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DISSERTATION

Impact of gram-negative bacteria on the allergic immune response in a mouse model of allergen-induced eczema

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1. Introduction

1.1. Atopic eczema

Atopy covers diseases like asthma, allergic rhinitis and eczema. It is characterized by a sensitization to foreign proteins and an elevated total IgE in serum.

Atopic eczema (AE) is a chronic relapsing, inflammatory skin disease which usually presents during early infancy and childhood. It is characterized by erythema, xerosis, lichenification, pruritus and typical skin lesions [1].

As an allergic skin disease, most of AE are related to the type I hypersensitivity, but they also have features characteristic of T cell dependent type IV hypersensitivity [2].

Type I hypersensitivity is mediated by IgE antibodies. They bind to mast cells and basophils via Fc ε RI. In the presence of an allergen mediators are released, leading to a series of clinical responses like an increased vascular permeability and smooth muscle contraction [2]. IgE-mediated allergy is triggered by proteins, so called allergens, which are presented by antigen-presenting cells (APC). This response favors a T helper 2 (Th2) cell differentiation under a polarizing cytokine and chemokine environment. The interaction between Th2 cells and B cells provokes the production of allergen-specific IgE which can bind to the high affinity receptor (Fc ε RI) expressed on tissue-localized mast cells and circulating basophils. Consequently, when encountering the antigen again, the IgE coated cells will rapidly release the mediators, like histamine and prostaglandins, which can cause allergic symptoms.

Type IV hypersensitivity can be elicited by either CD4+ or CD8+ cells. During the sensitization phase, antigens penetrate into the skin. These antigens can react with self proteins, creating haptens which are presented by cutaneous APC. Subsequently, T cells are activated into memory T cells which circulate into dermis. In the elicitation period, repeated exposure of sensitizing antigen initiates the reaction of T cells with a release of cytokines, promoting inflammation and attracting monocytes to skin [2].

The first onset of AE predominantly starts in infants and young children. 85% are affected in the first year of life, 95% of these children are affected before the age of 5 [3]. Two types of AE have been defined. Most of the patients (70-80%) have multiple IgE

sensitizations, which have been suggested as the "extrinsic" type. The other subgroup of AE (20-30%) belongs to non-IgE-associated, "intrinsic" type. Those patients rarely get asthma compared to "extrinsic" type [4-6]. The prevalence of the latter subgroup is considered to be higher in children than in adults [7].

Different factors are involved in pathogenesis of AE including genetics, infections, skin barrier defects, climate, allergens and immune dysfunctions [8,9]. The associated genetic alterations have been identified in several regions on different chromosomes, including 1q21, 17q25, 20p, 16q and 5q31 [6,10]. Multiple loss-of-function mutations in the FLG gene, which is located on chromosome 1q21, have been described most recently. This is an important link explaining the disrupted skin barrier function in AE [11,12]. FLG gene encodes the filaggrin (filament-aggregating protein), which is a main component of terminal keratinocyte differentiation [11-13]. SPINK5 and KLK 7 are also candidate genes associated with AE, leading to a skin barrier defect [14]. The variants of gene COL29A1 located on chromosome 3q21 were also identified. It is proven that COL29A1 encodes a novel epidermal collagen named collagen XXIX. Lack of this extracellular matrix protein can lead to the features of AE [15]. Th2-cytokine genes located on chromosome 5q22-23, like the IL-13 coding region, and the IL-4Rα subunit (16q12) also play a role in the genetic pathogenesis of AE [6,16].

1.1.1. Immunologic features in AE

As shown in figure 1, AE usually develops sequentially. During the acute period, due to the injury by scratching, environmental antigens invade into the skin and are presented by dendritic cells (DC), which release Th2-skewing cytokines. Mast cell-derived IL-4 and keratinocyte-derived thymic stromal lymphopoietin (TSLP) contribute to this reaction as well [17]. As a result, Th0 cells tend to a polarized differentiation into Th2 cells.

In the chronic phase of AE dendritic cells, mast cells and keratinocytes produce an increased amount of cytokines and chemokines due to their stimulation through scratching and microbial toxins. This leads to an enhancement of the expression of adhesion molecules and attracts macrophages and eosinophils to skin. Inflammatory dendritic epidermal cells (IDEC) are activated in the skin as well. The production of

proinflammatory cytokines promotes the differentiation of Th0 to Th1 which is associated with a high expression of IFN-γ [17]. The secreted IFN-γ induces apoptosis of keratinocytes, finally resulting in the eczematous lesions which is one characteristic of AE [18].

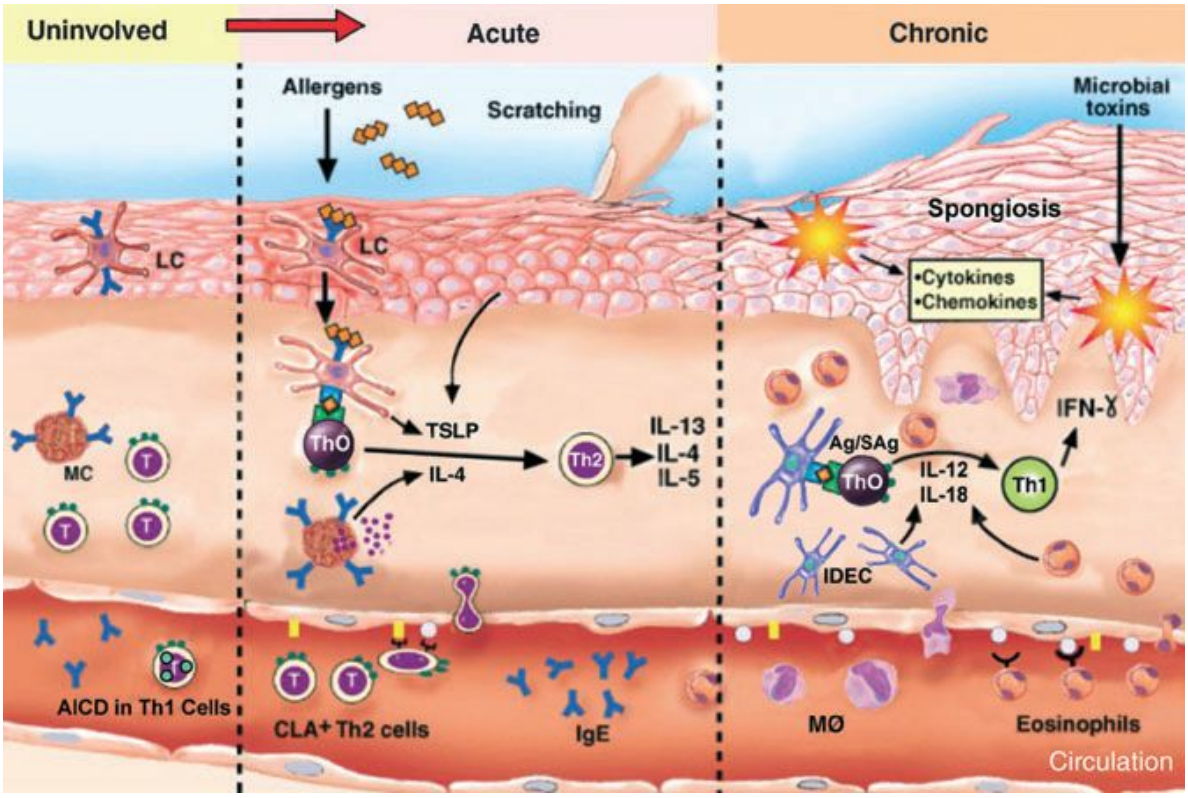

Figure 1: Immunological features of AE. CLA, cutaneous lymphocyte-associated antigen; Ag, antigen; SAg, superantigen; AICD, activation-induced cell death. Reprinted from Akdis CA, Akdis M, Bieber T, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. Allergy 2006;61:969-987. With permission from Wiley.

In a recent study, a dysregulation of effector T cells was found to be associated with an impaired CD4+CD25+Foxp3+ T regulatory (Treg) cell infiltration [19]. Treg cells have been demonstrated to suppress T cell activation and proliferation. A previous study suggested that elevated CD4+CD25+ Treg cells and CLA+CD4+CD25+ cells can be

detected in the peripheral blood of AE patients [20]. However, although increased numbers of CD25+ cells have been detected in AE lesions, no Foxp3 expression was detectable [19]. This indicates that CD25 can be expressed on other activated T cells as well and suggests that the determination of Foxp3 expression is important for identifying Treg cells. The defective expression of the transcription factor Foxp3 leads to immune dysregulation, like the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome which also has clinical features of atopic eczema [21].

1.1.2. Mouse models of atopic eczema

Mouse models provide several advantages compared to other experimental animals. They are of small size due to their identical genetic background and can be easily controlled regarding their environment. With the mouse model of AE, detailed knowledge about the skin physiology and immune system can be obtained and novel therapies can be evaluated *in vivo* [22].

Up to now, several types of AE mouse models have been reported, including the spontaneous manifestation of AE-like skin lesions in certain strains, transgenic mice, protein sensitization AE-like skin lesions and humanized mouse models of AE [22].

Epicutaneous sensitization with the allergen ovalbumin (OVA) can induce dermatitis, as described by Wang et al. [23], Spergel et al. [24] and our group [25]. OVA, as a protein allergen, can elicit an immune response with a specific character. The induced skin lesions in BALB/c mice exhibit significant epidermal and dermal thickening and a inflammatory cell infiltration, which consists of CD4+ T cells and eosinophils [26]. Elevated serum levels of total and antigen specific IgE and IgG1 as well as increased local expression of IL-4, IL-5 and IFN-γ were detected [22].

Due to the multiple genetic and environmental factors underlying AE, it is not possible to create a mouse model presenting all features of AE [27].

1.2. Immunomodulation by probiotics

In western countries, the prevalence of AE has risen over the past decades. It has been speculated in the hygiene hypothesis that the increase of AE is caused partly by a changed intestinal colonization pattern during infancy [28]. The increased hygienic practices, including more processed food, have an impact on the imprinting of the immune system [28]. This hygienic life style contributes to a reduction of Treg cells with exaggeration of Th2 and Th1 cells [29].

Probiotics are defined as live microorganisms which, when administered in an adequate amount, confer a beneficial health to the host (WHO) [30]. They contribute to human health by improving the microbiotical balance of the gut environment. Probiotics attract increasing interest because of their immunomodulatory properties [31-35].

In the following table the criteria for the use of probiotics in humans are listed:

- Identified at the genus, species and strain level.
- Safe for food and clinical use.
- Able to survive in intestinal environment (acid and bile tolerance).
- Able to colonize the human intestine or vagina (at least temporarily).
- Able to adhere to mucosal surfaces.
- Producing antimicrobial substance.
- Able to antagonize pathogenic bacteria.
- Possessing clinically documented health effects.
- Stable during processing and storage.

Table 1: Criteria for the use of probiotics in humans. Cited with kind permission from Springer Science+Business Media: < J Gastroenterol, Probiotics and immunity, 44, 2009, 26-46, Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME, table 1>.

According to the criteria mentioned above (Table 1), the most commonly used and studied probiotics are lactic acid bacteria (LAB). These include e.g. Lactobacilli and Bifidobacteria. Besides, some non-pathogenic strains of Escherichia coli (E. coli), like

E. coli Nissle 1917 (EcN 1917), belong to probiotics as well [36].

Probiotics are consumed orally. The main system encountering probiotics is the gastrointestinal (GI) tract. In consequence, the interaction with intestinal epithelium becomes the most important link for the further function of probiotics. As shown in Figure 2, probiotics interact with epithelial cells through toll-like receptors (TLRs), thereby increasing the production of chemokines and cytokines. These can regulate the antigen-presenting capacity of DC and activate lymphocytes in gut-associated lymphoid tissue (GALT) [37]. The luminal bacteria can be sampled by DC and macrophages below M cells in the Peyer's patch (PP). During this process, DC maturate, through the direct contact with probiotics, induces the differentiation of naïve T cells via the secretion of cytokines and direct interaction [37,38]. A local immune response or the generation of tolerance is developed by effector T cells in Peyer's patch and mesenteric lymph nodes (MLN) depending on the antigen. This efficacy of antigen translocation depends on the bacterial strain and is influenced by the amount of the intestinal flora [39]. In this process, DCs have been shown to be essential for the immunomodulatory effects of probiotics. It is supposed that the probiotics induce Treg cells through DC activation via binding to the lectin DC-SIGN (DC specific intercellular adhesion molecule 3-grabbing nonintegrin) [40].

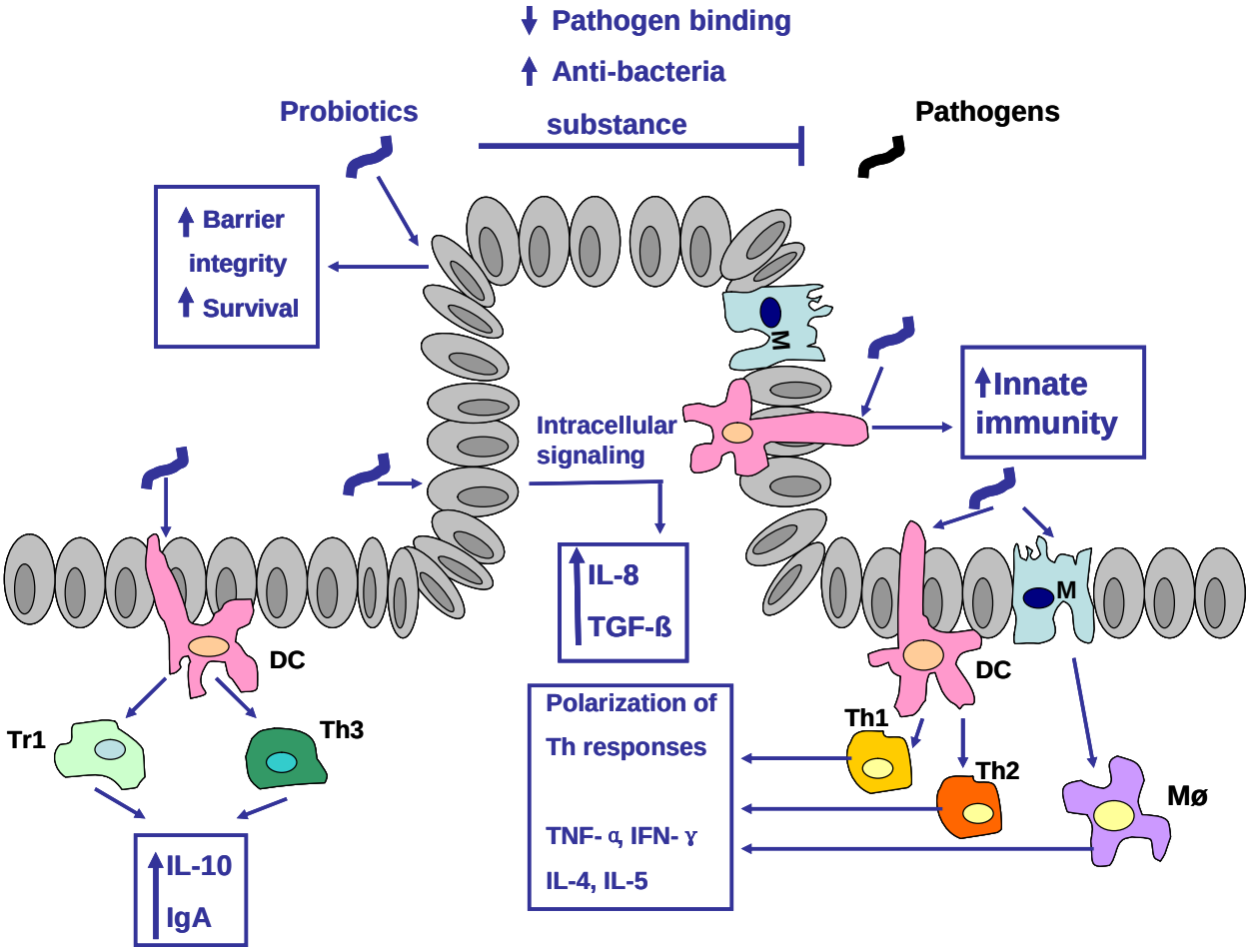

Figure 2: Pathways of action of probiotic bacteria via the intestinal mucosa. Modified from: 1. Corthesy B, Gaskins HR, Mercenier A. Cross-talk between probiotic bacteria and the host immune system. J Nutr 2007;137:781S-790S [38]. 2. Vanderpool C, Yan F, Polk DB. Mechanisms of probiotic action: Implications for therapeutic applications in inflammatory bowel diseases. Inflamm Bowel Dis 2008;14:1585-1596 [41].

Several mouse, but also human studies demonstrated that in particular IgA concentrations were elevated upon administration of probiotics [32,42-44]. Increased secretory IgA plays a critical role for mucosal immunity by providing protection against pathogens [45].

Several observations have led to the assumption that probiotics might be useful in the prevention or treatment of allergy e.g. by restoration of the Th1/Th2 imbalance. It has been proposed that probiotics can regulate the production of cytokines which play an

important role in reinstating the homeostasis [31-35]. In *in vitro* studies of allergic diseases, probiotics were found to suppress Th2 cytokines by stimulating Th1 cytokines, like IFN-γ and IL-12. However, *in vivo* studies have proven that these effects are more likely strain-dependent [32,46,47].

Another factor for the immunomodulation through bacteria is the promotion of CD4+Foxp3+ Treg cells which were found to be significantly increased upon probiotic administration [48,49]. It was suggested that Tregs in circulation transfer to draining lymph nodes, and then recruit to skin, contributing to the downregulation of cutaneous immune responses [50]. Their functional cytokines, IL-10 and TGF-ß, also contribute to the anti-inflammatory and immunomodulatory effects [51,52]. CD4+CD25+Foxp3+ cells are reduced in AE skin, which has been linked to a dysregulation of effector T cells [19]. These findings suggest that the promotion of Foxp3+ Treg cells by probiotics may be beneficial in AE.

1.3. EcN 1917

EcN 1917 was first isolated by Professor Alfred Nissle from the faeces of one soldier who did not develop an enterocolitis in the battlefield [53]. From then on, this new strain of E. coli, alias SK22 or DSM6601, was analyzed in many studies regarding its beneficial effects on human health [54-56].

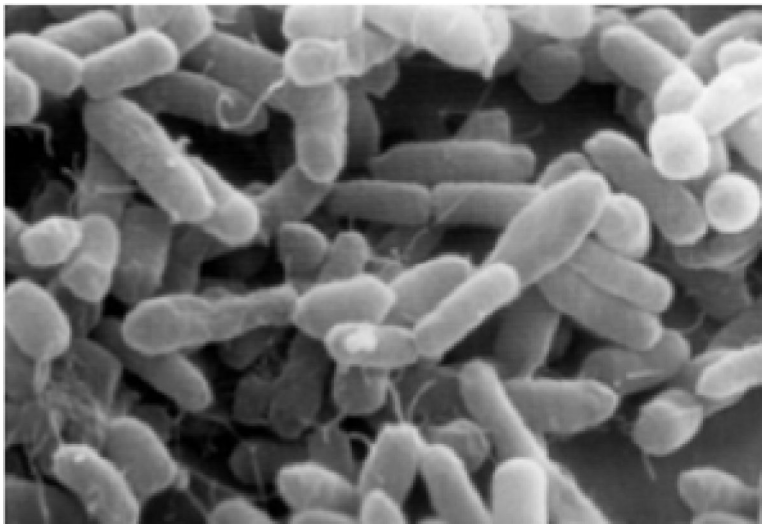

Figure 3: Electron micrograph of EcN 1917 at x 10000 magnification. Reprinted with kind permission of “mutaflor-the probiotic drug for life”.

1.3.1. Hallmarks of EcN 1917

EcN 1917, serotyping of O6: K5: H1, is a gram-negative strain of probiotics. As a part of gut flora, it can adhere to the intestine and antagonize other bacteria. In this regard, EcN 1917 presents features of probiotic bacteria by definition (Table 1).

Lipopolysaccharide (LPS) is an essential component of the outer membrane of gram-negative bacteria, consisting of lipid A, the oligosaccharide core and generally a long-chain polysaccharide O-antigen. EcN 1917 has a point mutation of the O-antigen polymerase wzy gene which results in a special “repeating unit” O-antigen. The core is directly connected to this special “repeating unit” O-antigen, instead of being connected by a long chain. This so-called semi-rough O6 LPS phenotype is responsible for the serum sensitivity and its ability of immunomodulation [57]. The serum sensitivity is

essential to eliminating the risk of sepsis [58].

A capsule protects many extraintestinal pathogenic E. coli strains from non-specific defense mechanisms, like phagocytosis and complement activation, which contribute to the serum resistance [59]. This is not the case with EcN 1917 which shows sensitivity due to the “semi-rough” LPS. EcN 1917 presents a capsule of the K5 type [60]. This K5 type capsule leads to a specific chemokine response following the interaction with gut epithelial cells [60]. This may contribute to the immunomodulatory effects of EcN 1917.

EcN 1917 possesses 3 different fimbriae, including F1A (type 1), F1C and a kind of so called “curli” fimbriae, contributing to the stable adhesion to epithelium and biofilm formation. F1C was even proven to contribute to the persistence in infant murine colonization [61]. In addition, these fimbriae take the place of the P- and S- fimbriae belonging to mannose-resistant hemagglutination (MRHA) attachment factors which are typically presented in pathogenic E. coli strain [58,61]. The absence of haemolysin and cytotoxic necrotizing factor (CNF) production also contributes to the non-pathogenic feature of EcN 1917 [58].

The iron uptake system and microcin are essential to antagonizing other bacteria, thereby acting as their “fitness factor”. EcN 1917 is shown to mainly produce microcins M and H47 which can inhibit pathogenic bacteria [62]. Since iron is essential for the important metabolism processes, 6 kinds of iron acquisition systems of EcN 1917 can assist to utilize the surrounding Fe^{3+} (enterobactin, yersiniabactin, aerobactin, salmochelin, ferric dicitrate transport system and the *chu* heme transport locus) [62-64].

The genomic peculiarities, including the lack of virulence factors, were detected in comparison to other E. coli strains [63].

All of these properties contribute to the probiotic nature of EcN1917. After successful competition with other bacteria and steady colonization in gut, EcN 1917 starts to interact with intestinal epithelial cells and to develop further features, like immunomodulation.

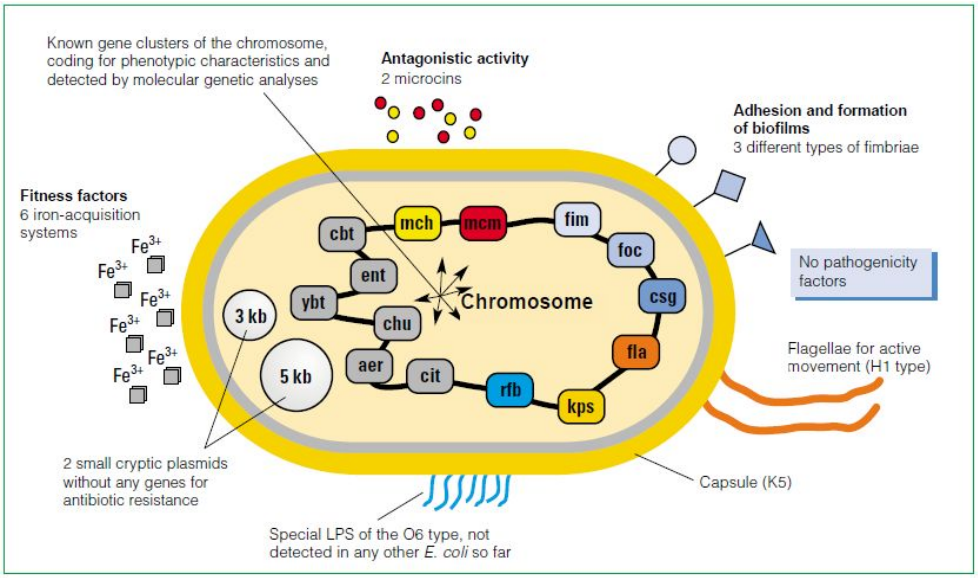

Figure 4: Phenotypical characteristics of EcN 1917 and its gene loci on the bacterial chromosome. Reprinted with kind permission of “mutaflor-the probiotic drug for life”.

1.3.2. Immunomodulation via EcN 1917

EcN 1917 has been used as a probiotic treatment for several intestinal immune disorders for decades, like inflammatory bowel disease (IBD) including ulcerative colitis and Crohn’s disease. This indicates that EcN 1917 exerts an immune regulating effect [65].

With respect to allergic diseases, EcN 1917 was rarely studied in models of hypersensitivities. Whereas in an allergen-induced Th2 response of airway inflammation, EcN 1917 was found to ameliorate severity by inducing a Th1 skewing cytokine pattern without affecting IgE production [66].

Most of the studies were designed to investigate the mechanisms of immunomodulation by EcN 1917 with an increased secretion of IgA [67], an alteration of pro- and anti-inflammatory cytokine production [68-72] and a modulation of T-cell expansion [73]. Non-pathogenic E. coli can inhibit degranulation of mast cells [74]. In addition, EcN 1917 has been shown to promote the production of ß-defensin 2 by flagellin via NF-κB- and AP-1-dependent pathways [75,76].

2. Objective

Due to the increased hygiene practices, the impact of the microflora on the immune system during infancy has been weakened. The use of bacterial supplementation has attracted considerable interest, since the composition of the intestinal microflora is different in humans with or without AE [77]. Therefore a supplementation with probiotics has been suggested as an innovative way to prevent or to treat AE.

Different strains of probiotics were applied in human and murine AE studies in order to determine their efficacy and their mechanisms [52,78,79]. However, no data is available about the impact on the immune response in an allergic skin model upon EcN 1917 administration. Orally administrated EcN 1917 is a potential way to prevent AE through immunomodulation in a local and/or systemic manner. Therefore in this thesis the preventive efficacy of oral EcN 1917 supplementation on the outcome of allergen-induced eczema was investigated. The following questions were addressed:

1) Does oral administration of ECN 1917 improve the outcome of allergen-induced eczema?
2) Is the local immune response in the skin altered by oral ECN 1917 administration?
3) What is the impact of EcN 1917 supplementation on development and outcome of the systemic immune response?
4) What are the mechanisms by which EcN 1917 supplementation acts on allergen-induced eczema?

3. Materials and methods

3.1. Materials

Reagents are listed in the appendix.

3.2. Methods

3.2.1. Mice sensitization and treatment

6-8-week-old female BALB/c mice (BfR, Berlin, Germany) were maintained under specific pathogen free conditions with standard laboratory food and water. The experiment was registered at the Landesamt für Gesundheit und Soziales Berlin (registration number G0040/08). As depicted in Figure 5, EcN 1917 (kindly provided by Ardeypharm GmbH, Germany) was supplemented preventively via oral feeding with daily amounts of 10^8 CFU in the high concentration subgroup (high EcN), 10^7 CFU in the low concentration subgroup (low EcN) and an electrolyte solution as the treatment control group (control).

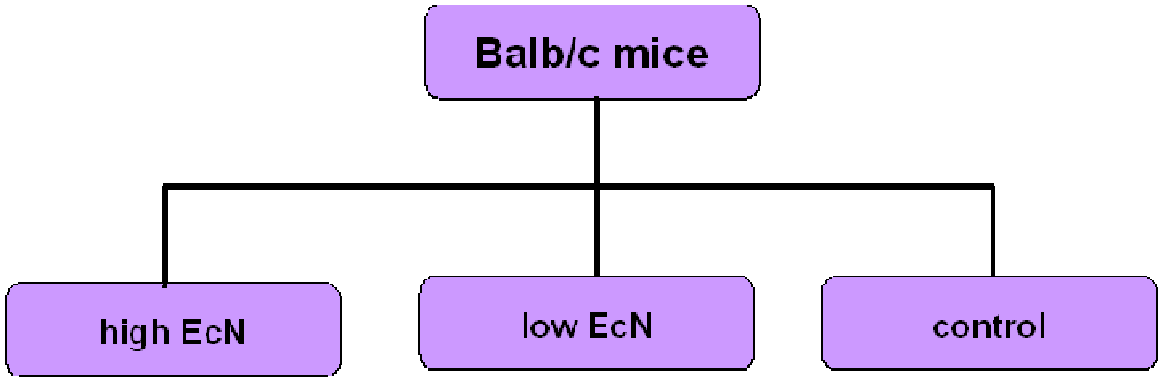

Figure 5: Treatment groups.

As shown in Figure 6, mice were sensitized intraperitoneally (i.p.) on days 1 and 14 with 100 µl of 10 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ (Alum). Epicutaneous (e.c.) allergen application was performed with OVA or with PBS as patch control. Each mouse was

patched by 3 times repeated application of OVA, placed for 1 week on the belly skin at 2-week intervals. Preventive EcN 1917 treatment was started 21 days before the first sensitization and was stopped at day 14. At day 64, mice were sacrificed and skin, spleen and blood samples were collected for further analysis.

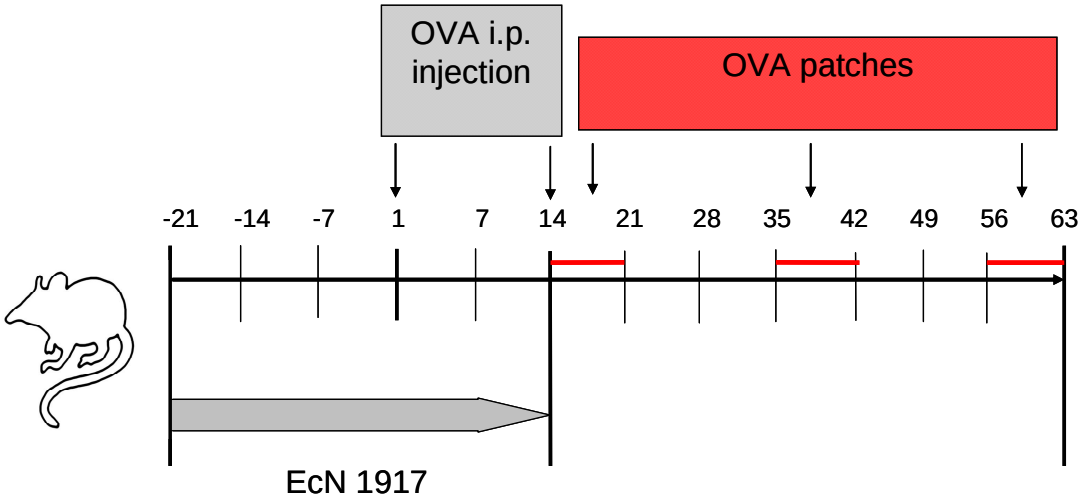

Figure 6: Induction of allergen-induced dermatitis and time schedule of EcN 1917 treatment. Systemic sensitization was performed on days 1 and 14, followed by three 1-week allergen patches. EcN 1917 supplementation was given from day -21 until day 14.

3.2.2. Clinical skin features

To evaluate the severity of allergen-induced lesions, a standardized skin scoring system consisting of 4 parameters (erythema, crusts, dryness and extension) was used. This clinical skin score (CSS) is based on the clinical features of human AE [80]. For calculating total CSS, the marked lesions were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) by 6 independent persons. The score ranged from 0 to 12. The total score was taken as an index of dermatitis severity.

3.2.3. Histological analysis

For histological analysis, 6 µm sections of skin samples were stained with hematoxylin for 20 seconds at room temperature (RT). Thickness of the epidermis was determined by Axiovision measuring-tools on the Axioplan light microscope at x 100 magnification and measured 5 times per mouse on average including the thickest and thinnest parts (Figure 7). The measurements were expressed in micrometers.

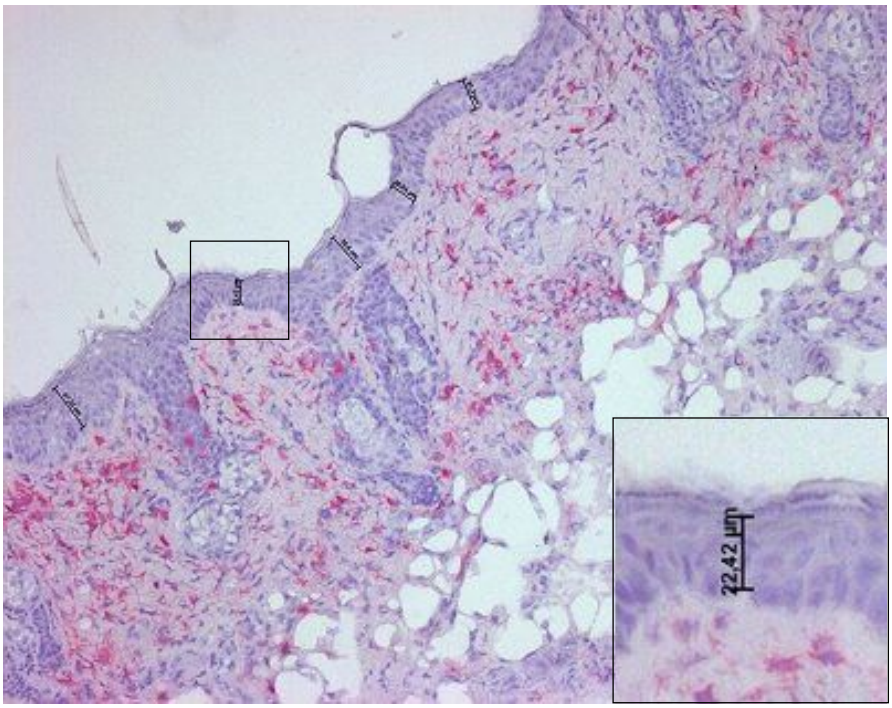

Figure 7: Example to demonstrate the epidermal thickness measurement.

3.2.4. Immunohistochemistry (IHC)

The area of sections with positively stained cells and the numbers of positive cells were calculated, evaluating positive cells per unit area (n/mm^2). If not mentioned, all procedures were performed at RT.

3.2.4.1. Staining of CD4+/CD8+ cells by IHC

Sections measuring 6 µm were prepared and used for staining by the streptavidin–biotin complex method.

Sections were blocked with 5% goat serum in tris-buffered saline (TBS) for 20 minutes and with avidin/biotin blocking kit for another 30 minutes. Afterwards, they were incubated with the rat anti-mouse CD4 or CD8 antibody for 1 hour, followed by a 30-minute incubation with anti-rat immunoglobulin (Ig)-biotin. Signals were detected by alkaline phosphatase/red detection kit containing streptavidine AP and CHROM/fast red. In the last step, sections were counterstained with hematoxylin. Between the staining steps, TBS washing steps were performed. CD4/CD8 antibody and anti-rat Ig-bio were diluted in reagent diluent. All the procedures were done at room temperature. Positively stained cells were counted at x 100 magnification and expressed as cells per mm^2.

3.2.4.2. Staining of mast cells

Skin sections were stained with 0.1% toluidine blue. Positively stained cells were counted at x 100 magnification and expressed as cells per mm^2.

3.2.4.3. Detection of Foxp3+ cells

The skin kept in formalin was dehydrated and hardened in an automatic tissue processor. Afterwards the paraffin-embedding of skin was carried out on a Tissue Tek embedding centre. For immunostaining, the sections were deparaffinized and subjected to a heat-induced epitope retrieval step before incubation with antibodies. Sections were immersed in sodium citrate buffer at pH 6.0 and heated in a high-pressure cooker. The slides were rinsed in cool running water, washed in TBS, blocked using a commercial peroxidase-blocking reagent and incubated with the rat antibody against Foxp3, followed by a secondary rabbit anti-rat antibody and the EnVision peroxidase kit against rabbit antibodies. Positively stained cells were counted at x 200 magnification and expressed as cells per mm^2.

3.2.5. Serum antibody levels

OVA-specific IgE, IgA, and total IgE levels were measured by ELISA. If not mentioned, all procedures were performed at RT.

3.2.5.1. Total IgE ELISA

Briefly, 96-well microtiter plates were coated overnight at 4^{o}C with sodium carbonate buffer containing anti-mouse IgE monoclonal antibodies (mAb), which was blocked for 2 hours in the following step. Afterwards, serum and standards were diluted and incubated overnight at 4^{o}C. The amount of bound antibody was determined with biotin-conjugated anti-mouse IgE, followed by application of streptavidin–horseradish peroxidase (HRP) and the 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution. Plates were washed with washing buffer 3 times between every two steps. Plates were read at 450 nm.

3.2.5.2. OVA-specific IgE ELISA

After overnight coating with anti-mouse IgE mAb at 4^{o}C, the plates were blocked with BSA in PBS for 2 hours. Serum samples and standards were diluted with BSA in PBS and incubated for 2 hours. Biotin-conjugated OVA were applied for detection, followed by treatment with streptavidin–HRP and with the TMB solution. Plates were read at 450 nm. Serum titers were expressed as laboratory units (LU) of serum dilution.

3.2.5.3. OVA-specific IgA ELISA

Procedures were basically similar to the ELISA of OVA-specific IgE. Plates were coated with OVA and blocked with skimmed milk. Serum samples and standards were diluted with skimmed milk and biotin-conjugated anti-mouse IgA was added for detection. Serum titers were expressed as laboratory units of serum dilution.

3.2.6. Splenocyte culture

The concentration of cell suspension was adjusted to 2 x 10^{6} cells/ml and stimulated with PMA/Ionomycin or OVA/anti-CD28. RPMI 1640 medium was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 µM glutamine and 10% fetal calf serum (FCS). For the stimulation with PMA/Ionomycin, final concentration was 20 ng/ml PMA and 2X10^{-7} M Ionomycin. Cells were cultured at 37°C in humidified air containing 5% CO_2 for 48 hours.

For stimulation with OVA/anti-CD28, 250 µg/ml OVA and 5 µg/ml anti-CD28 were used. Cells were cultured for 4 days. After the incubation time, supernatants were aliquoted after centrifugation at 340 x g and stored at -80 ^{o}C until further analysis.

3.2.7. Cytokine ELISA

Cytokines from splenocyte cultures were detected by ELISA. If not mentioned, all procedures were performed at RT.

3.2.7.1. IL-4, IFN-γ and IL-10 ELISA

96-well microplates were incubated with diluted capture antibody overnight and then blocked with blocking buffer for 1 hour. Afterwards, samples and standards were incubated for 2 hours. The incubation of detection antibody lasted for 2 hours, followed by streptavidine-HRP incubation for 20 minutes (avoiding direct light). In the last step, substrate solution was supplied and stopped after 5 minutes.

Washing with washing buffer was carried out between every two steps. Capture antibody was diluted in PBS. Standards, detection antibody and streptavidine-HRP were diluted in reagent diluent.

3.2.7.2. TGF-β ELISA

The procedures were similar to the previously described cytokine ELISA protocol except for the activation of samples. To activate the latent TGF-ß to immunoreactive TGF-ß, 100 µl samples were stimulated with 20 µl 1 N HCl for 10 minutes and neutralized by adding 20 µl 1.2 N NaOH/0.5 M HEPES.

3.2.8. RNA isolation from skin samples

Skin tissues were disrupted by pestle or ultratorax for homogenization. RNA isolation was performed by Nucleospin RNA II kit. Subsequently, 500 µl Buffer RA1 (lysis buffer), 5 µl ß-mercaptoethanol and 10% proteinase K were added for digestion of proteins. After

1 hour the lysate was transferred to NucleoSpin filter columns and centrifuged for 1 minute. The supernatant without any formed pellet was mixed with 500 µl ethanol (70%) for adjusting RNA binding conditions. Afterwards lysate was loaded onto Nucleospin RNA II column placed in a collection tube and centrifuged for 30 seconds. After adding membrane desalting buffer (MDB), centrifugation was applied to dry the column membrane. After DNase degradation centrifugation was performed and finally RNA was eluted into 40 µl of RNase-free water. The centrifugations were carried out at 11000 x g. All steps were performed at RT.

3.2.9. cDNA synthesis

cDNA synthesis was performed by Taq Man reverse transcription kit.

3.2.10. Quantitative PCR

Real-time qPCR was performed for gene expression quantification. After repeated cycles the target gene fragment is amplified.

Real-time qPCR uses a fluorescent probe, which can bind the double strands of DNA. After binding to DNA, the sample emits a much stronger fluorescent signal than when it is free. Thus, the fluorescence increases due to the PCR product accumulation. Double determinations were performed. Primers were designed using the internet databases UCSC, NCBI and online software Primer3.

The level of target gene expression was normalized to a housekeeping gene to correct the variances in RNA isolation, cDNA synthesis and efficiency of amplification. In this experiment, hypoxanthine guanine phosphoribosyltransferase (HPRT) was used.

The relative expression ratio (R) of a target gene is based on the efficiency (E) of the primer pairs and the crossing point value (Cp). It is expressed in comparison to the reference gene.

$$R = \frac{(E_{reference})^{\Delta Cp\ reference}}{(E_{target})^{\Delta Cp\ target}}$$

3.3. Statistical analysis

Statistical analysis and figures were done with “Graphpad Prism”. The non-parametrical, unpaired data were analyzed by non-parametric “Mann-Whitney U test”. All data are presented as median [min-max], except the CSS data which are presented as mean±SD due to their normal distribution. The statistical significances were shown as p-values. A p-value < 0.05 was considered to be statistically significant.

4. Results

4.1. Preventive EcN 1917 improved the clinical symptoms of allergen-induced eczema

The allergen patch induced a visible dermatitis which showed clinical signs of eczema, like dryness, erythema and excoriation. In addition, the extent of the lesions was considered as well. The administration of EcN 1917 ameliorated the clinical outcome in a dose-dependent manner, as the high concentration of EcN 1917 was more effective in reducing the clinical skin score compared to the low concentration (Figure 8a).

For objective evaluation, CSS was used as previously described [25]. The control group of preventive treatment reached a mean±SD CSS of 5.3±2.0 points. After the supplementation of high EcN, the skin score was reduced to a mean±SD of 3.6±1.2 ($p=0.032$) (Figure 8b). No statistical significance was determined between low EcN group and the control group.

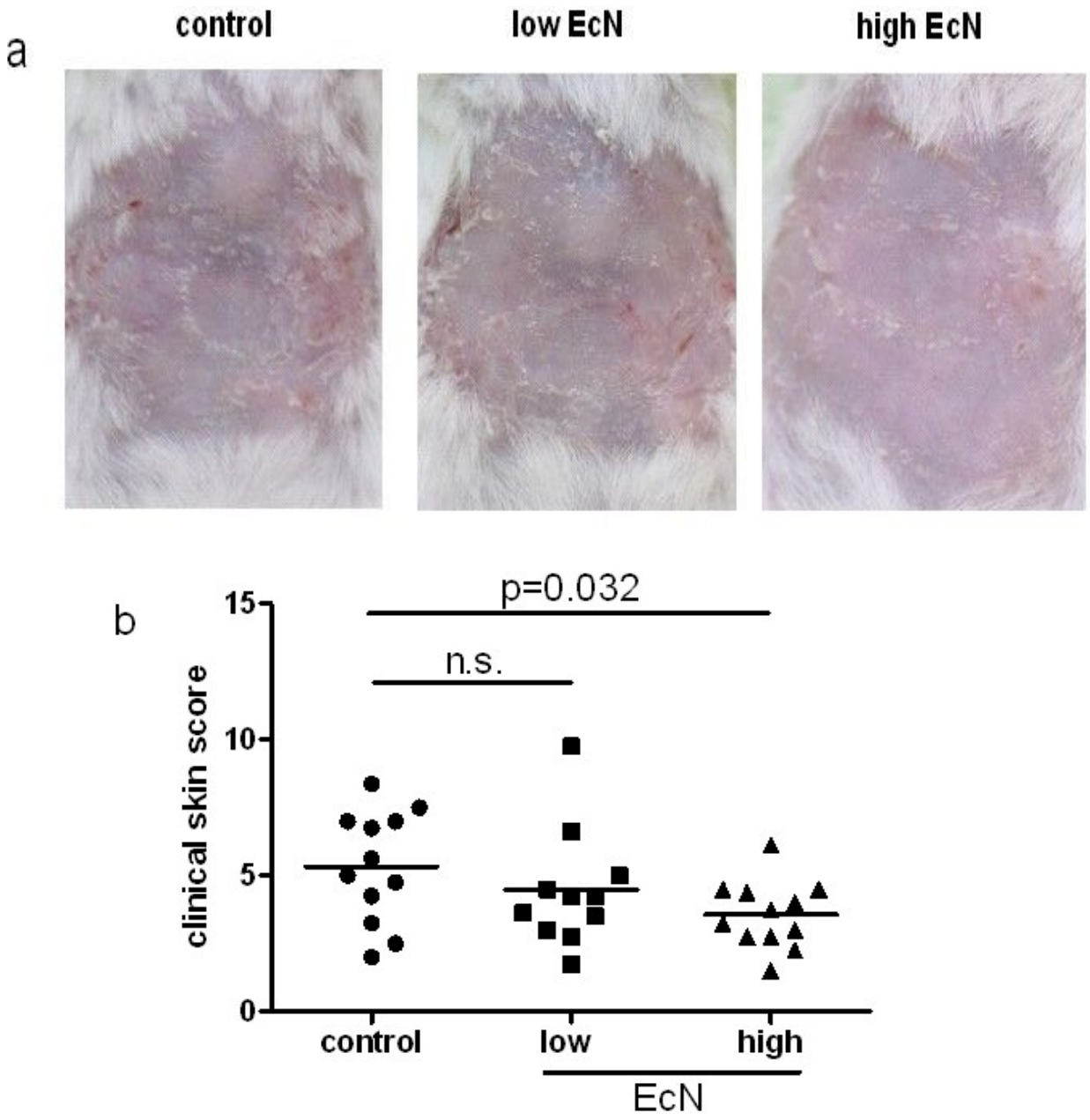

Figure 8: Clinical outcome of allergen-induced eczema upon preventive EcN 1917 supplementation. (a) In comparison to the control group, the high EcN supplemented group had reduced clinical severity of allergen-induced dermatitis. (b) The CSS were evaluated via the indicated 4 parameters and the mean was calculated in each group. Data are shown as single value with the mean as a bar. The statistical significance (p) is based on Mann-Whitney U test in comparison to control group (n>11).

4.2. High EcN supplementation resulted in a modulation of the local immune response

The local immune response within the eczematous skin lesion was studied by measurement of the epidermal thickness and the infiltration of inflammatory cells. This analysis was performed in the control and high EcN supplementation groups, as the clinical skin score was significantly different in these groups only.

In the high EcN supplementation group, the epidermal thickness was decreased upon the preventive treatment, from a median [min-max] thickness of 45.3 [29.5-65.2] µm in the control group to 36.8 [19.9-50.1] µm in the high EcN group (p=0.046) (Figure 10a).

To determine the cellular inflammation, infiltration of CD4+, CD8+ and mast cells was assessed in the sections of lesional skin (Figure 9).

CD4+ cells mainly appeared in the dermis. In the control group, infiltrated CD4+ cells reached a median [min-max] of 618.1 [416.8-994.9] per mm^2. By contrast, in the high EcN group fewer CD4 cells were observed (452.4 [326.2-511.2] per mm^2) (p=0.004). This indicated that high EcN supplementation led to a reduced infiltration of CD4+ cells in the preventive treatment group (Figure 10b).

CD8+ cells reached a median [min-max] of 263.4 [70.7-763.8] per mm^2 in the control group and 86.6 [20.4-469.1] per mm^2 in the high EcN group, respectively. However, we did not observe that CD8+ cells of high EcN supplemented mice were significantly different from the control group. However, a tendency of a reduction was observed upon preventive high EcN supplementation (Figure 10c).

Mast cells were mostly located in the dermis and among the subcutaneous tissue. Mast cell numbers were 89.4 [43.9-137.7] per mm^2 in high EcN treated mice and were significantly more numerous in the control group (p=0.001). The mast cell number in the control group was 133.7 [95.6-248.2] per mm^2 (Figure 10d).

In high EcN supplemented mice Foxp3+ cells reached a median [min-max] of 204.9 [77.7-355.6] per mm^2. Their number tended to increase in comparison to the control mice (149.7 [69.2-258.3] per mm^2), but did not reach statistical significance (Figure 11c). Interestingly, when the Foxp3+ cells were related to CD4+ cells, the ratio achieved 0.4 [0.2-0.7] in the high EcN group and 0.3 [0.1-0.6] in the control group, respectively. This

difference was statistically significant (p=0.005) (Figure 11d) and suggested a higher proportion of Foxp3+/CD4+ cells in high EcN mice compared to the control mice.

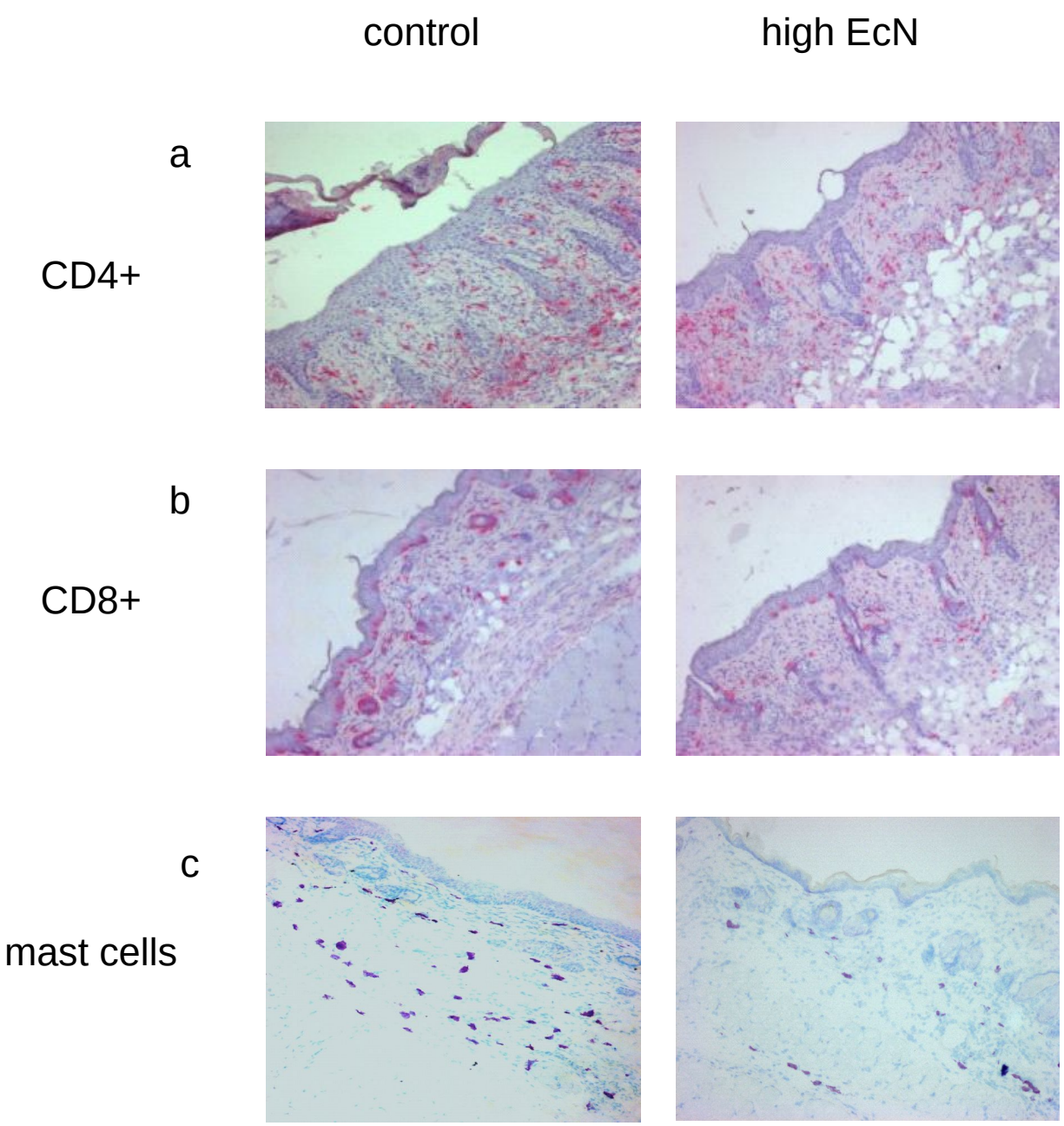

Figure 9: Cellular infiltration profile. The skin was cut into 6 µm sections and the inflammatory cells were stained. CD4+ cells (a), CD8+ cells (b) and mast cells (c) are shown. Pictures were taken at a magnification x 100.

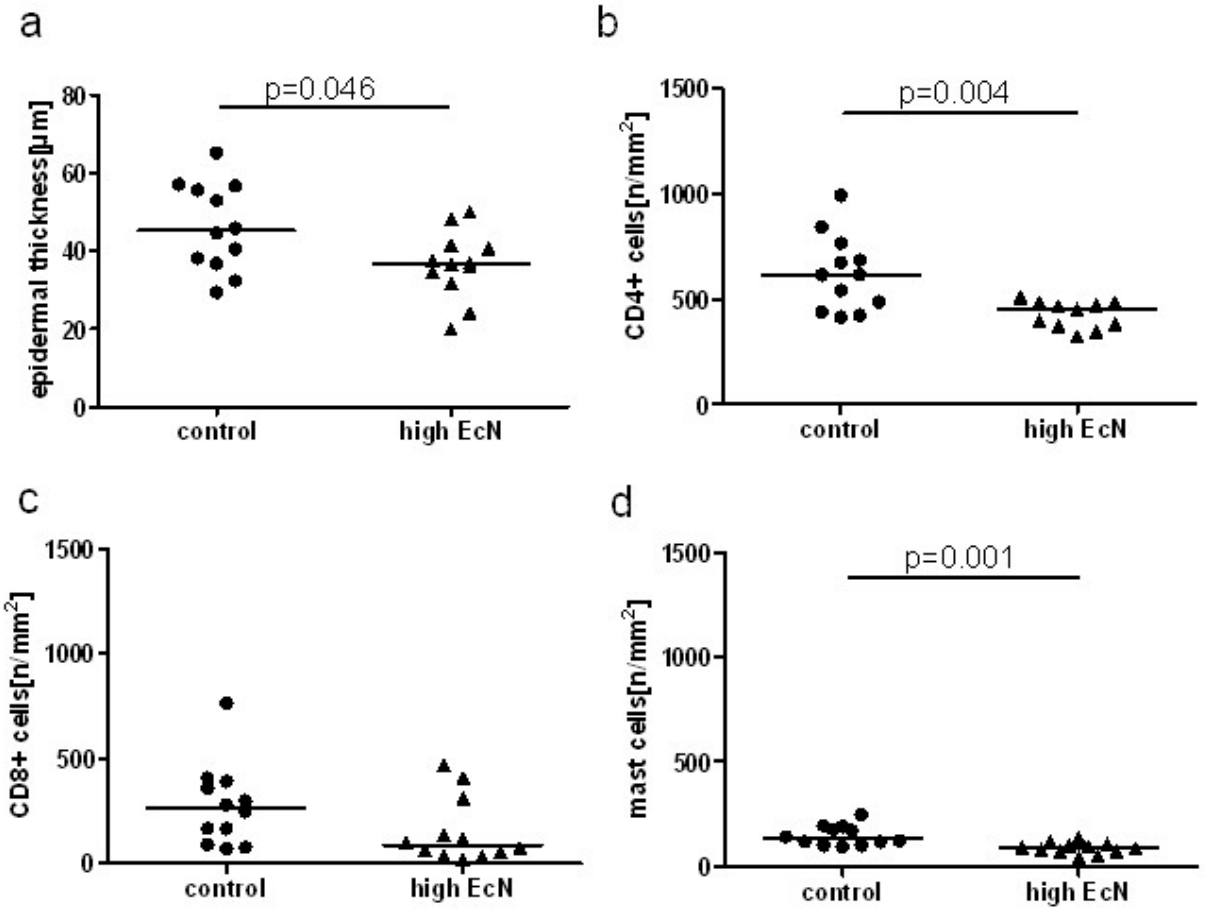

Figure 10: Epidermal thickness and the numbers of infiltrating cells in the dermis. The epidermal thickness (a) as well as the infiltrating cells (b, c and d) was determined in the preventive high EcN and the control group. Data are shown as single values with the median as a bar. The statistical significances (p) are based on Mann–Whitney U test in comparison to control group (n>11). Only if statistical difference was reached is p depicted.

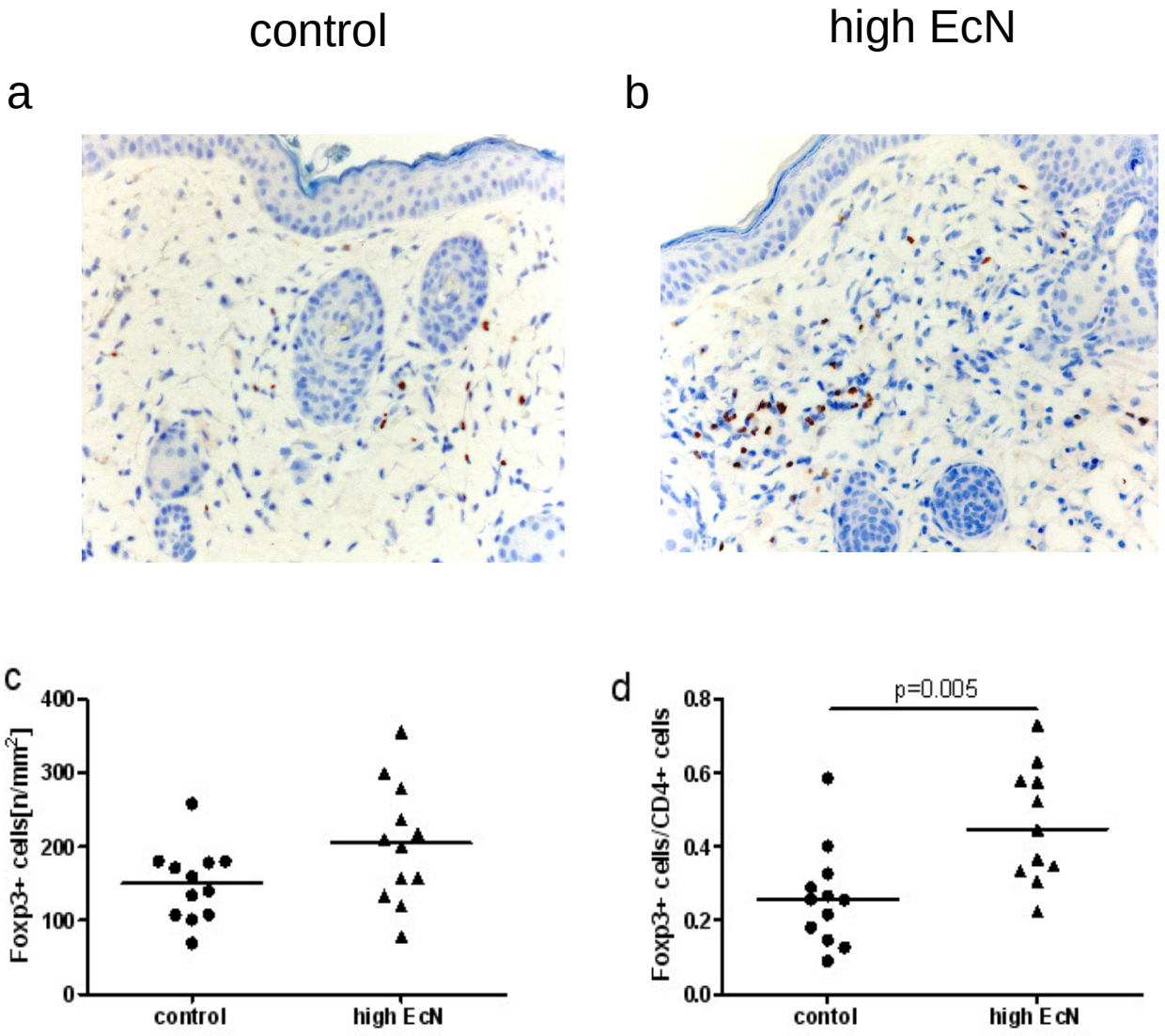

Figure 11: Number of Foxp3+ cells in lesional skin. Pictures were taken at a magnification x 200 (a, b). Foxp3+ cells (c) and Foxp3+ cells related to CD4+ cells (d) are shown. Data are presented as single values with the median as a bar. The statistical significance (p) is based on Mann–Whitney U test in comparison to the control group (n>11). Only if statistical difference was reached is p depicted.

4.3. Oral EcN 1917 administration increased systemic IgA response

In order to detect a potential systemic immunomodulation by EcN 1917, OVA-IgA, total IgE and OVA-IgE were analyzed by ELISA. OVA-IgA reached a median [min-max] concentration of 1542 [147.5-3614] LU/ml in high EcN group, 333.0 [27.4-1558] LU/ml in low EcN group and 273.8 [132.5-2069] LU/ml in the control group. This result in the high EcN group regarding IgA was significantly increased in comparison to the control group (p=0.028) (Figure 12).

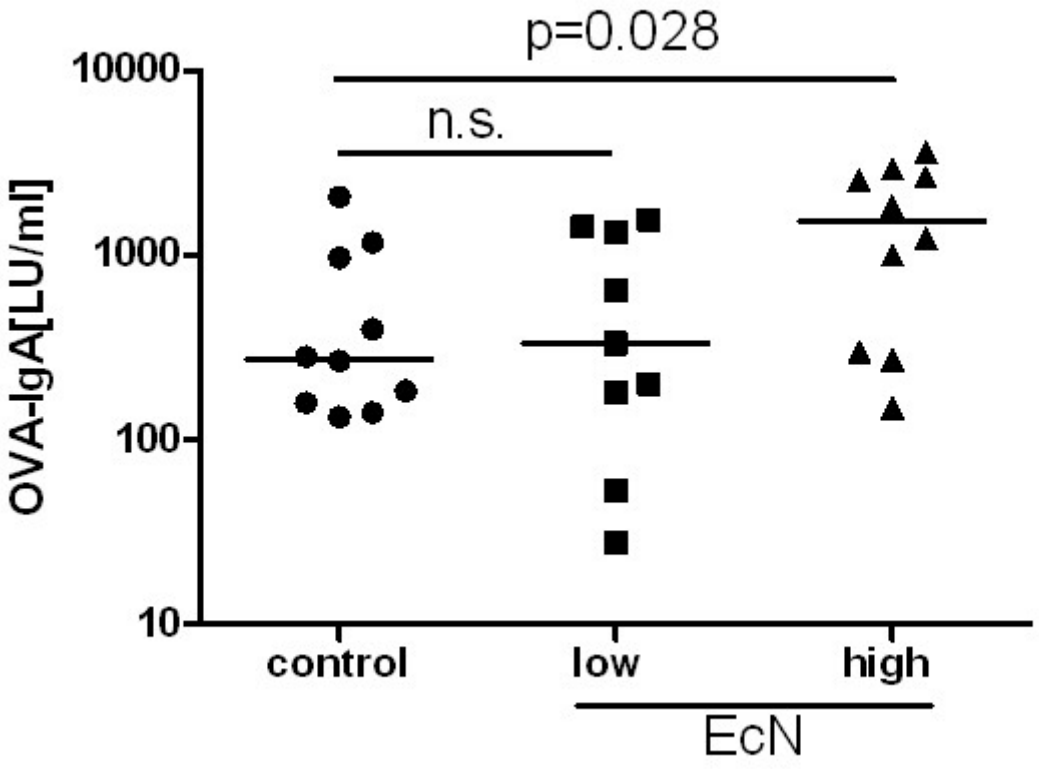

Figure 12: OVA-IgA response. OVA-IgA was analyzed by ELISA. High EcN administration resulted in a significant enhancement of the OVA-IgA production. Data are presented as single values with the median as a bar. The statistical significance (p) is based on Mann–Whitney U test in comparison to the control group (n>9).

Nevertheless, neither an alteration on total IgE nor on OVA-IgE was determined after EcN 1917 administration (Figure 13).

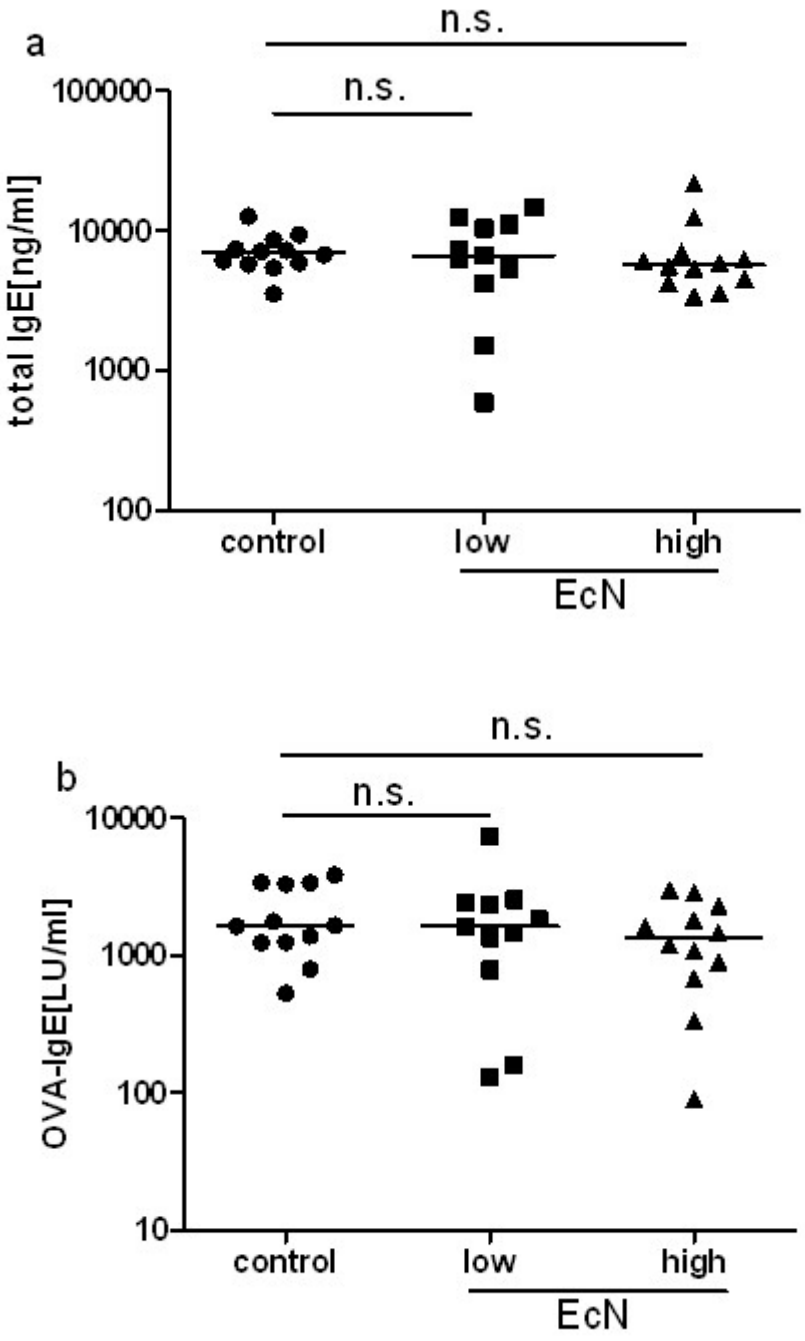

Figure 13: IgE responses in EcN 1917 supplemented and the control groups. Total IgE (a) and OVA-IgE (b) levels were analyzed by ELISA. Data are presented as single values with the median as a bar (n>11).

4.4. Cytokine profile

The responses of *ex vivo* restimulated T cells were detected in terms of cytokine production in splenocyte cultures. Cytokines produced by Th1 and Th2 cells including IL-4 and IFN-γ were measured (Figure 14). But also the regulatory cytokines like IL-10 and TGF-ß were determined by ELISA (Figure 15).

Splenocytes were cultured with either PMA/Ionomycin or OVA/anti-CD28. The cells stimulated with PMA/Ionomycin were considered as positive controls. OVA activates allergen-specific T cells and in the presence of the costimulatory signal provided by anti-CD28 an allergen-specific cytokine response can be measured. These stimulations significantly increased the secretion of the detected cytokines, except TGF-ß which tended to increase.

In PMA/Ionomycin-stimulated cell cultures, the concentration of IL-4 reached a median [min-max] value of 90.0 [72.3-120.5] pg/ml in the high EcN group and 102.4 [78.4-133.0] pg/ml in the control group. With respect to IL-10, a median [min-max] concentration of 41.0 [26.3-52.3] pg/ml in the high EcN group and 41.7 [24.7-101.8] pg/ml in the control group was observed. No remarkable differences between the high EcN and the control group regarding IL-4 and IL-10 secretion were detectable, but a decreased tendency of IL-4 production was observed. The high EcN group showed a reduced production of IFN-γ (2112 [925.6-2618] pg/ml) compared to 3087 [2010-4777] pg/ml in the control group ($p=0.003$). In contrast, TGF-ß showed a median [min-max] concentration of 144.2 [95.4-249.4] pg/ml in the high EcN group and 87.2 [48.4-238.4] pg/ml in the control group. Thus, a tendency of increased TGF-ß production was determined in the preventive high EcN group in comparison to the control group. These differences were observed in both unstimulated and stimulated samples.

After OVA/anti-CD28 stimulation, the concentration of IL-4 reached a median [min-max] value of 132.1 [4.1-315.7] pg/ml in the high EcN group and 311.3 [61.0-502.6] pg/ml in the control group. The concentration of IL-4 was significantly reduced in preventive high EcN mice ($p=0.014$). The high EcN group also showed a reduced production of IFN-γ (79.8 [0.0-234.0] pg/ml) compared to 151.5 [14.0-401.1] pg/ml in the control group ($p=0.03$). With respect to IL-10, the median [min-max] was 74.0 [29.2-310.6] pg/ml in the

high EcN and 47.9 [12.9-139.2] pg/ml in the control group. IL-10 production in the high EcN group was elevated compared to the control group (p=0.05). TGF-ß showed a median [min-max] of 589.0 [401.8-694.7] pg/ml in the high EcN group and 511.7 [327.2-654.7] pg/ml in the control group. A tendency of increased TGF-ß was observed in the high EcN group. By contrast, the basal TGF-ß secretion was higher in the high EcN group compared to the control group as well.

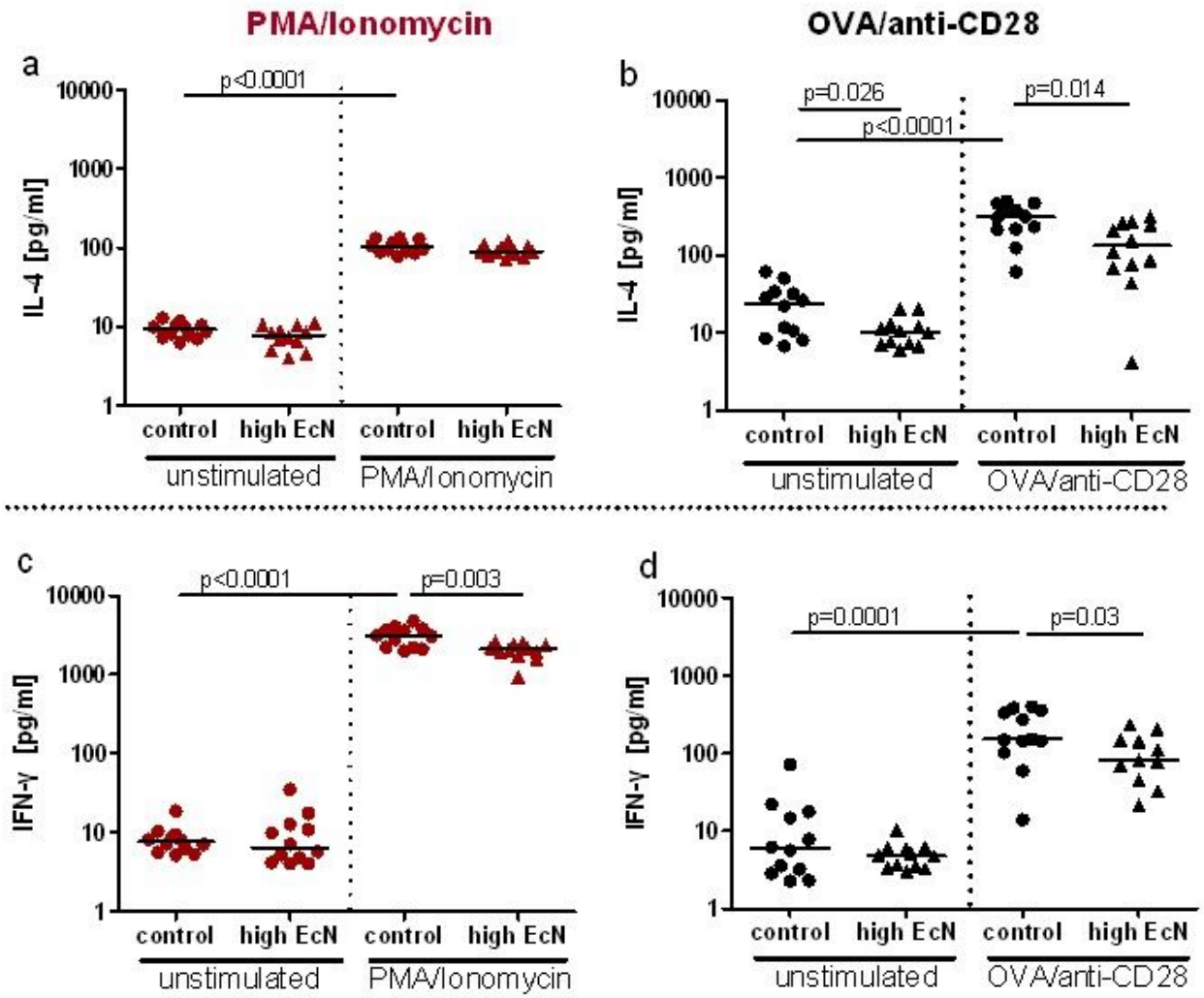

Figure 14: IL-4 and IFN-γ production upon two different stimulations (PMA/Ionomycin or OVA/anti-CD28). Splenocyte cultures were stimulated with PMA/Ionomycin (a, c) or OVA/anti-CD28 (b, d). IL-4 (a, b) and IFN-γ (c, d) concentrations were determined by ELISA. Data are presented as single values with the median as a bar. The statistical significances (p) are based on Mann–Whitney U test in comparison to control group (n=12). Only if statistical difference was reached is p depicted.

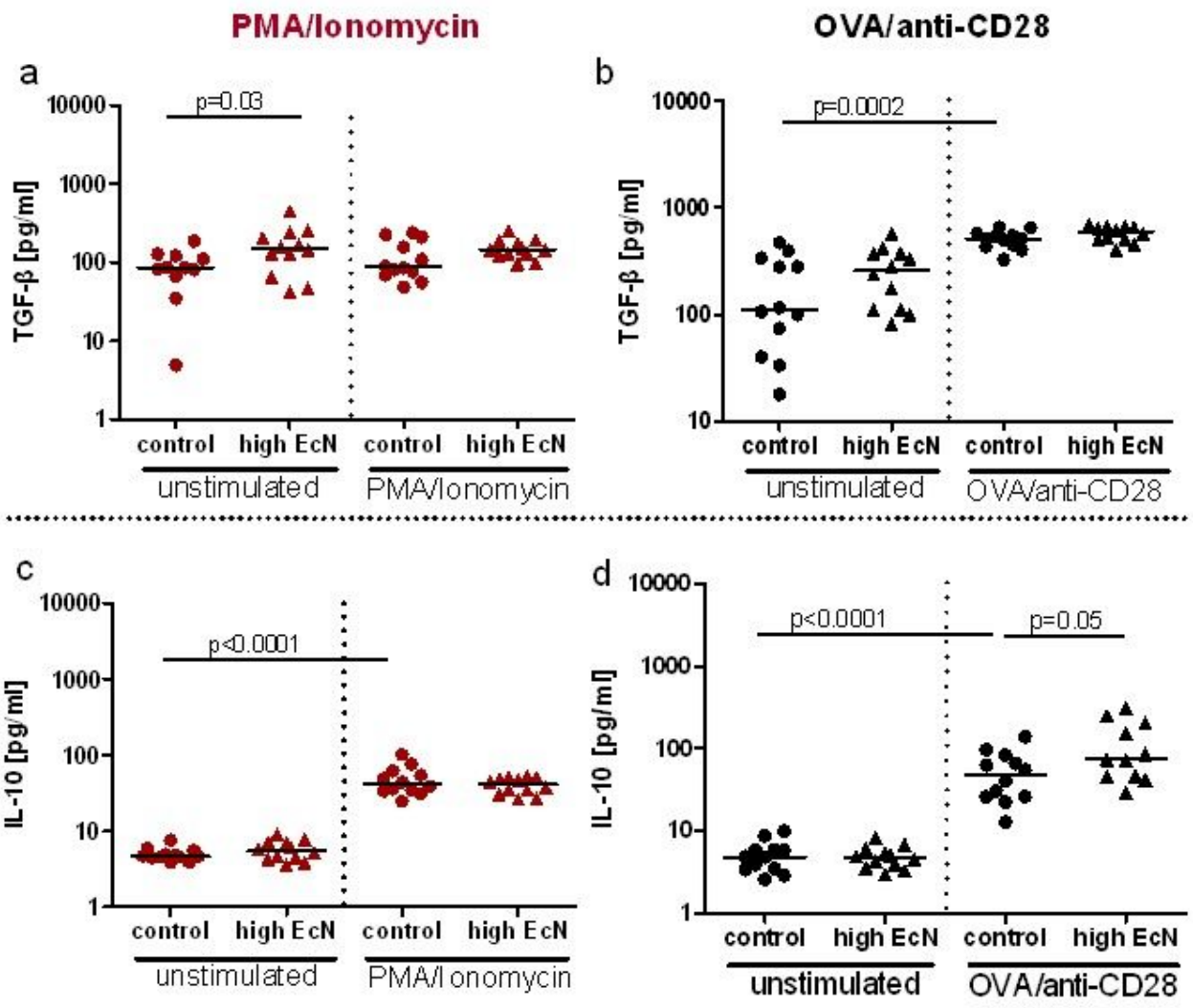

Figure 15: IL-10 and TGF-ß production upon two different stimulations (PMA/Ionomycin or OVA/anti-CD28). Splenocyte cultures were stimulated with PMA/Ionomycin (a, c) or OVA/anti-CD28 (b, d). TGF-ß (a, b) and IL-10 (c, d) concentrations were determined by ELISA. Data are presented as single values with the median as a bar. The statistical significances (p) are based on Mann–Whitney U test in comparison to control group (n>11). Only if statistical difference was reached is p depicted.

4.5. Quantitative PCR

The local cytokine response in skin was detected by measuring the mRNA expression of several cytokines. The data showed that the expression of IL-10, IFN-γ and TNF-α had no significant difference after EcN supplementation (data not shown).

5. Discussion

In western countries, the prevalence of AE has been rising in recent decades. The hygiene hypothesis was suggested to explain the increasing incidence of allergic diseases. Increased hygienic practices, including food hygiene, may contribute to this development. In the case of AE, the incidence has increased in the population who moved from undeveloped countries to industrial ones [81]. Defective microbial stimulation during infancy can lead to a loss of tolerance and raise the chance of sensitization to harmless antigens. This indicates that an appropriate stimulation of the immune system via bacteria may be beneficial.

Probiotics have become a safe option to investigate the capability of bacteria regarding immunomodulation. In this thesis, the probiotic EcN 1917 was studied. One study has shown that EcN 1917 is capable of presenting a virulent phenotype in a germ-free transgenic mouse strain which carries a defective TLR4-allele [82]. This may indicate that EcN 1917 is not suitable for immunosuppressed and immunodeficient patients. It may induce sepsis and multiple organ inflammation. Nevertheless, another study has proven that EcN 1917 can exert local and systemic anti-inflammatory effects in lipopolysaccharide-induced sepsis in mice [70]. Thus, further studies are needed to find out whether the virulent phenotype is due to the probiotic nature. In principle, EcN 1917 is a safe probiotic strain according to various studies on mice or humans [66,83,84].

EcN 1917, also known as SK22 or DSM6601, is a gram-negative strain of probiotics. As a part of gut flora, it adheres to the intestine and facilitates the crosstalk with the intestinal epithelium. Previously, EcN 1917 has been shown to have longer-term persistence and colonization in conventionally kept mice even after discontinuation of administration [56,85]. Also in humans, EcN 1917 can be detected a long time after discontinuation of administration [86]. All these findings indicate a good colonization of EcN 1917 in the gut environment.

An important step in this experiment was to set up the model of allergen-induced eczema. The murine model used was modified from the protocol previously described by Wang et al. [23] and Spergel et al. [24]. It was further optimized and used in this setting [25]. Macroscopically visible lesions with typical AE symptoms, including dryness, erythema, excoriation and extension, were induced. Due to this Th2-driven model of BALB/c mice, skin lesions exhibited epidermal thickening and inflammatory cell infiltration, like increased numbers of CD4+, CD8+ and mast cells. Thus, the well established protocol of allergen-induced eczema can contribute to increasing the knowledge of the preventive effects of EcN 1917 in an AE-like model.

Although different kinds of probiotics were previously used to treat allergic diseases like AE, little is known about the underlying mechanisms of EcN1917 on allergic skin inflammation. In this experiment, EcN 1917 was given in a preventive manner to mice before disease establishment. It was intended to determine whether this gram-negative probiotic can ameliorate AE and to reveal the underlying mechanisms. In previous studies, oral administration of EcN 1917 was commonly used at an amount of 10^8 CFU/day [54,66,87,88]. Thus, in this experiment the same amount was used as high concentration treatment and 10^7 CFU/day was applied as low concentration treatment. As live EcN 1917 required a balanced system, electrolyte was used to produce the probiotic solution. Thus, the control group was treated only by this electrolyte solution.

The major finding of this thesis is that EcN 1917 improved the clinical symptoms of allergen-induced eczema. In the control group, serious eczematous skin lesions were induced. By contrast, in preventively EcN 1917 treated mice, the skin lesions were significantly ameliorated in a dose dependent manner as confirmed by the CSS.

Due to the hypersensitivity which can be triggered by CD4+ and CD8+ cells, T cells are activated into memory T cells which circulate into the dermis upon allergen challenge. Thus, the local responses during the course of this clinical elicitation by EcN 1917 were determined. In immunohistochemical studies, mononuclear cell infiltration consisting of CD4+ and CD8+ T cells was demonstrated to play an important role in the local inflammation [89-91]. Increased epidermal thickness was considered as a sign of an

inflammation as well [91]. We observed a reduced epidermal thickness as well as reduced dermal T cell infiltration in the preventive high EcN group. In addition, mast cells were also found to be reduced in lesional skin of the high EcN group. All these findings confirm that preventive high EcN treatment led to an amelioration of allergen-induced eczema at the histological level as well. The next goal of this study was to identify possible underlying mechanisms of these observations. Previous studies revealed that the quantity of Foxp3+ Treg cells significantly increased when probiotics interacted with intestinal epithelium and DC in gut-associated lymphoid tissue, thereby inducing a suppression of the allergic response [92-95]. Treg cells are the most important CD4+ T cells to maintain the self-tolerance and have a suppressive impact on immunity. Human Treg cells mainly consist of natural Treg cells and adaptive Treg cells. Natural Treg cells, expressing Foxp3, are generated from CD4+ thymocytes by activation of thymic epithelial cells or DC. 5-10% of these natural Treg cells are present in peripheral circulation [96]. Adaptive Tregs are induced from CD4+ naïve T cells by antigen-presentation [96]. This interaction is usually located in peripheral lymphoid tissue, like mucosa-associated lymphoid tissue (MALT) and the gut-associated lymphoid tissue (GALT). Three subpopulations are known: 1) IL-10 secreting CD4+ Treg cells (Tr1), 2) TGF-ß secreting Treg cells (Th3) and finally the CD4+Foxp3+ induced Treg cells. The identification of Foxp3 as a key regulator of Treg cell development and function has facilitated their detection in the mouse [97]. Thus, Foxp3+ Treg cells were also analyzed in high EcN and control groups. As expected, the proportion of Foxp3+ cells was significantly higher in relation to CD4+ T cells after preventive high EcN supplementation. These data are supported by another study [92]. This study has also shown that Foxp3+ Treg cells were specifically elevated among CD4+CD25- T cells after probiotic administration [92]. Consequently, Foxp3+ Treg cells can in turn suppress the pathogenic effector cells through cell contacts (via e.g. membrane-tethered TGF-ß) at inflamed sites [92]. With respect to mast cells, Treg cells can impair their degranulation through cell-cell contact involving OX40-OX40L interactions [98]. Such interactions impair the influx of extracellular Ca^{2+} following Fc ε RI triggering [98]. It is also suggested that probiotics can directly suppress the inflammatory response of mast cells via TLR2

stimulation [99]. These effects of Foxp3 cells on mast cell function may contribute to the amelioration of the allergic skin response as well.

One of the major pathogenic factors of AE is the imbalance of Th cells. As cytokine production is essential for the differentiation and function of Th cells, the corresponding cytokine of Th2, Th1 and Treg cells, including IL-4, IFN-γ, lL-10 and TGF-ß, were analyzed. Depending on the strain, probiotics can induce a Th1 skewing to restore the balance of Th1 and Th2 [32,46,47]. In contrast, some probiotics were found to act as an immune response modulator by inducing IL-10 or TGF-ß secreting Treg cells [48,52,94]. Therefore, the influence of EcN 1917 at the cytokine level was investigated. IL-4 but also IFN-γ production of antigen-stimulated splenocytes was significantly reduced in the preventive high EcN group. These results suggest, at the systemic level, that both Th2- and Th1- related cytokines were reduced after the preventive high EcN treatment. Some Th2 cytokines are essential in enhancing mast cell proliferation and recruitment to effector sites, e.g. IL-4 [100]. Thus, such effects may be impaired if Th2 cytokines are reduced by EcN 1917 administration. However, the cytokine secretion in high EcN and control groups was not significantly different upon PMA/Ionomycin stimulation. PMA, phorbol-12-myristate-13-acetate, a kind of phorbol ester, activates protein kinase C. Ionomycin induces a Ca^{2+} influx. Both molecules are commonly used for polyclonal cell stimulation. Thus, all cells can be stimulated to release responding cytokines. In contrast, OVA/anti-CD28 is allergen-specific and probably more relevant for the allergen-induced skin inflammation. This may explain that no statistical significances were determined with the PMA/Ionomycin-stimulated cells. However, this finding excludes a more general T cell-related immunosuppression by EcN 1917, and suggests a rather reduced allergen-specific T cell effector response.

The regulatory cytokines IL-10 and TGF-ß were also detected in stimulated splenocytes. IL-10 is an anti-inflammatory acting cytokine [101]. It is capable of inhibiting antigen-specific proliferation, reducing cytokine production of Th cells and inducing the tolerogenic DCs [102,103]. TGF-ß is also an important regulatory cytokine. It is a protein that controls proliferation, cellular differentiation and many other functions in different cells. It is believed to be important in the regulation of the immune system by Foxp3+

Treg cells and the development of Foxp3+ Treg cells [104-106]. TGF-ß also plays a role in immunoglobulin synthesis by inducing IgA [107].

Interestingly, the allergen-specific IL-10 production in preventive high EcN mice was elevated. This may explain the reduction of Th effector cytokines after EcN 1917 treatment. By contrast, TGF-ß production did not differ significantly between the high EcN and control groups, but shows a tendency to increase with preventive high EcN administration. These data are supported by a previous study [71]. In that study the DCs from murine spleen were cultured with EcN 1917, and IL-10 was significantly elevated as well without increased TGF-ß. It has been suggested that Foxp3+ Tregs (ICOS+) or Foxp3- Tr1 cells produce IL-10 but not TGF-ß, which can induce a limitation of inflammation severity [21,108,109]. In this thesis the cytokine analysis also revealed a reduced Th1 and Th2 memory response which was associated with elevated levels of regulatory cytokines. In another study, some probiotic strain not only failed to prevent the development of atopic eczema but also increased the rate of sensitization in the infant recipient [110]. An animal study also observed this adverse effect with the supplementation of a lactobacillus strain during lactation [111]. It is known that some probiotic strain affects the immune response by increasing Th1 responses in Th2-mediated allergy [112]. Thus, this reaction can aggravate the Th1-mediated autoimmune diseases. To date, no study shows that EcN 1917 has this effect on developing the autoimmune disease. It may be associated with the suppressor activity of Treg cells instead of a Th1 skewing.

Remarkably, EcN 1917 ameliorated the local immune response, but had no effect on allergen-specific IgE production. This observation was also noted in human studies with some other probiotics [45,48,113]. However, allergen-specific IgA was significantly elevated in the context of high EcN administration. This is in line with other probiotic studies showing increased systemic IgA [32,114,115], but also secretory IgA [32]. TGF-ß, but also IL-10, may have contributed to this effect [107,116]. As previously described, secretory IgA was beneficial for protection against antigens and was even considered as a marker of immune maturation [117]. It was also proven that high intestinal IgA is

associated with reduced risk of IgE-associated allergic disease [118]. In this experiment, secretory IgA was not determined. However, IgA+ B cells induced by probiotics in the Peyer's patch can circulate through the mesenteric lymph nodes into the blood via the thoracic duct [119]. Thus, serum OVA-IgA, promoted by high EcN, was also considered to play an essential role in immunomodulatory effects. The process of class switch recombination and immunoglobulin synthesis are cytokine dependent, e.g. Th2 cytokines are required for induction of IgE [2]. However, allergen-specific IgE production was not significantly different in the EcN 1917 supplemented group compared to the control group. It is suggested that allergen-specific IgE played a limited role in this allergen-induced eczema model.

In this experiment, the cytokine mRNA expression profile in the skin including IL-10, IFN-γ and TNF-α was measured by real-time qPCR. There was no statistical significance between high EcN group and control group regarding the mRNA expression of these cytokines in the skin. These results may be due to the heterogeneity of cytokine-producing cells in the skin. These cytokines can be released by different cells in the skin. Furthermore, it needs to be considered that the skin samples have been taken at the end of the experiment. As the kinetics of cytokine production may differ between cells and also depend on the cytokine itself, we can not rule out that this kind of analysis is insufficient to reflect any changes in cytokine production, e.g. TNF-α is an early response gene and IL-10 is a more late response gene. EcN 1917 has been shown to inhibit peripheral T cell proliferation and to down regulate the expansion of newly recruited T cells into inflamed sites [73]. Furthermore, it was suggested that Foxp3+ Treg cells play a role in inflammatory sites through direct cell interaction with pathogenic effector cells [120,121]. All these mechanisms may explain our observation of a reduction of inflammatory cells without a change in cytokine expression level at the local site. However, a more detailed analysis of the different cytokines at a single cell level and at different time points of inflammation is required to prove this hypothesis.

So far our data obtained by immunohistochemical studies and systemic analysis indicate that preventive high EcN induced Foxp3+ Treg cells and suppressed the Th1 and Th2 response. This might be a potent mechanism by which the clinical improvement of the allergen-induced eczema is achieved. Taken together, the following scenario can be delineated for our system: After oral administration, EcN 1917 enters the gastrointestinal tract and interacts with the intestinal epithelium and gut-associated lymphoid tissue. Due to the exposure to probiotics, the intestinal lamina propria represents a suitable microenvironment for the generation of Treg cells [122]. In this step, DCs are essential to promote the immunological effects by presenting antigens from EcN 1917 in mesenteric lymph nodes. By interaction of activated DC with T and B cells, the immune responses are initiated [123]. DCs are activated by probiotics and drive differentiation of CD4+Foxp3− T cells into CD4+Foxp3+ Tregs by presenting the antigen [92]. After their generation, Treg cells can migrate to effector sites to function in the periphery. Recent investigations suggested that TLR2 expressed on Tregs directly regulate their expansion and function through a MyD88-dependent pathway [124]. Another study has shown that after oral administration of probiotics, chemokines (like CCL1 and CCL22) were highly expressed in the inflamed skin where increased numbers of CD4+Foxp3+ cells express the homing receptors CCR4 and CCR8 [92]. These findings indicate a potential direct regulation of Treg cell function, but also their homing profile by probiotics. Treg cells ameliorate the development of allergen-induced eczema by suppressing the proliferation of effector T cells, the differentiation of inflammatory DC and the inhibition of the inflammatory cell migration into the skin [125]. With respect to the reduction of effector T cells in inflamed sites, the main pathway was cell contact dependent [120,121], involving membrane-bound TGF-ß. In addition, Treg cells can counteract the production of Th2 cytokines which are associated with the proliferation and the recruitment of mast cells [100]. It was also suggested that Treg cells impair mast cell degranulation through direct cell contacts requiring OX40 and OX40L interaction [98]. At systemic level, the cytokine dependent effects were much stronger than cell-cell interactions [126]. Various cytokines were suggested to contribute to this process, like IL-10 and TGF-ß [127]. They are essential for suppressing Th responses. Allergen-specific Th2 cells were suppressed

with elimination of the Th2-cytokine IL-4, which is critical for the development of allergy [128-130]. It was also shown that the induction of Th1 cells was impaired by Treg cells [131]. Thus, correspondingly, the Th1 cytokines were reduced, like IFN-γ, associated with a reduced apoptosis of keratinocytes. EcN 1917 can reduce Th cytokine production by an inhibition of peripheral T cell proliferation via TLR2 as well [73]. Furthermore, it has been observed that these effects were not associated with an induction of CD4+ apoptosis [92]. As discussed earlier, the expression and function of Treg cells were determined to be impaired in AE [19]. Thus, the increased number of Foxp3+ Treg cells identified in our model can suppress inflammatory effector T cells at local and systemic level resulting in a protective effect by EcN 1917.

6. Conclusions

According to the hygiene hypothesis, a decreased contact with microbes was connected to the increasing prevalence of AE in western countries. Thus, in this experiment, the probiotic bacterium EcN 1917 was used to investigate its preventive impact on allergen-induced eczema. Indeed, the clinical outcome measured by determination of skin lesion severity was ameliorated by EcN 1917 supplementation. This macroscopic observation was paralleled by microscopic alterations including a reduction of epidermal thickness and a decrease of effector T cells and mast cells. Furthermore, an increase of Foxp3+ cells in the skin was observed. At the systemic level, an induction of allergen-specific IgA in serum of EcN 1917 supplemented mice was detected. In addition, Th1- and Th2-cytokine production of splenocytes was reduced, which was accompanied by an increased production of regulatory cytokines. Taken together, EcN 1917 can inhibit the severity of allergen-induced eczema by regulating the local and systemic immune response in a Treg dependent manner. Further studies are required to confirm the exact role of Foxp3+ cells in the skin, e.g. by targeted elimination of these presumable Treg cells.

In summary, the findings of this study reflect a novel strategy to improve the outcome of atopic eczema by EcN 1917 administration. However, although the local immune response was modulated in a positive manner, the systemic immune response regarding allergen-specific IgE was not altered at all. This suggests either a limited role of allergen-specific IgE in this allergen-induced eczema model or the relevance of Treg cells for the clinical improvement upon EcN 1917 treatment. More data are needed to understand the mechanisms of our observation in more detail. In further studies, EcN 1917 can also be supplemented in combination with other probiotics to potentially enhance the clinical effects on allergen-induced eczema. Furthermore, the time schedule as well as the dose of EcN 1917 administration should be explored to delineate the optimal preventive setting for EcN 1917 in AE.

Abstract

Atopic eczema (AE) is a common skin disease characterized by a chronic inflammation of the skin. Its incidence is increasing worldwide and this development is associated with western life style factors. Thus, it is claimed that the improved hygiene, the increased use of antibiotics and a decreased rate of microbial contacts during early life may be relevant. Probiotics offer an opportunity to balance the intestinal microflora and can provide beneficial factors for the prevention and treatment of different pathological conditions. Currently no data is available whether the oral application of E. coli Nissle 1917 (EcN 1917) is preventive of AE.

This project aimed to characterize the impact of non-pathogenic EcN 1917 on protein-induced eczema and to identify its immune modulating properties in mice.

The presented data show that high dose supplementation of EcN 1917 (10^8 CFU/day) leads to a clinical improvement of allergen-induced dermatitis. In more detail not only a down-regulation of clinical symptoms was observed, but also a reduction of the epidermal thickness, a decrease of infiltrating immune cells and increased numbers of Foxp3+ cells were determined in the skin of EcN 1917 supplemented mice in comparison to a control group. At the systemic level, an induction of allergen-specific IgA in serum of EcN 1917 mice was detected. In addition, IL-4 and IFN-γ production of splenocytes was reduced along with an elevation of IL-10 and a tendency of increased TGF-ß after allergen-specific stimulation. The local cytokine detection of IL-10, IFN-γ and TNF-α in the skin by real-time qPCR showed no significant alteration in EcN 1917 supplemented mice.

Taken together, these findings indicate that EcN 1917 ameliorated protein-induced eczema by an increase of Foxp3+ T cells, which may be responsible for the suppression of the clinical outcome. Further studies are required to confirm the exact role of Foxp3+ cells in the skin and to delineate the optimal preventive setting for EcN 1917 in AE.

Keywords:

Escherichia coli Nissle 1917, probiotics, eczema

Abstract

Das atopische Ekzem (AE) ist eine verbreitete, chronisch entzündliche Hauterkrankung, deren stetig steigende Inzidenz auf westliche Lebensstilfaktoren zurückgeführt wird. Vor allem der verstärkte Einsatz von Antibiotika sowie der verminderte mikrobielle Kontakt im frühen Kindesalter werden dabei laut Hygienehypothese als begünstigend beschrieben. Probiotika stellen eine potenzielle Möglichkeit dar, die intestinale Mikroflora positiv zu beeinflussen und somit Prävention und Behandlung verschiedener Erkrankungen effektiv zu unterstützen. Allerdings sind hinsichtlich des AE derzeit keine Daten über die klinische Wirksamkeit des nicht-pathogenen Probiotikums E. coli Nissle 1917 (EcN 1917) verfügbar.

Ziel dieses Projektes war die Untersuchung des klinischen Erscheinungsbildes der Allergen-induzierten Dermatitis sowie die Analyse der immunmodulatorischen Effekte nach präventiver EcN 1917-Supplementierung in Mäusen.

Die erhobenen Daten zeigen dass die tägliche Supplementierung von 10^8 Kolonien bildende Einheiten EcN 1917 zu einer Verbesserung des klinischen Erscheinungsbildes der Allergen-induzierten Dermatitis führte. Die Verminderung der klinischen Symptome war auf histologischer Ebene mit einer reduzierten Epidermisdicke, verminderter Infiltration von Immunzellen sowie einer Erhöhung Foxp3-positiver T-Zellen assoziiert. Systemisch führte die EcN 1917-Supplementierung zu einer erhöhten IgA-Serumkonzentration sowie einer verminderten IL-4 und IFN-γ-Produktion in Allergen-stimulierten Milzzellen. Parallel wurden eine vermehrte Allergen-induzierte IL-10- sowie eine tendenziell gesteigerte TGF-ß-Produktion detektiert. Im Gegensatz dazu war die lokale Expression von IL-10, IFN-γ und TNF-α in der Haut durch die Verabreichung von EcN 1917 nicht beeinflusst.

Zusammengefasst weisen diese Daten darauf hin, dass die Supplementierung mit EcN 1917 zu einer klinischen Verbesserung der Allergen-induzierten Dermatitis durch die Induktion von regulatorischen T-Zellen führt. Weitere Studien sind notwendig, um die exakte Rolle von regulatorischen T-Zellen in der Haut herauszuarbeiten und ein optimales präventives Behandlungsregime mit EcN 19617 im Rahmen der AE zu

entwickeln.

Schlüsselwörter:

Escherichia coli Nissle 1917, Probiotika, Ekzem

Appendix

Abbreviations

AE	Atopic eczema
APC	Antigen presenting cells
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte-associated antigen
CSS	Clinical skin score
CFU	Colony forming unit
DC	Dendritic cell
e.c.	Epicutaneous
ELISA	Enzyme-linked immnuosorbent assay
EcN 1917	E. coli Nissle 1917
Foxp3	Forkhead transcription factor box p3
GI	Gastrointestinal
GALT	Gut-associated lymphoid tissue
IFN	Interferon
Ig	Immunoglobulin
IDEC	Inflammatory dendritic epidermal cell
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
IBD	Inflammatory bowel disease
ICOS	Inducible costimulator
IHC	Immunohistochemistry
i.p.	Intraperitoneal
LU	Laboratory unit
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MLN	Mesenteric lymph nodes

Min	Minimum
Max	Maximum
mAb	Monoclonal antibodies
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PP	Peyer’s patch
PMA	Phorbol-12-myristate-13-acetate
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Th	T helper
TSLP	Thymic stromal lymphopoietin
Treg	T regulatory
TLR	Toll like receptor

Reagents

Reagent	manufacturer
Antibody diluent (Dako REAL$_{TM}$)	DAKO Diagnostika, Hamburg, Germany
Avidin/biotin blocking Kit	Vector, Burlingame, USA
Beta mercapto ethanol	Sigma-Aldrich, Steinheim, Germany
Bovine serum albumin (BSA), pH 7.0	SERVA, Heidelberg, Germany
Detection System (Dako REAL, K 5005, Alkaline Phosphatase/RED)	DAKO Diagnostika, Hamburg, Germany
DNase	Macherey-Nagel, Düren, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Goat serum (DakoCytomation, X0907)	DAKO Diagnostika, Hamburg, Germany
Peroxidase-blocking reagent	Dako, Glostrup, Denmark
Hydrogen peroxide, H_2O_2, 30%	Merck, Darmstadt, Germany
Magnesium chloride, $MgCl_2$	Merck, Darmstadt, Germany
Methanol, C_2H_5OH	Merck, Darmstadt, Germany
Milk powder	Roth, Karlsruhe, Germany
Nucleospin RNA II kit	Macherey-Nagel, Düren, Germany
Papanicolaou's solution (1a Harris' haematoxylin solution)	Merck, Darmstadt, Germany
Penicillin, 10.000 E	Biochrom, Berlin, Germany
Dulbecco´s Phosphate-Buffered Saline (PBS), without Ca^{2+}/Mg^{2+}, sterile	PAA, Pasching, Austria
Phorbol-12-myristate-13-acetate (PMA), sterile	Sigma-Aldrich, Steinheim, Germany
Potassium chloride, KCl	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate, KH_2PO_4	Merck, Darmstadt, Germany

Proteinase K	Macherey-Nagel, Düren, Germany
Reverse Transcription Kit	Applied Biosystems, Darmstadt, Germany
Real-time PCR kit	R&D, Minneapolis, USA
RPMI 1640, without Ca^{2+}/Mg^{2+}, sterile	Biochrom, Berlin, Germany
Sodium carbonate, Na_2CO_3	Merck, Darmstadt, Germany
Sodium chloride, NaCl	Merck, Darmstadt, Germany
Sodium hydrogen carbonate, NaHCO3	Merck, Darmstadt, Germany
Streptavidin-Horseradish Peroxidase (HRP), 100µg/mL	R&D, Minneapolis, USA
Streptavidin-Alkaline Phosphatase (AP)	ZYMED, San Francisco, USA
Streptomycin (10.000 g/ml)	Biochrom, Berlin, Germany
Sulphuric acid, H_2SO_4, 95-97%	Riedel de Haen, Seelze, Germany
Tetramethyl benzidine dihydrochloride, TMB	Sigma-Aldrich, Steinheim, Germany
Tris(hydroxymethyl)aminomethane (Tris-Base)	Sigma-Aldrich, Steinheim, Germany
Toluidine blue	Merck, Darmstadt, Germany
Tween20	Bio-Rad, Munich, Germany

Antibodies and secondary reagents

antibody	clone	manufacturer
Purified mouse IgE, κ	Clone C38-2	BD PharMingen, San Diego, USA
Purified rat anti-mouse IgE	Clone R35-72	BD PharMingen, San Diego, USA
Biotin-anti-mouse IgE	EM95.3-bio	DRFZ
Biotin-IgA	1040-08	Southern biot
Biotin-OVA		DRFZ
Rat antibody against Foxp3	Clone FJK-16s	eBioscience, San Diego, CA, USA,
Rabbit anti-rat antibody	# E0467	Dako, Glostrup, Denmark
EnVision peroxidase kit	#K4003	Dako, Glostrup, Denmark
Purified rat anti-mouse CD4 (L3T4)	Clone RM4-5	BD Pharmingen, San Diego, USA
Purified rat anti-mouse CD8a	Clone 53-6.7	BD Pharmingen, San Diego, USA
Goat anti-rat Ig-biotin	polyclonal	BD Pharmingen, San Diego, USA

References

1. Simpson EL. Atopic dermatitis: a review of topical treatment options. Curr Med Res Opin;26:633-640.

2. Kenneth M, Paul T, Mark W. Allergy and hypersensitivity. In: Janeway's Immunobiology. 7th ed. England: Garland Science, 2007:555-591.

3. Krafchik BR. Treatment of atopic dermatitis. J Cutan Med Surg 1999;3 Suppl 2:S2-16-S12-23.

4. Ott H, Stanzel S, Ocklenburg C, et al. Total serum IgE as a parameter to differentiate between intrinsic and extrinsic atopic dermatitis in children. Acta Derm Venereol 2009;89:257-261.

5. Leonardi S, Rotolo N, Vitaliti G, Spicuzza L, La Rosa M. IgE values and T-lymphocyte subsets in children with atopic eczema/dermatitis syndrome. Allergy Asthma Proc 2007;28:529-534.

6. Wuthrich B, Cozzio A, Roll A, et al. Atopic eczema: genetics or environment? Ann Agric Environ Med 2007;14:195-201.

7. Folster-Holst R, Pape M, Buss YL, Christophers E, Weichenthal M. Low prevalence of the intrinsic form of atopic dermatitis among adult patients. Allergy 2006;61:629-632.

8. Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. Curr Allergy Asthma Rep 2009;9:265-272.

9. Bieber T, Novak N. Pathogenesis of atopic dermatitis: new developments. Curr Allergy Asthma Rep 2009;9:291-294.

10. Palmer CN, Irvine AD, Terron-Kwiatkowski A, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006;38:441-446.

11. O'Regan GM, Sandilands A, McLean WH, Irvine AD. Filaggrin in atopic dermatitis. J Allergy Clin Immunol 2009;124:R2-6.

12. Vickery BP. Skin barrier function in atopic dermatitis. Curr Opin Pediatr 2007;19:89-93.

13. Susac A, Babic S, Lipozencic J. An overview on atopic dermatitis in children.

Acta Dermatovenerol Croat 2007;15:158-166.

14. Hubiche T, Ged C, Benard A, et al. Analysis of SPINK 5, KLK 7 and FLG genotypes in a French atopic dermatitis cohort. Acta Derm Venereol 2007;87:499-505.

15. Soderhall C, Marenholz I, Kerscher T, et al. Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. PLoS Biol 2007;5:e242.

16. Akdis CA, Akdis M, Bieber T, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. Allergy 2006;61:969-987.

17. Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. J Clin Invest 2004;113:651-657.

18. Trautmann A, Akdis M, Schmid-Grendelmeier P, et al. Targeting keratinocyte apoptosis in the treatment of atopic dermatitis and allergic contact dermatitis. J Allergy Clin Immunol 2001;108:839-846.

19. Verhagen J, Akdis M, Traidl-Hoffmann C, et al. Absence of T-regulatory cell expression and function in atopic dermatitis skin. J Allergy Clin Immunol 2006;117:176-183.

20. Akdis M, Akdis CA, Weigl L, Disch R, Blaser K. Skin-homing, CLA+ memory T cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated cytokine pattern: IgG4 counter-regulation by CLA- memory T cells. J Immunol 1997;159:4611-4619.

21. Ray A, Khare A, Krishnamoorthy N, Qi Z, Ray P. Regulatory T cells in many flavors control asthma. Mucosal Immunol. [Epub ahead of print].

22. Gutermuth J, Ollert M, Ring J, Behrendt H, Jakob T. Mouse models of atopic eczema critically evaluated. Int Arch Allergy Immunol 2004;135:262-276.

23. Wang LF, Lin JY, Hsieh KH, Lin RH. Epicutaneous exposure of protein antigen induces a predominant Th2-like response with high IgE production in mice. J Immunol 1996;156:4077-4082.

24. Spergel JM, Mizoguchi E, Brewer JP, et al. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. J Clin Invest 1998;101:1614-1622.

25. Dahten A, Koch C, Ernst D, et al. Systemic PPARgamma ligation inhibits allergic immune response in the skin. J Invest Dermatol 2008;128:2211-2218.

26. Suzuki H, Suzuki Y, Aizawa M, et al. Th1 polarization in murine IgA nephropathy directed by bone marrow-derived cells. Kidney Int 2007;72:319-327.

27. Scharschmidt TC, Segre JA. Modeling atopic dermatitis with increasingly complex mouse models. J Invest Dermatol 2008;128:1061-1064.

28. Ahuja A, Land K, Barnes CJ. Atopic dermatitis. South Med J 2003;96:1068-1072.

29. Romagnani S. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? Immunology 2004;112:352-363.

30. Guidelines for the evaluation of probiotics in food. FAO/WHO, 2002. (Accessed September 28, 2009, at http://www.who.int/foodsafety/publications/fs_management/probiotics2/en/index.html)

31. Bunselmeyer B. [Probiotics and prebiotics for the prevention and treatment of atopic eczema]. Hautarzt 2006;57:785-791.

32. Delcenserie V, Martel D, Lamoureux M, et al. Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol 2008;10:37-54.

33. Nova E, Warnberg J, Gomez-Martinez S, et al. Immunomodulatory effects of probiotics in different stages of life. Br J Nutr 2007;98 Suppl 1:S90-95.

34. Flinterman AE, Knol EF, van Ieperen-van Dijk AG, et al. Probiotics have a different immunomodulatory potential in vitro versus ex vivo upon oral administration in children with food allergy. Int Arch Allergy Immunol 2007;143:237-244.

35. Wichers H. Immunomodulation by food: promising concept for mitigating allergic disease? Anal Bioanal Chem 2009;395:37-45.

36. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J Gastroenterol 2009;44:26-46.

37. West NP, Pyne DB, Peake JM, Cripps AW. Probiotics, immunity and exercise: a review. Exerc Immunol Rev 2009;15:107-126.

38. Corthesy B, Gaskins HR, Mercenier A. Cross-talk between probiotic bacteria and the host immune system. J Nutr 2007;137:781S-790S.

39. Steffen EK, Berg RD. Relationship between cecal population levels of indigenous bacteria and translocation to the mesenteric lymph nodes. Infect Immun 1983;39:1252-1259.

40. Smits HH, Engering A, van der Kleij D, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 2005;115:1260-1267.

41. Vanderpool C, Yan F, Polk DB. Mechanisms of probiotic action: Implications for therapeutic applications in inflammatory bowel diseases. Inflamm Bowel Dis 2008;14:1585-1596.

42. Majamaa H, Isolauri E, Saxelin M, Vesikari T. Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. J Pediatr Gastroenterol Nutr 1995;20:333-338.

43. Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol 1994;10:55-63.

44. Fukushima Y, Kawata Y, Hara H, Terada A, Mitsuoka T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol 1998;42:39-44.

45. Miraglia del Giudice M, Jr., De Luca MG, Capristo C. Probiotics and atopic dermatitis. A new strategy in atopic dermatitis. Dig Liver Dis 2002;34 Suppl 2:S68-71.

46. West CE, Hammarstrom ML, Hernell O. Probiotics during weaning reduce the incidence of eczema. Pediatr Allergy Immunol 2009;20:430-437.

47. Prescott SL, Dunstan JA, Hale J, et al. Clinical effects of probiotics are associated with increased interferon-gamma responses in very young children with atopic dermatitis. Clin Exp Allergy 2005;35:1557-1564.

48. Ji GE. Probiotics in primary prevention of atopic dermatitis. Forum Nutr 2009;61:117-128.

49. del Giudice MM, Rocco A, Capristo C. Probiotics in the atopic march: highlights and new insights. Dig Liver Dis 2006;38 Suppl 2:S288-290.

50. Tomura M, Honda T, Tanizaki H, et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice.

J Clin Invest;120:883-893.

51. von der Weid T, Bulliard C, Schiffrin EJ. Induction by a lactic acid bacterium of a population of CD4(+) T cells with low proliferative capacity that produce transforming growth factor beta and interleukin-10. Clin Diagn Lab Immunol 2001;8:695-701.

52. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol 2005;174:3237-3246.

53. Schulze J, Schiemann M, Sonnenborn U. Alfred NISSLE's hypothesis of E coli antagonism and his "new therapeutic principle". In: 120 years of E. coli-Its importance in research and medicine. Hagen, Germany: Alfred-Nissle-Gesellschaft, 2006:25-28.

54. Grabig A, Paclik D, Guzy C, et al. Escherichia coli strain Nissle 1917 ameliorates experimental colitis via toll-like receptor 2- and toll-like receptor 4-dependent pathways. Infect Immun 2006;74:4075-4082.

55. Henker J, Laass M, Blokhin BM, et al. The probiotic Escherichia coli strain Nissle 1917 (EcN) stops acute diarrhoea in infants and toddlers. Eur J Pediatr 2007;166:311-318.

56. Westendorf AM, Gunzer F, Deppenmeier S, et al. Intestinal immunity of Escherichia coli NISSLE 1917: a safe carrier for therapeutic molecules. FEMS Immunol Med Microbiol 2005;43:373-384.

57. Grozdanov L, Zahringer U, Blum-Oehler G, et al. A single nucleotide exchange in the wzy gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of Escherichia coli strain Nissle 1917. J Bacteriol 2002;184:5912-5925.

58. Blum G, Marre R, Hacker J. Properties of Escherichia coli strains of serotype O6. Infection 1995;23:234-236.

59. Roberts IS. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu Rev Microbiol 1996;50:285-315.

60. Hafez M, Hayes K, Goldrick M, et al. The K5 capsule of Escherichia coli strain Nissle 1917 is important in mediating interactions with intestinal epithelial cells and chemokine induction. Infect Immun 2009;77:2995-3003.

61. Lasaro MA, Salinger N, Zhang J, et al. F1C fimbriae play an important role in biofilm formation and intestinal colonization by the Escherichia coli commensal strain

Nissle 1917. Appl Environ Microbiol 2009;75:246-251.

62. Patzer SI, Baquero MR, Bravo D, Moreno F, Hantke K. The colicin G, H and X determinants encode microcins M and H47, which might utilize the catecholate siderophore receptors FepA, Cir, Fiu and IroN. Microbiology 2003;149:2557-2570.

63. Grozdanov L, Raasch C, Schulze J, et al. Analysis of the genome structure of the nonpathogenic probiotic Escherichia coli strain Nissle 1917. J Bacteriol 2004;186:5432-5441.

64. Valdebenito M, Crumbliss AL, Winkelmann G, Hantke K. Environmental factors influence the production of enterobactin, salmochelin, aerobactin, and yersiniabactin in Escherichia coli strain Nissle 1917. Int J Med Microbiol 2006;296:513-520.

65. Schultz M. Clinical use of E. coli Nissle 1917 in inflammatory bowel disease. Inflamm Bowel Dis 2008;14:1012-1018.

66. Bickert T, Trujillo-Vargas CM, Duechs M, et al. Probiotic Escherichia coli Nissle 1917 suppresses allergen-induced Th2 responses in the airways. Int Arch Allergy Immunol 2009;149:219-230.

67. Cukrowska B, LodInova-ZadnIkova R, Enders C, et al. Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic E. coli strain Nissle 1917. Scand J Immunol 2002;55:204-209.

68. Cross ML, Ganner A, Teilab D, Fray LM. Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. FEMS Immunol Med Microbiol 2004;42:173-180.

69. Helwig U, Lammers KM, Rizzello F, et al. Lactobacilli, bifidobacteria and E. coli nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells. World J Gastroenterol 2006;12:5978-5986.

70. Arribas B, Rodriguez-Cabezas ME, Camuesco D, et al. A probiotic strain of Escherichia coli, Nissle 1917, given orally exerts local and systemic anti-inflammatory effects in lipopolysaccharide-induced sepsis in mice. Br J Pharmacol 2009;157:1024-1033.

71. Fink LN, Frokiaer H. Dendritic cells from Peyer's patches and mesenteric lymph nodes differ from spleen dendritic cells in their response to commensal gut bacteria. Scand J Immunol 2008;68:270-279.

72. Agrawal S, Agrawal A, Doughty B, et al. Cutting edge: different Toll-like

receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol 2003;171:4984-4989.

73. Sturm A, Rilling K, Baumgart DC, et al. Escherichia coli Nissle 1917 distinctively modulates T-cell cycling and expansion via toll-like receptor 2 signaling. Infect Immun 2005;73:1452-1465.

74. Magerl M, Lammel V, Siebenhaar F, et al. Non-pathogenic commensal Escherichia coli bacteria can inhibit degranulation of mast cells. Exp Dermatol 2008;17:427-435.

75. Schlee M, Wehkamp J, Altenhoefer A, et al. Induction of human beta-defensin 2 by the probiotic Escherichia coli Nissle 1917 is mediated through flagellin. Infect Immun 2007;75:2399-2407.

76. Wehkamp J, Harder J, Wehkamp K, et al. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infect Immun 2004;72:5750-5758.

77. Boyle RJ, Bath-Hextall FJ, Leonardi-Bee J, Murrell DF, Tang ML. Probiotics for the treatment of eczema: a systematic review. Clin Exp Allergy 2009;39:1117-1127.

78. Fink LN. Induction of regulatory T cells by probiotics: potential for treatment of allergy? Clin Exp Allergy;40:5-8.

79. Lomax AR, Calder PC. Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans. Curr Pharm Des 2009;15:1428-1518.

80. Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. Dermatology 1993;186:23-31.

81. Williams HC, Pembroke AC, Forsdyke H, et al. London-born black Caribbean children are at increased risk of atopic dermatitis. J Am Acad Dermatol 1995;32:212-217.

82. Bleich A, Sundberg JP, Smoczek A, et al. Sensitivity to Escherichia coli Nissle 1917 in mice is dependent on environment and genetic background. Int J Exp Pathol 2008;89:45-54.

83. Henker J, Laass MW, Blokhin BM, et al. Probiotic Escherichia coli Nissle 1917 versus placebo for treating diarrhea of greater than 4 days duration in infants and toddlers. Pediatr Infect Dis J 2008;27:494-499.

84. Ukena SN, Singh A, Dringenberg U, et al. Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One 2007;2:e1308.

85. Schultz M, Watzl S, Oelschlaeger TA, et al. Green fluorescent protein for detection of the probiotic microorganism Escherichia coli strain Nissle 1917 (EcN) in vivo. J Microbiol Methods 2005;61:389-398.

86. Lodinova-Zadnikova R, Sonnenborn U. Effect of preventive administration of a nonpathogenic Escherichia coli strain on the colonization of the intestine with microbial pathogens in newborn infants. Biol Neonate 1997;71:224-232.

87. Remer KA, Bartrow M, Roeger B, et al. Split immune response after oral vaccination of mice with recombinant Escherichia coli Nissle 1917 expressing fimbrial adhesin K88. Int J Med Microbiol 2009;299:467-478.

88. Hockertz S. Augmentation of host defence against bacterial and fungal infections of mice pretreated with the non-pathogenic Escherichia coli strain Nissle 1917. Arzneimittelforschung 1997;47:793-796.

89. Akdis M, Simon HU, Weigl L, et al. Skin homing (cutaneous lymphocyte-associated antigen-positive) CD8+ T cells respond to superantigen and contribute to eosinophilia and IgE production in atopic dermatitis. J Immunol 1999;163:466-475.

90. Sager N, Feldmann A, Schilling G, Kreitsch P, Neumann C. House dust mite-specific T cells in the skin of subjects with atopic dermatitis: frequency and lymphokine profile in the allergen patch test. J Allergy Clin Immunol 1992;89:801-810.

91. Matsumoto K, Mizukoshi K, Oyobikawa M, Ohshima H, Tagami H. Establishment of an atopic dermatitis-like skin model in a hairless mouse by repeated elicitation of contact hypersensitivity that enables to conduct functional analyses of the stratum corneum with various non-invasive biophysical instruments. Skin Res Technol 2004;10:122-129.

92. Kwon HK, Lee CG, So JS, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A;107:2159-2164.

93. Hacini-Rachinel F, Gheit H, Le Luduec JB, et al. Oral probiotic control skin inflammation by acting on both effector and regulatory T cells. PLoS One 2009;4:e4903.

94. Feleszko W, Jaworska J, Rha RD, et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T

regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy 2007;37:498-505.

95. de Roock S, van Elk M, van Dijk ME, et al. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. Clin Exp Allergy 2009.

96. Kenneth M, Paul T, Mark W. Regulatory CD4 T cells are involved in controlling adaptive immune response. In: Janeway's Immunobiology. 7th ed. England: Garland Science, 2007:354-356.

97. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-1061.

98. Gri G, Piconese S, Frossi B, et al. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. Immunity 2008;29:771-781.

99. Kasakura K, Takahashi K, Aizawa T, Hosono A, Kaminogawa S. A TLR2 ligand suppresses allergic inflammatory reactions by acting directly on mast cells. Int Arch Allergy Immunol 2009;150:359-369.

100. Lorentz A, Wilke M, Sellge G, et al. IL-4-induced priming of human intestinal mast cells for enhanced survival and Th2 cytokine generation is reversible and associated with increased activity of ERK1/2 and c-Fos. J Immunol 2005;174:6751-6756.

101. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol;10:170-181.

102. Del Prete G, De Carli M, Almerigogna F, et al. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 1993;150:353-360.

103. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271-275.

104. Huter EN, Punkosdy GA, Glass DD, et al. TGF-beta-induced Foxp3+ regulatory T cells rescue scurfy mice. Eur J Immunol 2008;38:1814-1821.

105. Andersson J, Tran DQ, Pesu M, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. J Exp Med 2008;205:1975-1981.

106. Zheng SG. The Critical Role of TGF-beta1 in the Development of Induced

Foxp3+ Regulatory T Cells. Int J Clin Exp Med 2008;1:192-202.

107. Stavnezer J. Regulation of antibody production and class switching by TGF-beta. J Immunol 1995;155:1647-1651.

108. Ito T, Hanabuchi S, Wang YH, et al. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity 2008;28:870-880.

109. Akbari O, Freeman GJ, Meyer EH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. Nat Med 2002;8:1024-1032.

110. Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. J Allergy Clin Immunol 2007;119:184-191.

111. Ezendam J, van Loveren H. Lactobacillus casei Shirota administered during lactation increases the duration of autoimmunity in rats and enhances lung inflammation in mice. Br J Nutr 2008;99:83-90.

112. Pohjavuori E, Viljanen M, Korpela R, et al. Lactobacillus GG effect in increasing IFN-gamma production in infants with cow's milk allergy. J Allergy Clin Immunol 2004;114:131-136.

113. Kim JY, Kwon JH, Ahn SH, et al. Effect of probiotic mix (Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial. Pediatr Allergy Immunol 2009. [Epub ahead of print].

114. Rautava S, Arvilommi H, Isolauri E. Specific probiotics in enhancing maturation of IgA responses in formula-fed infants. Pediatr Res 2006;60:221-224.

115. Yao TC, Chang CJ, Hsu YH, Huang JL. Probiotics for allergic diseases: Realities and myths. Pediatr Allergy Immunol 2009. [Epub ahead of print].

116. Mestecky J, Russell MW, Elson CO. Intestinal IgA: novel views on its function in the defence of the largest mucosal surface. Gut 1999;44:2-5.

117. Peebles RS, Jr., Hamilton RG, Lichtenstein LM, et al. Antigen-specific IgE and IgA antibodies in bronchoalveolar lavage fluid are associated with stronger antigen-induced late phase reactions. Clin Exp Allergy 2001;31:239-248.

118. Kukkonen K, Kuitunen M, Haahtela T, et al. High intestinal IgA associates

with reduced risk of IgE-associated allergic diseases. Pediatr Allergy Immunol 2009. [Epub ahead of print].

119. Galdeano CM, de Moreno de LeBlanc A, Vinderola G, Bonet ME, Perdigon G. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin Vaccine Immunol 2007;14:485-492.

120. Kim CH. Migration and function of FoxP3+ regulatory T cells in the hematolymphoid system. Exp Hematol 2006;34:1033-1040.

121. Huehn J, Hamann A. Homing to suppress: address codes for Treg migration. Trends Immunol 2005;26:632-636.

122. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007;204:1757-1764.

123. Huang FP, Platt N, Wykes M, et al. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. J Exp Med 2000;191:435-444.

124. Sutmuller RP, den Brok MH, Kramer M, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006;116:485-494.

125. Palomares O, Yaman G, Azkur AK, et al. Role of T regulatory cells in immune regulation of allergic diseases. Eur J Immunol. [Epub ahead of print].

126. Ring S, Schafer SC, Mahnke K, Lehr HA, Enk AH. CD4+ CD25+ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue. Eur J Immunol 2006;36:2981-2992.

127. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. Nat Rev Immunol 2007;7:610-621.

128. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;199:1567-1575.

129. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102:98-106.

130. Jutel M, Akdis M, Budak F, et al. IL-10 and TGF-beta cooperate in the

regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33:1205-1214.

131. Trautmann A, Akdis M, Kleemann D, et al. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. J Clin Invest 2000;106:25-35.

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Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of publications

1. 系统性红斑狼疮患者感染的病因学.
Yan Zhu, Zhenfu Liu. The etiology of infections in patients with systemic lupus erythematosus. Chin J Rheumatol Dec 2008;12(12):852-854.

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„Ich, Yan Zhu, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Impact of gram-negative bacteria on the allergic immune response in a mouse model of allergen-induced eczema. selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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