotype play central roles in shaping T-cell effector profiles,⁴⁴ cumulatively more antigenic exposure and increased infection rates might contribute to increased effector cells in adults with AD.⁴⁵⁻⁴⁸

T-cell activation through the T-cell receptor leads to a specific sequence of surface activation marker expression. CD69 is a very early activation marker (upregulated within 1 hour from cell activation and peaking by 16-24 hours). ICOS is an intermediate activation marker appearing within 24 hours, whereas HLA-DR is a late marker indicating chronic activation.⁴⁹⁻⁵¹

The ICOS costimulatory signal is required for T-cell activation and plays a role in shaping adaptive immunity.⁵²⁻⁵⁴ Mouse studies show that ICOS is vital in T-cell/B-cell interaction, immunoglobulin isotype switching (IgE production), and germinal center formation.^{55,56} Here we show that children with AD have higher ICOS expression, particularly among CD4 cells. These results might relate to the role of ICOS in T_H2 immunity expansion and IgE switching,⁵⁷ supporting an IgE-T_H2 mechanism in pediatric patients with AD. The only parameter that showed a trend of higher ICOS activation in children compared with adults with AD was among the CLA⁻ CD4 population, which further supports the role of ICOS in promoting noncutaneous atopy in children with AD. One possible implication is that ICOS suppression might be used as a therapeutic target in patients with extrinsic AD accompanied by a central T_H2 component.

These ICOS differences were not observed among CD8 cells in pediatric patients with AD. In addition to quantitative CD8

decreases relative to CD4 in infants,^{10,29} an alternative explanation might be that antigenic stimulation of AD in young populations is not presented by the MHC-I molecule.

HLA-DR expression on T cells indicates a state of chronic activation.⁵⁰ Although Bunikowski et al⁵⁸ have previously reported high HLA-DR⁺CD3⁺ T-cell numbers in children with AD older than 2 years compared with control subjects, another blood study showed no difference.²⁹ Antunez et al³⁰ subsequently showed no difference between children with AD and control subjects in CD4/CD8 HLA-DR expression, but results were not stratified to CLA⁺/CLA⁻ subsets, and all children were older than 7 years. Although we⁵⁹ and others^{60,61} reported high CD4/CD8 CLA⁺/CLA⁻ T_{CM}/T_{EM} cell HLA-DR activation in circulating skin-homing/CLA⁺ T cells of adults with AD, parallel pediatric data have been unavailable. Our current data indicate that all memory subsets show low HLA-DR activation in children, both control subjects and those with AD. For most subsets, HLA-DR activation is greater in adults versus children with AD but not in healthy adults versus children. This might result from the long-term, chronic activation in adults versus young children with newly diagnosed AD. Furthermore, differences between the adult groups were more striking in CD8 subsets (with higher values seen in CLA⁺ cells), indicating a possible role for CD8 in the pathogenesis of chronic long-standing AD.

CD69 also functions as an immunoregulatory receptor⁶² and has been reported to have opposing functional roles in inflammatory responses.⁶² In patients with AD, its expression on eosinophils was associated with high IgE levels and active inflammation,⁶³ and data from patients with other allergic diseases indicate that CD69 promotes the inflammatory process.⁶⁴ Our data show that CD69 is the only activation marker with higher levels in children versus adults with AD. This is in line with prior reports showing that CD69 was virtually absent on CLA⁺ T cells from adults with AD, probably because of its transient nature as a very early activation marker.^{60,65} Thus, not surprisingly, its levels are highest in the early developmental stages of AD in young children in whom inflammation is peaking.

Because conversion of naive to cytokine-producing memory subsets is an indication of T-cell activation, we have also assessed cytokine production.

In adults the T_H2/T_H22 profile predominates the skin phenotype with T_H1/T_H17 components in chronic lesions.³⁹ We have recently shown that the blood profile to a large extent resembles the skin phenotype, with skin-selective T_H2/T_C2/T_H22/T_C22 dominance and a T_H1/T_C1 defect.³³ Intracellular cytokine staining data in children are limited and mostly confined to total cytokine measurements (as opposed to CLA⁺/CLA⁻), largely showing high T_H2 frequency with conflicting results regarding T_H1/T_C1.^{20,23,24,26,28,32} The sole pediatric article including CLA cytokine data highlights only the IL-13⁺CLA⁺ predominance in CD4 cells.³⁰ Our results in children with AD display a skin-selective T_H1 defect accompanied by skin-selective increases in T_H2 and an imbalanced CLA⁺ T_H1/T_H2 ratio. Although similar trends were observed in adults, levels in and differences from control subjects were more striking in children. Increased migration to the skin,^{39,66} T_H1 exhaustion,⁶⁷ and high susceptibility to apoptosis⁶⁸ are all plausible explanations for the low IFN- γ levels in children. However, in light of the very high T_H2 signal and high ICOS activation, we suggest that the main cause for decreased T_H1 cells is suppression by T_H2 cells.^{69,70}

Current IgE models suggest that T_H2 T-cell differentiation is required to promote IgE class-switching, with a suppressive effect of IFN- γ .^{71,72} Thus increased ICOS expression that contributes to T_H2 differentiation and in turn to T_H1 suppression might all drive the allergic phenotype observed in young children with AD, including high IgE levels, increased food allergy, and other atopic diseases. Indeed, our data show that T_{CM}/T_{EM} cell subsets in adults with AD do not correlate with disease severity or IgE levels, but in children with AD, they strongly correlate with IgE levels. These data support the notion that T_H2-dependent IgE class-switching occurs early in the disease and persists into adulthood. Yet the pathogenic role of high IgE levels in patients with early-onset AD (seen in approximately 70% of affected children)^{29,73,74} remains unclear. Recent reports in adults suggest that IgE might serve to amplify immune activation rather than having a primary pathogenic role.⁷⁵

Our results show that although adults with AD demonstrate significantly higher IL-22⁺CLA⁺ values than adult control subjects or children with AD, no differences in IL-22 levels are observed between healthy children and children with AD. AD in infants and young children tends to be characterized by acute lesions with erythema and dryness, whereas adults with long-standing disease have marked thickening or lichenification of lesions. Our data, together with the previously recognized role of IL-22 in inducing epidermal hyperplasia,⁷⁶ support a role for IL-22 in disease chronicity and not in disease initiation. Immune reactions tend to have "epitope" spreading over time and in a similar way might involve T_H subset expansion to T_H22, which is seen in chronic AD in adults.

Previous cytokine data in children are discordant regarding relative CD4/CD8 immune dysregulation.^{20,23,25,30,32} Contrary to adults and similar to our observations with ICOS, T_H1/T_H2 imbalance is not evident in CD8 T cells.

We have shown that CD8 T cells are a major source of IL-22 in adult AD skin lesions and that CD8⁺IL-22⁺ T cells are correlated with disease severity.⁴⁰ Although AD was historically considered a CD4-driven disease, a pathogenic role has recently been elucidated for CD8 cells.⁷⁷⁻⁷⁹ Thus in infants and young children CD8 T-cell immaturity,⁸⁰⁻⁸² low counts,^{11,82} exhaustion from super/antigenic stimulation (eg, environmental, food, and bacterial) seen in patients with AD,^{46,83-86} or dysfunctional states⁸⁷ are all putative explanations for the low IL-22 signal in pediatric patients with AD.

In addition to the T_H2 bias at birth, bias toward T_H17 T-cell development has recently been described in healthy infants.⁸⁸ According to our results, adults with AD and control adults had higher IL-17 levels only among CLA⁻ cell subsets compared with their respective pediatric counterparts. This might be due to a selectively higher IL-17 signal in CLA⁺ cells in children, but the trend toward lower T_H17 values in children with AD compared with control subjects suggests that children with AD do not properly build this axis within the cutaneous compartment.

During the naive-to-memory cell conversion in infancy and early childhood, there is a physiologic increased susceptibility to infections.^{44,89} In addition, increased *Staphylococcus aureus* colonization, as evidenced by increased T-cell receptor V β expression on CLA⁺ T cells,⁹⁰ underdeveloped IL-22, reduced IL-17, and suppressed IFN- γ responses, all considered protective cytokines against pathogens,⁹¹⁻⁹⁸ might orchestrate the immune milieu, rendering children with AD more susceptible to skin infections.^{99,100}

Interestingly, IL-9 levels are higher in adults versus children with AD, but only in the skin-homing compartment. Ma et al¹⁰¹ have previously shown that T_H9 frequencies were higher in patients with AD compared with control subjects and that frequency correlated with SCORAD scores and IgE levels. Furthermore, we showed increased IL-9 levels in chronic AD lesional skin.³⁹ Alternately, higher T_H9 frequency in adults versus children with AD can be attributed to higher allergic contact dermatitis seen in this group, although this is speculative.¹⁰²

Studies have shown that approximately 75% of children “outgrow” their AD by 10 years of age.^{42,103-105} Based on our data, one possibility is that age-related disease resolution in children might reflect increased differentiation of T_H1 T cells to counterregulate increased T_H2 cell numbers, as well as reciprocal upregulation of T_H1/T_H17 cells, which synergistically suppress T_H2 production or T_H22 development.

Finally, these results might have relevant therapeutic implications. Although targeted anti-IL-22 treatment might be beneficial only in adults (ILV-094; [ClinicaTrial.gov](#) identifier: NCT01941537), investigating therapeutics that target T_H2 cytokines, such as anti-IL-13 mAb (Tralokinumab; [ClinicaTrial.gov](#) identifier: NCT02347176) and anti-IL-4 receptor α mAb (Dupilumab [REGN668/SAR231893]; [ClinicaTrial.gov](#) identifier: NCT01979016), might be particularly valuable in the pediatric population with AD.

Clinical implications: Therapeutic approaches to treat AD in children might be best directed to correction of T_H2/T_H1 imbalance, whereas adults might also benefit from IL-22-targeted therapies.

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METHODS

Isolation of PBMCs and multiparameter flow cytometric blood analyses

PBMCs were obtained by mean of gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). The blood was laid under the Ficoll gradient; after spinning, the PBMCs were collected at the interface between the plasma and the Ficoll gradient and washed with PBS before staining for flow cytometric analysis. For T-cell surface staining, PBMCs were washed and incubated for 30 minutes in ice with fluorochrome-conjugated mAbs to cell-surface molecules (CD3–phycoerythrin [PE] Alexa Fluor 610, CD8–V-500, CD4–Qdot800, CD45RO–PE-Cy7, CLA–fluorescein isothiocyanate, ICOS–PerCP-Cy5.5, CCR7–Alexa Fluor 700, HLA-DR–allophycocyanin-H7, and Live/dead-Blue Dye). Then the cells were fixed in ice with 4% paraformaldehyde (BD Biosciences, San Jose, Calif) for 20 minutes.

For intracellular cytokine staining, whole blood was activated with 25 ng/mL PMA and 2 μ g/mL ionomycin in the presence of 10 μ g/mL brefeldin A (protein transporter inhibitor) for 4 hours at 37°C. All reagents were

purchased from Sigma-Aldrich (St Louis, Mo). Nonactivated controls were only treated with brefeldin A. After this incubation, EDTA (2 mmol/L; Invitrogen, Grand Island, NY) was added for 15 minutes at room temperature to stop activation. The blood was then incubated with FACS lysing solution for 10 minutes at room temperature and washed with PBS twice. Then the cells were incubated for 20 minutes with fluorochrome-conjugated mAbs to cell-surface molecules (CD3–PE Alexa Fluor 610, CD4–Qdot800, CD69–Brilliant Violet 650, and CLA–fluorescein isothiocyanate). Subsequently, the cells were permeabilized (Perm/Wash; BD Biosciences) and incubated for 20 minutes with fluorochrome-conjugated mAbs (IL-13–PerCP-Cy5.5, IL-22–PE-Cy7, IL-9–PE, IFN- γ –Alexa Fluor 700, and IL-17–allophycocyanin eFluor 780) in 5% mouse serum (eBioscience, San Diego, Calif). All incubations for cell-surface and intracellular staining were done at room temperature. Appropriate FMO-isotype controls were used.

After incubations, cells were washed. This was followed by acquisition with a BD LSR II flow cytometer (BD Biosciences) and analysis with FlowJo software (TreeStar, Ashland, Ore).

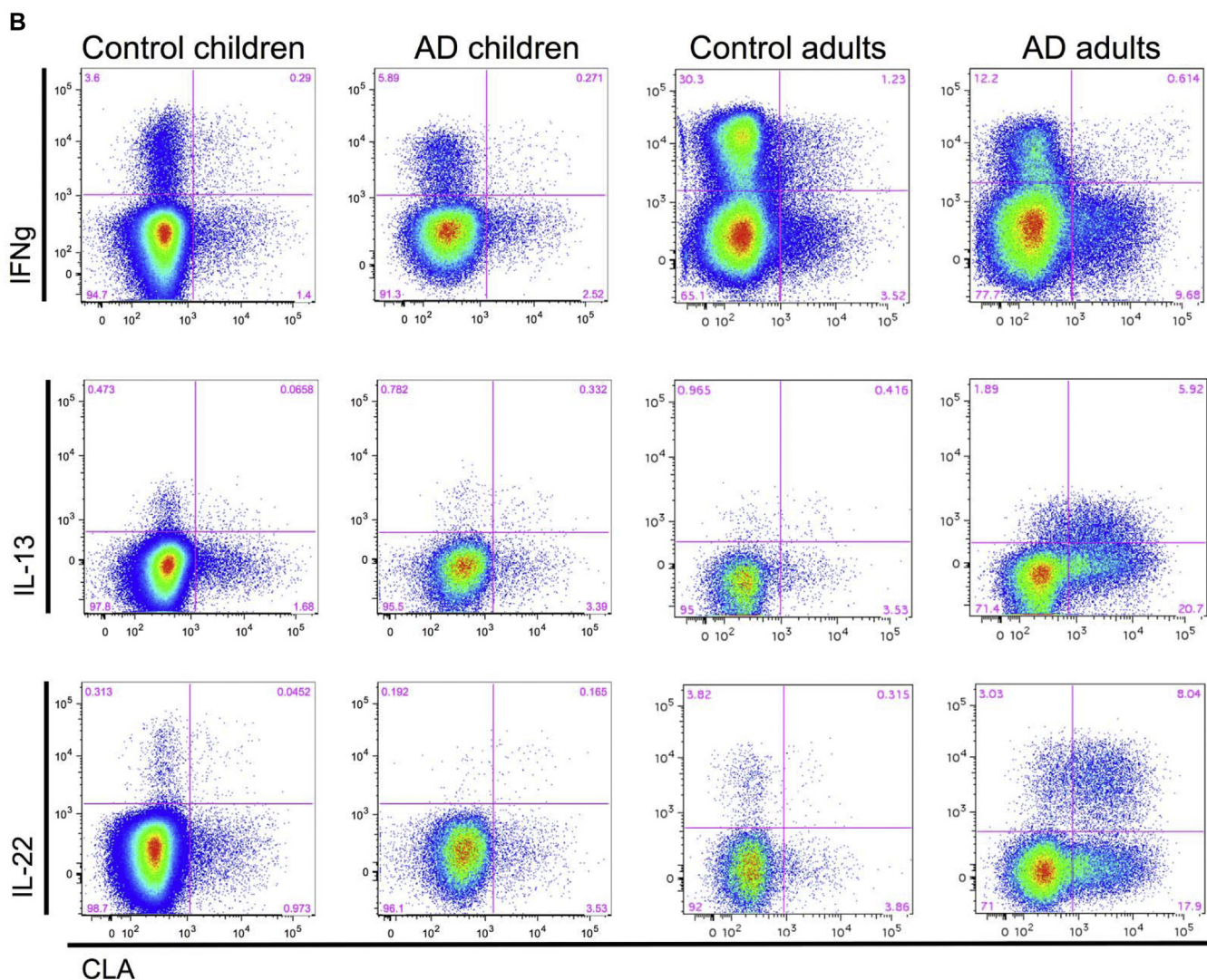
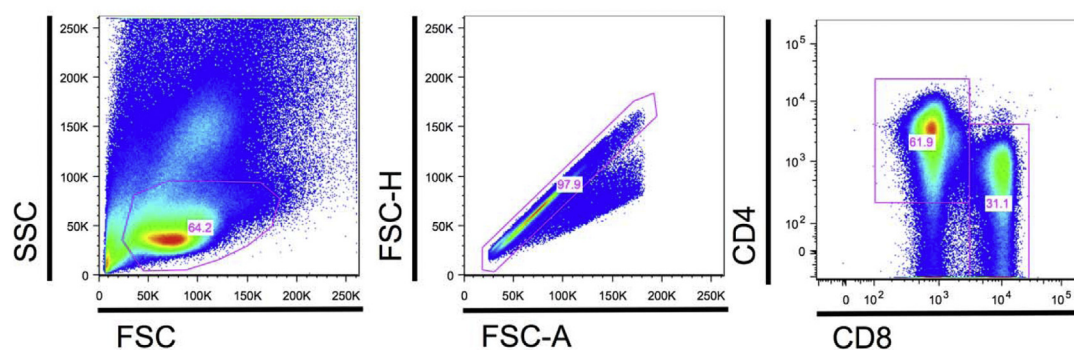
A Gating strategy

FIG E1. Gating strategy and frequency of IFN- γ , IL-13, and IL-22 versus CLA positivity in CD4⁺ T cells in pediatric and adult healthy control subjects and representative patients with AD. **A**, Gating strategy. *FSC*, Forward scatter; *SSC*, side scatter. **B**, Representative plots of IFN- γ , IL-13, and IL-22 and CLA expression, showing reduced IFN- γ ⁺CLA⁺ cells in patients with AD accompanied by increased IL-13⁺ cell percentages in both children and adults and enhanced IL-22⁺ cell percentages only in adults with AD.

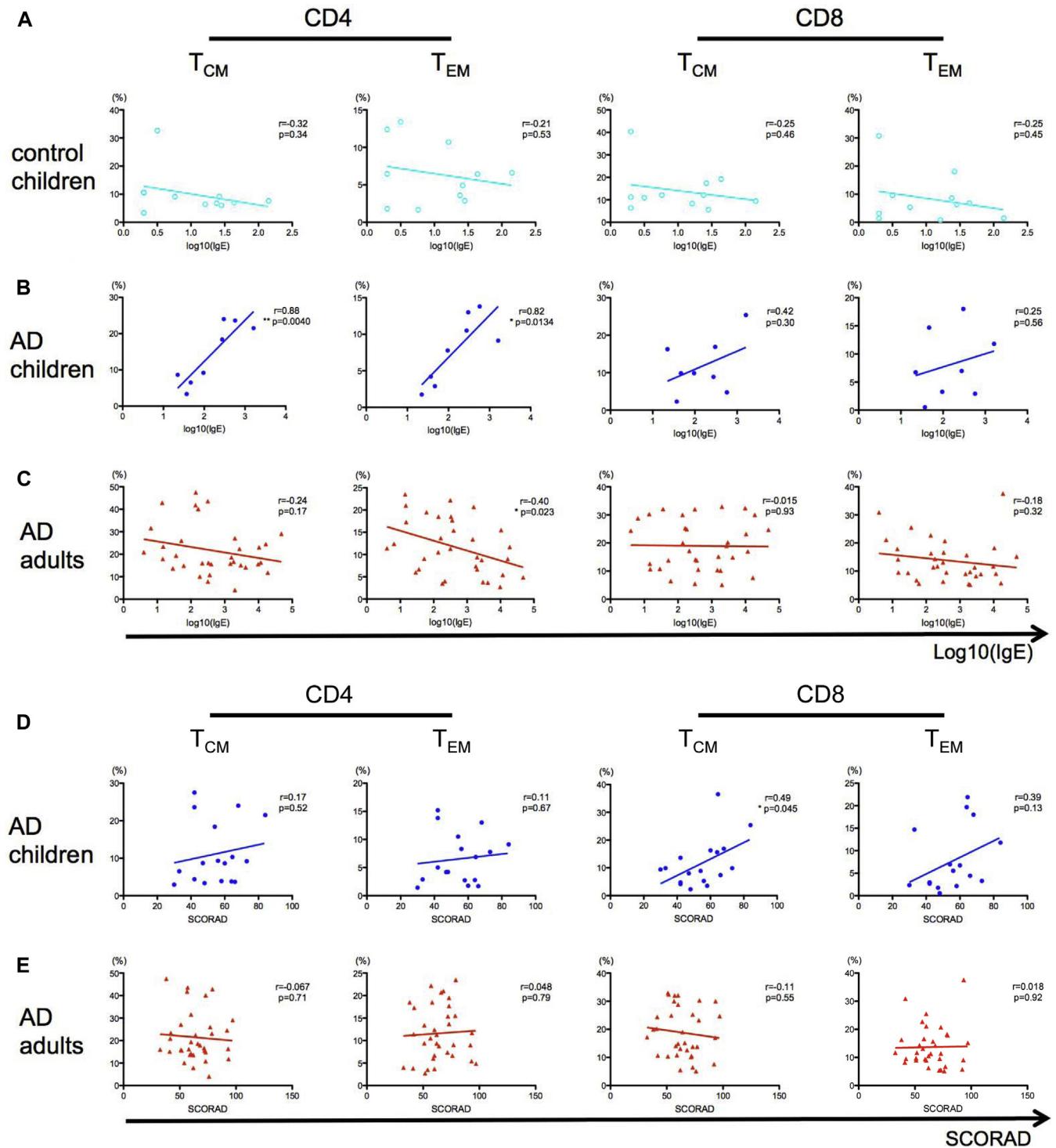


FIG E2. CD4 and CD8 T_{CM} and T_{EM} cell subset correlations with IgE levels and disease severity/SCORAD scores. **A**, No correlations were found between T_{CM}/T_{EM} memory cell subsets and IgE levels among control children. **B**, Positive correlations were observed in children with AD. **C**, T_{CM}/T_{EM} cell subsets were not correlated with IgE levels in adults with AD. **D** and **E**, SCORAD scores were not correlated with T_{CM}/T_{EM} cell frequencies in either children or adults with AD. * $P < .05$ and ** $P < .01$.