Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi: 10.1111/cei.13494

Childhood allergy is preceded by an absence of gut lactobacilli species and higher levels of atopy-related plasma chemokines

S. Björkander,*†
C. Carvalho-Queiroz,* J. Hallberg,†‡§
J.-O. Persson,¶ M. A Johansson,*
B. Nussbaum,* M. C Jenmalm,*
C. Nilsson†‡ and
E. Sverremark-Ekström *

*Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, †Department of Clinical Science and Education, Södersjukhuset, Karolinska Institutet, ‡Sachs' Children and Youth Hospital, §Institute for Environmental Medicine, Karolinska Institutet, ¶Department of Mathematics, Stockholm University, Stockholm, and **Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

Accepted for publication 30 June 2020
Correspondence: E. Sverremark-Ekström, Stockholm University, Department of Molecular Biosciences, The Wenner-Gren Institute, Svante Arrhenius Väg 20C, SE 106 91 Stockholm, Sweden.
E-mail: eva.sverremark@su.se

Summary

Alterations in the composition and reduced diversity of the infant microbiome are associated with allergic disease in children. Further, an altered microbiota is linked to immune dysregulation, including skewing of different T helper (Th) subsets, which is also seen in atopic individuals. The aim of this study was, therefore, to investigate the associations between gut lactobacilli and Th-related plasma factors in allergy development during childhood. A total of 194 children with known allergy status at 1 year of age were followed to 10 years of age. We used real-time polymerase chain reaction (PCR) to investigate the presence of three lactobacilli species (*Lactobacillus casei*, *L. paracasei*, *L. rhamnosus*) in infant fecal samples (collected between 1 week and 2 months of age) from a subgroup of children. Plasma chemokines and cytokines were quantified at 6 months and at 1, 2, 5 and 10 years of age with Luminex or enzyme-linked immunosorbent assay (ELISA). Fractional exhaled nitrogen oxide (FeNO) was measured and spirometry performed at 10 years of age. The data were analysed by non-parametric testing and a logistic regression model adjusted for parental allergy. An absence of these lactobacilli and higher levels of the chemokines BCA-1/CXCL13, CCL17/TARC, MIP-3α/CCL20 and MDC/CCL22 in plasma at 6 months of age preceded allergy development. The presence of lactobacilli associated with lower levels of atopy-related chemokines during infancy, together with higher levels of interferon (IFN)-γ and lower FeNO during later childhood. The results indicate that the presence of certain lactobacilli species in the infant gut may influence allergy-related parameters in the peripheral immune system, and thereby contribute to allergy protection.

Keywords: allergy, chemokines, child, gut lactobacillus, infant, microbiota, plasma

Introduction

A family history of allergy is a well-known risk factor for allergy development [1]. However, allergy is probably co-driven by improper environmental signals, including altered microbial exposures. After birth, children are colonized by a wide array of microbes, and bacteria present in the infant gut associate with systemic and mucosal immune responses as well as allergy development later in life [2–4]. Lactobacilli colonization peaks during infancy [5], and their presence is associated with being non-allergic [6–10]. The mechanisms by which lactobacilli mediate allergy protection are unknown. Both animal and human studies have shown their effects on gut and systemic immunity, suggesting a possible contribution to beneficial immune maturation during the crucial 'window of opportunity' early in life [11,12].

Allergy development is linked to immune dysregulation, including the skewing of different T helper (Th) subsets [13], where cytokines and chemokines are used as functional read-outs. The value of examining the Th phenotype and function in infancy as a predictor of later-life allergy has frequently been demonstrated. For instance, altered production of interleukin (IL)-4, IL-5, IL-10, IL-13 and interferon

(IFN)-γ from stimulated peripheral blood mononuclear cells (PBMC) associates with immunoglobulin (Ig)E-sensitization and allergy [14,15], and childhood allergy development is preceded by elevated levels of the Th2-related chemokines TARC/CCL17 and MDC/CCL22 at birth [16].

It is therefore of value to characterize the early-life plasma Th profile. In childhood, most cytokines are found in very low levels in the circulation, while chemokines are more readily detectable [16].

We have previously shown that the detection of a group of lactobacilli (*Lactobacillus casei*, *L. paracasei* and *L. rhamnosus*) in feces during infancy associates with a lower prevalence of allergy later in childhood [7,9]. In the present study, we followed children from birth to 10 years of age and investigated the interaction between infant lactobacilli and the systemic immune profile in the context of allergy development.

Methods

Study population

The material and data used in this study are from subjects participating in a prospective cohort in Stockholm, Sweden, where 281 children were born into the cohort between 1997 and 2000. All infants were full term and had birth weights within the normal range (data not shown). The children were followed from birth and were clinically examined by the same pediatrician (C.N.) at 1, 2, 5 and 10 years of age. Allergy development from 1 to 10 years of age in relation to heredity and environmental exposures in this cohort has recently been described in detail [17]. For the present study, we initially included 194 children with known allergy status at 1 year of age, and followed these children at 2 years (n = 185), 5 years (n = 167) and 10 years (n = 149) of age (Table 1a). These children had either two non-allergic parents (no heredity) or two allergic parents (double heredity). From these children, plasma samples were available at 6 months, 1 year, 2 years, 5 years and 10 years of age (Table 1b), and infant fecal samples were available from a subgroup of 65 children (Table 1c).

Ethical statement

The study was approved by the Human Ethics Committee at Huddinge University Hospital, Stockholm (no. 75/97, 113/97, 331/02, 2007/858-31/2) and the parents provided informed verbal consent. No written documentation of the participants informed approval was required, which was agreed to by the Human Ethics Committee and was according to the regulations at the time of the initiation of the study.

IgE-sensitization and allergy diagnosis in children

A detailed description of how allergy was defined can be found in Björkander *et al.* [17]. At 1, 2, 5 and 10 years of age, a skin-prick test (SPT) was performed against food and inhalant allergens, according to the manufacturer's instructions (ALK, Copenhagen, Denmark). The SPT was considered positive if the wheal diameter was ≥ 3 mm after 15 min. Serological analysis of allergen-specific IgE-antibody (sIgE-ab) to the selected allergens was performed using ImmunoCAP (Thermofisher Scientific, formerly Phadia AB, Uppsala, Sweden) and levels ≥ 0·35 kU/l were classified as positive. Children were considered to be allergic if a positive SPT or/and sIgE-ab was accompanied by one or several allergic symptom(s) (eczema, food allergy, asthma, rhinoconjunctivitis).

Table 1. Cohort characteristics

(a) Number and proportion of allergic subjects among total subjects included

1 year of age	2 years of age	5 years of age	10 years of age
29/194 (14·9%)	34/185 (18·4%)	53/167 (31·7%)	51/149 (34·2%)

(b) Number of plasma samples analysed for chemokines or cytokines at the indicated ages

6 months of age[a]	1 year of age[a]	2 years of age	5 years of age	10 years of age
Chemokines n = 116	Chemokines n = 107	Chemokines n = 121 Cytokines n = 172–176	Chemokines n = 71 Cytokines n = 141–150	Chemokines n = 72–73 Cytokines n = 141–144

(c) Subjects investigated for fecal lactobacilli (n = 65)

Non-detectable lactobacilli at 4 occasions[b]	Detectable lactobacilli at 1 or more occasions[b]	Detectable lactobacilli at 1–2 occasions[b,c]	Detectable lactobacilli at 3–4 occasions[b,c]
21	44	26	13

Allergic/total individuals at 1 year of age	Allergic/total individuals at 2 years of age[d]	Allergic/total individuals at 5 years of age[d]	Allergic/total individuals at 10 years of age[d]
11/65 (16·9%)	12/64 (18·5%)	19/60 (31·7%)	16/53 (30·2%)

[a]Cytokines were not measured at 6 months and 1 year of age due to sample limitations; [b]in feces collected at 1 week, 2 weeks, 1 month and 2 months of age; [c]five children could not be grouped due to missing data at one or several occasions; [d]one child at 2 years of age, five children at 5 years of age and 12 children at 10 years of age lack information regarding allergic disease.

Detection of lactobacilli in feces

Fecal samples were collected at 1 and 2 weeks and at 1 and 2 months of age from a subgroup of 65 non-allergic and allergic children (no heredity n = 28, double heredity n = 37) (Table 1c) and analysed for a group of lactobacilli (*L. casei*, *L. paracasei* and *L. rhamnosus*) [9]. DNA was extracted using the Qiamp DNA Stool Mini Kit™ (Qiagen, Hilden, Germany). Extracted nucleic acid concentrations were determined with Bio-Rad Smartspec (Bio-Rad Laboratories, Hercules, CA, USA) at 260 nm using Bio-Rad trUView Disposable Cuvettes (Bio-Rad). Primers were designed to detect a group of lactobacilli (*L. casei*, *L. paracasei* and *L. rhamnosus*). SYBR green real-time polymerase chain reaction (PCR) was performed in 96-well detection plates in ABI prism 7000 (Applied Biosystems, Stockholm, Sweden). All samples were analysed in triplicate with each well containing 2× power SYBR green mastermix (Applied Biosystems), primer pairs, sample DNA and water. Triplicates with computerized tomography (CT) values above 35 were considered negative. Reference lactobacilli DNA (Biotechon Diagnostics, Potsdam, Germany) were used as standard and positive controls. The standard curve ranged from 5 ng to 50 fg and was used to calculate the amount of bacteria-specific DNA. The amount of bacteria-specific DNA was related to the total amount of nucleic acids in each sample, giving relative amounts with a limit of detection at 5×10^{-6}%.

Measurements of lung function and fractional exhaled nitrogen oxide (FeNO)

At 10 years of age, FeNO was measured using the NIOX MINO (Aerocrine AB, Solna, Sweden) and lung function was evaluated by spirometry using the Jaeger MasterScreen-IOS system (Carefusion Technologies, San Diego, CA, USA), as described in detail in Björkander *et al*. [17].

Quantification of chemokines and cytokines in plasma

Chemokines (MIG/CXCL9, IP-10/CXCL10, I-TAC/CXCL11, BCA-1/CXCL13, TARC/CCL17, MIP-3α/CCL20 and MDC/CCL22) were quantified in plasma obtained from children at 6 months and 1, 2, 5 and 10 years of age and cytokines (IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-21 and IL-23) were quantified in plasma obtained at 2, 5 and 10 years of age (Table 1b). Chemokines were measured by Luminex using the Bio-Plex Pro Human Chemokine 7-plex kit (Bio-Rad) and cytokines were measured by enzyme-linked immunosorbent assay (ELISA) (Mabtech), according to the instructions from the manufacturers. Cytokine values below the detection limits were arbitrarily set to half the detection limit.

Statistics

The Chi-square , Fisher's exact or Mann–Whitney *U*-tests were used for non-parametric groupwise comparisons. The relations between allergy and chemokine levels in plasma were further analyzed with logistic regression adjusting for parental allergy. GraphPad Prism version 7 software was used for analyses and data presentation. Scatter dot-plots show medians with range. Results were considered significant if $P < 0{\cdot}05$ (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$, ****$P < 0{\cdot}0001$ in figures) and borderline significant if $P = 0{\cdot}05$–$0{\cdot}1$.

Results

The presence of *L. casei*, *L. paracasei* and *L. rhamnosus* in feces during infancy associates with a reduced allergy prevalence during childhood

We have previously shown that the presence of three lactobacilli species (*L. casei, L. paracasei* and *L. rhamnosus*, from here onwards referred to as lactobacilli) in feces at 2 weeks of age associates with a lower prevalence of allergy at 5 years of age, and that lactobacilli are less frequently detected during the first 2 months of life in children with double heredity compared to children without heredity [9]. Here, we used material from 65 children and further evaluated how the presence of these lactobacilli in feces at four occasions during the first 2 months of life relates to allergic disease at 1, 2, 5 and 10 years of age (Table 1c). For the 65 children, the proportion of allergic children at 1, 2 and 10 years of age was significantly lower in the group where lactobacilli were detected on least at one occasion during the first 2 months of life (Fig. 1a). Further, children where lactobacilli were detected more frequently (three to four occasions) were least likely to be allergic, significant for allergy at 1 and 2 years of age (Fig. 1b). Finally, this pattern was even more pronounced when only including children who were consistently allergic or consistently non-allergic at 1, 2, 5 and 10 years of age (Fig. 1c). In accordance with our previous findings [9], the proportion of lactobacilli-negative children was significantly higher within the double heredity group compared to the no heredity group (Fig. 1d), but importantly, the association between a presence of lactobacilli and being non-allergic at 1 and 2 years of age tended to persist when only children with double heredity were included in the analysis (Fig. 1e).

Higher levels of BCA-1/CXCL13, TARC/CCL17, MIP-3α/CCL20 and MDC/CCL22 in plasma obtained at 6 months of age precede allergy development

Next, we measured chemokines and cytokines in plasma from all children with known allergy status and available samples (Table 1a,b). Allergic children had higher levels of BCA-1/CXCL13, TARC/CCL17, MIP-3α/CCL20 and MDC/CCL22 in plasma obtained at 6 months of age, and the highest levels were found in plasma from children who were consistently allergic at 1, 2, 5 and 10 years of age [Fig. 2a: BCA-1/CXCL13; Fig. 2b: TARC/CCL17; Fig.

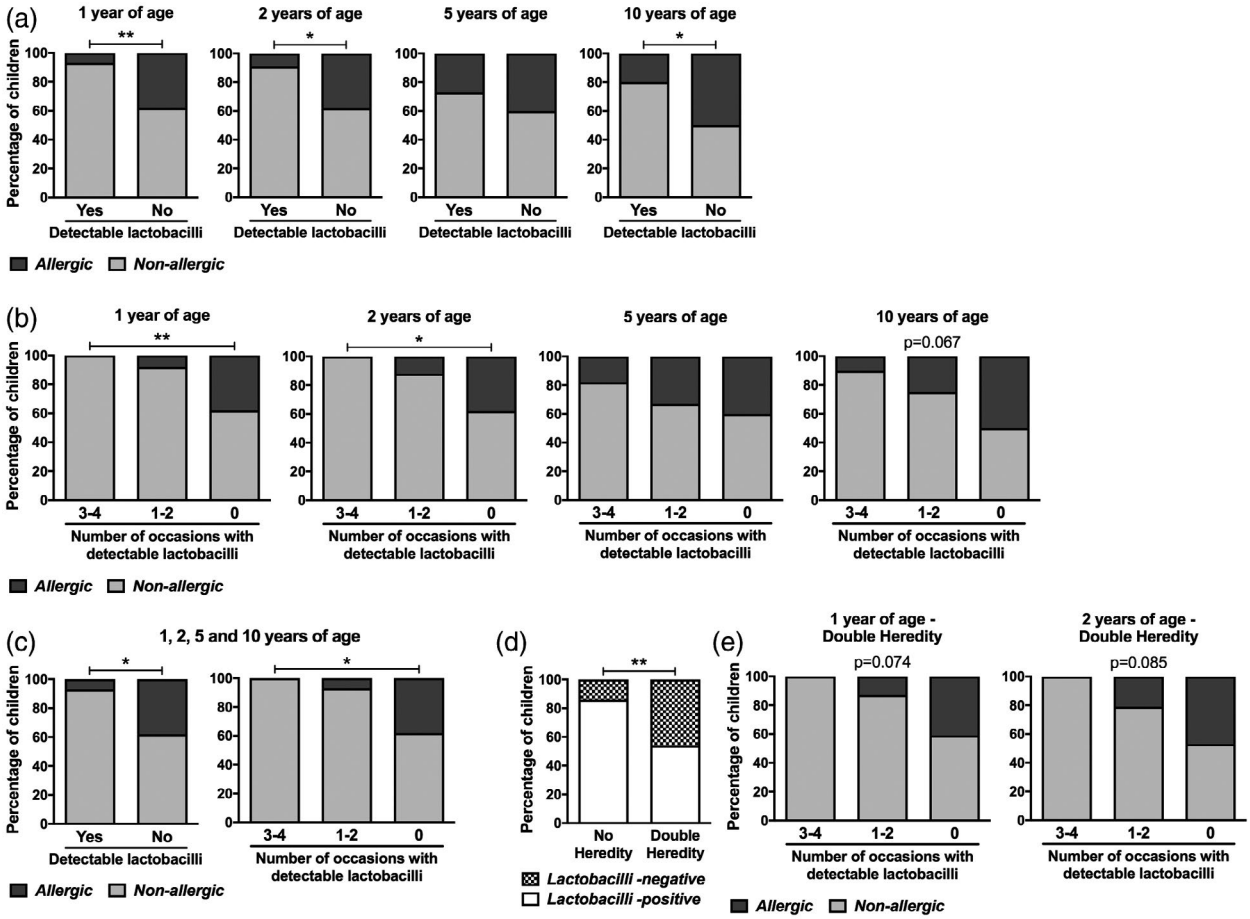

Fig. 1. The presence of lactobacilli was evaluated in feces collected at four time-points during infancy (1 and 2 weeks, 1 and 2 months of age). (a) The proportions of allergic and non-allergic children at 1, 2, 5 or 10 years of age within the groups where lactobacilli were detected at one or more time-points or were non-detectable at all time-points. (b) The proportions of allergic and non-allergic children at 1, 2, 5 or 10 years of age within the groups where lactobacilli were detected at three to four time-points, one to two time-points or were non-detectable at all time-points. (c) The proportions of children that were consistently allergic or non-allergic at 1, 2, 5 and 10 years of age within the groups where lactobacilli were detected at one or more time-points or were non-detectable at all time-points (left), or detected at three to four time-points, one to two time-points or were non-detectable at all time-points (right). (d) The proportions of children where lactobacilli were detected at one or more time-points or were non-detectable at all time-points within the groups with two allergic parents (double heredity) or two non-allergic parents (no heredity). (e) The same as in (b), including only 1- and 2-year-old children with double heredity. Fisher's exact test (a,c,d) or c^2 test (b,c,e) were used for statistical analysis.

2c: MIP-3α/CCL20; Fig. 2d: MDC/CCL22 (allergy at 1 or 10 years of age, and consistent allergy, and Supporting information, Table S1a (allergy at 1, 2, 5 or 10 years of age)]. The same pattern was observed for these chemokines when measured in plasma obtained at 1 year of age (Supporting information, Fig. S1a–d, Table S1a). Importantly, similar results were generated when these chemokines were analysed with a logistic regression model, adjusted for parental allergy (Table 2). The levels of MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 in plasma obtained at 6 months and 1 year of age did not consistently associate with allergy (Supporting information, Table S1a). The levels of chemokines and cytokines in plasma obtained at 2, 5 and 10 years of age did not correlate with allergic disease, except for IL-21 at 2 and 5 years of age (Supporting information, Table S1b). As we had access to a very limited plasma volume from 6 months and 1 year of age, cytokines were not measured at these time-points, considering both the relatively low levels that are usually found in plasma and a relatively high proportion of children who had cytokine values below detection limits at 2, 5 and 10 years of age. There were no consistent differences in chemokine and cytokine levels between the two heredity groups (Supporting information, Table S2).

Lactobacilli in infancy associates with lower levels of allergy-related chemokines at 6 months of age

Within the subgroup of 65 children investigated for lactobacilli, the levels of BCA-1/CXCL13, MIP-3α/CCL20 and

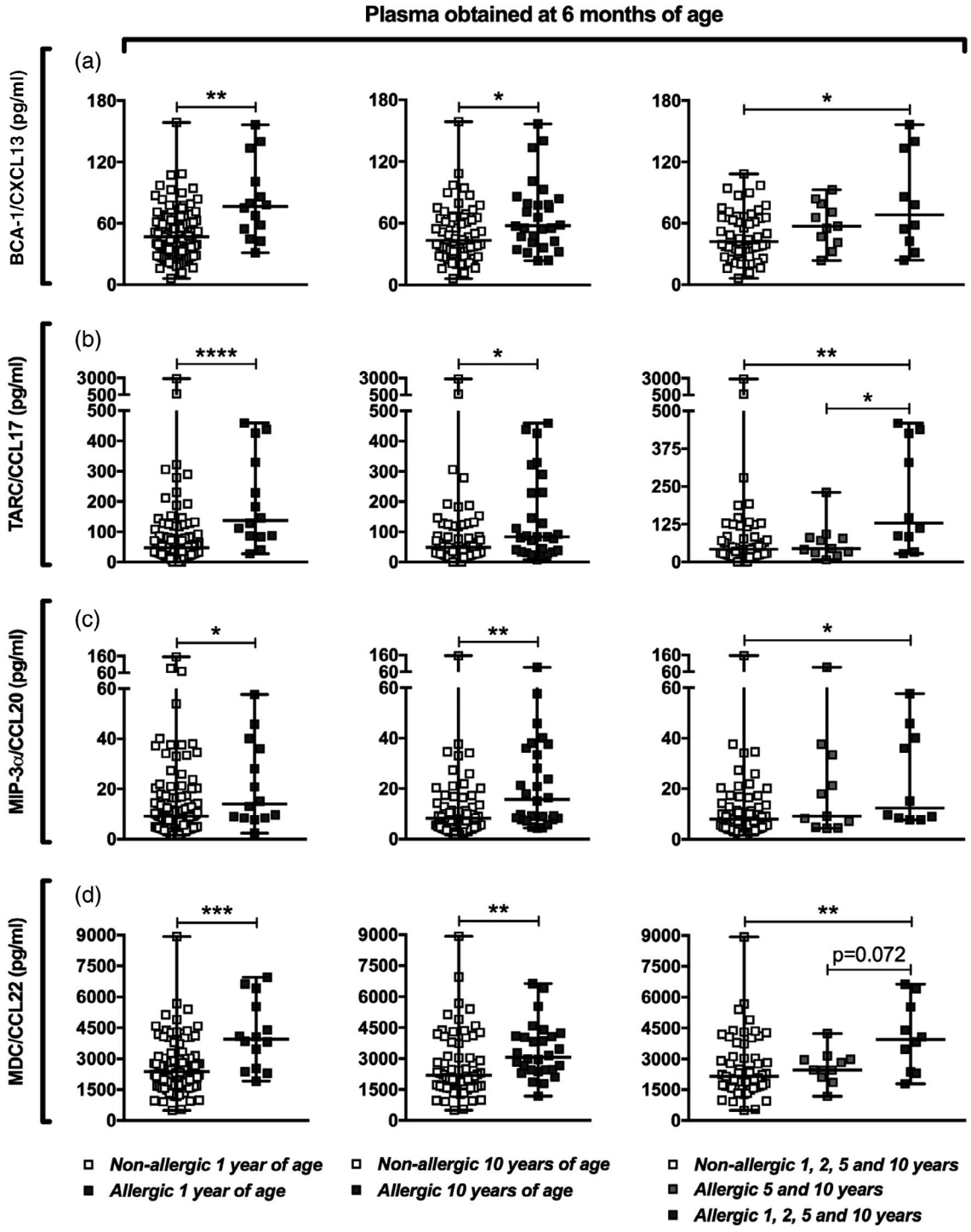

Fig. 2. The levels (pg/ml) of BCA-1/CXCL13 (a), TARC/CCL17 (b), MIP-3α/CCL20 (c) and MDC/CCL22 (d) in plasma obtained at 6 months of age in relation to allergic disease at 1 year of age (left column) or 10 years of age (middle column), or in relation to being consistently non-allergic at 1, 2, 5 and 10 years of age, allergic at 5 and 10 years of age or being consistently allergic at 1, 2, 5 and 10 years of age (right column). The Mann–Whitney *U*-test was used for statistical analysis.

MDC/CCL22 in plasma obtained at 6 months of age were highest in children where lactobacilli were detectable at no or one to two occasions, and subsequently lowest in children where lactobacilli were detected on three to four occasions (Fig. 3a, Table 3). This pattern persisted when including only children with double heredity (Fig. 3b) or

Table 2. The relation between plasma chemokine-levels and allergic disease was analysed with a logistic regression model adjusted for parental allergy

	1 year of age		2 years of age		5 years of age		10 years of age	
	OR (*P*)	OR_{adj} [a] (*P*)	OR (*P*)	OR_{adj} [a] (*P*)	OR (*P*)	OR_{adj} [a] (*P*)	OR (*P*)	OR_{adj} [a] (*P*)
(a) Chemokines in plasma obtained at 6 months of age and future allergy								
BCA-1/CXCL13	**10·31 (0·002)**	**9·43 (0·003)**	**3·19 (0·043)**	2·83 (0·075)	**4·34 (0·003)**	**4·25 (0·007)**	**3·33 (0·012)**	**3·09 (0·026)**
TARC/CCL17	**2·61 (0·002)**	**2·61 (0·001)**	1·64 (0·065)	**1·70 (0·047)**	1·49 (0·075)	1·53 (0·063)	1·42 (0·133)	1·43 (0·125)
MIP-3α/CCL20	1·78 (0·070)	1·75 (0·084)	1·16 (0·607)	1·12 (0·698)	1·47 (0·117)	1·51 (0·106)	**2·29 (0·004)**	**2·35 (0·004)**
MDC/CCL22	**12·69 (0·001)**	**13·64 (0·001)**	**3·73 (0·029)**	**3·51 (0·049)**	**3·50 (0·010)**	**3·40 (0·018)**	**3·39 (0·013)**	**3·20 (0·026)**
(b) Chemokines in plasma obtained at 1 year of age and present or future allergy								
BCA-1/CXCL13	**5·88 (0·014)**	**6·18 (0·017)**	1·99 (0·231)	1·85 (0·305)	2·07 (0·110)	1·99 (0·143)	2·22 (0·097)	2·22 (0·111)
TARC/CCL17	**2·79 (0·002)**	**2·78 (0·002)**	**2·40 (0·007)**	**2·41 (0·007)**	**1·79 (0·028)**	**1·89 (0·023)**	1·58 (0·091)	1·67 (0·075)
MIP-3α/CCL20	1·39 (0·300)	1·33 (0·359)	0·86 (0·641)	0·85 (0·598)	1·41 (0·199)	1·39 (0·218)	**2·52 (0·007)**	**2·62 (0·005)**
MDC/CCL22	**5·01 (0·019)**	**6·17 (0·017)**	1·85 (0·302)	1·94 (0·295)	1·72 (0·241)	1·83 (0·215)	2·57 (0·071)	3·09 (0·052)

Bold type indicates significance ($P < 0.05$).

OR = odds ratio;

[a]OR_{adj} = odds ratio after adjusting for parental allergy.

only non-allergic children (Fig. 3c). Importantly, also for this subgroup of children, parental allergy did not influence chemokine levels in plasma (Fig. 3d).

Lactobacilli in infancy associates with higher IFN-γ levels and lower FeNO later in childhood

Within the subgroup of 65 children investigated for lactobacilli, the levels of IFN-γ in plasma were higher in the lactobacilli-positive group at 5 and 10 years of age and highest when lactobacilli were detected on three to four occasions (Fig. 4a, Supporting information, Fig. S2a). Importantly, also for this subgroup of children parental allergy did not influence the levels of IFN-γ in plasma (Fig. 4b, Supporting information, Fig. S2b). With the exception of MIP-3α/CCL20, lactobacilli did not consistently correlate with cytokine or chemokine levels in plasma at 1, 2, 5 or 10 years of age (Table 3).

The presence of lactobacilli in infancy associated with lower FeNO at 10 years of age (Fig. 4c) and, importantly, this finding tended to persist when including only children with double heredity (Fig. 4d) or only non-allergic children (Fig. 4e). Lung function was evaluated by spirometry at 10 years of age and the forced expiratory volume in 1 s/forced vital capacity (FEV_1/FVC) *Z*-scores did not associate with the presence of lactobacilli (data not shown).

Discussion

In the present study, we followed the same children from birth to 10 years of age. Using this unique cohort material, we find that the presence of lactobacilli in feces during early infancy associates with allergy protection, lower levels of atopy-related chemokines and lower FeNO later in childhood (Fig. 5). Further, and importantly, the associations between lactobacilli, allergy protection and the systemic immune profile were also apparent within the subgroup of children with double allergic heredity.

The association between the presence of lactobacilli and being non-allergic was most obvious early in life, but the link was evident up to 10 years of age. Notably, children where lactobacilli were detected at several occasions in infancy were least likely to be allergic. Lactobacilli colonization has previously been associated with a reduced prevalence of allergy [6–10], while some studies have failed to find an association [18–20]. Factors that complicate the comparison between these studies included age of feces collection, age of allergy diagnosis and whether lactobacilli were investigated at genus- or species-level. Hence, it is difficult to find studies that are fully comparable to the findings presented in the current study. Parental atopy is a strong risk factor for child allergy, and the protective effect associated with lactobacilli becomes more complex considering that we and others show that lactobacilli are less frequently found in children with allergic heredity [9,21]. However, our findings in high-risk children support the idea that lactobacilli in the neonatal gut associate with being non-allergic later in childhood, despite heredity. Some have attributed the allergy-protective effects of lactobacilli to induction of Th1 immunity. Certain strains of lactobacilli induce IFN-γ and IL-12-secretion from human PBMC and skew DC to induce Th1 responses [22,23]. PBMC from lactobacilli-supplemented children secrete higher levels of IFN-γ, and this increase is associated with decreased severity of atopic dermatitis [24]. We observed a striking association between lactobacilli in the infant gut and higher levels of IFN-γ in plasma later during childhood. We could speculate that this reflects an early immune programming that becomes evident during childhood; however, the mechanism behind this is not known and beyond the scope of this study. Of note, IFN-γ plasma levels did not associate with allergy.

Here, we show that naturally occurring gut lactobacilli clearly associated with lower levels of three atopy-related

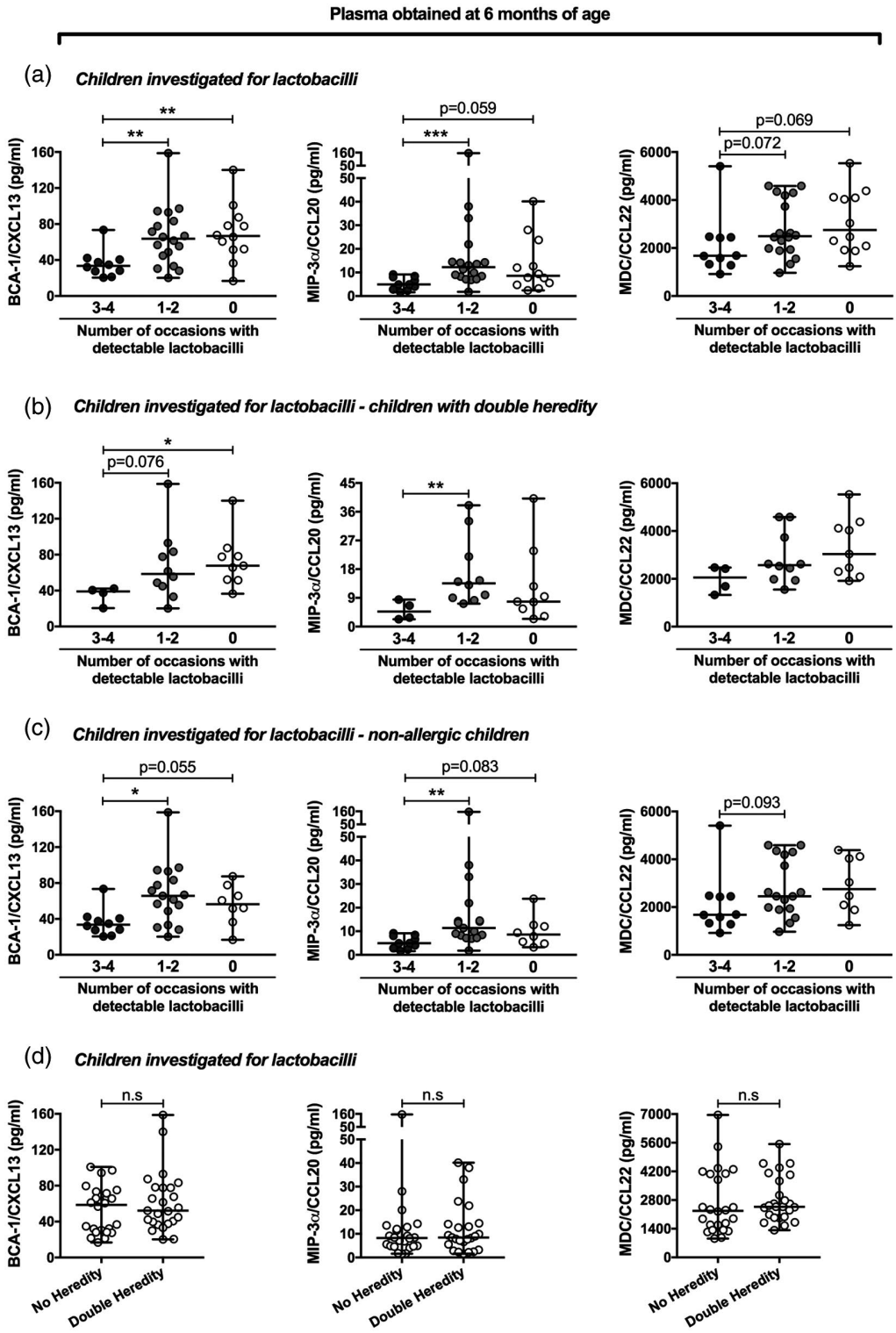

Fig. 3. (a) The levels (pg/ml) of BCA-1/CXCL13, MIP-3α/CCL20 and MDC/CCL22 in plasma obtained at 6 months of age from the groups where lactobacilli were detected at three to four time-points, one to two time-points or were non-detectable at all time-points. (b) Same as in (a), including only children with double heredity. (c) The same as in (a), including only non-allergic children. (d) Heredity and levels (pg/ml) of BCA-1/CXCL13, MIP-3α/CCL20 and MDC/CCL22 in plasma obtained at 6 months of age from the group with available lactobacilli data. The Mann–Whitney *U*-test was used for statistical analysis.

Table 3. Median levels (pg/ml) of cytokines and chemokines in plasma obtained at the indicated ages in relation to the number of occasions (0, 1–2, 3–4) when lactobacilli were detectable in fecal samples[a]

	6 months of age[b]				1 year of age[b]				2 years of age				5 years of age				10 years of age			
	3–4	1–2	0	(*P*)[c]	3–4	1–2	0	(*P*)[c]	3–4	1–2	0	(*P*)[c]	3–4	1–2	0	(*P*)[c]	3–4	1–2	0	(*P*)[c]
IL-4									0·5	0·5	0·5		0·6	3·1	0·5	**0·046**[d] 0·078[e]	0·5	0·5	3·7	
IL-5									2·0	2·0	2·0		4·6	2·0	2·2		2·0	2·0	2·0	
IL-10									176·5	51·2	89·0	**0·046**[f]	184·1	214·2	138·6		97·7	32·0	49·0	
IL-13									0·5	0·5	0·5		0·5	0·5	0·5		0·5	0·5	0·5	
IL-17A									1·5	1·5	1·5		1·5	1·5	1·5		1·5	12·6	1·5	0·074[d] **0·019**[f]
IL-21									916·2	566·5	793·5	0·062[f]	1280	834·6	649·5	0·072[e] 0·089[f]	358·4	143·3	270·5	
IL-23									2·0	24·3	5·5		2·0	24·8	13·5		2·0	16·3	3·6	0·050[f]
IFN-γ									51·7	58·7	54·9		143·9	97·0	54·5	**< 0·001**[e] 0·078[f]	81·8	53·3	25·2	
MIG	421·6	431·6	683		483·1	582·4	480·6		1044	759·6	910		341·4	816·3	502·6	0·088[f]	251·4	258·9	294·9	
IP-10	77·8	109·2	156·5	**0·021**[e]	71·4	90·0	63·0		72·6	90·0	92·9		48·1	52·3	51·4		36·7	40·0	28·2	
I-TAC	2·5	4·2	4·0		3·7	3·0	2·5		56·9	47·5	69·2		26·5	33·1	30·8		22·0	11·5	10·3	
BCA-1	33·5	63·6	66·7	**0·006**[e] **0·009**[f]	37·5	55·2	48·8	0·077[f]	47·2	66·2	67·3	0·085[f]	29·5	34·7	35·0		7·3	11·5	14·5	
TARC	44·3	54·9	51·7		27·4	36·6	34·4		519·3	617·1	523·2		344·6	387·3	442·6		47·1	58·0	84·5	**0·046**[d]
MIP-3α	4·9	12·2	8·6	0·059[e] **< 0·001**[f]	8·2	11·0	8·6		6·9	11·2	10·0	**0·032**[e]	3·5	5·9	2·6	**0·044**[d]	1·2	1·2	0·9	
MDC	1678	2491	2752	0·069[e] 0·072[f]	2311	1961	2330		2377	3202	2621		1464	1968	1650		819·3	675·4	833·1	

Bold type indicates significance ($P < 0.05$). The full chemokine names are omitted due to space limitation: MIG/CXCL9, IP-10/CXCL10, I-TAC/CXCL11, BCA-1/CXCL13, TARC/CCL17, MIP-3α/CCL20, IL = interleukin; IFN = interferon. [a] Detection of *Lactobacillus casei*, *L. paracasei* and *L. rhamnosus* in fecal samples obtained at 1 week, 2 weeks, 1 month and 2 months of age (four occasions); [b] cytokines were not measured at 6 months and 1 year of age due to sample limitations; [c] Mann–Whitney *U*-test; [d] comparison between no and one to two occasions, [e] Comparison between no and three to four occasions; [f]comparison between one to two and three to four occasions.

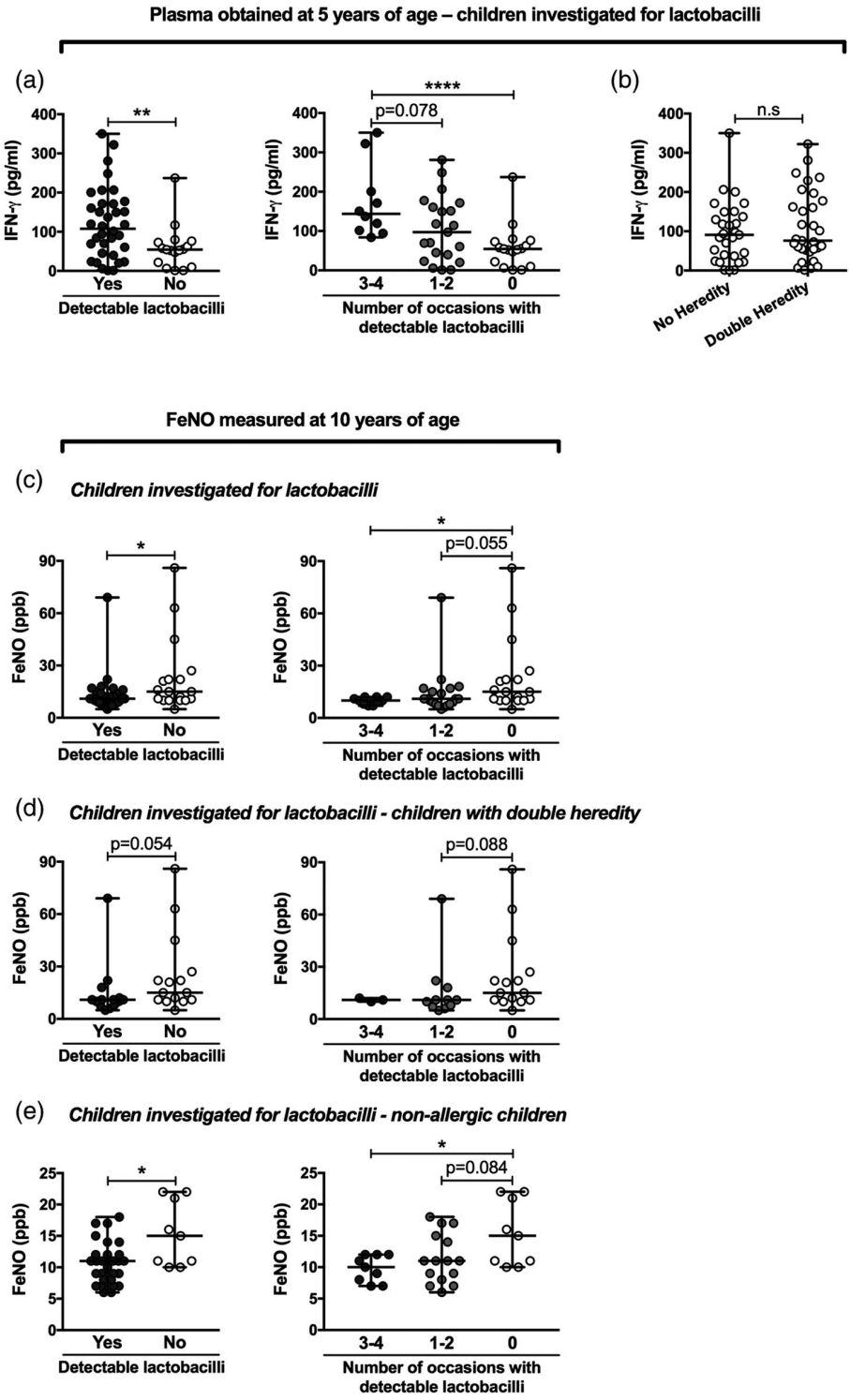

Fig. 4. (a) The levels (pg/ml) of interferon (IFN)-γ in plasma obtained at 5 years age from the groups where lactobacilli were detected at one or more time-points or were non-detectable at all time-points (left) or in the groups where lactobacilli were detected at three to four time-points, one to two time-points or were non-detectable at all time-points (right). (b) Heredity and levels (pg/ml) of IFN-γ in plasma obtained at 5 years age from the group with available lactobacilli data. (c) The levels of fractional exhaled nitric oxide (FeNO) at 10 years of age in the groups where lactobacilli were detected at one or more time-points or were non-detectable at all time-points (left) or in the groups where lactobacilli were detected at three to four time-points, one to two time-points or were non-detectable at all time-points (right). (d) Same as in (c), including only children with double heredity. (e) Same as in (c), including only non-allergic children. The Mann–Whitney *U*-test was used for statistical analysis.

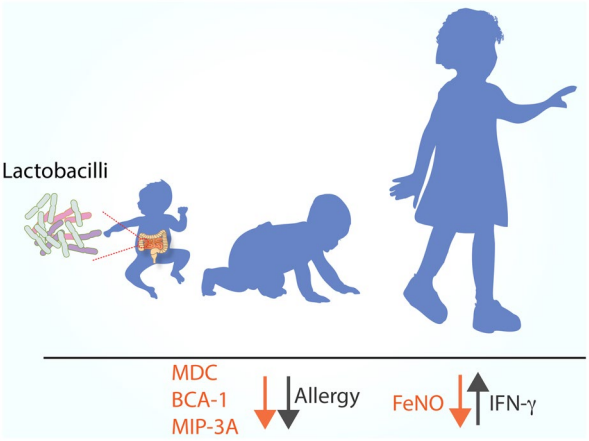

Fig. 5. Detection of lactobacilli (*L. casei*, *L. paracasei* and *L. rhamnosus*) in feces during the first 2 months of life associates with reduced allergy prevalence and lower levels of atopy-related chemokines in the first year(s) of life, and higher levels of interferon (IFN)-γ and lower fractional exhaled nitric oxide (FeNO) later in childhood. Graphic design: Fuad Bahram, FB Scientific Art Design.

chemokines: BCA-1/CXCL13, MIP-3α/CCL20 and MDC/CCL22. It has been shown that the presence of *L. reuteri* in fecal samples from 1-week-old children participating in a probiotic study associates with reduced levels of Th2 chemokines in infancy [16]. We found that allergic children had higher levels of the T follicular helper (Tfh)-related chemokine BCA-1/CXCL13, the Th17-related chemokine MIP-3α/CCL20 and the Th2-related chemokines TARC/CCL17 and MDC/CCL22 in plasma samples obtained at 6 months and 1 year of age. Children who were consistently allergic had the highest levels of these chemokines. Further, the levels of these chemokines later in life did not associate with allergic disease, suggesting that the early immune profile is more clearly connected to allergy development.

These results add to other studies where allergic disease/IgE-sensitization has been associated with Th2-related chemokines in early life [16,25]. Considering that chemokines are known influencers of Th subset polarization, they could serve as biomarkers for an increased risk to develop allergy. TARC/CCL17 and MDC/CCL22 direct Th2 cell responses and migration through binding to the chemokine receptor CCR4 [26]. BCA-1/CXCL13 binds to CXCR5 and directs the positioning of Tfh and B cells in lymph nodes, and also correlates with circulating Tfh cells, germinal center (GC) activity and the magnitude of antibody responses in humans [27]. Ovalbumin (OVA)-challenged mice up-regulate BCA-1/CXCL13, and bronchoalveolar lavage from asthmatic patients contain higher levels of this chemokine compared with healthy controls [28]. Importantly, chemokine levels in plasma to some extent only reflect the steady state of an individual and does not necessarily reflect how the given individual will respond upon allergen challenge. Further, the cellular source of chemokines in plasma is unknown. Hence, there might be cell subset-specific deviations in production between allergic and non-allergic children.

The cross-talk between the intestinal microbiota and the lungs is referred to as the gut–lung axis, and the link between microbiota composition and asthma has been established [29]. FeNO is considered as a marker of Th2-associated inflammation and we have recently shown that allergic disease associates with higher FeNO [17]. Here we found that infant gut lactobacilli associate with lower levels of FeNO at 10 years of age, and also in non-allergic children. Considering that the association between gut lactobacilli and absence of allergic disease was most pronounced early in life, it is likely that these bacteria will associate with reduced lung inflammation also before 10 years of age.

How could the presence of lactobacilli in the gut during infancy influence the peripheral immune system and subsequent allergy development later in life? The guts of children are more permeable and contain a lower diversity of microbes, indicating that individual species of bacteria can have a greater impact compared to later in life. Possibly, lactobacilli symbolize a microbiota that is beneficial for proper immune maturation. The gut microbiota influence key features of the immune system that are involved in allergic disease, including Th1/Th2 differentiation, induction of tolerance and T regulatory cell function [30]. Overall microbial diversity, microbial signatures as well as individual bacteria present during infancy, are linked to the development of allergic disease [3,19,31,32]. Microbial metabolites and their receptors are suggested as important effector molecules in gut microbiota-mediated effects on, and communication with, the peripheral immune system. The microbiome of children that later became allergic lacked genes that encode enzymes involved in the production of the short chain fatty acid (SCFA) butyrate [33], and children with high levels of butyrate and propionate were less likely to be sensitized at 1 year of age and to develop several types of allergic disease later in life [34]. Interestingly, intake of *L. acidophilus* associated with reduced hypersensitivity and increased concentrations of SCFAs in β-lactoglobulin-sensitized mice [35].

It is of great importance to evaluate how and if heredity skew results and measurements connected to allergy development. Importantly, heredity did not influence plasma chemokine levels; the presence of lactobacilli was connected to remaining non-allergic and also to lower FeNO when stratifying for heredity. The results obtained in this study indicate that gut lactobacilli are connected to remaining non-allergic during childhood, and that these bacteria

may influence allergy-related parameters of the infant peripheral immune system and thereby contribute to allergy protection.

Acknowledgements

This study was supported financially by the Swedish Research Council (2016-01715_3), the Cancer and Allergy Foundation, the Swedish Asthma and Allergy Association's Research Foundation, the Mjölkdroppen Foundation, the Hesselman Foundation, the Golden Jubilee Memorial Foundation, the Crownprincess Lovisa/Axel Tielman Foundations, the Engkvist Foundations, the Swedish Heart-Lung Foundation and Frimurare Barnhuset Foundation (Freemasons of Sweden). We wish to thank the participating families and research nurse Anna-Stina Ander.

Disclosures

The authors have no conflicts of interest to declare.

Author contributions

S. B. designed the study, analysed the data, performed statistical analysis and wrote the manuscript. C. C. Q. designed and performed experiments. J. H. analysed spirometry data and wrote the manuscript. J. O. P. performed statistical analysis. M. A. J. designed and performed experiments. B. N. performed experiments. M. C. J. designed experiments. C. N. initiated and recruited the cohort, performed clinical examinations and wrote the manuscript. E. S. E. envisioned and designed the study, interpreted the data and wrote the manuscript.

References

1 Portelli MA, Hodge E, Sayers I. Genetic risk factors for the development of allergic disease identified by genome-wide association. Clin Exp Allergy 2015; **45**:21–31.
2 Johnson CC, Ownby DR. The infant gut bacterial microbiota and risk of pediatric asthma and allergic diseases. Transl Res 2017; **179**:60–70.
3 Fujimura KE, Sitarik AR, Havstad S *et al*. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med 2016; **22**:1187–91.
4 Björkander S, Johansson MA, Hell L *et al*. FOXP3+ CD4 T-cell maturity and responses to microbial stimulation alter with age and associate with early life gut colonization. J Allergy Clin Immunol 2016; **138**:905–8.
5 Ahrné S, Lönnermark E, Wold AE *et al*. Lactobacilli in the intestinal microbiota of Swedish infants. Microbes Infect 2005; **7**:1256–62.
6 Björkstén B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy 1999; **29**:342–6.
7 Sjögren YM, Jenmalm MC, Böttcher MF, Björkstén B, Sverremark-Ekström E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. Clin Exp Allergy 2009; **39**:518–26.
8 Penders J, Thijs C, Mommers M *et al*. Intestinal lactobacilli and the DC-SIGN gene for their recognition by dendritic cells play a role in the aetiology of allergic manifestations. Microbiology 2010; **156**:3298–305.
9 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.
10 Simonyté Sjödin K, Hammarström ML, Rydén P *et al*. Temporal and long-term gut microbiota variation in allergic disease: a prospective study from infancy to school age. Allergy 2019; **74**:176–85.
11 Plaza-Diaz J, Gomez-Llorente C, Fontana L, Gil A. Modulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver by probiotics. World J Gastroenterol 2014; **20**:15632–49.
12 Petursdottir DH, Nordlander S, Qazi KR *et al*. Early-life human microbiota associated with childhood allergy promotes the T helper 17 axis in mice. Front Immunol 2017; **8**:1699.
13 Lambrecht BN, Hammad H. The immunology of the allergy epidemic and the hygiene hypothesis. Nat Immunol 2017; **18**:1076–83.
14 van der Velden VH, Laan MP, Baert MR, de Waal Malefyt R, Neijens HJ, Savelkoul HF. Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN-gamma, IL-4 and IL-10. Clin Exp Allergy 2001; **31**:997–1006.
15 Bullens DMA, Kasran A, Dilissen E *et al*. In vivo maturation of TH cells in relation to atopy. J Allergy Clin Immunol 2011; **128**:234–7.e7.
16 Abrahamsson TR, Sandberg Abelius M, Forsberg A, Björkstén B, Jenmalm MC. A Th1/Th2-associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization. Clin Exp Allergy 2011; **41**:1729–39.
17 Björkander S, Hallberg J, Persson J-O, Lilja G, Nilsson C, Sverremark-Ekström E. The allergic phenotype during the first 10 years of life in a prospective cohort. Immunity, Inflamm Dis 2019; **7**:170–82.
18 Murray CS, Tannock GW, Simon MA *et al*. Fecal microbiota in sensitized wheezy and non-sensitized non-wheezy children: a nested case–control study. Clin Exp Allergy 2005; **35**:741–5.
19 Penders J, Thijs C, van den Brandt PA *et al*. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut 2007; **56**:661–7.
20 Liwen Z, Yu W, Liang M, Kaihong X, Baojin C. A low abundance of *Bifidobacterium* but not *Lactobacillus* in the feces of Chinese children with wheezing diseases. Medicine 2018; **97**:e12745.

21 Koleva PT, Tun HM, Konya T *et al*. Sex-specific impact of asthma during pregnancy on infant gut microbiota. Eur Respir J 2017; **50**:1700280.
22 Miettinen M, Matikainen S, Vuopio-Varkila J *et al*. Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 1998; **66**:6058–62.
23 Mohamadzadeh M, Olson S, Kalina WV *et al*. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. Proc Natl Acad Sci USA 2005; **102**:2880–5.
24 Prescott SL, Dunstan JA, Hale J *et al*. Clinical effects of probiotics are associated with increased interferon-gamma responses in very young children with atopic dermatitis. Clin Exp Allergy 2005; **35**:1557–64.
25 Reubsaet LL, Meerding J, de Jager W *et al*. Plasma chemokines in early wheezers predict the development of allergic asthma. Am J Respir Crit Care Med 2013; **188**:1039–40.
26 Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 2014; **32**:659–702.
27 Havenar-Daughton C, Lindqvist M, Heit A *et al*. CXCL13 is a plasma biomarker of germinal center activity. Proc Natl Acad Sci USA 2016; **113**:2702–7.
28 Baay-Guzman GJ, Huerta-Yepez S, Vega MI *et al*. Role of CXCL13 in asthma: novel therapeutic target. Chest 2012; **141**:886–94.
29 Ver Heul A, Planer J, Kau AL. The human microbiota and asthma. Clin Rev Allergy Immunol 2019; **57**:350–63.
30 Gensollen T, Blumberg RS. Correlation between early-life regulation of the immune system by microbiota and allergy development. J Allergy Clin Immunol 2017; **139**:1084–91.
31 Kourosh A, Luna RA, Balderas M *et al*. Fecal microbiome signatures are different in food-allergic children compared to siblings and healthy children. Pediatr Allergy Immunol 2018; **29**:545–54.
32 Feehley T, Plunkett CH, Bao R *et al*. Healthy infants harbor intestinal bacteria that protect against food allergy. Nat Med 2019; **25**:448–53.
33 Cait A, Cardenas E, Dimitriu PA *et al*. Reduced genetic potential for butyrate fermentation in the gut microbiome of infants who develop allergic sensitization. J Allergy Clin Immunol 2019; **144**:1638–47.
34 Roduit C, Frei R, Ferstl R *et al*. High levels of butyrate and propionate in early life are associated with protection against atopy. Allergy 2019; **74**:799–809.
35 Wang JJ, Zhang QM, Ni WW *et al*. Modulatory effect of *Lactobacillus acidophilus* KLDS 1.0738 on intestinal short-chain fatty acids metabolism and GPR41/43 expression in β-lactoglobulin–sensitized mice. Microbiol Immunol 2019; **63**:303–15.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. The levels (pg/ml) of BCA-1/CXCL13 (A), TARC/CCL17 (B), MIP-3α/CCL20 (C) and MDC/CCL22 (D) in plasma obtained at 1 year of age in relation to allergic disease at 1 year of age (left column) or 10 years of age (middle column), or in relation to being consistently non-allergic at 1, 2, 5 and 10 years of age, allergic at 5 and 10 years of age, or being consistently allergic at 1, 2, 5 and 10 years of age (right column). The Mann-Whitney U-test was used for statistical analysis.

Fig. S2. (A) The levels (pg/ml) of IFN-γ in plasma obtained at 10 years age from the groups where lactobacilli were detected at one or more time-points or were non-detectable at all time-points (left) or in the groups where lactobacilli were detected at 3-4 time-points, 1-2 time-points or were non-detectable at all time-points (right). (B) Heredity and levels (pg/ml) of IFN-γ in plasma obtained at 10 years age from the group with available lactobacilli-data. The Mann-Whitney U-test was used for statistical analysis.

Table S1. Median levels (pg/ml) of cytokines and chemokines in plasma in relation to allergic disease

Table S2. Median levels (pg/ml) of cytokines and chemokines in plasma obtained from children at the indicated ages in relation to parental allergy

