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+CD127low 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 (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 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+ T-cell response to microbial stimuli. S aureus-CFS induced an increased percentage of CD25+FOXP3+CD127low 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 T1 transcription factor T-bet compared with CD4+ T cells in PBMC cultures (Fig 1, C). Naive cells did not express ROR-ycyt (T1β17) or GATA-3 (T1β2) (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.1 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-101 cells among the FOXP31 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.

Here, we show that FOXP31 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 FOXP31 CD4 T-cell responses as well as associations between the

FIG 1. The phenotype and functional responses of CD41CD251FOXP31CD127low cells mature with age. PBMC were analyzed after 2-hour resting (A and D) or after 24-hour stimulation with S. aureus-CFS (B and E). Naive CD41 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 CD251FOXP31CD127low (FOXP31) cells among the CD41 T-cell population. Middle and right: the percentages of CD45RA1 cells (middle) and HELIOS1 cells (right) among the FOXP31 population. Fig 1, B, Upper left: the percentage of FOXP31 cells among the CD41 T-cell population; upper right and lower panels: the percentages of IL-101, IFN-γ1, and IL-17A1 cells among the FOXP31 population. Fig 1, C, Representative intracellular stainings of FOXP3 and T-bet expression in isolated naive CD41 T cells or in CD41 T cells in whole PBMCs. Fig 1, D, The percentage of CD1611 cells among the FOXP31 population. Fig 1, E, The percentage of CD1611 cells and CD161 MFI values among the FOXP31 population. CB, Cord blood; MFI, mean fluorescence intensity; T-bet, T-box transcription factor. *P < .05, **P < .01, ***P < .001, and ****P < .0001.
early-life gut microbiota imply an important role for gut microbes in shaping immune responses in childhood.

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Disclosure of potential conflict of interest: C. Nilsson has received payment for lectures from ThermoFisher, Novartis, and MEDA. E. Sverremark-Ekström has consultant arrangements with AstraZeneca and has received payment for lectures from Nutricia. The rest of the authors declare that they have no relevant conflicts of interest.

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http://dx.doi.org/10.1016/j.jaci.2016.04.027
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.13 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.12 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) and then incubated with 10% human serum in FACS-wash (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 CD4 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 Asa 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.5,3

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 μg/mL), t-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 and Brefeldin A (BD Biosciences) present during the last 4 hours of incubation at 37°C in 5% CO2 atmosphere. Dynabeads Human T-Activator CD3/CD28-coated 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 × 10^5 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 FACSVersa instrument and the FACSVerse 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+C-D127low (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

**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-γt 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 ± L. reuteri-CFS. A, The percentage of CD161<sup>+</sup> cells among the FOXP3<sup>+</sup> population. B, Representative intracellular staining of IL-10 expression in FOXP3<sup>+</sup> 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 < .01, ***P < .001, and ****P < .0001.