Charles University in Prague
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Impact of probiotic bacteria on allergic sensitization in type I allergy model

Vliv probiotických bakterií na alergickou senzibilizaci v modelu alergie I. typu

Ph.D. Thesis

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‘They got stuff called allergy now. Used to call it hay fever, made you sneeze. Guy that figured out allergy should of got a patent. A allergy is, you get sick when there’s something you don’t want to do. I’ve knew dames that was allergic to dishwater.’

John Steinbeck, Sweet Thursday

Support bacteria - they're the only culture some people have.

Steven Wright

Statement of originality

I hereby declare that the material in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge this thesis is the product of my own work and contains no material previously published or written by another person, except where due acknowledgement is made.

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Martin Schwarzer

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ABSTRACT

The main goal in reversing the allergy epidemic is the development of effective prophylactic strategies. Early life events, such as exposures to microbes, have a major influence on the development of balanced immune responses. Due to their ability to interact with host immune system and to modulate host immune responses probiotics, mainly bifidobacteria and lactobacilli have been used with some success in prevention of allergic disease.

In order to be referred to as probiotic, bacterial strain has to undergo rigorous testing. We have selected three new *Lactobacillus* (*L.*) strains out of 24 human isolates according to their antagonistic activity against pathogenic bacteria, resistance to low pH and milieu of bile salts. Safety of these strains was proven upon intragastric administration to mice; moreover, we have shown their ability to shift cytokine Th1 - Th2 balance towards non-allergic Th1 response in isolated splenic cells.

Allergen specific prophylaxis using probiotics as vehicles for mucosal delivery of recombinant allergen is an attractive concept for development of well-tolerated and effective allergy vaccines. We have shown that neonatal mono-colonization of germ-free mice with the *L. plantarum* NCIMB8826 strain producing the major birch pollen allergen Bet v 1 attenuates the development of birch pollen allergy later in life. The mechanisms involve a shift towards a non-allergic Th1 phenotype accompanied by increased regulatory responses, which were antigen-specific as colonization by a wild type strain exerted no such effects.

Intrinsic immunomodulatory properties of the probiotic strain play a key role in its ability to interact with the immune system of the host. We have further shown that neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* CCDM367, a human isolate with Treg rather than Th1 immunomodulatory properties, was able to reduce allergic sensitization by activating regulatory responses via TLR2 and MyD88 signaling pathways.

Understanding what makes ‘allergen an allergen’ is a key in allergy prophylaxis or treatment. We have shown in a mouse model of food allergy that even minor irreversible changes in OVA secondary structure caused by thermal processing alter both its digestion and antigenic epitopes formation. This leads to activation of different T cell subpopulations, induces shift towards Th1 response and ultimately reduces its allergenicity.

Taken together, understanding the immunomodulatory potential of bacteria in the early host development can pave a new way for probiotic use in early nonspecific prevention of type I allergy. Combining the probiotics with relevant allergen can make this approach even allergen-specific.

ABSTRAKT

Hlavním cílem v boji proti alergické epidemii je vývoj účinných preventivních strategií. Události v raném postnatálním období, jako je kolonizace bakteriemi, mají významný vliv na rozvoj vyvážených imunitních reakcí. Probiotika, zejména bifidobakterie a laktobacily, jsou vzhledem ke svým schopnostem modulovat imunitní odpověď hostitele nadějnými kandidáty pro prevenci alergických onemocnění.

Aby mohl být bakteriální kmen označen jako probiotický, musí projít přísným testováním. Z 24 lidských bakteriálních izolátů jsme vybrali tři nové kmeny rodu *Lactobacillus* (*L.*) podle jejich antagonistické aktivity vůči patogenním bakteriím, odolnosti proti nízkému pH a prostředí žlučových solí. V myším modelu jsme prokázali jejich bezpečnost a ukázali jsme jejich schopnost ovlivnit cytokinovou produkci směrem k protialergické Th1 odpovědi v izolovaných slezinných buňkách.

Využití probiotik jako vektorů pro slizniční podání rekombinantního alergenu je atraktivním přístupem ve vývoji dobře tolerovaných a účinných vakcín proti alergickým onemocněním. Ukázali jsme, že neonatální monokolonizace bezmikrobních myší rekombinantním kmenem *L. plantarum* NCIMB8826 produkujícím Bet v 1 snižuje senzibilizaci k březovému pylu v pozdějším životě. Mechanismus zahrnoval posun směrem k protialergickému Th1 fenotypu, doprovázenému zvýšenou regulační odpovědí. Tento účinek byl antigen specifický, protože kolonizace nerekombinantním kmenem žádné podobné účinky nevykazovala.

Přirozené imunomodulační vlastnosti probiotického kmene hrají klíčovou roli v jeho schopnosti interagovat s imunitním systémem hostitele. Když jsme pro neonatální monokolonizaci bezmikrobních myší použili lidský izolát s Treg spíše než Th1 imunomodulačními vlastnostmi *Bifidobacterium longum* CCDM367, pozorovali jsme celkové snížení alergické senzibilizace s aktivací regulačních odpovědí, pravděpodobně za využití TLR2 a MyD88 signálních drah.

Pochopení toho, co dělá alergen alergenem, je klíčem jak k profylaxi, tak i léčbě alergií. V myším modelu potravinové alergie jsme ukázali, že i nevelké nevratné změny v sekundární struktuře ovalbuminu, způsobené tepelným opracováním, mění způsob jeho štěpení a tvorbu antigenních epitopů, což vede k aktivaci odlišných T-buněčných subpopulací, indukuje posun směrem k Th1 odpovědi a redukuje jeho alergenicitu.

Lze tedy shrnout, že pochopení imunomodulačního potenciálu bakterií v časném stádiu vývoje hostitele nám umožní lepší využití probiotik v nespecifické prevenci alergie I. typu. Jako vektory k alergen-specifické profylaxi pak mohou sloužit probiotika produkující příslušný alergen.

ABREVIATIONS

AAI	Allergic airway inflammation
AD	Atopic dermatitis
Alum	Aluminium hydroxide
APC	Antigen-presenting cells
ASIT	Allergen-specific immunotherapy
BALT	Bronchus-associated lymphoid tissue
Bet v 1	Major birch pollen allergen
BLG	Bovine β-lactoglobulin
BMDC	Bone marrow-derived dendritic cells
CALT	Conjunctiva-associated lymphoid tissue
CCR9	Chemokine (C-C motif) receptor 9
CD	Cluster of differentiation
CT	Cholera toxin
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
FAO/WHO	Food and Agricalture Organization/ World Health Organization
Fc	Antibody fragment, crystalizable
FcεR	IgE high affinity receptor
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GATA3	Transcription factor specific for Th2 lineage
GF	Germ-free
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
GSTP1	Glutathione S-transferase pi 1
HDM	House dust mite
HEV	High endothelial venules
h-OVA	OVA heated to 70°C
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

ILF	Isolated lymphoid follicles
JNK	One of the mitogen-activated protein kinases
kDa	Kilo Dalton
LAB	Lactic acid bacteria
LALT	Larynx-associated lymphoid tissue
LBIT	Bronchial immunotherapy
LNIT	Nasal immunotherapy
LTB	Heat-labile enterotoxin
MAD-CAM1	Mucosal vascular addressin cell adhesion molecule 1
MALT	Mucosa-associated lymphoid tissue
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MEK	One of the mitogen-activated protein kinases
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response gene 88
NALT	Nasopharynx (nose)-associated lymphoid tissue
NF-κB	Transcription factor Nuclear factor kappa B
NK cells	Natural-killer cells
NLR	Nucleotide oligomerization domain-like receptor
NOD	Nucleotide oligomerization domain
OIT	Oral immunotherapy
OVA	Ovalbumin
PP	Peyer's patch
PRR	Pattern recognition receptor
p38	One of the mitogen-activated protein kinases
RCTs	Randomized controlled trials
RIG-I	Retinoic acid inducible gene I
RLR	Retinoic acid inducible gene I-like receptor
RNA	Ribonucleic acid
SCIT	Subcutaneous immunotherapy
SIgA	Secretory IgA
SIgM	Secretory IgM

SLIT	Sublingual therapy
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory lymphocytes
TSLP	Thymic stromal lymphopoietin
VSL#3	Probiotic mixture
ZO	Zonula occludens

1. INTRODUCTION

1.1 Allergy

Originally, the term ‘allergy’ was first introduced in 1906 by von Pirquet as ‘a specifically changed reactivity of the host to an agent on the second or subsequent occasion’ (1). This covers a wide spectrum of immune responses, both protective and harmful. However, in the course of time, the definition of allergy has become restricted to those reactions that are harmful to the host.

1.1.2 Hypersensitivity reactions

The word ‘hypersensitivity’ was first used to describe the status of a mammalian organism after exposure to an infectious agent. The mammal is then ‘hypersensitive’ to this agent and therefore able to deal with it effectively on second exposure. In the last century, also the word hypersensitivity acquired the connotation of the reactions harmful to the host. In this context, hypersensitivity reactions are an exaggerated or misdirected immune response that results in local tissue injury or in systemic manifestations, including shock or death. They are initialized after the first contact with an antigen (e.g. inherently harmless antigens such as pollen, food or drugs). Upon subsequent encounter, the ‘sensitized’ immune system reacts by overwhelming, uncontrolled and pathophysiological reaction (2). According to the type of immune response and effector mechanisms, Gell and Coombes originally classified hypersensitivity diseases into four types (2-4). Although this classification doesn’t cover all recently known immunopathological mechanisms, it is satisfactory for the purpose of this work:

(A) **Type I** (immediate/ IgE-mediated hypersensitivity, type I allergy) reactions are mediated by interaction of IgE antibody and antigen. Allergen specific IgE binds to high affinity IgE-receptors on the surface of basophils and mast cells. Cross-linking of bound IgE antibodies after re-exposure to allergens leads to immediate release of inflammatory mediators (histamin, heparin, prostaglandin etc.), which leads to early-phase reactions such as hives, redness, angioedema (local level) or even an anaphylactic shock (systemic level). The late phase occurs 4-6 hours later due to the reaction mediated by recruited inflammatory cells (eosinophils, basophils) and Th2 lymphocytes (4).

(B) **Type II** (antibody-mediated/ cytotoxic hypersensitivity) reactions are due to antibody-antigen interactions on cell surfaces. IgG or IgM bind to host cells or tissues, activating either the classical pathway of complement system (resulting in cell lysis) or recruiting the inflammatory cells (phagocytes, natural-killer (NK) cells), that bind through their Fc receptor with Fc parts of antibodies resulting in elimination of these cells. Type II hypersensitivity reactions develop within several hours and can be involved in autoimmune diseases, transplantations or drug reactions (4).

(C) **Type III** (immune complex mediated) hypersensitivity reactions are local or general inflammatory responses due to formation of circulating immune complexes consisting of antigen and usually IgG or IgM antibodies and their deposition in tissues. Similar to the Type II hypersensitivity, induction of inflammatory response is mediated by reaction with Fc receptors on effector cells (macrophages, neutrophils) or complement activation (5). Systemic diseases such as serum sickness, systemic lupus erythematosus or farmers' lung belong to so called immune complex-mediated diseases. Type III reactions can take hours, days, or even weeks to develop (4, 5).

(D) **Type IV** (T-cell-mediated hypersensitivity, delayed type hypersensitivity - DTH) reactions are mediated by activated $CD4^+$ T lymphocytes or by $CD8^+$ T-cell mediated cytotoxicity. Delayed type reactions take 24 to 48 hours to develop, typical diseases are type I diabetes mellitus, rheumatoid arthritis and contact dermatitis (4).

1.1.3 Type I Allergy

Allergy, the commonest type of hypersensitivity, is often equated with type I hypersensitivity reactions mediated by IgE antibodies as described previously (4). They affect more than 25% of the population in the industrialized countries, thus becoming a substantial public health burden. Their prevalence has steadily increased over the last 50 years (6). The reason for the constant increase of type I allergies is still unclear. Although genetic factors contribute to the risk for developing allergy (7), environmental factors such as pollution, allergen levels, dietary changes, antibiotic overuse and changes in exposure to infectious diseases in early childhood seems to play a fundamental role (4). In almost all epidemiological studies comparing urban and rural areas, it was found that allergies are more prevalent in the city. Children who were raised in a farmhouse with heavy exposure to farm animals and endotoxin had less allergies than children living in the same area but not in a

farmhouse (8). This finding led to the identification of several rural bacterial strains, which were shown to prevent the onset of allergies and also to formulation of so called 'hygiene hypothesis' (9).

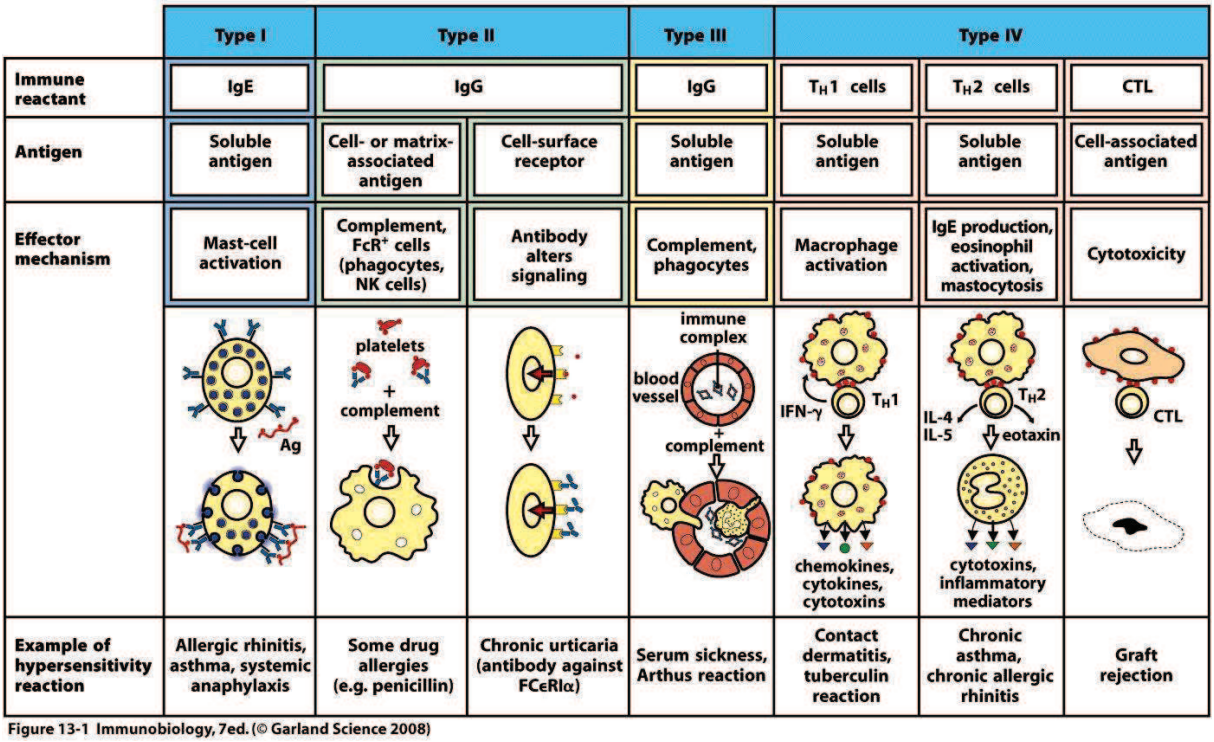

Fig.1: Four types of hypersensitivity reactions (4).

1.1.4 Gene environment

The increased risk of developing allergic diseases is connected with different genetic factors. Genetic linkage to bronchial responsiveness has been reported in humans (10) as well as in mice (11). Interestingly, a different impact of maternally and paternally inherited genetic polymorphisms on IgE production and asthma development has been suggested (12). Maternal atopy affects both pre- and postnatal IgE production, whereas paternal atopy affects mainly the latter. Maternally transmitted genes for antioxidant GSTP1 and high affinity IgE receptor β chain (FceRI-β) are associated with lung function and allergic sensitization, respectively, whereas paternally transmitted allele at chromosome 7p is linked to airway hyperresponsivnes (13).

1.1.5 Hygiene hypothesis

Originally described by Strachan, the 'hygiene hypothesis' (14) implies that less hygienic environments, especially environments that predispose to infections in early childhood, help to protect against atopy and asthma later in life. The underlying mechanistic hypothesis suggested, that infection induced Th1 stimuli balanced the Th2 predominant immune responses in childhood (15). Subsequently, 'hygiene hypothesis' was expanded to changes in the commensal intestinal flora in early childhood (16), which led to its modification to a 'counter-regulation hypothesis' (4). Altered intestinal microbial communities during early childhood may lead to lack of induction of both Th1 and Treg responses, leading to persistence of aberrant Th2 responses (17, 18). Recent studies showed that prenatal and early postnatal exposure to certain microbes or their components, helminth infections or contact with pets can have a protective effect in allergic diseases (19-23).

1.1.6 'Window of opportunity'

The first onset of allergy frequently occurs within the first months of life (24). This time seems to be crucial for proper activation of cells of the innate immune system via their so-called pattern recognition receptors and these cells have been shown to play a crucial role in early shaping of the immune system and suppression of the development of Th2-driven allergic immune responses (25). Although the exact 'window of opportunity' is not known, it appears to be located peri- and postnataly, closing at the end of the first year of life (26). In support of this hypothesis, several studies showed differences in gut microbiota in infants in whom atopy was and was not developing (27, 28). This led to the performance of interventional studies based on application of specific non-pathogenic bacterial strains (probiotics) to inhibit allergic immune responses (29, 30).

1.1.7 Epigenetics

We mentioned that genomic approaches have failed to explain the pathogenesis or the rise in allergic disease. Together with recent studies suggesting that programming of the fetal immune system occurs even before birth (prenatally) with mother playing an important role in this process (20, 31), the question arises: Is there a way to inheritably alter gene function

without altering the genetic code? The answer is yes and epigenetics represents a new field that explains the cross-talk between the environment and the genes (32). Indeed, it has been shown that the T cell differentiation is controlled by epigenetic mechanisms, e.g. methylation of INF-γ gene promoter as the main control of Th1 expression (33), or chromatin remodeling by transcription factor GATA3 for Th2 lineage commitment (34), and that maternal or perinatal exposure to bacterial antigens led to epigenetic changes connected with the allergy development (19, 35).

1.1.8 Mechanism of type I allergy

The initial phase in the development of IgE-mediated allergy is termed sensitization. During this reaction, an allergen enters the body through the epithelial barrier of the skin, airway or gut and is taken up by antigen-presenting cells (APC), mainly immature dendritic cells (DC). Subsequently, allergens are processed to peptides and presented via MHC II molecules to naïve $CD4^+$ T lymphocytes. It is still not clear how the Th2 immune responses are initiated. Recently an intimate association between innate and adaptive immunity has been suggested. Upon allergen exposure in a suitable environmental milieu, IL-25, IL-33 and TSLP are produced by epithelial cells. These cytokines act on innate lymphoid/myeloid type 2 cells that are capable of producing large amounts of Th2 cytokines, such as IL-4, IL-5, IL-9 and IL-13. Allergens are taken up and processed by TSLP and IL-33 primed DC and presented to naïve allergen-specific T cells in a Th2 milieu that drives their differentiation into Th2 cells that start to proliferate and further produce Th2 cytokines (36). These cytokines play a role in the production of allergen-specific IgE, eosinophilia, permissiveness of endothelium for the recruitment of inflammatory cells to inflamed tissues, production of mucus, and decreased threshold of contraction of smooth muscles (4). Allergen specific Th2 cells drive allergen specific B cells to produce allergen specific IgE, which circulates in plasma and binds to high affinity receptor (FcεRI) on effector cells such as mast cells, basophils and activated eosinophils (4). Re-exposure to the allergen induces activation of these cells by cross-linking of the allergen specific IgE antibodies and leads to secretion of proinflammatory mediators (biogenic amines including histamine and heparin; lipid mediators prostaglandins, leukotrienes and platelet-activating factor; enzymes such as tryptase and mast cells protease-1; and cytokines TNF-α, IL-4, IL-5 and IL-13). This immediate allergic reaction caused by mast cells degranulation is followed by a more sustained inflammation, known as the late-

phase responses. They are distinguished by recruitment of other effector cells, notably neutrophils, eosinophils, basophils and Th2 cells to the site of inflammation, which leads to prolonged inflammatory reactions (4).

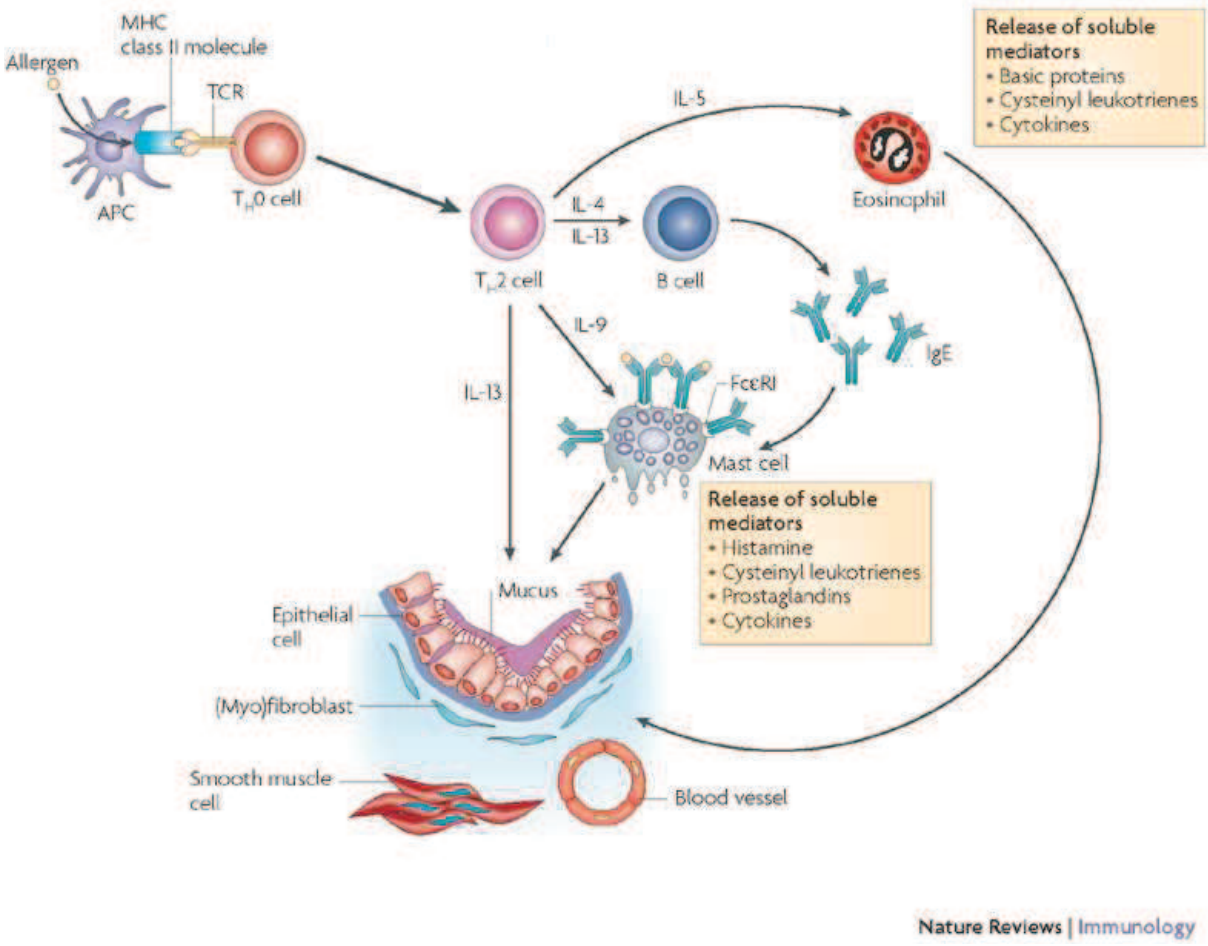

Fig 2: Schematic representation of pathogenic processes in allergic disease (modified from (37).

1.1.9 Pathophysiology of type I allergy

The manifestation of type I allergy depends on the type of allergen (e.g. inhaled pollen allergen, food allergen) and the localization of the allergic reaction (e.g. lung, skin, gut). **Atopic dermatitis** (AD) is a common chronically relapsing inflammatory skin disease, which occurs as a skin reaction to foods and environmental allergens. The prevalence of AD has increased exponentially over the years, with prevalence reaching up to 15% in children and 5% in adults. AD has its onset during the first 8 years of life in 85% affected individuals and

is regarded as the first atopic disease in so called 'atopic (allergic) march' with 30% of AD diagnosed children developing allergic rhinitis and/ or allergic asthma later in life (38). However, up to 50% of children outgrow this chronic disorder during adolescence (39). **Allergic rhinitis** and **conjunctivitis** are commonly known as hay fever. The most prevalent type I allergy results from the reaction of airborne (e.g. birch or grass pollen, house dust mite, animal dander) allergens with sensitized mast cells in the conjunctivae and nasal mucosa to induce the release of pharmacologically active mediators from mast cells; these mediators then cause localized vasodilatation and increased capillary permeability. The symptoms include watery exudation of the conjunctivae, nasal mucosa, and upper respiratory tract, as well as sneezing and coughing (4). **Allergic asthma** is a chronic inflammatory airway disease triggered by allergen induced activation of submucosal mast cells in the lower airways, leading to bronchial constriction and increased secretion of fluid and mucus. The prevalence in the United States of America reaches currently 8% for adults and up to 10% for children (40). Initially reversible airway obstruction changes after repeated allergen exposure to nonspecific airway hyper-responsiveness due to the continued presence of increased numbers of Th2 lymphocytes, eosinophils and neutrophils resulting in chronic inflammation, smooth muscle hyperplasia and airway remodeling (4, 41). **Systemic anaphylaxis** is an immediate systemic reaction caused by rapid, IgE-mediated release of potent immune mediators from tissue mast cells and peripheral basophils which is triggered by allergen introduced directly into the bloodstream (e.g. venom, penicillin) or rapidly absorbed from the gut (food allergy) (42). It is the most severe manifestation of type I allergy. The massive release of mediators leads to loss of blood pressure and airway constriction and may potentially lead to fatal syndrome called anaphylactic shock (4). **Food allergy** is induced by ingestion of allergens from common foods (e.g. peanuts, eggs, milk, shellfish etc.). They are highly digestion-resistant and activate the gastrointestinal mucosa associated mast cells to release mediators, which leads to manifestation of clinical symptoms such as vomiting and diarrhea (4). If the allergen is absorbed into the blood stream, activation of mast cells in dermis and subcutaneous tissue may occur resulting in urticaria (milk, penicillin) or systemic anaphylaxis (peanuts, shellfish) (43). It is estimated that food allergy currently affects up to 5% of the population of the westernized countries and 6-8% of young children (44). Interestingly, in some cases, children can outgrow the sensitivities to milk and eggs by adolescence (45).

1.1.10 Allergens

We are constantly exposed to different food or airborne antigens that do not induce the IgE production. This raises the crucial question: What is so unusual about those antigens that are allergens? Although we don't have a complete answer yet, there are some general properties common to many allergens: they are small proteins (mostly 10-50 kDa) often with side carbohydrate chain, highly stable with intrinsic enzymatic activity, highly soluble in body fluids and they are usually presented to the immune system in very low doses (4, 46). Besides these general properties environmental cofactors (e.g. tobacco smoke, diesel exhausts etc.) acting at the time of allergen exposure might be equally important as contributors to allergic sensitization and disease manifestation (47). Detailed characterization of biological functions and structure is important for predicting cross-reactivity, designing comprehensive diagnostic devices, and assessing the allergenic potential of novel proteins. Many allergens share similar biochemical and structural properties (48). They are distributed into a few protein families such as proteases, ligand-binding proteins, tropomyosins, albumins or Bet v 1 related proteins. Many pollen allergens belong to calcium-binding proteins and profilins whereas plant and animal food allergens are often lipid transfer proteins, profilins and tropomyosins (49).

As a consequence of structural and amino acid sequence similarity, allergen specific IgE recognizes structurally similar epitopes of allergens from close-related and unrelated species. This leads to so called cross-reactivity (50), with birch-food syndrome as an example (51, 52). Moreover, there are many proteins (presumably performing vital functions) that are tightly preserved throughout the evolutionary tree from plants to animals. These might function as so called pan-allergens. One of the first protein families described as pan-allergens were profilins, with homologous allergens in many different heterogeneous allergen sources (53).

1.1.11 Examples of clinically relevant allergens

In industrialized countries the major indoor airborne allergens are house dust mites (*Dermatophagoides pteronyssinus*) derived Der p 1 and Der p 2 and cats (*Felis domesticus*) derived Fel d 1. Outdoor airborne allergens include several trees allergens such as Bet v 1 from birch tree (*Betula verrucosa*) and grasses, e.g. Phl p 1 and Phl p 5 from timothy grass (*Phleum pratense*). Food related allergens are for example Ara h 1, 2, 3 and 6 from peanut (*Arachis hypogaea*), Gal d 1 (ovomucoid) and Gal d 2 (ovalbumin) from chicken eggs (*Gallus domesticus*) and pan-allergen Pen a 1 (tropomyosin) from shrimps (54).

1.1.12 Allergen-specific immunotherapy (ASIT)

In 1911, Noon and Freeman published their pioneering work on the treatment against allergic disease. They aimed at inducing tolerance by subcutaneous administration of increasing amounts of relevant allergen extracts (52). Nowadays, this treatment is known as allergen-specific immunotherapy (or desensitization, or hyposensitization) (52) and is defined as the administration of slowly increasing doses of specifically relevant allergen in the treatment of IgE-mediated allergic diseases, until a maintenance dosage is achieved or the patient is free of symptoms (55).

Currently, there are three common therapeutic approaches for allergic diseases:
(A) **Avoidance of the allergen** – not always possible, dependent on the type of allergy and allergen (e.g. house dust mite allergens vs. food allergens)
(B) **Symptomatic treatment** – pharmacological control of symptoms and prevention of exacerbations with a minimum of drug related side-effects (e.g. antihistamines, corticosteroids, anti-IgE antibodies (56).
(C) **Causative treatment** – allergen specific immunotherapy. Conventional subcutaneous immunotherapy (SCIT) has become the gold standard for the causative treatment for IgE-mediated allergic diseases for a large variety of allergens (52). In order to improve compliance to immunotherapy the change to a less invasive route of application via the mucosal surfaces (e.g. oral, nasal, sublingual) has been investigated in several studies (57-59). Of these the sublingual therapy (SLIT) seems to be the most promising alternative strategy (58).

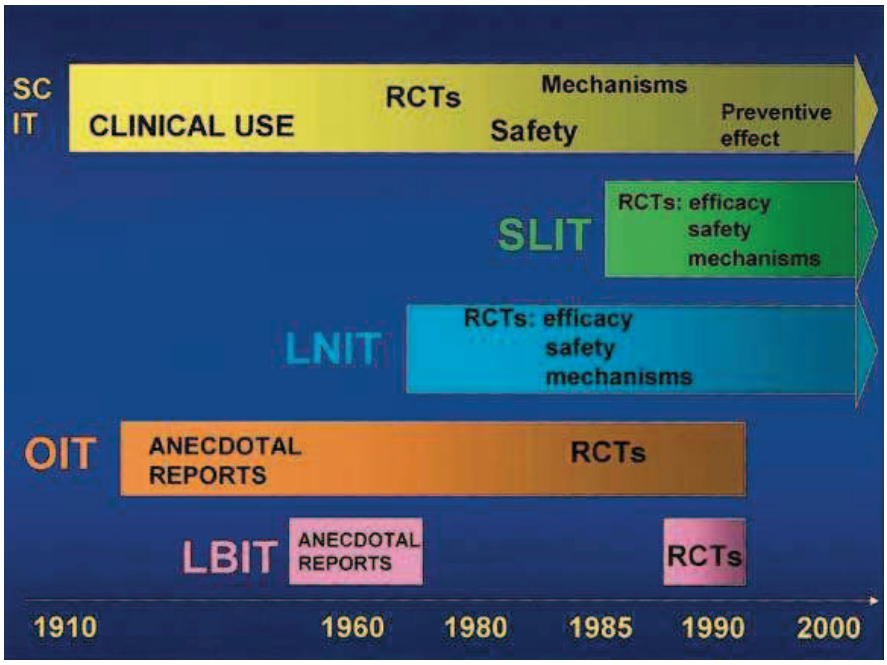

Fig. 3: The chronology of the different modalities of immunotherapy. RCTs - Randomized controlled trials, SCIT - Subcutaneous, SLIT - Sublingual, LNIT – Nasal, OIT – Oral, LBIT – Bronchial immunotherapy (58).

1.1.13 Subcutaneous immunotherapy (SCIT)

Subcutaneous immunotherapy (SCIT) is a well-established form of immunotherapy with proven efficacy under a variety of conditions. Immunotherapy is effective for the treatment of allergic rhinitis (including ocular symptoms), allergic asthma and stinging insect hypersensitivity and is effective in both adults and children (60). Typically, SCIT starts with weekly injections (12 – 16 weeks) of gradually increasing doses of standardized allergen extract up to a maintenance dose (5µg – 20µg of the major allergen), and then continuation of treatment at this dose (injection every 4 – 6 weeks) for a period of time (usually 3 – 5 years) (61). It has been shown that after completed SCIT treatment the protection can last up to 7 years (62).

Although SCIT is a well-established and highly effective therapy, it has several drawbacks. Severe side-effects can occur with the risk of developing anaphylactic shock (63), and during the long-lasting treatment many injections need to be applied by a skilled medical practitioner at specialized centers, which diminishes the compliance of the patients, particularly of children.

1.1.14 Sublingual immunotherapy (SLIT)

SLIT is a less invasive form of immunotherapy, which involves placing the vaccine in solution (drop preparation) or tablet form under the tongue for 1–2 min followed by swallowing (61). The optimum dosage, duration and frequency of administration have not yet been established. However, the commonly used protocol for aeroallergens involves daily treatment starting pre-seasonally and continuing perennially for 3 years (64). So far it has been successfully used against grass and tree pollen, house dust mite and cat allergy. Compared to SCIT the cumulative monthly dosage of SLIT has been 0.6 – 500 times greater (61). Described adverse reactions are rare, consisting mainly in local reactions in oral cavity (59).

1.1.15 Mechanism of allergen specific immunotherapy

Although exact mechanisms of ASIT are still not clearly known, in the last two decades major advances have been made in clarifying the underlying cellular and molecular events. In the recent review on understanding of the mechanisms of allergen-specific immunotherapy Akdis (65) suggests that regulatory T-lymphocytes (Treg) are the key regulator of allergen tolerance due to immunotherapy. Regional antigen-presenting cells (mainly dendritic cells), targeted by ASIT, induce Treg ($CD4^+CD25^+FoxP3^+$) cells and Tr1 cells ($IL\text{-}10^+$). These Treg cells and accompanying production of regulatory cytokines (such as IL-10 and TGF-β) contribute to the control of allergen-induced immune responses in several different ways: (1) suppression of antigen presenting cells that support the generation of effector T cells; (2) suppression of Th2 and Th1 cells; (3) suppression of allergen-specific IgE and induction of IgG4; (4) suppression of mast cells, basophils and eosinophils; and (5) interaction with resident tissue cells and remodeling (66).

One important aspect in tolerance induction via ASIT is an early and rapid increase in production of IgG4 and IgA antibodies, which is in line with the observation that IL-10 is a potential IgE suppressor and TGF-β is an IgA switching factor (65). IgG4 is thought to capture the allergen, acting as so called ‘blocking’ antibody and preventing the cross-linking of cell-bound allergen specific IgE and activation of mast cells and basophils (65).

The mechanisms of action in SLIT have been found to be similar as in injection immunotherapy, with focus on IL-10 induced Treg cells and IgG4 blocking antibodies. The initial step is represented by an allergen uptake by Langerhans cells within the mucosa via the

high affinity surface IgE receptors (67). In this respect, Bohle et al. (68) showed an initial Treg dominance and later on an upregulation of the expression of the Th1 cytokine INF-γ in birch pollen allergic individuals during the SLIT. Similar results have been reported in grass pollen SLIT: time dependent promotion of sublingual FoxP3-expressing cells, increase of allergen specific IgG4 and IgA and the inhibitory activity on IgE (69).

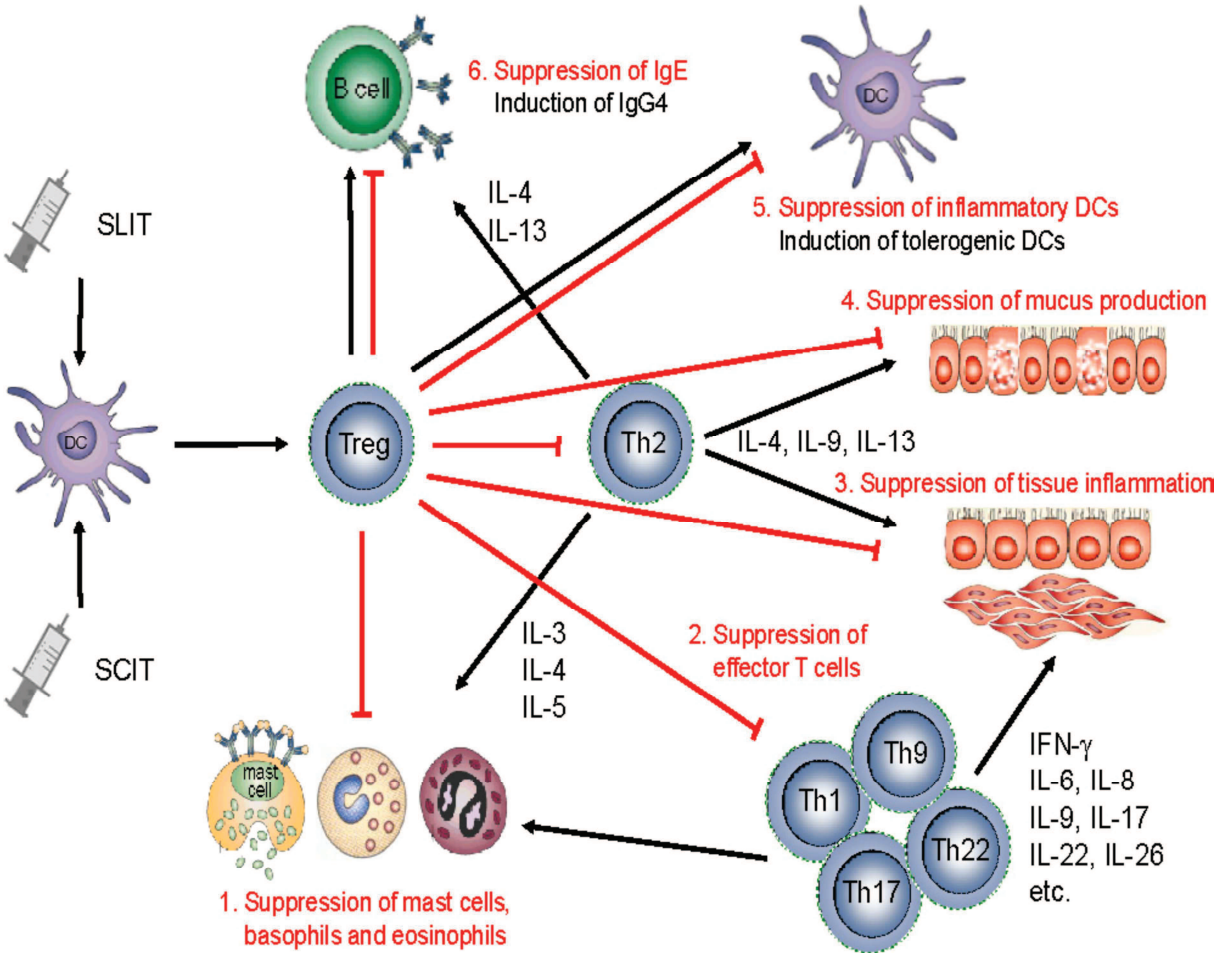

Fig. 4: Mechanisms of allergen-specific immunotherapy (66).

1.1.16 New treatment strategies of allergic diseases

Crude allergen extracts are common preparations used for ASIT. Although they show high efficacy with long lasting clinical effects (60), they have several drawbacks, including inconsistent composition or the presence of other antigens that can induce neo-sensitization during the course of treatment. Moreover, allergen extracts can also contain bioactive substances that could be potentially proinflammatory or possess Th2- promoting capacities (52, 70). Therefore, there is a need for new therapeutical agents and treatment strategies (52, 71):

Alergoids are allergens modified with glutaraldehyde or formaldehyde, which reduces IgE epitopes while preserving T-cell epitopes (72). In this respect, it has been shown that extensive heating destroys conformational IgE-binding epitopes in ovalbumin (73) and we have shown that even minor irreversible changes in OVA structure reduce its allergenicity (74). Another possibility is the use of **recombinant allergens**. These are purified proteins, produced by using the allergen's known molecular, immunological, and biological characteristics. They can be produced as a recombinant wild-type allergen (allergen is produced to mimic the properties of the natural allergen) or recombinant hypoallergens. **Recombinant hypoallergens** are characterized by a strong reduction or suppression of IgE reactivity and maintenance of T cell epitopes by site-directed or random mutagenesis, recombinant fragments, or deletion variants lacking conformational IgE epitopes (75). Recombinant proteins or mixtures of allergens have been studied in many clinical trials and animal studies (52). For example, studies with recombinant Bet v 1 and Bet v 1 fragments showed the immunogenic potential of these constructs (76, 77). Interesting option is the use of **peptide fragments** of corresponding T-cell epitopes of the specific allergens to induce immunological tolerance and decrease allergenicity. The advantage is the small size of the peptides, which reduces their ability to cross-link allergen-specific IgE on mast cells and thus prevent the IgE mediated side-effects. This approach shows promising results for animal, venom and grass allergy (71). Other promising approaches consist in creating **hybrid molecules**, where multiple allergens are produced as a fusion molecule and used in treatment of poly-sensitized individuals (75).

Genetic vaccination and **gene therapy** are as yet purely experimental treatment options. The first takes advantage of administration of allergen encoding nucleic acids, thus overcoming

the need to produce and purify corresponding antigen/antigens; the latter aims at administration of hematopoietic stem cells transduced to express the allergen (75).

In order to improve the efficacy of immunotherapy, **adjuvants** are used in most allergen preparations. After the World War II, aluminium hydroxide (Alum) was broadly introduced. However, it has been shown to induce Th2 responses and therefore other adjuvants with promising immunogenic properties are being tested (6): TLR4 ligand monophosphoryl lipid A (MPL) (78), virus like-particles (79), TLR9 ligand CpG oligonucleotides with strong Th1 inducing properties (80) or the cholera toxin B subunit (CTB) (81). Due to their intrinsic immunomodulatory properties several probiotics, mainly lactic acid bacteria (LAB) strains and mixtures have been co-administrated with allergens in mouse models (82, 83) and the use of LAB as vehicles for mucosal delivery of recombinant allergen is an attractive concept for development of well-tolerated and effective allergy vaccines (84).

1.1.17 Prophylactic vaccination against allergies

The conventional immunotherapy of allergic patients faces the inherent difficulty of attempting to counteract an already established pathological immune response (85). Therefore prophylactic treatment for prevention of allergic sensitization represents an attractive approach to stop the allergic pandemics (86). Two strategies seem to be feasible: 1) induction of high levels of allergen specific IgG, which then serves as an allergen blocking antibody or 2) induction of Treg cell and tolerance. Indeed, IgG antibody induction with hypoallergens or early tolerance induction via mucosal routes (e.g. oral, nasal) with T-cell epitope-containing peptides or recombinant hypoallergens has already been successfully tested in animal models (86). These proposed strategies would not be possible without determination of the time of allergic sensitization, which has been localized shortly after birth (24), thus directing the prophylactic interventions into the perinatal period (86).

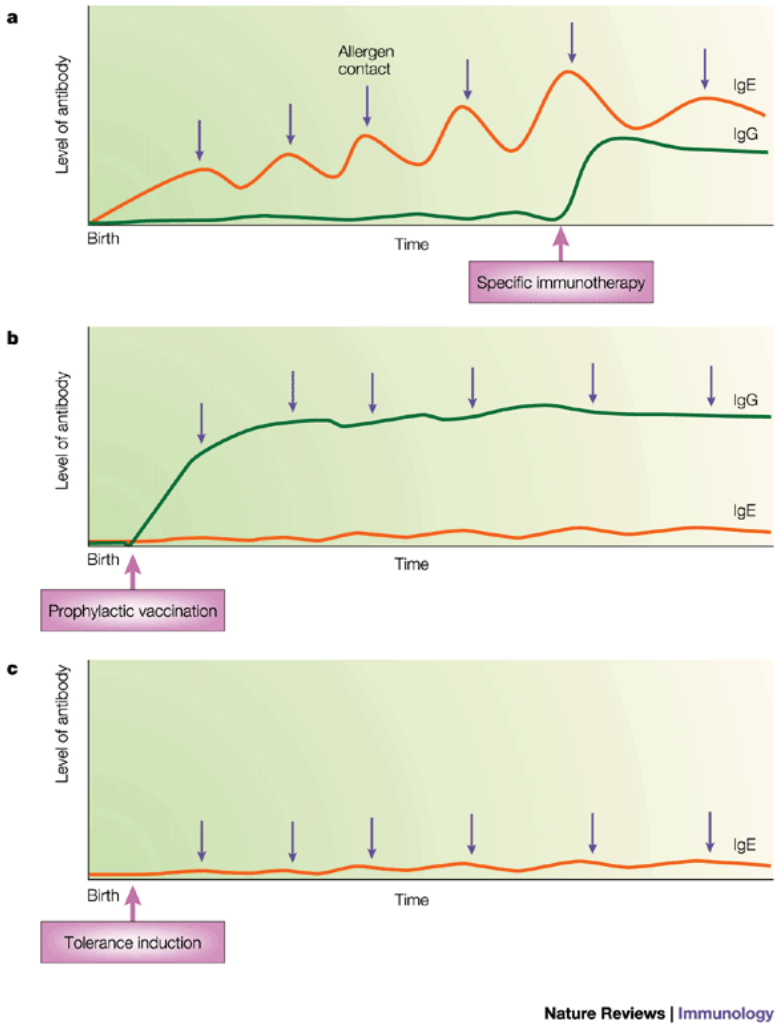

Fig. 5: Proposed action of prophylactic treatment for prevention of allergic sensitization (85). a) In allergic individuals, sensitization occurs shortly after birth due to allergen contact. Immunotherapy that counteracts established allergy is mostly associated with the strong induction of allergen-specific IgG antibodies and T-cell modulation. b) Prophylactic vaccination shortly after birth might induce high levels of allergen-specific IgG antibodies and prevent sensitization. c) Tolerance induction early in life might prevent the development of IgE responses after allergen contact.

1.2 Probiotics

The concept of using live bacteria to improve health was first conceived at the beginning of the 20th century by Elie Metchnikoff. Based on his study on health and longevity of Bulgarian peasant population he hypothesized that the regular consumption of lactic acid bacteria (useful microbes) in fermented dairy products such as yogurt can modify the flora in

our bodies and replace harmful microbes, thus improving health and promoting longevity (87, 88). The term 'probiotics' was first used in 1965 (89), albeit in a different context ('substances secreted by one organism which stimulate the growth of another'). Fifteen years later, Fuller (90) proposed that probiotics were 'live microbial supplements which beneficially affect the host animal by improving its microbial balance'. According to the FAO/WHO probiotics are currently defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (91). Over the last two decades the fundamental role of the commensal bacteria in modulating mucosal immune responses has been widely acknowledged and there is a great interest in the therapeutical potential of probiotics for many immune disorders, including allergies (92).

Characteristic	**Functional advantage**
Local GIT environment	Resistance to pH, bile, and digestive enzymes
Epithelial cell adherence	Prevent binding of pathogens or food antigens
Human origin	Increased likelihood of biological effectiveness
Anti-microbial activity	Direct toxicity to harmful bacteria, viruses, fungi, and parasites
Safety	Well-tolerated, important for clinical use

Table 1: Characteristics of an ideal probiotic strain (93).

1.2.1 Commonly used probiotic species

Many probiotics are lactic acid bacteria (LAB) including species of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Bifidobacterium* and *Leuconostoc*. They are widely distributed in the gastrointestinal tract; many species have been used in food processing and are generally regarded as safe (GRAS status). Besides these some nonpathogenic strains of *Escherichia coli* (e.g. *E. coli* Nissle, *E. coli* O83) and certain yeast strains (e.g. *Saccharomyces boulardii*) are used (26, 87). According to the definition, probiotics have to be alive and safe; therefore rigorous testing (e.g. pH tolerance, bile acid survival, pathogen-inhibiting properties) should be applied when selecting new strains (91). In

this respect we have selected three new probiotic strains and showed their safety and immunomodulatory properties upon administration to mice (94).

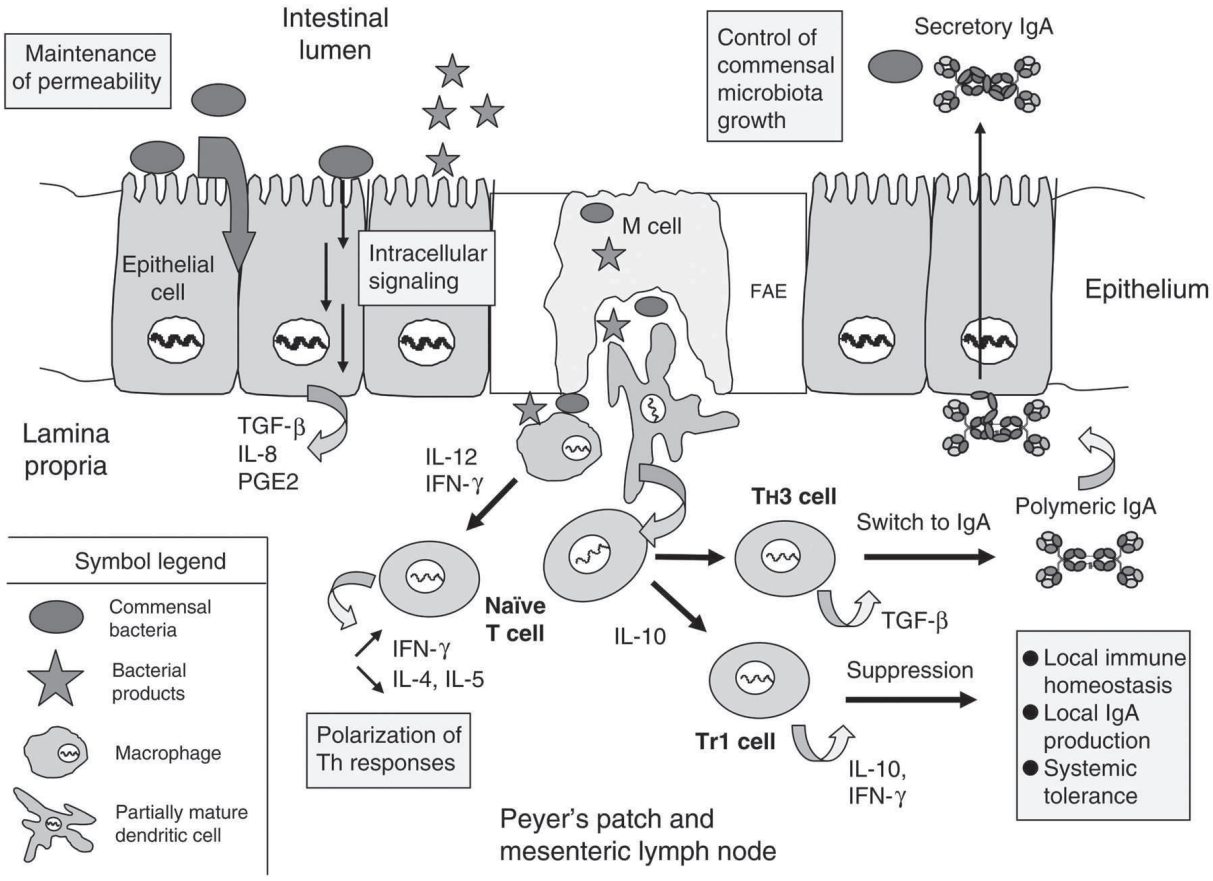

Fig. 6: Schematic representation of the multiple consequences of the cross-talk between the probiotic bacteria and the intestinal mucosa (95).

1.2.2 Probiotic mode of action

The effects of probiotics can in general be classified into two categories: 1) interaction of probiotics with other intestinal organisms (commensal and/ or pathogenic) and 2) interaction with the host by modulating epithelial barrier function and innate as well as acquired immune system (92). It has to be stressed that the probiotic effects vary between species and even strains; they depend on the metabolic properties, expression of surface molecules and secreted components (96).

Probiotics can directly affect other microorganisms in various ways (92). Production of bacteriocins with an antimicrobial effect towards Gram-positive as well as Gram-negative bacteria has been shown for bifidobacteria strains (97) as well as lactobacilli (98). To compete for the limiting iron availability in the gut, probiotic strain *E. coli* Nissle 1917 has been shown to utilize at least seven different iron uptake systems (99). The reported adherence blocking of pathogens is a result of competition between probiotic and pathogen for the same receptor, the induction of increased mucin production or formation of biofilm by probiotics (92). Moreover several strains including *E. coli* Nissle 1917 and *L. casei* DN114001, are able to prevent the invasion of epithelial cells by pathogens such as *S. typhymurium* (100).

Concerning the host the main targets of probiotic action are gut epithelial and gut-associated immune cells. Probiotics are able to reinforce the intestinal barrier as well as directly modulate epithelial cell functions including cytokine and chemokine release. *E. coli* Nissle 1917 and probiotic mixture VSL#3 have been shown to increase the expression and prevent the redistribution of ZO-1 and ZO-2, which are an important factor for preservation of tight-junction function in gut epithelium (101). Although it is a limited event, probiotics translocate to the lamina propria and affect innate and adaptive immunity (94). This translocation is supposed to happen in two ways: Sampling by M cells in Peyer's patches (PP) or by extending protrusions by DC present in lamina propria (102) (see the chapter Mucosal immune responses in the gut). DC as the main antigen presenting cells are the bridge between the innate and adaptive immune system, and present microbial antigens to naïve T cells in the PP and mesenteric lymph nodes (MLN). The IgA antibody-mediated mucosal response takes place and different T cell subsets are induced in a probiotic-strain dependent manner (95, 102).

1.2.3 Interaction of probiotics with immunocompetent cells

Probiotics can directly interact with monocytes, macrophages, lymphocytes and DC (103). Among these DC have the pivotal position at the intersection of innate and adaptive immunity with their ability to recognize and respond to bacterial components and to initiate primary immune responses (104). Probiotics are recognized by pattern recognition receptors (PRRs), which recognize conserved molecular structures known as microbe associated molecular patterns (MAMPs), and signal to induce the production of cytokines, chemokines

and maturation of antigen presenting cells. These receptors can be divided into three main groups: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors that recognize viral RNAs (RLRs) and nucleotide oligomerization domain-like (NOD) receptors (NLRs)(96). Selected ligands and sub-cellular localization are depicted in Table 2. Ligand binding to PPRs initiates a signaling cascade consisting of adaptor protein and cellular kinases which leads to initiation of downstream signaling cascades, i.e. mitogen-activated protein kinase (MAPK) and NF-κB pathway and other signaling pathways (104) with already mentioned consequences.

Recent reports show that certain probiotic strains of *Lactobacillus* and *Bifidobacterium* induce low maturation of DC accompanied by IL-10 and/ or TGF-β production. As a consequence, these DC promote the generation T regulatory cells (11, 105-107). On the other hand certain probiotic bacteria induce strong maturation of DC and production of high amounts of IL-12. These dendritic cells induce the polarization of naive Th cells into pro-inflammatory Th1 lineage (96, 108). Based on these reports, it has been recently suggested that according to their immunomodulatory properties probiotics can be divided into two groups: regulatory immune response inducers and pro-inflammatory immune response inducers (96, 109). In this respect we have shown striking differences between the effects of two bacteria (*B. longum* and *L. plantarum*) with different immunomodulatory properties on allergic sensitization in gnotobiotic model of allergic sensitization (83) (Schwarzer, unpublished results).

Receptor	Localization	Ligand	Origin of the ligand
TLR2	Cell surface	Lipopeptides	Bacteria
		Lipoproteins	G+ bacteria
		LTA	G+ bacteria
TLR2/1	Cell surface	Triacylated lipopeptide	G-bacteria, mycoplasma
TLR2/6	Cell surface	Diacylated lipopeptides	G+ bacteria, mycoplasma
TLR3	Intracellular compartment	dsRNA	Viruses, virus infected cells
TLR4/MD2	Cell surface, intracellular compartment	LPS	G- bacteria
TLR5	Cell surface	Flagellin protein	Bacteria
TLR7	Intracellular compartment	ssRNA	Viruses
TLR8	Intracellular compartment	ssRNA	Viruses
TLR9	Intracellular compartment, cell surface	DNA	DNA viruses, bacteria
TLR11	Cell surface	Uropathogenic bacterial components	Uropathogenic bacteria
NOD1	Cell cytoplasm	Meso-DAP	PG from G-, some G+, mycobacterium
NOD2	Cell cytoplasm	MDP	PG from G-, G+ bacteria, mycobacterium

PG, peptidoglycan; LTA, lipoteichoic acid; LPS, lipopolysaccharide; DAP, diaminopimelic acid; MDP, muramyl dipeptide; G+, Gram positive; G-, Gram negative; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA.

Table 2: Pattern recognition receptors, sub-cellular localization and respective ligands (96).

1.3 Mucosal immune system

The mucosal surface comprises the gastrointestinal tract, upper and lower respiratory tracts and the urogenital tract, extending to an area of more than 400 m^2, which is 200 times the area of skin (4) and the mucosal-associated immune system, the largest and most complex part of the immune system, harbors almost 80% of the immunologically active cells of the body (110).

To fulfill its function the mucosal immune system has to discriminate between harmful and beneficial antigens (111), which means it has to mount strong protective immunity against invasion and colonization by pathogens and at the same time prevent the development of potentially harmful immune responses to antigens derived from food, airborne material and commensal microorganisms.

The mucosal immune system can be principally divided into **inductive sites,** where antigens sampled from mucosal surfaces stimulate cognate naive T and B lymphocytes, and **effector sites**, where the effector cells after extravasation, retention, and differentiation perform their action. The mucosal immune responses are based on the recirculation of immunocytes between these two sites (112).

The **inductive sites** for mucosal immunity consist of mucosa-associated lymphoid tissue (MALT) as well as local and regional mucosa-draining lymph nodes (LNs) (112). Subdivided according to anatomical location, they include gut-associated lymphoid tissue (GALT) with the Peyer's patches (PP), isolated lymphoid follicles (ILF), mesenteric LNs and the appendix; the Bronchus-associated lymphoid tissue (BALT, not generally present in healthy individuals) and nasopharynx (nose)-associated lymphoid tissue (NALT). Other not yet well characterized potential MALT structures have been described (e.g. CALT – conjunctiva-associated, LALT – larynx-associated) (112).

On the other hand, the **effector sites** consist of distinctly different compartments including the lamina propria of various mucosae and surface epithelia (113).

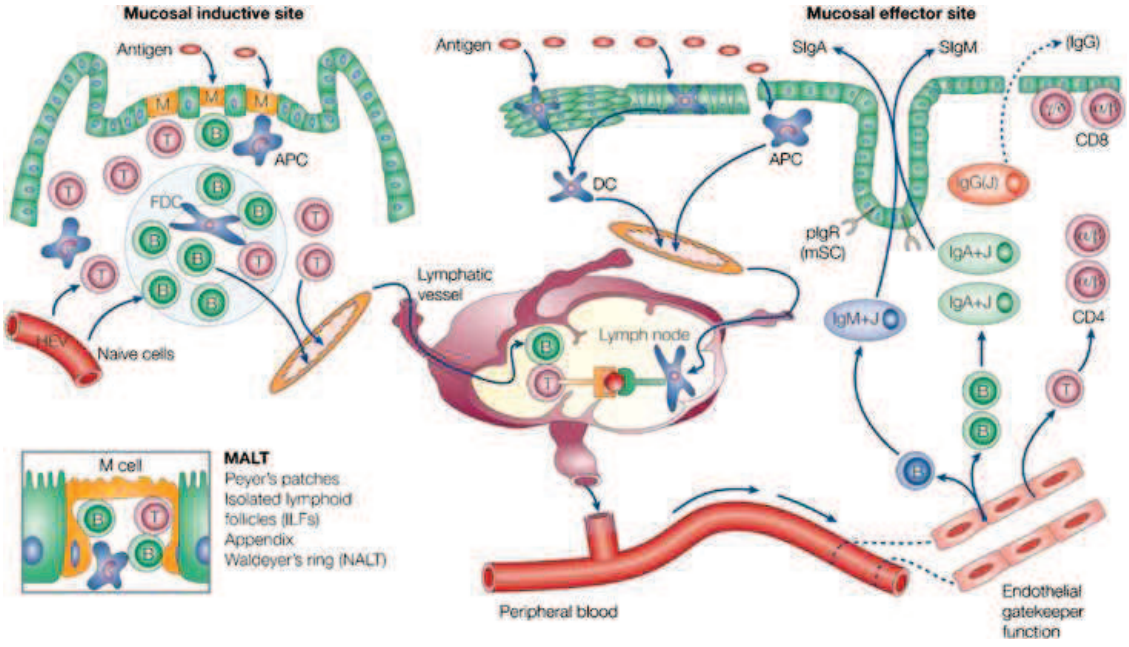

Fig. 7: Human mucosal immune system. APCs, antigen-presenting cells; DC, dendritic cells; FDCSs, follicular dendritic cells; HEVs, high endothelial venules; MALT, mucosa-associated lymphoid tissue; pIgR, polymeric Ig receptor; SIgA, secretory IgA; SIgM, secretory IgM (112).

1.3.1 Mucosal immune responses in the gut

Main goal of mucosal immunity is to reduce the need for elimination of penetrating exogenous antigens by proinflammatory systemic immunity. The first line of defense is provided by the microbiota itself, consisting in exclusion of pathogenic bacteria due to the limited resources and receptors (110). The primary function of the epithelial cells is to create a physical barrier (‘castle wall’) and several epithelial cell types with distinct function such as goblet cells (secretion of the protective mucus layer) or Paneth cells (secretion of anti-microbial peptides, such as defensins, cathelicidins, and calprotectins) contribute to host protection (114). In recent years there has been a shift in the perception of epithelial cells as ‘passive barrier’ (111): they are MHC II positive but lack the expression of co-stimulatory molecules which makes them good candidates for tolerogenic APCs. Moreover, they have been shown to produce TSLP and IL-25, thus contributing to immunotolerant milieu of the mucosa (115).

Antigens and microorganisms that cross this barrier are caught by APCs, mainly DC. This translocation is assumed to happen in two main ways:
(A) In the specialized lymphoid aggregates called Peyer's patches. They consist of large B-cell follicles and intervening T-cell areas. These are separated from intestinal lumen by specialized epithelial cells – microfold (M) cells, which lack microvilli on the luminal surface and normal thick layer of mucus. M-cells are cells specialized cells for endocytosis and transepithelial transport and they closely collaborate with APCs, mainly DC, macrophages and B-cells which are located in their basolateral pockets (116, 117).
(B) Intra- or sub-epithelial DC can directly sample the lumen content across the intestinal epithelium by migrating into the narrow spaces between epithelial cells and extending protrusions (102).

Antigen loaded DC might directly present the antigen to the T cells in PP or alternatively, they might migrate to the MLN and present the antigen to the naive T cells. Activated T cells differentiate into helper T cells or Treg cells and migrate to the mucosa to induce local immune responses. MLNs are now viewed as a crossroad between the peripheral and mucosal circulation pathways and thus T cell activation in MLNs might explain the induction of systemic immunity or tolerance in response to intestinal antigens (111). Activated T cells also interact with antigen primed B cells and secrete TGF-β to induce B cell class switching to IgA (4). Lymphocytes primed in the GALT up-regulate the expression of 'homing receptors', which are tissue specific adhesion molecules (e.g. α4β2 integrin) and chemokine receptors (e.g. CCR9), which guide them back to the mucosal effector sites. In the mucosal vasculatures high levels of α4β2 integrin ligand MAD-CAM1 are expressed, thus allowing effector T cells and IgA secreting plasma cells to migrate to the mucosa (118).

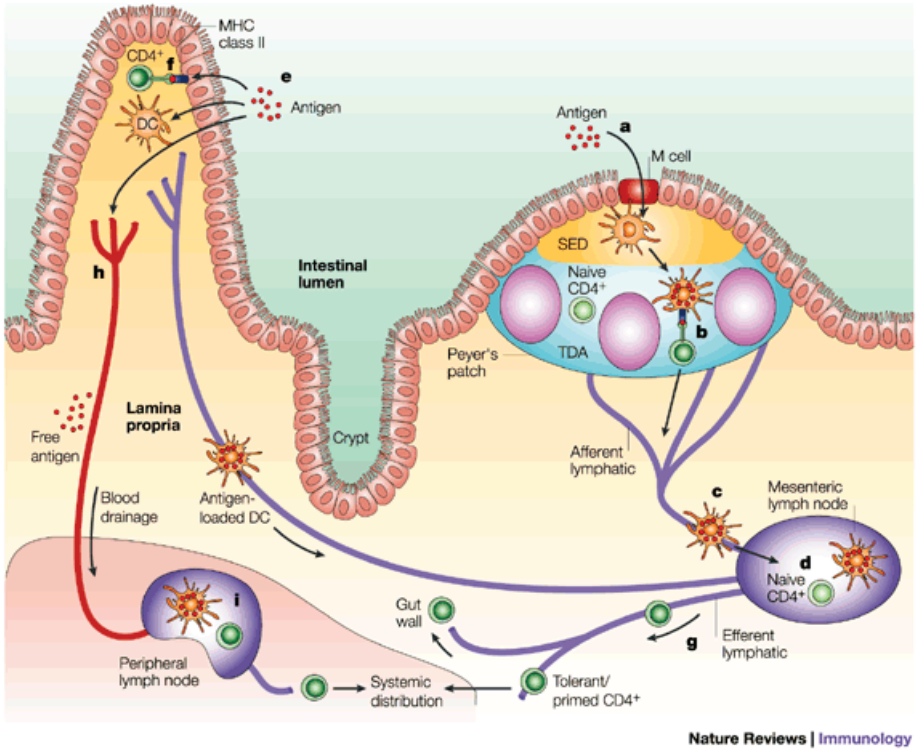

Fig. 8: Antigen uptake in the gut. Antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (a), and after transfer to local dendritic cells (DC), might be presented directly to T cells in the Peyer's patch (b) or DC can flow with the draining lymph (c) and subsequently present the antigen to T cell in the mesenteric lymph nodes (MLNs) (d). A similar process might occur if antigen enters through the epithelium covering the villus lamina propria (e), but in this case, there is the further possibility that MHC class II^+ enterocytes might act as local APCs (f). In all cases, the antigen-responsive $CD4^+$ T cells acquire expression of the alpha4β7 integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (g) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Antigen might also gain direct access to the bloodstream from the gut (h) and interact with T cells in peripheral lymphoid tissues (i) (111).

1.3.2 Adaptive anti-inflammatory mechanisms of the mucosal immune system

The mucosal immune system operates two main adaptive anti-inflammatory mechanisms: (1) immune exclusion performed by secretory (s)IgA (and sIgM) antibodies and (2) immunosuppressive mechanisms to avoid local and peripheral hypersensitivity to

innocuous antigens. The latter strategy is commonly called mucosally induced tolerance and in the gut it is often referred to as ‘‘oral tolerance” (119).

1.3.3 IgA production

IgA is the most abundant antibody subclass produced at the mucosal surface. In contrast to the IgA in internal fluids the majority of externally secreted IgA is present in the form of dimers and tetramers, in which IgA monomers are linked via an additional small polypeptide termed J chain (4). Due to the polymerization and extensive glycosylation, sIgA is protease-resistant to avoid degradation in the intestinal lumen. IgA is actively transported across the epithelial barrier by the epithelial polymeric immunoglobulin receptor (pIgR), and its extracellular part then creates the so-called secretory component of the sIgA (120). IgA protects mucosa by inhibiting uptake and neutralizing biologically active antigens and induces immune exclusion by specific and non-specific binding to bacterial and viral surfaces (110). Additionally, sIgM and IgG are also produced at the mucosal sites and in IgA deficient individuals can to some extent replace the function of sIgA (120).

1.3.4 Mucosal tolerance

Originally in 1911 Wells and Osborne (121) showed that anaphylactic reactions to ovalbumin (OVA) in guinea pigs could be prevented by prior feeding of OVA and they defined this specific suppression as ‘oral tolerance’. Due to the fact that antigen specific unresponsiveness can be induced by other mucosal routes (e.g. nasal, sublingual, rectal) it is now called mucosal tolerance (4). The suppression of systemic immune responses is long lasting and antigen specific: responses to other antigens are not affected. There are two main effector mechanisms of mucosal tolerance 1) clonal anergy (functional unresponsiveness) or apoptosis (deletion) of Ag-specific T cells and 2) active suppression through the induction of Ag-specific regulatory T cells (122). The primary factor that determines which form of tolerance develops following oral administration of antigen is the dose of antigen: Low doses favor the generation of regulatory cell-driven tolerance, whereas high doses of antigen favor anergy and apoptosis driven tolerance (123). Based on its mechanism mucosal tolerance has

been suggested as a potent therapeutic strategy for treatment of human autoimmune as well as other diseases (124).

Induction of regulatory cells via the mucosal delivery of antigens is essential for development of tolerance (125). In mouse several types of Treg cells have been distinguished:

Thymic derived FoxP3$^+$ Tregs (nTregs) are CD4$^+$CD25$^+$FoxP3$^+$ cells produced during CD4$^+$ T cell differentiation in thymus due to their TCR high affinity interaction with self-antigens. They suppress immune responses in cell-cell dependent manner, by acting as an IL-2 sink or by production of IL-10 and/ or TGF-β (126).

Mucosally induced FoxP3$^+$ Tregs (iTregs) are CD4$^+$CD25$^+$FoxP3$^+$ cells induced by antigen feeding. Their induction has been shown to be IL-2, retinoic acid and TGF-β dependent (126).

Tr1 type Tregs are CD4$^+$CD25$^-$FoxP3$^-$ cells exerting their immunosuppressive effect by producing regulatory cytokine IL-10 and TGF-β. They can be induced from naive cells in the presence of IL-10 and antigen. They have been shown to regulate tolerance in small intestine as the main producers of IL-10 (127).

Th3 type Tregs are CD4$^+$CD25$^-$ cells that produce TGF-β, a potent immunoregulatory cytokine involved in immunosuppression, cell differentiation and IgA class switching (125).

CD8 Tregs have been shown to play a role in oral tolerance induction in murine model, although it seems that they are not essential, contrary to the CD4$^+$ T cells (128). They have also been shown to maintain epithelial integrity in mouse colitis model in IL-10 dependent manner (129).

iNKT cells represent phenotypically and functionally heterogeneous group positive for NK1.11abTCR1. They have been shown to exert regulatory functions in intestinal tissue homeostasis through IL-10 and possibly TGF-β production (130).

γδ T cells are intestinal intraepithelial cells positive for γδ -T cell receptor. They contribute to oral tolerance and downregulate exaggerated inflammatory responses by TGF-β production (131).

1.3.5 Mucosal delivery systems

To improve immunogenicity or tolerance induction, several mucosal delivery systems have been studied as promising candidates for mucosal vaccination (132). The proposed advantages of delivery systems should be: 1) protection of the vaccine from physical

elimination and enzymatic digestion, 2) facilitating antigen uptake across the epithelium and target subepithelial APCs, 3) stimulating and modulating the antigen induced immune responses.

The non-living systems include various inert systems (e.g. liposomes, biodegradable particles etc.), linking antigens to proteins with binding affinity for epithelial surfaces, such as *E. coli* heat-labile enterotoxin (LTB) or cholera toxin (CTB) and several attenuated viruses and virus-like particles. Two main categories of live bacterial vectors can be distinguished, one based on attenuated pathogens such as *Salmonella* or *Bordetella*, and the other using commensal bacteria, such as lactobacilli, streptococci or staphylococci as vectors (132).

Several reports have demonstrated the great potency of lactic acid bacteria (LAB) as mucosal delivery vehicles and adjuvant system. LAB have been shown to modulate T-cell responses to an expressed or co-administered antigen, and genetically modified LAB thus represent an attractive vaccination approach (82, 84, 108, 133). LAB have been manipulated to produce several allergens such as Der p 1, cow's milk allergen BLG and Bet v 1 and they were shown to effectively prevent the allergic sensitization in mice (108, 133, 134). In this respect we have shown that neonatal colonization of germ-free mice with *L. plantarum* constitutively producing Bet v 1 reduced allergen-specific sensitization and induced a Th1-biased cytokine profile upon subsequent systemic sensitization (83).

1.4 Gut microbial community

Each human adult harbors approximately 10^{14} bacteria in the gut, which is about 10 times the number of cells making up the human body (135). There are at least 400–500 different bacterial species and these species can be divided into different strains, thus even enhancing the great complexity of intestinal microbiota (26). From these bacteria only one third is cultivable and only recently we have been getting acquainted with the uncultivable members by molecular-biological techniques and next-generation sequencing (136).

The germ-free status of the fetus changes rapidly after birth. Upon delivery, the gut is colonized with *Escherichia coli*, *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp., *Bacteroides* spp. and *Bifidobacterium* spp. The last has been shown to predominate in the fecal flora in breast fed infants (137). In neonates the composition of colonizing microbiota fluctuates and it becomes stable after weaning resembling the microbiota of adults. The initial

colonization pattern can be influenced by multiple factors such as individuals' genetic background, mode of delivery, dietary changes, high hygiene or over-use of antibiotics (138).
According to Isolauri, the promotion of specific strains of the healthy gut microbiota provides several benefits: 1) Balance in the gut microbiota, 2) Enhanced nutrition uptake (i.e. increased energy efficiency), 3) Enhanced macrophage activity and phagocytosis, 4) Tightening of the intestinal epithelium and strengthened gut barrier function, 5) Competitive exclusion of pathogens by impeding their adhesion potential, 6) Induction of mucin production, 7) Stimulation of gut humoral immunity (IgA), 8) Augmentation of antigen-specific immune responses, lowering the risk of reinfection (137).
In the light of events discussed above (e.g. epigenetics, window of opportunity etc.) the development of gut microbiota in early life is crucially related to health later in life, influencing the maturation, structure and function of our immune system, metabolic health and microbiological programming (137). Hence, given the complexity of the host – microbiota interaction, there is a fundamental need for appropriate model(s).

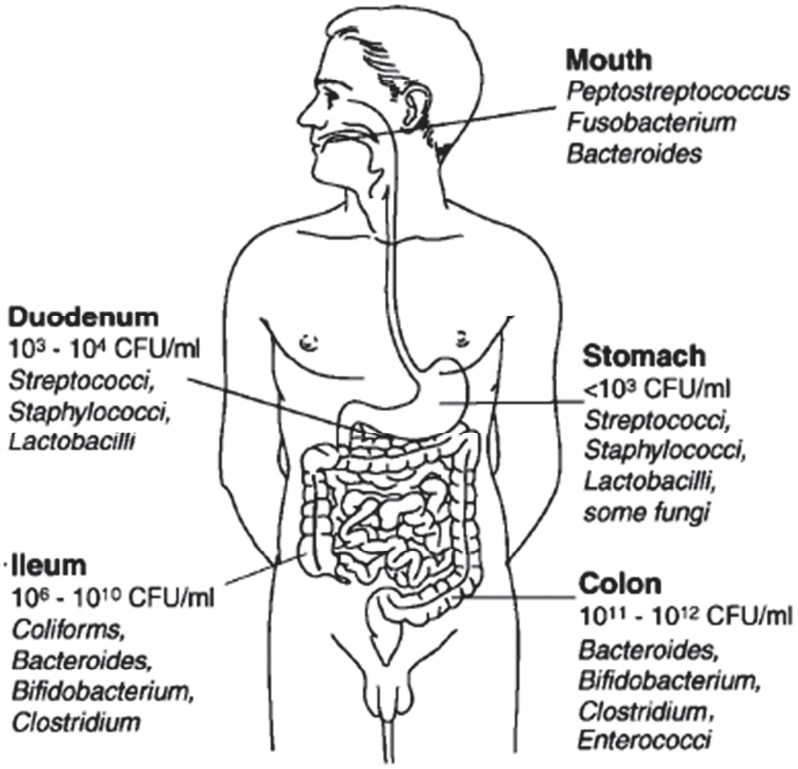

Fig. 9: Bacterial densities increase throughout the gut (139).

1.5 Gnotobiology

Much of our current understanding of microbiota - host interactions has been acquired from studies of germ-free animals (140). These so-called axenic animals are reared in sterile isolators and they can be studied in their sterile state or they can serve as living test tubes after colonization with single microbial species or defined species mixtures (141). Today rodents, particularly mice, are the most commonly used gnotobiotic animals (142).

Theoretical basis for gnotobiology (a term derived from Greek meaning 'sciences about the known life') has been established by Pasteur in 1885, who postulated that microbes would be essential for long-term viability of both plants and animals (142). Indeed, he was right. Although it was shown in 1895 by Nuttall and Thierfelder, that mammals can survive for short period under germ-free conditions and, after the World War I, that they can be even bred for generations, subsequent studies revealed that microbiota is the driving force in the evolution of immune system and that bacteria are the key players needed for proper function of the intestine (rewieved in (142, 143)). For example, axenic mammals have lower numbers of lymphocytes present in organized and diffuse lymphatic tissues (144), oligoclonal T cell receptor diversity (145) and lower numbers of IgA secreting B cells in lamina propria and consequently lower amount of secretory IgA in the intestine (146). The most prominent morphological sign of axenic mice is enlarged cecum as a consequence of mucus accumulation due to the absence of mucolytic bacteria. Several studies have shown that these immunological and morphological changes in axenic animals are reversible depending on the bacteria strain(s) used for colonization (147, 148).

Gnotobiology has a long lasting scientific tradition in the Czech Republic with Gnotobiological laboratory in Nový Hrádek established in 1953 (originally a Biological station of the Academy of Sciences, it was named Gnotobiological laboratory in 1969) by Jaroslav Šterzl. In 1957 the program of rearing of GF animals has started. Subsequently, the production of GF piglets was technologically mastered and also rats and rabbits were transferred to GF conditions (149, 150). Currently we breed several strains of GF and gnotobiotic mice in 38 flexible-film isolators ventilated with HEPA-filtered sterile air under positive pressure. They are provided with irradiation-sterilized diet and bedding, water is sterilized by autoclaving (Schwarzer, personal observation). GF piglets are still used in our laboratory in time limited experiments (151).

Gnotobiotic animals were and still are an invaluable tool for determining which host immune functions are genetically encoded and which require interactions with microbes (141). By

using the germ-free mice, we were able to establish a model of neonatal mother-to-child colonization. We showed, that after single gavage of bacteria from human origin mice became colonized and that this colonization remains stable thereafter (83).

1.6 Animal models of type I allergy

'*The best model to use to study human asthma are patients*'

Roger M. Perlmutter (152)

In allergy research, particularly research concerning food allergy, asthma, and anaphylaxis, where experimentation on humans often bears too high risks, great advancements in understanding pathogenesis have been achieved in animal models (153). Also, in these models novel approaches of potential prophylactic/ therapeutic benefit can be safely tested.

Besides guinea-pigs, rats, sheep, dogs, monkeys (154) and newly emerging *Drosophila* spp. (155), the mouse has long become a prime model organism for research into allergies and acute and chronic inflammation for understandable reasons: it is easy to breed, maintain and handle, a wide array of specific reagents are available for analysis of the cellular and mediator responses, and genetically engineered transgenic or gene-knockout mice for modeling airway disease are available (156). Moreover, germ-free and gnotobiotic mice provide us with attractive and genetically defined, simplified models to study the *in vivo* homeostatic responses between the microbiota and immune system as well as deviations in these interrelations that affect the onset and severity of the disease. They allow us to examine the role of genetic and environmental factors in early events during disease development and to elucidate the pathogenic mechanisms (110).

It needs to be stressed that the animal models of human diseases, although still crucial for understanding the diseases and for therapy development, have certain limitations which need to be taken into account when interpreting the results and extrapolating them to humans (157). For example current mouse models of asthma, a uniquely human disease, suffer from the well documented profound disparity in the airway branching pattern, smooth muscle mass, and type and location of cells within human and mouse lungs (158).

Mouse models of food allergy (Fig. 10)

Many different mouse models for food allergy are in use. The biggest differences are the use of model allergens and the sensitization strategy prior to oral challenge (159). For oral sensitization, addition of an adjuvant (or other method to manipulate the intestinal epithelium) is needed in most cases to break the tolerance in the gut. Cholera toxin (CT) is most commonly used. Alternatively, mice are systemically sensitized (i.p. or i.d.) prior to oral challenge, resulting in anaphylactic reactions. In our model we used a systemic sensitization model in the presence of alum adjuvant (74), systemic sensitization models without adjuvant are also established (153).

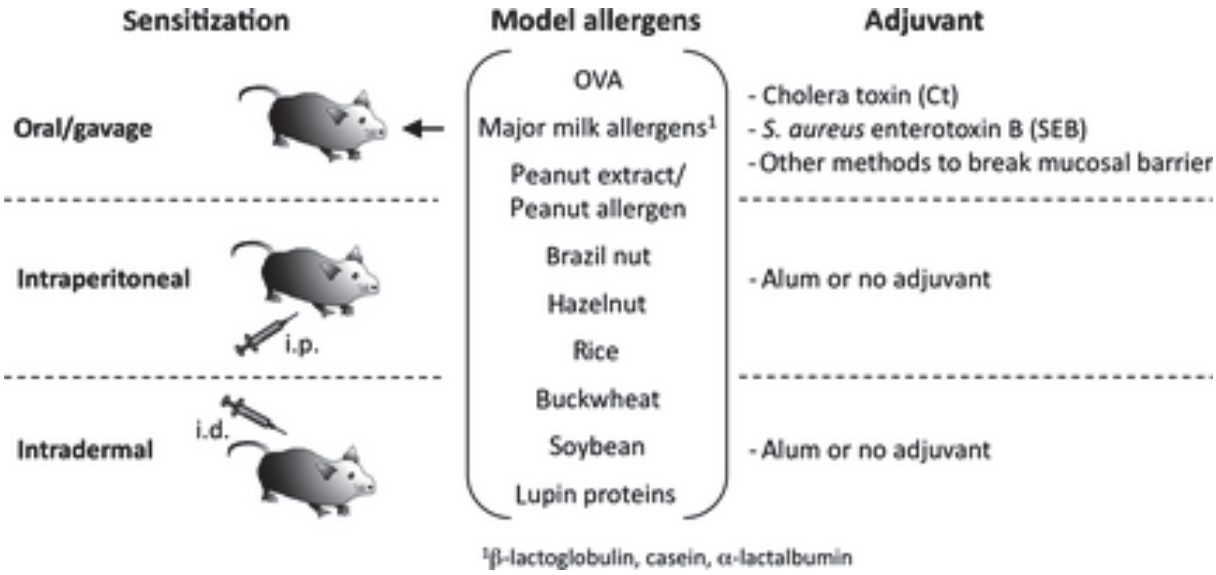

Fig. 10: Examples of mouse food allergy models (153).

Mouse models of allergic airway inflammation (AAI) (Fig. 11)

(A) Antigens commonly used as model allergens to trigger AAI. The OVA is most commonly used to study the AAI in mouse. However, OVA lacks clinical relevance for human asthma and more relevant and naturally occurring antigens are increasingly used. (B) Typical example of acute and chronic AAI models triggered by sensitization and challenge with OVA. In the acute model, an adjuvant (typically aluminium hydroxide, alum) is co-administered during the sensitization phase to enhance the antigenicity of the antigen. Chronic AAI is elicited in the absence of adjuvant. Alternative models to OVA-induced AAI follow similar schemes but rely on more clinically relevant antigens, such as HDM or Bet v 1, or a

combination of multiple antigens (153). In our gnotobiotic model, we have concentrated on the sensitization stage using Bet v 1 and alum as adjuvant (83).

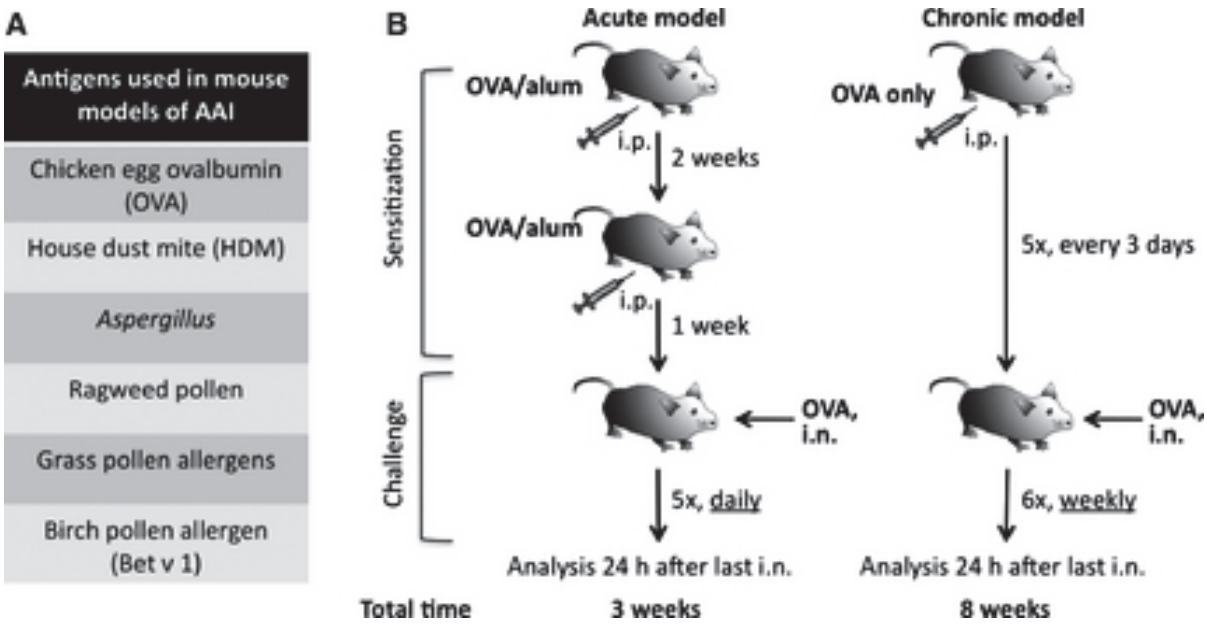

Fig. 11: Mouse models of allergic airway inflammation (153).

2. SIGNIFICANCE AND AIMS OF THE THESIS

Allergy has become a significant health burden in the industrialized world with the prevalence reaching a magnitude of epidemic. The observed rise in allergic disease cannot be explained by genomic changes; therefore it is commonly associated with reduced exposure to microbes and altered microbial communities (microbial dysbiosis) early in life. In line with the fact that the immune programming is initiated in prenatal and early postnatal life, this period represents a 'window of opportunity' for the prevention of allergic sensitization. In this respect, early interventions using probiotics appear as a valuable approach for prophylaxis of allergies.

Aims of the thesis:

- **Selection of new probiotic bacterial strains and testing of their safety and immunomodulatory properties.**

- **Testing the feasibility of a probiotic as mucosal antigen delivery vector in allergy prevention in gnotobiotic neonatal mother-to-offspring colonization mouse model.**

- **Prevention of allergic sensitization by probiotic strains in neonatal mother-to-offspring colonization mouse model and characterization of their immunomodulatory properties.**

- **Testing the impact of changes in allergen secondary structure on its allergenicity in mouse food allergy model.**

3. RESULTS AND DISCUSSIONS

3.1 Comments on presented publications and manuscript

Publication I

Cukrowska B., Motyl I., Kozakova H., **Schwarzer M.**, Gorecki R. K., Klewicka E., Slizewska K., Libudzisz Z.: Probiotic *Lactobacillus* strains: *in vitro* and *in vivo* studies. ***Folia Microbiol*** **(Praha), 2009. 54(6): p. 533-7.**

There is a great interest in using live bacteria to improve the bacterial dysbiosis in early life, which is probably the key trigger in the increase of prevalence of allergic diseases. According to WHO (91), bacteria should be alive, although there are reports showing, that even dead bacteria or lysates can modulate the host immune system (160). In order to survive the passage into the intestine, potential probiotics must be resistant to gastric acids and bile salts. Further, to be used in human medicine, they must be safe for host organisms (91). We have shown, that out of 24 *Lactobacillus* strains, isolated from healthy breast fed infants, only five strains presented *in vitro* both acid and bile salt resistance. In addition to surviving the harsh environment in the GIT, bacterial adherence to intestinal epithelial cells is considered to be a desirable feature of a probiotic strain, as it can promote the gut residence time, pathogen exclusion, and interaction with host epithelial and immune cells (161). Therefore only three strains that showed the capacity to adhere to the epithelial Caco-2 cells were selected. We have shown that all three selected strains possess antagonistic activity against several pathogenic strains of *E. coli* and *Salmonella*, suggesting they might play an important positive role in the modulation of intestinal microbiota.

Regarding their safety, the mixture of bacteria was given to mice by intragastric gavage for seven consecutive days. At the end of the experiment, we observed no translocation through the intestinal barrier to blood and internal organs; the bacteria translocated only to the MLN. The translocation of bacteria to MLN was well described in mice before (162); due to the fact that MLNs are now viewed as a crossroads between the peripheral and mucosal circulation pathways, the translocation of bacteria might be necessary for exerting their immunomodulatory functions on systemic immunity (111). Indeed, when restimulated with

the bacteria, spleen lymphocytes of lactobacilli-treated mice were activated to a higher production of anti-allergic Th1 cytokines and lower production of pro-allergic IL-5 than cells obtained from control mice.

By sequencing of 16S rDNA the bacterial strains were identified as *L. casei* LOCK 0900 and LOCK 0908 and *L. paracasei* LOCK 0919, although by subsequent analysis the *L. casei* strains were reclassified as *L. rhamnosus* (Spanova, personal communication).

Taken together we have shown that selected *Lactobacilli* strains are able to survive in the gut and to interact with the immune system of the host. *In vivo* mouse experiments showed the safety of the strains and their ability to shift the cytokine balance in favor of anti-allergic immune responses. These findings make them promising candidates for prevention/ therapy of allergic diseases.

Publication II

Schwarzer M.[*], Repa A.[*], Daniel C., Schabussova I., Hrncir T., Pot B., Stepankova R., Hudcovic T., Pollak A., Tlaskalova-Hogenova H., Wiedermann U., Kozakova H.: Neonatal colonization of mice with *Lactobacillus plantarum* producing the aeroallergen Bet v 1 biases towards Th1 and T-regulatory responses upon systemic sensitization.

***Allergy*, 2011. 66(3): p. 368-75.**

[*] equally contributed

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The use of probiotic bacteria with intrinsic Th1-promoting or immunomodulatory properties as vehicles for mucosal delivery of recombinant allergen is an attractive concept for development of well-tolerated and effective allergy vaccines (84). These interventions are of increasing interest early in life, when so called 'window of opportunity' is localized and the immune programming is initiated (163). In this study we cloned the major birch pollen allergen *bet v 1* gene under the control of a strong promoter leading to constitutive expression of the allergen in the well characterized strain *L. plantarum* NCIMB8826 with strong Th1 immunomodulatory properties. In order to intervene at an early developmental stage and by using germ-free mice, we established a model of mother-to-offspring neonatal monocolonization. After confirming that the colonization and Bet v 1 production is stable, we found that the recombinant strain was able to induce a nonallergic Th1 response with

significant IFN-γ but absent IL-4 and IL-5 production in spleen cells upon allergen challenge *in vitro*. This showed us the importance of strain selection as well as timing of colonization, because previous reports with *Lactobacillus casei* secreting beta-lactoglobulin led to both Th1 and Th2 cytokine production after colonization of adult GF mice (133). Moreover, when using Gram-negative bacteria as ovalbumin-producing vector, Dahlman *et* al. previously demonstrated an induction of allergen-specific IgE thus showing aggravation and potential participation in the induction of allergic symptoms (164).

When neonatally colonized mice were subsequently sensitized with the birch pollen allergen Bet v 1, we found a shift towards a nonallergic Th1 phenotype on the cellular level accompanied by increased regulatory responses as seen from decreased levels of both Bet v 1 specific IgG1 and IgG2a in sera, and up-regulated mRNA of the regulatory marker FoxP3 in spleen.

To sum up, mono-colonization with recombinant *L. plantarum* specifically induced immunomodulation of allergic immune responses, supporting the concept of early immuno-imprinting using recombinant probiotic bacteria. Our results therefore promote the use of recombinant lactic acid bacteria for early allergen-directed prevention of type I allergy.

Manuscript I

Schwarzer M., Srutkova D., Schabussova I., Hudcovic T., Akgün J., Wiederman U., Kozakova H.: Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to Bet v 1.

Manuscript in preparation.

Recent reports suggest that probiotic strains with the capacity to induce regulatory responses, rather than Th-1-promoting strains might be more successful in suppression of allergic responses (20, 165). Due to the fact that *L. plantarum* is a strain with strong Th1 immunomodulatory properties (108) and that the wild-type *L. plantarum* itself did not exert any suppressive effects on the allergic immune response in our previous work (83), we aimed at testing a different probiotic strain with rather Treg immunomodulatory capacities. We selected *Bifidobacterium longum* spp. *longum* CCDM367 (*B. longum*), a healthy breast fed infant isolate, which has been shown to induce regulatory responses *in vitro* and to suppress

the inflammatory responses in mouse models of experimental colitis (Srutkova, unpublished results). Upon neonatal mother-to-offspring mono-colonization of GF mice and subsequent sensitization, *B. longum* significantly reduced the development of allergen-specific immune responses, which was associated with induction of regulatory milieu. On the humoral level we observed suppression of Bet v 1-specific IgE as well as levels of total IgE and allergen-specific IgG1 and IgG2a in serum compared to sensitized GF controls. Accordingly, allergen-specific recall proliferation of splenocytes and production of both Th1- and Th2-associated cytokines in spleen cell cultures were significantly reduced in these mice; this was associated with increased levels of regulatory cytokines IL-10 and TGF-β in serum. Our data are supported by recent finding that certain bacterial strains can instruct DC to induction of FoxP3 positive regulatory T cells and enhanced IL-10 production (105); induction of IL-10 producing Treg cells has been shown upon bifidobacteria administration to mice (166). *In vitro*, we showed that *B. longum* induces BMDC to produce IL-10 and TGF-β, with only low levels of IL-12. It is well documented that probiotic bacteria are recognized by PRRs on different cell types (96, 108). By using the BMDC from KO mouse we showed that *B. longum* induced IL-10 production is dependent on TLR2 and MyD88 and independent of TLR4 and that downstream signaling involves MEK, JNK, p38 and NFκB.

Taken together, we showed that *B. longum* is a strain with the ability to induce regulatory cytokines/ T cells with the ability to prevent allergic sensitization. These properties make it a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans.

Publication III

Golias J.[*], **Schwarzer M.**[*], Wallner M., Kverka M., Kozakova H., Srutkova D., Klimesova K., Sotkovsky P., Palova-Jelinkova L., Ferreira F., Tuckova L.: Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy.

***PLoS One*, 2012. 7(5): p. e37156.**

[*] equally contributed

There is a great deal of interest in understanding precisely how traditional food processing methods influence food allergenicity (167). Although baking, cooking, roasting, grilling, drying, pasteurization and sterilization were reported to reduce allergenicity, in some cases these methods may increase allergenic potential or may reveal ‚neo'-epitopes which were masked in the native protein (167).

Forming approximately 60% of the total egg white proteins OVA is by far the most abundant of them. Allergies to eggs belong among the most frequent food allergies and their prevalence, severity and persistence has been steadily increasing during the last decades (168). We showed that heating of hen egg allergen OVA to 70°C (h-OVA) has only minor effect on its secondary structure compared to OVA heated to 95°C. However, these minor changes had a high impact on the immunological behavior of the allergen. Protein hOVA was more resistant to proteolytic digestion and after 20 minutes of digestion peptides similar to OVA heated to 95°C were observed; moreover, they had a similar *in vitro* Treg inducing capacity. Similar to our results, suppressive effects of some OVA T cell epitope peptides on allergic immune responses via $FoxP3^+$ T cell generation were recently described (169). In line with the *in vitro* data we showed that the ability to induce the allergic immune responses is diminished in hOVA. Heating of OVA significantly decreased clinical symptoms (allergic diarrhea) and immune allergic response on the level of IgE, IL-4, IL-5, IL-13. Furthermore, h-OVA induced lower activities of serum mast cell protease-1 and enterocyte brush border membrane alkaline phosphatase as compared to native OVA. On the other hand, h-OVA stimulated higher IgG2a in sera and IFN-γ secretion by splenocytes. The importance of structural epitopes in specific antibody formation was revealed, when we changed the coupling allergen (h-OVA was used for OVA sensitized sera and vice versa) for specific Abs determination. Moreover, the binding was significantly higher when h-OVA antigen was used for specific IgG1 antibody determination. We assume that this is caused by heating uncovered linear epitopes (supplementing the loss of the conformational ones). On the other hand, when the OVA heated to 95°C was used, we observed a strong drop in the signal in all OVA-specific antibodies.

In conclusion, we showed that even a mild change in the secondary structure of OVA after thermal processing has far-reaching consequences concerning its antigenic properties. After digestion of h-OVA, fragments with different immunogenic properties are formed leading to the shift from Th2 to Th1-type response as compared to native OVA. Nevertheless, the h-OVA fragments still have the ability to induce allergic symptoms, but these are less pronounced and need longer time to develop.

3.2 Presented publications and manuscript

3.2.1 **Probiotic *Lactobacillus* strains: *in vitro* and *in vivo* studies**

Cukrowska B., Motyl I., Kozakova H., Schwarzer M., Gorecki R. K., Klewicka E., Slizewska K., Libudzisz Z.

Folia Microbiol (Praha), 2009. 54(6): p. 533-7.

Folia Microbiol. **54** (6), 533–537 (2009) **http://www.biomed.cas.cz/mbu/folia/**

Probiotic *Lactobacillus* Strains: *in vitro* and *in vivo* Studies

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ABSTRACT. Three *Lactobacillus* strains (LOCK 0900, LOCK 0908, LOCK 0919) out of twenty-four isolates were selected according to their antagonistic activity against pathogenic bacteria, resistance to low pH and milieu of bile salts. Intragastric administration of a mixture of these strains to Balb/c mice affected cytokine T_H1–T_H2 balance toward nonallergic T_H1 response. Spleen cells, isolated from lactobacilli-treated mice and re-stimulated *in vitro* with the mixture of heat-inactivated tested strains, produced significantly higher amounts of anti-allergic tumor necrosis factor-α and interferon-γ than control animals whereas the level of pro-allergic interleukin-5 was significantly lower. *Lactobacillus* cells did not translocate through the intestinal barrier into blood, liver and spleen; a few *Lactobacillus* cells found in mesenteric lymph nodes could create antigenic reservoir activating the immune system. The mixture of *Lactobacillus* LOCK 0900, LOCK 0908 and LOCK 0919 strains represents a probiotic bacterial preparation with possible use in prophylaxis and/or therapy of allergic diseases.

Abbreviations

CFU	colony forming units	PBS	phosphate-buffered saline
Con A	concanavalin A	T_H	T helper (lymphocytes)
IFN	interferon	TGF	transforming growth factor
IL	interleukin	TNF	tumor necrosis factor
MLN	mesenteric lymph nodes		

The evidence of the importance of intestinal microflora on the development of immune responses leads to the theory that well-balanced microbial composition can improve the health of the host. Application of probiotics, *i.e*. viable nonpathogenic microorganisms exhibiting beneficial health effects when consumed in adequate amount, seems to be a perspective way for maintenance of balanced microflora (Schrezenmeier and de Vrese 2001). Probiotics are believed to trigger innate and adaptive immune responses and suppress colonization of the intestine by pathogenic bacteria by competing with them for a limited number of receptors present on the surface epithelium (Ostad *et al*. 2009). They also influence enhancement of mucosal barrier function, increase production of secretory IgA (Vanciková *et al*. 2003) and elicit production of short-chain fatty acids with health benefits related to colitis and cancer prevention (Fedorak and Madsen 2004; Kokesova *et al.* 2006).

Adaptive mucosal immune defense is induced by $CD4^+$ T lymphocytes, which differ in their secreted cytokine profile: T_H1 lymphocytes (IFN-γ, IL-2, TNF-α), T_H2 lymhocytes (IL-4, IL-5, IL-13, IL-9, IL-6) and regulatory $CD25^+$ $CD4^+$ T cells (TGF-β, IL-10). Disturbed balance between T_H1 and T_H2 lymphocyte subpopulations with elevated production of T_H2 cytokines leads to allergy development (Umetsu and DeKruyff 2006). On the other hand, production of T_H1 cytokines correlates with allergy prophylaxis and amelioration of allergic symptoms (Pohjavuori *et al.* 2004).

Lactic acid bacteria – mostly strains of the genus *Lactobacillus* – and bacteria of the genus *Bifidobacterium* are the most widely used probiotic bacteria in allergy therapy studies for their potency to modulate the T_H1–T_H2 balance (Ljungh and Wadstrom 2006). In clinical trials, probiotics have been shown to improve clinical syndromes of food allergy (Isolauri *et al*. 2000), strengthen T_H1 response in children with IgE mediated atopic dermatitis (Pohjavuori *et al.* 2004), protect the development of allergic diseases in infants

*These authors contributed equally to the work.

(Lodinova-Zadnikova *et al*. 2003, Kocourkova *et al*. 2007) and even protect infants from developing atopic dermatitis when given to atopic mother during pregnancy (Kalliomaki *et al*. 2001*a*).

In order to be used in human medicine for treatment and/or prevention of allergies, probiotic bacteria must be safe for the host organism, resistant to gastric acids and bile salts in order to survive the transit into the gut, and modulate T_H1–T_H2 balance in favor of anti-allergic T_H1 immune responses. In this study we report a selection of probiotic *Lactobacillus* strains by *in vitro* tests and subsequent verification of their immunomodulating properties in *in vivo* mouse model.

MATERIAL AND METHODS

Survival of Lactobacillus *strains at low pH and in the presence of bile salt. Lactobacillus* strains isolated from feces of healthy infants were obtained from *Pure Culture Collection of Technical University*, Lodz (LOCK). Bacteria were cultivated for 1 d in MRS medium (*Oxoid*, UK), centrifuged and re-suspended to final concentration 10^7–10^8 CFU/mL in PBS. Bacteria were incubated in 0.85 % NaCl at pH 1.5, 2.5 and 3.5 for 2 h to study the impact of pH. Effect of the bile was estimated in 2 and 4 % bile salt solutions (Ox gall powder; *Sigma*, Germany) after a 2-d treatment. The bacteria were then plated on MRS agar medium for 2 d at 37 °C. The measurement was repeated at least three times in two independent experiments.

Lactobacillus survival (in %) was calculated as follows: $N_i/N_x \times 100$, where N_i = log CFU/mL after incubation, N_x = log CFU/ mL before incubation.

Antagonistic activity against pathogens. Antagonistic properties toward pathogens were analyzed by a slab method. Agar slabs of 10 mm in diameter were aseptically cut off the MRS agar, overgrown with respective *Lactobacillus* strain and placed on plates with the agar media (Nutrient agar; *Merck*, Germany) inoculated with the indicator strain (10^5–10^6 CFU/mL). After a 18-h incubation, the diameter of growth-inhibition zones around the agar slabs was measured. The following strains were used as indicator pathogens: *Escherichia coli* LOCK 105, *Enterococcus faecalis* LOCK 105, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* 018 (obtained from the *Serum and Vaccine Producer Biomed*, Cracow, Poland), *Salmonella enterica* subsp. *enterica* sv. Enteritidis (clinical isolate obtained from the *Institute of Sea and Tropical Medicine*, Gdynia, Poland), *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* sv. Typhimurium, *Listeria monocytogene*s, *L. innocua* (clinical isolates obtained from *Voivodeship Sanitary-Epidemiological Station*, Bydgoszcz, Poland). The measurement was repeated four times in two independent experiments.

Sequencing of 16S rDNA. Total DNA was extracted (Tailliez *et al*. 1998) to analyze rDNA sequences for 16S ribosomal RNA. The 1063-bp fragment of the 16S rDNA gene was amplified by PCR reaction using primers: 1406R and 343F (5′-TAC GGG AGG CAG CAG-3′), the inverse complementary to primer 343aR (all described by Salama *et al.* 1991). Sequencing of the PCR products was done using BigDye Terminator Cycle Sequencing (*Applied Biosystems*, USA). Sequences were read by ABI 377 apparatus (*Applied Biosystems*) and subjected to the BLAST search program (NCBI) (Krauthammer *et al*. 2000).

Experimental design in vivo. Eight-week-old inbred BALB/c mice (n = 5 per group) were used for *in vivo* experiments. Strains selected in *in vitro* tests were mixed in equal proportions, lyophilized with hydrolyzed milk formula and resuspended in PBS in final concentration 10^9 CFU/mL. The mixture of bacteria (0.5 mL) was given intragastrically by soft rubber tubing twice daily for 7 consecutive days. Control mice received PBS-reconstituted hydrolyzed milk.

The experiment was approved by the *Animal Experimentation Ethics Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic* and conducted in accordance with the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (CETS no. 123).

Bacteria translocation. MLN, spleen and liver were removed aseptically, weighed and diluted 5× in sterile PBS. After homogenization, suspensions were plated onto MRS agar and incubated for 3 d at 37 °C. The blood was taken intracardially and plated undiluted. The number of *Lactobacillus* CFU was also established in colon and cecum contents. Results were expressed as the number of CFU/g of intestine contents or the number of CFU per organ or pooled MLN from each group.

Lymphocyte cultivation for cytokine production. Cytokine production was measured in supernatants after cultivation of spleen lymphocytes. Spleens were minced in RPMI 1640 medium (*Sigma-Aldrich*, USA), filtered by nylon mesh and washed thrice in the medium. Cell concentration was adjusted to 5×10^6 per mL in complete RPMI 1640 medium supplemented with 5 % fetal calf serum and antibiotics (*Sigma-Aldrich*). Lymphocytes (100 μL) were applied to 96-well cultivation microplates (*Nunc*, Denmark) and cultivated in RPMI 1640 medium with a mixture of heat inactivated (30 min, 80 °C) *Lactobacillus* cells (10^6 per mL in

total volume of 300 μL of RPMI 1640 medium). Polyclonal lymphocyte activator Con A (*Sigma-Aldrich*) in 5 μg/mL final concentration was used as a positive control. Cells cultivated with RPMI 1640 medium only were used as negative controls. At least 10 replicates for each activator from one mouse were done. Plates were incubated for 72 h under 5 % CO_2 atmosphere at 37 °C; the supernatants were then collected and stored at –40 °C until cytokine production analysis.

Detection of cytokine production. The levels of IL-5, TNF-α and IFN-γ were measured in culture media. Cytokine production was determined by mouse ELISA kits (*R&D System*, USA) according to manufacturer's instructions. The results were read on Infinite M200 (*Tecan Group Ltd.*, Austria) and expressed in pg/mL.

Statistical analysis was done using nonparametric Mann–Whitney test; $p < 0.05$ was considered as statistically significant.

RESULTS

Characterization of selected strains. Only five out of 24 tested strains presented *in vitro* both acid and bile salt resistance. As we have previously reported that three out of these five strains also adhere to Caco-2 epithelial cell line (Marewicz *et al*. 2000), these three strains (LOCK 900, LOCK 0908, LOCK 0919) were selected for *in vivo* experiments.

The survival of selected strains after incubation at pH 2.5 did not change markedly and remained 67.5–84.2 % (10^6–10^8 CFU/mL) (Table I), whereas in other strains it decreased to 1–10^5 CFU/mL. All 24 examined strains exhibited high survival rate at pH 3.5 (>90 %), whereas pH 1.5 was lethal (*data not shown*). The incubation with 2 % bile salt solution resulted in survival >80 % for all examined strains, whereas 4 % solution markedly decreased the amount of living bacteria (*data not shown*). However, the surviving of LOCK 0900, LOCK 0908 and LOCK 0919 after incubation in 4 % solution was ≥88.8 % (*i.e.* ≥10^6 CFU/mL) (Table II). Selected strains showed antagonistic activity against all used pathogenic bacteria (Table III) but no antagonistic properties towards each other were observed.

Table I. The survival (%[a])of selected *Lactobacillus* strains after a 2-h incubation at low pH

Strain	pH 1.5	pH 2.5	pH 3.5
LOCK 0900	32.8 ± 3.6	67.5 ± 4.6	99.3 ± 3.6
LOCK 0908	13.2 ± 4.1	80.9 ± 3.5	93.5 ± 4.1
LOCK 0919	13.1 ± 3.7	84.2 ± 5.2	98.3 ± 3.9

[a]Arithmetical means ±SD.

Table II. The survival (%[a])of selected *Lactobacillus* strains after 48 h of incubation with 2 and 4 % bile salt solution

Strain	2 %	4 %
LOCK 0900	97.2 ± 3.8	88.8 ± 5.1
LOCK 0908	94.6 ± 4.2	90.8 ± 4.9
LOCK 0919	97.0 ± 5.6	95.8 ± 4.4

[a]Arithmetical means ±SD.

Table III. Antagonistic activity of selected *Lactobacillus* strains[a]

Strain LOCK	*Ec*A	*Ec*L	*Ec*0	*Pa*	*S*E	*S*T	*Ef*	*Sa*	*Lm*	*Li*
0900	15.5 ± 1.8	11.0 ± 1.6	14.0 ± 2.0	21.5 ± 1.3	10.0 ± 1.8	12.0 ± 1.9	8.5 ± 1.7	17.5 ± 1.8	11.0 ± 0.8	11.0 ± 1.1
0908	16.0 ± 1.8	13.0 ± 1.5	12.0 ± 1.6	18.0 ± 1.6	14.0 ± 1.6	13.0 ± 1.6	9.5 ± 1.9	12.0 ± 1.6	12.0 ± 1.0	12.0 ± 1.3
0919	13.5 ± 1.2	18.0 ± 1.4	16.0 ± 1.0	19.5 ± 1.2	13.0 ± 1.9	14.0 ± 1.5	6.5 ± 2.1	14.0 ± 1.8	11.0 ± 1.1	9.0 ± 0.7

[a]Arithmetical means ±SD of inhibition zones (mm).

*Ec*0 *E. coli* 018
*Ec*L *E. coli* LOCK 105
*Ec*A *E. coli* ATCC 25922
Ef *Enterococcus faecalis*
Li *Listeria innocua*
Lm *Listeria monocytogenes*
Pa *Pseudomonas aeruginosa* ATCC 27853
Sa *Staphylococcus aureus* ATCC 25923
*S*E *Salmonella enterica* ssp. *enterica* sv. Enteritidis
*S*T *Salmonella enterica* sv. Typhimurium

Analysis of 16S rDNA sequences demonstrated that the studied strains belong to the genus *Lactobacillus* and, more precisely, to the group of closely related species including *L. casei*, *L. rhamnosus* and *L. paracasei.* The strains LOCK 0900 and LOCK 0908 exhibited 97 and 98 % homology, respectively, to the sequence in *GenBank* accession no. D16552 (*L. casei*), whereas LOCK 0919 showed 99 % similarity to the sequence D79212 (*L. paracasei*).

Bacteria translocation through the intestinal barrier. The number of *Lactobacillus* CFU in colon ($3.3 \pm 0.5 \times 10^9$) and cecum ($2.3 \pm 0.2 \times 10^9$) of lactobacilli-treated mice was similar to control animals (colon – $2.8 \pm 0.3 \times 10^9$, cecum – $3.2 \pm 0.5 \times 10^9$). No bacteria were cultivated from blood, liver and spleen of both experimental and control groups. However, in MLN of lactobacilli-treated mice, 225 CFU per pooled MLN were grown in contrast to control group where no bacteria were detected.

Cytokine production in cultures of spleen lymphocytes. Lymphocytes from both lactobacilli-treated mice and control group were stimulated by Con A to a high secretion of anti-allergic T_H1 cytokines (IFN-γ and TNF-α) and pro-allergic IL-5 whereas stimulation with inactivated lactobacilli induced mainly secretion of T_H1 cytokines (Table IV). There were no significant differences in the cytokine levels between experimental groups after stimulation of spleen lymphocytes with Con A. Nevertheless, after co-cultivation of cells with lactobacilli a significant increase in IFN-γ and TNF-α levels in lactobacilli-treated mice was found compared with control animals. In contrast, these cells produced significantly lower amounts of IL-5 than control cells.

Table IV. The cytokine production in spleen cell cultures[a]

Cytokine	Lactobacilli-treated mice		Control mice	
	Lm	Con A	Lm	Con A
IFN-γ	442 ± 98**	4054 ± 1002	151 ± 23	4001 ± 987
TNF-α	1012 ± 223*	449 ± 85	881 ± 132	444 ± 93
IL-5	29 ± 3‡	1146 ± 289	37 ± 3	1285 ± 298

[a]The level of cytokine (pg/mL) was measured by ELISA in supernatants of spleen lymphocytes cultivated with the heat inactivated mixture of tested *Lactobacillus* strains (Lm) or Con A. Spleen cells of lactobacilli-treated mice incubated with Lm produced significantly higher amounts of IFN-γ (**$p = 0.01$), TNF-α (*$p = 0.04$) and significantly lower of IL-5 (‡$p = 0.04$) than cells of control mice. There were no statistical differences between experimental groups after Con A activation. Values are expressed as aritmetical means ± SD.

DISCUSSION

In the selection of prospective probiotic *Lactobacillus* strains we used the criteria (pH and bile resistance) similar to those published by Kurman (1988), who stated that the resistance of bacteria to 4 % bile can be considered a good characteristic for probiotics.

Selected strains presented different levels of antagonistic activities against tested pathogens (11 strains of 8 species) but, finally, the strains were chosen to ensure the highest antagonistic activity in the mixture. The main antagonistic metabolites produced by the tested strains were lactic and acetic acids (*unpublished data*), both of which were shown to reduce pH and thus inhibit growth of pathogenic bacteria (Ljungh and Wadstrom 2006). Moreover, the LOCK 0900 and LOCK 0919 strains also synthesize bacteriocins resistant to proteolytic enzymes, *e.g.*, biosurfactants with described antimicrobial function (Walencka *et al.* 2008).

The disturbances in gut ecosystem that occur in early life are supposed to cause a misbalanced development of the immune system favoring pro-allergic reactions. As an increased number of genus *Clostridium* was found in the intestine of allergic patients (Kalliomaki *et al*. 2001*b*), antagonistic properties of *Lactobacillus* bacteria can play an important positive role in the modulation of the gut microbiota.

We observed that in human blood-cell cultures the selected strains (LOCK 0900, LOCK 0908, LOCK 0919) induced higher synergistic effects on cytokine production compared with individual strains (*unpublished results*); the mixture of bacteria was, therefore, used for *in vivo* experiment in mice. During studies in mouse model the selected strains were safe and affected significantly the cytokine profile toward anti-allergic response. They did not translocate through the intestinal barrier to blood and internal organs (CFU of *Lactobacillus* were found only in MLN of lactobacilli-treated mice). The translocation of bacteria to MLN was described in mice given high doses of these bacteria (Macpherson and Uhr 2004). After being caught by dendritic cells, bacteria are shuttled to MLN where they can activate the immune system. Thus, the presence of probiotic bacteria in MLN creates the antigenic reservoir with an ability to induce immune responses. We also showed that the spleen lymphocytes of lactobacilli-treated mice are activated to a higher production of anti-allergic T_H1 cytokines and lower production of pro-allergic IL-5 than cells obtained from control mice. The systemic immune responses to ingested antigens are considered to be probably generated

in MLN (Mowat 2003) and thus the observed shift in the T_H1–T_H2 balance can be attributed to translocated lactobacilli.

Our results showed that the *Lactobacillus* strains LOCK 0900, LOCK 0908 and LOCK 0919 are able to withstand the gut conditions and to interact with the immune system of the host. *In vivo* mouse experiments showed that, on the cellular level, they can shift the cytokine balance in favor of anti-allergic immune response.

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3.2.2 **Neonatal colonization of mice with *Lactobacillus plantarum* producing the aeroallergen Bet v 1 biases towards Th1 and T-regulatory responses upon systemic sensitization**

Schwarzer M.*, Repa A.*, Daniel C., Schabussova I., Hrncir T., Pot B., Stepankova R., Hudcovic T., Pollak A., Tlaskalova-Hogenova H., Wiedermann U., Kozakova H.

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Neonatal colonization of mice with *Lactobacillus plantarum* producing the aeroallergen Bet v 1 biases towards Th1 and T-regulatory responses upon systemic sensitization

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Keywords
allergy; germ-free mice; mucosal immunity; primary prevention; recombinant lactic acid bacteria.

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Abstract

Background: The use of recombinant lactic acid bacteria (LAB) as vehicles for mucosal delivery of recombinant allergens is an attractive concept for antigen-defined allergy prevention/treatment. Interventions with LAB are of increasing interest early in life when immune programming is initiated. Here, we investigated the effect of neonatal colonization with a recombinant LAB producing the major birch pollen allergen Bet v 1 in a murine model of type I allergy.

Methods: We constructed a recombinant *Lactobacillus* (*L.*) *plantarum* NCIMB8826 strain constitutively producing Bet v 1 to be used for natural mother-to-offspring mono-colonization of germ-free BALB/c mice. Allergen-specific immunomodulatory effects of the colonization on humoral and cellular immune responses were investigated prior and after sensitization to Bet v 1.

Results: Mono-colonization with the Bet v 1 producing *L. plantarum* induced a Th1-biased immune response at the cellular level, evident in IFN-γ production of splenocytes upon stimulation with Bet v 1. After sensitization with Bet v 1 these mice displayed suppressed IL-4 and IL-5 production in spleen and mesenteric lymph node cell cultures as well as decreased allergen-specific antibody responses (IgG1, IgG2a, and IgE) in sera. This suppression was associated with a significant up-regulation of the regulatory marker Foxp3 at the mRNA level in the spleen cells.

Conclusion: Intervention at birth with a live recombinant *L. plantarum* producing a clinically relevant allergen reduces experimental allergy and might therefore become an effective strategy for early intervention against the onset of allergic diseases.

Allergic diseases have become a substantial public health burden in industrialized countries, and their prevalence has steadily increased over the last decades. At present, allergen-specific immunotherapy (SIT) is the only curative and disease-modifying treatment in the majority of patients (1), which however has some drawbacks: SIT vaccines contain crude extracts from natural sources containing a mixture of allergens and contaminant components, which may pose a risk for *de novo* sensitization to new allergens (2). In this respect, standardized recombinant allergens could improve the safety profile and efficacy of SIT (3). To keep the allergens at the injection site, traditional SIT utilizes aluminium hydroxide as an adjuvant. Although aluminium adjuvants are widely used with good results in humans, they promote Th2-like responses (4), which might reduce SIT efficacy. Recently, lactic acid bacteria (LAB) have been introduced

into the field of SIT (5). We and others have shown that certain LAB strains shift the allergen-specific Th2 response towards a more balanced Th1/Th2 profile (5). In this regard, the use of such nonpathogenic LAB with intrinsic Th1-promoting or immunomodulatory properties as vehicles for mucosal delivery of recombinant allergen is an attractive concept for development of well-tolerated and effective allergy vaccines (6).

The indigenous flora of the gastrointestinal tract is required to maintain immune homeostasis within the gut and promotes the maturation of the host's immune system. Meanwhile, it is assumed that a balanced constitution of the microflora is one of the factors promoting protection from allergy and asthma (7). Several clinical studies indicated that a reduced presence of lactobacilli or bifidobacteria in the early intestinal microbiota of atopic children precedes the occurrence of allergic diseases (8). Along these lines, human trials using specific LAB strains for oral interventions have pointed out a potential for primary prevention of allergic diseases and encouraged further research (9).

Germ-free (GF) mice, born and raised in sterile isolators lacking microbial communities, enable the introduction and investigation of specific single microbial strains to study their interaction with the host's immune system both within the intestinal tract and in peripheral tissue. Studies employing GF animals have shown that the intestinal microbiota shapes the development, distribution, differentiation and inflammatory profile of immune cells in the gut and peripheral sites within the host (10).

Recently, it has been proposed that many factors affecting the initiation and course of allergic disorders act within a narrow window of opportunity, either pre-, peri- and/or postnatally (11). Thus, preventive strategies intervening at an early developmental stage seem promising to modulate immune responses with a sustained effect.

The aim of our study was to test a strategy of early prophylaxis of type I allergy based on live recombinant LAB producing a specific allergen. We here show that neonatal mono-colonization of GF mice with the *Lactobacillus* (L.) *plantarum* NCIMB8826 strain producing the major birch pollen allergen Bet v 1 attenuates the development of birch pollen allergy later in life. The mechanisms involve a shift towards a nonallergic Th1 phenotype accompanied by increased regulatory responses.

Methods

Recombinant *Lactobacillus plantarum* constitutively producing Bet v 1

Strain construction

Lactobacillus plantarum NCIMB8826 was used as the final host to carry a plasmid for constitutive intracellular Bet v 1 production and grown at 37°C in MRS medium (Difco, Becton Dickinson, Franklin Lakes, NJ, USA). *Escherichia* (E.) *coli* MC1061 was used as intermediate host for cloning and cultured at 37°C in Luria broth. Antibiotics were used at following concentrations: for *E. coli*, ampicillin (100 μg/ml) and for *L. plantarum*, erythromycin (5 μg/ml). Molecular biology techniques, electrotransformation and cloning techniques were previously described (12). Briefly, a polymerase chain reaction (PCR) was performed on the *E. coli* expression vector pMW175 carrying the Bet v 1-encoding cDNA fragment using the sense OMEC190 and antisense OMEC191 primers as described in (12). Subsequently, the amplified fragment was subcloned into the pZero-2 plasmid (Invitrogen, Groningen, Netherlands), verified by DNA sequencing, the insert digested with the restriction enzymes *Nco*I–*Xba*I and subcloned into the *Nco*I–*Xba*I digested plasmid pGIT032 (13) carrying the constitutive *L. plantarum* promoter for lactate dehydrogenase (pldhL) and an erythromycin resistance gene. The resulting construct (pMEC181) carried the Bet v 1-encoding sequence under control of pldhL. Finally, *L. plantarum* NCIMB8826 was electrotransformed with pMEC181 giving rise to an erythromycin-resistant *L. plantarum* constitutively producing intracellular Bet v 1 (recLp). To generate a control strain (conLp), *L. plantarum* NCIMB8826 was electroporated with the empty plasmid pGIT032. *In vitro* stability was demonstrated after growth over 100 generations as described (12).

Analysis of Bet v 1 production

Analysis of Bet v 1 production was performed as previously described (12). Briefly, cell extracts of recLp and conLp were mixed with a protease inhibitor (Complete, Boehringer Mannheim, Germany). For western blot, cell extracts or purified Bet v 1 (Biomay, Vienna, Austria) were separated by gel electrophoresis, blotted onto nitrocellulose and detected with a mouse monoclonal anti-Bet v 1 antibody followed by HRP-conjugated goat anti-mouse antibodies. For protein quantification by ELISA, recLp extracts were compared with a Bet v 1 standard curve and expressed as $\mu g/10^9$ colony forming units (CFU) and percentage of protein content measured using a kit (Bio-Rad, Munich, Germany).

Animal experiments

Animals

Germ-free BALB/c mice were kept under sterile conditions with a 12- h light–dark cycle at 22°C. Sterile pellet diet (ST1, Bergman, Czech Republic) and water were fed *ad libitum*. Faecal samples were weekly controlled for microbial contamination (14). Experiments were approved by the local ethics committee.

Colonization and in vivo strain stability

Germ-free animals were colonized with 2×10^8 CFU recLp or conLp by intragastric tubing. Drinking water was supplemented with erythromycin (50 μg/ml) to ensure long-term stability of the recombinant strain *in vivo*. Colonization and presence of the plasmid pMEC181 were analysed weekly by plating on MRS agar ± 5 μg/ml erythromycin and expressed as log CFU/g faeces ± standard error of the mean (SEM). Conformational integrity of the protein was assessed by western blot analyses of 2×10^9 CFU recLp freshly grown from faeces.

Immune responses induced by colonization with recLp (experiment I)
Eight-week-old GF mice were inoculated with recLp and mated 20 days later (Fig. 1A). The offspring ($n = 6$) mono-colonized with recLp via their mothers (group 1) was killed at 56 days to analyse cellular and humoral immune responses in sera, spleens and small intestines. Age-matched GF animals served as controls (group 2).

Influence of recLP on birch pollen allergy (experiment II)
Mice neonatally mono-colonized with recLp (group 1), conLp (group 2) or noncolonized GF mice (group 3) were intraperitoneally (i.p.) sensitized at 56 days of age three times in a 10-day interval using 1 μg Bet v 1 adsorbed to 2 mg aluminium hydroxide (Serva, Heidelberg, Germany) in 200 μl saline (Fig. 1B). Mice were killed 7 days after the last immunization. Age-matched untreated GF mice served as controls when appropriate. Experiments were performed in two independent sets of 5–7 mice/group, and results were pooled for analysis.

Sampling
At the killing process, serum was collected for antibody analyses. Pooled mesenteric lymph nodes (MLN, experiment II only) and spleens were removed for *in vitro* cytokine assays and real-time RT-PCR (a spleen segment was stored in RNA*later*® Solution (Ambion, Austin, TX, USA) at −20°C until RNA isolation). Spleen and MLN cells were prepared as previously described (15). Small intestines were excised, weighed and gut lavages performed for the determination of Bet v 1–specific IgA (12).

Read outs

Cellular immune responses
Cytokine production upon *in vitro* Bet v 1 restimulation of spleen or pooled MLN cells was assessed as described in (15). Briefly, 5×10^6 cells/500 μl were incubated in 48-well plates (Corning, Oneonta, NY, USA) ± Bet v 1 for 48 h. Interleukin (IL)-4, IL-5, IL-10 and interferon (IFN)-γ were measured from supernatants using ELISA kits (RnD, Mineapolis, MN, USA) with sensitivities < 5 pg/ml (IL-4, IL-5), < 12 pg/ml (IL-10) and < 2 pg/ml (IFN-γ) and reported in pg/ml after subtraction of baseline levels of nonstimulated cultures. Values below assay sensitivity were considered "not detectable" (n.d.).

Humoral immune responses
Allergen-specific serum IgG1, IgG2a and IgA levels were determined by ELISA as described in (15). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) coated with Bet v 1 were applied with sera at appropriate dilutions (experiment I: 1/10 for IgG1, IgG2a, IgE and IgA; experiment II: 1/10000 for IgG1, 1/100 for IgG2a and 1/10 for IgE, 1/10 for IgA in both serum and intestinal lavages). Rat anti-mouse IgG1, IgG2a and IgA antibodies (1/500, Pharmingen, San Diego, CA, USA) and peroxidase-conjugated mouse anti-rat IgG antibodies (1/2000; Jackson, Immuno Labs., West Grove, PA, USA) were used for the detection. As it was shown that allergen-specific IgG interferes with allergen-specific IgE detection (16), total IgE was additionally measured using an ELISA kit (Bethyl, Montgomery, TX, USA) Antibody levels were reported as optical density or absolute units (total IgE). Bet v 1–specific IgA in intestinal lavages was reported as optical density/gram.

RT-PCR
RNA was isolated from spleens of recLp colonized or GF mice sensitized to Bet v 1 (groups 1 and 3, experiment II) using a RNeasy® Minikit (Quiagen, Valencia, CA, USA). After DNase treatment, RNA integrity and purity was determined by gel electrophoresis and photometry (260/280 nm). Reverse transcription into cDNA was performed using oligo$(dT)_{15}$ primers (ImProm-II™ Reverse Trancription

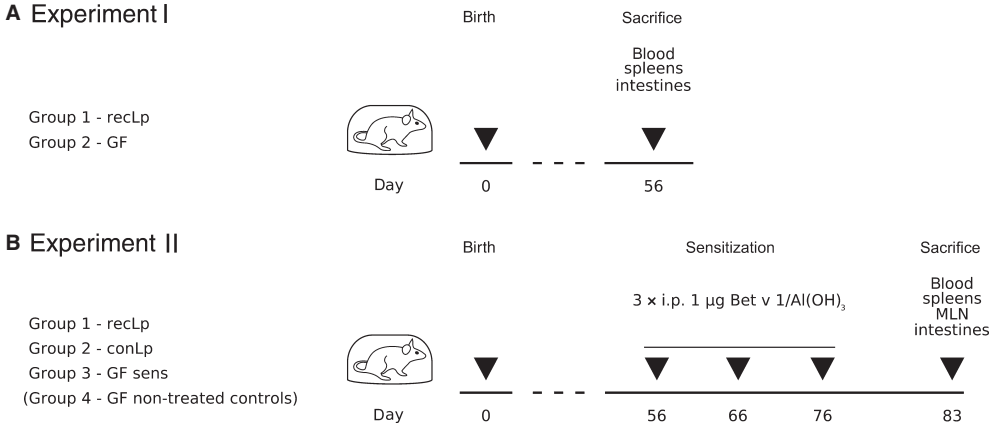

Figure 1 Experimental design. (A) Experiment I: Mice were neonatally colonized with Bet v 1 producing recombinant *Lactobacillus plantarum* (recLp), a group of germ-free (GF) mice served as controls. After 56 days, blood samples and small intestines were taken for antibody analysis, and spleens were removed for cytokines assays. (B) Experiment II: Mice neonatally colonized with Bet v 1 producing recLp, control wild-type *L. plantarum* (con Lp) and a group of GF mice were three times intraperitoneally (i.p.) sensitized with recombinant Bet v 1/Al(OH)3. Blood samples and small intestines were taken for antibody analysis and spleens and mesenteric lymph nodes for cytokines assays and qPCR.

System, Promega, Madison, WI, USA). Universal Probe Library (Roche, Mannheim, Germany) probes were used for the quantification of TGF-β1 (UPL#15), IL-10 (UPL#13), Foxp3 (UPL#13) and β-actin (UPL#101). Gene expression was determined using FastStart TaqMan® Probe Master Mix (Roche) at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C. Relative quantification was performed using GENEX software (MultiD Analyses AB, Göteborg, Sweden).

Statistics

Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Student's *t*-test for comparison between groups. Data were expressed as mean ± SEM.

Results

Recombinant *L. plantarum* constitutively produces Bet v 1 *in vitro* and after colonization

Bet v 1 detected in cell extracts of recLp, but not the control strain is shown in Fig. 2A. 10^9 CFU recLp contained 0.8 ± 0.3 μg of Bet v 1 corresponding to 0.5–0.7% of total soluble cellular protein. The *in vivo* presence of recLp and pMEC181 in recLp in experiment II was stable, as demonstrated by equal bacterial counts seen after plating in the presence and absence of antibiotics (Fig. 2B) and preserved Bet v 1 production (Fig. 2B, insert).

Mono-colonization with recombinant *L. plantarum* induces allergen-specific cellular Th1 but no humoral response in naïve mice (experiment I)

Spleen cells from mice mono-colonized with recLp displayed high IFN-γ production upon Bet v 1 stimulation *in vitro*, while Th2 cytokines (IL-4 and IL-5) were undetectable (Table 1). IL-10 production did not differ from noncolonized GF mice. At the humoral level, no induction of allergen-specific antibodies (IgG1, IgG2a, IgA or IgE) or changes in total IgE levels were detectable in sera of mono-colonized mice compared with GF controls. Furthermore, no significant production of allergen-specific specific IgA was detectable in gut lavages.

Mono-colonization with recombinant *L. plantarum* suppresses allergen-specific antibody responses in Bet v 1–sensitized mice (experiment II)

Allergen-specific IgG1 and IgG2a responses induced by sensitization with Bet v 1 were significantly suppressed by mono-colonization with recLp, but not with conLp or in naive GF mice (Fig. 3E, F). Allergen-specific IgE was reduced, and total IgE levels were significantly suppressed (Fig. 3H, G). No significant changes occurred in allergen-specific IgA in sera (group 1, recLp: 0.32 ± 0.05 OD, group 2, conLp: 0.25 ± 0.03 OD, group 3, GF sens: 0.27 ± 0.03 OD, group 4, GF naive: 0.02 ± 0.01 OD, experiment II) or intestinal lavages (group 1, recLp: 0.09 ± 0.02 OD, group 2, conLp: 0.11 ± 0.03 OD, group 3, GF sens: 0.13 ± 0.02 OD, group 4, GF naive: 0.05 ± 0.02 OD, experiment II).

Mono-colonization with recombinant *L. plantarum* suppresses allergen-specific Th2 responses in a model of type I allergy (experiment II)

In spleen cell cultures, IL-4 and IL-5 production (Fig. 3A, B) was significantly suppressed and IFN-γ (Fig. 3C) levels were increased in mice colonized with recLp, but not the control strain (conLp) compared with GF animals sensitized to the

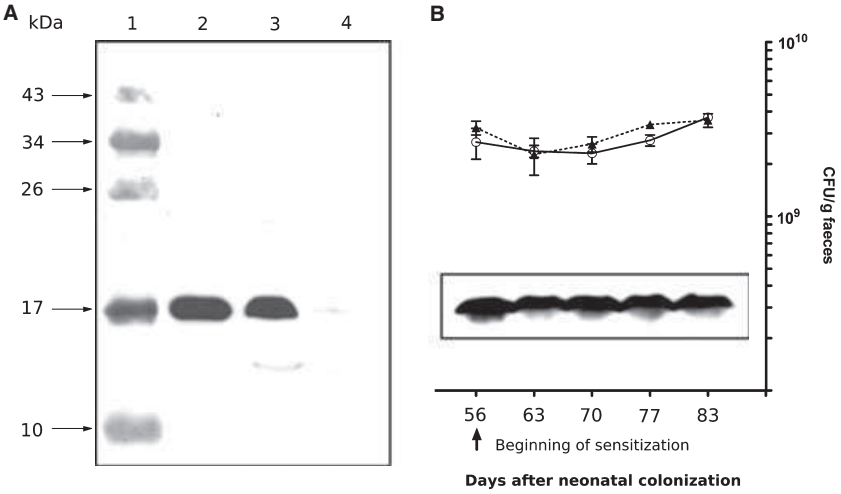

Figure 2 Western blot analyses of Bet v 1 production by recombinant *Lactobacillus plantarum* (recLp) and characterization of *in vivo* colonization. (A) Bet v 1 production from total cell extracts. Lane 1: molecular weights, lane 2: recombinant Bet v 1, lane 3: Bet v 1 produced by recLp, lane 4: control *L. plantarum* (con Lp) (B) Bacterial counts in faecal pellets from mice colonized with recLp (Experiment II) pooled and plated on MRS medium with (○) or without (▲) erythromycin (5 μg/ml) in triplicates. The data are expressed as log CFU/g faeces ± SEM. Frame insert: Western blot analysis of Bet v 1 production from bacteria from pooled faeces at the indicated day of life.

Table 1 Characterization of immune responses after mono-colonization (experiment I)

					Serum					
	Cytokines (pg/ml)				(OD)				(μg/ml)	Gut lavage (OD)
Colonization	IFN-γ	IL-4	IL-5	IL-10	IgG1	IgG2a	IgA	IgE	Total IgE	IgA
recLp	419.3 ± 48.8*	n.d.	n.d.	69.1 ± 5.5	0.34 ± 0.06	0.16 ± 0.01	0.50 ± 0.11	0.06 ± 0.01	222.8 ± 35.4	0.10 ± 0.0
None	5.7 ± 4.9	n.d.	n.d.	52.3 ± 10.6	0.29 ± 0.04	0.19 ± 0.03	0.44 ± 0.08	0.06 ± 0.02	224.4 ± 28.6	0.09 ± 0.0

Mice (n = 6 per group) were colonized with the indicated strain and killed at 56 days of age. Cytokines are measured from spleen cell culture supernatants stimulated with Bet v 1. Serum antibody levels are Bet v 1 specific unless otherwise indicated (total) and reported as optical density (OD) or μg/ml for total IgE. Values are reported as mean ± SEM. *P < 0.05; n.d., not detectable.

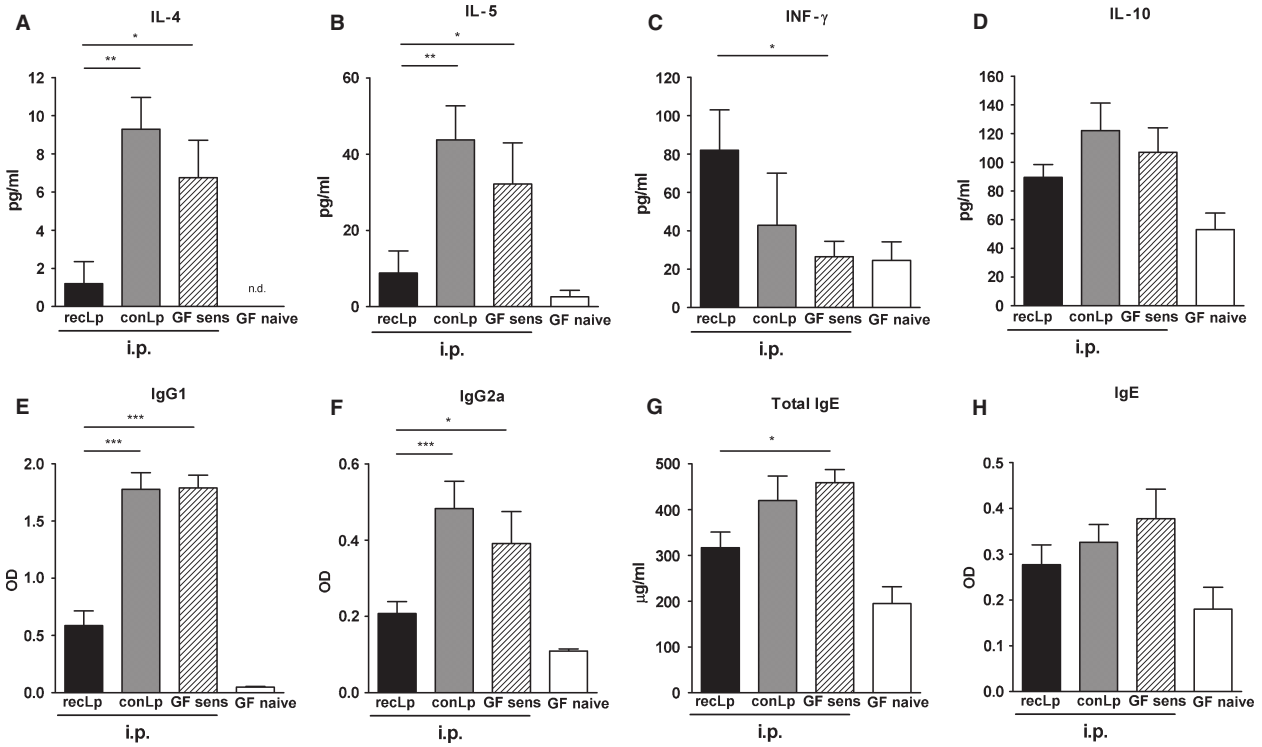

Figure 3 Influence of mono-colonization with recLp on cytokine and serum antibody levels. Experiment II, n = 5–7/group, data pooled from two independently performed experiments. Mice colonized with recombinant *Lactobacillus plantarum* (recLp, black bars), control wild-type *L. plantarum* (con Lp, grey bars) and a group of uncolonized germ-free (GF sens, dashed bars) mice were intraperitoneally (i.p.) sensitized with Bet v 1/Al(OH)3. Naive GF mice (GF naive, white bars) served as untreated controls. (A) IL-4, (B) IL-5, (C) INF-γ and (D) IL-10 cytokine levels were measured from supernatants of spleen cells of the respective groups after *in vitro* stimulation with Bet v 1. Cytokine levels are expressed after subtraction of base line levels of unstimulated splenocytes. Bet v 1–specific IgG1 (E), IgG2a (F) and IgE (H) are reported as optical density (OD) units, total IgE (G) as μg/ml. Data shown are mean values ± SEM. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, n.d. = not detectable.

allergen (GF sens). IL-10 levels did not differ statistically between the groups (Fig. 3D).

Also in pooled MLN cells, suppressed IL-5 and enhanced IFN-γ production was noted in mice mono-colonized with recLp and sensitized to the allergen. IL-4 production was not detectable. Interleukin-10 seemed reduced in mice colonized with recLp or conLp compared with GF-sensitized mice (GF sens) (Table 2).

Regulatory markers are increased in spleen cells of mice mono-colonized with recombinant *L. plantarum* (experiment II)

A significant up-regulation of mRNA of the regulatory marker Foxp3 in spleen cells of mice mono-colonized with recLp was detectable compared with GF-sensitized controls. (Fig. 4). IL-10 and TGF-β mRNA was enhanced, though without statistical significance.

Table 2 Cellular immune responses in mesenteric lymph nodes (experiment II)

	Cytokines (pg/ml)			
Experimental set-up	IFN-γ	IL-4	IL-5	IL-10
recLp (group 1) + sens	36.0 ± 0.2	n.d.	16.4 ± 3.4	14.1 ± 3.6
conLp (group 2) + sens	17.8 ± 0.7	n.d.	53.1 ± 22.5	17.2 ± 4.0
None (group 3) + sens	13.1 ± 4.9	n.d.	58.5 ± 20.4	30.7 ± 4.1
None (group 4)	9.5 ± 5.6	n.d.	5.4 ± 3.8	18.9 ± 1.6

Mice were colonized with the indicated strain, groups 1–3 were sensitized (sens) to Bet v 1. Mice were killed at 83 days of age. Pooled mesenteric lymph node cells (n = 5–7 per group) were stimulated with Bet v 1 for 48 h, and cytokines were measured from supernatants. Mean values of two independent are given in pg/ml. n.d., not detectable.

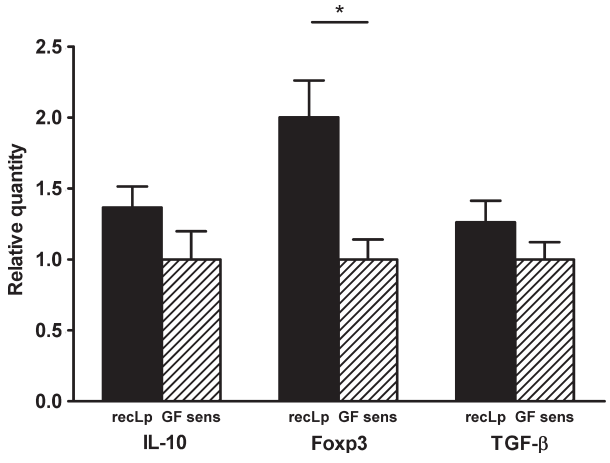

Figure 4 Regulatory markers in spleen of mice mono-colonized with recLp. Mice colonized with recombinant *Lactobacillus plantarum* (n = 6, recLp, black bars) and uncolonized germ-free mice (n = 6, GF sens, dashed bars) were intraperitoneally sensitized with Bet v 1/Al(OH)3. IL-10, Foxp3 and TGF-β mRNA expression in spleen cells was determined by real-time (RT)-PCR. Data shown are mean values ± SEM. *$P \leq 0.05$.

Discussion

The hygiene hypothesis (16) has brought up new perspectives for the prevention of allergy preparing the ground for application of LAB for modulation of the intestinal microflora to influence allergy development (7). Furthermore, LAB have been demonstrated to act as potent mucosal adjuvants and/or antigen-delivery systems (6). In the light of this, recombinant LAB strains genetically engineered to produce and deliver allergens to mucosal surfaces to induce tolerance have emerged as new tools for mucosal intervention against type I allergy (5, 12, 17–24). We previously evaluated two LAB strains, *L. plantarum* and *L. lactis,* for their potential to modulate allergic immune responses in a model of birch pollen allergy in conventional mice, showing a strong Th1 inducing capacity and induction of counter-regulatory immune responses when admixed with Bet v 1 (25). Aiming at standardized antigen delivery to the mucosal surfaces by the bacteria, we constructed a recombinant LAB producing Bet v 1. Accordingly, prophylactic intranasal application of the recombinant strains succeeded in suppressing systemic and local (within the airways) allergen-specific Th2 responses in adult mice (12).

Evidence is accumulating that programming of the immune system, particularly with respect to tolerance induction, starts already at birth and is under close control of the maternal immune system (11), suggesting this period important for processes of immuno-programming. To take advantage of this early "window of opportunity" (11) for modulating the onset of type I allergy, we mono-colonized GF mice from birth with a Bet v 1 producing *L. plantarum* by natural colonization via their mothers. To be used for neonatal colonization, our previously published recombinant *L. plantarum* strain (12) seemed unsuitable, as it carries a plasmid with an inducible promoter that is only highly active *in vitro*, but not under colonizing conditions. We therefore recloned the *bet v 1* gene under the control of a strong promoter leading to constitutive expression of the allergen. After demonstrating stable colonization and excluding a loss of the plasmid *in vivo*, we first analysed the effects of neonatal colonization on the immune system in the absence of allergic sensitization. At the cellular level, the recombinant strain induced a nonallergic Th1 response with significant IFN-γ but absent IL-4 and IL-5 production in spleen cells upon allergen challenge *in vitro*. These findings differ from Hazebrouck et al. (26) who found that colonization of adult GF mice with a *Lactobacillus casei* secreting beta-lactoglobulin leads to both Th1 and Th2 cytokine production, which may suggest an advantage of early neonatal intervention over application of recombinant LAB in adulthood. At the humoral level, we did not detect the induction of allergen-specific serum antibodies, which is similar to findings in adult mice colonized with recombinant LAB (26). A report by Dahlman et al. (27) showing that colonization with recombinant Gram-negative bacteria–induced allergen-specific IgE underlines the suitability of Gram-positive LAB as delivery vector for interventional strategies against allergy (25). However, significant strain-specific differences are even the case within these Gram-positive probiotics, as diverse effects on allergic sensitization have been shown after ingestion (28) or sublingual application (29) of a panel of probiotic LAB strains, which argues for careful strain selection. When neonatally colonized mice were subsequently sensitized with the birch pollen allergen Bet v 1, we found suppressed allergen-specific Th2 responses (IL-4/IL-5) and enhanced levels of IFN-γ in spleen and mesenteric lymph nodes. In contrast to our previous studies in conventional mice using repeated oral applications of recombinant LAB that did not succeed in a suppression of Th2 responses (30), the early and long-term intervention used here thus seems to be more efficient.

Notably, the wild-type *L. plantarum* itself did not exert any suppressive effects on the allergic immune response. Therefore, the effects elicited by our vector system depended

on the expression of the specific allergen and indicates that the bacteria represent an adjuvant system without immunosuppressive effects in the absence of expression of a specific allergen, as shown in previous studies with this strain (12, 25). This aspect seems particularly important for interventions at early developmental stages.

The mechanisms of immunomodulation/-regulation exerted by different recombinant LAB strains are complex. A shift towards Th1 (5) but also induction of regulatory cells (5, 22) were described. As we found both a shift towards a Th1 cytokine pattern and the suppression of antibody responses and Th2 cytokine responses, we also examined regulatory markers. According to previous findings in adult mice tolerized against Bet v 1 (31), we found increased mRNA expression of Foxp3 in splenocytes of neonatally colonized and sensitized mice. This finding suggests that the induction of counter-regulatory cellular Th1 responses was paralleled by development of regulatory T cells after colonization from birth and bacterial persistence in the gut.

Taken together, mono-colonization with recombinant *L. plantarum* specifically induced immunomodulation of allergic immune responses, supporting the concept of early immuno-imprinting using recombinant LAB. Our results therefore promote the use of recombinant lactic acid bacteria for early allergen-directed prevention of type I allergy.

This new research area of immuno-imprinting would, however, definitely benefit from examination of the predictive value of mouse models, e.g. by analysing correlations between preclinical and clinical data. Moreover, aiming at treating neonates with a positive family history of atopy, suitable treatment protocols including environmental containment strategies and a broader range of protection to a panel of allergens are desirable.

Acknowledgments

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Conflict of interest

All authors have contributed to the work; the authors have no conflict of interest.

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3.2.3 Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to Bet v 1

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Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to Bet v 1

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Running title: Colonization by *B. longum* prevents allergic sensitization

Key words: allergy, *Bifidobacterium*, germ-free mice, probiotics, TLR2

ABSTRACT

Background: The main goal in reversing the allergy epidemic is the development of effective prophylactic strategies. Early life events, such as exposures to microbes, have a major influence on the development of balanced immune responses. Here we investigated the effect of neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* ssp. *longum* CCDM367 on subsequent allergic sensitization.

Methods: Adult male and female germ-free (GF) mice were mono-colonized with *B. longum,* mated and their offspring, as well as age-matched GF controls, were sensitized with Bet v 1. Furthermore, signaling pathways involved in the recognition of *B. longum* were investigated.

Results: Neonatal mono-colonization of GF mice with *B. longum* significantly suppressed Bet v 1-specific IgE-dependent β-hexosaminidase release as well as levels of total IgE and allergen-specific IgG1 and IgG2a in serum compared to sensitized GF controls. Accordingly, allergen-specific recall proliferation of splenocytes and production of both Th1- and Th2-associated cytokines in spleen cell cultures were significantly reduced in these mice. The general suppression of Bet v 1-specific immune responses in *B. longum* colonized mice was associated with increased levels of regulatory cytokines IL-10 and TGF-β in serum. *In vitro, B longum* induces low maturation status of bone marrow-derived dendritic cells and production of cytokines in TLR-2 and MyD88 dependent manner.

Conclusion: Our data demonstrate that neonatal mono-colonization with *B. longum* reduces allergic sensitization by activating regulatory responses, likely via TLR2 and MyD88 signaling pathways. Thus, *B. longum* might be a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans.

INTRODUCTION

There has been a remarkable increase in allergic diseases over the past few decades, especially in Western countries (1). It has been suggested that environmental changes, rather than genetic factors, are driving the epidemic character of allergy and events acting within a narrow window of opportunity, either prenatally or early in life, might have major effects (2, 3).

Germ-free status of fetus changes rapidly after birth and the composition of colonizing microbiota can be influenced by multiple factors such as the mode of delivery, dietary changes, high hygiene or over-use of antibiotics (4). The exposure to microbial stimuli is crucial for development, maturation and function of the immune system and association between intestinal disbiosis and allergic disease has been proposed (5). Several prospective studies have shown lower neonatal colonization by bifidobacteria and lactobacilli species accompanied by higher counts of *Clostridium difficile* in neonates who developed allergy later in life (6-8).

Due to their ability to interact with host immune system and to modulate host immune responses, bifidobacteria and lactobacilli have been used with some success in prevention or treatment of allergic disease in infants at risk. Several randomized clinical trials have shown that combined prenatal/postnatal probiotic interventions reduced the cumulative incidence of eczema while less beneficial effects were found in trials using exclusively postnatal or exclusively prenatal treatment approaches (recently reviewed in (9)). These data highlight the importance of timing of probiotic interventions, where the prenatal period seems to be the most significant component.

Dendritic cells are pivotal in early bacterial recognition through pattern recognition receptors (PRRs), which leads to induction of distinct innate responses that shape the type of T helper cell responses (10, 11). In this respect, Konieczna *et al.* recently showed that preconditioning of DC with probiotic *B. infantis* led to induction of FoxP3 positive regulatory T cells and enhanced and IL-10 production (12). Due to the fact that allergic diseases have been associated with a deficiency in regulatory T cells numbers and/or function (13), specific probiotic strains inducing regulatory immune responses might be beneficial in the prevention of allergic disorders. Indeed, neonatal application of *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb-12 suppressed allergic sensitization and airway inflammation by induction of T regulatory cells associated with increased TGF-β production (14). Similarly, we have previously shown that perinatal administration of *L. paracasei* to pregnant/lactating

mice protected against the development of airway inflammation in offspring by activating regulatory pathways (15).

Germ-free (GF) animals represent a powerful model to study the interaction of single bacterial strain or defined mixture of microbial strains with the host immune system (16). Taking the advantage of this model we have previously shown, that neonatal mono-colonization of GF mice with the recombinant bacterial strain *L. plantarum* producing Bet v 1 reduced the development of allergic responses upon systemic sensitization (17).

Bifidobacterium longum subspecies *longum* CCDM367 (*B. longum*) is a commensal bacterial strain originally isolated from the feces of healthy breast fed infant. *B. longum* has been shown to induce regulatory responses *in vitro* and to suppress the inflammatory responses in mouse models of experimental colitis (Srutkova, unpublished results). In the present study we investigated whether neonatal mother-to-offspring mono-colonization of GF mice with *B. longum* is able to protect mice against allergic sensitization to Bet v 1 in gnotobiotic model of type I allergy.

MATERIALS AND METHODS

Animals

Germ-free mice on a BALB/c background were kept under sterile conditions and were supplied with water and sterile pellet diet (ST1, Bergman, Czech Republic) *ad libitum*. Fecal samples were weekly controlled for microbial contamination as previously described (18). Wild-type and TLR2$^{-/-}$, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice on a C57BL/6 background, obtained from M. Müller (Vienna, Austria), were kept under SPF conditions. All animal experiments were approved by local ethics committee.

Bacterial strain

Probiotic strain *Bifidobacterium longum ssp. longum* CCDM367 (*B. longum*), originally isolated from the faeces of breast-fed healthy child, was provided by Culture Collection of Dairy Microorganisms (Milcom, Czech Republic). *B. longum* cultures were grown anaerobically in MRS medium (Oxoid, UK) supplemented with L-cysteine-hydrochloride (0.5 g/l) for 48 hours at 37°C. For *in vitro* experiments, *B. longum* was inactivated with 1% formaldehyde-PBS as described before (19).

Colonization and experimental design

Eight-week-old GF male and female mice were colonized with 2 x 10^8 CFU of *B. longum* in 200 µl of sterile PBS by intragastric administration and mated 20 days later. Colonization was checked by plating of feces as described previously (17). Female offspring, neonatally colonized via their mothers, and control GF mice were subcutaneously (s.c.) sensitized on days 1, 14 and 28 with 1 µg of Bet v 1 emulsified in 2 mg of $Al(OH)_3$ (Serva, Germany). Seven days after the last immunization, mice were killed by CO_2 asphyxia and samples were taken for further analysis (Fig. 1A).

Humoral immune responses

Blood samples were taken at sacrifice and serum levels of anti-Bet v 1 IgG1 and IgG2a were measured by ELISA as previously described (17). Levels of Bet v 1 specific IgE in sera (final dilution 1:90) were measured by rat basophile leukemia cells degranulation assay as described before (19). Levels of total IgE and IgA in sera (dilution 1:10 and 1:400, respectively) were measured using a commercial ELISA kit as recommended by the manufacturers (Bethyl, USA). Levels of IL-10 and TGF-β in sera (final dilution 1:1000) were measured by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instruction. Small intestine was excised and gut lavage was prepared as described previously (15). Levels of total IgA and TGF-β in gut lavage were measured as described above with final dilution 1:2500 and 1:1000, respectively and reported as absolute units.

Cellular immune responses

Spleen and mesenteric lymph node (MLN) single cell suspensions from *B. longum* mono-colonized and control mice were prepared and cultured as previously described (15). Mononuclear cells (3 x 10^6 cells/ml) were stimulated with Bet v 1 (20 µg/ml) or media alone in 96-well plates at 37°C for 60 hours in culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin). Levels of cytokines in culture supernatants were measured by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, USA). Values are expressed as pg/ml after subtraction of baseline levels of unstimulated cultures. Proliferative responses of spleen cultures with/ without Bet v 1 restimulation were determined by scintillation counting after addition of ^{3}H-Thymidine (0.5 µCi/well; Lacomed, Czech Republic) for the last 16 h of 76 h cultivation. Values are shown as stimulation index.

Preparation and activation of bone marrow-derived dendritic cells

Mouse bone marrow-derived dendritic cells (BM-DC) were prepared as previously described (15). BM-DC (10^6 cells/well) were stimulated with 10^6 or 10^7 CFU of *B. longum* for 18 h. As controls, BM-DC were incubated with Pam3C (Pam3CSK4, 1 µg/ml, InvivoGen, USA) or ultra-pure LPS (LPS-EB, 1 µg/ml, InvivoGen, USA). Levels of IL-10, TGF-β, and IL-6 in culture supernatants were determined by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions. Levels of IL-12p70 were measured with matched antibody pairs (BD Pharmingen, USA). Where indicated, BM-DC were pretreated with mitogen-activated protein kinase (MAPK) specific inhibitors (MEK: PD98059; p38: SB203580; JNK: SP600125) or NF-κB (BAY 11-7082) at 10 µM dissolved in DMSO for 1 h at 37°C.

Flow-cytometry analysis

Single cell suspensions of spleens or MLN were stained for regulatory T cells with Foxp3 Staining Buffer Set (eBioscience, USA) using fluorochrome-conjugated monoclonal antibodies for CD4 (FITC) and Foxp3 (PE) (eBioscience, USA). Dendritic cells were labeled with monoclonal antibodies for CD11c (FITC), MHCII (APC), CD40, CD80 or CD86 (PE) (eBioscience, USA). Cells were analyzed using FACSCalibur flow cytometer (Becton-Dickinson, USA) and analyzed with FlowJo 7.6.2 software (TreeStar, USA).

Stimulation of HEK293 cells stably transfected with TLRs

HEK293 cells stably transfected with plasmid carrying human (h)TLR2/CD14 gene were kindly provided by M. Yazdanbakhsh (Leiden, Netherlands) and cells transfected with hTLR4/MD2/CD14 were a gift of B. Bohle (Vienna, Austria). Cells were stimulated with formalin-inactivated *B. longum* (10^6, 10^7, or 10^8 CFU/ml). TLR2 lignad Pam3C (1 µg/ml) and and TLR4 ligand LPS (1 µg/ml) were used as positive controls. After the 20-h incubation period, culture supernatants were harvested and concentration of human IL-8 was analyzed by ELISA (Thermo Scientific, USA) according to the manufacturer's instructions.

Statistics

Data are expressed as means ± SEM. Statistical analysis was performed by Student's t-test (GraphPad Prism 5.04, GraphPad Software, San Diego, CA). Differences were considered significant at p value of <0.05.

RESULTS

Neonatal mono-colonization with *B. longum* prevents systemic sensitization to Bet v 1

Colonization of mice with B. longum remained stable throughout the experiments and reached a level of approximately 5 x 10^9 CFU/g of feces (data not shown). Sensitization of GF offspring?mice derived from GF parents induced high levels of Bet v 1-specific antibodies in serum; neonatal mono-colonization of GF mice with *B. longum* abrogated this process. Both the Th2-related IgG1 as well as Th1-related IgG2a in serum were significantly reduced in mice colonized with *B. longum*, compared to GF controls (Fig. 1C, D). Furthermore, the presence of *B. longum* led to reduction of the IgE-dependent and Bet v 1-driven basophil degranulation (Fig. 1B). Similarly, *B. longum* suppressed levels of total IgE in sera of mono-colonized mice in comparison to GF controls (Fig. 1E). Concomitantly, *B. longum* neonataly colonized mice showed elevated levels of IL-10 in sera (Fig. 1F) and TGF-β both in sera (Fig. 1G) and gut lavage (Supplementary Fig. 1). No changes were observed in production of total IgA antibody neither in serum (data not shown) nor in gut lavage (Supplementary Fig. 1).

Mice neonataly mono-colonized with *B. longum* are protected from the development of cellular allergic responses

Neonatal mono-colonization with *B. longum* reduced the development of systemic allergen-specific recall responses. Production of Th2-related IL-5, IL-13 and IL-4 (Fig. 2 A-C) as well as production of pro-inflammatory cytokines IFN-γ, TNF-α and IL-17 (Fig. 2 E-G) in Bet v 1-stimulated splenocyte cultures was significantly lower in *B. longum* mono-colonized mice compared with control GF mice. Interestingly, the production of regulatory cytokine IL-10 was also significantly lower than for control GF mice (Fig. 2D). Similar effect was observed in Bet v 1-stimulated mesenteric lymph node cell cultures (data not shown). Moreover, neonatal colonization with *B. longum* led to reduced Bet v 1-induced proliferative responses of spleen cells *ex vivo* (Fig. 2H) and significant increase of CD4+Foxp3+ cells in MLN (Supplementary Fig. 2).

B. longum induces low maturation status of dendritic cells and production of regulatory cytokines

Stimulation of BM-DC with *B. longum* induced only moderate and dose dependent induction of maturation markers CD40, CD80 and CD86 when compared with LPS (TLR4 ligand) or Pam3C (TLR2 ligand) (Fig. 3A). However, *B. longum* induced high levels of regulatory

cytokines IL-10 and TGF-β (Fig. 3B and C) and also IL-6 (Fig. 3E). On the contrary, only small amounts of IL-12p70 were detected (Fig. 3D).

Role of innate receptors in recognition of *B. longum*

In order to study the role of TLRs in *B. longum* mediated signals, human embryonic kidney cells (HEK) 293 stably transfected with TLR2 or TLR4 were stimulated with different concentrations of formalin inactivated *B. longum*. Pam3C was used as positive control for TLR2 and LPS for TLR4. High levels of IL-8 in *B. longum* stimulated HEK293/TLR2 indicated that *B. longum* signals trough TLR2 in a dose dependent manner (Fig. 4A). There was no production of IL-8 after stimulation of HEK293/TLR4 with *B. longum* (Fig. 4B).

***B. longum* signals through TLR2 and MyD88 to induce regulatory cytokines by dendritic cells**

We have shown that stimulation of wild-type BM-DC with *B. longum* induced up-regulation of surface markers CD40, CD80 and CD86 (Fig. 5A). This effect was significantly reduced, when BM-DC from TLR2- or MyD88-deficient mice were used. On the other hand, the lack of TLR4 had no impact on induction of these surface markers by *B. longum*. Similarly, BM-DC stimulation with *B. longum* induced production of IL-10 and IL-12p70 in wild-type and TLR4-deficient cells but production of these cytokines was abrogated in BM-DC derived from TLR2- and Myd88-deficient mice (Fig. 5 B and C). *B. longum*-induced production of IL-10 and IL-12p70 from TLR4-deficient BM-DC remained unchanged compared to wild-type cells. The production of surface markers (Fig. 5A) and cytokines (data not shown) induced by TLR2 ligand Pam3C was abrogated in cells derived from TLR2- and MyD88-deficient mice.

Activation of MAPK and* NF-κB in dendritic cells is critical for the production of IL-10 by *B. longum

In order to investigate the functional role of ERK, p38, JNK or NF-κB pathways in cytokine production induced by *B. longum*, BM-DC were pretreated with a selective inhibitor of the ERK (PD98059), p38 (SB203580), JNK (SP600125) or NF-κB (BAY 11-7082) pathway, prior to incubation with *B. longum*. As shown in Fig. 6, production of IL-10 induced by *B. longum* markedly decreased in the presence of inhibitors of the ERK, p38, JNK or NF-κB pathways. This data indicates that all four pathways are important for IL-10 production. In contrast, the IL-10 production induced by TLR2 ligand Pam3C was dependent on ERK, p38,

and NF-κB only, as selective blockade of JNK activation induced by Pam3C had no significant impact on IL-10 production (Fig. 6).

DISCUSSION

In this report, we demonstrate that neonatal mother-to-offspring mono-colonization with *B. longum* CCDM367 significantly reduces the development of allergen-specific immune responses in a gnotobiotic mouse model of type I allergy, which was associated with induction of regulatory milieu. Furthermore, we show that TLR2 and subsequent downstream signaling via Myd88 and MAPK are crucial for the recognition of *B. longum* and induction of IL-10.

Clinical studies differ greatly in their outcomes regarding the role of probiotic interventions in strategies for prevention of allergic diseases (reviewed in (20)). However, most of the studies claim that the probiotic intervention in prenatal/perinatal period appears to be crucial for manifestation of beneficial effects, confirming the existence of "window of opportunity" in the programming of the immune system in early life (14, 15, 21).

In order to investigate the interaction between single bacteria and the host immune system, as well as the impact of the specific strain on the reprograming and the maturation of offspring's immune system, we established a mouse model of neonatal mother-to-offspring mono-colonization using germ-free mice. By that we previously showed that constitutive delivery of Bet v 1 by *Lactobacillus plantarum,* but not the wild-type bacteria alone, was able to modulate subsequent systemic sensitization to Bet v 1 (17). Here we show that mother-to-offspring monocolonization of GF mice with *B. longum* significantly decreased the levels of Th2- as well as Th1-associated Bet v 1-specific antibodies in serum. This data suggest that the inhibition of sensitization was not achieved simply by immune shift toward the Th1 mode, but rather that general reduction of immune responses might take a place.

Consistent with the results observed in sera, on cellular level *B. longum* inhibited antigen-specific recall responses in spleen cells. Both Th1- and Th2- associated cytokines, as well as antigen specific proliferation, were dramatically reduced in mice colonized with *B. longum* in comparison to controls. We observed similar general suppression in antigen specific cytokine production after prophylactic intranasal application of a different *B. longum* strain (22) and similarly, *Bifidobacterium* has been shown to inhibit T-cell proliferation (23) and antigen-specific cytokine production (24) in OVA food allergy model. It is necessary to stress out, that although the reduction of humoral and cellular immune responses observed with *B. longum* is

feasible in the context of sensitization to allergens, it remains important to evaluate, whether the perinatal application of *B. longum* or other strains with immunosuppressive properties could interfere with immune responses against infections, vaccines or anti-tumor immunity.

On the basis of previous reports suggesting that certain probiotic bacteria induce the generation of Foxp3 regulatory T cells both in humans and animals (12, 23), which interferes with Th2 cell effector mechanisms, we postulated that the suppressive effects of *B. longum* on Bet v 1-induced sensitization may be mediated by these cells. Indeed, in MLNs of *B. longum*-monocolonized mice we found small, but significant, increase in FoxP3+ Treg cells. Moreover, we found significantly increased levels of IL-10 and also of TGF-β in sera. Interleukin-10 and TGF-β are regarded as regulatory cytokines, that are produced by a broad variety of cells, including tolerogenic DC and they are indispensable for induction of regulatory T cells (10). Interestingly, it has been recently shown, that besides induction of FoxP3+Treg cells (23), bifidobacteria are able to induce another subset of FoxP3 negative Treg cells, IL-10-producing type 1 regulatory cells (Tr1) (25). We therefore speculate, that *B. longum* is exerting its immunoregulatory properties both through induction of Tr1 and FoxP3+ Treg cells. However, the precise role of *B. longum*-induced IL-10 and TGF-β in prevention of sensitization in our model remains to be investigated.

Dendritic cells are regarded as the key players in routing the immune responses and induction of different T cell subsets (12, 25). *B. longum* stimulated BM-DC showed only mild upregulation of co-stimulatory markers accompanied by increased production of IL-10 and TGF-β. Indeed, immature DC and DC producing IL-10 have been regarded as tolerogenic (26) and they were shown to be able to induce regulatory T-cells (12, 23). Recent reports show that the stimulation of TLR2 on dendritic cells enhances their ability to induces the Treg cells (27) and in humans, TLR2 signaling on antigen presenting cells inhibits the allergen specific Th2 responses (28). In this respect, we have shown that *B. longum* engages TLR2 but not TLR4 on transfected HEK cells and confirmed these findings by showing that cytokine production induced by *B. longum* was abrogated in TLR2-/- and MyD88-/-, while it was retained in TLR4-/- deficient DC. It is thus tempting to speculate, that the immunomodulatory effect of *B. longum* observed *in vivo* is due to its strong interaction with TLR2 receptor leading to the IL-10 production.

Since TLR2 has been shown to activate MAPK and NF-κB, we further investigated their roles in IL-10 production induced by *B. longum*. We show that the *B. longum* induced IL-10 is critically dependent on NF-κB activation. Further, the data obtained with MAPK inhibitors stress out the importance of p38 and ERK, and surprisingly also JNK signaling pathways.

This is in contrast with the results obtained for the TLR2 ligand Pam3C, where JNK pathway was dispensable for IL-10 induction. To our knowledge there are no other studies showing the involvement of MAP-kinase pathways in IL-10 induction by *Bifidobacterium*.

Taken together, our data shows that *B. longum* is a strain with the ability to induce regulatory cytokines/ T cells with the ability to prevent allergic sensitization. These properties make it a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans. Moreover, our results stress out the general importance of intrinsic immunomodulatory properties of bacterial strain used for intervention and shed light on the function of bifidobacteria in shaping the immune system in early human ontogeny.

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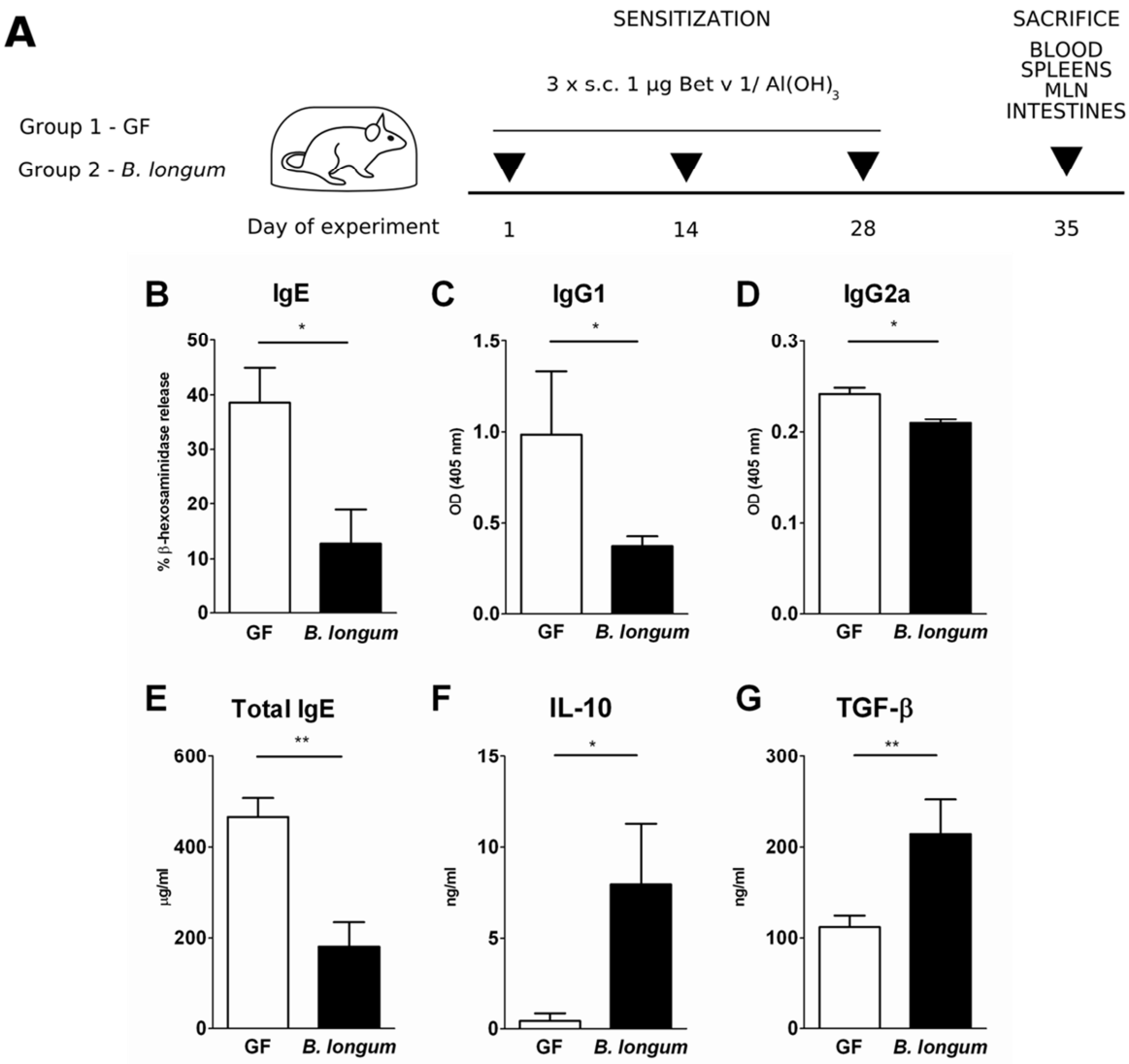


Figure 1: **Systemic sensitization to Bet v 1 in mice neonataly colonized with *B. longum*.** Experimental design: Eight-week old germ-free (GF) mice (n=5) and age-matched mice neonataly colonized with *B. longum* (n=5) were three times subcutaneously (s.c.) sensitized with 1 µg of recombinant Bet v 1 in Alum (Bet v 1/Al(OH)3). At sacrifice blood, small intestine, spleen and mesenteric lymph nodes were collected for further analysis (A). Bet v 1-specific IgE (B) in sera was measured by rat basophil leukemia (RBL-2H3) cell degranulation assay, IgG1 (C), IgG2a (D), total IgE (E), IL-10 (F) and TGF-β (G) in sera were measured by ELISA. Germ-free mice (GF, white bars) and *B. longum* colonized mice (black bars). Data are plotted as mean values ± SEM. One representative experiment out of two is shown. *$P \leq 0.05$, **$P \leq 0.01$.

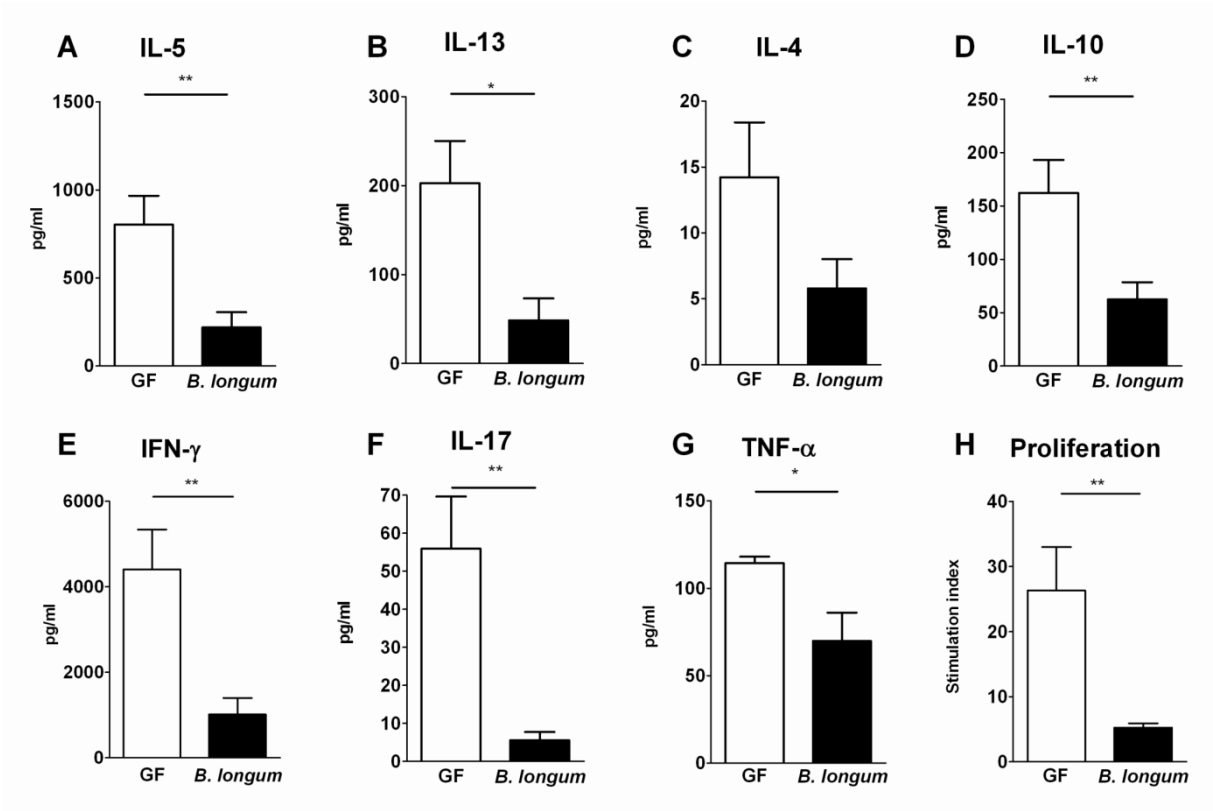


Figure 2: **Influence of *B. longum* neonatal colonization on Bet v 1 specific cytokine production and proliferative response in splenocytes.** Spleen cells from germ-free mice (GF, white bars) and *B. longum* colonized mice (black bars) were cultured *in vitro* 60 h with 20 µg/ml of Bet v 1. Production of IL-5 (A), IL-13 (B), IL-4 (C), IL-10 (D), IFN-γ (E), IL-17 (F) and TNF-α (G) were measured in culture supernatants by MILLIPLEX MAP Mouse Cytokine/Chemokine Panel. Cytokine levels are expressed after subtraction of base line levels of unstimulated splenocytes. Proliferative responses of splenic cells were assessed by incorporation of ^{3}H-Thymidine for the last 16 h of 76 h cultivation (H). Values are shown as stimulation index. Pooled values from two independent experiments (n=5 mice/group) are shown as mean values ± SEM. *P ≤ 0.05, **P ≤ 0.01.

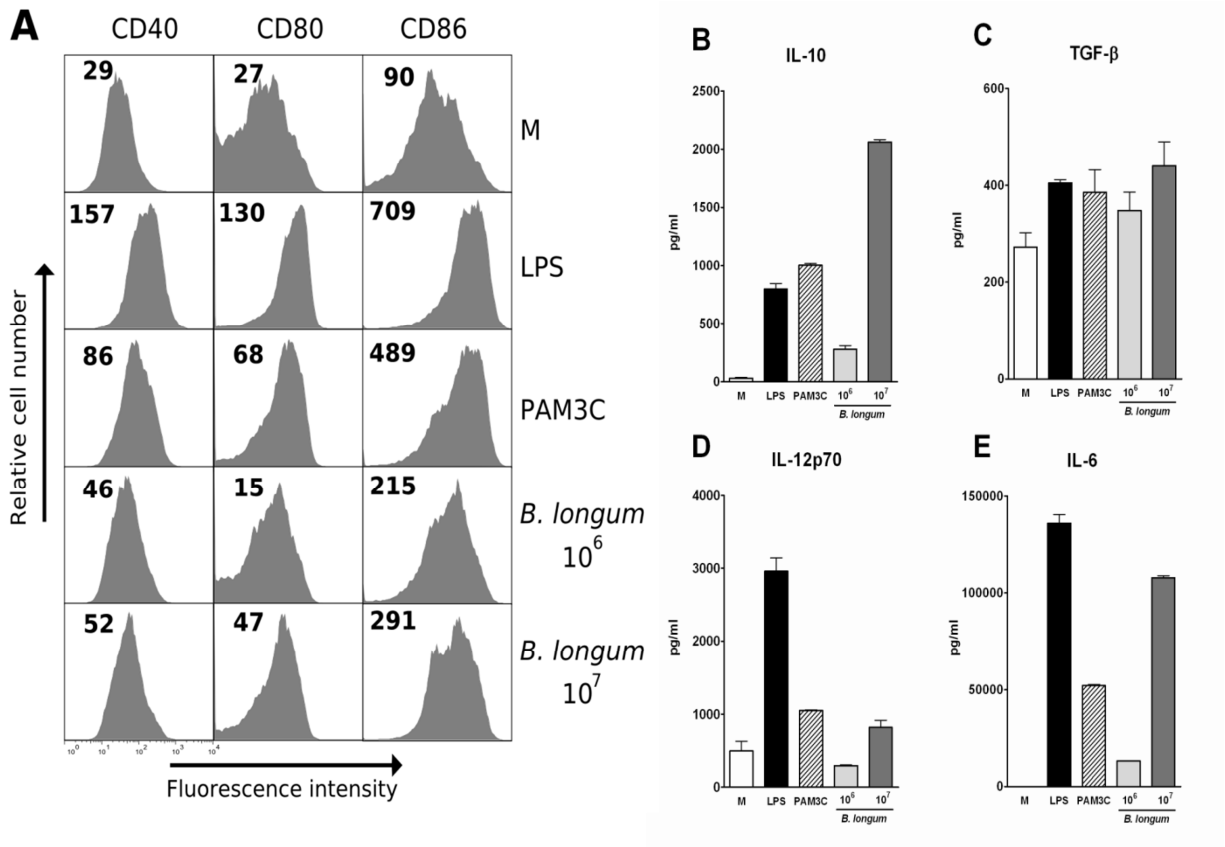


Figure 3: **Maturation of dendritic cells and cytokine production induced by *B. longum.*** Bone marrow-derived dendritic cells from BALB/c mice were cultured with media alone (M), ultra-pure lipopolysaccharide from *E. coli* (LPS, 1 μg/ml), Pam3CSK4 (PAM3, 1 μg/ml) or two different concentrations of formalin-inactivated *B. longum* (10^6 or 10^7 CFU/ml) for 18 hours. BMDC were analyzed by flow cytometry for CD40, CD80 and CD86 expression (A). Numbers shown are median florescence units. One representative out of three experiments is shown. Production of IL-10 (B), TGF-β (C), IL12p70 (D) and IL-6 (E) in culture supernatant were determined by ELISA. Mean values ± SEM are shown. One representative out of three experiments is shown.

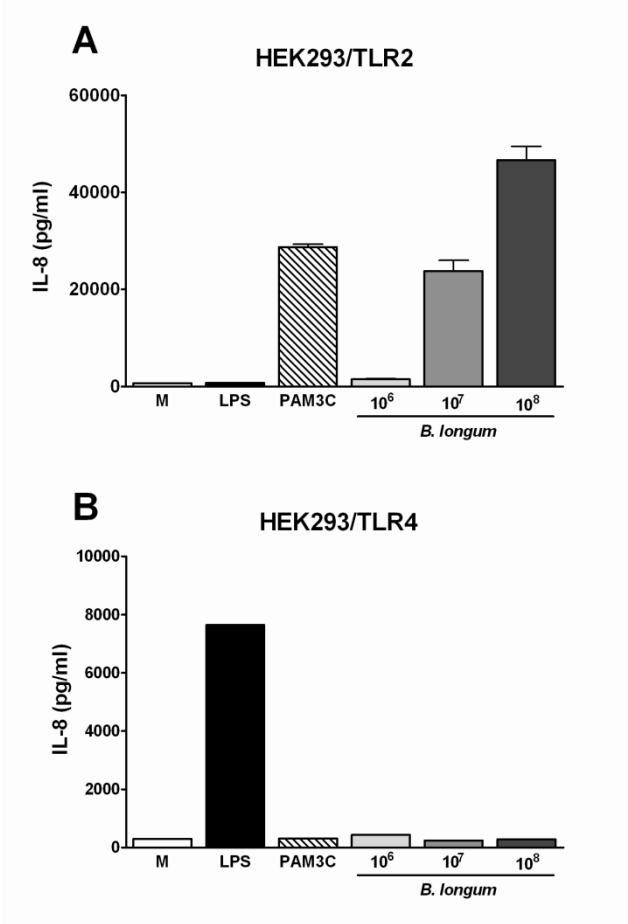


Figure 4: **Activation of TLR receptors by *B. longum*.** Human embryonic kidney cells (HEK293) stably transfected with an expression vector for human TLR2 (293-hTLR2/CD14) (A) and TLR4 (293-hTLR4/MD2/CD14) (B) were cultured for 20 h with different concentrations of formalin inactivated *B. longum* (10^6, 10^7, 10^8 CFU/ml) or left unstimulated. Ultra-pure lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) and Pam3CSK4 (PAM3, 1 μg/ml) were used as positive controls for TLR4 and TLR2, respectively. Unstimulated cells (M) were used as control. Results are expressed as mean values ± SEM, one representative experiment out of at least three is shown.

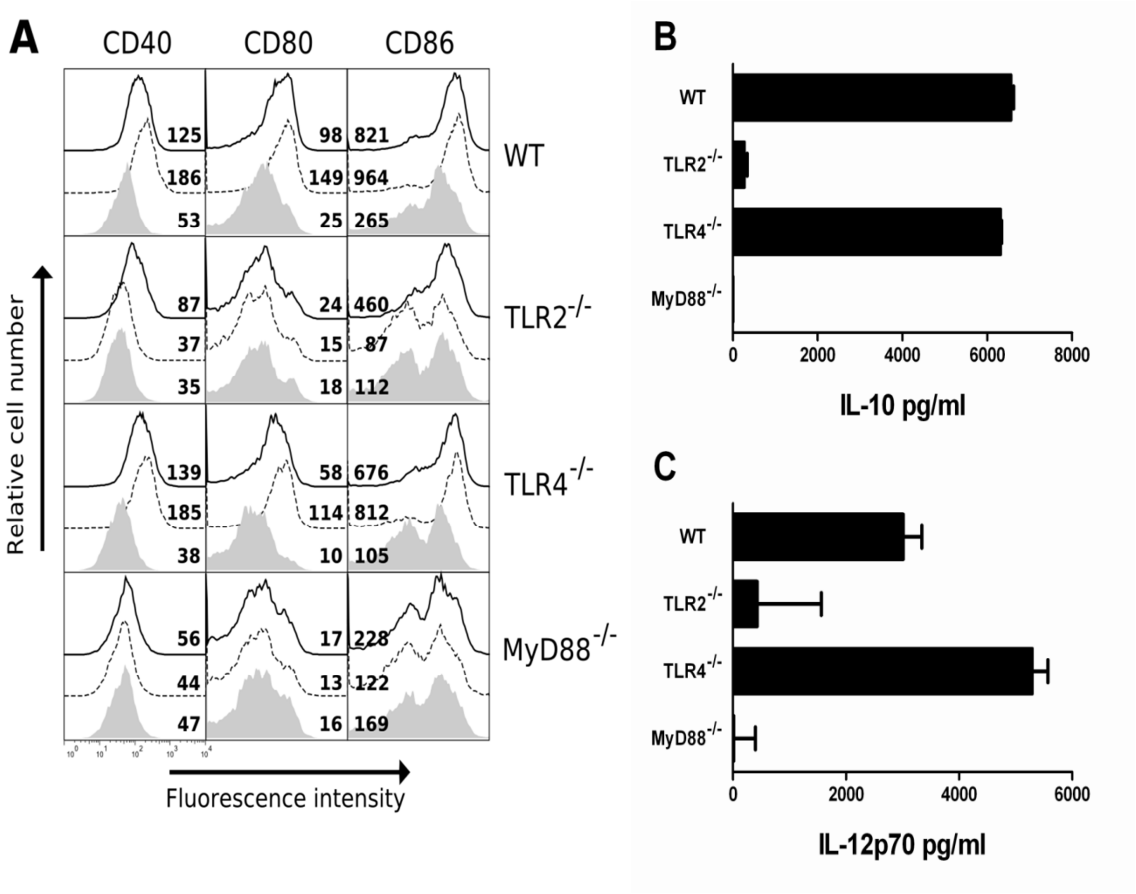


Figure 5: **Involvement of TLR in BMDC maturation and cytokine production induced by *B. longum*.** BMDC generated from wild-type (WT), TLR2-, TLR4- and MyD88-deficient mice with C57BL/6 background were cultured with media (gray histograms), Pam3CSK4 (1 μg/ml, dashed lines) or formalin inactivated *B. longum* (10^7 CFU/ml, solid line) for 18 h (A). Expression of maturation markers CD40, CD80 and CD86 was assessed by flow cytometry. Numbers shown are median florescence units. Production of IL-10 (B) and IL12p70 (C) after stimulation with *B. longum* (10^7 CFU/ml) was measured by ELISA. Mean values ± SEM after subtractions of baseline levels from unstimulated cells are shown. One representative out of two experiments yielding similar results is shown.

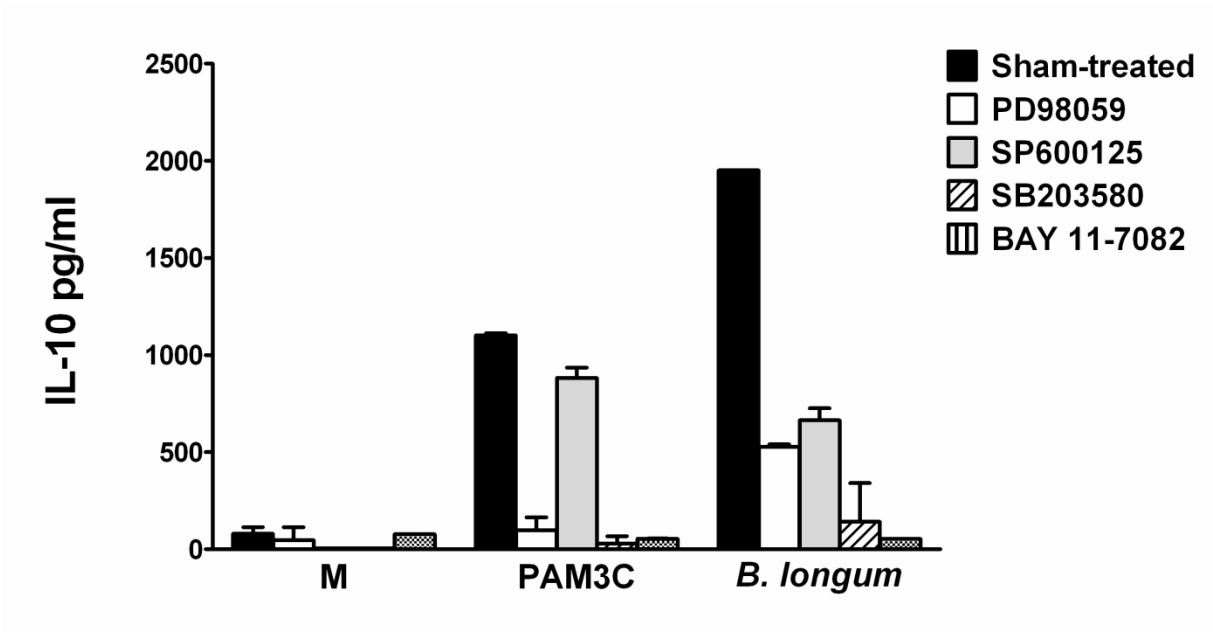
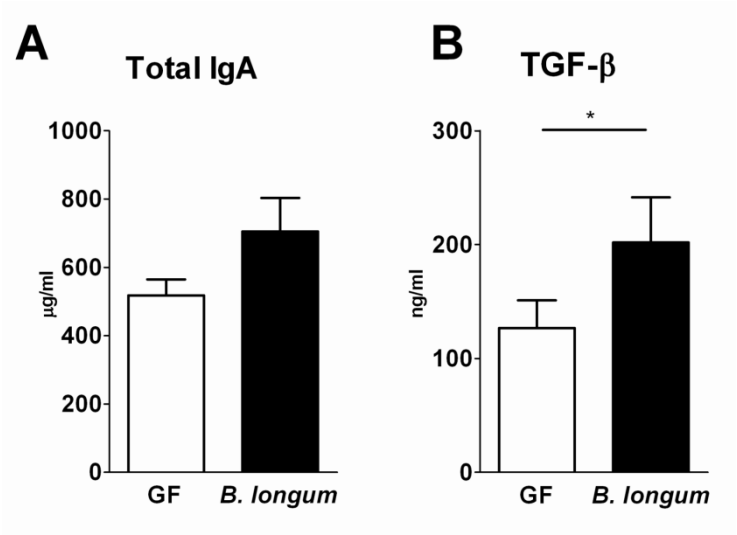
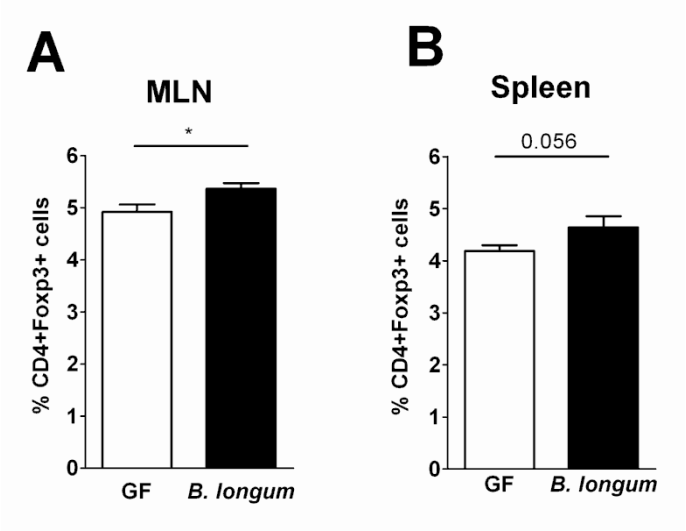


Figure 6: **Involvement of MAPK pathways and NF-κB in *B. longum* induced production of IL-10.** BMDC were incubated with DMSO only (Sham-treated, black bars) or with MEK 1 (PD9859, white bars), JNK (SP600125, gray bars), p38 (SB203580, dashed bars) and NF-κB (BAY 11-7082, dotted bars) pathway inhibitors for 1 h prior to stimulation with formalin inactivated *B. longum* (10^7 CFU/ml) or Pam3CSK4 (PAM3, 1 μg/ml). Unstimulated cells (M) were used as controls. Levels of IL-10 were determined by ELISA. Results are expressed as mean values ± SEM, one representative experiment out of two is shown.



Supplementary figure 1: **Levels of IgA and TGF-β in gut lavage.** Total IgA (A) and TGF-β (B) in gut lavage were measured by ELISA. Germ-free mice (GF, white bars) and *B. longum* colonized mice (black bars). Data are plotted as mean values ± SEM. Pooled results from two experiments (n=10 mice) are shown. *P ≤ 0.05.



Supplementary figure 2: **Numbers of CD4+FoxP3+ cells in splenocytes and mesenteric lymph nodes cells.** The percentage of Tregs in cell suspension isolated from mesenteric lymph nodes (A) and spleens (B) of germ-free mice (GF, white bars) and *B. longum* colonized mice (black bars). Representative data from one out of two independent experiments are shown. *P ≤ 0.05.

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3.2.4 Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy

Golias J.*, Schwarzer M.*, Wallner M., Kverka M., Kozakova H., Srutkova D., Klimesova K., Sotkovsky P., Palova-Jelinkova L., Ferreira F., Tuckova L.

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Correction: Heat-Induced Structural Changes Affect OVA-Antigen Processing and Reduce Allergic Response in Mouse Model of Food Allergy

Jaroslav Golias, Martin Schwarzer, Michael Wallner, Miloslav Kverka, Hana Kozakova, Dagmar Srutkova, Klara Klimesova, Petr Sotkovsky, Lenka Palova-Jelinkova, Fatima Ferreira, and Ludmila Tuckova

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Footnotes

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Heat-Induced Structural Changes Affect OVA-Antigen Processing and Reduce Allergic Response in Mouse Model of Food Allergy

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Abstract

Background and Aims: The egg protein ovalbumin (OVA) belongs to six most frequent food allergens. We investigated how thermal processing influences its ability to induce allergic symptoms and immune responses in mouse model of food allergy.

Methodology/Principal Findings: Effect of increased temperature (70°C and 95°C) on OVA secondary structure was characterized by circular dichroism and by the kinetics of pepsin digestion with subsequent HPLC. BALB/c mice were sensitized intraperitoneally and challenged with repeated gavages of OVA or OVA heated to 70°C (h-OVA). Levels of allergen-specific serum antibodies were determined by ELISA (IgA and IgGs) or by β-hexosaminidase release test (IgE). Specific activities of digestive enzymes were determined in brush border membrane vesicles of jejunal enterocytes. Cytokine production and changes in regulatory T cells in mesenteric lymph nodes and spleen were assessed by ELISA and FACS. Heating of OVA to 70°C caused mild irreversible changes in secondary structure compared to boiling to 95°C (b-OVA), but both OVA treatments led to markedly different digestion kinetics and Tregs induction ability in vitro, compared to native OVA. Heating of OVA significantly decreased clinical symptoms (allergic diarrhea) and immune allergic response on the level of IgE, IL-4, IL-5, IL-13. Furthermore, h-OVA induced lower activities of serum mast cell protease-1 and enterocyte brush border membrane alkaline phosphatase as compared to native OVA. On the other hand h-OVA stimulated higher IgG2a in sera and IFN-γ secretion by splenocytes.

Conclusions: Minor irreversible changes in OVA secondary structure caused by thermal processing changes both its digestion and antigenic epitopes formation, which leads to activation of different T cell subpopulations, induces shift towards Th1 response and ultimately reduces its allergenicity.

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Introduction

Food allergy is a serious health concern affecting 6–8% of young children and about 2–4% of adults. Allergies to eggs, milk and peanut are currently the most frequent food allergies and their prevalence, severity and persistence has been increasing during the last decades. Food allergy is considered mainly as an IgE-mediated type I hypersensitivity, characterized by an increased production of IgE antibodies and Th2 cytokines, common markers found both in human disease and in experimental models [1–4].

Depending on the route of exposure, dose of allergen and the presence of suitable adjuvant, the immune response can result in either sensitization or oral (mucosal) tolerance induction [4–6]. In mouse models of food allergy, oral administration of allergen usually results in oral tolerance induction, but its co-administration with strong mucosal adjuvant such as cholera toxin or with anti acid drugs (increasing gastric pH) could be used for allergic sensitization [7–10]. Another reliable and effective approach to overcome the oral tolerance induction is pretreatment of mice by systemic intraperitoneal (*i.p.*) administration of allergen with aluminum hydroxide (alum) as adjuvant followed by repeated

intra-gastric treatments. This experimental model mimics a mild form of human allergy with IgE–mediated mast cell degranulation causing increased small intestine permeability [2,11,12] with diarrhea as one of the symptoms of anaphylaxis. Histological examination of small intestine reveals changes of epithelium, *e.g.* alteration in number of goblet cells and mucin production and the damage of tips of villi, as well as changes of lamina propria, *e.g.* increased cell infiltration and/or activation [13–15].

The mucosa of small intestine is an actively metabolizing, rapidly proliferating, absorptive epithelium with nutritional and homeostatic functions. The activity of brush border enzymes is sensitive marker of intestinal cell differentiation and postnatal development, reflecting both dietary changes and microbial colonization [16–18]. Partial and subtotal atrophy of the villous apparatus was shown to correlate with the activity and expression of alkaline phosphatase [19]. Moreover, this enzyme may be also involved in host's defense against pathological stress-induced damage, such as during inflammation and infection [20].

Egg white contains several allergens such as ovalbumin (OVA), ovomucoid, ovotransferin and lysozyme. Forming approximately 60% of the total egg white protein, OVA is by far the most abundant of them [21]. Like the majority of food allergens OVA is consumed after thermal processing and it has been shown that after heating its molecular structure as well as allergenicity is altered [22,23]. However, it should be considered that egg allergens are processed at different temperatures (baked, scrambled or soft/hard boiled eggs or even native as whipped egg white) and these processing conditions can have a major impact on the secondary structure, susceptibility to enzymatic digestion in the gastrointestinal tract and allergenicity. Partial decrease of IgE binding after OVA thermal processing suggested that both linear and conformational epitopes participate in the OVA-IgE specific interactions [22–24]. Moreover, heating of allergens can lead to their aggregation, which reduces their absorption and transport through epithelial layer and thus decreases their allergenicity [25]. However, the impact of different temperature treatment on the changes in the secondary structure of OVA and on its ability to induce clinical symptoms of food allergy hasn't been studied in detail.

In the present study we show that heating of hen egg allergen OVA to 70°C has only minor effect on its secondary structure. However, these minor changes lead to different kinetics and occurrence of fragments after digestion. This result in activation of different T cell subpopulations and changes in both cytokine production and specific antibody formation, which leads to significant reduction of egg allergy symptoms.

Materials and Methods

Ethics Statement

All animal experiments were approved by the Laboratory Animal Care and Use Committee of the Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic, approval ID: 94/2006 and 244/2009.

Animals

Two month-old female BALB/c mice (*H-2b*) (Animal facility of the Institute of Physiology ASCR, Czech Republic) were kept under standard conditions, fed by OVA-free diet and water *ad libitum*.

Ovalbumin Preparation

For *i.p.* sensitization, OVA (Worthington, Lakewood, NJ, USA) and heated OVA (h-OVA; prepared by exposure of OVA to 70°C for 10 minutes, enabling accurate and reproducible dosing) were dissolved in phosphate-buffer saline (PBS) to a final concentration of 300 μg/ml containing 5 mg/ml of alum adjuvant (Sigma, Steinheim, Germany). For oral administration, OVA and h-OVA were dissolved in PBS to a final concentration of 100 mg/ml. For *in vitro* studies boiled OVA (b-OVA) was prepared by exposure of OVA to 95°C for 10 minutes. EndoGrade® Ovalbumin (Hyglos GmbH, Germany) with endotoxin content <1 EU/mg was used for enzymatic digestion and *in vitro* stimulation.

Circular Dichroism

Protein secondary structure elements were determined by CD spectroscopy. Spectra were recorded in 5 mM sodium phosphate buffer (pH 7.4) with a JASCO J-815 spectropolarimeter fitted with a PTC-423S Peltier single position cell holder (Jasco, Tokyo, Japan). All spectra are baseline-corrected and presented as mean residue molar ellipticity $[\Theta]_{MRW}$ at a given wavelength. Thermal denaturation of proteins was monitored from 20°C to 70°C or from 20°C to 95°C at the fixed wavelength of 222 nm with a temperature slope of 1°C/min. The melting point (Tm) was calculated from the inflection point of the resulting sigmoid curve [26].

Enzymatic Digestion and HPLC Separation of Ovalbumin Fragments

Peptides of OVA, h-OVA or b-OVA were prepared using pepsin-agarose gel similarly as described previously [27]. Briefly, digestion of proteins was stopped after 20, 40, or 60 minutes by removing the pepsin-agarose gel by centrifugation (10 min; 1500 g) and by neutralization with 1 M NaOH to final pH 7. Digested or undigested proteins were separated using SP 250/10 NUCLEOSIL 300-7 C18 column (Macherey-Nagel, Düren, Germany) on the HPLC system Gold 125NM Solvent Module (Beckman Coulter, Miami, FL, USA). Samples were applied on columns and separated as described previously [27]. For *in vitro* stimulations, digests were dissolved in complete RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 500 μg/ml.

Experimental Protocol

Mice were divided into the three groups according to the treatment – OVA, h-OVA and PBS (controls). Mice were sensitized *i.p.*, with a two week interval, with 60 μg of either OVA or h-OVA together with 1 mg of alum in a final volume of 200 μl PBS on day 1 and 14. Control mice received only 200 μl PBS containing 1 mg of alum. Two weeks later, the mice were challenged 10 times at 2–3 days intervals by *i.g.* gavages of 15 mg of OVA in a final volume of 150 μl PBS. Diarrhea was assessed visually by monitoring mice for 30 minutes after each *i.g.* exposure. Body weight was recorded before gavage and rectal temperature both before and 30 minutes after each *i.g.* exposure.

Quantification of OVA-specific Antibodies and Mast Cell Protease-1

Blood samples were collected before the first *i.p.* injection, during the experiment and at sacrifice. Allergen-specific serum IgG1, IgG2a and IgA levels were determined by ELISA [28]. Briefly, 96-well microtiter plates were coated either with OVA, h-OVA or b-OVA (5 μg/ml). Serum samples were diluted 1/10000 for IgG1, 1/100 for IgG2a and 1/10 for IgA. Rat anti-mouse IgG1, IgG2a and IgA antibodies (Abs) (1 μg/ml Pharmingen, San Diego, CA, USA) were applied, followed by peroxidase-conjugated mouse anti-rat IgG Abs (1/1000; Jackson, Immuno Labs., West

Grove, PA, USA) for detection. Antibody levels were reported as optical density (OD). As it was shown that allergen-specific IgG interferes with allergen-specific IgE detection [29], allergen-specific IgE levels in sera were quantified by degranulation of rat basophil leukemia (RBL-2H3) cells (originally described by [30], kindly provided by prof. Ursula Wiedermann). RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 cells/per well) and passively sensitized by incubation with mouse sera in a final dilution of 1/90 for 2 hours. After washing, OVA, h-OVA or b-OVA (0.6 μg/ml) were added for 30 min at 37°C to induce degranulation. Supernatants were incubated with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) for analysis of β-hexosaminidase using a fluorescence microplate reader (λ_{ex}:360 nm/λ_{em}:465 nm) Infinite M200 (Tecan Group Ltd., Grödig, Austria). Results are reported as percentage of total β-hexosaminidase release from cells after disruption with 1% Triton X-100.

Levels of serum mouse mast cell protease-1 (MMCP-1) enzyme were determined by commercial kit (eBioscience, San Diego, USA) according to manufacturer's instructions. Sacrifice sera were diluted 1/250 and the MMCP-1 levels are reported as ng/ml.

Cell Culture and Cytokine Evaluation

Mesenteric lymph nodes (MLN) and spleens were removed at sacrifice. Single-cell suspensions were prepared in RPMI-1640 containing 10% fetal bovine serum (BioClot GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). Cells (6×10^5/well) were cultured in a flat-bottom 96-well plate (TPP, Trasadingen, Switzerland) without any stimuli or in the presence of either OVA or h-OVA (100 μg/well) for 72 hours (37°C, 5% CO_2). Supernatants were collected and stored at –40°C until analyses. IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, INF-γ and TNF-α were determined by the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Panel (Millipore, Billerica, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, Hercules, USA) with sensitivities <0.3 pg/ml for IL-4, <0.8 pg/ml for IL-5, <2.1 pg/ml for IL-6, <2.6 pg/ml for IL-10, <12.4 pg/ml for IL-13, <0.7 pg/ml for IL-17, <1.1 pg/ml for IFN-γ and <3.1 pg/ml for TNF-α. Values are reported in pg/ml after subtraction of baseline levels of non-stimulated cultures. Values below assay sensitivity were considered non-detectable (n.d.). In order to measure the capacities of OVA, h-OVA and b-OVA and their peptic digests (100 μg/well) to induce Tregs, we cultivated them with naïve mouse splenocytes for 48 hours.

Flow Cytometry Analysis

Single-cell suspensions of spleens or MLN were stained for regulatory T cells using Foxp3 Staining Buffer Set (eBioscience, San Diego, CA, USA) with fluorochrome labeled anti-mouse monoclonal Abs: CD3e-Fluorescein isothiocyanate (eBioscience; clone 145-2C11), CD4-Qdot® 605 (Invitrogen, clone RM4-5), CD25-Alexa Fluor® 700 (eBioscience; clone PC61.5) and Foxp3-phycoerythrin (eBioscience; clone FJK-16s) according to the manufacturer's recommendation. Flow cytometric analysis was performed on LSRII (BD Biosciences, San Jose, CA, USA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Determination of Enterocyte Brush-border Enzyme Activities

Jejunum was removed, washed with cold saline and brush border membrane vesicles (BBMV) were prepared from jejunal scrapings as described by Kessler *et al.* [31]. Protein concentration in BBMV was determined by the method of Lowry *et al.* [32] using bovine serum albumin, fraction V (Serva, Heidelberg, Germany) as standard. The activity of alkaline phosphatase (EC 3.1.3.1), γ-glutamyltranspeptidase (EC 2.3.2.2), dipeptidyl peptidase IV (EC 3.4.14.5), lactase (EC 3.2.1.23/62/108) and sucrase (EC 3.2.1.48/10) were determined as described previously [33]. Enzyme activities were expressed in nkat/mg protein, 1 nkat being the amount of the enzyme that converts 1 nmol of substrate per second under the given conditions.

Histology and Morphometry

Intestinal tissue sections were fixed immediately in 4% formalin. The fixed tissues were cut and processed using routine methods. Paraffin sections (5 μm) were deparaffinized in xylene, rehydrated through an ethanol gradient to water and stained by hematoxylin-eosin. Villus height was evaluated under the Olympus BX 40 microscope equipped with Photo camera DP 70 using program QuickPhoto Micro 23 program (Olympus, Japan). The mean height of 20–30 villi ± SEM was calculated.

Statistical Analysis

Differences between multiple experimental groups were evaluated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and differences between two groups were evaluated using unpaired two-tailed Student's *t*-test. Data were expressed as the mean ± SEM unless otherwise stated. GraphPad Prism statistical software (version 5.03 GraphPad Software, La Jolla, CA, USA) was used for analyses.

Results

The Effect of Thermal Processing on OVA Secondary Structure and Enzymatic Digestion

Since eggs could be consumed after various kinds of processing, we analyzed the effect of different temperatures on the secondary structure of OVA allergen. Employing the circular dichroism technique, we found that heating to 70°C or 95°C causes irreversible changes in secondary structure of OVA allergen (Fig. 1). The structural changes induced by heating were accompanied by different susceptibility to pepsin digestion. HPLC elution profiles of pepsin-digested OVA, h-OVA or b-OVA were documented after 20 and 40 minutes (Fig. 2). The majority of native OVA was split to fragments after 20 min, while the majority of both forms of heated OVA remained undigested. However, while both h-OVA and b-OVA had similar peptide profiles after 20 or 40 min of digestion, these were both quite different from those of untreated OVA (Fig. 2). The profiles after 40 min of digestion remained almost unchanged after 60 min of digestion (data not shown).

Experimental Allergic Diarrhea Induced by OVA and Heated-OVA

Allergic diarrhea appeared in about 70% of mice already after the 5th *i.g.* dose of OVA, but only in 20% of those fed with h-OVA. After 7 *i.g.* doses, the disease symptoms were found in more than 90% of OVA fed animals, but only in 35% of those fed with h-OVA. At the end of the experiment (10 *i.g.* doses), the diarrhea was found in all mice fed with OVA, but only in 70% of mice fed with h-OVA (Fig. 3a, b). There were small, non-significant differences in body weight and in rectal temperature after each *i.g.* dose of either OVA or h-OVA and PBS control group (data not shown). Morphometry analysis of histological

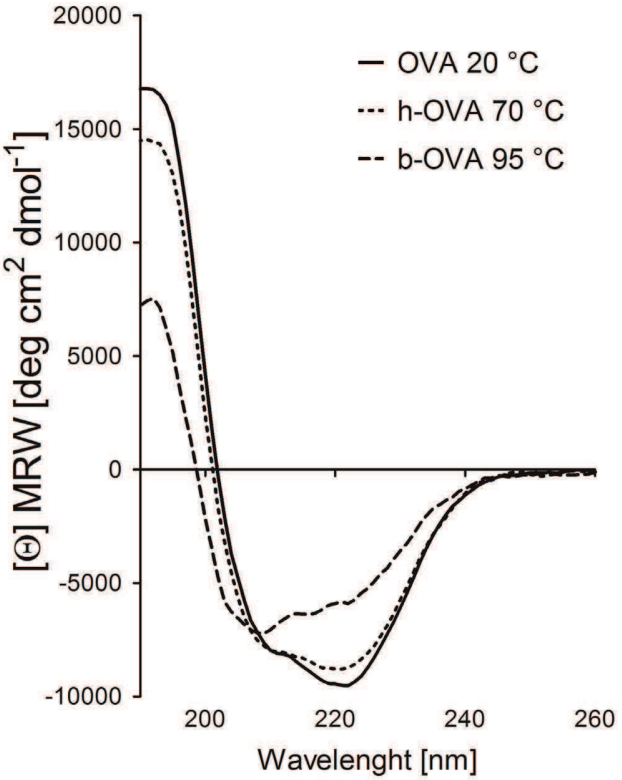

Figure 1. Circular dichroism spectra of native and heated-OVA. Circular dichroism spectra showed only minor irreversible structural changes of hen egg ovalbumin-OVA heated for 10 minutes at 70°C (h-OVA, dotted line) as compared to OVA heated at 95°C (b-OVA, dashed line). Spectra were taken after renaturation at 20°C, native conformation of OVA at 20°C is shown as control (solid line).
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pictures documented shortening of villi in mice treated with either form of OVA, as compared to PBS-treated controls (PBS 190.2±5.1 μm, OVA 157.7±14.0* μm, h-OVA 161.4±6.0** μm).

OVA and h-OVA Treatment Changes Activity of Brush-border Hydrolases

The brush-border membrane hydrolases are enzymes involved in the final steps of digestion processes. We tested if these enzymes are involved in small intestine homeostasis and could be therefore considered as new markers in food allergy. We determined their activities in the jejunum of OVA-, h-OVA- and PBS-treated mice (Table 1). We found that the specific activity of alkaline phosphatase was significantly higher in mice treated with native OVA but only slightly increased in those exposed to h-OVA, as compared with PBS-treated mice. On the other hand, as compared to PBS-treated controls, both OVA and h-OVA treatments significantly decreased the specific activity of dipeptidyl peptidase IV. We did not observe any significant changes among the three experimental groups in the levels of glutamyl transpeptidase, lactase or sucrase (Table 1).

Thermal Processing of OVA Changes the Kinetics of OVA-specific Antibody Responses and the Levels of Serum MMCP-1

To determine the effect of thermal processing of the allergen on the level and specificity of anti-OVA antibodies, the serum levels of IgE, IgG1, IgG2a and IgA against either OVA or h-OVA were determined in the course of the experiment. As shown in Fig. 3c the level of IgE anti-OVA Abs was higher in response to native OVA than to h-OVA. In contrast, OVA-specific IgG2a was significantly higher after h-OVA feeding. The levels of the other two isotypes (IgG1 and IgA) were increased compared to controls but the differences corresponding to the two OVA forms were diminished towards the end of experiment. At the end of the

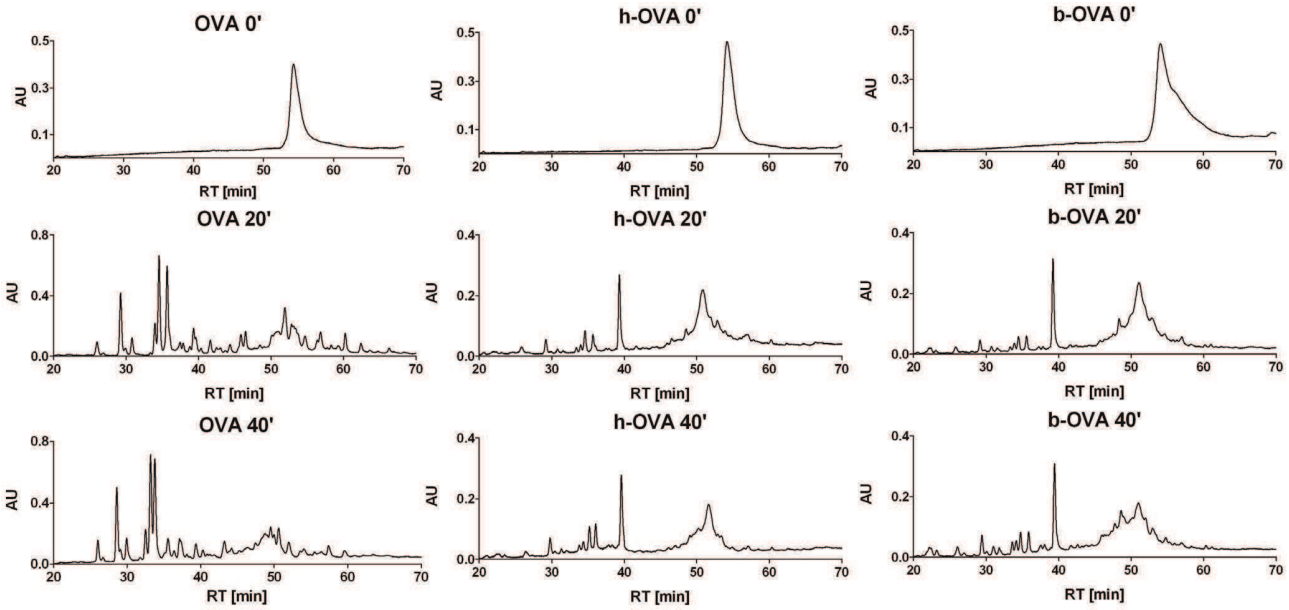

Figure 2. RP-HPLC separation profile of native-OVA and heated-OVA peptic digests. RP-HPLC separation profile monitored at 280 nm corresponds to OVA and OVA heated at 70°C (h)-OVA or boiled at 95°C (b)-OVA undigested (0′) and after 20 (20′) and 40 minutes (40′) of digestion by pepsin. RT – retention time.
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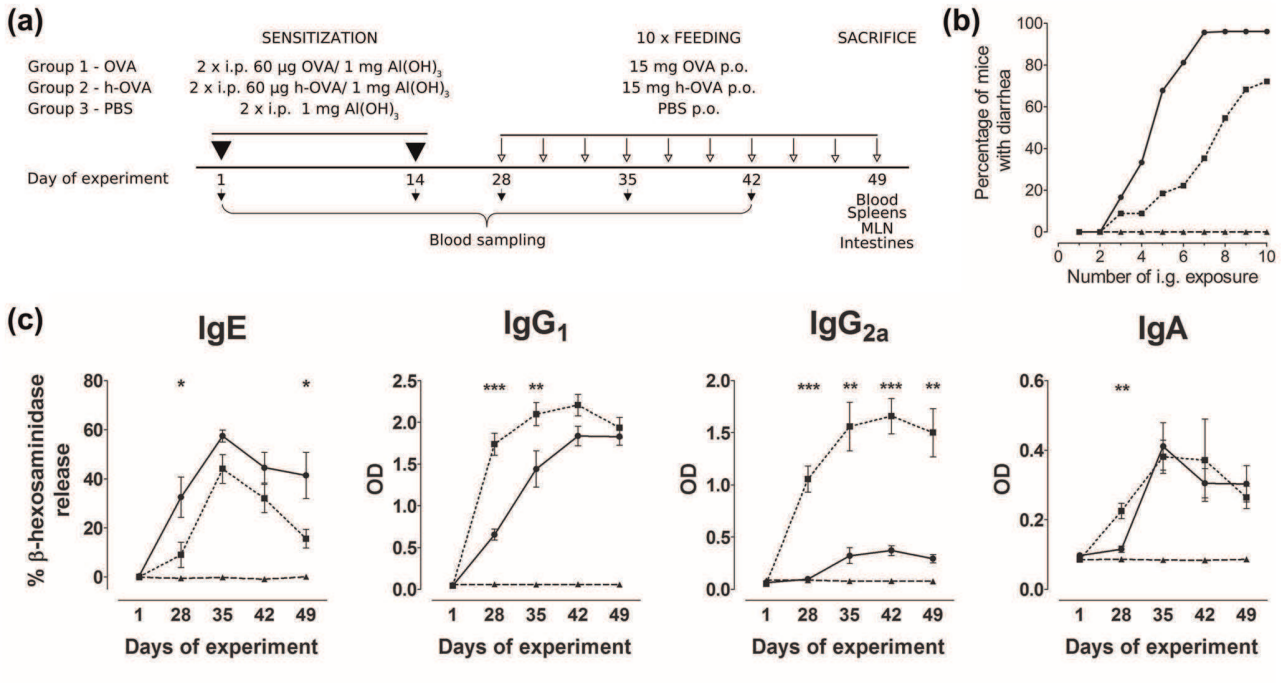

Figure 3. Impact of heating on OVA-induced allergic response. Experimental design (a). Mice were sensitized twice intraperitoneally (*i.p.*) with OVA/Al(OH)$_3$, heated OVA (h-OVA)/Al(OH)$_3$ or PBS/Al(OH)$_3$ alone and subsequently challenged with ten doses of OVA, h-OVA or PBS by intragastric tubing (*i.g.*). Blood samples were taken at indicated time points for antibody analysis. At the end of the experiment, spleens and mesenteric lymph nodes were taken for FACS and cytokine assays, small intestine for histology and enterocyte brush border for enzyme activity analysis. **Occurrence of allergic diarrhea** (**b**). Occurrence of allergic diarrhea in OVA (solid line) or h-OVA (dotted line) challenged mice, data pooled from three independent experiments. PBS controls are shown as dashed line. **The kinetics of specific Abs formation** (**c**). Levels of specific antibodies in sera from mice exposed to OVA (solid line), h-OVA (dotted line) or PBS (dashed line) were detected by ELISA (IgA, IgG1and IgG2a) or by β-hexosaminidase release assay (IgE). Data are represented as mean ± SEM (n = 10 mice/group), representative data from one out of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001.
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experiment, we characterized the specificity and the degree of cross-reactivity of anti-OVA antibodies using ELISA with OVA, h-OVA or b-OVA bound as an antigen (Fig. S1). The levels of OVA-specific antibodies remained unchanged, when we used h-OVA or OVA as a coating antigen, except for IgG1, which levels were significantly higher, when h-OVA instead of OVA was used. When b-OVA was used as coating antigen, the response of both OVA- and h-OVA treated mice decreased significantly in all measured isotypes.

Table 1. Specific activities of enterocyte brush-border enzymes (nkat/mg protein) in jejunum of treated mice.

Enzyme (nkat/mg protein)	OVA	h-OVA	PBS
Alkaline phosphatase	14.26±1.09***	10.08±0.84$^{\#}$	8.27±0.29
GGT	10.79±3.33	8.64±1.59	9.74±2.02
DPP IV	4.61±0.50**	5.39±0.45*	7.39±0.77
Lactase	9.19±0.63	8.37±0.59	9.99±1.61
Sucrase	32.16±8.04	36.00±4.10	27.30±4.24

GGT – gamma-glutamyltranspeptidase, DPP IV – Dipeptidyl peptidase IV. Values are expressed as the mean ± SEM.
*$P<0.05$ ovalbumin-treated group (OVA) *vs.* PBS-treated group.
**$P<0.01$ ovalbumin-treated group (OVA) *vs.* PBS-treated group.
***$P<0.001$ ovalbumin-treated group (OVA) *vs.* PBS-treated group.
$^{\#}P<0.05$ heated-ovalbumin-treated group (h-OVA) *vs.* ovalbumin-treated group (OVA).
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Increase of allergen specific IgE is essential for mast cell activation and development of allergic diarrhea symptoms. We determined the level of MMCP-1 enzyme as the marker of mast cell activation and degranulation. In this case, the reducing effect of thermal processing was clearly demonstrated; the h-OVA induced only half the level of serum MMCP-1 compared to the native OVA (Fig. 4).

Ex vivo Cytokine Production by MLN and Splenocytes Induced by OVA Allergens

Local and systemic cell responses to OVA and h-OVA were evaluated in all three groups of animals as *in vitro* cytokine production by MLN and splenocytes after exposure to corresponding allergens. Cytokine production from controls (PBS group) was low or not detectable and did not change after exposure to either form of OVA (data not shown). As shown in Fig. 5a, the levels of TNF-α, IL-4, IL-5, IL-10 and IL-13 were higher in culture media obtained from MLN exposed to native OVA. The differences in cytokine secretion were less pronounced in the experiments with splenocytes cultures (Fig. 5b). Only the production of IFN-γ was higher after exposure to h-OVA as compared to OVA. Levels of IL-6 and IL-17 didn't differ among the groups neither in MLN nor in spleen (data not shown).

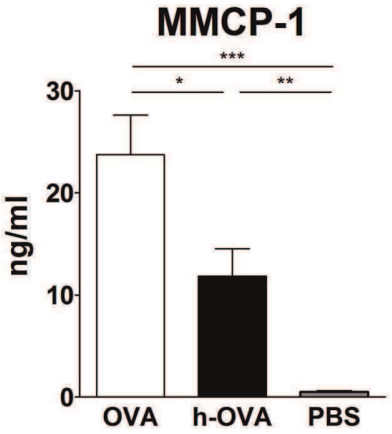

Figure 4. Decreased mast cell protease induction by heated-OVA. Heated OVA (h-OVA, black bar) induced significantly lower amounts of mast cell protease (MMCP-1), the marker of mast cell activation, compared to mice fed with native OVA (white bar). Data are represented as mean ± SEM (n = 10 mice/group), representative data from one out of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001.
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Differentiation of CD4+CD25+Foxp3+ T Cells in OVA and h-OVA Fed BALB/c Mice

Since regulatory T cells (Tregs) are known to be crucial for induction of oral tolerance to protein antigens [34], we analyzed the changes in Tregs in spleen and MLNs of OVA-, h-OVA- and PBS-treated mice at the end of the experiment. In spleen we observed a decrease in Tregs in h-OVA treated mice, as compared to OVA- and PBS-treated mice (Fig. 6). Only a non-significant increase was found in MLNs of h-OVA treated mice.

Induction of CD4+Foxp3+ T Cells by h-OVA and b-OVA in vitro is Increased After a 20-min Pepsin Digestion

To characterize the effect of heating and enzymatic digestion on T cell subpopulations, especially on regulatory T cell differentiation in more detail, splenocytes from naïve (untreated) BALB/c mice were cultured *in vitro* either with OVA, h-OVA or b-OVA as well as with their peptic digests. As shown in Fig. 7, the *in vitro* stimulation of splenocytes with undigested heated proteins led to a slight increase in proportion of CD4+Foxp3+ Treg cells compared to native form of OVA. Interestingly, 20 min peptic digests of heated forms of OVA induced increased proportion of Tregs, but this ability decreased again after 40 min of digestion. In contrast the pepsin digestion did not change the ability of OVA to slightly increase the proportion of Tregs as compared to undigested OVA.

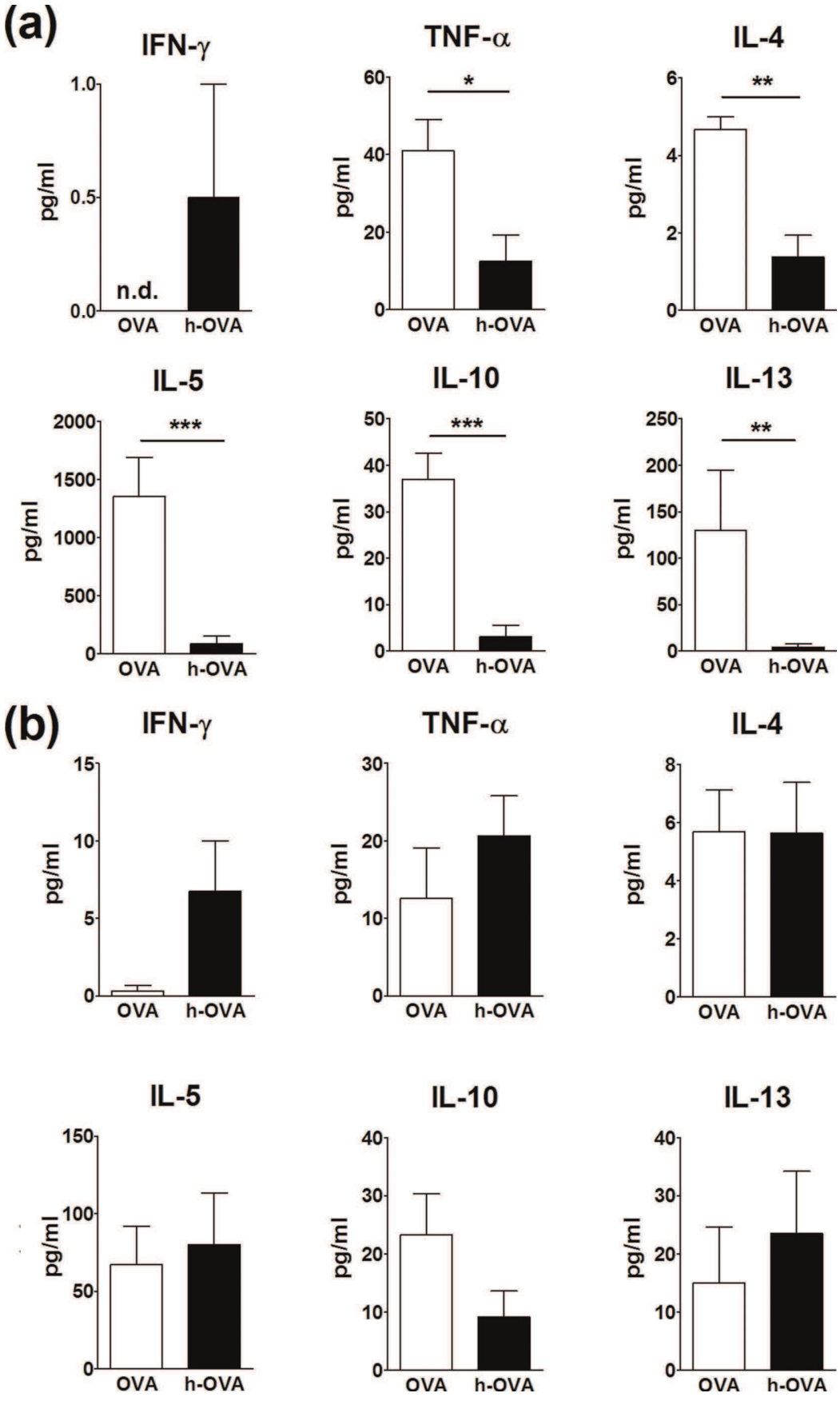
Discussion

In this study, we showed that small irreversible changes in secondary structure of egg allergen OVA caused by thermal processing significantly affect its digestion by gut enzymes and decrease its allergenicity in the mouse model of food allergy. While both heated and native OVA induced allergic diarrhea in BALB/c mice, the disease symptoms appeared much earlier and with a higher frequency in OVA fed mice than in those fed with h-OVA. As compared to OVA-treated group, the sera of h-OVA-treated mice contained also significantly lower levels of specific IgE and MMCP-1, known markers of mast cell activation and degranulation [35]. It seems that even slight changes in the secondary structure elements have a high impact on the immunological behavior of the allergen. This could be explained by differences either in allergen absorption, which could lead to a decrease in allergen exposure, or in allergen digestion, which leads to production of peptides with different allergenicity and to a partial loss of conformational epitopes and/or exposure of new linear epitopes to immune cells.

The small intestine is noted for its plasticity in response to various dietary changes, which may be reflected in activation of enterocyte brush-border enzymes. Here we demonstrated for the first time that alkaline phosphatase (ALP) can be used as a new marker in food allergy, because its specific activity was significantly increased in OVA-treated group compared to controls. This is in line with the recent findings that ALP has a crucial role in regeneration of enterocytes and that its activity correlates with villous atrophy [19,36]. We can speculate that the increased level of ALP contributes to restoration of homeostasis in the enterocyte membranes after long-term stimulation with OVA allergens. On the contrary, dipeptidyl peptidase IV (CD26 - that cleaves L-alanine or L-proline residues in the penultimate N-terminal position) was significantly reduced in both OVA- and h-OVA-treated groups, as compared to PBS controls. Interestingly, a decrease in CD26 was found in patients with celiac disease induced by gluten, which belongs to wheat components responsible for food- or wheat-dependent exercise-induced allergy and for occupational asthma [37,38].

The changes in secondary structure by heating could influence antibody response *in vivo*. Here we report that OVA induced significantly higher levels of OVA-specific IgE and lower levels of IgG2a, as compared to h-OVA. High levels of potentially "blocking" IgG2a (mouse homolog of human IgG4) may compete for allergen [22]. The ability of heat-denatured allergens to induce Th1 associated IgG2a was also shown for other allergens, such as bee venom or birch pollen [39]. However, the effect cannot be generalized, because in a recent study by van der Ventel [15] a higher sensitizing potential was shown for cooked fish proteins. Surprisingly, when we changed the coupling allergen (h-OVA was used for OVA sensitized sera and vice versa) the binding of specific Abs was retained. Moreover, the binding was significantly higher when h-OVA antigen was used for specific IgG1 antibody determination. We assume that this is caused by heating-uncovered linear epitopes (supplementing the loss of the conformational ones), which are then presented after processing by antigen-presenting cells to T and B lymphocytes. On the other hand, when the extensively heated b-OVA was used, we observed a strong drop in the signal in all OVA-specific antibodies, which correlated with observed circular dichroism structural changes, and suggested the importance of structural epitopes in specific antibody formation.

Next, we addressed the question if the differences in OVA and h-OVA-specific antibody responses are also associated with cytokine milieu. On the local level in MLNs, we found a significantly higher production of Th2 cytokines in the OVA-treated mice, accompanied by proinflammatory TNF-α production after an *in vitro* exposure to OVA. Surprisingly, we determined an up-regulation of regulatory cytokine IL-10, which could be a result of a biological feedback aimed at dampening down the local inflammation, similar to chronic experimental colitis [40]. OVA-treatment did not significantly influence cytokine production in splenocytes, except for IFN-γ, which was produced predominantly by h-OVA stimulated splenocytes. The same observation was recently made by van der Ventel [15], who showed an increased IFN-γ production by splenocytes of mice challenged with heated fish extract. Our findings suggest that heating of OVA induces


(a)
IFN-γ
pg/ml
1.0
0.5
0.0
n.d.
OVA
h-OVA
TNF-α
60
40
20
0
*
IL-4
6
4
2
**
IL-5
2000
1500
1000
500
***
IL-10
50
40
30
20
10
IL-13
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200
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100
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(b)
15
10
5
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8
6
4
IL-5
150
100
50
IL-10
IL-13

Figure 5. Cytokine production after *in vitro* restimulation with OVA. The cytokine production from mesenteric lymph nodes (a) and splenocytes (b) of BALB/c mice fed with OVA (white bars) or h-OVA (black bars) and stimulated *in vitro* with appropriate allergen. Cytokine levels are expressed after subtraction of base line levels of unstimulated lymph node cells or splenocytes. Data shown are mean values ± SEM (n = 4–7 mice/group), representative data from one out of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001, n.d. = not detectable.
doi:10.1371/journal.pone.0037156.g005

changes in its digestion and processing by immune cells that lead to changes in the local cytokine environment ultimately leading to a shift from Th2- toward Th1-type response, reduction in the level of specific IgE and an increased production of blocking IgG2a antibodies [22]. These data fit well with clinical symptoms observed in allergic subjects in response to heated egg allergens [5,24].

Moreover, our results support recent data showing that thermal processing interferes with OVA stability [23]. Here, we show that h-OVA and b-OVA are initially (at 20 min) more resistant to proteolysis than native OVA. The difference in degradation kinetics could be explained by partial aggregation of heated forms of OVA, which makes the target structures less accessible for the enzyme. Nevertheless, after 40 min digestion the number of h-OVA and b-OVA fragments was even higher and their spectrum differed from those obtained from OVA. However, the spectra of h-OVA and b-OVA fragmented peptides were similar, differing only in the region corresponding to retention time of 50 min. Surprisingly, when we stimulated splenocytes from naïve mice *in vitro* we found an increase in the percentage of regulatory T cells in response to h-OVA and b-OVA. The capacity of both heated forms of OVA to induce Tregs was increased after 20 min of pepsin digestion and decreased again after 40 min digestion. The prolonged digestion had no effect on Treg inducing capacity of

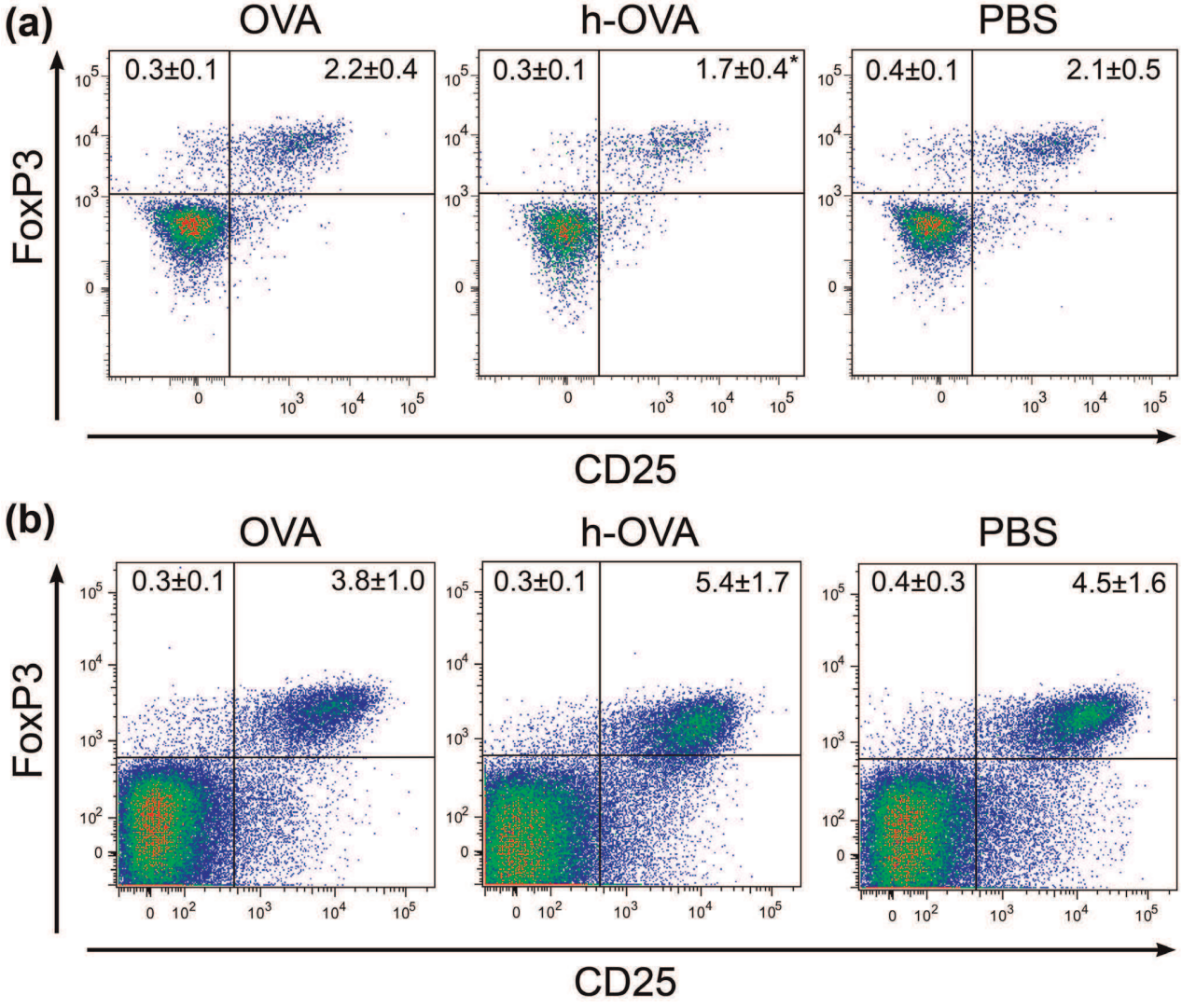

Figure 6. Numbers of Tregs in splenocytes and mesenteric lymph nodes of OVA treated mice. Typical plots depicting numbers of Tregs in mouse splenocytes (a) and mesenteric lymph node (b) in gated CD3+CD4+CD8– T helper cells after feeding with OVA, h-OVA or PBS, respectively. Numbers in upper quadrants shows proportions (mean ± SD) of either CD25–Foxp3+ or CD25+Foxp3+ Th cells out of all cells. Representative data from one out of three independent experiments. *P≤0.05.
doi:10.1371/journal.pone.0037156.g006

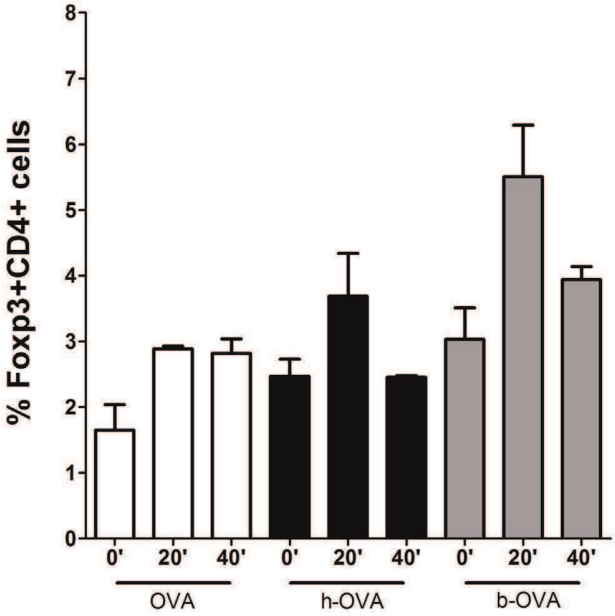

Figure 7. Number of Tregs in spleen cell suspensions co-cultured *in vitro* with OVA digests. The percentage of Tregs in cell suspension isolated from spleens of non-stimulated (naïve) BALB/c mice cultured *in vitro* for 48 hours with undigested (0′) and after 20 (20′) and 40 minutes (40′) peptic digest of OVA (white bars), h-OVA (black bars) or b-OVA (grey bars). The data represent the percentage of CD4+Foxp3+ cells out of all live cells as measured by FACS. Representative data from one out of three independent experiments are shown. Data are represented as mean ± SEM.
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native (heat untreated) OVA digests. These data are supported by recent evidence in experimental mouse model of suppressive effects of some OVA T cell epitope peptides on allergic immune responses via Foxp3+ T cell generation [41].

A direct continuation of the study would be the analysis of intestinal DC subsets and goblet cells [42–44] in initial steps of allergen sensitization in our model, which should contribute to understanding how the tolerance or allergic response is achieved. The analysis of the role of enzymes in brush-border membrane of epithelial cells (activated after OVA gavages) will shed light on allergen digestion and immunogenicity of fragments (esp. dipeptidases) and on regeneration of gut epithelium (ALP). Moreover, it would be of great importance to apply this model for verification of hygiene hypothesis using animals kept under conventional and/or germ-free condition and subsequently colonized with various bacterial strains.

In conclusion, we showed that even a mild change in the secondary structure of OVA after thermal processing has far-reaching consequences concerning its antigenic properties. After digestion of h-OVA, fragments with different immunogenic properties are formed leading to the shift from Th2 to Th1-type response as compared to native OVA. Nevertheless, the h-OVA fragments still have the ability to induce allergic symptoms, but these are less pronounced and need longer time to develop.

Supporting Information

Figure S1 Cross-reactivity of anti-OVA specific antibodies. At the end of the experiment we determined the levels of OVA-specific antibodies in OVA and heated (h)-OVA treated mice against OVA, h-OVA (70°C) and boiled (b)-OVA (95°C). The levels were retained for IgE, IgG2a and IgA (a, c, d) when we used OVA as coating antigen for h-OVA-treated mice or h-OVA as coating antigen for OVA treated mice. In case of IgG1 (b) the levels were significantly higher when we used h-OVA as coating antigen for either OVA- or h-OVA- treated mice. When we used b-OVA we observed a significant drop in the signal for all measured antibodies. Representative data from one out of three experiments (n = 8). Repeated measures ANOVA with Tukey's multiple comparison test was used for analysis of differences between antibody levels of the same sample measured either against OVA, h-OVA and b-OVA antigen. n.s. non-significant, *P≤0.05, **P≤0.01, ***P≤0.001.
(TIF)

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Author Contributions

Conceived and designed the experiments: JG LT FF MS HK. Performed the experiments: JG MS MK DS HK MW KK PS. Analyzed the data: MS JG MK DS LT HK MW KK PS LP-J. Contributed reagents/materials/analysis tools: LT MW HK FF. Wrote the paper: LT MS JG MK MW HK PS. Obtained permission for use of animal model: LT.

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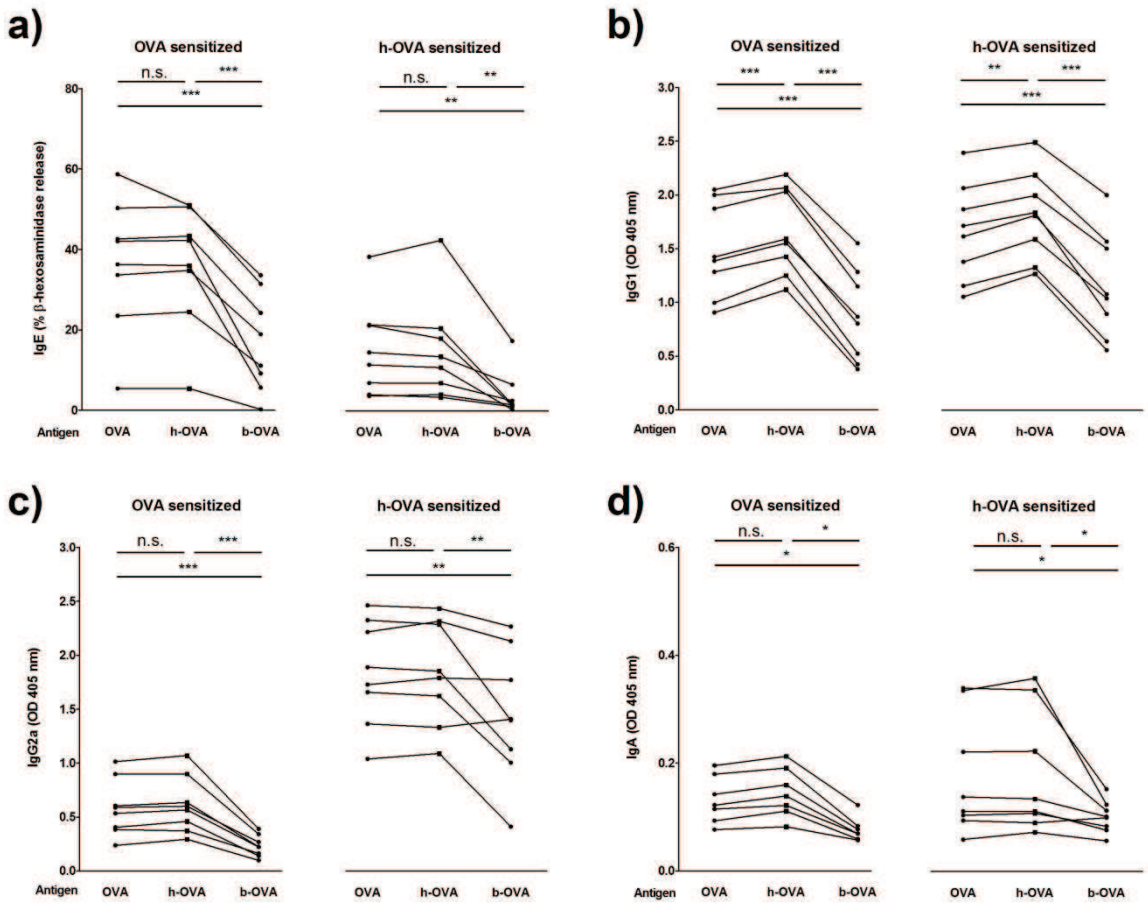

Figure S1.

Cross-reactivity of anti-OVA specific antibodies. At the end of the experiment we determined the levels of OVA-specific antibodies in OVA and heated (h)-OVA treated mice against OVA, h-OVA (70°C) and boiled (b)-OVA (95°C). The levels were retained for IgE, IgG2a and IgA (a, c, d) when we used OVA as coating antigen for h-OVA-treated mice or h-OVA as coating antigen for OVA treated mice. In case of IgG1 (b) the levels were significantly higher when we used h-OVA as coating antigen for either OVA- or h-OVA- treated mice. When we used b-OVA we observed a significant drop in the signal for all measured antibodies. Representative data from one out of three experiments (n = 8). Repeated measures ANOVA with Tukey's multiple comparison test was used for analysis of differences between antibody levels of the same sample measured either against OVA, h-OVA and b-OVA antigen. n.s. non-significant, *P≤0.05, **P≤0.01, ***P≤0.001.

4. CONCLUSSIONS

(A) We show here that the *Lactobacillus* strains LOCK 0900, LOCK 0908 and LOCK 0919 are able to withstand gut antimicrobial environment and to interact with the immune system of the host. *In vivo* mouse experiments showed cytokine shift in favor of anti-allergic immune response. Thus the mixture of *Lactobacillus* strains represents a probiotic bacterial preparation with possible use in prophylaxis and/ or therapy of allergic diseases.

(B) We have demonstrated that neonatal mono-colonization of germ-free mice with the *Lactobacillus plantarum* NCIMB8826 strain producing the major birch pollen allergen Bet v 1 attenuates the development of birch pollen allergy later in life. The mechanisms involved a shift towards a non-allergic Th1 phenotype accompanied by increased regulatory responses. Thus intervention at birth with a live recombinant *L. plantarum* producing a clinically relevant allergen reduces experimental allergy and might therefore become an effective strategy for early intervention against the onset of allergic diseases.

(C) We showed in neonatally colonized gnotobiotic mice, that *Bifidobacterium longum* was a strain with the ability to induce regulatory cytokines/ T cells via TLR2 and MyD88 signaling pathways and thus prevent allergic sensitization. Therefore, *B. longum* might be considered as a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans. Moreover, our results stress out the general importance of intrinsic immunomodulatory properties of bacterial strain used for intervention and shed light on the function of bifidobacteria in shaping the immune system in early human ontogeny.

(D) Aiming at elucidating the importance of secondary structure for antigen allergenicity in mouse food allergy model we have established that heating of hen egg allergen OVA to 70°C has only minor effect on its secondary structure. Nevertheless, these minor irreversible changes in secondary structure changed both its digestion and antigenic epitopes formation, which led to activation of different T cell subpopulations, induced shift towards Th1 response and ultimately reduced its allergenicity.

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transcription factor 3- and transforming growth factor-beta-associated mechanisms. *Clin Exp Allergy* 40, 668-678

Appendix A – Curriculum vitae

Born: January 28, 1980, Nový Jičín, Czech Republic

Education and Employment:

1998 – 2004 Faculty of Science, Masaryk University in Brno

2005 – now Institute of Microbiology of the AS CR, v.v.i. Department of Immunology and Gnotobiology, Nový Hrádek. PhD project – Impact of probiotic bacteria on allergic sensitization in type I allergy model. Supervisor: RNDr. Hana Kozáková, CSc.

Memberships, Honors:

2004 Member of Czech Immunological Society

2009 Member of European Academy of Allergology and Clinical Immunology

2013 Member of Czech Society of Allergology and Clinical Immunology

2009 Prize for the best poster at JMA poster session, XXVIII EAACI Congress of the European Academy of Allergy and Clinical Immunology, Warszawa, Poland

2009 Prize for the best presentation, XXVIII EAACI Congress of the European Academy of Allergy and Clinical Immunology, Warszawa, Poland

2010 Prize for the best poster, Annual Meeting of the Austrian Society for Allergology and Immunology (ÖGAI), 2010, Vienna, Austria

2011 Prize for the best paper awarded by the Czech Society for Probiotics and Prebiotics

2012 Prize for the best paper by a young immunologist in 2011 awarded by the Czech Immunological Society

Stays:

2010 Institute for Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Vienna, Austria

2012 Functional genomics of host/ intestinal bacteria interactions group, Institut of Functional Genomics Lyon (IGFL), Lyon, France

Projects:

2011 – 2013 **Danone – Institute**. Relationship between immunomodulatory properties of lactobacilli *in vitro* and their ability to influence allergic sensitization in gnotobiotic model of type I allergy.

Publications:

Total: 10

Sum of citations: 50

H-index: 5 (WoS)

Appendix B – Publications

Schwarzer M, Srutkova D, Schabussova I, Hudcovic T, Akgün J, Wiederman U, Kozakova H: Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to Bet v 1. Manuscript in preparation

Golias J, **Schwarzer M**, Wallner M, Kverka M, Kozakova H, Srutkova D, Klimesova K, Sotkovsky P, Palova-Jelinkova L, Ferreira F, Tuckova L: Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. PLoS ONE 2012;7(5): e37156. doi:10.1371/journal.pone.0037156

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Stepankova R, Tonar Z, Bartova J, Nedorost L, Rossman P, Poledne R, **Schwarzer M**, Tlaskalova-Hogenova H: Absence of microbiota (germ-free conditions) accelerates the atherosclerosis in ApoE-deficient mice fed standard low cholesterol diet. J Atheroscler Thromb 2010;17:796-804.

Cukrowska B, Rosiak I, Klewicka E, Motyl I, **Schwarzer M**, Libudzisz Z, Kozakova H: Impact of heat-inactivated *Lactobacillus casei* and *Lactobacillus paracasei* strains on cytokine responses in whole blood cell cultures of children with atopic dermatitis. Folia Microbiol (Praha) 2010;55:277-280.

Kolinska J, Zakostelecka M, **Schwarzer M**, Stepankova R, Hudcovic T, Kozakova H: Effect of nonpathogenic *Escherichia coli* monoassociation on small intestinal brush border glycoconjugate moieties and cytokine production after colonization in ex germ free rats and pigs. Int J Interferon Cytokine Mediator Res 2010;2:73-84.

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