Characterization of Candidate Anti-Allergic Probiotic Strains in a Model of Th2-Skewed Human Peripheral Blood Mononuclear Cells

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Key Words

Allergy · Anti-CD40 · Interleukin-4 · Peripheral blood mononuclear cells · Probiotics · Th2 cytokines

Abstract

Background: Pre-clinical and clinical studies have evaluated the efficacy of probiotics in allergy. However, predictive in vitro systems for rational strain selection are still missing. **Methods:** We developed a novel in vitro screening system for the characterization of probiotics with anti-allergic potential. In this model, human peripheral blood mononuclear cells (PBMC) from healthy donors (n = 68) were skewed towards a Th2 cytokine phenotype by culture with IL-4 and anti-CD40, to resemble cells from allergic donors. Th2-skewed cells were then co-cultured with probiotics; a total of 35 strains were tested. Levels of IFN- γ , IL-10, IL-5 and 7 additional cytokines in culture supernatants were determined by ELISA or multiplex assay. Gene expression was assessed by real-time PCR. For validation, splenocytes from ovalbumin-primed mice and PBMC from grass-allergic donors were re-stimulated with respective antigen and co-cultured with probiotics, and cytokine profiles were correlated. **Results:** Culture with IL-4 and anti-CD40 antibody induced secretion of IL-5 from PBMC, indicative of induction of a Th2 pheno-

type. Cytokine profiles induced by probiotics were strain specific even though species- and genus-specific clustering was observed for many strains by principal component analysis. This was paralleled by mRNA levels of the corresponding genes such as increased Tbet and reduced GATA-3 gene expression. Cytokine profiles induced by probiotics in PBMC stimulated with IL-4 and anti-CD40 correlated with those obtained from allergen-stimulated murine splenocytes or human PBMC from grass-allergic donors. **Conclusions:** Cytokine profiling of probiotic strains with IL-4-/anti-CD40-stimulated PBMC allowed to determine the effect of probiotics on Th2-skewed cells and thus to classify probiotic strains with anti-allergic potential.

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Introduction

The allergy pandemic nowadays affects 20–30% of the population in developed countries [1]. The spectrum of allergic symptoms is wide and ranges from atopic dermatitis (AD)/eczema in food-sensitized babies and young children to respiratory symptoms (such as allergic rhinitis and asthma) in adolescents and adults sensitized to grass and tree pollen, house dust mites and animal dan-

der. Simple and cost-effective prevention strategies could help to lower the medical and economic burden associated with allergic diseases.

Clinical trials to evaluate the efficacy of probiotics in the prevention or treatment of various allergic conditions have yielded sometimes controversial results. Some studies indicated beneficial effects in the primary prevention of atopic eczema in infants [2–6] while others failed to confirm these findings [7–9] (see also meta-analysis by Lee et al. [10]). Results were generally less encouraging for treatment of AD [11] even though some studies showed positive results [reviewed in ref. 12–14]. Moreover, a few studies have demonstrated the efficacy of probiotics in symptom reduction of respiratory allergy in adult patients [15–17]. The interplay of numerous factors, including the environment (e.g. microbiota composition [18] and birth delivery mode), the host (e.g. genetic predisposition) and the probiotic strain itself (strain properties, dose and time of administration) are critical in determining the success of probiotic intervention against allergy [19]. Generally, for many clinically applied probiotic strains a compelling rationale for its use in a particular indication is not evident.

Thus, simple and predictive *in vitro* screening models could greatly facilitate rational strain selection and would enhance the chances of success in subsequent clinical trials. Determination of cytokine profiles ('immunoprofiles') induced by probiotics in human peripheral blood mononuclear cells (PBMC) [20, 21] or dendritic cells (DC) [22] has been applied in several studies for the characterization of probiotics. For example, an anti-inflammatory PBMC cytokine profile (low IL-12, high IL-10 along with low IFN- γ and TNF- α) was predictive of a protective anti-inflammatory effect *in vivo* in a mouse colitis model [23]. Cytokine profiles prognostic of an anti-allergic effect need to be established yet. One limitation to this end is the fact that normal human PBMC or CD4+ T cells do not secrete Th2 cytokines, which are a hallmark of an allergic state. Fujiwara et al. [24] partly overcame these limitations by using Th2-skewed splenocytes from ovalbumin (OVA)-primed mice. Notably, a strain of *Lactobacillus paracasei* selected on the basis of its ability to induce IL-12 and inhibit IL-4 secretion in this model showed a benefit in a subsequent clinical trial in humans suffering from allergic rhinitis [25]. Alternatively, Vissers et al. [26] recently showed that co-culture of specific probiotic strains with PBMC from human allergic donors decreased IL-13 production along with strain-specific effects on induction of Th1 cytokines and cell surface markers.

Here we report the development and use of a model of Th2-skewed human PBMC from normal donors for screening and immunological characterization of probiotic strains with anti-allergic potential. We show that strain-specific profiles were obtained and that various potentially anti-allergic immunoprofiles were found. Importantly, we show that cytokine profiles correlated well between this model and two other *in vitro* models based on allergen-primed Th2-skewed cells, namely OVA-specific murine splenocytes as well as PBMC from grass-allergic human donors. Linking these findings with *in vivo* effects may help to more rationally select probiotic strains for intervention against allergy.

Materials and Methods

Reagents and Bacterial Biomass

Bacterial biomass (kindly provided by the Food and Health Microbiology Group, Nestlé Research Center: B. Bourqui, D. de Malprade and N. Pagé) was produced by culture of each strain under optimal conditions in liquid cultures. Lactic acid bacterial strains were grown in standard growth conditions described in the literature, i.e. MRS $\pm$ cysteine or M17 at 30, 37 or 40°C under anaerobiosis or not, depending on the species. *Escherichia coli* was grown in Luria broth. Growth kinetics were determined for each strain and according to these, biomass was harvested 3 h after reaching the stationary phase. At this time point, cultures were washed in cold PBS and frozen in PBS 20% glycerol at -80°C in 50- μ l aliquots. Further details on probiotic strains are given in table 1. Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was purchased from Sigma (Buchs, Switzerland).

Isolation and Culture of Th2-Skewed Human PBMC from Normal, Healthy Donors

Regular blood donations of healthy volunteers were collected and filtered to separate plasma from cells at the Transfusion Center of the Lausanne University Hospital (Centre Hospitalier Universitaire Vaudois). Human PBMC were isolated from these filters by flushing the cells trapped in the filters back into the blood collection bag with 90 ml of Hanks balanced salt solution (HBSS; Sigma). The cells were diluted 1:2 with HBSS and the PBMC were isolated by density gradient centrifugation on Histopaque (Sigma). The cells at the interphase were collected and washed two times with HBSS. The PBMC were resuspended in complete Iscove's modified Dulbecco's medium (cIMDM; Sigma) containing 10% fetal bovine serum (FBS; Bioconcept, Paris, France), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma) and 0.1% gentamycin (Sigma). The cells were cultured in 48-well plates (Milian, Meyrin, Switzerland) at 1.5×10^6 cells/ml in the presence of 50 ng/ml of IL-4 (Bioconcept) and 1 μ g/ml of anti-CD40 antibody (R&D Systems, Abington, UK) in cIMDM to induce a Th2 cytokine phenotype. LPS was used at 1 μ g/ml. After 3 days of culture, probiotics were added at 10^7 , 10^6 and 10^5 CFU/ml. After adding ingredients, PBMC culture was continued for an additional 48 h resulting in total culture duration of 5 days.

Isolation and Culture of Th2-Skewed, OVA-Primed Murine Splenocytes

Female BALB/c mice, 6–8 weeks old (Charles-River, l'Abresles, France), were sensitized intraperitoneally with 50 µg/ml OVA (Sigma) and aluminum sulfate (alum; 1 mg/mouse; Merck, Dietikon, Switzerland) in a volume of 200 µl/mouse. On day 8, a second sensitization was carried out by the subcutaneous route with OVA and alum as described above. The mice were sacrificed on day 15 by cervical dislocation. The spleens were collected into complete RPMI 1640 (Sigma) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 0.1% gentamycin and 50 µM 2-mercaptoethanol (Sigma). Spleens were homogenized in cRPMI to obtain a single-cell solution. Erythrocytes were lysed with red blood cell lysis solution (Roche, Rotkreuz, Switzerland). After washing, the cells were cultured in 48-well plates at 2×10^6 cells/ml in the absence or presence of OVA at 1,000 µg/ml. Probiotics were added concomitantly for a total culture duration of 5 days. Analysis of cell viability during development of the various in vitro cell culture systems confirmed that cell viability at the given concentrations of OVA and bacteria was not compromised.

Isolation and Culture of PBMC from Grass-Allergic Human Donors

Heparinized, venous blood (50 ml) from male and female adults (18–40 years old) with confirmed allergy (allergic rhinoconjunctivitis or asthma) to grass pollen were collected at the beginning of pollen season 24–48 h after the first occurrence of symptoms. PBMC were isolated as described above. Cells were resuspended in RPMI (Sigma) complemented with 8% of human serum AB (Blutspendedienst SRK, Bern, Switzerland), 1% L-glutamine, 1% penicillin/streptomycin, 1% of non-essential amino acids (Invitrogen, Lucerne, Switzerland), 1% sodium pyruvate (Invitrogen) and 0.1% gentamycin (Sigma). Cells were cultured in 96-well plates (Milian) at 1×10^6 cells/ml in three different conditions: (i) in medium, (ii) in the presence of 90 µg/ml grass allergen pollen extract (ALK-Abello wässrig 6 grass, Volketswil, Switzerland) or (iii) in the presence of 50 ng/ml of IL-4 and 1 µg/ml of anti-CD40. After 2 days of culture, probiotics were added at 10^7 CFU/ml. PBMC culture was continued for an additional 3 days resulting in total culture duration of 5 days.

Cytokine ELISA

Human IFN-γ, human IL-5, human IL-10, mouse IFN-γ, mouse IL-13 and mouse IL-10 were measured using DuoSet kits from R&D systems according to the manufacturer's instructions.

Evaluation of ELISA Results

Optical density values were transformed into pictograms per milliliter using the standard curves. To adjust for the large donor-to-donor variation generally observed with human PBMC, data were standardized according to an arbitrary internal standard. The value of IFN-γ (pg/ml) obtained for each donor by stimulation with LPS was set to 100%. For normalization of IL-10 values, the amount of IL-10 induced with strain *Bifidobacterium lactis* NCC 2818 (CNCM-I3446 deposited by Nestlé) was considered as 100%. Finally, the amount of IL-5 induced with IL-4 and anti-CD40 antibody in medium only (i.e. in the absence of probiotics) was set to 100%. As the effect of the different candidate probiotics was highly strain dependent, the concentration (10^5 , 10^6 or 10^7) yielding optimal stimulation (for IFN-γ and IL-10) or optimal inhibition (for IL-5) was used for the calculation of percentages as shown in table 1.

Multiplex Cytokine Assay

The concentrations of human IFN-γ, IL-10, IL-5, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p70, TNF-α and TNF-β were measured using the human Th1/Th2 11-plex multiplex kit (Bender MedSystems, Lucerne, Switzerland) according to the manufacturer's instructions. Samples were measured on a flow cytometer (FACS-Calibur; Becton Dickinson, Franklin Lakes, N.J., USA), and analyzed with FlowCytomix Pro 2.1 software (BMSFFS/2.1; Bender MedSystems).

Quantitative Gene Expression Levels by Real-Time PCR

PBMC (1.5×10^6 cells/ml) were cultured in cIMDM with or without IL-4 (50 ng/ml) and anti-CD40 (1 µg/ml) for 3 days. Thereafter, probiotic candidates were added to PBMC and cells were collected after 10 and 24 h. Total RNA was extracted from stimulated PBMC with the SV total RNA isolation system kit (Promega, Wallisellen, Switzerland) including a DNase treatment according to the manufacturer's instructions. Total RNA was quantified using the Ribogreen RNA quantification kit (Molecular Probes, Basel, Switzerland). Reverse transcription was performed on 1 µg of total RNA by using the Multiscribe reverse transcriptase kit (Applied Biosystems, Foster City, Calif., USA). Total RNA was mixed with 50 µM of random hexamers, 0.5 mM of dNTPs, 20 U of RNase inhibitor (Applied Biosystems), 62.5 U of Multiscribe reverse transcriptase, $1 \times$ RT buffer and 5.5 mM of MgCl₂ in a final volume of 100 µl. Human IFN-γ, IL-10, IL-5, Tbet, GATA3 and FoxP3 (Applied Biosystems) were quantified by real-time PCR (ABI PRISM 7900HT; Applied Biosystems) using the TaqMan gene expression assays. Quantification was normalized with the mean of 3 housekeeping genes: β-actin, GAPDH and HPRT (Applied Biosystems).

Based on the cycle threshold (C_t) values obtained, a relative and normalized mRNA expression was determined for each gene using ΔC_t . The C_t value for each gene was corrected by the C_t mean of the three housekeeping genes. The results were calculated as a relative expression using the formula $2^{-\Delta C_t} \times K$.

Principal Component Analysis

Numerical cytokine values (pg/ml) determined by multiplex cytokine assay (see above) for each strain were used to perform a principal component analysis (PCA, dimensioning technique) using R v2.14.1 Development Core Team software 2011 (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org/>). Data were standardized (subtraction of the averages and division by the standard deviation over the individuals, the bacteria) in order to give the same weight to each variable, the cytokines.

Results

Probiotics Modulate Cytokine Production in a Model of Th2-Skewed Human PBMC

To induce a Th2 cytokine profile, human PBMC from normal, healthy donors were incubated with IL-4 and anti-CD40 antibody. This did not lead to spontaneous IFN-γ or IL-10 production, while secretion of IL-5 became detectable after 3–4 days of culture (fig. 1). Levels of IL-5

Table 1. Cytokine profiles of Th2-skewed human PBMC stimulated with probiotics

NCC	Strain	IFN- γ ¹		IL-10		IL-5		IFN- γ /IL-10 ratio
		n	%	n	%	n	%	
251	<i>B. adolescentis</i>	12	139.9 $\pm$ 32.9	12	121.8 $\pm$ 25.9	6	53.6 $\pm$ 10.3	1.1
264	<i>B. adolescentis</i>	12	86.0 $\pm$ 23.1	12	88.4 $\pm$ 16.8	6	24.1 $\pm$ 5.4	1.0
2950	<i>B. breve</i>	10	238.0 $\pm$ 62.1	11	35.6 $\pm$ 10.3	9	84.4 $\pm$ 13.6	6.7
453	<i>B. bifidum</i>	8	247.3 $\pm$ 30.6	10	30.5 $\pm$ 10.2	7	84.0 $\pm$ 18.4	8.1
2818	<i>B. lactis</i>	62	74.9 $\pm$ 14.7	66	100.0 $\pm$ 0	43	63.9 $\pm$ 4.7	0.7
435	<i>B. longum</i>	8	19.1 $\pm$ 8.6	9	4.3 $\pm$ 1.2	8	44.8 $\pm$ 8.5	4.4
2705	<i>B. longum</i>	9	60.4 $\pm$ 15.4	10	47.6 $\pm$ 19.4	8	45.9 $\pm$ 11	1.3
3001	<i>B. longum</i>	10	63.4 $\pm$ 8.3	11	13.6 $\pm$ 1.9	10	81.9 $\pm$ 8.1	4.7
2912	<i>B. longum</i>	11	56.5 $\pm$ 18.1	12	33.7 $\pm$ 6.6	6	43.8 $\pm$ 5.5	1.7
90	<i>L. acidophilus</i>	9	26.4 $\pm$ 5.3	10	4.8 $\pm$ 3	7	67.2 $\pm$ 20.3	5.5
3009	<i>L. acidophilus</i>	10	151.9 $\pm$ 29.7	12	63.8 $\pm$ 9.1	7	61.4 $\pm$ 5.3	2.4
2531	<i>L. crustorum</i>	10	27.7 $\pm$ 10.4	11	5.1 $\pm$ 1.3	5	37.2 $\pm$ 6.6	5.4
315	<i>L. fermentum</i>	10	39.2 $\pm$ 13.5	12	48.7 $\pm$ 8.7	5	53.9 $\pm$ 14.4	0.8
714	<i>L. helveticus</i>	12	105.0 $\pm$ 19.2	12	37.6 $\pm$ 8.7	6	38.6 $\pm$ 10.3	2.8
1176	<i>L. helveticus</i>	12	101.3 $\pm$ 29.7	12	75.0 $\pm$ 11.6	6	39.4 $\pm$ 3.4	1.4
2849	<i>L. helveticus</i>	12	77.2 $\pm$ 17.4	12	16.0 $\pm$ 10.2	6	79.3 $\pm$ 11.1	4.8
4016	<i>L. helveticus</i>	9	102.6 $\pm$ 25.7	10	39.0 $\pm$ 6	8	52.2 $\pm$ 17.2	2.6
533	<i>L. johnsonii</i>	9	73.5 $\pm$ 9.8	10	15.9 $\pm$ 8	7	33.2 $\pm$ 12.6	4.6
1825	<i>L. paracasei</i>	9	16.2 $\pm$ 8.9	11	5.0 $\pm$ 3.6	10	70.3 $\pm$ 8.5	3.2
2461	<i>L. paracasei</i>	11	33.6 $\pm$ 11.3	14	11.0 $\pm$ 1.7	12	49.4 $\pm$ 8	3.1
4014	<i>L. paracasei</i>	11	22.4 $\pm$ 11.4	11	3.5 $\pm$ 0.7	6	43.3 $\pm$ 5.8	6.4
1107	<i>L. plantarum</i>	5	29.4 $\pm$ 13.2	7	5.7 $\pm$ 1.2	5	19.1 $\pm$ 7.1	5.1
2785	<i>L. plantarum</i>	11	60.9 $\pm$ 12.3	10	6.2 $\pm$ 3.9	9	67.2 $\pm$ 12.2	9.9
2581	<i>L. reuteri</i>	10	26.4 $\pm$ 5.1	9	5.4 $\pm$ 1.4	6	15.2 $\pm$ 4.3	4.9
2455	<i>L. rhamnosus</i>	10	60.0 $\pm$ 19.6	9	19.1 $\pm$ 5.5	8	39.5 $\pm$ 8	3.1
4007	<i>L. rhamnosus</i>	10	28.5 $\pm$ 3.1	11	11.9 $\pm$ 2.3	10	48.2 $\pm$ 9.5	2.4
1260	<i>L. sakei</i>	11	162.8 $\pm$ 7.8	11	121.0 $\pm$ 18.7	7	23.9 $\pm$ 7.7	1.3
3018	<i>L. salivarius</i>	9	47.8 $\pm$ 16.1	10	5.6 $\pm$ 2.2	8	37.1 $\pm$ 11.4	8.6
2180	<i>L. lactis</i>	12	86.2 $\pm$ 61.8	12	53.0 $\pm$ 13.3	6	40.8 $\pm$ 8.4	1.6
2287	<i>L. lactis</i>	10	274.1 $\pm$ 78.4	9	97.4 $\pm$ 25.3	8	51.5 $\pm$ 6.6	2.8
2799	<i>S. thermophilus</i>	10	128.9 $\pm$ 41.5	9	34.7 $\pm$ 12.5	6	48.7 $\pm$ 9.4	3.7
2019	<i>S. thermophilus</i>	11	112.4 $\pm$ 30.8	12	28.7 $\pm$ 5.9	9	39.3 $\pm$ 4	3.9
2768	<i>E. faecium</i>	10	104.4 $\pm$ 14	12	32.7 $\pm$ 8.8	7	52.9 $\pm$ 11.8	3.2
- ²	<i>E. coli</i>	10	198.3 $\pm$ 53.6	11	63.1 $\pm$ 22.4	8	27.4 $\pm$ 3.7	3.1
Nissle	<i>E. coli</i>	10	153.5 $\pm$ 46.5	11	50.2 $\pm$ 11.6	9	25.1 $\pm$ 4.6	3.1

Data are expressed as medians $\pm$ SEM. NCC = Nestlé Culture Collection; *B.* = *Bifidobacterium*; *L.* = *Lactobacillus* or *Lactococcus*; *S.* = *Streptococcus*; *E. faecium* = *Enterococcus faecium*; *n* = number of donors.

¹ To equalize large donor-to-donor variations, pg/ml values were normalized to % according to internal references as described in the Materials and Methods Section.

² This probiotic strain of *E. coli* was first described in Lodinová-Zádníková et al. [40].

were highly donor dependent (fig. 1–3), including a considerable proportion of ‘non-responders’. Based on these initial results, Th2-skewing of PBMC was induced for 3 days before adding probiotics. After addition of probiotic candidates or LPS, culture was continued for 2 more days, thus leading to a total culture period of 5 days. As shown in figure 2a for a single donor, co-culture with probiotics according to this protocol led to secretion of IFN- γ and

IL-10, and to inhibition of IL-5 secretion in a strain-specific fashion. Interestingly, IL-4/anti-CD40 conditioning rendered cells more reactive to IFN- γ secretion upon addition of probiotics (grey bars) compared to unconditioned (medium; white bars) PBMC (fig. 2a, left). In contrast, capability of IL-10 secretion was reduced in Th2-skewed cells compared to PBMC cultured in medium alone (fig. 2a, middle). As shown before, IL-5 production

Fig. 1. Characteristics of a model of Th2-skewed human PBMC. Human PBMC were cultured in medium only (**a–c**) or in medium with IL-4 and anti-CD40 antibody (**d–f**). Kinetics of cytokine production (measured by ELISA) for 9 randomly chosen donors are shown.

was a unique feature of IL-4/anti-CD40-conditioned PBMC (fig. 2a, right). Induction of IFN- γ and IL-10 as well as inhibition of IL-5 secretion was dependent on the dose of probiotics, as shown for 5 individual strains (fig. 2b).

Immunoprofiling of Probiotic Bacterial Strains on Th2-Skewed Human PBMC

PBMC from a total of 68 donors were used in this study and results of 61 (IFN- γ), 65 (IL-10) and 45 (IL-5) donors could be evaluated; the number of IL-5 data sets was lower due to several non-responders, i.e. cells from donors producing no or only minute amounts of IL-5. As shown in figure 3, there were large interindividual differences in the absolute amounts of cytokines induced. To allow direct comparison and pooling of data obtained from different donors, results were normalized according to internal standards used in each experiment: IFN- γ levels obtained from each donor by LPS stimulation were set to 100%, IL-10 was normalized according to levels induced by *B. lactis* NCC 2818 (= 100%) and IL-5 levels induced in the absence of probiotics (medium) were set to 100%.

Immunoprofiles of 35 strains (9 bifidobacteria, 19 lactobacilli, 2 lactococci, 2 streptococci, 1 enterococcus and 2 *E. coli*) are shown in table 1. Large variability in the induced levels of IFN- γ and IL-10 were observed between the strains. Nevertheless, bifidobacteria were overall more robust IL-10 inducers than lactobacilli (median of 35.6 vs. 11.9%). The 2 strains of *Lactococcus*, particularly *L. lactis* NCC 2287, were robust inducers of IFN- γ and

IL-10 whereas the 2 strains of *E. coli* were efficient inducers of IFN- γ . Likewise, the efficacy of reducing IL-5 secretion (% residual production of IL-5 is shown) varied greatly between the strains. Nevertheless, while some rules applying to genera or species were identified, often exceptions to the rules were found, underlining the strain specificity of the candidate probiotics. The cytokine protein profiles for IFN- γ , IL-10 and IL-5 were confirmed on gene expression levels by real-time PCR (fig. 4a). In addition, expression of Tbet, FoxP3 and GATA-3 was quantified. A slight increase in Tbet was observed, whereas FoxP3 and GATA-3 tended to be reduced (fig. 4b). However, generally the differences between strains were less marked on the level of these master transcription factors compared to the corresponding prototypic cytokines.

For a short list of strains, some were selected based on their ability of inhibiting IL-5 production, others on the basis of their documented anti-allergic effect in humans (see Discussion); a broader panel of cytokines was determined by multiplex assay (table 2). PCA revealed that based on the profile of 10 cytokines a certain level of clustering by genus was observed (fig. 5). The profiles of lactobacilli and bifidobacteria overlapped partially, whereas lactococci had a distinct profile, probably driven by their strong IL-12 production (table 2).

Correlation of Cytokine Profiles between Th2-Skewed Human PBMC and OVA-Specific Murine Splenocytes and Grass-Allergen-Specific Human PBMC

To determine whether comparable cytokine profiles were induced by probiotics in artificially Th2-skewed

Fig. 2. Dose-dependent modulation of cytokine production by probiotics. **a** Modulation of cytokine production by probiotics (10^7 CFU/ml) in Th2-skewed vs. non-skewed (medium) cells. Data from 1 representative donor are shown. **b** Human PBMC cultured in the presence of IL-4 and anti-CD40 antibody were stimulated with probiotic strains at various doses. Cytokines were determined by ELISA. Data (mean $\pm$ SEM) from 13–55 donors (numbers of donors in parentheses) are shown (depending on the strain and cytokine).

PBMC versus allergen-stimulated cells, a selection of strains (again mainly chosen based on their ability of inhibiting IL-5 production) was tested on OVA-specific murine splenocytes [24] and on PBMC collected from grass-allergic donors at the beginning of the pollen season and restimulated with grass pollen. Strong correlation of IFN- γ and IL-10 levels between Th2-skewed PBMC and OVA-specific splenocytes (fig. 6a) was observed for most but not all strains (non-correlating strains excluded post hoc are shown in white). Similarly, levels of murine IL-13 correlated with human IL-5 induced with IL-4/anti-CD40. Profiles obtained from grass-allergen-

specific PBMC (fig. 6b) correlated with Th2-skewed PBMC for IFN- γ and IL-10. In contrast, no correlation of IL-5 levels was observed, which was most likely due to the fact that strains were chosen based on their ability to inhibit IL-5 secretion, resulting in low variability in this parameter. Taken together, these data demonstrate that similar cytokine profiles were induced by individual probiotics in artificially Th2-skewed PBMC in the absence of allergen and ‘naturally’ Th2-skewed PBMC or splenocytes under allergen-specific restimulation. These findings validate the use of IL-4-/anti-CD40-stimulated cells to mimic allergen-specific cells.

Fig. 3. Range of cytokine production by all donors included in the study: IFN- γ induced by *E. coli* LPS, IL-10 induced by probiotic strain *B. lactis* NCC 2818 and IL-5 production induced by IL-4 + anti-CD40 without addition of probiotics. Cytokines were measured by ELISA. These stimuli were included in all experiments to allow normalization of values between donors as described in the text. Grey diamonds represent individual donors (medians $\pm$ interquartile ranges).

Fig. 4. Gene-expression of Th2-skewed human PBMC stimulated with probiotics. Human PBMC cultured in medium with IL-4 and anti-CD40 antibody were stimulated with probiotic strains at optimal doses. Cells were sampled 10 and 24 h after addition of probiotics, and gene expression levels were quantified by RT-PCR.

Relative gene expression (in arbitrary units, AU) was normalized to 3 housekeeping genes. Data are means $\pm$ SEM from 2 donors for NCC 2818, NCC 2705 and NCC 1176, and 4 donors for NCC 2461 and NCC 2287.

Fig. 5. PCA of 10 cytokines produced by Th2-skewed human PBMC stimulated with probiotics. Human PBMC cultured in medium with IL-4 and anti-CD40 antibody were stimulated with probiotics. Ten cytokines were determined by multiplex assay. Medians of cytokines were calculated for each strain on 7 donors. The bi-plot shows the first 2 principal components of the PCA (PC1 and PC2). Bacteria are represented on these 2 axes, and an-

other plot (top right) shows the directions of each variable (cytokines) on these same axes. The aim of this plot is to provide a visual representation of the data, in only 2 dimensions, to summarize the information (40 + 26 = 66% of the variance of the data is explained on this plot). Details on NCC codes shown in the figure are described in table 1.

Discussion

In this paper, we describe an in vitro model for immunological characterization of probiotics for anti-allergic intervention. It allowed us to determine unique cytokine profiles for probiotic strains of different genera and species. Particularly, it permitted to address the ability of dif-

ferent strains to interfere with Th2 cytokine (IL-5) production by PBMC.

It has been shown previously that culture of PBMC with IL-4 and anti-CD40 leads to Th2-skewing and eventually to IgE antibody production in cell culture supernatant [27]. On the other hand, co-culture of PBMC with bacteria has frequently been used for characterization of

Table 2. Cytokines by Th2-skewed human PBMC stimulated with probiotics (multiplex analysis)¹

NCC	Strain	IL-12p70	IFN- γ	IL-2	IL-10	IL-8	IL-6	IL-5	IL-1 β	TNF- α	TNF- β
	Medium	2 $\pm$ 1	3 $\pm$ 16	35 $\pm$ 13	16 $\pm$ 4	321 $\pm$ 132	191 $\pm$ 39	22 $\pm$ 74	0 $\pm$ 1	17 $\pm$ 8	10 $\pm$ 81**
	LPS	20 $\pm$ 11	582 $\pm$ 281	40 $\pm$ 11	88 $\pm$ 21	2,897 $\pm$ 1,813	6,163 $\pm$ 2,176	13 $\pm$ 44	4 $\pm$ 5	80 $\pm$ 273	147 $\pm$ 97**
264	<i>B. adolescentis</i>	187 $\pm$ 238	277 $\pm$ 168	523 $\pm$ 48	1,078 $\pm$ 221*	4,229 $\pm$ 2,137	6,286 $\pm$ 3,042*	16 $\pm$ 92	194 $\pm$ 626*	1,385 $\pm$ 5,569	2,867 $\pm$ 407**
2818	<i>B. lactis</i>	20 $\pm$ 61	637 $\pm$ 178	61 $\pm$ 36	902 $\pm$ 183*	3,562 $\pm$ 1,130	10,255 $\pm$ 3,665*	20 $\pm$ 110	155 $\pm$ 585*	1,243 $\pm$ 4,893	212 $\pm$ 319**
3001	<i>B. longum</i>	214 $\pm$ 292	292 $\pm$ 102	53 $\pm$ 39	69 $\pm$ 35*	9,768 $\pm$ 2,048	2,683 $\pm$ 3,113*	22 $\pm$ 105	20 $\pm$ 508*	618.2 $\pm$ 4,583	298 $\pm$ 170**
2705	<i>B. longum</i>	51 $\pm$ 137	205 $\pm$ 200	41 $\pm$ 35	225 $\pm$ 92*	4,751 $\pm$ 1,531	3,501 $\pm$ 3,409*	14 $\pm$ 110	94 $\pm$ 396*	927 $\pm$ 5,431	333 $\pm$ 159**
435	<i>B. longum</i>	12 $\pm$ 20	68 $\pm$ 48	56 $\pm$ 16	36 $\pm$ 16*	7,107 $\pm$ 2,086	710 $\pm$ 1,175*	22 $\pm$ 76	9 $\pm$ 30*	97 $\pm$ 583	165 $\pm$ 86**
1176	<i>L. helveticus</i>	61 $\pm$ 366	369 $\pm$ 415	69 $\pm$ 39	741 $\pm$ 319*	3,073 $\pm$ 1,169	4,952 $\pm$ 3,743*	27 $\pm$ 85	156 $\pm$ 481*	1,609 $\pm$ 5,146	366 $\pm$ 264**
533	<i>L. johnsonii</i>	585 $\pm$ 527	704 $\pm$ 121	51 $\pm$ 45	306 $\pm$ 71*	11,161 $\pm$ 2,055	64,104 $\pm$ 2,952*	18 $\pm$ 75	28 $\pm$ 231*	974 $\pm$ 3,819	279 $\pm$ 262**
4014	<i>L. paracasei</i>	125 $\pm$ 287	68 $\pm$ 84	40 $\pm$ 22	35 $\pm$ 22*	6,275 $\pm$ 1,420	1,452 $\pm$ 1,450*	18 $\pm$ 91	5 $\pm$ 219*	134 $\pm$ 3,875	306 $\pm$ 126**
2461	<i>L. paracasei</i>	51 $\pm$ 49	137 $\pm$ 78	44 $\pm$ 24	62 $\pm$ 20*	11,584 $\pm$ 2,521	805 $\pm$ 1,269*	9 $\pm$ 68	8.2 $\pm$ 56*	3 24 $\pm$ 3,451	175 $\pm$ 164**
1107	<i>L. plantarum</i>	339 $\pm$ 278	310 $\pm$ 264	40 $\pm$ 14	169 $\pm$ 963*	6,039 $\pm$ 1,749	11,328 $\pm$ 2,883*	13 $\pm$ 85	1,512 $\pm$ 84*	2,448 $\pm$ 2,439	188 $\pm$ 66**
4007	<i>L. rhamnosus</i>	49 $\pm$ 55*	69 $\pm$ 77*	16 $\pm$ 31*	53 $\pm$ 19*	6,406 $\pm$ 2,921*	691 $\pm$ 1,016*	18 $\pm$ 76*	12 $\pm$ 49*	906 $\pm$ 5,957*	218 $\pm$ 116**
2287	<i>L. lactis</i>	2,466 $\pm$ 479	949 $\pm$ 137	28 $\pm$ 23	640 $\pm$ 324*	5,368 $\pm$ 2,142	21,424 $\pm$ 678*	13 $\pm$ 66	58 $\pm$ 107	2,410 $\pm$ 3,520	252 $\pm$ 202**
2180	<i>L. lactis</i>	3,530 $\pm$ 1034*	1,332 $\pm$ 183*	47 $\pm$ 20*	300 $\pm$ 523**	9,826 $\pm$ 1,914*	28,677 $\pm$ 4,762**	20 $\pm$ 100*	217 $\pm$ 6**	16,250 $\pm$ 4,165*	209 $\pm$ 77**

Data are expressed as pg/ml or medians $\pm$ SEM. NCC = Nestlé Culture Collection; *B.* = *Bifidobacterium*; *L.* = *Lactobacillus* or *Lactococcus*.

¹ Seven donors were tested, for some cytokines only 5 (*) or 3 (**) data sets could be evaluated.

probiotics and other bacteria. A limitation of these systems when used for identification of potentially anti-allergy probiotics is the absence of spontaneous Th2-cytokine production by normal human PBMC. An alternative to overcome this limitation is the use of allergen-specific cells from laboratory animals [19] or humans [26]. While allergen-specific, Th2-skewed cells from laboratory animals are readily available, results obtained with them might not be representative for humans. In contrast, human allergen-specific PBMC can be obtained as well [26], but this includes logistic hurdles, such as restrictions for optimal bleeding time points and large donor-to-donor variability due to differences in the degree of sensitization and specificity for various allergens. Particularly for screening purposes such limitations are certainly relevant, e.g. for the characterization of the 35 strains described in this paper, a total of 68 donors were needed. Therefore, we chose artificial skewing with IL-4/anti-CD40 to assure a more readily available and reproducible source of PBMC secreting an 'allergy-like' Th2 cytokine profile.

Our data show that similar cytokine profiles were obtained by co-culture of probiotics with IL-4-/anti-CD40-conditioned PBMC versus allergen-specific human PBMC or allergen-specific murine splenocytes (fig. 6). Even though the three models operate under different conditions (e.g. culture time or dosing) and thus cannot be directly compared, establishing this correla-

tion was critical for the validation of the artificial, non-antigen-specific model we used for screening. The high correlations of IFN- γ and IL-10 for most of the strains deliver this proof. Likewise, a good correlation was observed between IL-5 from Th2-skewed human PBMC and IL-13 from OVA-specific splenocytes. In contrast, no correlation existed between IL-5 from Th2-skewed and grass-allergen-specific human PBMC. This lack of correlation is likely explained by the choice of bacterial strains for this sub-analysis: based on the first screening (table 1) only strains with a strong capability of IL-5 inhibition were further analyzed. This led to the clustering of results at approximately 200 ng/ml for IL-4-/anti-CD40-stimulated IL-5 and 100–200 ng/ml for grass-allergen-induced IL-5 (fig. 6). In these narrow boundaries, small variations may lead to large reductions in correlations. Importantly, our data confirm very recent results by another laboratory showing that PBMC from allergic donors upon restimulation with anti-CD3/anti-CD28 antibody or with allergen secreted IL-13, which could be inhibited by co-culture with probiotics [26].

Comparison of cytokine profiles in IL-4-/anti-CD40 versus unconditioned cells showed increased IFN- γ and reduced IL-10 reactivity by Th2-skewed cells after probiotic stimulation (fig. 2a). It may be speculated that induction with anti-CD40 antibody led to a generally higher activation state of CD4⁺ T cells in PBMC which may result in a higher IFN- γ response upon exposure to probi-

Fig. 6. Correlation of cytokine expression after probiotic stimulation of Th2-skewed human PBMC vs. allergen-specific human PBMC and murine splenocytes. Normal human PBMC were Th2 skewed with IL-4 and anti-CD40 antibody. **a** Mice were primed with OVA, splenocytes were restimulated with OVA. Data from 2 independent mouse experiments are included; number of human

PBMC donors for each strain and cytokine are shown in table 1. **b** PBMC from human allergic donors were restimulated with grass allergen or IL-4 and anti-CD40 antibody. All cells were co-cultured with probiotics; cytokines were measured by ELISA. Data from 4–13 donors are included (depending on the cytokine and strain).

otics. However, it has to be noted that IL-4/anti-CD40 conditioning did not lead to spontaneous IFN- γ production (fig. 1). Reduced production of IL-10 by IL-4-treated cells has been described previously [28]. Yao et al. showed that treatment of DC with IL-4 in the presence of LPS or CpG reduced IL-10 production and thus promoted Th1 differentiation of CD4 $^{+}$ T cells. The differences in reactivity of allergic versus non-allergic cells have also been described in vivo. Dunstan et al. [29] showed that PBMC from patients with AD produced lower amounts of IFN-

γ , IL-10 or TNF- α in response to polyclonal or antigen-specific stimuli than cells from healthy counterparts. Similarly, a cross-over study comparing a mix of *L. paracasei* Lpc-37, *L. acidophilus* 74-2 and *Bifidobacterium animalis* subsp. *lactis* DGCC 420 (*B. lactis* 420) to placebo revealed differences in reactivity of PBMC from healthy subjects compared to patients suffering from AD [30]. However, clearly the unique feature of the model presented here is the induction of IL-5 secretion allowing the study of the effect of individual probiotic strains on Th2

cytokine secretion in vitro. Indeed, the ability of re-directing a Th2 state may be one of the critical components for achieving primary prevention of allergy.

Comparison of the cytokine profiles stimulated by the studied strains revealed some inter-genus and inter-species differences. For example, overall bifidobacteria were more robust inducers of IL-10 compared to lactobacilli, which is reflected in the lower IFN- γ /IL-10 ratio. Similarly, some recurring traits of particular species were observed, such as robust induction of IFN- γ by (most) *L. acidophilus* and *L. helveticus* strains (tables 1, 2). However, often cytokine profiles were strain specific, for example revealing 2 strains of *Bifidobacterium longum* (NCC 435 and NCC 3001) with the capacity of inducing relatively high amounts of IFN- γ along with low IL-10, resulting in a high IFN- γ /IL-10 ratio while 2 other strains of *B. longum* (NCC 2705 and NCC 2912) had a low IFN- γ /IL-10 ratio. Taken together these data strongly underline the strain specificity of probiotics in their immunomodulatory capacity, including differences in their ability to potentially counterbalance an established Th2 cytokine profile. They also emphasize the need for screening and characterization of strains before proceeding to in vivo preclinical models or clinical studies in humans.

In contrast to the large variations between IFN- γ and IL-10 protein and mRNA levels induced by different probiotics strains (fig. 4a), the differences between the mRNA levels for Tbet, FoxP3 and GATA-3 were minor (fig. 4b). This indicates that for immunological strain characterization in vitro determination of individual cytokines (or other yet to be defined markers) is currently a good way forward and that measurement of master transcription factors determining Th1 (Tbet), Treg (FoxP3) or Th2 (GATA-3) orientations are not sensitive enough in our assay for classification of probiotic strains. It has been suggested that Treg induction might be one of the protective mechanisms of probiotics [reviewed in ref. 31]. *Lactobacillus* signalling through DC-SIGN effectively induced DC that were able to drive Treg differentiation of CD4+ T cells [32]. Our analyses did not indicate induction of FoxP3 expression by probiotics in PBMC. Moreover, it has been shown that induction of FoxP3 in vivo was actually positively associated with AD development. This implies that FoxP3 expression is not necessarily correlated to protection, but may rather illustrate the bodies' attempt to counteract allergic sensitization/inflammation. In addition, PBMC might not be the optimal cellular source for inducing Treg.

To valorize the in vitro characterization model we need to learn how to translate the in vitro cytokine pro-

files into in vivo effects and thus to determine the predictive value of the cytokine profiles, i.e. we need to determine a 'correlate of protection' for allergy. Thus, which in vitro cytokine profile may predict protection in vivo? A study for prevention of allergy showed that neonates born to mothers that had consumed *Lactobacillus rhamnosus* DR20 during pregnancy developed less AD in the first 2 years of life and that these infants had increased cord blood and serum IFN- γ levels [33]. Likewise, in two intervention studies for treatment of established AD it was shown that positive clinical outcomes were associated with mild inflammatory reactions induced by the probiotic intervention. Intervention with *L. rhamnosus* LGG resulted in increased serum levels of CRP and IL-6 [34] and similar results were obtained with a mixture of 4 probiotics [35]. In addition, successful intervention with *Lactobacillus fermentum* PCC in children suffering from AD was accompanied by increased IFN- γ and TNF- α production by PBMC in response to polyclonal stimuli [36]. Finally, a recent study by Snel et al. [37] showed that after consuming *Lactobacillus plantarum* CBS125632 for 4 weeks, PBMC from allergic donors produced less IL-5 and IL-13 but more IL-10. Collectively, these findings suggest that induction of mild inflammation and particularly of IFN- γ secretion could be characteristic of a protective anti-allergic response, a hypothesis that has been put forward previously [38]. In our present study, we included strains which had proven anti-allergic activity in vivo either in human or in pre-clinical animal studies, for example *L. rhamnosus* NCC 4007 [2], which is indistinguishable by molecular typing from *L. rhamnosus* ATCC 53103, *L. paracasei* NCC 4014 which is equivalent to strain KW3110 [25], *B. longum* NCC 3001 which corresponds to strain BB536 [15], or *L. paracasei* NCC 2461 who was recently shown to reduce symptoms of allergic rhinitis [39]. Interestingly, most of these strains shared a 'discrete' cytokine profile, with low IFN- γ and IL-10 but efficient inhibition of IL-5 production (table 1, fig. 5). Thus, a 'low-key' cytokine pattern (possibly associated with a 'homeostatic' effect conferred by the probiotic strain) might be an alternative cytokine profile with promise for clinical success. Overall, these considerations indicate that not one single but several cytokine profiles from PBMC might be predictive of protection in vivo. Evidently, anti-allergic effects of probiotics may not solely be mediated via immunomodulation, e.g. direct re-enforcement of the intestinal barrier may translate in an anti-allergic effect as well. To identify such non-immune characteristics, cytokine profiling will not be the tool of choice.

In summary, we established a simple in vitro assay that allowed immune classification of a selection of candidate probiotic strains. This tool will be instrumental in selecting a small number of potential anti-allergic strains for further evaluation in more sophisticated in vitro models such as DC/T-cell assays and in vivo allergy models. We hypothesize that careful in vitro and in vivo characterization of probiotics will facilitate the design of clinical studies and will help to explain contradictory results observed in human studies in the area of probiotics and allergy.

Acknowledgments

We thank Bertrand Bourqui, Dominique De Malprade and Nicolas Pagé for harmonization of bacterial biomass. We thank Marietta Weiss and Audrey Demont for technical help, and Dr. Maurice Beaumont and Sylviane Oguey-Araymon for the clinical coordination. We greatly appreciate the helpful discussions and critical inputs from Guenolée Prioult, Anurag Singh, Laurence van Overtfeldt (Stallergènes SA, Paris, France) and Philippe Moingeon (Stallergènes). This project has been funded by NEST-EC SA.

Disclosure Statement

The authors have no conflicts of interest.

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