

ORIGINAL PAPER

Effects of *Lactobacillus* GG treatment during pregnancy on the development of fetal antigen-specific immune responsesR. J. Boyle^{*†}, L.-J. Mah^{*}, A. Chen^{*}, S. Kivivuori^{*}, R. M. Robins-Browne[‡] and M. L.-K. Tang^{*†}^{*}Department of Allergy and Immune Disorders, Murdoch Children's Research Institute, University of Melbourne, Parkville, Vic., Australia, Departments of [†]Paediatrics and [‡]Microbiology and Immunology, University of Melbourne, Parkville, Vic., AustraliaClinical and
Experimental
Allergy

Summary

Background Several clinical trials suggest that probiotics may have a role in the prevention of eczema. The optimal timing and mechanisms underlying this intervention are not clear. In particular it is not known whether such treatment works during pregnancy or whether postnatal exposure is important.

Objective We investigated whether the probiotic *Lactobacillus rhamnosus* strain GG (LGG) influences fetal immune responses when administered to pregnant women, as a possible mechanism for its protective effects against the development of eczema.

Methods Peripheral blood mononuclear cell from 11 adults treated with LGG, and cord blood mononuclear cells (CBMCs) from 73 women participating in a randomized controlled trial of LGG treatment were cultured with heat-killed LGG, ovalbumin (OVA) or without stimulus. Cells were analysed by flow cytometry and real-time PCR for markers of dendritic cell (DC) phenotype, T cell proliferation and regulation. Cytokine secretion was analysed in culture supernatants by multiplex cytokine assay.

Results LGG treatment of adults led to systemic immune responses suggestive of antigen-specific tolerance including reduced CD4⁺ T cell proliferation to heat-killed LGG (30% reduction; $P = 0.03$). LGG treatment of pregnant women did not influence CD4⁺ T cell proliferation, forkhead box P3 expression, DC phenotype or cytokine secretion in CBMCs cultured with heat-killed LGG or OVA.

Conclusion LGG treatment of pregnant women fails to influence fetal antigen-specific immune responses. This suggests that modulation of fetal immune responses may not be a major mechanism by which probiotics such as LGG prevent eczema.

Keywords eczema, immune regulation, pregnancy, probiotic, T cell proliferation

Submitted 10 June 2008; revised 18 July 2008; accepted 29 July 2008

Correspondence:

Mimi L.-K. Tang, Department of Allergy and Immunology, Royal Children's Hospital, Parkville, Vic., 3052, Australia.
E-mail: mimi.tang@rch.org.au

Introduction

Allergic diseases such as asthma, eczema, rhino-conjunctivitis and food allergy are now the commonest chronic diseases of childhood in many countries, and represent an ever-increasing burden to human health [1]. Recent clinical trials have indicated that the administration of probiotic bacteria to pregnant women and their infants may be an effective way to prevent allergic diseases such as atopic eczema, with beneficial effects persisting to at least 7 years age [2–6]. The mechanisms mediating these protective effects are not yet clear, but transplacental induction of fetal immune tolerance to the probiotic and related antigens may

be a contributory factor. In support of this probiotics commenced postnatally do not appear to be effective in preventing eczema, whereas probiotics commenced prenatally may be effective [2–5, 7–9]. It is a well-established observation that maternal infection or immunization during pregnancy can influence fetal immune development, and recent data from a large cross-sectional European study showed a close relationship between farming exposures during pregnancy and the risk of subsequent allergic sensitization [10–17]. These studies all suggest that the manipulation of fetal immune development using non-pathogenic microbes during pregnancy could be an important therapeutic target for the prevention of allergic disease [18].

We chose to explore this possibility by investigating the transplacental immune effects of a non-pathogenic intestinal microbe of human origin. We selected the well-studied probiotic bacterium *Lactobacillus rhamnosus* strain GG (LGG; American Type Culture Collection 53103) for this work, because two out of three randomized controlled trials have indicated that perinatal exposure to LGG may reduce the risk of allergic disease development in the infant, and one possible mechanism for this effect is transplacental immune priming [3, 4, 19]. We initially examined the immune effects of LGG in healthy adults, and then evaluated the ability of LGG to induce pro-inflammatory or regulatory immune effects in the fetus when administered to pregnant women.

Materials and methods

Subject recruitment, treatment and sample collection

First 11 healthy non-atopic adults were treated with 1.8×10^{10} CFU LGG (Dicoflor, Dicofarm SpA, Rome) daily for 7 days. Ten millilitres of venous blood was harvested before treatment and at day 7. Then cord blood mononuclear cell (CBMC) samples were harvested from the infants of 73 women enrolled in a randomized-controlled trial of prenatal LGG supplementation for the prevention of infant eczema (Probiotic Eczema Prevention Study registered with Cochrane Skin Group <http://www.nottingham.ac.uk/ongoingskintrials>, Trial no. 36). Each participant had a history of doctor-diagnosed asthma, allergic rhinitis, food allergy or atopic eczema in themselves, their partner or a previous child. Exclusion criteria included significant fetal abnormality, maternal or fetal immune deficiency and previous probiotic use during the current pregnancy. The Ethics Committees of the Royal Children's Hospital and the Mercy Hospital for Women, Melbourne approved the studies, and written informed consent was obtained from all participants. Treatment allocation using a computer-generated randomization list was done by a third party, with no involvement in participant recruitment. Participants were allocated to take 1.8×10^{10} CFU LGG daily or maltodextrin placebo from 36 weeks gestation until delivery. Participants, clinical trial staff and laboratory staff were not aware of individual treatment allocation through the study. Blood samples were collected in polypropylene tubes containing heparinized RPMI tissue-culture medium (Invitrogen, Carlsbad, CA, USA).

Preparation of blood samples and cell culture

Mononuclear cells were separated by density centrifugation and cryopreserved for future batched analyses [20]. Two million cells per millilitre were incubated with or without antigen in AIM-V serum-free medium (Invitrogen)

for 48 h, or in RPMI with 10% autologous plasma for 6 days in proliferation assays. Heat-killed LGG (HKL) was prepared by incubating LGG at 75 °C for 45 min and was used at 5 : 1 HKL to mononuclear cell ratio. Ovalbumin (OVA) (Sigma, St Louis, MO, USA) was used at 100 µg/mL [21]. IFN- γ -1b (Boehringer Ingelheim, Ingelheim, Germany) and LPS (Sigma) were both used at 10 ng/mL. All cell culture reagents were tested for endotoxin contamination (Cape Cod Associates, E. Falmouth, MA, USA). OVA required endotoxin removal over polymyxin B columns before use (Pierce, Rockford, IL, USA).

Flow cytometry

Cell pellets were stained with fluorochrome-conjugated monoclonal antibodies in 50 µL staining volumes. Lineage cocktail-fluorescein isothiocyanate (FITC) (anti-CD3, 14, 16, 19, 20, 56), HLA-DR-Peridinin chlorophyll protein (PerCP), CD123-PE and CD11c-Allophycocyanin (APC) were used to identify dendritic cell (DC) phenotypes as CD11c^{hi}CD123^{w^{lo}} myeloid DC (mDC), CD123^{hi}CD11c^{lo} plasmacytoid DC (pDC) and CD11c^{lo}CD123^{w^{lo}} immature DC (iDC). CD3-APC, CD4-PerCP, CD25-PE-Cy7 (BD Bioscience, San Jose, CA, USA), CD25-FITC and FoxP3-PE (E-Bioscience, San Diego, CA, USA) were used to identify CD25^{hi}FoxP3^{hi} T cell populations. CFSE was used as a cell tracking dye, and aminostilbamidine methanesulphonate as a viability dye (Molecular Probes, Eugene, OR, USA). CBMC or peripheral blood mononuclear cell (PBMC) were incubated with fluorochrome-labelled antibodies or isotype controls for 30 min, and for intracellular staining cells were subsequently permeabilized, fixed and stained with FoxP3-PE antibody or isotype control (E-Bioscience). Data were acquired on a four-colour LSR II (BD Bioscience) and analysed with FACSDiva v4.1 software using well-defined gating strategies.

Enzyme-linked immunosorbent assay

Concentrations of IL-10, IFN- γ , IL-13, IL-12p40 and TNF- α were determined in CBMC culture supernatants harvested at 48 h by multiplex cytokine bead assay using a Luminex 100 Analyzer (Luminex Corporation, Austin, TX, USA). Anti-cytokine beads and matched anti-cytokine biotinylated reporters were used according to the manufacturer's instructions (Millipore, Billerica, MA, USA). Data were analysed with Luminex IS 2.3 software using a five-parameter regression formula to calculate sample concentrations from standard curves. Concentrations of TGF- β 1 were evaluated using a commercial human TGF- β 1 ELISA kit according to the manufacturer's protocol (BD Biosciences). Supernatants were analysed undiluted in duplicate with recombinant cytokine as a positive and culture medium as a negative control. TGF- β 1 concentrations were determined based on a standard curve generated using the

KCjunior v1.40.3 program (Bio-Tek Instruments, Winooski, VT, USA) with a four-parameter equation. ELISA data were analysed both as dichotomous data – detected vs. not detected; and as continuous data – mean level in each group.

Real-time polymerase chain reaction

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed to cDNA using the Superscript First Strand Synthesis System and oligo(dT) primers (Invitrogen). All reactions included a 'RT minus' control with no RT to control for the possibility of contaminating DNA. FoxP3 and IL-4 mRNA were quantified by real-time PCR using FAM-labelled Taqman Gene Expression Assays on an ABI Prism 7300HT system (Applied Biosystems, Foster City, CA, USA). Eukaryotic translation elongation factor 1 α (EEF1A1), which is stably expressed in human mononuclear cell cultures was used as a reference gene [22]. The mean level of gene expression in cDNA samples was expressed as a ratio to mean EEF1A1 expression.

Statistics

The clinical trial was designed with a sample size of 250 in order to have 90% power to detect a 40% difference in eczema risk between probiotic and placebo groups. Secondary outcomes were the immune outcomes reported herein, derived from CBMC of participants who delivered at the Mercy Hospital for Women. All available CBMC samples were evaluated and primary analyses were by intention to treat. Data were assessed using histograms and skewed data were $\log_{10}$ transformed. Parametric paired data were analysed using the paired *t*-test, and non-parametric paired data using the Wilcoxon signed rank test and sign test. Parametric unpaired data were analysed using the independent *t*-test, and non-parametric unpaired data using Mann–Whitney *U*-test. Continuous data are presented as arithmetic mean $\pm$ 1 standard error of the mean (SEM), or medians with interquartile ranges. Categorical data were analysed using χ^2 test or Fisher's exact test. *P*-value < 0.05 was considered statistically significant, with due caution in interpreting the results of multiple comparisons. Where the significance of findings was unclear a sensitivity analysis was performed by excluding participants in whom (i) treatment compliance data (returned capsule counts) were not available ($n = 9$), (ii) capsule counts suggested compliance levels $< 50\%$ ($n = 2$) or (iii) no treatment capsules were taken due to premature delivery between randomization and 36 weeks gestation ($n = 1$). Analyses were performed using SPSS v 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Effects of Lactobacillus rhamnosus strain GG treatment on peripheral blood mononuclear cell proliferation

The effects of orally administered LGG were first evaluated in healthy adults. PBMC were harvested from 11 adults before and upon completion of 7 days LGG treatment. Treatment was associated with a 30% reduction [95% confidence interval 11–50%; $P = 0.03$] in mean $CD4^+$ T cell proliferative response to HKL compared with proliferative responses from the same subjects before LGG treatment (Fig. 1). In contrast, there was no change in $CD4^+$ T cell proliferation to OVA ($P = 0.2$) or medium alone ($P = 0.6$) after LGG treatment.

Effects of Lactobacillus rhamnosus strain GG treatment on dendritic cell phenotype

We investigated DC phenotype in cultured PBMC harvested from adults before and after LGG treatment. pDC increased from 3.20% to 5.29% of total DC ($P = 0.02$) after LGG treatment, in PBMC cultured for 48 h with HKL. A trend towards increased ratio of pDC to mDC was also seen after LGG treatment in PBMC cultured with HKL (mean ratio 0.36 pre-treatment, 0.58 post-treatment; $P = 0.07$). LGG treatment was not associated with any significant change in DC phenotype in PBMC cultured with OVA or medium alone (data not shown).

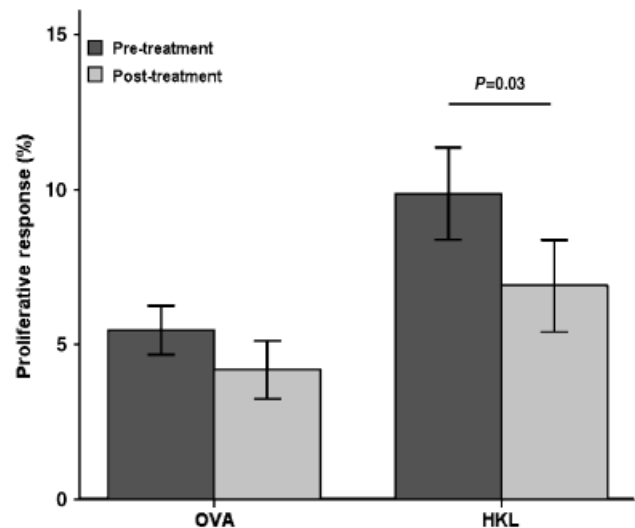


Fig. 1. $CD4^+$ T cell proliferation in 11 adults treated with *Lactobacillus rhamnosus* strain GG (LGG) for 7 days. The mean percentage viable $CD4^+$ T cells that divide (CFSE¹⁰) after 6 days culture with ovalbumin (OVA) or heat-killed LGG (HKL) are shown at day 0 (pre-treatment) and day 7 (post-treatment). Bars represent ± 1 SEM. *P*-value is for paired *t*-test. SEM, standard error of the mean.

Table 1. Characteristics of women treated with the probiotic *Lactobacillus rhamnosus* strain GG ($N = 35$) or placebo ($N = 38$)

Characteristic	Probiotic	Placebo	<i>P</i> -value*
Maternal age [†]	33.6	33.0	0.54
No. previous pregnancies [†]	1.24	1.34	0.73
Maternal eczema (%)	55.3	48.6	0.57
Maternal smoking (%)	11.8	12.9	0.99
Caesarean delivery (%)	25.7	36.4	0.34
Maternal infection [‡] (%)	8.6	6.3	0.99
Treatment compliance (%)	94.1	87.9	0.15

**P*-values are for unpaired *t*-test where continuous data are presented as means, and χ^2 or Fisher's exact test where categorical data are presented as %.

[†]Numbers shown are arithmetic means.

[‡]Self-reported infection during the treatment period, i.e. between 36 weeks gestation and delivery.

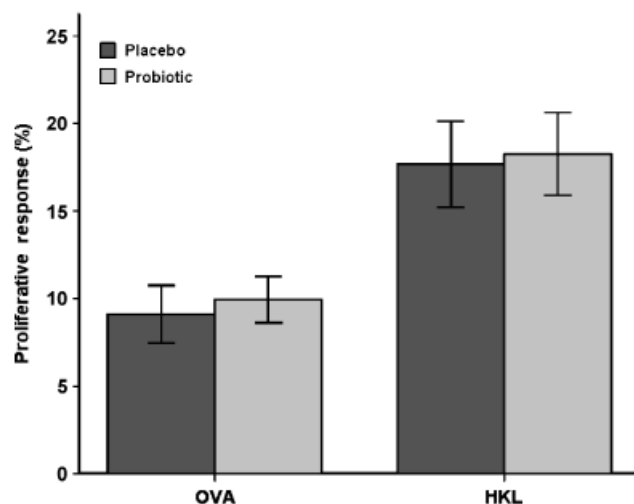


Fig. 2. Mean CD4⁺ T cell proliferation in 52 cord blood mononuclear cell samples from infants whose pregnant mothers were treated with the probiotic *Lactobacillus rhamnosus* strain GG (LGG) or placebo for 36 weeks gestation until delivery. The mean percentage viable CD4⁺ T cells that divide (CFSE^{lo}) after 6 days culture with ovalbumin (OVA) or heat-killed LGG (HKL) is shown. Bars represent ± 1 SEM. There was no significant difference between treatment groups (unpaired *t*-test). SEM, standard error of the mean.

Effects of prenatal *Lactobacillus rhamnosus* strain GG on cord blood mononuclear cell proliferation

The effects of prenatal treatment with LGG on fetal immune responses at birth were examined. No significant difference was seen between randomization groups in perinatal factors, which might influence fetal immune responses (Table 1). Returned capsule counts showed that a mean of 91.0% (SEM 2.1) of expected capsules were taken over a mean of 25 days by participants in the randomized-controlled trial of prenatal LGG treatment. CBMC from 52 infants (27 placebo treated; 25 probiotic treated) were evaluated for proliferative

responses to HKL and OVA (Fig. 2). We found no difference in mean CD4⁺ T cell proliferation between placebo-treated and probiotic-treated groups when CBMC were cultured with HKL ($P = 0.9$), OVA ($P = 0.7$) or medium alone ($P = 0.5$). There was no difference between groups in mean number of CD25^{hi}FoxP3^{hi} CD4⁺ T cells in the non-proliferating T cell population after culture with either HKL (6.58% placebo, 7.85% probiotic; $P = 0.3$), OVA (5.46% placebo, 6.48% probiotic; $P = 0.2$) or medium alone (3.86% placebo, 4.29% probiotic; $P = 0.4$).

Effects of prenatal *Lactobacillus rhamnosus* strain GG on cord blood dendritic cell phenotype

We evaluated CBMC harvested from 73 infants (35 placebo treated; 38 probiotic treated) for DC numbers and phenotype after 48 h culture with HKL and OVA (Table 2). There was no difference in the number of total DC, pDC, mDC, iDC or pDC : mDC ratio between probiotic and placebo groups after culture with HKL, OVA or medium alone.

Effects of prenatal *Lactobacillus rhamnosus* strain GG on cord blood mononuclear cell FoxP3 expression

We evaluated CD25^{hi}FoxP3^{hi} CD4⁺ T cell numbers and FoxP3 mRNA expression in CBMC from 73 infants cultured with HKL, OVA or medium alone for 48 h. The data are summarized in Figs 3 and 4. There was no difference between groups in the number of CD25^{hi}FoxP3^{hi} T cells after culture with HKL (mean 9.54% placebo, 7.66% probiotic; $P = 0.2$), OVA (mean 5.77% placebo, 4.76% probiotic; $P = 0.2$) or medium alone (mean 5.35% placebo, 4.65% probiotic; $P = 0.2$). There was no difference between groups in mean fluorescence intensity of FoxP3 or CD25 staining in the CD4⁺ T cell population after culture with HKL, OVA or medium alone (data not shown). There was no difference between treatment groups in total CBMC FoxP3 mRNA expression after culture with HKL ($P = 0.8$), OVA ($P = 0.7$) or medium alone ($P = 0.9$; Fig. 4). We also evaluated FoxP3 expression in uncultured CBMC – there was no difference between treatment groups in number of CD25^{hi}FoxP3^{hi} T cells ($P = 0.80$), in mean fluorescence intensity of FoxP3 staining in the CD4⁺ T cell population ($P = 0.24$) or in total CBMC FoxP3 mRNA expression ($P = 0.98$).

Effects of prenatal *Lactobacillus rhamnosus* strain GG on cord blood mononuclear cell cytokine secretion

CBMC culture supernatants from 63 infants were harvested at 48 h and evaluated for secretion of IL-10, IL-12p40, IL-13, TNF- α , TGF- β 1 and IFN- γ . We first analysed the data dichotomously. There was no significant difference between probiotic and placebo groups in the proportion of samples with detectable cytokine after culture with

Table 2. DC numbers and phenotype in 73 infants whose pregnant mothers had received the probiotic *Lactobacillus rhamnosus* strain GG (LGG) ($N = 35$) or placebo ($N = 38$)

Cell type*	Stimulus†	Probiotic	Placebo	‡P-value
Total DC%	Nil	1.98	2.24	0.54
pDC%	Nil	4.53	4.62	0.94
mDC%	Nil	5.26	3.76	0.27
pDC:mDC	Nil	2.41	2.89	0.66
Total DC%	OVA	2.07	2.13	0.90
pDC%	OVA	8.27	9.45	0.51
mDC%	OVA	4.40	3.56	0.54
pDC : mDC	OVA	5.10	4.93	0.92
Total DC%	HKL	2.15	2.27	0.80
pDC%	HKL	15.37	14.92	0.87
mDC%	HKL	3.32	4.16	0.36
pDC : mDC	HKL	9.05	12.06	0.54

*DC numbers are shown as mean % total viable lymphocyte/monocytes; plasmacytoid dendritic cell (pDC) and myeloid DC (mDC) numbers are shown as mean percentage total DC population.

†Cells were cultured in AIM-V serum-free medium alone, with ovalbumin or with heat-killed LGG (HKL) for 48 h.

‡P-values are for unpaired *t*-test.

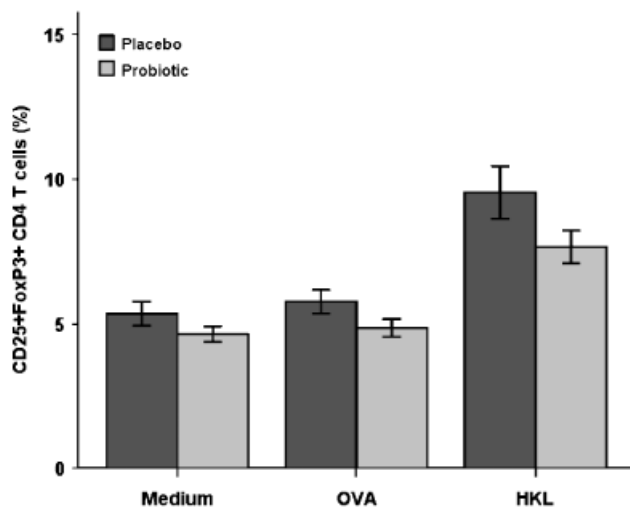


Fig. 3. CD25^{hi}FoxP3^{hi} CD4⁺ T cell number in 73 cord blood mononuclear cell samples from infants whose pregnant mothers were treated with *Lactobacillus rhamnosus* strain GG (LGG) or placebo. The mean percentage CD4⁺ T cells that stained CD25^{hi}FoxP3^{hi} after 48 h culture with OVA, heat-killed LGG (HKL) or medium alone is shown. Bars represent ± 1 SEM. There was no significant difference between treatment groups (unpaired *t*-test). SEM, standard error of the mean.

HKL, OVA or medium alone. An exception was the detection of IL-12p40 in medium only culture (6% probiotic group; 25% placebo group; $P = 0.04$). For all cytokines other than IL-12p40, there were sufficient samples with detectable cytokine to compare mean levels between treatment groups (Fig. 5). There was no significant difference between probiotic and placebo groups in mean

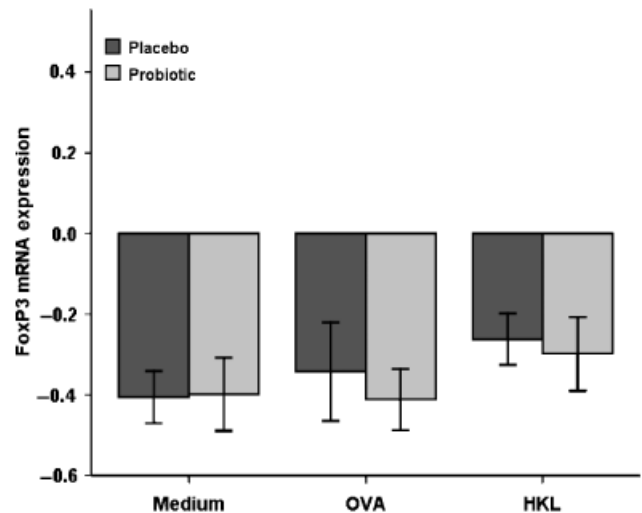


Fig. 4. FoxP3 mRNA expression in 73 cord blood mononuclear cell samples from infants whose pregnant mothers were treated with *Lactobacillus rhamnosus* strain GG (LGG) or placebo. Mean log₁₀(FoxP3 mRNA) is shown, where FoxP3 mRNA is expressed as a ratio relative to EEF1A1 mRNA. Cells were harvested after 48 h culture with ovalbumin, heat-killed LGG (HKL) or medium alone. Bars represent ± 1 SEM. There was no significant difference between probiotic and placebo groups (unpaired *t*-test). SEM, standard error of the mean.

cytokine levels, except for IFN- γ levels in medium only culture (mean log₁₀IFN- γ level 1.42 pg/mL probiotic; 1.87 placebo; $P = 0.02$). When CBMC were primed with IFN- γ for 3 h and stimulated with LPS for 24 h to augment IL-12 secretion there was no significant difference between placebo and probiotic groups (mean log₁₀IL-12 level 1.87 pg/mL placebo, 1.87 pg/mL probiotic; $P = 0.96$). IL-4 mRNA expression was evaluated in CBMC harvested at 48 h from the same cultures, with no significant difference seen between placebo and probiotic groups in culture with HKL ($P = 0.5$), OVA ($P = 0.1$) or medium alone ($P = 0.6$).

Discussion

Probiotic bacteria have diverse *in vitro* effects on cellular immune responses, but until now their *in vivo* immune effects in humans have not been well characterized [23]. We have shown for the first time that oral administration of LGG to healthy adults can lead to systemically detectable changes in T cell proliferative responses and DC phenotype. Both the decrease in CD4⁺ T cell proliferation and the increase in pDC numbers in PBMC cultured with HKL are consistent with antigen-specific tolerance induction [24]. We subsequently evaluated the transplacental immune effects of LGG. In contrast to the data for healthy adults we found that prenatal administration of LGG to pregnant women did not induce detectable changes in T cell proliferation, FoxP3 expression, DC phenotype, pro-inflammatory or regulatory cytokine production in cord blood. One other group has recently reported that LGG

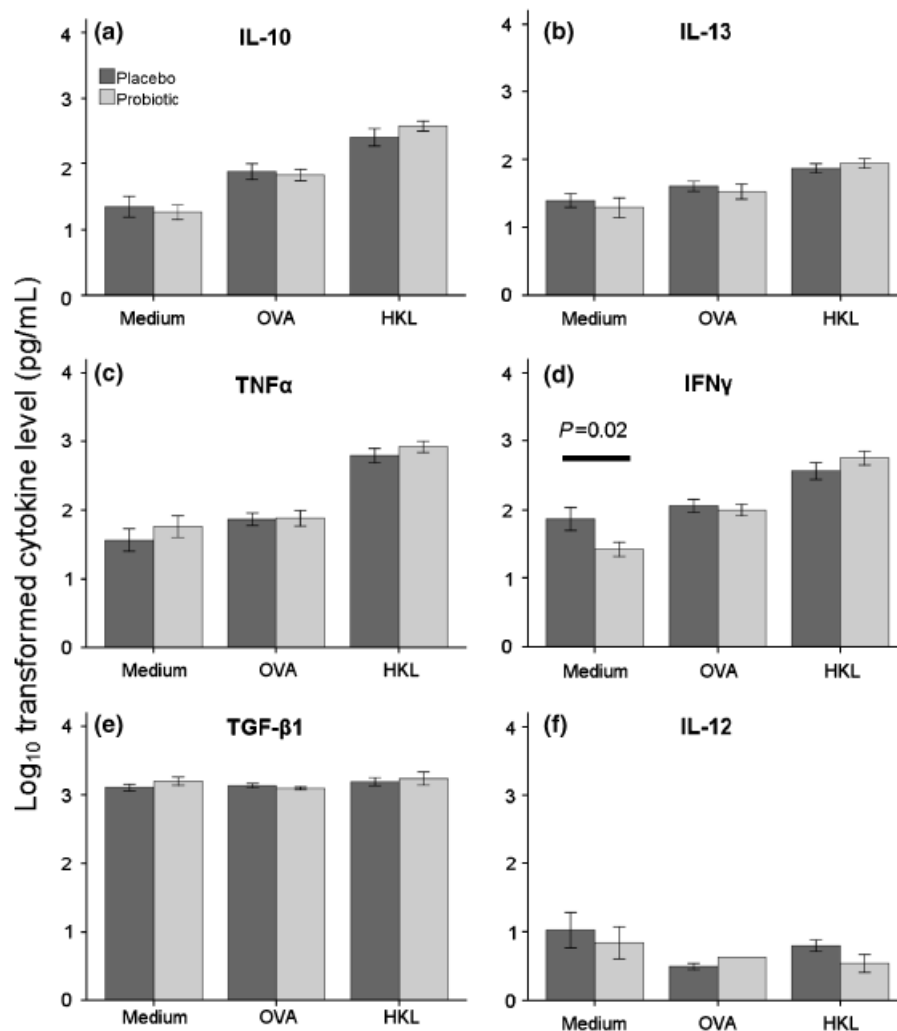


Fig. 5. Levels of cytokines IL-10 (a); IL-13 (b); TNF- α (c); IFN- γ (d); TGF- β 1 (e) and IL-12p40 (f) in supernatants of 63 cord blood mononuclear cell (CBMC) samples harvested from infants whose pregnant mothers were treated with LGG or placebo from 36 weeks gestation until delivery. Mean cytokine levels $\pm$ 1 SEM are shown for CBMC cultured with ovalbumin, heat-killed LGG (HKL) or medium alone. There was a reduced level of IFN- γ in unstimulated culture supernatants at 48 h ($P=0.02$; unpaired t -test). SEM, standard error of the mean.

fails to cause transplacental immune effects, although this group's immune assays appear to be less sensitive as that they were unable to detect any immune effects even in treated adults [25]. A recent study of probiotic administration during pregnancy also failed to show significant immune effects in the neonate despite significant effects on the composition of the maternal intestinal microbiota [26]. These data suggest the possibility of a class effect, with well-tolerated non-pathogenic micro-organisms failing to influence fetal immune responses trans-placentally, in contrast to pathogenic microbial exposures [10–17]. The results suggest that transplacental induction of antigen-specific tolerance is not a major mechanism for the protective effects that probiotics may have against the development of eczema.

Our finding that LGG has immune effects suggestive of antigen-specific tolerance induction in adults differs from

previous studies in atopic infants, where LGG induced systemic inflammatory responses with increased C-reactive protein and IL-6 secretion [27, 28]. The difference may be explained by the age of participants because in infancy the intestinal mucosal barrier is more permeable, immune responses are less mature and the intestinal microbiota is less stable than during adulthood [29]. Two other studies have failed to demonstrate antigen-specific tolerance induction by LGG [25, 30]. Schultz et al. [30] treated 5 healthy adults with 2×10^9 CFU/day LGG for 5 weeks and found no change in a marker of T cell activation, but a significant decrease in IL-6 secretion in whole blood cultured with HKL at the end of treatment. We included a larger number of subjects, a higher dose of LGG and a more sensitive method to identify proliferating CD4⁺ T cells after 6 days in culture. Kopp et al. [25] treated 105 pregnant women with a family history of

allergic disease with 10^{10} CFU/day LGG or placebo and evaluated proliferative response to LGG in PBMC from 68 of them at delivery. They found no significant difference in proliferative response between LGG and placebo-treated women; however, there are significant differences between their study and ours, which might account for this discrepant finding. They used a between-subject rather than a within-subject design, a lower ratio of LGG to PBMC in culture (1 CFU LGG : 1.25–25 PBMC, compared with 5 CFU LGG : 1 PBMC in our studies) and importantly a different proliferation assay. They used a 7-day tritiated thymidine assay so that variations in actual cell numbers included in cultures, in cell viability after culture and in total CD4⁺ T cell number in each sample may have reduced their ability to detect the differences in CD4⁺ T cell proliferative responses that we found. Notably proliferation to LGG was decreased in the LGG-treated group in their study (median 4666.5 counts per minute placebo; 2359 LGG; statistics not presented), although when analysed as stimulation index this difference was no longer evident. Finally, it is possible that Kopp et al. failed to reproduce our findings because their adult subjects were pregnant women rather than healthy non-pregnant adults. Pregnancy clearly represents a state of altered immune responsiveness, and it is possible that this may include a resistance to the immune changes we have seen in LGG-treated adults [31].

In view of previous reports of the transplacental immune effects of *in utero* microbial exposures, our finding that maternal LGG exposure does not alter fetal antigen-specific proliferative responses must be assessed critically [10–17]. The relevance of cord blood T cell proliferative responses to long-term T cell memory is a subject of debate, with some work suggesting they are not representative of conventional T cell memory responses [32]. Other work using tetramers has identified priming of CD4⁺ memory T cells in human cord blood following maternal immunization, but a relationship between cord blood proliferative responses *in vitro* and later T cell proliferation *in vivo* has still not been established [17]. Therefore the failure of prenatal LGG exposure to influence cord blood T cell proliferative responses does not, on its own, prove that LGG has failed to induce immune effects in the fetus. For this reason we also explored other markers of tolerance induction in cord blood samples – namely numbers of CD25^{hi}FoxP3^{hi} CD4⁺ T cells, total CBMC FoxP3 mRNA expression, induction of different DC phenotypes and the secretion of T-helper type 1 (Th1), Th2 and regulatory cytokines in 48 h culture. The data showed no effect of prenatal probiotic exposure on FoxP3 expression following antigen stimulation, on pDC or mDC numbers or on CBMC cytokine secretion in culture with antigen. In CBMC cultured with medium alone, a lower number of culture supernatants had detectable IL-12p40 in the probiotic group (6% probiotic; 25% placebo;

$P=0.04$) and mean IFN- γ levels were lower in the probiotic group (mean log₁₀IFN- γ level 1.42 pg/mL probiotic; 1.87 placebo; $P=0.02$). These may, however, be chance findings because the differences were not statistically significant when IL-12 data were analysed as a continuous variable or IFN- γ data were analysed dichotomously, the level of statistical significance was not high given the number of separate comparisons made, and when data were analysed with poorly compliant participants excluded these differences became non-significant ($P=0.17$ for difference in log₁₀ IFN- γ level in culture with medium alone; $P=0.14$ for detectable IL-12p40 in culture with medium alone).

Our data effectively exclude certain transplacental immune effects as the mechanisms through which probiotic bacteria prevent allergic disease. They do not, however, exclude all possible transplacental immune effects – for example we did not evaluate the effects of prenatal probiotic treatment on fetal B cell development. Our data do, however, suggest that probiotics are likely to act through different mechanisms in the prevention of allergic disease, and these merit investigation in future studies. Possible mechanisms include effects on maternal breast milk composition, on neonatal intestinal microbiota development or immune effects related to postnatal administration directly to infants [33].

In summary we have found evidence of systemically detectable immune effects following oral administration of LGG to healthy adults. However, we did not find evidence of any immune effects in infants born to mothers receiving the same treatment. These results suggest that the protective effects of perinatal probiotic supplementation against the development of allergic disease are not mediated by transplacental modulation of fetal antigen-specific immune responses.

Acknowledgements

We are grateful to the participants for volunteering for these studies. The studies were funded by grants from the Jack Brockhoff Foundation, the Murdoch Children's Research Institute and the Ilhan Food Allergy Foundation. Mimi Tang was supported by a Murdoch Children's Research Institute Salary Grant. Robert Boyle was supported by a University of Melbourne Baillieu Scholarship and a Murdoch Children's Research Institute Postgraduate Scholarship. LGG and placebo capsules were manufactured and supplied by Dicoform Ltd (Roma, Italy). We are grateful to Dr Susan Donath of the Murdoch Children's Research Institute Clinical Epidemiology and Biostatistics Units for advice regarding statistical analyses, to Professors Patrick Holt and Susan Prescott for advice regarding laboratory methodology, to Professors Roy Robins-Browne and Seppo Salminen for advice regarding study design and to Matt Burton, Noel Cranswick, Kay Hynes,

Christine Plover, Michael Permezel, Val Bryant, Christine Axelrad, Sally Moore, Sampo Lahtinen and Amy Bin Chen for their assistance in the conduct of these studies.

References

- Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med* 2006; **355**:2226–35.
- Abrahamsson TR, Jakobsson T, Bottcher MF *et al*. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:1174–80.
- Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001; **357**:1076–9.
- Kukkonen K, Savilahti E, Haahtela T *et al*. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:192–8.
- Osborn DA, Sinn JK. Probiotics in infants for prevention of allergic disease and food hypersensitivity. *Cochrane Database Syst Rev* 2007; CD006475.
- Kalliomaki M, Salminen S, Poussa T, Isolauri E. Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:1019–21.
- Taylor A, Hale J, Wiltschut J, Lehmann H, Dunstan JA, Prescott SL. Evaluation of the effects of probiotic supplementation from the neonatal period on innate immune development in infancy. *Clin Exp Allergy* 2006; **36**:1218–26.
- Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. *J Allergy Clin Immunol* 2007; **119**:184–91.
- Taylor AL, Hale J, Wiltschut J, Lehmann H, Dunstan JA, Prescott SL. Effects of probiotic supplementation for the first 6 months of life on allergen- and vaccine-specific immune responses. *Clin Exp Allergy* 2006; **36**:1227–35.
- Brustoski K, Moller U, Kramer M *et al*. Reduced cord blood immune effector-cell responsiveness mediated by CD4+ cells induced in utero as a consequence of placental *Plasmodium falciparum* infection. *J Infect Dis* 2006; **193**:146–54.
- Desai M, ter Kuile FO, Nosten F *et al*. Epidemiology and burden of malaria in pregnancy. *Lancet Infect Dis* 2007; **7**:93–104.
- Ege MJ, Bieli C, The Parsifal Study Team *et al*. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006; **117**:817–23.
- Gill TJ III, Repetti CF, Metlay LA *et al*. Transplacental immunization of the human fetus to tetanus by immunization of the mother. *J Clin Invest* 1983; **72**:987–96.
- Gray GE, McIntyre JA. HIV and pregnancy. *BMJ* 2007; **334**:950–3.
- Malhotra I, Mungai P, Wamachi A *et al*. Helminth- and Bacillus Calmette-Guerin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *J Immunol* 1999; **162**:6843–8.
- Petersen E. Protozoan and helminth infections in pregnancy. Short-term and long-term implications of transmission of infection from mother to fetus. *Parasitology* 2007; **134**:1855–62.
- Rastogi D, Wang C, Mao X, Lendor C, Rothman PB, Miller RL. Antigen-specific immune responses to influenza vaccine in utero. *J Clin Invest* 2007; **117**:1637–46.
- Blumer N, Sel S, Virna S *et al*. Perinatal maternal application of *Lactobacillus rhamnosus* GG suppresses allergic airway inflammation in mouse offspring. *Clin Exp Allergy* 2007; **37**:348–57.
- Kopp MV, Hennemuth I, Heinzmann A, Urbanek R. Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: no clinical effects of *Lactobacillus* GG supplementation. *Pediatrics* 2008; **121**:e850–6.
- Dunstan JA, Mori TA, Barden A *et al*. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. *J Allergy Clin Immunol* 2003; **112**:1178–84.
- Dunstan JA, Hale J, Breckler L *et al*. Atopic dermatitis in young children is associated with impaired interleukin-10 and interferon-gamma responses to allergens, vaccines and colonizing skin and gut bacteria. *Clin Exp Allergy* 2005; **35**:1309–17.
- Hamalainen HK, Tubman JC, Vikman S *et al*. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem* 2001; **299**:63–70.
- Smits HH, Engering A, van der Kleij D *et al*. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* 2005; **115**:1260–7.
- Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 2004; **5**:1219–26.
- Kopp MV, Goldstein M, Dietschek A, Sofke J, Heinzmann A, Urbanek R. *Lactobacillus* GG has in vitro effects on enhanced interleukin-10 and interferon-gamma release of mononuclear cells but no in vivo effects in supplemented mothers and their neonates. *Clin Exp Allergy* 2008; **38**:602–10.
- Shadid R, Haarman M, Knol J *et al*. Effects of galactooligosaccharide and long-chain fructooligosaccharide supplementation during pregnancy on maternal and neonatal microbiota and immunity – a randomized, double-blind, placebo-controlled study. *Am J Clin Nutr* 2007; **86**:1426–37.
- Marschan E, Kuitunen M, Kukkonen K *et al*. Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation. *Clin Exp Allergy* 2008; **38**:611–8.
- Viljanen M, Pohjavuori E, Haahtela T *et al*. Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema-dermatitis syndrome. *J Allergy Clin Immunol* 2005; **115**:1254–9.
- Tannock GW, Munro K, Harmsen HJ, Welling GW, Smart J, Gopal PK. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl Environ Microbiol* 2000; **66**:2578–88.

- 30 Schultz M, Linde HJ, Lehn N *et al.* Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. *J Dairy Res* 2003; **70**:165–73.
- 31 Poole JA, Claman HN. Immunology of pregnancy. Implications for the mother. *Clin Rev Allergy Immunol* 2004; **26**:161–70.
- 32 Thornton CA, Upham JW, Wikstrom ME *et al.* Functional maturation of CD4+CD25+CTLA4+CD45RA+ T regulatory cells in human neonatal T cell responses to environmental antigens/allergens. *J Immunol* 2004; **173**: 3084–92.
- 33 Rautava S, Kalliomaki M, Isolauri E. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *J Allergy Clin Immunol* 2002; **109**:119–21.