

susceptibility to histoplasmosis is difficult. There are no reports of histoplasmosis in X-linked agammaglobulinemia, suggesting that antibodies may not be an important part of the protective host response against *Histoplasma*. The recent identification of a variety of combined immune defects in patients previously diagnosed as suffering from CVID leaves open the possibility that those patients diagnosed with CVID, in fact, had more complex underlying immune defects, including such entities as STAT1 gain of function or GATA2 deficiency. Similarly, CMC has been linked to a heterogeneous group of immunologic disorders that also predispose to histoplasmosis, including dominant negative STAT3 mutations, gain-of-function STAT1 mutations, and IL-12Rβ1 deficiency,^{E1} indicating that patients with known CMC and histoplasmosis likely have a primary immune defect.

Cases of ICL were included in this review because disseminated histoplasmosis itself can cause CD4 lymphocytopenia due to bone marrow infiltration, and successful treatment of histoplasmosis can restore CD4 T-cell counts.⁹ In many cases, sequential measurements of cell counts before and after infections support the diagnoses of ICL; however, CD4 lymphocytopenia subsequent to uncontrolled histoplasmosis may be misinterpreted as the predisposing condition to disseminated histoplasmosis, and another immune defect may be overlooked.

Our review of severe histoplasmosis suggests that in the absence of a known acquired or secondary immune deficiency, patients with disseminated histoplasmosis should be investigated for selected PIDs. Most immune defects identified molecularly in patients with disseminated histoplasmosis converge on the pathways involving IL-12/IFN-γ, NF-κB, and STAT3.

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REFERENCES

1. Kauffman CA. Histoplasmosis. *Clin Chest Med* 2009;30:217-25.
2. Grabbe C, Husnjak K, Dikic I. The spatial and temporal organization of ubiquitin networks. *Nat Rev Mol Cell Biol* 2011;12:295-307.
3. Courtois G, Israël A. IKK regulation and human genetics. In: Karin M, editor. *NF-κB in health and disease. Current topics in microbiology and immunology. Volume 349*. Berlin: Springer; 2011. pp. 73-95.
4. Filipe-Santos O, Bustamante J, Haverkamp MH, Vinolo E, Ku CL, Puel A, et al. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. *J Exp Med* 2006;203:1745-59.

5. Orange JS, Jain A, Ballas ZK, Schneider LC, Geha RS, Bonilla FA. The presentation and natural history of immunodeficiency caused by nuclear factor kappa B essential modulator mutation. *J Allergy Clin Immunol* 2004;113:725-33.
6. Braue J, Murugesan V, Holland S, Patel N, Naik E, Leiding J, et al. NF-kappa B essential modulator deficiency leading to disseminated cutaneous atypical mycobacteria. *Mediterr J Hematol Infect Dis* 2015;7:e2015010.
7. Mihu MR, Nosanchuk JD. Histoplasma virulence and host responses. *Int J Microbiol* 2012;2012:268123.
8. Subramanian Vignesh K, Landero Figueroa JA, Porollo A, Caruso JA, Deepe GS Jr. Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. *Immunity* 2013;39:697-710.
9. Rhew DC, Goetz MB, Louie MH. Reversible CD4+ T lymphocyte depletion in a patient who had disseminated histoplasmosis and who was not infected with human immunodeficiency virus. *Clin Infect Dis* 1995;21:702-3.

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FOXP3⁺ CD4 T-cell maturity and responses to microbial stimulation alter with age and associate with early-life gut colonization



To the Editor:

The immune system is immature at birth, and environmental exposures are crucial to ensure proper immune maturation. Interestingly, the composition of the gut microbiota in early life is linked to the development of immune-mediated diseases such as allergy.¹ Forkhead box P3 (FOXP3)⁺ CD4 regulatory T (Treg) cells control the course and duration of immune responses. A failure in these regulatory processes could lead to exaggerated immune responses, host damage, and immune-mediated diseases such as allergy.^{2,3} IL-10-producing Treg cells are present in the human colon and suggested to suppress inflammatory responses, indicating a connection between the microbiota and Treg cell-function.⁴ Also, peripheral Treg cells are educated by the colonic microbiota⁵ and microbiota-derived metabolites affect Treg cell-function and generation in the periphery.⁶ Today, we know very little about FOXP3⁺ CD4 T-cell function during early childhood because most studies have been conducted on cord blood, which indeed is informative of the immune status *in utero* but not of postnatal life, when the child has been directly exposed to environmental factors such as the microbiota.

Lactobacilli are present in the infant gut and correlate with a lower risk of allergy later in life.⁷ In addition, lactobacilli dampen *in vitro* immune activation induced by *Staphylococcus aureus* (*S aureus*), a bacterium prevalent in the gut in early life and associated with enhanced immune activation.^{8,9}

Here, we investigated the maturation of peripheral CD4⁺CD25⁺FOXP3⁺CD127^{low} T cells in early life in a cross-sectional study, and whether colonization with lactobacilli and *S aureus* during infancy associates with the maturation and functional responses of FOXP3⁺ cells at 2 years of age. Furthermore, we studied how soluble products from these bacteria affect FOXP3⁺ cells *in vitro*.

PBMCs from 25 two-year-old children were selected from a prospective birth cohort. Children were included on the basis of the presence of DNA from *S aureus* and a group of *Lactobacillus*

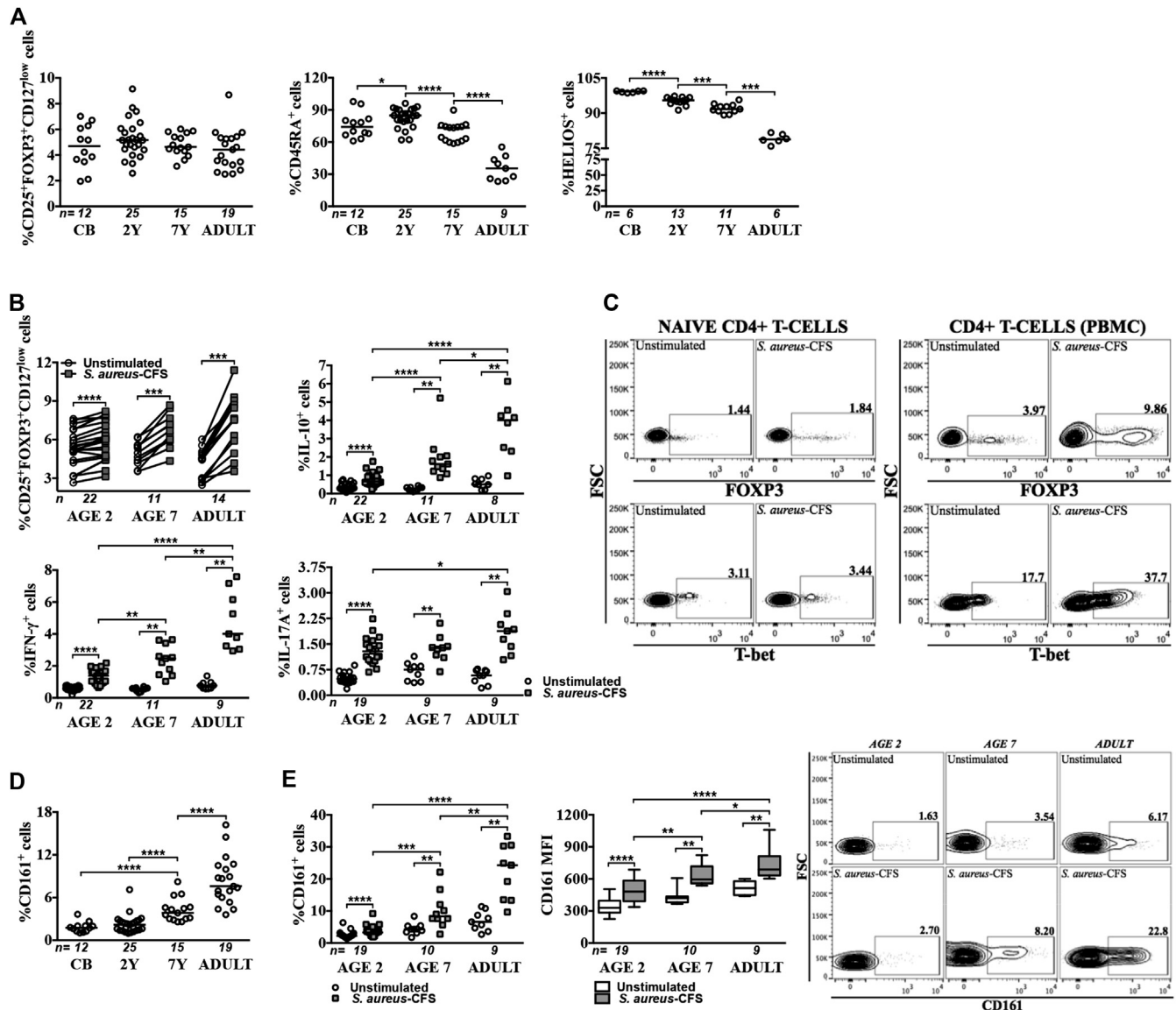


FIG 1. The phenotype and functional responses of CD4⁺CD25⁺FOXP3⁺CD127^{low} cells mature with age. PBMCs were analyzed after 2-hour resting (**A** and **D**) or after 24-hour stimulation with *S aureus*-CFS (**B** and **E**). Naive CD4⁺ T cells were cultured with autologous monocytes and analyzed after 48-hour stimulation with *S aureus*-CFS (**C**). Fig 1, **A**, *Left*: the percentage of CD25⁺FOXP3⁺CD127^{low} (FOXP3⁺) cells among the CD4⁺ T-cell population. *Middle and right*: the percentages of CD45RA⁺ cells (*middle*) and HELIOS⁺ cells (*right*) among the FOXP3⁺ population. Fig 1, **B**, *Upper left*: the percentage of FOXP3⁺ cells among the CD4⁺ T-cell population; *upper right and lower panels*: the percentages of IL-10⁺, IFN-γ⁺, and IL-17A⁺ cells among the FOXP3⁺ population. Fig 1, **C**, Representative intracellular stainings of FOXP3 and T-bet expression in isolated naive CD4⁺ T cells or in CD4⁺ T cells in whole PBMCs. Fig 1, **D**, The percentage of CD161⁺ cells among the FOXP3⁺ population. Fig 1, **E**, The percentage of CD161⁺ cells and CD161 MFI values among the FOXP3⁺ population. CB, Cord blood; MFI, mean fluorescence intensity; T-bet, T-box transcription factor. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

(*L*) strains (*L rhamnosus*, *L casei*, *L paracasei*) in fecal samples at 3 occasions during infancy: age 1 week, 2 weeks, and 2 months.⁷ In addition, PBMCs from healthy donors were collected at birth (cord blood, *n* = 12), 7 years of age (*n* = 15), and adulthood (*n* = 19) to investigate immune maturation. For some experiments, naive CD4 T cells or CD14⁺ monocytes were isolated by magnetic separation. PBMC or isolated cell populations were analyzed after 2 hours of resting (basal phenotyping) or after 24-hour stimulation with cell-free

supernatants (CFSs) derived from *S aureus* 161:2 and/or *L reuteri* DSM 17938. Secreted cytokine levels in PBMC cultures were measured with ELISA. For a complete description of [subjects](#), [materials](#), and [methods](#), see this article's Online Repository at www.jacionline.org.

To characterize the FOXP3⁺ CD4 T-cell population during childhood, we investigated these cells in CBMCs and in PBMCs from children at 2 and 7 years of age and from adults by flow cytometry. There were no differences in the percentage of

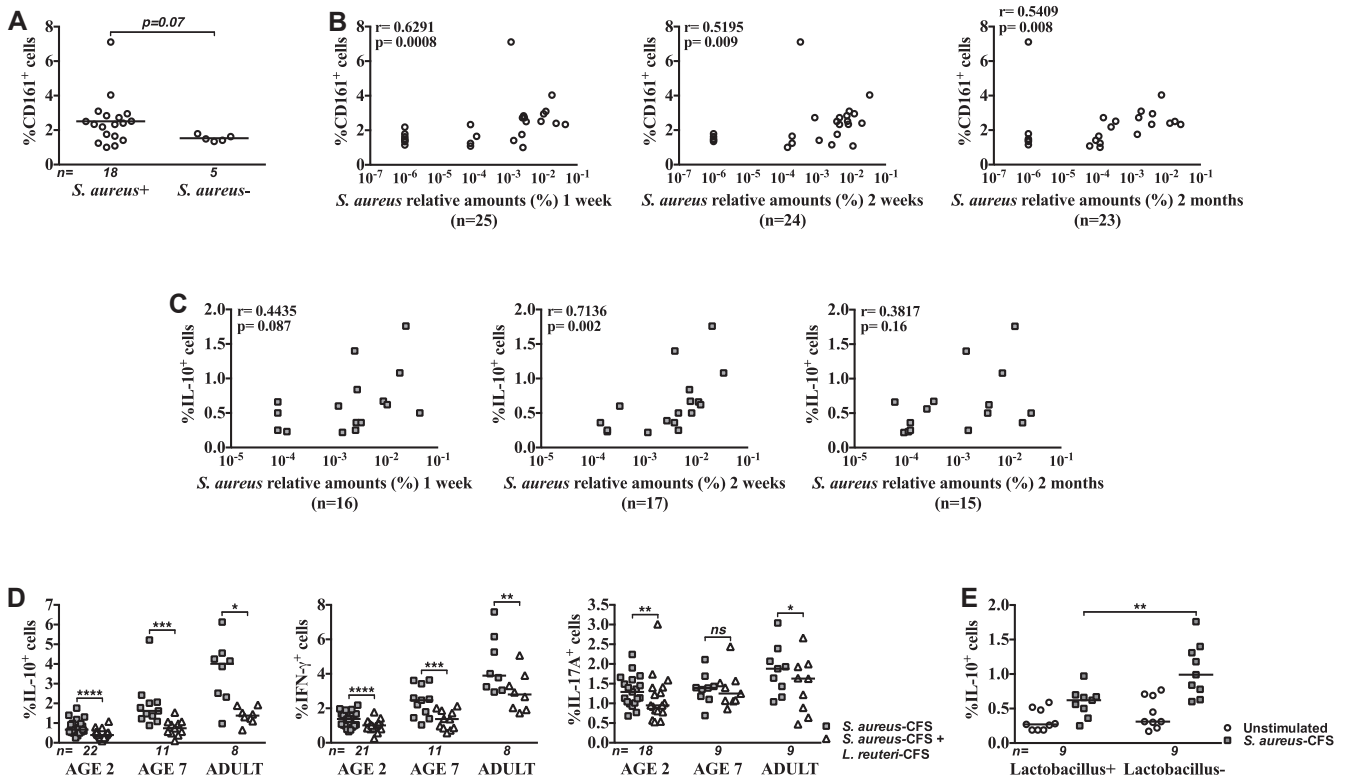


FIG 2. Infant colonization with *S. aureus* and lactobacilli associates with CD161 expression and cytokine production by FOXP3⁺ cells at 2 years of age. PBMC were analyzed after 2-hour resting (**A** and **B**) or after 24-hour stimulation with *S. aureus*-CFS ± *L. reuteri*-CFS (**C-E**). Immunological data at 2 years of age were correlated with the presence/absence of gut bacteria early in life (age 1 week, 2 weeks, 2 months). Fig 2, **A** and **B**, The percentage of CD161⁺ cells among the FOXP3⁺ population in 2-year-old children colonized (+) or not colonized (–) by *S. aureus* or in correlation with the relative amounts of *S. aureus* in stool expressed as percent bacterial DNA of total nucleic acids. Fig 2, **C**, The percentage of IL-10⁺ cells among the FOXP3⁺ population in correlation with the relative amounts of *S. aureus* in stool. Fig 2, **D**, The percentage of FOXP3⁺ cells expressing IL-10, IFN-γ, and IL-17A. Fig 2, **E**, The percentage of IL-10⁺ cells among the FOXP3⁺ population in 2-year-old children in relation to lactobacilli colonization. **P* < .05, ***P* ≤ .01, ****P* ≤ .001, and *****P* ≤ .0001.

FOXP3⁺ cells within the CD4 T-cell population between the investigated age groups. Within the FOXP3⁺ population, the percentage of CD45RA⁺ cells significantly decreased after 2 years of age. The percentage of HELIOS⁺ cells seemed to decline with age, supporting the idea that HELIOS expression could represent thymic origin¹⁰ (Fig 1, A; see Fig E1, A, in this article's Online Repository at www.jacionline.org). Still, usage of continuous, longitudinal samples would be required to fully explore age-related aspects of FOXP3⁺ T-cell maturation. Declining percentages of HELIOS⁺ and CD45RA⁺ cells were also observed in the total CD4⁺ and CD4⁺FOXP3⁺ T-cell populations (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). We then investigated how age influences the FOXP3⁺ cell response to microbial stimuli. *S. aureus*-CFS induced an increased percentage of CD25⁺FOXP3⁺CD127^{low} cells among the CD4 T-cell population in all age groups in all but 1 individual (Fig 1, B; Fig E1, B). There were lower percentages of FOXP3⁺ cells with an early (24-hour) expression of IL-10, IFN-γ, and IL-17A in children than in adults (Fig 1, B; Fig E1, C). We speculated that this could depend on the higher percentage of naive CD4 T cells in children. Because low cell numbers precluded kinetic analyses of the infant cell responses, we purified naive CD4 T cells from healthy adult donors and could show that following

stimulation, these naive cells produced less cytokines (data not shown) and had a lower induction of both FOXP3 and the T_H1 transcription factor T-bet compared with CD4⁺ T cells in PBMC cultures (Fig 1, C). Naive cells did not express ROR-γt (T_H17) or GATA-3 (T_H2) (Fig E1, D).

CD161 expression has been connected to the cytokine-producing capacity of FOXP3⁺ cells.¹¹ Here, we demonstrate that the percentage of CD161⁺ cells and CD161 expression among the FOXP3⁺ population are significantly related to age (Fig 1, D and E; Fig E2, C). Notably, *S. aureus*-CFS induced an increased expression of CD161 by FOXP3⁺ cells (Fig 1, E). We therefore investigated whether early-life *S. aureus* colonization associated with CD161 expression at 2 years of age. Noncolonized children tended to have a lower percentage of CD161⁺ cells within the FOXP3⁺ population (Fig 2, A) and the amounts of *S. aureus* at 3 different time points in early life positively correlated with the percentage of CD161⁺ cells at 2 years of age (Fig 2, B). *S. aureus* amounts also positively correlated with the percentage of IL-10⁺ cells among the FOXP3⁺ population after stimulation (Fig 2, C). We suggest that the age-related increased percentage of CD161⁺ cells reflects a capacity for enhanced cytokine production by FOXP3⁺ cells after microbial stimulation, supported by the observed

increase in CD161 expression after *S aureus* stimulation in all age groups.

We have previously shown that lactobacilli-derived factors are able to dampen *S aureus*-induced activation of lymphocytes *in vitro*.⁹ After costimulation of PBMCs with both *S aureus*-CFS and *L reuteri*-CFS, the activation of FOXP3⁺ cells was dampened. This was evident as a lower percentage of FOXP3⁺ cells (data not shown) and a reduced percentage of FOXP3⁺ cells expressing CD161 (Fig E3, A) and cytokines (Fig 2, D; see Fig E3, B, in this article's Online Repository at www.jacionline.org). Furthermore, *L reuteri*-CFS dampened the secretion of IFN- γ and IL-17A from PBMC cultures (Fig E3, C). Of note, the capacity of *L reuteri*-CFS to dampen *S aureus*-induced activation was not related to age. Finally, the percentage of IL-10⁺ cells among the FOXP3⁺ population was lower in 2-year-old children that were colonized by lactobacilli in infancy (Fig 2, E). Indeed, lactobacilli colonization associates with fewer IL-10-producing PBMCs after PHA stimulation.⁸

Here, we show that FOXP3⁺ T cells from children are impaired in microbial-induced cytokine-production, which possibly connects to a lower CD161 expression. The clear influence of bacterial products derived from *S aureus* and lactobacilli, which are present in the early gut of infants, on peripheral FOXP3⁺ CD4 T-cell responses as well as associations between the early-life gut microbiota imply an important role for gut microbes in shaping immune responses in childhood.

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REFERENCES

- West CE, Renz H, Jenmalm MC, Kozyskij AL, Allen KJ, Vuillermin P, et al. The gut microbiota and inflammatory noncommunicable diseases: associations and potentials for gut microbiota therapies. *J Allergy Clin Immunol* 2015; 135:3-13.
- Hoeppli RE, Wu D, Cook L, Levings MK. The environment of regulatory T cell biology: cytokines, metabolites, and the microbiome. *Front Immunol* 2015;6:61.
- Zhang H, Kong H, Zeng X, Guo L, Sun X, He S. Subsets of regulatory T cells and their roles in allergy. *J Transl Med* 2014;12:125.
- Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, et al. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol* 2006;177:5852-60.
- Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio C-W, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 2011;478:250-4.

- Arpaia N, Campbell C, Fan X, Dikly S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 2013;504:451-5.
- Johansson MA, Sjögren YM, Persson J-O, Nilsson C, Sverreemark-Ekström E. Early colonization with a group of Lactobacilli decreases the risk for allergy at five years of age despite allergic heredity. *PLoS One* 2011;6:e23031.
- Johansson MA, Saghaian-Hedengren S, Haileselassie Y, Roos S, Troye-Blomberg M, Nilsson C, et al. Early-life gut bacteria associate with IL-4-, IL-10- and IFN- γ production at two years of age. *PLoS One* 2012;7:e49315.
- Haileselassie Y, Johansson MA, Zimmer CL, Björkander S, Petersdottir DH, Dicksved J, et al. Lactobacilli regulate *Staphylococcus aureus* 161:2-induced pro-inflammatory T-cell responses *in vitro*. *PLoS One* 2013;8:e77893.
- Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 2010;184:3433-41.
- Björkander S, Hell L, Johansson MA, Forsberg MM, Lasaviciute G, Roos S, et al. *Staphylococcus aureus*-derived factors induce IL-10, IFN- γ and IL-17A-expressing FOXP3+CD161+ T-helper cells in a partly monocyte-dependent manner. *Sci Rep* 2016;6:22083.

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Epigenome-wide association study links site-specific DNA methylation changes with cow's milk allergy



To the Editor:

IgE-mediated cow's milk allergy (CMA), which affects 2% to 3% of young children, is among the most common types of food allergy. The molecular mechanisms underlying the development, persistence, and resolution of CMA remain largely unknown. Several recent studies have suggested that epigenetic alterations are involved in allergy development.^{1,2} DNA methylation (DNAm), a type of epigenetic mechanism, regulates gene expression. Alterations in DNAm play a key role in T-cell differentiation and in maintaining T_H1/T_H2 balance,^{1,2} and thus may offer a potential mechanistic explanation for the natural history of CMA. However, to date, research on the epigenetics of food allergy,³ or CMA in particular,⁴ has been very limited. Most of the available studies are based on small sample sizes (<60 children), and were not replicated in an independent sample.

We performed the first epigenome-wide association study of CMA in US children using a 2-stage approach. In the discovery stage, we measured DNAm at 485,512 genomic loci in whole blood samples from 106 white children with CMA (cases) and 76 nonallergic and nonatopic whites (controls) using the Illumina HumanMethylation450 arrays. All the cases and controls were enrolled by the Chicago Food Allergy Study using identical protocols. We then sought to validate the top significant DNAm loci in 2 independent replication samples including (1) childhood whole blood samples of 25 white children (5 CMA cases and 20 controls) from the same Chicago Food Allergy Study (Chicago replication sample) and (2) cord blood samples from 140 black children (8 cases and 132 controls) from the Boston Birth Cohort (Boston replication sample) (see this article's **Methods** section in the Online Repository at www.jacionline.org). Population characteristics of the discovery and replication samples are presented in **Tables E1** and **E2** in this article's Online Repository at www.jacionline.org, respectively.

CMA cases were defined if the children met the following criteria: (1) a convincing history of symptoms indicative of an

METHODS

Ethics statement

Ethical permissions were obtained from the Regional Ethics Committee in Stockholm, Sweden (2014/2052-32; Dnr 04-106/1) and from the Human Ethics Committee at Huddinge University Hospital, Stockholm (Dnr 331/02 and Dnr 117/97). All study subjects or legal guardians gave their informed consent. All samples were handled as instructed in the approved ethical applications. It will not be possible to connect published data to any individual.

Subjects

In this study, we used material from healthy newborn children, 2-year-old children, 7-year-old children, and adults as described below. Twenty-five 2-year-old children were selected from a prospective birth cohort described in detail elsewhere.^{E1} Children were included on the basis of the presence of DNA from *S aureus* and a group of *Lactobacillus* strains (*L rhamnosus*, *L casei*, and *L paracasei*) in fecal samples at 3 occasions during infancy: age 1 week, 2 weeks, and 2 months.^{E2} Immunological data were correlated with the presence/absence of lactobacilli or *S aureus* or with the relative amounts of *S aureus* in stool collected at the indicated time points of age expressed as percent bacterial DNA of total nucleic acids. All infants from the prospective birth cohort were born vaginally at full term, with normal birth weight, were exclusively breast-fed, and did not receive antibiotics during the first 3 months of life. The infants were without known health conditions, except for 7 children who were IgE-sensitized at 2 years of age; however, these children did not differ in any of the investigated immune parameters. In addition, mononuclear cells from healthy donors were collected at birth (cord blood, $n = 12$, all vaginally delivered), 7 years of age ($n = 15$), and adults ($n = 19$, age 18-65 years) to investigate immune maturation in relation to age.

Cord/peripheral blood mononuclear cell isolation

Venous blood was collected in heparinized vacutainer tubes (BD Biosciences Pharmingen, San Jose, Calif). CBMCs/PBMCs were isolated by Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient separation. PBMCs were used directly or washed in RPMI-1640 and diluted in freezing medium containing 40% RPMI-1640, 50% FCS (Life Technologies-Gibco, Carlsbad, Calif), and 10% dimethyl sulfoxide (Sigma Aldrich, St Louis, Mo) and stored in liquid nitrogen until analyzed.

Purification of human naive CD4 T cells and CD14⁺ monocytes

The human naive CD4 negative selection kit (StemCell Technologies, Grenoble, France) was used to isolate naive CD4⁺ T cells from freshly prepared PBMCs. The mean percentage of CD45RA⁺CD45RO⁻ cells among live CD4⁺ T cells was 99.6 ± 0.39 after purification. The human CD14 positive selection kit (Stem Cell Technologies) was used to purify monocytes from PBMCs; the mean percentage of CD14⁺ cells was 97.4 ± 1.67 after purification. Overall, the viability of the purified cells was more than 90%.

Bacterial strains

CFSs from *S aureus* 161:2 (expressing genes for staphylococcal enterotoxin A and H, kind gift from Åsa Rosengren, the National Food Agency, Uppsala, Sweden) and *L reuteri* DSM 17938 (kind gift from Biogaia AB, Stockholm, Sweden) were kindly provided by Stefan Roos, the Swedish University of Agricultural Sciences. *S aureus* 161:2 was cultured in BHI broth (Merck, Darmstadt, Germany), and *L reuteri* DSM 17938 was cultured in MRS broth (Oxoid, 149, Hampshire, United Kingdom) and the CFSs were prepared as previously described.^{E3}

In vitro activation of PBMCs

CBMCs/PBMCs were thawed and washed before counting and exclusion of nonviable cells by Trypan blue staining. The cell viability was more than

90%. Cells were resuspended to 10^6 cells/mL in cell culture medium: RPMI-1640 supplemented with 20 mM HEPES, penicillin (100 U/mL), streptomycin (100 μ g/mL), L-glutamine (2 mM) (all from HyClone Laboratories, Inc, South Logan, Utah), and 10% heat-inactivated FCS. The PBMCs were either rested for 2 hours (basal phenotyping) or incubated for 24 hours with cell culture medium alone or with 2.5% of CFS from either *S aureus* 161:2 or *L reuteri* DSM 17938 or both in combination in flat-bottomed 96-well cell-culture plates (Costar, Cambridge, UK) with Monensin or Brefeldin A (BD Biosciences) present during the last 4 hours of incubation at 37°C in 6% CO₂ atmosphere. Dynabeads Human T-Activator CD3/CD28-beads (Life Technologies, Carlsbad, Calif) used at 2:1 (cell:bead) ratio served as positive control. Alternatively, 2.5×10^5 PBMCs diluted to 1×10^6 cells/mL or 1×10^5 isolated, naive CD4⁺ T cells diluted to 0.5×10^6 cells/mL in cell culture medium were stimulated with *S aureus*-CFS for 48 hours. Naive CD4⁺ T cells were plated with 12,000 autologous monocytes.

Flow cytometry

Cells were stained with the LIVE/DEAD Fixable Dead Cell Stain Kit-Aqua (Life Technologies) and then incubated with 10% human serum in FACS-wash buffer (PBS, 2 mM EDTA and 0.1% BSA). Cells were stained with cell surface antibodies: CD4 FITC, CD25 APC-H7, CD45RA PE-Cy7, CD127 PE-Cy7, CD127 BV421 (all from BD Biosciences) or CD45RA FITC (ImmunoTools, Friesoythe, Germany) or CD161 PerCP-Cy5.5 (Biolegend, San Diego, Calif). For intracellular staining, the Transcription factor buffer set (BD Biosciences) was used according to instructions from the manufacturer. Cells were stained with FOXP3 PE, IFN- γ PerCP-Cy5.5, IFN- γ APC, IL-17A V450 (all from BD Biosciences) or IL-10 APC, HELIOS APC (Biolegend). The FACSVerse instrument and the FACSSuite software (BD Biosciences) were used to acquire data. Lymphocytes were gated on the basis of forward and side scatter properties. After gating on live CD4⁺ T cells (the percentage of live cells was generally above 95%), cells were divided into being CD25⁺FOXP3⁺CD127^{low} (FOXP3⁺ cells) or FOXP3⁻ cells. Unstimulated cells or corresponding isotype-matched antibodies were used as negative controls. The results show either the percentage of positive cells within a given population or the mean surface expression of receptors per cell defined as geometrical mean fluorescence intensity. Analysis was done with FlowJo Software (TreeStar, Ashland, Ore).

Statistics

The GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif) was used for statistical analysis. Groups were compared with the nonparametric Kruskal-Wallis test and if significant, comparisons between 2 groups were made with the Mann-Whitney *U* test. Comparisons of parameters within the same individual were made with the Wilcoxon matched pairs test or the Spearman rank correlation test. The differences were considered significant if *P* was less than .05 (**P* < .05, ***P* ≤ .01, ****P* ≤ .001, *****P* ≤ .0001). For all figures, shown statistics relate to comparisons of 2 parameters. Dot plots show median values represented as the horizontal line, boxes cover data values between the 25th and 75th percentiles, with the central line as median, and bars show median with interquartile range.

REFERENCES

1. Nilsson C, Linde A, Montgomery SM, Gustafsson L, Näsman P, Blomberg MT, et al. Does early EBV infection protect against IgE sensitization? *J Allergy Clin Immunol* 2005;116:438-44.
2. Johansson MA, Sjögren YM, Persson J-O, Nilsson C, Sverremark-Ekström E. Early colonization with a group of *Lactobacilli* decreases the risk for allergy at five years of age despite allergic heredity. *PLoS One* 2011;6:e23031.
3. Haileselassie Y, Johansson MA, Zimmer CL, Björkander S, Petursdottir DH, Dicksved J, et al. *Lactobacilli* regulate *Staphylococcus aureus* 161:2-induced pro-inflammatory T-cell responses in vitro. *PLoS One* 2013;8:e77893.

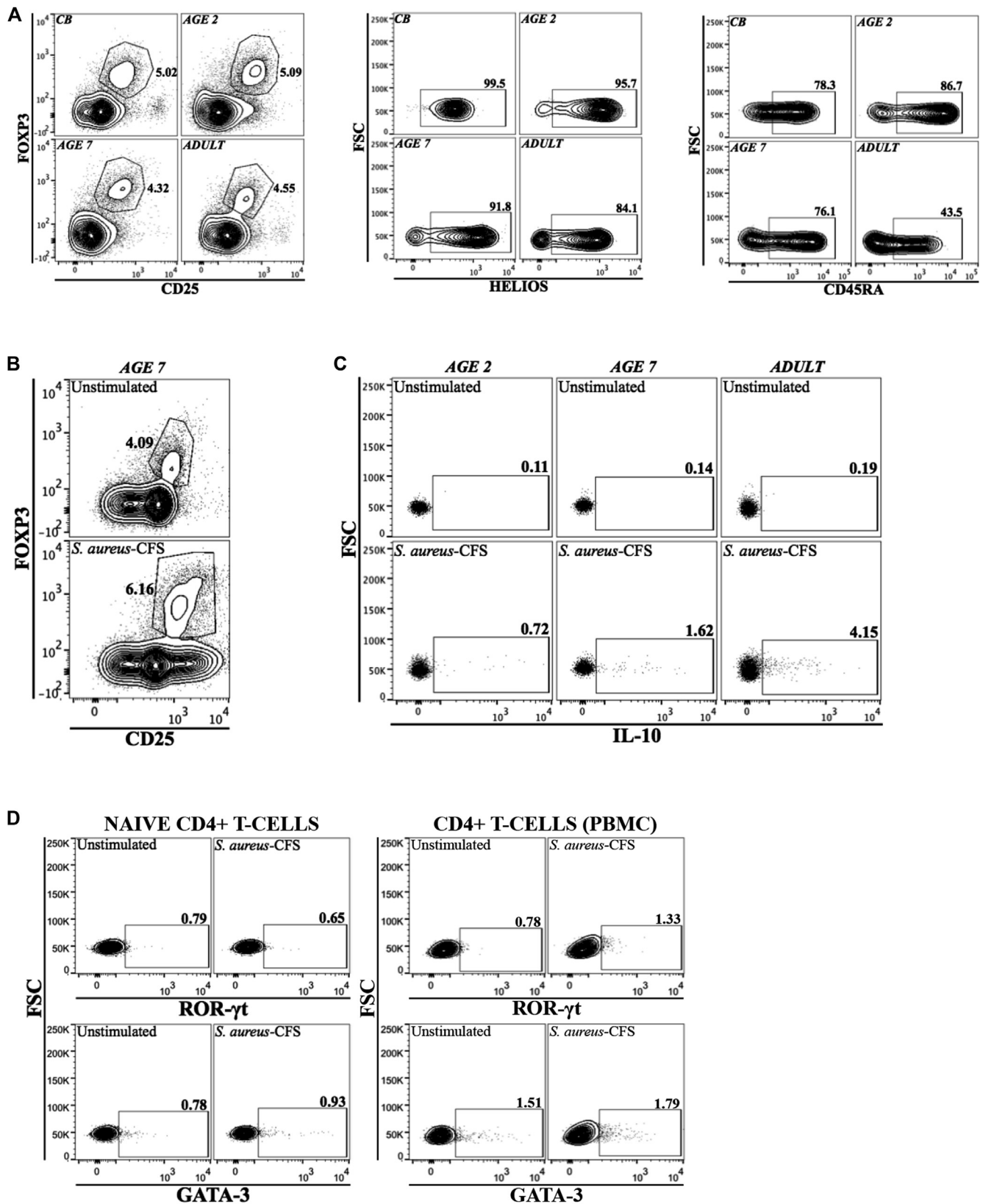


FIG E1. Representative dot plots of flow cytometry data. **A**, PBMCs were rested for 2 hours before being stained and analyzed by flow cytometry. *Left*: staining of FOXP3 and CD25 gated from live CD4⁺ T cells; *middle and right*: staining of HELIOS (*middle*) and CD45RA (*right*) gated on CD4⁺CD25⁺FOXP3⁺CD127^{low} cells. **B**, PBMCs were left unstimulated or cultured in the presence of *S. aureus* 161:2-CFS. Representative staining of FOXP3 and CD25 exemplified by PBMCs from one 7-year-old donor. **C**, PBMCs were left unstimulated or cultured in the presence of *S. aureus*-CFS. Representative intracellular staining of IL-10 expression in FOXP3⁺ cells. **D**, Representative intracellular stainings of ROR- γ t and GATA-3 expression in isolated naive CD4 T cells cultured with autologous monocytes or in CD4 T cells in whole PBMCs after stimulation with *S. aureus* 161:2-CFS. CB, Cord blood; FSC, forward scatter; GATA, trans-acting T-cell-specific transcription factor GATA-3; ROR, RAR-related orphan receptor.

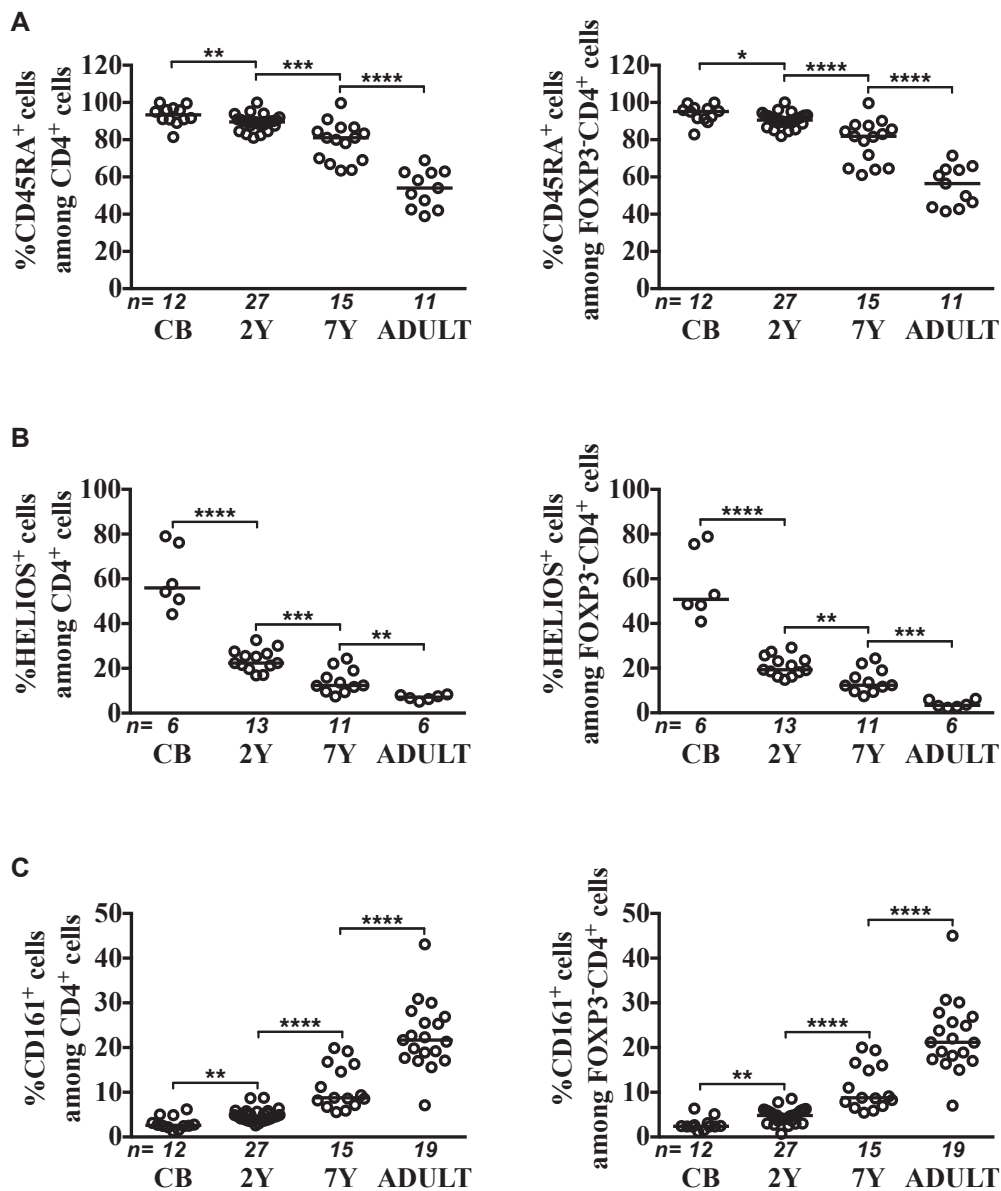


FIG E2. The percentage of CD4⁺ and CD4⁺FOXP3⁺ T-cells that express CD45RA, HELIOS, and CD161 alter with age. PBMC were rested for 2 hours before being stained and analyzed by flow cytometry. **A-C,** The percentage of CD45RA⁺ cells (Fig E2, A), HELIOS⁺ cells (Fig E2, B), and CD161⁺ cells (Fig E2, C) among the total CD4⁺ (left column) or the CD4⁺FOXP3⁺ (right column) T-cell populations. CB, Cord blood. **P* < .05, ***P* ≤ .01, ****P* ≤ .001, and *****P* ≤ .0001.

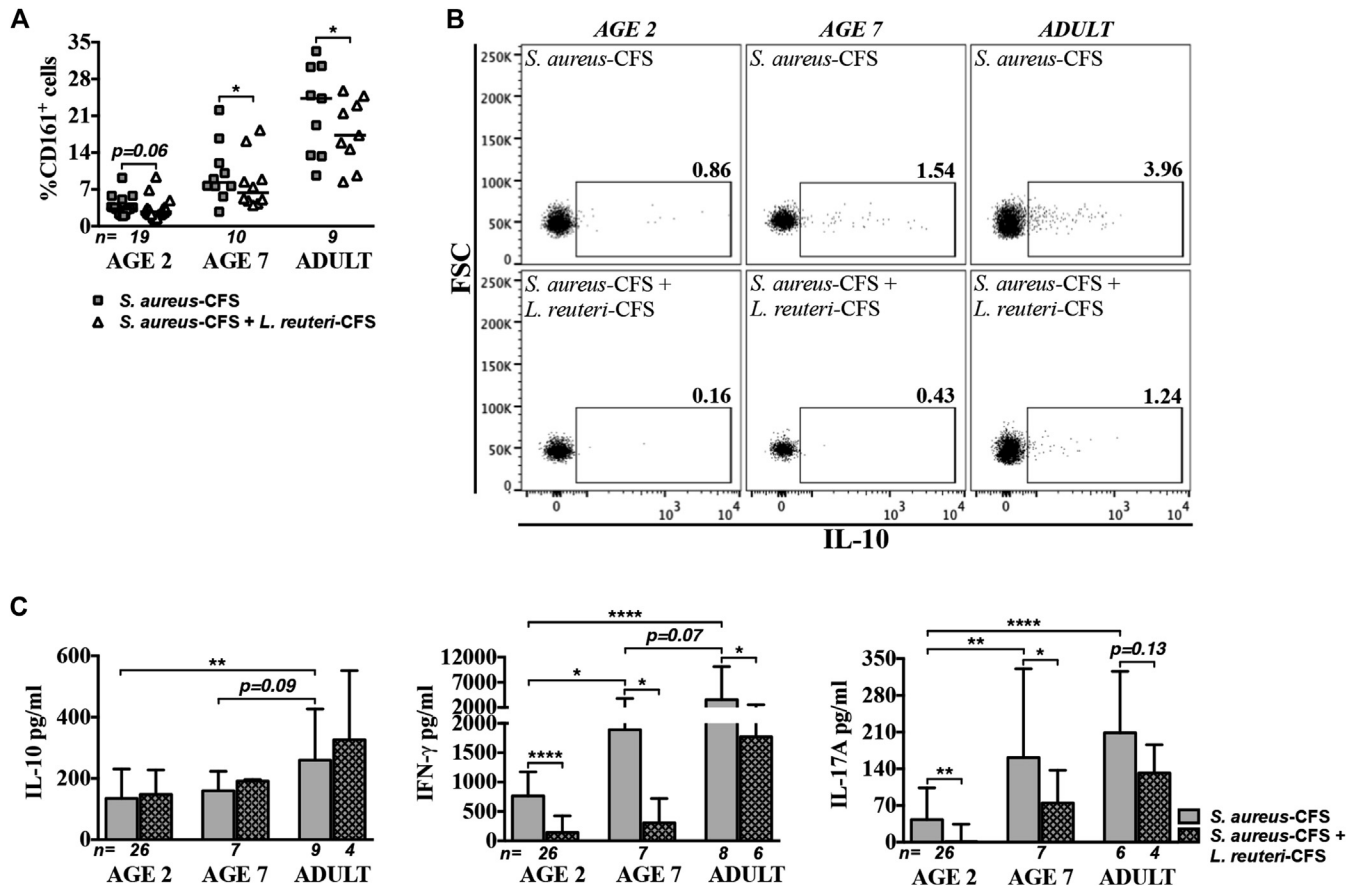


FIG E3. *L. reuteri*-CFS dampen *S. aureus*-CFS–induced cytokine production. PBMCs were left unstimulated or cultured in the presence of *S. aureus*-CFS $\pm$ *L. reuteri*-CFS. **A**, The percentage of CD161⁺ cells among the FOXP3⁺ population. **B**, Representative intracellular staining of IL-10 expression in FOXP3⁺ cells. **C**, Secreted levels of IL-10, IFN- γ , and IL-17A in PBMC cultures measured with ELISA. Secreted levels of all cytokines at unstimulated conditions were with no exceptions below the detection limits (not shown). FSC, Forward scatter. * $P < .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.