

Probiotic *Escherichia coli* Nissle 1917 Suppresses Allergen-Induced Th2 Responses in the Airways

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Key Words

Asthma • Probiotics • *Escherichia coli* Nissle 1917 • T helper-1/2 • Lipopolysaccharide • Toll-like receptor-4

Abstract

Background: Recent clinical trials, epidemiological studies and animal experiments have suggested that probiotics may help suppress the development of allergic responses. **Objective:** To investigate whether the application of the probiotic *Escherichia coli* strain Nissle 1917 (EcN) protects mice from developing ovalbumin (OVA)-specific T helper-2 responses in the airways. **Methods:** OVA-specific Th2 responses were induced by 2 intraperitoneal (i.p.) injections with OVA/alum followed by 1 intranasal (i.n.) challenge with OVA. EcN was given orally during the entire sensitization and challenge period, together with OVA/alum during the i.p. sensitizations, or i.n. before or during the airway challenge with OVA. **Results:** We found that when the bacteria were given together with OVA/alum airway eosinophilia, airway hyper-reactivity, goblet cell metaplasia and IL-5 levels in the bronchoalveolar lavage and mediastinal lymph node cell cultures were reduced. This effect was associated with increased numbers of

IFN- γ producing T helper-1 cells and IFN- γ levels in the airways and strongly increased OVA-specific IgG_{2a} titers in the serum. The suppressive effect on airway eosinophilia was dependent on IFN- γ but not TLR-4. Applying EcN i.n. or orally did not reduce the development of allergen-specific Th2 responses. **Conclusions:** Our results suggest that EcN can inhibit the development of allergic responses when the bacteria are present at the site of Th2 cell priming and that this immunomodulatory effect is due to a shift from Th2 to Th1 response. The data support the hypothesis that probiotics may help reduce allergic responses and that EcN may also be used as adjuvant therapy to induce allergen-specific Th1 responses.

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Introduction

Allergic immune responses to common environmental antigens lead to clinical disorders such as allergic asthma, hay fever, eczema and allergic rhinitis. It is clear that the development of allergies involves both genetic and environmental factors. Naive T cells are activated by an-

tigen – presenting cells and IL-4 through IL-4 receptor/Stat6 – mediated signals and the activation of specific downstream transcription factors. These events lead to the development of allergen-specific CD4⁺ T helper-2 cells (Th2) in the lymph nodes or spleen. Once generated, effector Th2 cells migrate to the site of allergen exposure, under the guidance of specific chemokines and adhesion molecules, where they produce IL-4, IL-5, IL-9 and IL-13. The secretion of these cytokines induces the production of allergen-specific IgE by B cells, the development of eosinophilia, smooth-muscle contraction and mucus secretion. IgE cross-linking induces the degranulation of eosinophils and mast cells leading to the release of preformed or de novo synthesized inflammatory mediators, which are also critical factors inducing the development of allergic disorders [1].

Currently, only very few effective therapies are available. The most effective ones are steroids, antihistamines, anti-IgE antibodies, leukotriene receptor antagonists and long-acting β agonists together with inhaled steroids (for the treatment of asthma). However, these treatment regimens do not show any disease-modifying effects. The only well established disease-modifying treatment available is the allergen-specific immunotherapy [1]. However, this treatment does not improve allergic symptoms in all patients, in particular asthmatics, and may cause severe side effects. Although somewhat controversial [2, 3], recent clinical studies indicate that a further therapeutic and preventive treatment for allergic disorders may be the use of probiotics in particular *Lactobacillus* species [4–14]. How probiotics colonizing the gut protect from the development of allergic disorders in humans is not clear. However, it has been shown that protection is in some cases associated with increased proinflammatory Th1 responses and inflammation [12, 13, 15] and has been speculated to also be mediated by induction of regulatory T (Tr) cells in the gut [9, 10].

Experiments in animals also support the view that probiotics help suppress the development of allergic responses by enhancing Th1 and/or Tr responses [16–21]. Most studies in animals and humans were performed with probiotic *Lactobacillus* and *Bifidobacterium* species. Thus far little is known about whether other probiotic bacteria can also suppress allergic disorders in humans and animals. For this reason we investigated whether the application of *Escherichia coli* strain Nissle 1917 (EcN), a serum-sensitive probiotic strain (currently used to treat diarrhea and colitis ulcerosa in humans) can protect mice from developing OVA-specific Th2 responses. EcN was given orally during the entire sensitization and challenge

period, together with OVA/alum during the 2 intraperitoneal (i.p.) sensitizations, or intranasally (i.n.) before or during the airway challenge with OVA. We found that only when EcN was given together with OVA/alum i.p., were airway eosinophilia, airway hyper-reactivity (AHR) and goblet cell metaplasia reduced. Applying EcN i.n. or orally had no effect on the development of allergen-specific Th2 responses. Our results support the view that probiotics may generally help to reduce the development of allergen-specific Th2 responses, but their effects may depend on their presence at the site of Th2 cell priming.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). IFN- γ -deficient mice (on a C57BL/6 genetic background) were generously provided by O. Liesenfeld (Berlin, Germany). TLR-4-deficient mice (on a C3H/HeJ genetic background) were also purchased from Charles River. Animals were maintained under conventional conditions in an isolation facility. At the onset of the experiments, animals were between 6 and 8 weeks of age. All experiments were performed according to the guidelines of the local and government authorities for the care and use of experimental animals.

Induction of OVA-Specific Th2 Responses

In order to induce OVA-specific Th2 responses, mice were treated twice i.p. with a mixture of 2 μ g OVA (Sigma Chemical Co., St. Louis, Mo., USA) in 200 μ l alum adjuvant (Serva, Heidelberg, Germany) on the days 0 and 14. Subsequently, mice were anesthetized by an i.p. injection of ketamine/xylazine (Sigma) and challenged i.n. with 50 μ l PBS containing 100 μ g OVA on day 24. Analyses were performed 6 days after the i.n. challenge. An identical protocol has been used previously to address similar questions [22, 23].

Bronchoalveolar Lavage

Six days after the i.n. OVA challenge, mice were sacrificed, blood samples collected, the trachea cannulated and a bronchoalveolar lavage (BAL) performed by flushing the lungs and airways 4 times with 1 ml PBS containing 10% FCS (PAA Laboratories, Linz, Austria). BAL cells were counted and spun onto glass slides using a cytospin centrifuge (Shandon, Pittsburgh, Pa., USA). The cells were then fixed and stained with Diff Quick (Dade Behring, Marburg, Germany) according to the manufacturer's instructions. The total numbers of eosinophils, neutrophils, lymphocytes and macrophages were determined by light microscopy.

Treatment of Mice with EcN and Lipopolysaccharide

EcN was grown in LB medium to an OD_{600nm} of 1, corresponding to 6.538×10^8 bacteria per ml [24]. The bacteria were spun down, washed 3 times and resuspended in sterile PBS. For the i.n. and i.p. experiments 2×10^6 EcN [in some experiments also heat-killed (hk) EcN] or 30 μ g lipopolysaccharide (LPS; *E. coli* 026:B6, Sigma) per mouse were given either alone (4 weeks and 1

week prior to OVA challenge), together with OVA (during airway challenge) or together with OVA/alum during sensitization at the indicated time points. To kill EcN, the bacteria were incubated for 1 h at 75°C. Plating of these bacteria on agar plates showed that all bacteria were killed. For the feeding experiments bacteria containing a plasmid with a green fluorescent protein construct and kanamycin resistance were used (EcN^{GFP}) [25] to control for the viability of the bacteria in the gut. EcN^{GFP} was resuspended in a salt solution containing 2.5 g/l NaCl, 2.5 g/l KCl, 0.2 g/l MgSO₄, 0.2 g/l CaCl₂ and 6.4 mg/l NaOH, with a pH of 6.5. Mice were fed daily with 1×10^8 live EcN bacteria, starting 5 days prior to the first i.p. sensitization with OVA (fig. 1a). To detect EcN^{GFP} in the feces, 1 mg of fresh feces from the fed mice were collected, diluted in PBS and serial dilutions were plated on LB agar plates containing 30 µg/ml kanamycin. The amount of EcN^{GFP}-positive cells present in the feces were detected using the fluorescence imager Typhoon 8600 and Image Quant software (Molecular Dynamics, Piscataway, N.J., USA).

Culture Condition of Cells

Single-cell suspensions from the mediastinal lymph nodes (MLN) of the different groups of mice were prepared. The cell preparations were resuspended in click RPMI 1640 medium (Biochrom, Cambridge, UK) complemented with 10% FCS (PAA), 2 mM L-glutamine, 10 mM HEPES (Biochrom), 0.35% 2-mercaptoethanol, 50 mg/l gentamycin and 100 U/ml penicillin-G (Sigma), added to cell culture plates (2×10^6 cells/ml) and left in medium alone, stimulated with 40 µg/ml OVA or with a plate-bound monoclonal antibody to CD3 (145-2C11, BD Pharmingen, 25 µg/ml) together with 200 U/ml recombinant human IL-2 (Novartis, Basel, Switzerland) at 37°C in a humidified atmosphere containing 5% CO₂. After 48 h, the culture supernatants were harvested and tested for the presence of cytokines by ELISA.

ELISA for the Detection of Immunoglobulins and Cytokines

For the detection of OVA-specific Ig levels in the serum or cytokines in the BAL fluid and cell culture supernatants, sandwich ELISAs were performed as previously described [22, 23].

FACS Analysis

CD4⁺ T cells from the BAL producing IL-4, IL-5 or IFN-γ or bone marrow-derived dendritic cells (BM-DCs) expressing MHC class II, B7.1, B7.2 or CD40 were detected by using 2- and 3-color FACS analysis as described previously [22, 23].

Histological Analysis

Lungs were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections (2–3 µm) were cut and stained using standard histological protocols with hematoxylin and eosin or periodic acid-Schiff reagent (PAS). The stained sections were blinded and then visualized by light microscopy and scored from 0 to 3 for mucus production.

Airway Hyper-Reactivity Measurements

AHR was assessed by methacholine-induced airflow obstruction, using a whole-body plethysmograph (model PLT UNR MS, EMKA Technologies, Paris) as previously described [22, 23]. For the measurement of the AHR, the murine asthma model was slightly modified, as described previously [22, 23]. Instead of only 1 OVA i.n. challenge, OVA was given 6 times on days 24–26 and

31–33 and AHR was measured at day 34. This modification is necessary since the mice only challenged with OVA once did not show any AHR at this time point (data not shown).

Stimulation of BM-DCs

BM-DCs were generated from bone marrow of C57BL/6 wild-type mice in RPMI 1640 (Gibco BRL) complemented with 10% FCS (PAA), 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Sigma) and 200 U/ml GM-CSF (granulocyte macrophage colony stimulating factor; PeproTech Inc., London, UK) as previously described [23]. After 10 days, nonadherent cells were harvested, counted, resuspended at 2×10^6 cells/ml and stimulated in 24-well plates (NUNC, Denmark) for 24 h with different amounts of live or hk-EcN or 1 µg/ml LPS (*E. coli* 026:B6, Sigma). BM-DCs resuspended only with supplemented RPMI medium were used as controls. The culture supernatants were harvested and tested for the presence of cytokines by ELISA. In parallel, the expression levels of MHC class II, B7.1, B7.2 and CD40 on the surface of the BM-DCs were determined by FACS analysis.

Statistical Analysis

Results were evaluated using the unpaired Student's t test or 1-way ANOVA, where applicable.

Results

The Oral Application of EcN Does Not Inhibit the Development of OVA-Specific Th2 Responses

We investigated whether the oral application of EcN can protect mice from developing OVA-specific Th2 responses. For this purpose, mice were fed daily with 1×10^8 live EcN bacteria, starting 5 days prior to the first i.p. sensitization with OVA (fig. 1a) and then analyzed if Th2 responses were reduced in these animals in comparison to the control mice. For these experiments we used an EcN strain that contained a plasmid with a green fluorescent protein construct (EcN^{GFP}), in order to check that when the EcN bacteria reached the gut they were alive and present in sufficient numbers to possibly have an impact on the immune response initiated against OVA. This mutant strain has been previously shown to react in an immunologically similar manner to EcN lacking the green fluorescent protein [25]. Figure 1h shows that significant amounts of EcN^{GFP} could be detected in the feces of mice fed with these bacteria ($1.66 \times 10^6 \pm 0.35$ bacteria/mg feces during the entire feeding period). However, lung eosinophilia, Th1 and Th2 cytokine levels in the BAL or supernatants of MLN cell cultures and OVA-specific antibody levels in the serum were similar in the EcN-treated mice compared with OVA controls (fig. 1b–e). Furthermore, the percentage and the total numbers (data not shown) of Th1 and Th2 cells detected in the

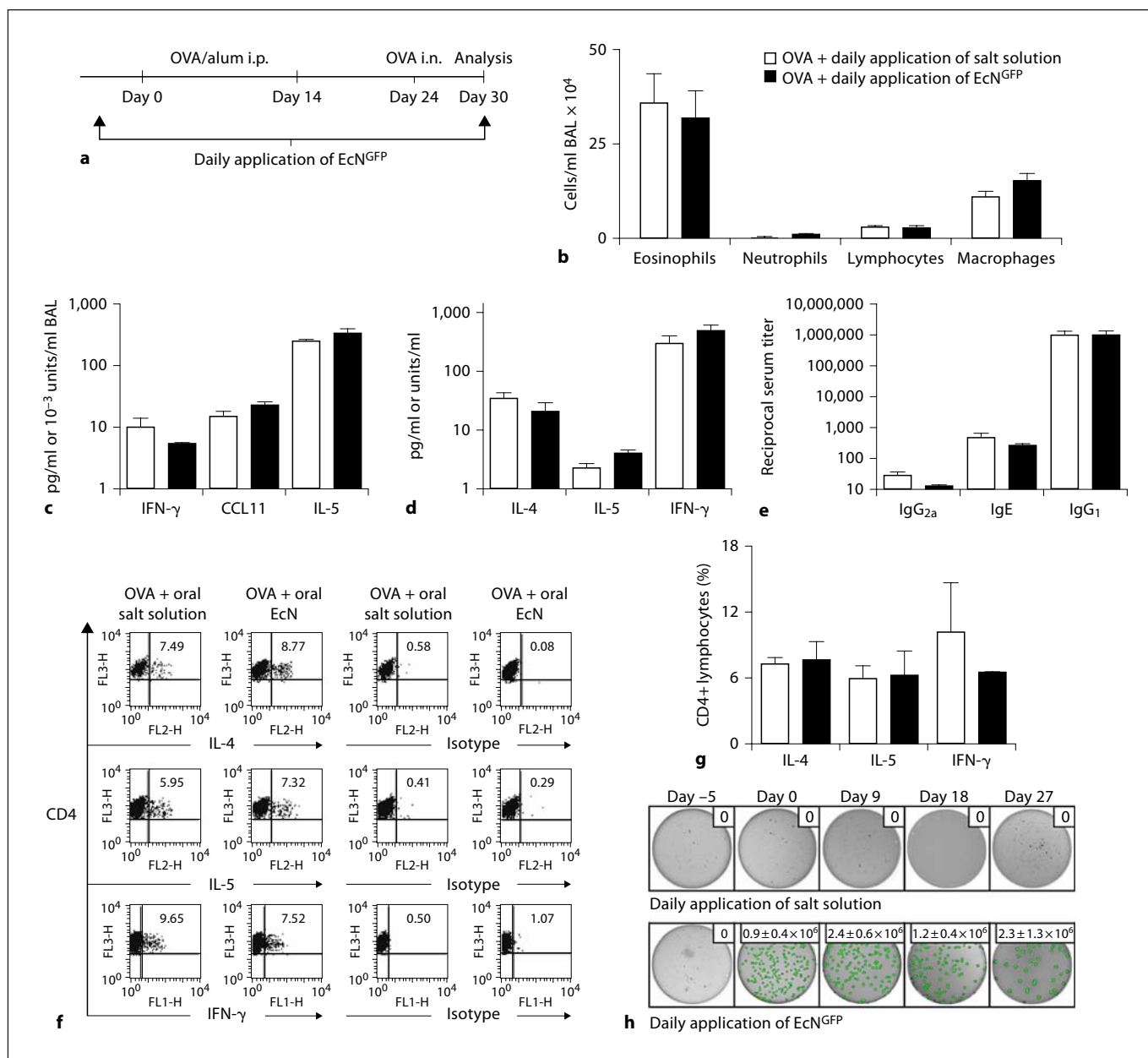


Fig. 1. The development of allergen-specific Th2 responses is not inhibited by the oral application of EcN. **a** Mice were i.p. treated with OVA/alum on days 0 and 14 and then challenged with OVA on day 24. Analysis was performed on day 30. In addition, mice were treated daily with 1×10^8 live EcN^{GFP} or a salt solution, administered orally, starting 5 days before the first immunization and continuing until the end of the experiment. **b** Amount of different cell types detected in the BAL fluid. **c** IFN-γ, CCL11 and IL-5 levels in the BAL fluid. **d** IL-4, IL-5 and IFN-γ levels detected in the supernatants of MLN cell cultures stimulated with OVA

for 48 h. **e** Serum levels of OVA-specific antibodies. **f** Representative examples of intracellular FACS staining for IL-4, IL-5 and IFN-γ, gated on CD4+ T cells, obtained from the BAL fluid of the different groups of mice. **g** Percentage of CD4+ T cells, producing IL-4, IL-5 or IFN-γ. **h** The amount of EcN^{GFP} bacteria detected in 1 mg of feces from the different groups of mice. The mean value ± SEM (from 3 independent experiments; 12 mice/group) is indicated in the upper right corner and representative agar plates are shown.

Table 1. Airway eosinophilia is reduced in TLR-4- but not IFN- γ -deficient mice immunized with OVA and treated with EcN

	OVA	OVA/alum/ EcN i.p.
C57BL/6 mice	817.6 $\pm$ 161.0	121.9 $\pm$ 28.6**
IFN- γ ^{-/-} mice	215.2 $\pm$ 109.7	110.0 $\pm$ 46.8
TLR-4 ^{-/-} mice	547.3 $\pm$ 108.7	114.2 $\pm$ 18.4**

Data are the means $\pm$ SEM of total eosinophil numbers ($\times 10^3$) in the BAL fluids of the different groups of mice (n = 6/group). C57BL/6 control, IFN- γ - and TLR-4-deficient mice were treated as described in figure 2a. ** p < 0.01 vs. OVA-only-treated mice.

BAL fluid of the different groups of mice were also not changed (fig. 1f, g). Similar results were obtained when lymphocytes from the mediastinal lymph node were analyzed (data not shown). The size of the mesenteric lymph nodes was not increased in the mice treated with EcN (data not shown). Taken together, these results clearly show that the presence of EcN in the gut of mice does not reduce the development of OVA-specific Th2 responses initiated by an i.p. immunization followed by an i.n. challenge with OVA.

The Application of EcN Together with OVA/Alum i.p. Strongly Inhibited the Generation of OVA-Induced Th2 Responses

The results above suggest that EcN may not have sufficient immune modulatory capacities when given orally to have an impact on the development of Th2 responses initiated at a different site. For this reason, we analyzed whether EcN could reduce the initiation of Th2 responses when applied together with the allergen (fig. 2a). We found that in the lungs of mice treated with live EcN, the numbers of eosinophils were more than 6-fold lower than in OVA-only-treated mice (fig. 2b). Mice treated with PBS/alum or EcN/PBS/alum and then challenged with OVA or PBS developed no airway eosinophilia and showed no significant increase in total cell numbers in comparison with the OVA/alum-sensitized and OVA-challenged mice (data not shown). The reduction of airway eosinophilia correlated with goblet cell hyperplasia and cellular infiltration in the airways (fig. 3) and decreased levels of CCL11 present in the BAL fluid (fig. 2c). While the amount of IL-5 in the BAL was also decreased, albeit not significantly, the level of IFN- γ in EcN-treated mice was significantly increased (fig. 2c). No IL-4 or IL-

10 could be detected in any of the BAL fluid samples (data not shown). Furthermore, MLN cells from mice treated with EcN produced significantly less IL-5 and had increased levels of IFN- γ after stimulation with OVA in vitro (fig. 1d). No effect on IL-4 levels was observed. Similar results were obtained, when the cells were restimulated with anti-CD3/IL-2 and no cytokines were detected when the cells were cultured in medium only (data not shown). EcN-treated mice also showed increased numbers of Th1 and decreased numbers of Th2 cells in the BAL fluid (fig. 2g, h) and strongly increased OVA-specific IgG_{2a} levels in the serum (fig. 2e). There was no difference in the number of CD4⁺ T cells expressing IL-10 between the OVA and OVA/EcN groups in the BAL fluid (data not shown). Surprisingly, although OVA-specific IgE levels were reduced, the difference did not reach significance and IgG₁ antibody levels were not reduced in these animals (fig. 2e).

For the measurement of AHR, the model was slightly modified as described previously [22, 23]. Figure 2f shows that the application of EcN i.p. together with alum also significantly reduced AHR after allergen challenge. Eosinophil numbers in the BAL were also strongly reduced compared to the OVA-sensitized and challenged group (OVA/alum group $2.78 \times 10^6 \pm 0.74$ vs. OVA/alum/EcN group $0.29 \times 10^6 \pm 0.08$, p < 0.001, mean $\pm$ SEM of 8 mice/group).

The reduction of the OVA-induced type 2 response by the application of live EcN was associated with increased Th1 responses. This suggests that IFN- γ -secreting Th1 cells may at least in part be responsible for this effect. To confirm this hypothesis, we repeated the experiments described in figure 1a in IFN- γ -deficient mice and analyzed whether EcN was still able to reduce the development of airway eosinophilia. Table 1 shows that the suppressive effect was at least in part mediated by IFN- γ .

We were also interested to investigate whether the i.n. application of EcN before or together with OVA airway challenge but after OVA i.p. sensitization (fig. 4a) could reduce the development of allergic responses in the airways. Figure 4b shows that EcN had no effect on the development of allergen-induced airway eosinophilia when given 4 weeks and 1 week before the allergen challenge, and also when given together with OVA. However, increased numbers of lymphocytes and macrophages were detected in the BAL of the mice treated with EcN together with OVA i.n., indicating that EcN had a proinflammatory effect.

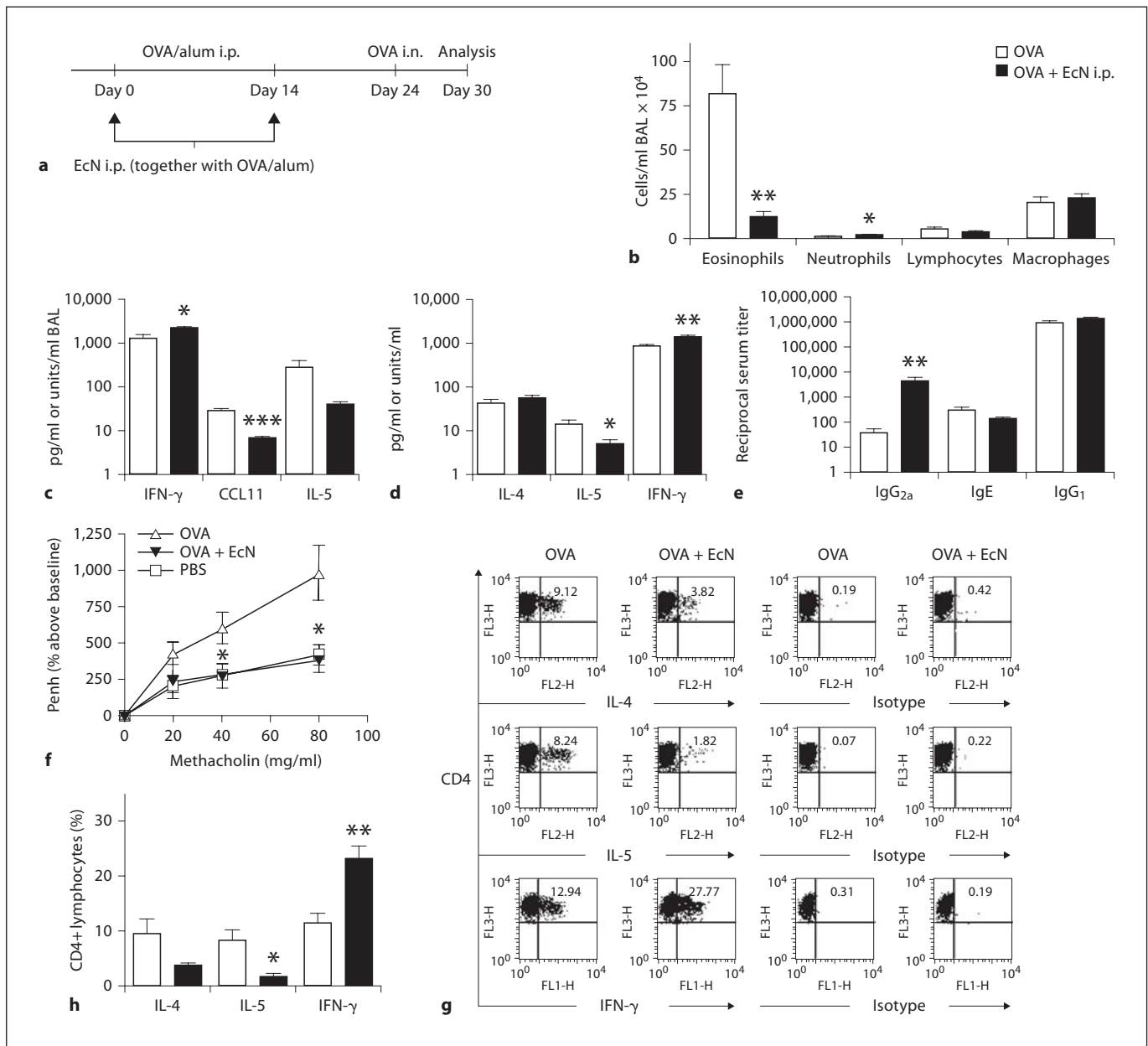


Fig. 2. Application of EcN simultaneously with OVA/alum i.p. inhibits the development of Th2 responses. **a** Mice were i.p. treated with OVA/alum, or OVA/alum/EcN on days 0 and 14 and then challenged with OVA on day 24. Analysis was performed on day 30. **b** Amount of different cell types detected in the BAL fluid. **c** IFN- γ , CCL11 and IL-5 levels in the BAL fluid. **d** IL-4, IL-5 and IFN- γ levels detected in the supernatants of MLN cell cultures stimulated with OVA. **e** Serum levels of OVA-specific antibodies. **f** AHR detected in the different groups of mice after methacholine challenge. In order to measure AHR, mice had to be challenged

with OVA on days 24, 25, 26, 31, 32 and 33, and they were then analyzed on day 34. **g** Representative examples of intracellular FACS staining for IL-4, IL-5 and IFN- γ gated on CD4+ T cells, obtained from the BAL fluid of the different groups of mice. **h** Percentage of CD4+ T cells, producing IL-4, IL-5 or IFN- γ . Shown are mean values $\pm$ SEM of 2 independent experiments with 10 mice/group, with the exception of the AHR measurement where 6 mice/group were analyzed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with OVA-only-treated mice.



Fig. 3. Goblet cell metaplasia is reduced in mice treated with EcN. Mice were treated as shown in figure 2a. The lungs were fixed, cut into 2- to 3- μ m sections and stained with PAS. Shown are lung sections of untreated mice (a), OVA only immunized mice (b) and

mice immunized with OVA and treated with EcN (c). Representative examples of 2 independent experiments with 10 mice/group are shown.

Color version available online

The Suppressive Effect of EcN on the Development of Airway Eosinophilia Was Not Dependent upon LPS or Mediated by TLR-4

Numerous studies have shown that the application of LPS derived from *E. coli* can inhibit the development of allergic responses [5, 26–29]. Therefore, we wanted to investigate whether the LPS of EcN may be responsible for our observed suppressive effects on allergic Th2 responses. We also used commercially available LPS in control experiments, since the LPS of EcN has a different composition (responsible for the serum sensitivity of this strain) than that of normal *E. coli* bacteria [30]. We treated EcN for 1 h at 75°C to kill the bacteria (hk-EcN) and applied them together i.p. with OVA/alum on days 0 and 14. We found that hk-EcN also reduced the allergic responses in the airways, albeit not significantly (fig. 5a). Interestingly, IFN- γ in the BAL fluid of mice treated with hk-EcN were also significantly increased, whereas CCL11 and IL-5 levels in the BAL were not altered (data not shown). Additionally, hk-EcN did not change the amount of OVA-specific IgG_{2a}, IgG₁ and IgE present in the serum compared to the OVA-only-treated control group (data not shown). These results argue against the hypothesis that the observed effects were only due to the LPS component of the EcN, since LPS is not destroyed by this treatment. Supporting this view was our finding that applying 30 μ g of *E. coli* LPS together with OVA/alum during the sensitization also did not reduce the development of OVA-induced lung eosinophilia (fig. 5b), which was in contrast to the results with live EcN. Furthermore, the suppressive effect of live EcN on airway eosinophilia was not dependent on TLR-4 (table 1), strongly supporting the view that EcN LPS was not mediating the suppressive effects. We

also found that – in contrast to live EcN – LPS was able to reduce airway eosinophilia when applied i.n. together with OVA (fig. 5b). This effect correlated with significantly increased IFN- γ levels and numbers of IFN- γ -producing CD4-positive Th1 cells and a reduction in IL-5-producing Th2 cells in the BAL compared to OVA-only-treated mice (data not shown). When LPS was given at earlier time points before OVA airway challenge, no effect was seen on the numbers of eosinophils present in the BAL (fig. 5b).

Maturation and Cytokine Production of BM-DCs after in vitro Stimulation with Live or hk-EcN

Our observation that live but not hk-EcN could significantly reduce Th2 cell development was surprising. A possible explanation for our results is that live EcN induces stronger Th1 responses than hk-EcN. To test this hypothesis, we incubated live and hk-EcN together with BM-DC, since efficient Th1 cell priming is dependent upon the maturation status of DCs and the cytokines produced by them [29]. We found that the highest amounts of live and hk-EcN induced the upregulation of MHC class II and the costimulatory molecules B7.1, B7.2 and CD40 on BM-DCs in vitro to the same extent as LPS, which served as a positive control (fig. 6a). In the same experiment the highest doses of live and hk-EcN induced the production of several proinflammatory cytokines, known to promote Th1 cell development, e.g. TNF- α , IL-1 β and IL-12 (fig. 6b). However, again, hk-EcN was as potent as live EcN in inducing the production of these cytokines. Taken together, these data indicate that both live and hk-EcN have the capacity to induce the maturation of BM-DCs in vitro.

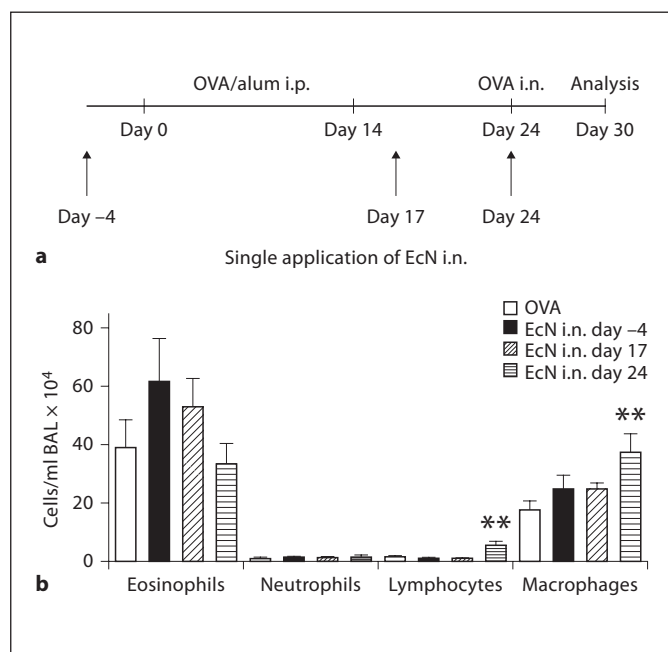


Fig. 4. The i.n. application of EcN has no effect on airway eosinophilia. **a** Mice were i.p. treated with OVA/alum on days 0 and 14 and then challenged with OVA on day 24. Additionally, EcN was applied either 4 weeks before OVA challenge (day -4), 1 week before (day 17) or together with OVA i.n. on day 24. Analysis was performed on day 30. **b** Amount of different cell types detected in the BAL fluid of mice treated with EcN i.n. Shown are the mean values $\pm$ SEM from 5 mice/group (EcN i.n. days -4 and 17) or of 2 independent experiments with 10 mice/group (EcN i.n. day 24). ** p < 0.01 compared to OVA-only-treated mice.

Discussion

Numerous clinical trials, epidemiological and experimental animal studies suggest that supplementing children's diets with probiotic bacteria may lead to a reduction in the severity and incidence of allergic diseases. The clinical studies, however, are not conclusive and it remains to be seen if probiotics can help prevent or treat allergic disorders in the majority of at-risk patients or patients already suffering from allergic diseases. The vast majority of these studies used different *Lactobacillus* or *Bifidobacterium* strains [4, 5, 14]. To our knowledge, there is little or no information on whether any other probiotic bacteria have suppressive effects on the development of allergic Th2 responses in the lungs of animals. For this reason, we investigated whether the application of the probiotic EcN can protect mice from developing OVA-specific Th2 responses in the airways. For this purpose,

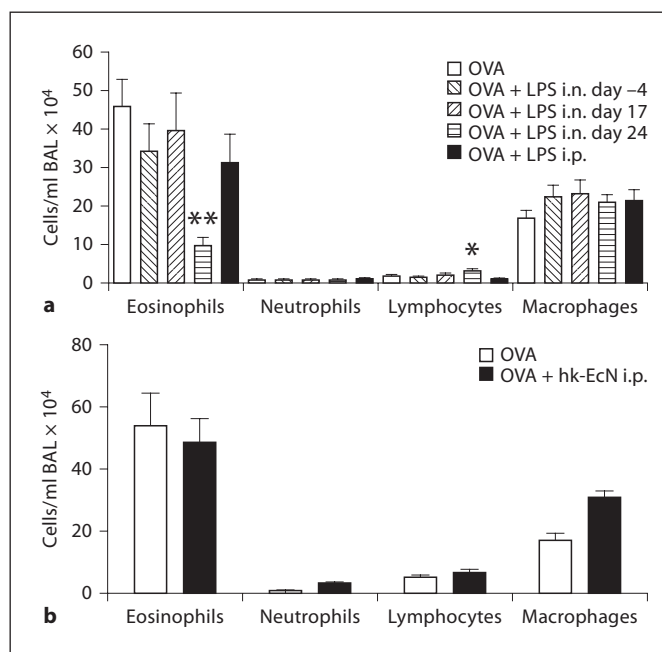


Fig. 5. LPS alters airway eosinophilia only when applied i.n. together with the OVA challenge, whereas hk-EcN i.p. has no impact on the numbers of eosinophils. **a** Mice were treated as described in figure 2a using hk-EcN. Mean amounts of different cell types detected in the BAL fluid of 10 mice/group from 2 independent experiments are shown. **b** Mice were treated as described in figure 2a or 4a, with the exception that LPS was used for the experiments. Shown are the mean amounts of the different cell types $\pm$ SEM detected in the BAL fluid of the different groups with 9–12 mice/group. * p < 0.05; ** p < 0.01 compared to the OVA control group.

mice were fed daily with 1×10^8 live EcN bacteria, starting 5 days prior to the first i.p. sensitization with OVA and finishing 1 day before sacrificing the animals. We found that lung eosinophilia, Th1 and Th2 cytokine levels in the BAL or supernatants of MLN cell cultures and OVA-specific antibody levels in the serum were similar in the EcN-treated mice and the OVA controls, clearly demonstrating that feeding the mice with EcN had no impact on the development of allergen-specific Th2 responses. We controlled for the presence of EcN in the gut by counting the fluorescent bacteria in the feces and found that significant numbers of bacteria could be detected. This argues against a lack of suppression due to low numbers of live bacteria reaching or staying in the gut.

We also investigated if the presence of EcN at the site of Th2 cell priming had an impact on the development of allergic responses. We found that when EcN was given

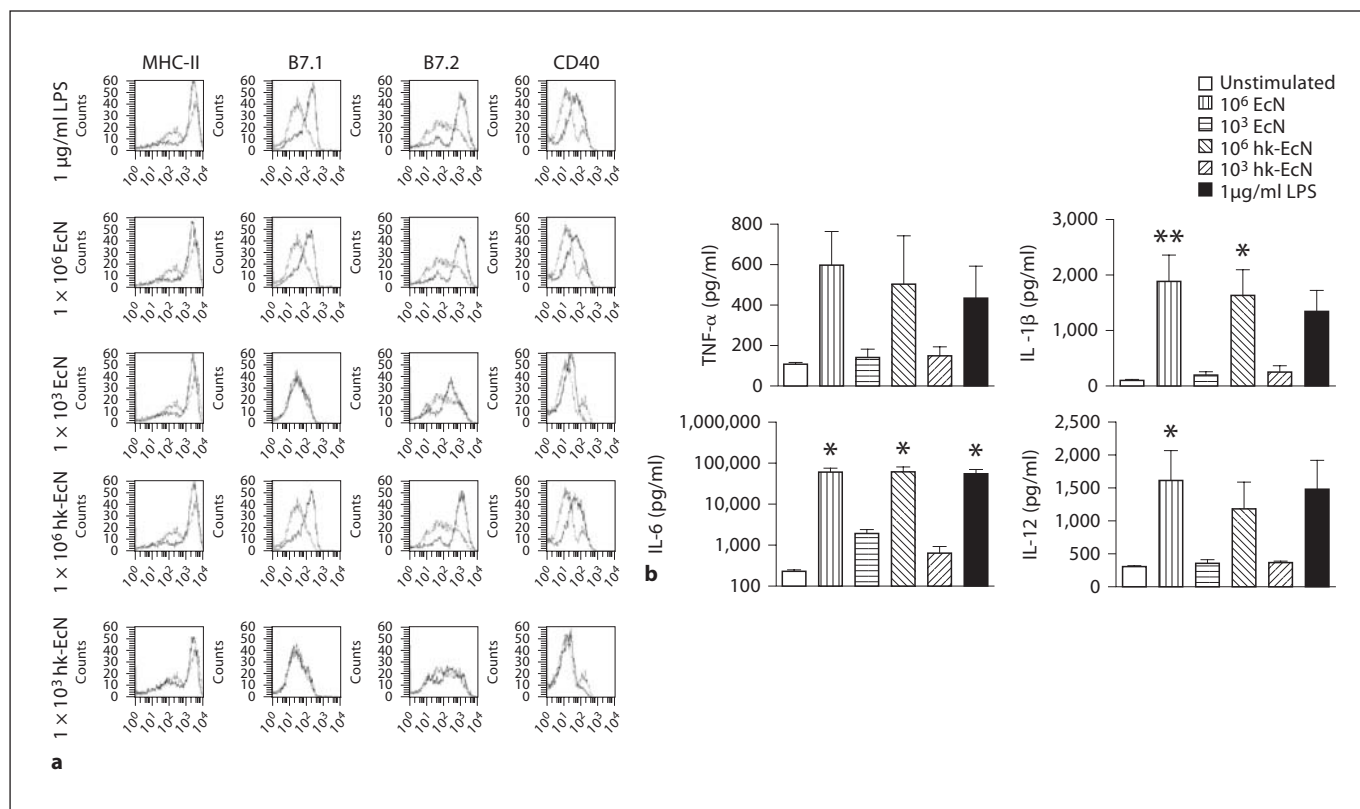


Fig. 6. Stimulation of BM-DCs with EcN, hk-EcN or LPS. BM-DCs generated from wild-type mice were stimulated with either 1×10^6 or 1×10^3 live or hk-EcN/ml or with 1μ g/ml LPS as a positive control. 24 h later the cells and the supernatants were analysed. **a** FACS stainings for MHC class II (MHC-II), B7.1, B7.2 and CD40 of the cells obtained from the different cell cultures. The dotted lines represent the stainings of unstimulated BM-DCs.

b Amounts of TNF- α , IL-1 β , IL-6 and IL-12 detected in the supernatants from the different cell cultures. Shown are representative stainings from 3 separate experiments (**a**) and the mean amount $\pm$ SEM of cytokines detected in the supernatants from 3 separate cell cultures/group (**b**). * $p < 0.05$; ** $p < 0.01$ compared to unstimulated BM-DCs.

together with OVA/alum i.p. airway eosinophilia, AHR and goblet cell metaplasia were strongly reduced. These effects correlated with decreased amounts of IL-5 and CCL11 being detected in the BAL fluid of the EcN-treated mice. Numbers of Th2 cells were also decreased in the BAL fluid clearly indicating that the presence of EcN in the peritoneum reduced allergen-specific Th2 responses. However, total OVA-specific IgG₁ and IgE levels in the serum were not significantly reduced. This finding is surprising since it could be expected that a reduction of Th2 typical pathology in the lung correlates with reduced OVA-specific IgE and IgG₁ levels. We previously observed similar effects when we treated mice with a combination of OVA/alum/and hk BCG or CpG oligonucleotides [22], suggesting that, in general, airway inflammation can more readily be suppressed than the production of allergen-specific IgE or IgG₁ in this model.

What is the mechanism of how EcN reduces Th2 responses in the lung without affecting IgE or IgG₁ serum levels? The levels of IFN- γ in the BAL of EcN-treated mice were significantly increased, and MLN cells from mice treated with EcN produced increased levels of IFN- γ after stimulation with OVA in vitro. EcN-treated mice also had increased numbers of IFN- γ -producing Th1 cells in the BAL fluid. These findings suggest that EcN induced the development of allergen-specific Th1 cells. Supporting this view are the strongly increased levels of OVA-specific IgG_{2a} detected in the serum of these mice. Allergen-specific Th1 cells have been reported to directly suppress the development of allergen-induced Th2 responses in the lung through the production of IFN- γ [29]. We also found that when we applied 5×10^6 OVA-specific IFN- γ -producing CD4⁺ Th1 cells (derived in vitro from CD4⁺ T cells isolated from OT-2 transgenic mice)

intravenously 1 day before starting the OVA immunization protocol or 1 day before OVA airway challenge, airway eosinophilia was significantly reduced (data not shown). Our data suggest that EcN induced the development of allergen-specific Th1 cells which, through the production of IFN- γ , reduced Th2 cell-mediated inflammation in the lungs. Supporting this hypothesis was our finding that the suppressive effect on the development of airway eosinophilia was not observed in IFN- γ -deficient mice. Apparently, the switch from an allergen-specific Th2 to a Th1 response by EcN was not strong enough to reduce OVA-specific serum IgE and IgG₁ levels. A further possibility is that the presence of EcN induced the generation of Tr cells which interfered with the development of Th2 responses in the lung. A similar effect was reported in mice treated with probiotics [18].

The strong induction of OVA-specific Th1 responses was somewhat surprising since EcN is nonpathogenic, serum sensitive and is rapidly killed in the animals in the peritoneum, and Th1 responses are therefore not needed to clear the bacteria. However, EcN has immunomodulatory capabilities which favor the induction of Th1 responses. One component may be LPS, since numerous studies have reported that the application of *E. coli*-derived LPS inhibits the development of allergen-induced Th2 responses [27, 31]. Our finding that TLR-4 deficient mice showed no significant reduction in airway eosinophilia when EcN was added to OVA/alum suggests that LPS was not responsible for the observed effects since LPS mediates immunomodulatory effects through TLR-4 [32]. Considering that LPS is such a strong immune modulator, the lack of a detectable LPS involvement was surprising. However, the LPS from EcN only contains one O6 antigen repeating unit instead of multiple repeats found in both pathogenic and nonpathogenic *E. coli*. The lack of multiple O6 repeats is also believed to be responsible for the serum sensitivity of EcN [30]. One possible explanation is that although the lipid-A part, the core oligosaccharide and one O6 antigen are expressed on the surface of EcN, this type of LPS may no longer bind to or signal through TLR-4. However, when we added 30 μ g of *E. coli* LPS to OVA/alum, we also found (in contrast to other reports using Balb/c and not C57BL/6 mice [27]) no reduction in airway eosinophilia. Taken together, these data indicate that EcN LPS is not responsible for the reduced Th2 response.

A further possible mediator of the observed effects may be EcN DNA. A recent report by Takahashi et al. [33] reported that CpG oligonucleotides derived from probiotic *Bifidobacterium longum* inhibited the development

of allergen-induced Th2 responses in mice [33] and the antiallergic effects of *Lactobacillus reuteri* were dependent upon TLR-9 [21]. Since the DNA of EcN contains unmethylated CPG motifs, it is possible that degraded DNA from EcN may be initiating the development of Th1 responses by inducing IL-12 and other factors (e.g. IL-23, IL-27) favoring Th1 cell development through a TLR-9-mediated mechanism. Numerous studies, including our own, have shown the strong Th1-inducing and Th2-suppressing capacity of CpG ODN in mice [5, 22, 29]. A further possibility is that the flagellin expressed by EcN may also induce Th1 responses by binding and signaling through TLR-5. Supporting this view is a report showing that flagellin induces the production of IL-12 of human dendritic cells through a TLR-5-dependent mechanism [34] and that the flagellin from EcN was responsible for the induction of β -defensin 2 by Caco-2 cells [35]. Currently it is not clear by which mechanism(s) EcN induces Th1 responses – DNA, flagellin, both or some other not yet identified components of EcN.

A very surprising finding of our study was that when EcN was heat killed, it was no longer as efficient in inhibiting allergen-induced airway eosinophilia in comparison to live EcN (eosinophilia was still reduced and enhanced Th1 responses were detected, but the effects were no longer significant). However, since we heat-inactivated EcN for 1 h at 75°C, most proteins were denatured. This finding suggests that proteins (possibly flagellin, as discussed) may be at least in part responsible for the induction of Th1 and suppression of Th2 responses. However, surprisingly we found that live as well as hk-EcN induced the upregulation of MHC class II and the costimulatory molecules B7.1, B7.2 and CD40 on BMDCs in vitro, indicative of dendritic cell maturation. Live and hk-EcN induced the production of several proinflammatory cytokines, known to promote Th1 cell development (e.g. TNF- α , IL-1 β and IL-12) to the same degree. These data indicate that hk-EcN should be as potent as live EcN in inducing Th1 responses in vivo. However, this is not the case and, possibly, EcN needs to be alive to have the greatest Th1-inducing and Th2-suppressing effect. Although serum sensitive, EcN may still be able to survive some time in the host, and this environment may trigger the induction of bacterial survival mechanisms (e.g. heat shock proteins) which in turn may increase the Th1-cell-inducing capacity of EcN. HSP-65-specific Th1 responses have been reported in mice infected with *E. coli* [36].

In conclusion, our data show that EcN applied together with OVA/alum during the sensitization phase switch-

es the immune response from Th2 to Th1 in the lung but not in the periphery, where allergen-specific IgE and IgG₁ levels were not significantly reduced. Applying EcN orally before and during allergen sensitization or i.n. after allergen sensitization had no suppressive effect on the development of allergen-specific Th2 responses.

These data indicate that EcN may be able to prevent the development of allergies in humans when present at the site of allergen exposure before allergen sensitization has occurred. However, in humans, EcN may also reduce the development of allergies when applied orally, since premature newborns showed immune responses after oral treatment with EcN [37] and applying another probiotic *E. coli* strain reduced the incidence of allergic disease over a long time period after the oral application [38].

Furthermore, our studies also support the hypothesis that the exposure of small children to commensal bacteria or bacterial products [39] may help suppress allergen sensitization, although increased incidence of atopy has been associated with the colonization of the gut with *E. coli* [11].

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