

Oral administration of *Escherichia coli* Nissle 1917 prevents allergen-induced dermatitis in mice

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Abstract:

Background: The prevalence of allergies has been linked to Western life style factors including a decrease of microbial exposure. Probiotics, such as *Escherichia coli* Nissle 1917 (EcN), have been shown to be beneficial for prevention and treatment of several chronic inflammatory diseases.

Objective: The aim of this study was to investigate the impact of oral EcN administration on development and outcome of allergen-induced dermatitis.

Methods: In sensitized BALB/c mice, skin inflammation was induced by topical allergen application. EcN was administered orally in a preventive manner. Severity of dermatitis was analysed by evaluation of skin score, local cellular and cytokine profile. The systemic immune response was assessed by analysis of immunoglobulins and allergen-dependent cytokine response.

Results: Oral EcN administration improved allergen-induced dermatitis dose-dependently. In parallel, a reduction of epidermal

thickness and infiltrating immune cells together with an enhanced number of forkhead box P3 (Foxp3)⁺ cells and a trend of increased IFN γ , IL-10 and TGF β expression was detected in eczematous skin. In allergen-stimulated splenocytes reduced IL-4 and IFN γ along with an elevated IL-10 production and a tendency to an increased TGF β secretion were observed.

Conclusions: Our findings indicate that EcN alters the local allergen-induced immune response by increase of Foxp3⁺ cells and by favouring an immunoregulatory cytokine pattern. Thus, oral administration of EcN might be an effective strategy in prevention and potentially therapy of allergic inflammatory skin diseases.

Key words: allergy – eczema – *Escherichia coli* Nissle 1917 – probiotics – regulatory T cells

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Introduction

Atopic eczema is a chronic inflammatory skin disease and often associated with IgE-dependent diseases such as allergic asthma and rhinitis (1). The observed increase of allergic diseases in the last decades has been associated with the hygiene hypothesis (2). Although the underlying mechanisms are complex and not completely understood, the increased hygienic practices and less microbial exposure may promote the immunological disorder prevalence in Western countries and more recently in developing countries (2). Due to the T helper type (Th) 2-polarized immune environment in placenta, the immune system of a newborn is Th2-dominated and has to be redirected during the first year of life, the time when the initial allergic sensitization takes place (3). Bacterial products favour the shift from Th2 to a Th1 profile resulting from Toll-like receptor (TLR)-stimulated production of IL-12 and interferons by innate immunity cells (4). Thus, a limited microbial contact in early life would not induce Th1 sufficiently to counter-regulate the Th2 response (4). However, beside Th2-related allergies, the prevalence of Th1-mediated autoimmune diseases such as diabetes mellitus has been increasing during latest decades as well (5). Hence, regulatory T (Treg) cells and Th17 cells may be involved in modulating the Th1/Th2 balance and thus contribute to the development of both allergic and

autoimmune diseases (5). Beside effector cells, cells of the innate immune system such as dendritic cells are discussed to essentially participate in controlling immune responses (5).

The intestinal micro flora is important for the maintenance of the normal gut barrier function and the development of a tolerogenic immune status (3). Following this line, germfree mice do not develop oral tolerance and show a persistent Th2-dominant immune response (6). Neonatal reconstitution of these mice with intestinal bacteria reverses this immune deviation (6). It has been shown in humans that allergic infants exhibit a diminished intestinal colonization by *Lactobacillus* or *Bifidobacterium* strains and increased *Clostridium* and *Staphylococcus aureus* counts in comparison to infants without allergies (7). At the molecular level, certain compounds of intestinal bacteria induce tolerance via up-regulation of inhibitory Toll-interacting protein and down-regulated surface expression of TLR (8). Further data indicate that specific intestinal microorganisms induce Treg cells and hence suppress allergic disorders (9,10).

One of the best characterized probiotic bacteria is the Gram-negative, serum-sensitive *Escherichia coli* Nissle 1917 (EcN, serotype O6:K5:H1) which has been shown to efficiently colonize the intestine of humans, piglets and mice (11). EcN has been used as a probiotic treatment for several intestinal inflammatory immune

disorders, such as ulcerative colitis and Crohn's disease (12). Thereby, alteration of the intestinal micro flora, barrier enhancement, local and systemic immunomodulation are described as potential mechanisms (13). We could previously show that EcN can directly modify lymphocyte functions (14). However, EcN was rarely studied in *in vivo* models of allergic diseases and in particular robust data on EcN and inflammatory skin disorders are missing. In this study, we investigated the immunomodulatory impact of oral EcN administration in a mouse model of allergen-induced dermatitis and analysed the possible underlying mechanisms.

Materials and methods

Mouse model of allergen-induced dermatitis

Female BALB/c mice (8–10 weeks) were purchased from the Federal Institute of Risk Assessment (Berlin, Germany). Mice were kept within an accredited animal facility at the Charité-Universitätsmedizin (Berlin, Germany) and maintained under specific pathogen-free conditions at constant room temperature ($21 \pm 1^\circ\text{C}$), relative humidity ($45 \pm 5\%$) and 12 h light–dark cycle. Procedures were performed in compliance with protocols approved by the local State Office of Health and Social Affairs. For induction of allergen-induced dermatitis, mice were treated as previously described (15). As shown in Fig. 1a, mice were sensitized i.p. on days 1 and 14 with Ovalbumin (OVA, Grade V; Sigma-Aldrich,

Taufkirchen, Germany) adsorbed to alum (Pierce, Rockford, IL, USA) or phosphate buffered saline (PBS) as control. As atopy patch test application induces the infiltration of significant higher numbers of activated leucocytes (16), e.c. application of OVA/Alum or PBS/Alum was performed using this method between the days 14–21, 35–42 and 56–63. Each mouse had a total of three 1-week exposures to the same skin site separated by 2-week intervals without application. For treatment evaluation, non-sensitized mice were used as negative control and PBS-patched mice as patch control.

The probiotic strain *Escherichia coli* Nissle 1917 (EcN) is used as the active component of a microbial preparation (Mutaflor®; Ardeypharm GmbH, Herdecke, Germany) licensed for the medical use in Germany and some other European countries for treatment of inflammatory bowel diseases (11). Originally, this *E. coli* strain was discovered by Alfred Nissle in 1917 during the First World War and was named by him accordingly. Nissle isolated EcN from faeces of a soldier who did not develop infectious diarrhoea while staying in a heavily with enteropathogens contaminated region (11). Living EcN was given to mice orally via drinking water with a daily dose of 10^7 colony-forming units (CFU) (17) and 10^8 CFU (18). The electrolyte solution in which the EcN was diluted served as control. The application was preventively performed between day –14 and day +21 of the sensitization protocol. On day 64 the phenotypical outcome was evaluated, animals were sacrificed and samples were taken.

Assessment of skin inflammation

To evaluate the severity of OVA-induced eczematous lesions a skin scoring system was used (15). Typical skin features of human eczema (erythema, excoriation/crusting, dryness and extension) were considered (19) and graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) by five blinded persons. For calculating total clinical skin score, the parameters were summed up. The clinical skin score was taken as index of dermatitis severity ranging from 0 (none) to 12 (severe).

Immunohistochemistry

The tissue sections ($5\ \mu\text{m}$) were blocked with 5% goat serum and avidin/biotin blocking kit (Vector, Burlingame, CA, USA). After 1 h of incubation with rat anti-mouse CD4 (RM4-5; BD Pharmingen, San Diego, CA, USA), CD8 (53-6.7; BD) or Ki67 (TEC-3 M7249; DAKO Diagnostika, Hamburg, Germany) specimens were incubated with biotinylated goat anti-rat IgG (BD) for 30 min. Detection was performed using DAKO REAL™ Detection System Alkaline Phosphatase/RED (DAKO) and using haematoxylin counterstaining (Merck, Darmstadt, Germany). For detection of Foxp3, the rat anti-mouse clone FJK-16s (eBioscience, San Diego, CA, USA) was applied and slides were blocked using peroxidase-blocking reagent (DAKO) followed by a rabbit anti-rat antibody (DAKO) and the EnVision peroxidase kit (K4003; DAKO). To visualize the mast cell granules, samples were stained with 0.1% toluidine blue (Merck) in 0.5 M HCl for 1 h.

Dermal CD4⁺, CD8⁺, Foxp3⁺ and mast cells, epidermal thickness and Ki67 expression were analysed by Axiovision tools on the Axio-plan light microscope (Carl Zeiss, Berlin, Germany) at $\times 100$ magnification. Thereby, positively stained cells were counted per area.

Immunoglobulin measurement

OVA-specific IgE, IgG1 (15) and total IgE (20) levels were measured using ELISA as previously described. For determination of OVA-specific IgA, plates were coated overnight with $5\ \mu\text{g}/\text{ml}$

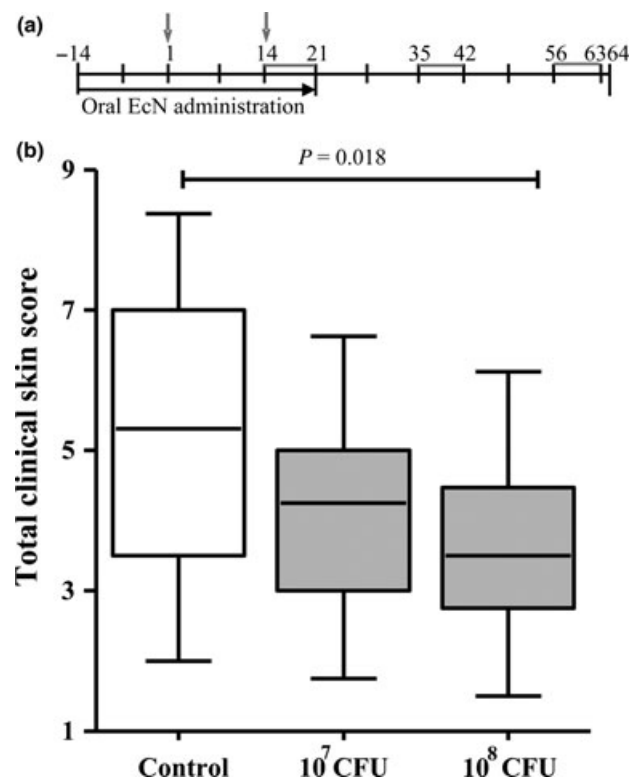


Figure 1. Reduced severity of allergen-induced dermatitis upon oral EcN administration. (a) Sensitization schedule for allergen-induced dermatitis and oral EcN administration in mice. Mice were sensitized i.p. with OVA/Alum at days 1 and 14 (grey arrows) and e.c. with OVA/Alum comprising three 1-week exposures (grey lines). EcN (black arrow) was orally applied from day –14 to day +21. On day 64, mice were sacrificed and samples were collected. (b) Clinical skin score was defined as sum of individual scores graded from 0 (none) to 3 (severe) for erythema, crusts, dryness and extension. Data are shown as box plots (25th and 75th percentile, median); $n \geq 8$.

OVA in sodium carbonate buffer. After blocking with 3% milk powder in PBS, serial dilutions of sera were incubated overnight and detected with biotinylated goat anti-mouse IgA (Southern Biotech, Birmingham, AL, USA). The reaction was developed with streptavidin peroxidase and tetra methyl benzidine and stopped with sulphuric acid (all Sigma-Aldrich). The plates were measured at 450/690 nm and the amount of OVA-specific IgA was calculated according to the standard serum pool.

Measurement of allergen-specific cytokine production in splenocyte cultures

Splenocytes were resuspended in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% heat-inactivated fetal calf serum (all Biochrom/Seromed, Berlin, Germany). 10^6 cells/ml were either left in medium alone, stimulated with 250 µg/ml OVA and 5 µg/ml anti-CD28 (37.51; BD) for 96 h or with 20 ng/ml phorbol-12-myristate-13-acetate and 200 nM ionomycin for 48 h at 37°C, 5% CO₂. Finally, IL-4, IL-10, IFN γ and TGF β secretion was analysed in cell-free supernatants using ELISA according to manufacturer's instructions (R&D Systems, Wiesbaden, Germany).

Analysis of cytokine expression in eczematous skin

Total RNA was extracted from frozen skin samples using RNA preparation kit from Macherey-Nagel (Düren, Germany) and reversely transcribed into cDNA according to manufacturer's guidelines (Applied Biosystem, Darmstadt, Germany).

PCR amplification was performed with specific primers (IFN γ : forward: AACTATTTTAACTCAAGTGGCATAGAT, reverse: TGC-TGTTGTGTAAGAAGGTAG; IL-10: forward: TTTAAGGGT-ACTTGGGTTGC, reverse: AGGGTCTTCAGCTTCTCACCC; TGF β : forward: GCAACAACGCCATCTATGAG, reverse: AGACAGCC-ACTCAGGCGTAT). The relative mRNA expression level was quantified based on the reference gene hypoxanthine phosphoribosyltransferase (HPRT; forward: CGTCGTGATTAGCGATGATG, reverse: AATCCAGCAGGTCAGCAAAG) by real-time quantitative RT-PCR using LightCycler 1.5 (Roche Diagnostics, Penzberg, Germany). As negative control, the template was replaced by sterile water. The melting curve analysis was used to control the melting point specificity. Efficiency corrected relative quantification was performed using 2- $\Delta\Delta$ CT method (21).

Statistical analysis

Due to non-Gaussian distribution of the data, Mann-Whitney-U-test for non-parametrical, unpaired data was performed by using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). A *P*-value <0.05 was considered to be statistically significant.

Results

Improvement of allergen-induced dermatitis upon oral EcN administration

First, we analysed the impact of oral EcN on clinical outcome of inflammatory skin response in this murine model of allergen-induced dermatitis. All mice showed comparable liver, spleen and body weight. No unwanted side effects were observed.

Our data show that EcN administration led to a significant, dose-dependent clinical improvement of allergen-induced dermatitis (Fig. 1b). Thereby, 10^8 CFU EcN was most effective, achieving a reduction of the clinical skin score by one-third (control 5.3 ± 0.6 vs 10^8 CFU EcN: 3.6 ± 0.4 , *P* = 0.018, Fig. 1b). The lower EcN dose of 10^7 CFU also improved the dermatitis, but failed to reach statistical significance (4.5 ± 0.6 ; NS).

Decreased local inflammatory immune response

Next, we investigated whether the reduced clinical skin score was accompanied with changes of the local inflammatory response. Indeed, we observed a decrease of dermal CD4⁺ cells (control: 627 ± 52 CD4⁺/mm² vs 10^8 CFU EcN: 428 ± 20 CD4⁺/mm², *P* = 0.004; Fig. 2a). A tendency to a reduced number of dermal CD8⁺ cells was detected as well (control: 276 ± 56 vs EcN: 152 ± 45 CD8⁺/mm², *P* = 0.069). EcN-treated mice had significantly lower mast cell numbers (89 ± 8 mast cells/mm²) in the dermis in comparison with control fed mice (149 ± 14 mast cells/mm², *P* = 0.001). Furthermore, a reduced epidermal thickness was measured in the EcN group (control: 46 ± 3 µm vs EcN: 37 ± 2 µm, *P* = 0.046; Fig. 2b) which was associated with a reduced keratinocyte Ki67 expression (control: 3.3 ± 0.2 vs EcN: 2.0 ± 0.2 , *P* = 0.017; Fig. 2c).

Interestingly, we observed a trend to an increase of dermal Foxp3⁺ cells (control: 149 ± 15 Foxp3⁺/mm² vs EcN: 204 ± 23 Foxp3⁺/mm², *P* = 0.099; Fig. 2a), which reached statistical significance if the total number of CD4⁺ cells was considered (control group: 0.26 ± 0.04 vs EcN: 0.46 ± 0.05 , *P* = 0.005; Fig. 2d).

Local inflammatory cytokine profile

As the cytokine expression at the local sites is important for the outcome of the allergen-induced dermatitis, we analysed the expression of cytokines which are important for the development of eczema.

As depicted in Table 1, upon treatment with 10^8 CFU EcN a tendency towards an increased mRNA expression of IFN γ , IL-10 and TGF β was determined, but due to great individual differences in local mRNA expression, the results did not reach statistical significance.

Allergen-specific T cell responses

To investigate the immunomodulatory impact of oral EcN administration in more detail, allergen-stimulated cytokine response was analysed next.

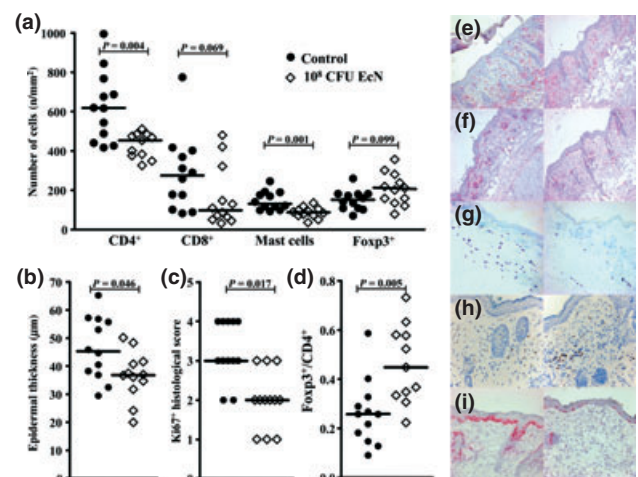


Figure 2. Immunohistochemical analysis of eczematous skin from mice of control versus 10^8 CFU EcN group. Number of (a) CD4⁺, CD8⁺, mast cells and Foxp3⁺ cells; (b) Epidermal thickness; (c) Ki67 expression; (d) Foxp3⁺/CD4⁺ ratio. Positively stained cells were counted per mm² and epidermal thickness was determined in 5 µm haematoxylin-counterstained skin sections at $\times 100$ magnification using digital measuring tools. Data are shown as single values, bars represent median; *n* $\geq$ 8. Representative illustrations of (e) CD4, (f) CD8, (g) mast cell, (h) Foxp3 and (i) Ki67 staining. The specimens of control mice are shown in the left panel, whereas illustrations of EcN fed mice are depicted in the right panel.

Table 1. Relative mRNA expression in eczematous skin upon oral EcN application

Oral application	IFN γ	IL-10	TGF β
Control	0.04 [0.01–0.92]	0.17 [0.01–2.68]	0.02 [0–0.52]
10 ⁸ CFU EcN	0.10 [0.02–0.54]	0.40 [0.07–2.31]	0.27 [0–1.02]

Expression of cytokine mRNA was determined using qRT-PCR. Relative expression of IFN γ , IL-10 and TGF β in eczematous skin from control versus EcN-treated mice. Data are shown as median [minimum – maximum]; $n \geq 8$.

As shown in Fig. 3, splenocytes from mice which had received 10⁸ CFU EcN exhibited a reduced OVA-dependent IL-4 (control: 305 $\pm$ 4 pg/ml vs EcN: 154 $\pm$ 30 pg/ml, $P = 0.007$; Fig. 3a) but also IFN γ secretion (control: 209 $\pm$ 38 pg/ml vs EcN: 97 $\pm$ 21 pg/ml; $P = 0.018$; Fig. 3b). In addition, cells showed a significantly increased IL-10 secretion (control: 95 $\pm$ 10 pg/ml vs EcN: 128 $\pm$ 30 pg/ml, $P = 0.027$; Fig. 3c). Although splenocytes from mice with EcN administration did not exhibit an enhanced OVA-induced TGF β production (Fig. 3d), an increased basal TGF β production at day 2 (control: 90 $\pm$ 15 pg/ml vs EcN: 168 $\pm$ 32 pg/ml, $P = 0.040$; data not shown) was measured. Moreover, we observed a trend to an increased TGF β production after mitogenic stimulation (control: 120 $\pm$ 20 pg/ml vs EcN: 151 $\pm$ 13 pg/ml, $P = 0.099$; data not shown).

Increased systemic IgA response in EcN-treated mice

As we observed profound changes of the allergen-specific T cell cytokine responses, we hypothesized that also a modulation of the humoral immune response might take place upon EcN administration. Not only total IgE but also OVA-specific IgE and IgG1 remained stable in serum of EcN-treated mice (Fig. S1). However, upon oral EcN administration, the OVA-specific IgA in serum was significantly elevated (1656 $\pm$ 394 LU/ml) in comparison with control fed mice (576 $\pm$ 202 LU/ml, $P = 0.029$; Fig. S1).

Discussion

In this study, we investigated the effect of oral EcN administration on the severity of the inflammatory skin response in allergen-induced dermatitis.

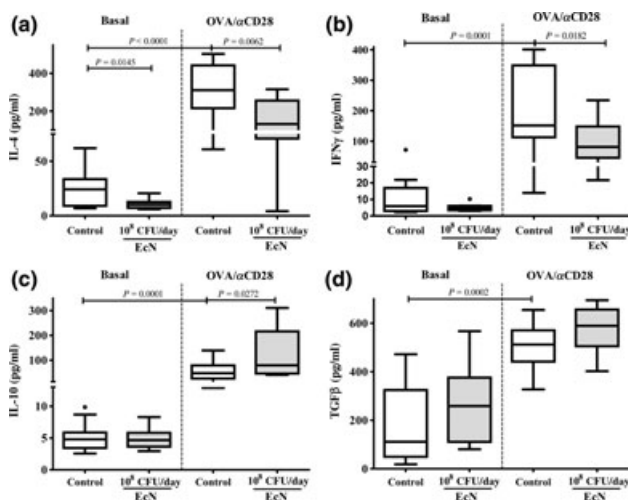


Figure 3. Cytokine production of splenocytes from mice treated with control versus 10⁸ CFU EcN. Basal and OVA/ α CD28 induced (a) IL-4; (b) IFN γ ; (c) IL-10 and (d) TGF β secretion. Cytokines were determined 96 h after stimulation using ELISA. Data are shown as box plots (25th and 75th percentile, median); $n \geq 8$.

The results demonstrate that EcN administration prior to and during allergen sensitization led to an amelioration of the allergen-induced dermatitis. This improvement was associated with a decreased number of inflammatory cells and reduced epidermal thickness in eczematous skin. We assumed an induction of immune regulatory mechanisms by oral EcN administration, as an increased proportion of dermal Foxp3⁺ cells was detected in lesions. In line with this observation, a local but also systemic up-regulation of the regulatory cytokines IL-10 and TGF β was determined.

Previous reports have shown that probiotics favour indeed the onset of Treg cells (9,10). Treg cells play an important role in regulating immune responses to allergens and in maintaining functional tolerance (22). Thus, our results are of great importance as they support the notion that Treg cells are detectable at inflammatory sites due to their ability to migrate to and to stay in inflamed tissues. This ability is a prerequisite for their regulatory function *in vivo* (10,23). The increased presence of Foxp3⁺ cells in the dermis may favour a suppression of effector cells, such as T cells, mast cells or keratinocytes by cell–cell contact, involving, e.g. OX40 or membrane-bound TGF β (24–26). Cell–cell contact may be the main pathway of Treg cell action at inflamed sites (25,26). The inhibitory capacity of naturally occurring Treg cells requires not only cell–cell contact, the cells can function through inhibitory cytokines as TGF β and IL-10 (27). Besides naturally occurring CD4⁺CD25⁺Foxp3⁺ Treg cells, other Foxp3⁺ Treg cells, e.g. allergen-specific inducible Treg, Tr1 and Th3 cells may have contributed to the local TGF β and IL-10 production as well as to the observed immunomodulatory mechanisms (27). Kwon et al. (10) have recently demonstrated in an experimental atopic dermatitis model that a probiotic mixture induced the conversion of T cells into Treg cells which were recruited into inflamed skin where they promoted regulatory effects. It was further shown that tolerogenic dendritic cells expressing typical regulatory characteristics, such as IL-10, TGF β , cyclooxygenase-2 and indoleamine-2,3-dioxygenase are generated, which in turn promote the generation of Treg cells in mesenteric lymph nodes (10). Oral probiotic treatment may not only increase the number of Treg cells but also may enhance their regulatory capacity (28). In addition, it has been suggested that EcN can translocate into mesenteric lymph nodes and modulate the cytokine secretion of mesenteric cells directly (29). At the molecular level, TLR-2 and TLR-4-dependent pathways are important signalling pathways for EcN-mediated immune modulation (17,30). TLR signalling has been shown to play a decisive role in Treg cell homeostasis. Furthermore, Treg cells themselves express different TLRs, such as TLR-2 and TLR-4 (31). Consequently, interaction with TLR ligands may promote Treg cells at different levels (32). How EcN administration augmented the regulatory function of the Treg cells in this study needs further investigations, e.g. in controlled transgenic animal models.

In addition, EcN can activate other cells like dendritic cells to produce high amounts of IL-10 (30). Our study demonstrates an increase of IL-10 and TGF β expression in skin, but also an induction of IL-10 and a trend to an enhanced TGF β production in splenocytes. Both cytokines are known to induce IgA production in B cells (33). We determined an increased OVA-specific IgA in serum upon EcN treatment, supporting our observation about the induction of regulatory mechanisms at the systemic level. The increase of regulatory cytokine production was associated with a

reduction of the allergen-induced Th1 and Th2 cytokine production in splenocyte cultures, supporting previous findings that probiotic microorganisms can inhibit Th effector functions (9). However, OVA-specific IgE and IgG1 levels in serum were stable upon EcN treatment. This finding is surprising as a reduction of the Th2 cytokine response should result in reduced IgE and IgG1 levels. It has to be considered that endpoint immunoglobulin measurements were performed 40 days after EcN administration. An impact of EcN on serum IgE and IgG1 may be covered by turn-over processes due to half-life of antibody molecules in serum (34). However, immunoglobulin class switching was induced by OVA i.p. sensitization during EcN treatment. The stable serum IgE and IgG1 upon EcN administration may reflect the fact that, in contrast to IgA, the isotype switching towards these two immunoglobulin classes was not modulated in EcN-treated mice. Nevertheless, similar observations were reported previously in a murine model of airway hyper-responsiveness (18). In this study, serum IgE and IgG1 remained unchanged although the systemic Th2 response was reduced by systemic EcN treatment (18). The authors speculate that the allergen-specific Th2 cytokine response might be more sensitive to external factors like triggers of the innate immune response than the B cell response resulting in allergen-specific IgE or IgG1 (18). Whether this hypothesis can also apply for our model needs further clarification.

We observed not only a reduced allergen-specific IL-4 but also reduced IFN γ production upon preventive EcN administration. In line with this, previous data show a reduced IFN γ production upon preventive EcN supplementation in a mouse model of colitis (29). By contrast, other investigators reported an induction of IFN γ and IgG2a upon EcN treatment (18,30). However, in this study, the inhibition of allergen-specific IL-4 production upon

EcN administration seems not to be mediated by counter-regulation through specific Th1 cells. The observation of an increased allergen-stimulated IL-10 production suggests rather an involvement of Treg cells in inhibition of Th2 response.

Finally, probiotics have been shown to reduce allergic inflammation in different experimental settings (9,28,30). In addition, different clinical trials have suggested the benefit of probiotic supplementation for the prevention and treatment of atopic eczema (35–37). Although the results are heterogeneous potentially due to differences in study design (35–37), there is encouraging evidence that probiotics are efficient for prevention of atopic eczema in children (35,36). Accordingly, the results of our study demonstrate that the immunomodulatory properties of EcN protect against allergen-induced dermatitis by induction of regulatory mechanisms which may balance the systemic and the local immune responses as well. Taken together, oral administration of EcN might be an effective strategy in prevention and potentially therapy of allergic inflammatory skin diseases.

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Conflict of interest

The authors state no conflict of interest.

Author contributions

C.W., M.W. designed the study; C.W., Y.Z., A.K., D.E. performed the research; C.W., Y.Z., A.K., D.E. analysed the data; C.W., Y.Z. wrote the paper; A.K., M.W. revised the paper critically; all authors have approved the paper before submission.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Serum concentrations of OVA specific (a) IgA, (b) IgG1 (c) IgE and (d) total IgE of mice treated with control versus EcN.

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Supplementary Figure: Serum concentrations of OVA specific a) IgA, b) IgG1 c) IgE and c) total IgE of mice treated with control vs. EcN. Data are shown as single values, bars represent median values; $n \geq 9$.