Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema–dermatitis syndrome

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Background: The immunomodulating mechanisms of *Lactobacillus* GG (LGG) and other probiotics are poorly understood.
Objective: We studied *in vivo* the immunologic effects of probiotics in infants with atopic eczema–dermatitis syndrome (AEDS) and cow's milk allergy (CMA).
Methods: Two hundred thirty infants with AEDS and suspected CMA received, concomitant with elimination diet, either LGG, a mixture of 4 probiotic strains (MIX), or placebo for 4 weeks. All available paired pretreatment and posttreatment plasma samples (n = 132) were analyzed for concentrations of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, soluble intercellular adhesion molecule 1, soluble E-selectin, TGF-β1, TGF-β2, and C-reactive protein.
Results: In infants with IgE-associated AEDS, treatment with LGG induced higher C-reactive protein levels than in the placebo group (geometric mean, 0.83 μg/mL [95% CI, 0.56-0.81] vs 0.42 μg/mL [95% CI, 0.27-0.65]; $P = .021$). Concomitantly, IL-6 levels increased after treatment with LGG ($P = .023$) but not with MIX or placebo. Soluble E-selectin levels were higher after probiotic than after placebo treatment in infants with IgE-mediated CMA (LGG geometric mean, 86.7 ng/mL [95% CI, 75.2-100]; MIX geometric mean, 91.6 ng/mL [95% CI, 74.8-111.9]; and placebo geometric mean, 64.9 ng/mL [95% CI, 53-79.3]; analysis of covariance, $P = .035$; LGG vs placebo, $P = .023$; MIX vs placebo, $P = .020$). Use of MIX induced an increase in plasma IL-10 levels ($P = .016$).
Conclusion: Probiotics induced systemically detectable low-grade inflammation, which might explain the clinical effects of probiotics in AEDS and CMA. (J Allergy Clin Immunol 2005;115:1254-9.)

Key words: *Allergy, atopic eczema–dermatitis syndrome, cytokines, human, inflammation, probiotics, tolerance*

A highly hygienic environment with lower microbial load and fewer feco-oral infections might provide less stimulation to the gut immune system, favoring allergy-prone immunity. Atopic children have been reported to harbor more coliforms and clostridia and fewer bifidobacteria and lactobacilli in their gut flora than nonatopic children.[1-3] Probiotic bacteria are suggested to prevent and treat allergies by counteracting these changes.[4,5] *Lactobacillus* GG (LGG) is the most studied probiotic bacteria. Its ability to survive passage through the gastrointestinal tract in newborns has been shown in fecal samples[6] and in colonic biopsy specimens showing LGG attaching to colonic epithelial cells.[7]

Studies in animal models suggest that intestinal colonization plays a role in the regulation of oral tolerance.[8,9] The effect of colonization is seen in the number of T cells in Peyer's patches, with T-cell numbers being limited in germ-free mice when compared with mice in a pathogen-free environment.[8,9] An increase in the number of T cells is seen when mice are colonized by gram-positive or gram-negative bacteria, such as bifidobacteria or *Escherichia coli*. Furthermore, the insufficient number or absence of T cells in Peyer's patches was related to the failure to induce oral tolerance in germ-free mice. These findings suggest that intestinal microflora is important for the development of the gut immune system and the induction of oral tolerance, which is aberrant in food allergies.

Probiotics have been shown to alleviate symptoms of atopic eczema–dermatitis syndrome (AEDS) and intestinal inflammation, but clinical studies indicate no direct evidence for the mechanisms involved.[4,10-12] We showed probiotic bacteria LGG to alleviate AEDS symptoms and to increase the IFN-γ response of peripheral lymphocytes in infants with IgE-associated AEDS.[13,14]

We have now studied the direct effect of probiotics on infants' intestinal immune systems by measuring changes in systemic levels of cytokines and inflammatory markers. In this double-blind placebo-controlled study, we gave

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Abbreviations used
AEDS: Atopic eczema–dermatitis syndrome
cfu: Colony-forming units
CMA: Cow's milk allergy
CM: Cow's milk
CRP: C-reactive protein
ICAM: Intercellular adhesion molecule 1
LGG: *Lactobacillus* GG
MIX: Mixture of 4 probiotic strains
SPT: Skin prick test

LGG, a mixture of 4 probiotics (MIX), or placebo to infants with AEDS suspected to have cow's milk allergy (CMA) and investigated the effects of the probiotic bacteria on the posttreatment plasma inflammation marker C-reactive protein (CRP), the soluble adhesion molecules E-selectin and intercellular adhesion molecule 1 (ICAM-1), and plasma levels of the cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, TGF-β1, and TGF-β2.

METHODS

Subjects

A group of 230 infants suspected of having CMA were recruited between November 1999 and March 2002 to study the effects of probiotics on AEDS symptoms at the Skin and Allergy Hospital of Helsinki University Central Hospital.[13] We acquired paired plasma samples of 132 infants (age, 1.4-11.5 months; mean age, 6.5 months; 65% male subjects) for analysis. At the first visit, cow's milk (CM) elimination was started for infants and their breast-feeding mothers. All infants received extensively hydrolyzed whey formula (Peptidi-Tutteli; Valio Ltd, Helsinki, Finland). At the same visit, infants were randomly allocated according to computer-generated block randomization of 6 infants to receive one of 3 products in a double-blind manner mixed with food twice a day for 4 weeks: (1) the LGG group (n = 52) received capsules containing *Lactobacillus rhamnosus* GG (ATCC 53103; 5×10^9 colony-forming units [cfu]), (2) the MIX group (n = 42) received a mixture of probiotics (LGG, 5×10^9 cfu; *Lactobacillus rhamnosus* LC705, 5×10^9 cfu; *Bifidobacterium breve* Bbi99, 2×10^8 cfu; and *Propionibacterium freudenreichii ssp. shermanii* JS, 2×10^9 cfu), and (3) the placebo group (n = 38) received an inert matrix material, microcrystalline cellulose. These products (supplied by Valio Ltd) looked, smelled, and tasted identical. Parents were urged not to feed any other probiotic preparations to the infants during the study.

Clinical improvement was evaluated by using the Severity Scoring of Atopic Dermatitis at the second visit, after the 4-week treatment, and at the third visit (4 weeks after treatment ended).[13] After a successful elimination lasting 8 weeks, 4 weeks after the end of probiotic treatment, all patients had good remission of AEDS symptoms, and we performed a double-blind placebo-controlled CM challenge, as described earlier.[13] CMA was confirmed in 70 infants.

At the first visit, skin prick tests (SPTs) with commercial allergen extracts of egg white (Alyostal prick test; Stallergenes SA, Antony, France), cat, dog, and birch (Soluprick; ALK-Abelló, Hørsholm, Denmark) were performed according to the standard technique. Duplicate tests were performed with fat-free CM, with commercially available adapted CM and hypoallergenic infant formulas, and with cereal grains and purified gliadin. A mean wheal diameter of at least 3 mm larger than that elicited by the negative control was considered positive. Concentrations of serum wheat- and CM-specific IgE were measured by using the Pharmacia CAP system RAST FEIA (Pharmacia Ltd, Uppsala, Sweden).[13] Infants with a positive SPT response or an antigen-specific IgE concentration of 0.7 kU/L or greater to any antigen tested were considered to have IgE-associated AEDS (n = 82). Infants with positive CM challenge and a positive SPT response to CM or CM-specific IgE concentration of 0.7 kU/L or greater were considered to have IgE-mediated CMA (n = 32). One parent of each infant provided written informed consent. The local ethics committee approved the study protocol.

ELISA

Pretreatment blood samples were collected at the first visit, and posttreatment samples were collected after the 4-week treatment at the second visit. CRP concentrations were determined by using the immunoturbidimetric ultrasensitive CRP assay (Orion Diagnostica, Espoo, Finland) with a detection limit of 0.29 μg/mL. The soluble (s)ICAM-1 and sE-selectin protein levels were determined with commercial sandwich ELISA kits (Parameter; R&D Systems, Abingdon, United Kingdom). Detection limits for the assays were 37.2 ng/mL and 10.6 ng/mL. TGF-β1 and TGF-β2 in plasma samples were activated, and their levels were measured according to the manufacturer's instructions with human TGF-β1 and TGF-β2 ELISA kits (Quantikine, R&D Systems), with detection limits of 84 pg/mL and 54.6 pg/mL, respectively. All assays were performed in duplicate, with intensity of color measured with multiscan MS version 8.0 (Labsystems Oy, Helsinki, Finland).

Cytometric bead array

Plasma samples of peripheral blood were obtained by means of centrifugation of the heparinized blood. Paired plasma samples of 121 infants were available for analysis. IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ capture beads were incubated with plasma samples or standards and mixed with the phycoerythrin-conjugated detection antibodies, according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, Calif). Protein concentrations were measured on a FACScan flow cytometer (CellQuest software, BD), with a detection limit 2.6 pg/mL for IL-2 and IL-4, 3.0 pg/mL for IL-6, 2.8 pg/mL for IL-10 and TNF-α, and 20 pg/mL for IFN-γ. Results were analyzed on BD CBA analysis software MAC OS version 9.

Statistical analyses

All plasma sE-selectin and sICAM-1 values were greater than the detection limits. In terms of CRP, 19% of samples were less than the detection limit. Because distributions of CRP, sE-selectin, and sICAM-1 were skewed, these comparisons were made after logarithmic transformation. Analysis of covariance was used for multiple comparisons in which treatment groups were compared with each other with respect to posttreatment values. Because differences existed in baseline values (pretreatment values), these were taken as covariates in the analysis. An antilogarithm was taken from baseline-adjusted geometric means to receive the initial unit of measurement. The Fisher least-significant-difference test served for comparisons between the probiotic groups and the placebo group. Data are presented as geometric means and 95% CIs.

Because all plasma IL-2 and TNF-α values were less than detection limits, they were not further analyzed. In terms of IL-6, 79% of pretreatment samples and 73% of posttreatment samples were less than the detection limit. In terms of IL-10 and IL-4, 65% of pretreatment samples and 58% of posttreatment samples were less than the detection limit; 74% of IFN-γ samples were less than the detection limit. None of the TGF-β1 samples, 36% of pretreatment

TABLE I. Comparison of serum posttreatment values of CRP and sE-selectin adjusted by pretreatment values in all infants with AEDS, IgE-associated AEDS, and IgE-mediated CMA treated with LGG, MIX, or placebo for 4 weeks

	n	CRP (μg/mL)	n	sE-selectin (ng/mL)
AEDS				
LGG	52	0.63 (0.48-0.84)	52	86.3 (79.8-93.1)
MIX	42	0.67 (0.49-0.91)	41	88.9 (81.5-96.8)
Placebo	38	0.43 (0.31-0.60)	36	86.5 (78.7-94.8)
P value, ANCOVA		.130		.865
IgE-associated AEDS				
LGG	31	0.83 (0.56-0.81)*	31	92.9 (84.3-102.3)
MIX	21	0.79 (0.49-1.27)	20	101.2 (89.7-114)
Placebo	26	0.42 (0.27-0.65)*	24	88.9 (79.6-99.3)
P value, ANCOVA		.047		.288
IgE-mediated CMA				
LGG	16	0.88 (0.46-1.68)	16	86.7 (75.2-100)†
MIX	8	1.32 (0.52-3.36)	8	91.6 (74.8-111.9)†
Placebo	8	0.46 (0.18-0.85)	8	64.9 (53-79.3)†
P value, ANCOVA		.293		.035

Values are expressed as geometric means (95% CIs).
ANCOVA, Analysis of covariance.
*Fisher least-significant-difference test: LGG versus placebo, $P = .021$.
†Fisher least-significant-difference test: LGG versus placebo, $P = .023$; MIX versus placebo, $P = .020$

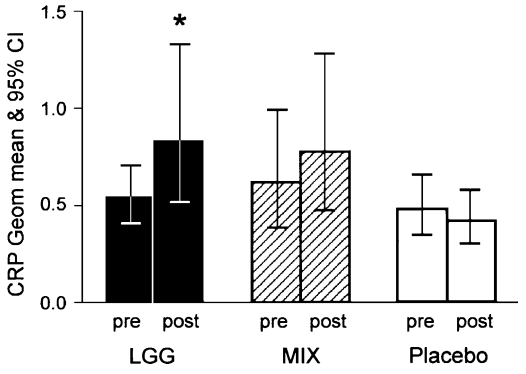

FIG 1. Unadjusted pretreatment and posttreatment serum CRP geometric means and 95% CIs in 3 treatment groups of infants with IgE-associated AEDS. Posttreatment serum CRP adjusted by pretreatment value. *Fisher least significant difference test: LGG versus placebo, $P = .021$.

TGF-β2 samples, and 37% of posttreatment TGF-β2 samples were less than the detection limit. Nonparametric tests were used for cytokine analysis because of the great number of values less than the detection limit and the nonnormal distribution of data. The Wilcoxon signed-rank test was used to compare follow-up samples, and the Kruskall-Wallis and Mann-Whitney *U* tests were used for comparisons of pretreatment and posttreatment values. Nonparametric tests were 2-tailed. Data are presented as means, ranges, and upper quartiles. All statistical analyses were performed with SPSS for Windows. Statistical significance was defined as a *P* value of .05 or less.

RESULTS

After treatment of infants with IgE-associated AEDS, pretreatment-adjusted serum CRP levels were greater in the LGG group than in the placebo group (Table I and Fig 1). However, after treatment, in the whole study population and in infants with IgE-mediated CMA, pretreatment-adjusted serum CRP levels did not differ significantly after probiotic or placebo treatment (Table I).

Among infants with IgE-mediated CMA, adjusted posttreatment serum sE-selectin was higher in the LGG and MIX groups than in the placebo group (Table I). For the whole study population and for the IgE-associated AEDS subgroup, adjusted sE-selectin levels showed no differences between treatment groups (Table I). Adjusted serum sICAM-1 levels showed no significant differences between treatment groups in the whole study population or in any subgroup analyses (data not shown).

Among infants with IgE-associated AEDS, secretion of IL-6 increased during the treatment period in the LGG group ($P = .023$, Wilcoxon signed-rank test) but not in the MIX or placebo groups (Table II and Fig 2). Thus after analysis with the Mann-Whitney *U* test, the posttreatment IL-6 level was higher in the LGG group than in the placebo group ($P = .036$, Table II).

Among all infants with AEDS, the level of IL-10 in the MIX group increased significantly ($P = .016$), whereas changes in the LGG or placebo groups were not significant. However, posttreatment IL-10 levels were higher in the LGG ($P = .046$) and MIX ($P = .039$) groups than in the placebo group (Table II). In infants with IgE-associated AEDS, no significant changes occurred in IL-10 levels in follow-up samples (Table II).

Different treatments had no effects on IL-4, TGF-β1, and TGF-β2 responses in follow-up samples in the whole study population or in infants with IgE-associated AEDS (data not shown). IFN-γ levels were low in all subgroups, with no changes during follow-up in any of the treatment groups (data not shown).

TABLE II. Comparison of serum pretreatment and posttreatment values of IL-6 and IL-10 in all infants with AEDS and with IgE-associated AEDS treated with LGG, MIX, or placebo for 4 weeks

		IL-6 (pg/mL)			IL-10 (pg/mL)		
	n	Before	After	*P* value*	Before	After	*P* value*
AEDS							
LGG	47	1.1 (0.0, 0-15.4)	2.4 (3.8, 0-20.3)	.070	1.6 (3.0, 0-7.9)	2.4 (4.4, 0-11.3)‡	.158
MIX	38	2.3 (3.9, 0-22.6)	2.6 (3.6, 0-35.8)	.897	1.3 (3.4, 0-4.9)	2.5 (3.8, 0-16.8)‡	.016
Placebo	36	0.7 (0.0, 0-7.4)	2.5 (0.0, 0-61.2)	.610	1.2 (3.0, 0-6.7)	1.5 (2.5, 0-19.0)‡	.877
IgE-associated AEDS							
LGG	26	0.9 (0.0, 0-8.7)	3.4 (6.9, 0-20.3)†	.023	1.5 (3.0, 0-5.0)	2.3 (4.4, 0-11.3)	.338
MIX	19	2.1 (3.9, 0-18.5)	2.0 (4.1, 0-17.0)	.866	1.2 (3.3, 0-4.9)	2.1 (3.9, 0-5.5)	.075
Placebo	24	1.0 (0.0, 0-7.4)	0.9 (0.0, 0-9.5)†	.499	1.2 (3.2, 0-6.7)	2.0 (3.4, 0-19.0)	.593

Values are expressed as means (upper quartiles, ranges).
*Wilcoxon signed-rank test.
†Mann-Whitney *U* test: posttreatment LGG versus placebo, $P = .036$.
‡Mann-Whitney *U* test: posttreatment LGG versus placebo, $P = .046$; MIX versus placebo, $P = .039$.

DISCUSSION

The data presented document a significant increase in CRP levels in LGG-treated IgE-associated infants with AEDS compared with those seen in the placebo group. CRP is a marker of inflammation, with low levels of CRP reflecting subclinical inflammation, such as in atherosclerosis.[15] IL-6 induces gene activation of CRP in hepatocytes and stimulates CRP secretion.[16] Here we demonstrated concomitantly increased IL-6 and CRP levels in LGG-treated infants with IgE-associated AEDS. In acute allergic reactions, CRP and IL-6 levels correlated with each other,[17,18] and IL-6 correlated with the extent of erythema.[17] We found, however, that symptoms of AEDS alleviated more in the LGG group than in the placebo group in infants with IgE-associated AEDS,[13] suggesting a possible mechanism of treatment (ie, intestinal inflammation affecting the healing of eczema). The biologic functions of CRP are diverse. It activates complement, which mediates phagocytosis.[19] CRP does not promote formation of C5 convertase, and therefore complement activation initiated by CRP does not mediate proinflammatory reactions and membrane damage.[19] Human CRP inhibits production of different inflammatory cytokines, such as TNF-α and IFN-γ, and of chemokines, but CRP enhances IL-10 production on cultured cells.[20]

During treatment with LGG, plasma IL-6 concentrations increased, and the posttreatment level was higher in the LGG group than in the placebo group among infants with IgE-associated AEDS, suggesting a direct effect of LGG on intestinal epithelial cells or monocytes-macrophages. Interestingly, even though both probiotic preparations contained the same amount of LGG, the MIX did not show effects on IL-6. An explanation for this might be competition between probiotic strains in the gut flora just as lactobacilli have been demonstrated to replace bacterial pathogens in epithelial cell line surfaces.[21] Different strains might compete for space or receptors or have negative additive effects on each other. Direct stimulation of PBMCs with LGG has resulted in IL-6 production, likely by monocytes.[22] In the gut mucosa IL-6 regulates

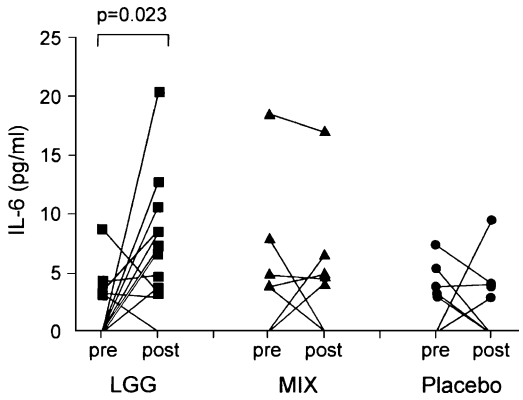

FIG 2. Pretreatment and posttreatment serum IL-6 levels in 3 treatment groups of infants with IgE-associated AEDS. *P* values are determined by using the Wilcoxon signed-rank test.

mucosal total protein synthesis, as well as IgA production by Peyer's patch B cells, and induces an increase in the number of IgA-producing cells in B cells of the human appendix.[23,24] This might explain our earlier findings that LGG treatment resulted in an increase in fecal IgA levels in infants with IgE-associated CMA.[25] IL-6 also acts on endothelial cells to promote upregulation of sICAM-1 and sE-selectin.[26] IL-6 might also have anti-inflammatory effects in healthy human subjects by inducing release of IL-10, IL-1 receptor antagonist, and plasma cortisol.[27] IL-6 signaling through the soluble IL-6 receptor has also been reported to regulate the epidermal barrier repair after skin injury, which also might be of importance in the healing of AEDS.[28]

Here sE-selectin levels were higher after treatment with probiotics than with placebo in infants with IgE-mediated CMA. Soluble E-selectin can be detected in plasma of both healthy and atopic children and has a significant correlation with the severity of AEDS.[29] Inflammatory cytokines, such as TNF-α, induce upregulation of adhesion molecules, including sE-selectin, on endothelial cells located in the target organs of the inflammatory process.

After shedding from endothelial cells, it has been suggested that these soluble adhesion molecules might retain their capacity to bind receptors, thereby potentially limiting the inflammatory process.[30] We also found that in the same patients probiotics caused an enhanced secretion of IFN-γ by peripheral lymphocytes *in vitro*.[14]

In the whole study population, *in vivo* IL-10 levels increased during treatment with MIX. After treatment, IL-10 levels were higher in both probiotic groups than in the placebo group. In children with AEDS, LGG treatment for 4 weeks increased the ability of PBMCs to secrete IL-10.[11] In children with AEDS treated with a combination of 2 lactobacillus strains, no effect was evident on production of IL-10 by *in vitro*–stimulated PBMCs.[12] Moreover, *in vitro* stimulation of PBMCs with LGG only weakly induced IL-10.[22] It should be emphasized that our findings reflect *in vivo* changes in IL-10, and other researchers have studied *in vitro*–induced production of IL-10. We did not see a beneficial clinical effect of MIX in the alleviation of AEDS or CMA,[13] which induced, however, an increased IL-10 production in infants. The negligible clinical effect of MIX, which contains LGG but also other probiotics, might actually be explained by the activation of IL-10, which might contribute to the development of AEDS. Human AEDS lesions show overexpression of IL-10 mRNA, and monocytes from patients with AEDS spontaneously produce higher IL-10 levels than those from control subjects.[31] In a murine model of allergic dermatitis, IL-10 promotes T_H2 responses, suppresses T_H1 responses, and enhances eosinophil accumulation in the skin.[32] On the other hand, increased IL-10 levels are associated with induction of oral tolerance in animal models.[33,34] In human subjects IL-10 suppresses production of T_H1 and T_H2 cytokines during specific immunotherapy and in normal immunity to mucosal respiratory allergens.[35] IL-10 also mediates the control of intestinal inflammation in mice by regulatory T cells.[36] The induction of T-regulatory cells was also recently shown to be associated with recovery from CMA in children.[37] IL-10 as an immunomodulating cytokine seems to be controversial and might also be disease specific.

We showed no significant changes in IL-4, IFN-γ, TGF-β1, and TGF-β2 levels in plasma during treatment. Even though these cytokines have a balanced and pivotal role for immune responses and allergic reactions, our failure to demonstrate changes by no means excludes the possibility that they are affected by probiotics. By nature, cytokines and chemokines are local mediators, and only major changes might spill over into the circulation and be apparent in plasma. Furthermore, the half-life of these substances varies and might be quite short.

This is, to the best of our knowledge, the first study to show *in vivo* effects of probiotic bacteria on inflammatory markers in allergic patients. We showed, somewhat unexpectedly, an upregulation of inflammatory markers related to the activation of the innate immune system, which we propose to be part of the effect of probiotics in AEDS. Treatment with LGG increased levels of IL-6 and CRP, but these changes were restricted to the group of children with IgE-associated AEDS, suggesting that the immunologic effect of LGG is modified by host-released factors.

Our studies in the infants are limited to peripheral blood samples, and it has not been possible for us to monitor the changes in the intestinal target cells. The activation of IL-6 suggests that the target cells of probiotic action are cells of the innate immune system (ie, monocytes–macrophages–dendritic cells); Peyer's patch–derived dendritic cells especially are potent producers of IL-6.[38] IL-6 is also produced by epithelial cells, but *in vitro* studies show that lactobacilli, including LGG, are not able to stimulate IL-6 production from an epithelial cell line,[39] supporting the view that monocyte-lineage cells are responsible for IL-6 activation induced by administration of LGG in the infants. Our results indirectly suggest that the dysfunction of the intestinal antigen-presenting cells plays a fundamental role in the development of IgE-associated AEDS, especially because the clinical and immunologic effects of LGG were restricted to these patients.

Interestingly, inflammatory mechanisms have been linked to intestinal immune response to oral antigen stimulation.[40] The activation of COX-2–dependent arachidonic acid metabolites, such as prostaglandin E_2, have been shown to be responsible for the downregulation of inflammatory intestinal immune response to dietary antigen in a T-cell receptor transgenic mouse model.[40] In this model oral antigen stimulation of antigen-specific T cells present in the lamina propria leads to intestinal inflammation, when antigen is given together with COX-2 inhibitors, known as anti-inflammatory drugs. COX-2 expression is high in macrophages and is inducible by bacterial products, such as LPS. It is tempting to speculate that the activation of macrophages with probiotics[22,41] could induce a COX-2–dependent downregulation of response to dietary antigens. Our results support the view that stimulation of the intestinal immune response and induction of low-grade inflammation by probiotics might paradoxically alleviate allergic symptoms.[13,25]

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