

FOXP3 mRNA expression at 6 months of age is higher in infants who develop atopic dermatitis, but is not affected by giving probiotics from birth

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Factors that influence immune regulation in early life may be implicated in the rise in allergic disease, including reduced microbial burden. The aim of the study was to examine the infant regulatory T-cell function in relation to (a) probiotic supplementation for the first 6 months of life and (b) the subsequent development of an early allergic phenotype. Two hundred and thirty-one allergic, pregnant women were recruited into a randomized, controlled trial. The infants received either a probiotic or placebo daily for the first 6 months of life. One hundred and seventy-eight children completed the study, with blood samples available from 118 (60 placebo; 58 probiotic). CD4+CD25+CTLA4+ T-regulatory phenotype and allergen-induced FOXP3 mRNA expression were compared in relation to this intervention as well as according to evidence of early disease (atopic dermatitis). The administration of probiotics was not associated with any significant differences in the proportion of circulating CD4+CD25+CTLA4+ cells, or in the resting expression of FOXP3. There were also no relationships between these parameters and patterns of gut colonization, and this probiotic did not reduce the risk of atopic dermatitis. Children who developed atopic dermatitis ($n = 36/118$) had significantly higher induced FOXP3 expression following stimulation with both house dust mite (HDM) ($p = 0.017$) and ovalbumin (OVA) allergens ($p = 0.021$) than those that did not develop atopic dermatitis. Although this relationship was seen in both the probiotic and placebo groups, it was more pronounced in the probiotic group. However, regression analysis demonstrated that higher allergen-induced FOXP3 expression was predicted by the presence of atopic dermatitis ($p = 0.018$) rather than probiotics supplementation ($p = 0.217$). The higher levels of allergen-induced FOXP3 in atopic dermatitis suggest activation of these compensatory mechanisms rather than a primary defect in this pathway. Probiotic supplementation and gut colonization did not appear to substantially modify these regulatory pathways, or the risk of developing atopic dermatitis.

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Interactions between the microbial environment and the host immune system are critical for normal immune development. It has been pro-

posed that microbial-induced activation of key regulatory pathways [including antigen presenting cells (APC) and regulatory T cells] plays a

central role in inhibiting type 2 (Th2)-mediated allergic responses (1). This has provided an immunologic basis for the 'hygiene hypothesis'. Although there is still no conclusive evidence that alterations in microbial flora or regulatory function are responsible for the rising rates of allergic disease at the population level, there is still mounting interest in the role of bacteria products such as probiotics in the treatment and prevention of allergic disease.

There is accumulating evidence that probiotics and other bacterial products can have immunomodulatory effects (2). While the mechanisms are still not well understood, effects on regulatory cell populations are among the main candidate pathways. Recent animal studies support the notion that intestinal flora can induce regulatory T-cell populations (3) and human studies have also shown increased *in vitro* production of regulatory cytokines (IL-10) after ingestion of probiotic bacteria (4).

The role of regulatory T cells in allergic disease is still unclear. As these cells are important for controlling effector responses, there has been logical speculation that deficient function in these populations could be a primary defect in allergic disease. However, although several studies suggest deficiencies in allergen-specific T-regulatory numbers or function in allergic disease (5, 6), others have shown the opposite (7). While the role of these cells in the primary pathogenesis of disease is still controversial, there is better evidence that T-regulatory cells play a role in the secondary control of allergic responses (8, 9) and environmental factors that can enhance their activity (such as microflora) are of central interest for therapeutic reasons.

In this context, the purpose of this study was to examine the effects of probiotic supplementation, given from birth, on the expression of regulatory T-cell markers at 6 months of age. While there is a heterogeneous group of T cells with regulatory properties, our study focused on well-described populations that constitutively express CD25 (the α chain of the IL-2 receptor) and other suppressive molecules including CTLA-4 (10, 11). CD4, CD25, CTLA4 are not individually exclusive to these thymus-derived regulatory cells; however, clustering of these markers has been used to identify these cells, and we have used this strategy in our study. We also examined expression of the forkhead transcription factor, FOXP3, which plays a critical role in the development and function of regulatory T cells (12) and is now an established marker for naturally occurring T-regulatory cells in both mice and humans

(12). Human CD4+CD25+ T cells up-regulate FOXP3 after *in vitro* activation (12, 13) providing further indirect evidence that regulatory pathways can be activated or induced in the periphery. Here, we examined the effects of probiotics on the capacity of allergen-specific T-regulatory cells to up-regulate FOXP3 expression. Although functional assays have been developed to assess suppressive activity of these cells *in vitro* (using mixed lymphocyte reactions), this is not possible in young infants because of restricted volumes of blood that can be ethically collected.

We also examined T-regulatory cells in relation to early emerging evidence of an atopic phenotype (atopic dermatitis). It has been previously noted that adults with atopic dermatitis have significantly increased numbers of peripheral blood T-regulatory cells with normal immunosuppressive activity (7) suggesting that these cells are involved in active compensatory mechanisms rather than a primary defect. To our knowledge, this is also the first study to assess this issue in infants.

Methods

Study population

A population of 231 pregnant women with diagnosed allergic disease and documented allergen sensitization [to house dust mite (HDM), grass pollens, cat pelt, dog hair, feathers, moulds; or cockroach extracts] were recruited into a randomized-controlled trial. Their infants received either a probiotic (3×10^9 *Lactobacillus acidophilus* LAVRI-A1 in maltodextrin) or placebo (maltodextrin) supplement daily for the first 6 months of life. One hundred and seventy-eight infants completed the supplementation period and blood samples were available from 118 of these (58 in the probiotic group and 60 in the placebo group). T-regulatory cell phenotyping and FOXP3 mRNA expression were examined in the infants at the end of the supplementation period.

Choice of probiotic strain

This study was designed and initiated before the study by Isolauri et al. (14) was published. At that stage, there was little information about the relative effects of different probiotics in this context. The choice of *L. acidophilus* LAVRI-A1 was based on promising effects *in vitro* (initially unpublished) and evidence that the strain conformed to the FAO/WHO expert panel guidelines for probiotics (resistance to acid and bile; adherence to cells of the intestinal

epithelium and colonization in the intestinal tract; antagonistic activity towards enteric pathogens; and maintenance of strain identity and viability throughout shelf life) (15, 16). Both *in vitro* and *in vivo* immunological effects of this strain have been subsequently published (22, 23). Preliminary studies in animals have shown that co-administration of LAVRI-A1 with allergen is associated with down regulation of immediate hypersensitivity reactions (17).

Clinical assessment of atopic dermatitis at 6 and 12 months

Infants had a clinical assessment at 6 and 12 months of age, when they were specifically assessed for evidence of doctor diagnosed atopic dermatitis. This diagnosis was made in infants with typical skin lesions (19).

Compliance

Compliance was assessed by the use of a dose chart (completed by parents) and dose counts (returned sachet packets counted by the pharmacy department). Stools were also monitored for differences in colonization (below).

Sample collection and initial processing

Infant blood samples were collected into heparinized tubes, and peripheral blood mononuclear cells (PBMC) were isolated within 2 h using Lymphoprep (Nycomed Pharma, Asker, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis.

Flow cytometric analysis of regulatory T-cell phenotype

Peripheral blood mononuclear cells were thawed and stained immediately (prior to culture) to assess the expression of T-regulatory phenotype. Cells were washed and stained with a commercial three-colour antibody kit (BD Biosciences, Sydney, Australia) containing fluorescein isothiocyanate-conjugated CD4, CD25-phycoerythrin and CD3-PerCpCy5.5. Intracellular staining for CTLA4 was performed with allophycocyanin-conjugated CTLA4 using Cell Fix/Perm (BD Biosciences). Stained cells were resuspended in PBS/0.2% BSA/0.05% sodium azide and analysed immediately on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). At least 100,000 mononuclear cells were acquired on the flow cytometer in order to obtain sufficient numbers of regulatory T cells for accurate enumeration. Data analysis were performed with the FLOWJO software (Tree Star).

Cell culture

The technique used to culture infant cells is described in detail elsewhere (20). Briefly, 2×10^6 PBMC/ml was cultured in duplicate in 96-well round-bottom plates in AIM V (Gibco, Life Technology Ltd, Paisley, Scotland) serum-free medium for 48 h with or without (control) allergens. The allergens included whole dust mite extract (HDM) 20 μ g/ml (CSL, Melbourne, Australia) and ovalbumin (OVA) 100 μ g/ml (Sigma, Castle Hill, Australia). These plates were then placed in a 37°C 5% CO₂ incubator for 48 h.

mRNA isolation and analysis

Following a 48-h culture period, supernatants were collected and stored at -80°C for cytokine analysis. mRNA was isolated from the cell pellets of allergen (OVA and HDM) and control cultures. Briefly, total RNA was isolated using the RNeasy 96 Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. Reverse transcription was performed by using the Omniscript kit (Qiagen) according to the manufacturer's protocol with oligo-dT (Promega, Madison, WI, USA) and RNasin[®] Ribonuclease Inhibitor (Promega). Reverse transcribed RNA samples were diluted 1/5 and FOXP3 expression was quantitated by real-time PCR by using QuantiTect SYBR Green Master Mix (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems, Uppsala, Sweden). Relative standard curves were prepared from serial diluted RT-PCR products or plasmid standards and normalized to the reference housekeeping gene (HKG) UBE2D2 (21). Data are expressed as expression level above background (level in stimulated cells minus unstimulated cells) and multiplied by a scaling factor to obtain whole numbers.

Cytokine assays

Cell culture supernatants were analysed for IL-10, IL-13 and IFN- γ using an in-house time resolved fluorometry detection system (DELFI, Wallac, Turku, Finland). Briefly, the DELFIA method was followed by using paired antibodies (Pharmingen, Sydney, NSW, Australia) and the biotinylated secondary antibody was detected using Europium-labelled streptavidin (Wallac) and fluorescence was quantified using a fluorometer (Wallac VICTOR2; PerkinElmer Life Sciences, Boston, MA, USA). Standard curves generated using serial dilutions of recombinant human IL-10, IL-13 and IFN- γ (Pharmingen)

were linear between 3 and 30,000 pg/ml. Cytokine data are expressed as the difference between the stimulated culture and control (pg/ml). The limit of detection was 3 pg/ml for all cytokines. TGF- β was determined by using an ELISA kit (R&D Systems Europe, Abingdon, UK) for detection of latent TGF- β 1. The limit of detection was 3.9 pg/ml. TGF- β cytokine data are expressed as the difference between the stimulated culture and control (pg/ml).

CD4+CD25+ T cells depletion experiments

To remove CD25+ T cells from the PBMC population, CD25 Dynabeads® (Dyna, Vic, Australia) were used according to manufacturers instructions with minor alteration. Briefly, 25 μ l of washed Dynabeads were added to PBMCs resuspended in Roswell Park Memorial Institute 1640 (RPMI, Life Technology, UK) culture medium plus 2% heat inactivated foetal calf serum (Australian Biosearch, Perth, Australia) and incubated for 30 mins at 4°C with gentle rotation. Beads were removed, supernatant was transferred to a new tube and this procedure was repeated. Following the double depletion to remove the CD25+ T cells, remaining cells were washed and resuspended in AIM V (Gibco, Life Technology Ltd, Paisley, Scotland) serum-free medium. Monitoring by flow cytometry revealed that >90% of CD25+ cells were depleted from the total PBMC pool.

Stool analysis

Stool samples were collected 1 and 6 months after commencing the supplement. There were 106 infants with available stool and PBMC samples to assess the association between colonization and T-regulatory cells, 54 in the placebo and 52 in the probiotic group. Lactobacilli, bifidobacteria and coliforms were enumerated in these samples as follows: 10-fold diluted homogenates of the stool samples (w/v) in half strength Wilkin-Chalgren broth (Oxoid, Basingstoke, UK) were prepared by thawing the frozen samples in an anaerobic chamber and carrying out the homogenization in the chamber. Homogenates were serially diluted in 10-fold steps and aliquots (10 μ l) were drop inoculated on MRS, Prioionic acid agar, or MacConkey agar for lactobacilli, bifidobacteria and coliforms, respectively. After incubation at 37°C for 48 h for lactobacilli and bifidobacteria, and 24 h for coliforms, the colonies were counted and results expressed as the number of colonies per gram wet weight of faeces (CFU per gram).

Statistical analysis

Statistical analysis was performed by using SPSS software (version 11.0 for Mac OS X). Non-parametric data were analysed by using the Mann-Whitney *U*-test to calculate differences between the groups. Where the data could be normalized by log transformation, the data were expressed as geometric mean and 95% CI and differences between the groups were assessed by the student's *t*-test. Correlations were assessed by using Kendall's tau (τ) b (where a proportion of the variables of interest shared 'zero' values) in order to avoid the problems associated with 'ties' within the data. Linear regression was used to assess weighted effects (e.g. relative influence of atopic dermatitis or probiotics supplementation) on the dependent variable (e.g. FOXP3) where the dependent variable was normally distributed or normalized by logarithmic transformation. The Kolmogorov-Smirnov test and probability plots were used to confirm that data were normally distributed prior to inclusion in the linear model. A *p*-value of <0.05 was considered statistically significant for all analyses.

Results

The characteristics of the study population are described in detail elsewhere in detail (22, 23). Briefly, 178 mothers and their healthy full-term infants completed the study; 89 in the probiotic group and 89 in the control group. There were no significant differences in the baseline characteristics including gestational age, birth weight, or gender of the neonates in the two groups. Approximately, 80% of doses were administered with no differences between the groups (*p* = 0.607).

The effects of probiotic supplementation on the risk of atopic dermatitis

At 6 months of age, at the end of the supplementation period, the rates of atopic dermatitis were similar in the probiotic (*n* = 23/89, 25.8%) and placebo (*n* = 20/88, 22.7%) groups (*p* = 0.629). Similarly at 12 months of age, no differences were seen in the rate (*p* = 0.581) or severity (*p* = 0.995) of atopic dermatitis between the two groups. Thus, the administration of probiotics did not reduce the risk of atopic dermatitis, at least in the first year of life as described in more detail elsewhere (23).

As indicated above, peripheral blood samples were available from 58 infants from the probiotic group and 60 infants from the placebo group at

6 months of age; these samples were used for the following immunological analyses.

The effects of probiotics on stool colonization patterns

At the end of the study period (6 months of age), infants in the probiotic group had significantly higher rates of *Lactobacillus* colonization (36%) compared with the placebo group (21.6%) ($p = 0.039$). This trend was also evident at 1 month of age, with almost twice the rate of colonization in the probiotic group (23%) compared with the placebo group (13%) ($p = 0.123$). There were no differences in the rates of colonization with *Bifidobacterium* or coliforms between the groups.

FOXP3 expression in the CD4+ CD25+ cell fraction

In preliminary experiments, we demonstrated that $>90\%$ depletion of CD4+ CD25+ T cells using magnetic beads resulted in significant reduction in FOXP3 expression (Fig. 1). For the purpose of subsequent assays, this indicated that FOXP3 expression occurs largely, although not exclusively in the CD4+CD25+ cell fraction. Similar reductions in FOXP3 mRNA expression following depletion of CD4+CD25+ cells have also been seen in adult cells, and by reintroducing the CD25+ cells back into the

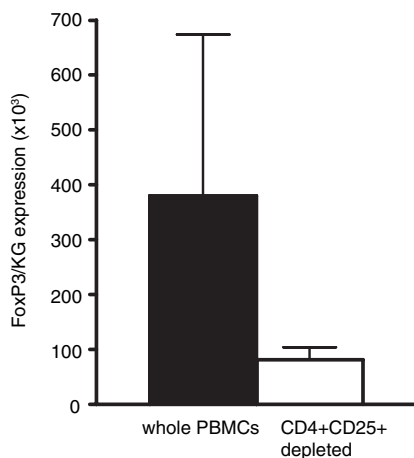


Fig. 1. Preliminary data: FOXP3 expression following CD4+CD25+ cell depletion: the expression of FOXP3 mRNA expression was assessed in peripheral blood mononuclear cells (PBMCs) (shaded) and CD4+CD25+ depleted PBMC cultures (clear) following stimulation with ovalbumin (OVA) allergen for 48 h. Quantitative gene expression was assessed with real-time RT-PCR and the mRNA level is shown as FOXP3 gene expression relative to the housekeeping gene UBE2D2 (HKG). Levels shown are the difference between allergen stimulated and control culture. Following CD4+CD25+ depletion FOXP3 mRNA levels were dramatically reduced.

culture system, FOXP3 expression is reconstituted (unpublished data).

T-regulatory cell markers in children who developed atopic dermatitis

We determined whether children (independent of intervention) who developed atopic dermatitis showed differences in either the circulating number of putative T-regulatory cells and/or the capacity of these cells.

No differences in the frequency of putative CD4+CD25+CTLA4+ regulatory T cells in children who developed atopic dermatitis. Flow cytometry data were only available on 77 of the children. We compared the frequency of CD4+CD25+CTLA4+ T cells, as a marker of T-regulatory cells, as a percentage of circulating total CD4+ T cells in children (with available flow cytometry data) who did ($n = 24$) and did not ($n = 53$) have evidence of physician diagnosed atopic dermatitis in the first 6 months of life. There were no differences between infants who developed atopic dermatitis and those that did not (data not shown).

Higher allergen-induced FOXP3 expression in children who developed atopic dermatitis. We then examined for differences in allergen-induced FOXP3 mRNA up-regulation (after 48 h) in 118 children with cell samples available. Most children (77.1% following stimulation with OVA and 84.7% following stimulation with HDM) showed increased FOXP3 mRNA expression following allergen stimulation. However, children with atopic dermatitis ($n = 36$) had significantly greater increases in FOXP3 expression after stimulation with both food allergen (OVA; $p = 0.021$) and inhalant allergen (HDM; $p = 0.017$) compared with children who did not develop atopic dermatitis ($n = 82$), as shown on Fig. 2. There were no differences in the levels of unstimulated FOXP3 expression (data not shown). As noted further below, the trend for higher FOXP3 up-regulation with atopic dermatitis was evident in *both* the probiotic and the placebo groups (Fig. 5). Although this was only statistically significant in the probiotic group, the trend is also clearly evident in the placebo group and suggests that this effect is not primarily due to the probiotic. This was confirmed in a linear regression model, which examined the relative predictive effects of both 'atopic dermatitis' and 'probiotic supplementation' on FOXP3 expression. In this model, only atopic dermatitis (regression coefficient 0.219; $p = 0.018$) and not probiotic

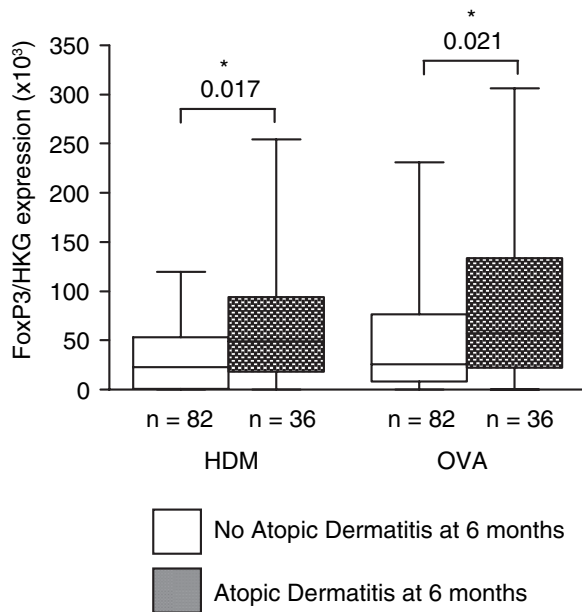


Fig. 2. FOXP3 expression in infants with and without atopic dermatitis: the increase in FOXP3 mRNA expression was assessed following the stimulation of peripheral blood mononuclear cells with house dust mite and ovalbumin (OVA) allergens for 48 h as noted in the Methods. Quantitative gene expression was assessed with real-time RT-PCR and the mRNA level is shown as FOXP3 gene expression relative to the housekeeping gene UBE2D2 (HKG). The levels shown are the difference between levels in the allergen stimulated and unstimulated cultures and are displayed as the median and interquartile ranges. Infants who developed atopic dermatitis at 6 months of age demonstrated significantly higher FOXP3 mRNA levels than infants who did not develop atopic dermatitis. * $p < 0.05$ is considered statistically significant.

supplementation (regression coefficient 0.113; $p = 0.217$) significantly predicted OVA-induced FOXP3 upregulation. Similarly, HDM-induced FOXP3 upregulation was significantly related to the presence of atopic dermatitis (regression coefficient 0.20; $p = 0.033$) but not probiotic supplementation (regression coefficient 0.07; $p = 0.42$).

The effects of probiotic supplementation on regulatory T-cell phenotype and FOXP3 expression

Although probiotics did not reduce the risk of developing disease at 6 months of age, we examined whether this intervention had any effects on underlying T-regulatory cell markers.

Probiotics did not influence the frequency of putative CD4+CD25+CTLA4+ regulatory T cells. Children who were supplemented with *L. acidophilus* for 6 months did not show any significant differences in the frequency of

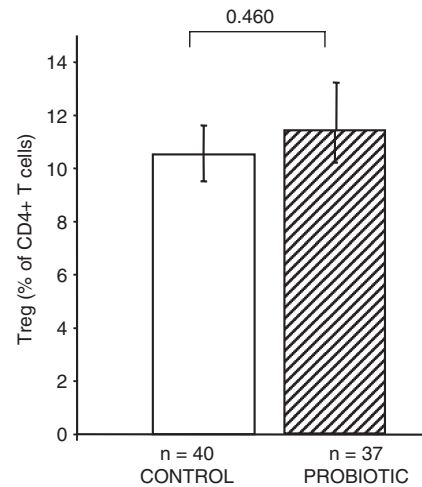


Fig. 3. Enumeration of T-regulatory cell frequency: using flow cytometry, CD4+CD25+CTLA4+ T-regulatory cells were enumerated and expressed as a percentage of CD4+ T cells. No differences in T-regulatory cell frequency were seen between infants who received probiotics (shaded bars) compared with the placebo group (clear bars). Data are represented as geometric mean and 95% CI. The groups were compared by Student's *t*-test.

CD4+CD25+CTLA4+ expressing T-regulatory cells compared with the placebo group (Fig. 3).

Effects of probiotics on FOXP3 expression. Probiotics had no effect on resting expression of FOXP3 (data not shown). There were also no significant differences in FOXP3 upregulation following 48 h culture with HDM or OVA in children who had received daily probiotics since birth (Fig. 4).

As noted above, we performed a subgroup analysis to determine if probiotic supplementation modified FOXP3 expression (and/or CD4+CD25+CTLA4+ co-expression) in children who developed early evidence of an atopic phenotype with the development of atopic dermatitis. Children with atopic dermatitis in both groups showed a trend for increased FOXP3 upregulation following allergen stimulation, although this was only statistically significant in children who had been receiving regular probiotics since birth (Fig. 5). However, regression analysis demonstrated that higher allergen-induced FOXP3 was more strongly predicted by the presence of atopic dermatitis (OVA: $p = 0.018$; HDM: $p = 0.033$) rather than probiotics supplementation (OVA: $p = 0.217$; HDM: $p = 0.42$) as noted above.

Together these findings suggest that probiotic supplementation did not have a significant effect on FOXP3 expression in this population.

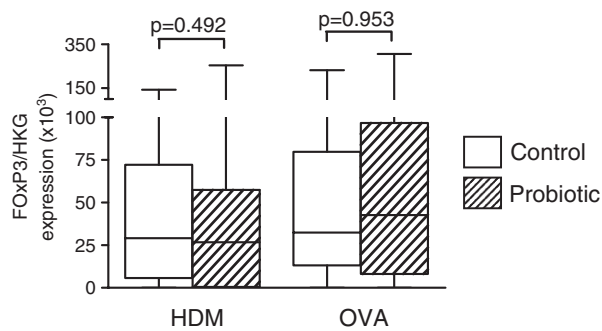


Fig. 4. Analysis of FOXP3 mRNA expression following stimulation with house dust mite (HDM) and ovalbumin (OVA) allergens: the increase in FOXP3 mRNA expression was assessed following a 48-h peripheral blood mononuclear cell culture with allergens, and compared in children receiving probiotics (shaded bars) with those receiving placebo (clear bars). Quantitative gene expression was assessed with real-time RT-PCR and the mRNA level is shown as FOXP3 gene expression relative to the housekeeping gene UBE2D2 (HKG). The levels shown are the difference between levels in the allergen stimulated (HDM) and (OVA) and unstimulated cultures and are displayed as the median and interquartile ranges. No differences were seen between the probiotic and control group for FOXP3 mRNA expression.

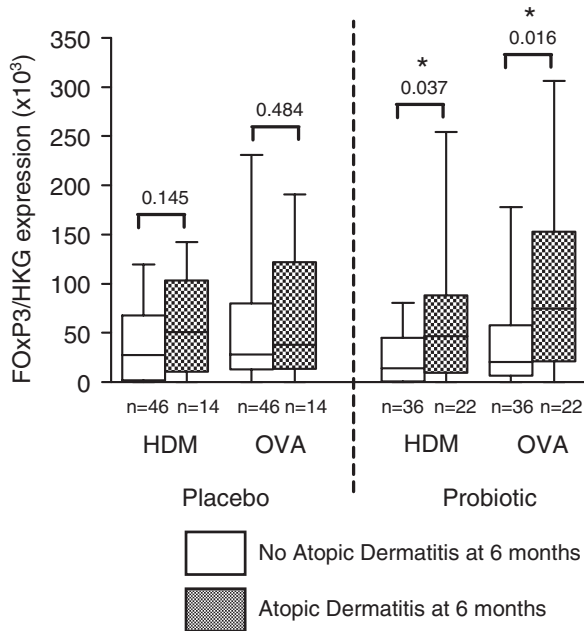


Fig. 5. Comparison of allergen-stimulated FOXP3 expression in atopic dermatitis comparing infants supplemented with probiotics vs. placebo: allergen-induced FOXP3 mRNA expression was assessed following a 48-h peripheral blood mononuclear cell culture. Quantitative gene expression was assessed with real-time RT-PCR and the mRNA level is shown as FOXP3 gene expression relative to the housekeeping gene UBE2D2 (HKG). The levels shown are the difference between levels in the allergen stimulated (house dust mite and ovalbumin) and unstimulated cultures. The responses of children with dermatitis (shaded bars) are compared with those with no disease (clear bars) for children in the probiotic and the placebo group, and are displayed as the median and interquartile ranges. * $p < 0.05$ is considered statistically significant.

The relationship between T-regulatory markers and cytokine responses

We observed significant but weak positive correlations between allergen-specific Th2 (IL-13) responses and the level of FOXP3 expression after stimulation with the corresponding allergen (OVA: $p = 0.19$, $p = 0.008$; HDM: $p = 0.13$, $p = 0.048$), but there were no relationships for IL-10 or IFN- γ responses. When the infants were compared based on their clinical phenotype, the significant relationships between FOXP3 induction and HDM induced IL-13 responses (Kendall $\tau b = 0.23$; $p = 0.008$) and OVA (Kendall $\tau b = 0.18$; $p = 0.023$) were only seen in children with atopic dermatitis. Similarly if the groups were compared on the basis of probiotic supplementation, this relationship with HDM IL-13 responses was only seen in children on probiotics (Kendall $\tau b = 0.21$; $p = 0.041$). The only other significant correlation was a negative correlation in the probiotic group between FOXP3 expression and TGF- β following HDM stimulation (Kendall $\tau b = -0.22$; $p = 0.028$). We also examined the relationship between the numbers of CD4+CD25+CTLA4+ expressing T-regulatory cells and cytokine responses, but did not see any consistent relationships.

The relationship between colonization patterns and regulatory cell markers

There were no significant relationships between the colonization (with Lactobacilli, bifidobacteria and coliforms) and circulating CD4+CD25+CTLA4+ cells or FOXP3 expression at rest or following stimulation. However, it is recognized that the stool culture techniques used in this study may not be as sensitive as molecular techniques.

Discussion

The most significant finding of this study is that infantile atopic dermatitis is associated with greater FOXP3 up-regulation following allergen stimulation, presumably by allergen-specific T-regulatory cells. This trend is clearly evident in *both* groups, although only statistically significant in the probiotic group (Fig. 5). Regression analysis also confirmed that higher allergen-induced FOXP3 expression was significantly predicted by the presence of atopic dermatitis rather than by probiotic supplementation *per se*.

In this population, probiotic supplementation did not reduce the risk of developing atopic dermatitis in predisposed children, and had no

major effects on T-regulatory cell markers. Specifically, probiotics did not appear to have significant effects on CD4+CD25+CTLA4+ cell numbers or FOXP3 expression in high-risk children. It was of note that higher-induced FOXP3 expression in children with atopic dermatitis was more apparent in the probiotic group (Fig. 5). Although it could be argued that probiotics may have further enhanced the potential compensatory mechanisms in affected individuals, this was not associated with any reduction in disease expression, and higher FOXP3 induction was more a function of atopic dermatitis than probiotic supplementation, as already noted. Thus, the significance of this is not clear, and it is possible that the unexplained lower FOXP3 expression in unaffected children in the probiotic group (although not statistically significant) could be contributing to this effect (seen of Fig. 4). It remains to be determined if this has any effect on the subsequent severity of disease expression and the development of sensitization.

FOXP3 is now an established marker for regulatory T cells in both mice and humans (12), and the level of expression has been shown to correlate with suppressive activity (24, 25). Because of the limited cell numbers available in infants, we therefore used this as a surrogate marker of regulatory activity. The increased expression of FOXP3 following PBMC stimulation in this study demonstrates that human regulatory T cells up-regulate this important functional gene following allergen exposure. The observations that this allergen-induced increase in FOXP3 expression was greater in children who had early evidence of an allergic phenotype is consistent with previous observations in adults. Ou *et al.* (7) recently noted that CD4+CD25+ regulatory T cells expressing FOXP3 are increased in patients with atopic dermatitis compared with normal individuals. This suggests that regulatory cell populations are up-regulated as part of secondary compensatory mechanisms, even though this response may be insufficient to overcome disease. Although it is possible that suboptimal function of these cells could contribute to disease, CD4+CD25+ cells from atopic dermatitis patients have demonstrated normal suppressive properties *in vitro* compared with unaffected individuals (7, 26). Thus, while there have been preliminary reports of reduced CD4+CD25+ regulatory cell function in adults with established allergic respiratory disease (5, 6), the role of these cells in the initiation of disease processes has not yet been determined.

Atopic dermatitis is frequently the first manifestation of the atopic phenotype and our findings appear to challenge the notion that there is a primary defect in T-regulatory cell numbers or FOXP3 activity in affected children during this early period. The observation that higher allergen-induced FOXP3 was associated with higher IL-13 (rather than lower) Th2 responses to both HDM ($\tau = 0.23$; $p = 0.008$) and OVA ($\tau = 0.18$; $p = 0.023$) in children with atopic dermatitis also suggests a compensatory rather than causal relationship. However, we acknowledge that all of the infants in our study had mothers with allergy and it is possible that this conferred pre-existing differences in FOXP3 expression, as noted recently by Schaub *et al.* (24) at birth, which could not be detected in our more selected high-risk population. Although FOXP3 expression has been shown to correlate with suppressive function (24, 25), ideally more detailed functional studies are needed to confirm if the greater allergen-induced increases FOXP3 expression seen in children with atopic dermatitis reflect suppressive activity. It remains difficult to do this in small children, as blood sample volumes are limited.

While the role of regulatory T cells in allergy pathogenesis is unclear, there is some evidence for a compensatory role in tolerance induction. Increases in the numbers or function of putative CD4+CD25+ regulatory T cells have been associated with the development of grass pollen tolerance following immunotherapy (9) and oral tolerance in milk reactive children (with non-IgE mediated disease) following allergen exposure (27). There is also growing interest in other environmental factors, such as intestinal flora and other microbial factors, that can activate these regulatory pathways for potential therapeutic effect. Earlier animal studies suggest that gut flora are essential for normal tolerance (28), and more recent studies suggest that this may be at least in part because of induction of local regulatory T-cell populations (3) through Toll-like receptor-mediated activation (29). Because of speculation that more 'hygienic' environments may be leading to less microbial induced activation of the regulatory immune system, the effects of probiotics have been explored for allergy prevention. The first study to do this claimed a 50% reduction in atopic dermatitis by giving probiotics (*Lactobacillus rhamnosus* GG) to a population at high allergy risk during late gestation and the first 6 months of life (14). Using an *L. acidophilus* species (LAVRI, Probiomix; Sydney, Australia), we were unable to confirm this effect [as discussed

in more detail in (23)]. Here, we demonstrate that probiotics did not significantly alter the number of circulating CD4+CD25+ regulatory T cells or their capacity to up-regulate FOXP3 in response to allergen stimulation. The fact that dermatitis-associated allergen-induced FOXP3 was more enhanced in the probiotic group (Fig. 5) might lend some support to the popular theory that microbial products promote regulatory function (or further enhance the compensatory regulatory pathways that appear activated in atopic dermatitis). However, this effect was marginal at best (with no significant difference compared with children who developed atopic dermatitis in the placebo group) and did not appear to have any clinical relevance in this population.

In summary, this is the first study to explore the effects of probiotic supplementation on markers of regulatory immune function. There was no evidence that this intervention altered the number of putative CD4+CD25+CTLA4+ regulatory cells, or allergen-induced FOXP3 mRNA expression compared with high-risk children who did not receive the *L. acidophilus* supplement. This is also the first study to show that the development of atopic dermatitis in infancy is associated with increased allergen-induced FOXP3 mRNA expression. This may reflect secondary compensatory mechanisms and supports observations that CD4+CD25+ regulatory cells are increased in established atopic dermatitis in adults. Biological differences between probiotic strains are well recognized (30), and it is possible that other strains could produce different clinical and immunological effects in this context. For this reason, the findings of other researchers currently investigating the effects of other probiotics will be of great interest.

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