INVESTIGATION OF INTERVENTION STRATEGIES FOR Ig-E MEDIATED FOOD ALLERGY IN A MURINE MODEL OF COW'S MILK ALLERGY.

by

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ABSTRACT

Food allergy, an immune-mediated adverse hypersensitivity reaction to ingested food, is an emerging global health problem that not only causes the disease to susceptible individuals but also causes psychological impacts and financial damage to their families. Food allergy incidence is significantly increased in the past decade and currently estimated at 6-8% in 1 year old children, and 2-4% in older children and adults. Food allergy is the leading cause of anaphylaxis in children and severe cases may lead to fatal anaphylaxis reactions. Although continuous efforts have been made to better understand the nature of allergens, predisposing environmental factors, and the host's immune response mechanisms, there is no cure available for food allergy today. In this study, we have investigated three food allergy reducing strategies by supplementing probiotics, low doses of allergens and omega-3 poly unsaturated fatty acids (ω-3 PUFAs) in cow's milk protein sensitized Balb/c mice. *Lactobacillus rhamnosus* GG (LGG) supplementation had tendency to promote Th1 response while VSL#3 provided more potent allergy reducing effects via inducing intestinal secretory IgA (sIgA). Low doses of allergen administration offered suppression of allergen-specific immune responses via Treg-mediated active suppression, indicated by suppressing both allergen-specific Th1 response [reduced BLG-specific serum IgG2a and elevated IL-12(p40)], and Th2 response [lower BLG-specific serum IgE and IgG]. Interestingly, mice received both VSL#3 and low doses of allergen exhibited both allergen-specific active suppression effects and higher sIgA production. We then investigated the effects of different levels of ω-3 and ω-6 PUFAs in the energy and fat rich Western-style diet on food allergy development. Observation of elevated BLG-specific serum immunoglobulins in all experimental mice indicated that both ω-3 and ω-6 PUFAs failed to prevent the development of allergen-specific immune response. However, ω-3 PUFAs alleviated anaphylactic reactions and the severity of allergic reaction as indicated by the unchanged rectal temperature, lower hypersensitivity scores, and Th1-favoured immune responses in BLG-sensitized O3H mice. In general, this study revealed the promising strategies for treatments and prevention against food allergy in the near future.

RÉSUMÉ

Les allergies alimentaires, réactions dues à une hypersensibilité du système immunitaire après ingestion d'un certain type d'aliments, constituent un problème de santé publique croissant qui, en plus de dégrader la santé des individus susceptibles, engendre un impact psychologique et financier pour les familles touchées. L'incidence des allergies alimentaires a significativement augmenté ces dernières années et le taux est actuellement estimé être de 6-8% chez les jeunes enfants d'1 an et de 2-4% chez les autres enfants et les adultes. Les allergies alimentaires représentent la première cause d'anaphylaxie chez l'enfant et plusieurs cas d'allergies peuvent causer une réaction anaphylactique fatale. Bien que des efforts continus aient été faits pour tenter de mieux comprendre la nature des allergènes, les facteurs environnementaux prédisposant aux allergies et les mécanismes de la réponse immunitaire, aucun traitement contre les allergies alimentaires n'est disponible à l'heure actuelle. Dans cette étude, nous avons testé trois différentes stratégies visant à réduire l'allergie alimentaire, à savoir l'utilisation de probiotiques, de faibles doses d'allergènes et d'acides gras polyinsaturés oméga-3 chez des souris Balb/c sensibilisées aux protéines de lait de vache. L'administration de *Lactobacillus rhamnosus* GG (LGG) a montré une tendance à promouvoir une activation des cellules Th1 alors que VSL#3 a engendré une plus forte réduction de l'allergie via l'induction de la sécrétion intestinale d'IgA (sIgA). L'administration de faibles doses d'allergènes a provoqué une suppression de la réponse immunitaire dirigée contre les allergènes via l'activation des cellules Treg, ceci étant suggéré par la suppression des réponses immunitaires Th1 spécifique [diminution des IgG2a BLG-spécifiques et augmentation des IL-12(p40)] et Th2 spécifique [diminution des IgE et IgG BLG-spécifiques]. De plus, les souris ayant reçu à la fois VSL#3 et des faibles doses d'allergènes ont montré une suppression des effets de la réponse allergénique et une production plus élevée de sIgA. Nous avons ensuite étudié les effets de différents niveaux d'oméga-3 et d'oméga-6 dans un régime alimentaire de type occidental riche en énergie et en matières grasses sur le développement des allergies alimentaires. L'observation d'une élévation des immunoglobulines BLG-spécifiques chez toutes les souris expérimentales ont indiqué que les oméga-3 et -6 n'ont pas réussi à empêcher le développement de la réponse

immunitaire dirigée contre les allergènes. Cependant, les oméga-3 ont diminué les réactions anaphylactiques et le degré de sévérité de la réaction allergique, ceci étant suggéré par un score d'hypersensibilité plus faible, une absence de changement de la température rectale et une réponse immunitaire favorisée par les cellules Th1. Pour conclure, cette étude a révélé des stratégies prometteuses pour le traitement et la prévention des allergies alimentaires dans les années à venir.

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CONTRIBUTION TO KNOWLEDGE

Chapter II: This experiment observed the effects of LGG probiotics on CMA development in Balb/c mice that were sensitized via gavage or i.p. route. While gavage sensitization of CMP with cholera toxin-B subunit (CTB) failed to induce allergic reaction, i.p. sensitization with CMP alone (adjuvant free) induced CMP-specific immediate immune responses. We here report that mice with adjuvant-free i.p. sensitization followed by oral challenge with CMP were suitable to be used as a model for food allergy study. In addition, we revealed that LGG probiotic contributed an immunomodulation effect in CMP-sensitized mice by promoting Th-shifting toward Th1-dominated responses.

Chapter III: We have examined the effects of single or co-administration of VSL#3 and low doses of allergen in BLG-sensitized mice. To our best knowledge, this is the first experiment that determined the combined effects of supplementing VSL#3 and low doses of allergens in BLG-sensitized mice. We found that VSL#3, unlike LGG, significantly alleviated allergic reaction by producing sIgA while low doses of allergen supplementation suppressed the allergen-specific immune responses by Treg mediated active suppression. Interestingly, mice received the combined treatments (VSL#3 and low doses of allergen) suppressed allergen-specific immune responses and demonstrated a higher sIgA production.

Chapter IV: This experiment, for the first time, investigated the effects of different ratios of ω-6 and ω-3 fatty acids in Western-style diets on BLG-sensitized mice. Observation of BLG-specific immunoglobulins in all groups suggested that inclusion of both ω-3 and ω-6 PUFAs in Western-style diet did not avert development of food allergy. We further revealed that ω-3 had prevented development of anaphylaxis reaction and reduced the severity of allergic symptom in BLG-sensitized mice.

Overall, this study has examined the effects of probiotics, low doses of allergen, and supplementation of different ratios of ω-6 and ω-3 fatty acids to diets on cow's milk protein allergy development and results from three experiments have potential implication for development of novel treatments and prevention of food allergy.

CONTRIBUTION OF AUTHORS

In this thesis, three co-authored manuscripts are presented.

Authors of Manuscript 1 (Chapter II): C. L. Thang, B. Baurhoo, J. I. Boye, B. K. Simpson, and X. Zhao.

C. L. Thang designed and carried out all experiments. C.L. Thang and X. Zhao performed data analysis and wrote the manuscript. B. Baurhoo assisted with microbiological and statistical analyses. J. I. Boye assisted in milk protein purification. B. K. Simpson and J. I. Boye reviewed the manuscript. This manuscript has been published in Allergy, Asthma & Clinical Immunology journal. *Allergy Asthma Clin Immunol.* 2011 Dec 6;7:20.

Authors of Manuscript 2 (Chapter III): C. L. Thang, J. I. Boye, and X. Zhao.

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Authors of Manuscript 3 (Chapter IV): C. L. Thang, J. I. Boye, H. N. Shi and X. Zhao. C. L. Thang designed and carried out experiments. . C.L. Thang and X. Zhao analyzed data, interpreted results and wrote the manuscript. J. I. Boye and X. Zhao assisted in experimental design. H. N. Shi assisted in cytokines analysis from lymphocytes culture supernatants. J. I. Boye and H. N. Shi reviewed the manuscript. The revised manuscript has been submitted to Molecular Nutrition and Food Research.

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LIST OF ABBREVIATIONS

AA..... Arachidonic acid

ALA..... Alpha- Linolenic acid

BLG……... β-lactoglobulin

BW ……Bodyweight

CMA……. Cow's milk allergy

CMP…….. Cow's milk proteins

CTB…….. Cholera toxin B-subunit

DHA……. Docosahexaenoic acid

EoE..... Eosinophilic esophagitis

EPA..... Eicosapentaenoic acid

FI ……. Feed intake

Foxp3……... Transcription factor forkhead p3

Ig…….…. Immunoglobulin

IL …… Interleukin

IP (i.p.)……. Intraperitoneally

LA..... Linoleic acid

LGG……. *Lactobacillus rhamnosus* GG

LPS ……. Lipopolysaccharides

MCP-1……. Monocyte chemoattractant protein-1

MLN..... Mesenteric lymph nodes

ω-3.....Omega-3 fatty acid

ω-6.....Omega-6 fatty acid

OT…….…….. Oral tolerance

RAST……... Radioallergosorbent test

sIgA…….… Secretary IgA

SPT……...Skin prick test

TCA…… Trichloroacetic acid

Th…… T helper cell

Treg……. Regulatory T cell

CHAPTER I. GENERAL INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION

Food allergy is the body's response to allergens through immune-mediated hypersensitive reactions, while non-immunologic adverse reactions to foods are named as food intolerance (e.g. lactose intolerance). In allergic individuals, a food protein is mistakenly identified by the immune system as being harmful, while the same protein is normally regarded as being harmless to many others. The immune reactions involved in food allergy can be IgE-mediated, non-IgE-mediated, or both (Wang and Sampson, 2011). The clinical symptoms of food allergy may be seen in various body systems; including digestive (nausea, diarrhoea, vomiting), respiratory (nasal congestion, pruritus, cough, wheezing), cardiovascular (hypotension, fainting, dizziness, tachycardia), and cutaneous (erythema, urticarial, pruritus, angioedema) systems. Severe cases of food allergy may lead to life threatening anaphylaxis reactions (Boyce et al., 2011).

The global incidence of food allergy is significantly increased in the past decade and currently estimated at 6-8% in 1 year old children, and 2-4% in older children and adults (Sicherer et al., 2012). In fact, food allergy is the principal cause of anaphylaxis in children (Santos and Lack, 2012) and food anaphylaxis is the most commonly treated anaphylactic reaction in emergency departments of industrialized countries (Sampson, 2000). A recent study estimated that in USA there were 203,000 visits per year to emergency departments due to food allergy with about 90,000 episodes of anaphylaxis (Clark et al., 2011).

Although over 170 foods have been known to cause allergy, 90% of food allergy cases are related to a handful of foods including milk, egg, peanut, tree nuts, shellfish, fish, wheat, and soy (Boye, 2012). Severity and resolution rates of food allergies may vary, depending on many factors such as type of foods, age, and family background. Most allergic individuals may resolve allergies to milk, egg, wheat, and soy within a few years but allergic reactions to peanuts, tree nuts, sesame seeds, buckwheat, and sea food usually persist to life (Sicherer et al., 2012). Although FAO/WHO has identified eight foods/ substances (milk, egg, fish, crustacean, cereals, peanut, tree nut, and sulphites) and their products as major allergens (also known as Codex standard), incidence and severity

of allergic reactions to these foods may vary worldwide. In Canada, there are currently ten "priority food allergens": milk, egg, peanut, tree nuts, sesame seed, seafood (fish, crustaceans, and shellfish), soy, wheat, mustard, and sulphites while USA adds soybeans, and the European Union regulations includes soybeans, celery, mustard, sesame seeds, and lupin in addition to Codex standard (Alvarez and Boye, 2012). Epidemiological studies have suggested that higher prevalence of food allergy in Western countries is associated with the genetic factors (e.g., family history of allergy, filaggrin mutation), and environmental factors induced by lifestyle changes; such as microbial factors (e.g., lower microbial exposure in early life), allergen exposure (e.g., exposure to food allergens in the maternal and in the infant's diet and in the environment), and dietary factors (e.g., lower intake of vitamin D and omega-3 fatty acids) (Santos and Lack, 2012). However, recent increases of food allergy cases are thought to be more related with lifestyle changes since observed allergy incidences are developing much faster than the genomic change can contribute. There are no medications available at the moment for treatment of food allergy. Strict avoidance of specific-allergen and intra-muscular (IM) injection of epinephrine on severe food allergy caused by accidental allergen exposure are regarded as the first line of therapy (Santos and Lack, 2012). Together, all these demonstrate the urgent necessity to unveil the mechanism and develop new treatments of food allergy. The aim of this thesis, therefore, was to investigate intervention strategies of food allergy in a mouse model by adjusting the microbial factor (probiotics supplementation), allergen exposure (oral tolerance induction), and dietary factor (different ratios of omega-3 and -6 enrich diets). We have used cow's milk protein as the food allergen throughout our studies since cow's milk protein allergy is the most frequent food allergy particularly in infants and children. To determine the effectiveness of such treatments, we have examined hypersensitivity scores, anaphylactic responses, allergen-specific and total serum immunoglobulins (IgE, IgG1, IgG2a, IgA), cytokines from spleen and mesenteric lymph nodes.

LITERATURE REVIEW

1.1 Food allergy

Food allergy can be generally described as an adverse immune response to foods (Johansson et al., 2004). Food intolerance, on the other hand, is an adverse food reaction that does not involve the immune system (e.g., lactose intolerance). Over the past three decades, food allergy was a concern mainly for the urban population of the developed countries. Today, it is equally important for other parts of the world (Table 1.1). In fact, 250 million people worldwide are currently considered as potential food allergy patients that include approximately 1.2 million Canadians (Pawankar et al., 2012). Food allergy has been developed as a major public health problem. Furthermore, individuals with food allergy have increased susceptibility to other types of allergy including allergic rhinitis, eosinophilic esophagitis and asthma.

To protect allergic individuals from serious anaphylaxis attack through accidental exposure of offending food allergens, authorities from many countries have recently updated and enforced the Prepackaged Foods labelling regulations (Alvarez and Boye, 2012). In Canada, Health Canada also has made Amendments to the Food Allergen Labelling Regulations, which require enhanced labelling food allergen and gluten sources and added sulphites (Health Canada, 2013). To date, avoidance of causative foods and immediate treatment of symptoms when they arise are the best management practices for food allergic individuals (Han et al., 2012). Nevertheless, avoidance of offending allergen(s) can limit the availability of nutritious food choices especially in children. Moreover, constant avoidance of food allergens is almost impossible since some allergy-causing foods, such as milk and eggs, are extensively used in prepared foods.

Table 1.1 Estimates of global food allergy prevalence through population studies[1]

Country	Age (years)	Population (n)	Overall (%)	Methodology	Reference
North America					
USA	(1-5)	909	4.3	IgE levels	Liu et al., 2010
	(6-19)	2,869	3.8		
	(20-39)	1,672	2.4		
USA	(0-2)	5,429	6.3	Report	Gupta et al., 2011
	(6-10)	9,911	7.6		
	(14-18)	10,514	8.6		
Canada	Children	2,198	7.14	Report	Soller et al., 2012
	Adults	7,469	6.56		
	All	9,667	6.67		
South America					
Argentina	Adults	Nationwide	5	Phone survey	Boye, 2012
Europe					
UK	By 3	891	2.9	Report, SPT, DBPCFC	Venter et al., 2008
Denmark	3	486	2.26	Report, SPT, OFC	Osterballe et al., 2005
Asia					
China	(0-2)	1,604	6.2	Report, SPT, FE, DBPCFC	Chen et al., 2012
Hong Kong, China	(2-7)	3,677	4.62	Report/Doctor-diagnosed	Leung et al., 2009
Japan	(0-6)	101,322	5.1	Food avoidance	Noda, 2010
Korea	<1	1,177	5.26	Convincing history	Kim et al., 2011
Taiwan	(4-18)	15,169	7.65	Convincing history/SPT/IgE	Wu et al., 2012
Australia	Adults	5,729	11	Report	Oktaria et al., 2013
Middle East					
Israel	(0-2)	9,070	1.2	IgE levels	Dalal et al., 2002
Africa					
Zimbabwe	All	14,000	~10	Doctor-diagnosed	Sibanda, 2003
South Africa	Teenagers	211	5	SPT	Levin et al., 2011
Ghana	(5-16)	1,431	5	SPT	Obeng et al., 2011

[1]Adapted and modified from Boye (2012), Lee et al. (2013), Oktaria et al. (2013), Soller et al. (2012). Report: self/parent-reported based on symptoms provided only; Convincing history: symptoms occurring in less than 2 h. SPT, skin prick test; FE, food elimination; DBPCFC, double-blinded placebo-controlled food challenge; OFC, open food challenge.

1.1.1 Classification of food allergy

1.1.1.1 IgE-mediated food allergy (Immediate hypersensitivity)

Since the onset of symptoms in IgE-mediated allergy usually occurs within minutes to an hour after allergen exposure, it is also termed as "immediate hypersensitivity". In general, IgE-mediated allergy consists of two phases and commences when the allergens cross the cutaneous, respiratory or intestinal epithelial barriers of the body. These allergens are further processed and presented by antigen-presenting cells (APCs), mostly dendritic cells (DCs), to the naïve CD4+ T cells. This stage is commonly known as "sensitization phase". DCs have a unique ability to induce tolerance to self-antigens and many harmless proteins while producing immune responses to pathogens and certain allergens by differentiation of the naïve CD4+ antigen-specific T cells into effector cells, such as Th1, Th2 or Th17, or regulatory T cells (Treg). In the case of IgE-mediated allergy, the signals from APCs cause differentiation of naïve CD4+ antigen-specific T cells into Th2 cells which produce IL-4 and IL-13. These Th2 cells will signal B cells to produce allergen-specific IgE. The secreted IgE will bind to FcεRI receptors of mast cells and basophils in the tissue (Dombrowicz et al., 1993). When the same allergen is reintroduced subsequently, it will cause cross-linking of the allergen with IgE bound to mast cells and result in mast cell degranulation and release of potent inflammatory mediators including histamine, leukotrienes, prostaglandines and proteases, leading to exhibition of allergy symptoms. This stage is known as "activation phase". Affected individual may manifest a single or combination of the following symptoms arising from cutaneous, gastrointestinal or respiratory systems; eczema, urticarial, angioderma, nausea, vomiting, diarrhoea, rhinoconjunctivitis and asthma (Hill et al., 1997). In severe cases, IgE-mediated food allergy may lead to life-threatening anaphylactic reactions within a few minutes after exposure of offending allergens. Immediate gastrointestinal (GI) hypersensitivity and oral allergy syndrome are the two commonly seen IgE-mediated food allergy particularly in infants and young children. In immediate gastrointestinal hypersensitivity, GI symptoms are predominant but other symptoms such as urticarial may also be observed. The most common culprit foods are milk, egg, wheat, soy, peanut and seafood (Sicherer, 2002). Oral allergy syndrome, on the other hand, is more common in patients with seasonal

allergic rhinitis and major allergens are heat-labile raw apples, pears, cherries, carrots, potatoes, celery, hazelnuts and kiwis.

1.1.1.2 Non-IgE-mediated food allergy (Delayed hypersensitivity)

In comparison with IgE-mediated allergy, the mechanism of non-IgE-mediated allergy is less understood. In this type of allergy, production of interferon-gamma (IFN-γ) and activation of macrophages during Th1-mediated inflammation have been widely accepted as potential mechanisms. But, other mechanisms, such as formation of immune complexes leading to activation of the complement system, or T-cell/mast cell/neuron interactions inducing functional changes in smooth muscle action and intestinal motility (Bauer and Razin, 2000), have also been proposed. Patients with this type of food allergy exhibit no allergen-specific serum IgE and do not response to skin prick test (**SPT**) and radioallergosorbent test (**RAST**) (Pelto et al., 1999). Non-IgE-mediated allergy is also called as "delayed hypersensitivity" because the onset of symptoms usually takes from an hour to several days. Food protein-induced enterocolitis syndrome (FPIES) is a non-IgE-mediated food allergy (Nowak-Wegrzyn and Muraro, 2009). Although cereal grains, fish, poultry and vegetables may cause non-IgE-mediated food allergy, cows' milk and soy are the most common offending foods (Sicherer et al., 2012).

1.1.1.3 Mixed IgE- and non-IgE-mediated food allergy

Although food allergies have been conventionally classified into two distinct types; IgE- and non-IgE-mediated food allergy, increasing evidence and newly recognized clinical aspects suggest the existence of another type of food allergy in which both IgE- and non-IgE-mediated immune reactions were observed (Jyonouchi et al., 2009; Pratt et al., 2008; Reda, 2009; Sicherer, 2003). This type of food allergy mainly affects the GI tract and is marked by eosinophilic infiltration. Depending on the site of eosinophilic infiltration, mixed immune-mediated food allergies in the gut are classified as allergic eosinophilic esophagitis (EoE), eosinophilic gastroenteritis (EG), and allergic eosinophilic proctocolitis (Reda, 2009).

1.1.2 Symptoms and diagnosis

IgE-mediated reactions are observed immediately or within a few minutes after exposure of offending allergens and the reactions could be local (as in oral allergy syndrome) or systemic (as in anaphylaxis). The symptoms may include urticaria, angioedema, acute rhinoconjunctivitis, bronchospasm, pruritis, tingling in the mouth, and anaphylaxis.
Non-IgE-mediated (T-cells mediated) reactions usually take several days to fully develop and weeks to completely resolve. These reactions may be seen in skin, respiratory tract and gastrointestinal tract and are named according to the affected location and/or allergens involved for the clinical convenience. These reactions include food protein-induced enterocolitis, food protein-induced proctocolitis (the most commonly seen), and food protein-induced enteropathy (Pratt et al., 2008). Celiac disease, gluten-sensitive enteropathy caused by stimulation of alcohol-soluble portion of gluten, known as gliadin, found in wheat, rye, and barley, is an example of a food protein-induced enteropathy and is associated with gastrointestinal symptoms, dermatitis herpetiformis, dental enamel defects, osteoporosis, delayed puberty and persistent iron deficiency anemia (Hill et al., 2005). Symptoms associated with non-IgE-mediated food allergy are mainly gastrointestinal symptoms including nausea, bloating, intestinal discomfort and diarrhoea and sometimes mixed with respiratory symptoms. However, anaphylactic reactions have not been reported in non-IgE-mediated reactions.

Mixed IgE and cell-mediated allergic reactions, unlike the previous two reactions, are more difficult to be diagnosed since the symptoms are less obvious (e.g. non-specific abdominal pain), and easy to misdiagnose with other disorder (e.g. EoE versus gastroesophageal reflux). However, allergy cases that involve both IgE and cell-mediated reactions are frequently seen in the gastrointestinal tract, such as allergic EoE, eosinophilic gastroenteritis, and allergic eosinophilic proctocolitis; in lungs, such as asthma; or in the skin, such as atopic dermatitis (Pratt et al., 2008).

Food allergy should be considered when (1) an individual suffers anaphylaxis or any combination of immediate symptoms listed in Table 1.2 that occur within minutes to hours of ingesting food, (2) allergy symptoms have followed ingestion of a specific food on more than 1 occasion, (3) infants and children were diagnosed with atopic dermatitis,

EoE, enterocolitis, enteropathy, and allergic proctocolitis, and (4) adults were diagnosed with EoE (Boyce et al., 2011). In fact, food allergy diagnosis can be very confusing since initial identification of food allergy as a possible cause, during the differential diagnosis process, requires thorough medical history and physical examination, and a series of tests are necessary to confirm food allergy, identify the offending allergen and the most likely mechanism (such as IgE-dependent, IgE associated, or non-IgE-mediated) (Sicherer, 2002). Moreover, the fact that most of the patients with positive immunological reactions to food allergens (skin prick test and serum IgE) exhibit no relevant clinical symptoms when they consume that foods makes diagnosis more difficult (Santos et al., 2012).

The diagnosis can be achieved by performing a combination of appropriate tests from the following list; elimination of the suspected food from the diet, oral food challenge tests, laboratory and skin test, and gastrointestinal tests. Elimination of doubtful food from the diet is useful in confirmatory diagnosis. **SPT** is commonly used to detect specific allergens in IgE-mediated allergy. When **SPT** is unavailable, *in vitro* detection of allergen-specific serum IgE can be employed by using either **RAST** or the fluorescent enzyme immunoassay (**FEIA**) test (Reda, 2009). However, clinical history that leads to the suspected allergen should also be taken into consideration when interpreting these results.

For detection of non-IgE-mediated food allergies, the atopy patch test (**APT**), a fairly new diagnostic tool, can be used and its diagnostic accuracy is higher with the use of fresh food in comparison with the use of freeze-dried purified food (Canani et al., 2007). However, oral food challenges by feeding small amounts of the suspected food and gradually increasing unless there is a reaction under observation of a physician should be performed to confirm the results of **APT**. Oral food challenges are useful to determine the degree of tolerance or to exclude the suspected food from the allergy diagnosis. Combination of **SPT** and **APT** are reported to give positive predictive values in detecting mixed IgE and cell-mediated allergic reactions (Lack, 2008a). Additionally, gastrointestinal tests, such as endoscopic studies, biopsies and esophageal pH monitoring are valuable in performing differential diagnosis among non-IgE-mediated food protein-induced proctocolitis, mixed IgE and cell-mediated EoE, and other gastrointestinal disorder such as gastroesophageal reflux (Reda, 2009).

Table 1.2 Food allergy symptoms[1]

Target organ	Immediate symptoms	Delayed symptoms
Cutaneous	Erythema	Erythema
	Pruritus	Pruritus
	Urticaria	Flushing
	Angioedema	Angioedema
	Morbilliform eruption	Morbilliform eruption
		Eczematous rash
Ocular	Pruritus	Pruritus
	Conjunctival erythema	Conjunctival erythema
	Tearing	Tearing
	Periorbital edema	Periorbital edema
Upper respiratory	Nasal congestion	
	Pruritus	
	Rhinorrhea	
	Sneezing	
	Laryngeal edema	
	Hoarseness	
	Dry staccato cough	
Lower respiratory	Cough	Cough
	Chest tightness	Dyspnea
	Dyspnea	Wheezing
	Wheezing	
	Intercosral retractions	
	Accessory muscle use	
GI (Oral)	Angioedema of the lips, tongue, or palate	
	Oral pruritus	
	Tongue swelling	
GI (Lower)	Nausea	Nausea
	Colicky abdominal pain	Abdominal pain
	Reflux	Reflux
	Vomiting	Vomiting
	Diarrhea	Diarrhea
		Irritability and food refusal with weight loss (young children)
Cardiovascular	Tachycardia (occasionally bradycardia in anaphylaxis)	
	Hypotension	
	Dizziness	
	Fainting	
	Loss of consciousness	
Miscellaneous	Uterine contractions	
	Sense of "impending doom"	

[1]Adapted from Boyce et al. (2011).

1.1.3 Major food allergens

In principle any food can generate an allergic reaction. Currently more than 170 foods have been identified as causative agents for allergic reactions, either from plant or animal sources. The Pfam database, a large collection of protein families, classifies plant proteins into almost 4000 families based on their sequence homology (related to conserved 3D structures and function), although observation of their structural and functional features do not lead to distinguish allergens from nonallergens (Pulendran and Artis, 2012). From the database, 31 plant food protein families involved in food allergy development and proteins from 4 families represent approximately 65% of total plant food allergens (Chapman et al., 2007). These families include the cereal prolamin superfamily (proline- and glutamine-rich α-helical proteins with a 8 cysteine residues), the cupins (contains 2 groups of seed storage proteins called vicilins and legumins), Bet v 1 (homologues of the major birch pollen allergen, such as Mal d 1 in apple and Api g 1 in celery); and profilins (such as Api g 4 from celery) (Jenkins et al., 2005) (Table 1.3). In fact, Bet v 1 and profilins homologues are also the major culprits for pollen-related food allergy, oral allergy syndrome (Vieths et al., 2002). Similarly, major animal food allergens are derivatives of three animal foods; milk (α-Lactalbumin, β-Lactoglobulin, and the casein family), egg (ovomucoid), and seafood (tropomyosins and parvalbumins) (Chapman et al., 2007; Wang and Sampson, 2011) (Table 1.3). Food proteins derived from afore mentioned four plant food protein families and three animal foods, such as peanuts, milk, eggs, wheat, nuts (e.g., hazelnuts, walnuts, almonds, cashews, pecans, etc.), soybeans, fish, crustaceans and shellfish, are considered as "priority food allergens" and are responsible for over 90% of the reported food allergy cases (Boye, 2012). However, the prevalence of certain food allergies, order of priority food allergens and sensitivity to food allergens may vary with age, geographic location, and ethnic background (Boye, 2012; Hong and Wang, 2012; Lee et al., 2013). For example, recent review indicated that those born in Asia, regardless of ethnicity, had lower risk of peanut and tree nut compared to those born in Western countries (Lee et al., 2013). In south east Asia, shellfish allergy is the most common food allergy in older children and adults, and also the leading cause of food-induced anaphylaxis (Lee et al., 2013).

Table 1.3 Major food allergens[1]

Food allergen family	Food source	Allergen examples
Animal food protein families		
Caseins and β-Lactoglobulin	Cow's milk	αS1, αS2, β, κ-casein
Ovomucoid	Egg white	Gal d 1
Seafood		
Parvalbumin	Fish	Gad c 1- cod
Tropomyosin	Crustaceans and mollusks	Pen a 1- shrimp
Plant food protein families		
Bet v 1 super family	Fruits, vegetables, soy	Gly m 4- soy
		Mal d 1- apple
Cupin superfamily		
7S globulin	Peanut, tree nuts, legumes, seeds	Ara h 1- peanut,
		β-conglycinin-soy
11S globulin	Peanut, tree nuts, legumes	Ara h 3-peanut;
		glycinin-soy
Profilins	Fruits, vegetables, legumes	Ara h 5-peanut
		Api g 4-celery
Prolamin superfamily		
Prolamins	Cereals	α- and γ-gliadin-wheat
Nonspecific lipid-transfer proteins	Fruits and vegetables	Mal d 3- apple
		Pru p 3-peach
α-Amylase/trypsin inhibitors	Barley and rice	Hor v 1-barley
2S albumins	Peanut, tree nuts, seeds	Ara h 2-peanut

[1] Adapted and modified from Chapman et al. (2007), and Wang and Sampson (2011).

1.1.4 Predisposing factors

Food allergy is caused by a complex interaction of multiple genetics and environmental factors.

Children (up to 4 years) with a family history of allergic diseases have a higher risk of food allergy, compared to children of allergy-free parents, and the risk is even higher if both parents have allergic diseases (Pyrhönen et al., 2011). Similarly, a recent expert panel report declared that family history of atopy and the presence of atopic dermatitis are risk factors for food allergy development (Boyce et al., 2011). Further, a twin-pairs study (58 twin pairs) on peanut allergy revealed that the concordance rate of peanut

allergy in monozygotic twins (64.3%) were significantly higher than dizygotic twins (6.8%) (Sicherer et al., 2000). A larger twin pairs (826 Chinese twin pairs) study also revealed that monozygotic twins had higher concordant sensitization rates for peanut and shellfish than dizygotic twins (Liu et al., 2009). The hunt for common genetic variants across the genome that are risk factors for food allergy is going on. A number of genes, such as HLA class II gene family (*HLA-DRB1, HLA-DQB1, HLA-DPB1*), *CD14*, *forkhead box P3 (FOXP3), signal transducer and activator of transcription 6 (STAT6), SPINK5, IL10, IL13, NLRP3,* and *FLG* genes have been associated with food allergy (Hong and Wang, 2012). These genes may be involved in the antigen presentation, a shift of the immune system towards a Th2 response and suppression of Th1 and Th2 responses by regulatory T (Treg).

A majority of food allergy cases start in first a few years of life indicating the food allergy susceptible nature of early life. Introduction of changes in lifestyle, such as a smaller family size, a higher rate of C-section birth, and the diet, such as consumption of energy rich high fat diet, in Western and developed countries was followed by increased food allergy prevalence. Several hypotheses related to environment have been proposed as risk factors for the recent rapid rise of food allergy (Lack, 2008b). These hypotheses include (1) dual-allergen-exposure hypothesis, (2) hygiene hypothesis, (3) dietary fat hypothesis, which suggests that higher intakes of ω-6 and lower intakes of ω-3 fatty acids lead to increased production of prostaglandin E2 (PGE2) and thus the development of FA; (4) antioxidant hypothesis, which stresses the anti-inflammatory effects of antioxidants in allergic diseases; and (5) vitamin D hypothesis (either low or high intake).

1.1.5 Attempts to reduce severity of food allergy

Although strict avoidance of allergy causing foods from the diet is the only effective treatment for food allergies (Sicherer and Sampson, 2010), it is extremely difficult to completely avoid the contact of food allergens. Therefore, various attempts in different areas, such as food processing techniques to reduce the allergenicity of foods, medications that can provide relief for allergic symptoms, and newly developed

therapeutic regiments and strategies, have been applied to reduce severity of food allergy symptoms.

1.1.5.1 Attempts to reduce allergenicity of major allergens

Several food processing techniques have been introduced to reduce the allergenic properties of major food allergens, e.g. cow's milk protein. These techniques include thermal treatment, biochemical processes (enzymatic digestion), irradiation (Lee et al., 2001) and high pressure treatments (Bonomi et al., 2003). Currently, these processing methods give inconsistent results and more extensive clinical studies are needed to elucidate the efficiency of such treatments.

1.1.5.2 Medications for food allergy symptoms relief

Antihistamines, corticosteroids, and epinephrine are commonly used medications for relief of food allergy associated clinical symptoms. During allergic reactions, many potent inflammatory mediators, such as histamine, IL-4, IL-5, LTB4, are released by degranulated mast cells. Upon released, histamine binds rapidly to a variety of cells via two major receptors, H1 and H2. When histamine binds to H1 receptors in smooth muscle, it causes constriction of smooth muscle around the bronchi in the lung and results in difficulty of breathing (asthma). Similarly, binding of H1 receptors on endothelial cells may cause separation of cell junctions and thus increase vascular permeability and extravasation of fluid into tissue spaces, result in dropping in blood pressure. However, H2 receptors are associated with mucus secretion, increased vascular permeability, and release of acids from stomach mucosa. Antihistamines (e.g. Benadryl) can relieve urticaria, pruritus, flushing and mild angioedema by competitive blocking to H1 receptors. H2 receptors can be blocked by other drugs, such as cimetidine (Coico, 2009).
Corticosteroids, such as prednisone and prednisolone, are potent anti-inflammatory agents. Through binding to the intracellular steroid receptors, corticosteroids down-regulate expression of several genes that code for inflammatory cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-8, TNF-α,and GM-CSF (Coico, 2009). Although the application of corticosteroids in urgent situations is limited by their delayed onset of

initial anti-inflammatory effects, it is effective in allergic eosinophilic esophagitis or gastroenteritis (Atkins and Furuta, 2012). Prompt administration of epinephrine is essential to reverse anaphylaxis reactions in patients with severe food allergy (Sicherer and Sampson, 2010).

1.1.5.3 Newly developed therapeutic strategies

1.1.5.3.1 Allergen-specific therapy

In allergen-specific therapy, native food allergens, modified recombinant engineered food proteins, or overlapping peptides, have been administered with the goal to achieve reduced allergic reactions and increased oral food tolerance upon later exposure to the specific food (Nowak-Węgrzyn et al., 2012). The mechanisms, effects and concerns of such therapies have been summarized in Table 1.4. More detailed information can be accessed from the following literatures (Nowak-Węgrzyn et al., 2012; Wang and Sampson, 2011, 2012; Yang and Chiang, 2013).

1.1.5.3.2 Allergen-nonspecific therapy

Recent research advances in food allergy studies have also led to promising allergen-nonspecific therapies. These therapies include administration of monoclonal anti-IgE and monoclonal anti-IL-5, TLR-9 therapy, *Lactococcus lactis* transfected with murine IL-10, and Chinese herbal medicine that contains a mixture of 9 herbs (Table 1.5). More detailed information can be accessed from the following literatures (Nowak-Węgrzyn and Sampson, 2012; Wang and Sampson, 2011, 2012; Yang and Chiang, 2013).

Table 1.4 Allergen-specific therapies for food allergy[1]

Allergen-specific therapies	Mechanism	Effects	Concerns
Oral Immunotherapy (OIT)	Gradual exposure to allergens to induce desentization or tolerance	Improved clinical tolerance	Unclear whether the effects are desentization or induction of tolerance; side effects are common and unpredictable
Sublingual immunotherapy (SLIT)	Gradual exposure to allergens to induce desentization or tolerance	Improved clinical tolerance	Unclear whether the effects are desentization or induction of tolerance; side effects are common
Recombinant vaccines	Mutate IgE-binding sites; proteins stimulate T cells to proliferate, but have greatly reduced IgE-binding capacity	Protection against peanut anaphylaxis in mice	Improved safety profile compared with conventional IT; requires identification of T cell epitopes for each allergen
Peptide immunotherapy (PIT)	Peptide fragments contain T cell epitopes, but are not of sufficient length to cross-link IgE and therefore cannot trigger mast cell or basophil activation	Protection against peanut anaphylaxis in mice	Improved safety profile compared with conventional IT; requires identification of T cell epitopes for each allergen
ISS-conjugated protein immunotherapy	ISS bound to proteins can act as adjuvants to promote switching to a Th1 response	Protection against peanut sensitization in mice	Concern for excessive Th1 stimulation and potential for autoimmunity
Plasmid DNA immunotherapy	Allergen gene immunization to promote endogenous allergen production resulting in possible induction of tolerance	Less severe and delayed peanut-induced anaphylaxis in a murine model	Serious concerns regarding safety in view of strain-dependent effects in mice

[1]Adapted from Wang and Sampson (2011). ISS:Immunostimulatory sequences.

Table 1.5 Allergen-nonspecific therapies for food allergy[1]

Therapy	Mechanism of action	Effects	Comments
Monoclonal anti-IgE (TNX-901 and Omalizumab)	Binds to circulating IgE and prevents IgE deposition on mast cells and blocks degranulation. Interferes with the facilitated antigen presentation by B cells and dendritic cells.	Improves symptoms of asthma and allergic rhinitis; provides protection against peanut anaphylaxis in 75% of treated patients	Subcutaneous at monthly or 2-week intervals, unknown long-term consequences of IgE elimination; food non-specific; may be used in combination with specific food allergen oral immunotherapy
Monoclonal anti-IL-5 antibody (Mepolizumab)	Block proallergic cytokines	Anti–IL-5 causes reduction in tissue eosinophils, but does not induce resolution of histologic or clinical features of eosinophilic esophagitis (EoE).	Mepolizumab was well tolerated and had an acceptable safety profile, even at the high 1500-mg dose level. Mepolizumab is currently being evaluated in children with EoE.
Chinese herbal medicine	Upregulation of Th1 cytokines (IFN-γ, IL-12); downregulation of Th2 cytokines (IL-4, IL-5, IL-13); decreased allergen-IgE and T cell proliferation to peanut	Reverses allergic inflammation in the airways; protects mice from peanut anaphylaxis	Oral, generally safe and well tolerated, current studies focus on identification of the crucial active herbal components in the multiherb formulas and establishing optimal dosing in phase I and II clinical trials
Lactococcus lactis transfected with murine IL-10	Decreased serum IgE and IgG1; increased IgA in the gut; increased gut and serum IL-10	Pretreatment of young mice prior to sensitization with β-lactoglobulin in the presence of cholera toxin protected against anaphylaxis on the oral food challenge	This approach was only tested in the mouse model; however, the concept of probiotic bacteria may be applied to delivery of engineered allergens in human studies
TLR-9	Induction of Th1-type immune response	Protect from peanut anaphylaxis in a murine model	Concern for excessive Th1 stimulation and potential for autoimmunity

[1]Adapted and modified from Wang and Sampson (2011), and Nowak-Węgrzyn and Sampson (2012).

1.2 Cow's milk allergy

Cow's milk allergy (CMA) is one of the most common food allergies affecting infants and children. The incidence rate of CMA in infants and children is higher than adults and is usually one of the most prevalent food allergies in developed countries (Table 1.6) because cow's milk protein is usually the first foreign food introduced to infants while their gut mucosal barrier function and immune system are still in a developmental immaturity stage (Chehade et al., 2005).

In North America, incidence of CMA is estimated at 2.5% in children and about 1% in adults with a 75% outgrowing rate at the age of 16 years of age (Sicherer and Sampson, 2010). In addition, CMA has gained the name "complex disorder" due to the occurrence of multiple allergenic epitopes in many milk proteins and its ability to develop in IgE-mediated and/or non-IgE-mediated mechanism.

Table 1.6 Estimates of cow's milk allergy prevalence through population studies[1]

Country	Age (years)	Population (n)	CMA (%)	Methodology	Reference
USA	<1	2,441	3.8	Doctor-diagnosed, symptoms-based criteria	Luccioli et al., 2008
Canada	Children	2,198	2.23	Report	Soller et al., 2012
	Adults	7,469	1.89		
UK	0-3	891	0.4	Report, SPT, DBPCFC	Venter et al., 2008
Denmark	3	486	0.6	Report, SPT, OFC	Osterballe et al., 2005
Japan	0-6	101,322	1.42	Food avoidance	Noda, 2010
Korea	<1	1,177	1.69	Convincing history	Kim et al., 2011
China	<1	477	2.7	SPT	Chen et al., 2011
Taiwan	<3	813	1.1	Convincing history/SPT/IgE	Wu et al., 2012

[1] Adapted and modified from Lee et al. (2013), Luccioli et al. (2008), Soller et al. (2012).

1.2.1 Cow's Milk in general

Milk is a special biological fluid secreted from the mammary gland of female mammalians and it is primarily intended to provide nutritional requirements of neonates. Human beings are the only known mammals that consume the milk of other mammals particularly from cows. In fact, cow's milk and its associated food have been deeply rooted as an important part of regular diets and the dairy industry plays a huge role in supporting nutrition to human and continues to be a backbone of the Agro-Food sector in many countries. In developed countries, bovine milk and milk-derived products contribute about 19% of total dietary protein intake and 73% of calcium intake (Tome et al., 2004). However, for some people, consumption of cow's milk and cow's milk-derived food has to be avoided due to milk allergy, an inflammatory response to milk proteins.

1.2.2 Milk proteins/allergens

Milk protein is a very complex mixture of molecules. Thanks to advanced analytical techniques, over 200 types of various proteins have been identified from bovine milk (Ng-Kwai-Hang, 2011). These proteins can be arranged into five groups: caseins, whey proteins, milk fat globule proteins, enzymes and minor miscellaneous proteins. Caseins and whey proteins make up almost all of the total milk protein composition whereas the other 3 groups are found in trace amounts (Table 1.7). Caseins can be separated from the other proteins by acid precipitation at pH 4.6 that forms coagulum. The ratio of casein and whey in the milk, however, is different among the species. In human milk the ratio is 40:60, in equine the ratio is 50:50 and in major dairy animals (cows, goats, sheep and buffaloes) the ratio is 80:20. Major bovine milk proteins are discussed below.

Casein (CN) in bovine milk forms the main fraction of milk proteins and is subdivided into a number of families, including α-S1-, α-S2-, β-, κ-, γ-caseins.

The ***α-S1-casein*** (α-S1-CN) is a single-chain phosphoprotein with 199 amino acid residues and represents about 40% of total casein. α-S1-CN is characterized by a high content of proline residues without disulfide bonds and the presence of a small amount of secondary structures, such as α-helix or β-sheets. The several epitopic regions of α-S1-

CN recognised by human IgE antibodies have been identified. These identified major epitopic regions include aa19-30, aa86-103 and aa141-150 (Spuergin et al., 1996), aa181-199 (Nakajima-Adachi et al., 1998) and 9 regions from amino acid residues 17 through 194 (Chatchatee et al., 2001). The possible reasons for different dominant epitopic regions reported by different researchers may be due to racial (Kaminogawa and Totsuka, 2003), dietary, breeds and environmental differences.

The ***α-s2-Casein*** is comprised of 207 amino acids and has one disulfide bond (Wal, 1998). The α-S2-CN family accounts for 12.5% of the casein fraction and are the most hydrophilic among all caseins due to the presence of clusters of anionic groups. Ten IgE-binding regions have been identified between amino acid positions (31-200) of α-s2-casein (Busse et al., 2002).

Table 1.7 Characteristics of the Major Proteins in Human and Cow's Milk[1]

Proteins	Human (mg/mL)	Cow (mg/mL)	Molecular Weight (kDa) of Cow's Milk Proteins	Number of Amino Acids in Cow's Milk
Whole caseins				
α-s1-Casein	0	11.6	23.6	199
α-s2-Casein	0	3	25.2	207
β-Casein	2.2	9.6	24	209
κ-Casein	0.4	3.6	19	169
γ-Casein	0	1.6	11.6–20.5	
Whey proteins				
β-Lactoglobulin	0	3	5.3	162
α-Lactalbumin	2.2	1.2	4.8	123
Immunoglobulins	0.8	0.6	<150	
Serum albumin	0.4	0.4	66.4	582
Lactoferrin	1.4	0.3	76.2	703
Other	1.3	0.6		

[1]Adapted from Boye et al. (2012).

The ***β-casein (β-CN)*** represents 35% of the total caseins. ***β-casein*** can be cleaved by plasmin into γ1- γ2- γ3- CN fragments. β-CN is the most hydrophobic component among casein fractions. Six major and three minor IgE-binding epitopes, as well as eight major and one minor IgG binding regions, have been identified on β-casein (Chatchatee et al., 2001).

The ***κ-casein (κ-CN)*** accounts for 12.5% of the total casein fraction. Eight major IgE-binding epitopes, as well as two major and two minor IgG-binding epitopes, have been detected on κ -casein (Chatchatee et al., 2001).

Bovine **β-lactoglobulin (BLG)** is the most abundant whey protein and represents 50% of total whey proteins. It has no homologous counterpart in human milk. It possesses three disulfide bridges. BLG is relatively resistant to proteases and acid hydrolysis. BLG belongs to the lipocalin superfamily and is capable of binding a wide range of molecules including retinol, β-carotene, saturated and unsaturated fatty acids and aliphatic hydrocarbons (Breiteneder and Mills, 2005). The following BLG epitopes have been reported: aa1-16, aa31-48, aa47-60, aa67-78 and aa75-86 (Inoue et al., 2001; Jarvinen et al., 2001).

Bovine **α-lactalbumin (ALA)** is characterised by four disulfide bridges and possesses a high-affinity binding site for calcium, which stabilizes its secondary structure. Bovine ALA shows a 72% of amino acid sequence homology to human ALA and thus ALA becomes an ideal protein for the nutrition of human infants. Four different linear IgE-binding peptides, aa1-16, aa3-26, aa47-58 and aa93-102, have been identified by epitope mapping in children who outgrow CMA later in life (Jarvinen et al., 2001).

Bovine serum albumin (BSA) accounts for about 5% of the total whey proteins. BSA is physically and immunologically very similar to human blood serum albumin. BSA has 17 disulfide bonds and most of the disulfide bonds are protected in the core of the protein and are therefore not easily accessible (Restani et al., 2004). This may be the reason for having relatively stable tertiary structure in BSA. Interestingly, Canadian patients with insulin-dependent diabetes mellitus have elevated BSA-specific serum IgG, but not of antibodies to other milk proteins, suggesting these antibodies are capable of reacting with the pancreatic beta cell-specific surface protein and could participate in the development of islet dysfunction (Karjalainen et al., 1992).

Lactoferrin (LF) is a milk-specific iron-binding protein. Although LF in cow's milk is homologous to human LF, the content is lower than that of human's milk. Its main function is to defend the host against infections and inflammations through its ability to sequester iron from the environment that inhibit availability of essential nutrient for bacterial growth (Ward et al., 2002).

1.3 Overview of immune system

The mammalian immune system comprises two components; innate immune system and adaptive (acquired) immune system. The key functions of immune system are to recognize and differentiate environmental agents (e.g. microorganisms or their products, foods, chemicals, drugs, pollens, dander) upon contact and generate appropriate responses including elimination of foreign antigens, formation of immunologic memory, and development of tolerance to self-antigens, gut-microbiota and harmless foods (Luckheeram et al., 2012). The innate immunity is congenital and its main function is to provide the host first line of defense against pathogens and environmental stimuli. Features of innate immunity include physical and chemical barriers (e.g. skin, secreted fatty acids, pH), cellular barriers (e.g. phagocytes), pattern recognition molecules (e.g. Toll-like receptors), and serum proteins (e.g. β-lysin, lysozyme) (Coico, 2009).

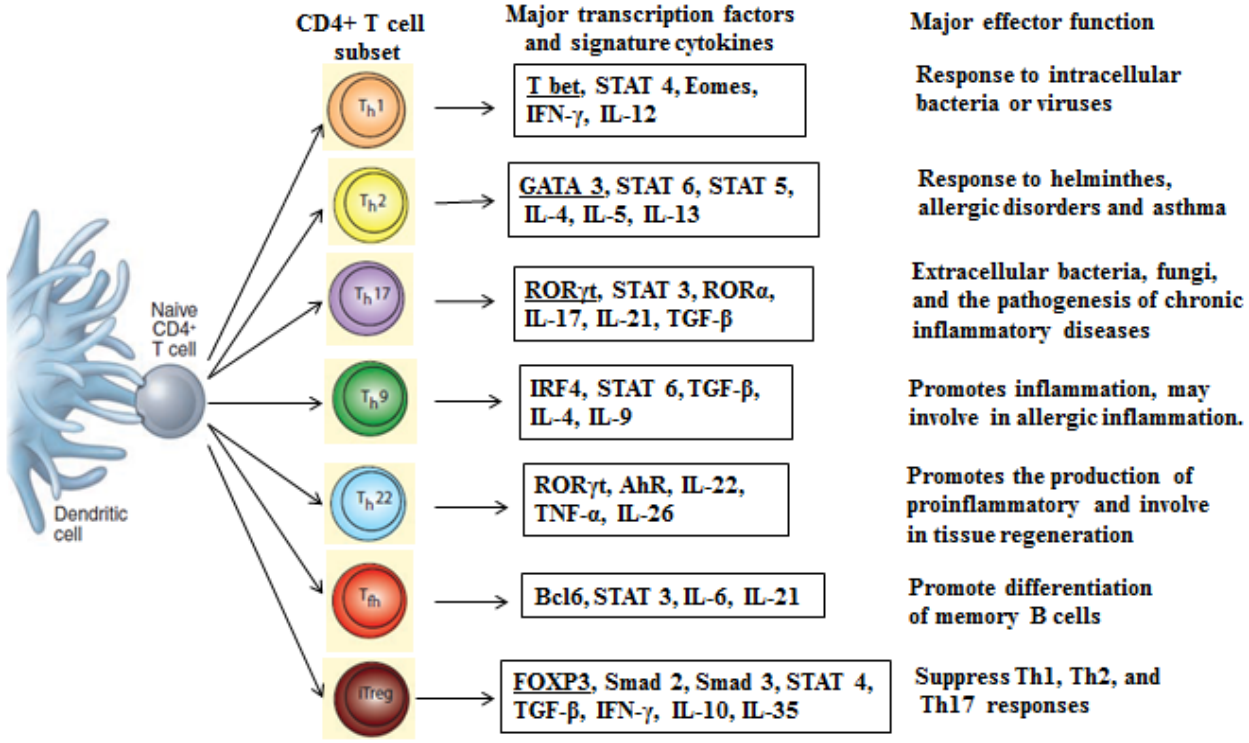
The adaptive immunity has two separate arms of defense, namely humoral (antibody-mediated) immunity and cellular (cell-mediated) immunity. Innate immune system can neutralize or eliminate foreign pathogen within minutes to hours since the system response in non-specific manner. However, it may take days to weeks to get adaptive immune responses because this system requires a series of events, such as antigenic stimulation by DCs to T and B cells, proliferation of activated cells, and clonal expansion of the cells, in order to respond to pathogens in an antigen-specific manner. The unique quality of adaptive immunity is the involvement of a long lasting memory response through which the individual will have immunity to subsequent insult by same offending agent.

1.3.1 The role of T cells in immunity

T cells play a key role in the adaptive immunity. In thymus, T cells are equipped with antigen receptors, (such as αβ and γδ chains) known as T cell receptor (TCR), and differentiated and later released into periphery and secondary lymphoid organs as αβ+ CD4+ or CD8+ T cells (majority), γδ T cells, NKT cells, NK cells, and nTreg. γδ T cells are found commonly at mucosal epithelial sites, such as the skin, gut, and lung, and are thus regarded as provider for first line of defense against pathogens. Most γδ T cells do

not have either CD4 or CD8 coreceptor molecules but some express CD8. NKT cells represent about 1% of peripheral blood monocytes and express both αβTCR and a surface molecule, NK 1.1 (Coico, 2009). NKT cells do not respond to MHC molecules but respond to glycolipids presented by CD1d. Another type of cells emerge from thymus is natural killer (NK) cells. Since NK cells leave the thymus during the early developmental stage, they lack TCR and are considered as a part of the innate immune defenses (Coico, 2009). The most interesting and beneficial types of T cells are CD4+ and CD8+ T cells which provide host defence against pathogens and various environmental stimuli (Palm et al., 2012). CD8+ T cells are known as killer T cells or cytotoxic T cells (CTC) and activated CD8+ T cells kill the bacteria or virus infected target cells by releasing granules containing perforin and granzymes (serine proteases) (Coico, 2009). Activation and proliferation of CD8+ T cells require antigen presentation by DC through MHC class I molecule, costimulatory signals (B7-CD28 and CD40-CD40L), and cytokines, especially IL-12. Although complete mechanism of CD8+ T cells is currently not well understood, two distinct subsets of CD8+ T cells; high T-box transcription factors (T-bet^{hi}) and high Eomesodermin (Eomes^{hi}), have been reported recently (Paley et al., 2012). Similarly, in order to activate and proliferate, CD4+ T cells require a series of signals, such as antigen presentation by DC through MHC class II molecule, costimulatory signals (B7-CD28 and CD40-CD40L), adhesion molecules ***intracellular adhesion molecule 1 (ICAM-1, CD54)*** expressed on APC and ***the leukocyte function associated antigen 1 (LFA-1, CD11a/CD18)*** expressed on T cell and ***CD58 (LFA-3)*** expressed on the human APC and ***CD2*** expressed on the T cell and cytokines, especially IL-2 (Coico, 2009). Differentiation of naïve CD4+ T cells to specific CD4+ T cell subset is determined by type of encountering antigen and microenvironments during activation (Oh and Ghosh, 2013). To date, seven subsets of CD4+ T cells, Th1, Th2, Th17, Th9, Th22, Tfh and inducible regulatory T (iTreg) cells, have been reported (Figure 1.1) (Oh and Ghosh, 2013).

Figure 1.1 CD4+ T-cell subsets. The master regulators are underlined.[1]



[1]Adapted and modified from Jabeen and Kaplan (2012) Kumar et al. (2013), Luckheeram et al. (2012), Oh and Ghosh (2013). AhR -Aryl hydrocarbon receptor; RORγt- retinoic acid-related orphan receptor transcription factor.

1.3.2 Immune response to infectious microorganisms

The immune system provides immunity against invading germs including viruses, bacteria, fungi and protozoa through innate response and adaptive arm such as CT8+ T cells and CD4+ T cells derived Th1, Th17 cells. This broad spectrum antimicrobial immunity is collectively known as type 1 immunity (Palm et al., 2012). Depending on the pathogen, type 1 immunity may involve induction of phagocytosis, and immunoglobulin M (IgM), IgA, and different classes of IgG.

1.3.3 Immune response to infectious macroparasites and non-infectious stimuli

The body provides immunity against the attack of parasites (e.g. helminthes and ticks), and non-infectious environmental stimuli including food allergens through Th2 cells mediated immune responses. This type of immunity is known as type 2 immunity (Palm et al., 2012). Almost half of the world's population (over 3 billion people) is estimated currently to be either infested with parasites or suffered with asthma, and various types of allergies (Pulendran and Artis, 2012). Th2 cells express the transcription factors GATA-binding protein-3 (GATA-3), signal transducer and activator of transcription-5 (STAT-5), and STAT-6, and are mediated by IgE and IgG1 antibodies. Typical Th2 cells produce IL-4, IL-5, IL-9, IL-13, and IL-10 (Paul and Zhu, 2010; Pulendran and Artis, 2012). Although Th2 immune response against parasite infestation has been widely accepted as beneficial defense mechanism, hypersensitivity reactions observed in food allergies and asthma have been regarded as untended type 2 immunity. In their recent review, Palm and colleagues proposed that extremely sensitive response to environmental stimuli (such as helminthes, noxious xenobiotics, venoms and haematophagous fluids, and irritants) via allergic reaction is not a pathological consequence but an intended and beneficial response for host defence (Palm et al., 2012). The authors further suggested that features of Th2 immunity, such as innate lymphoid cells (ILCs), Th2 cells and released mediators from degranulated mast cells and basophils, provide allergic defences through barrier defense, expulsion, inactivation and destruction, restriction, and repair mechanisms.

1.4 Immune response to gut microbiota

1.4.1 Establishment of gut microbiota

Foetal gut is sterile, however; microbial colonization, predominantly bacteria, in the gastrointestinal tract begins during or immediately after birth. Co-existence of external microbial population in the host's gut lumen as commensal flora involves a gradual developmental process. The composition of gut microbiota is more or less different in each individual at the species level and is influenced by many factors including modes of delivery, feeding, hygiene levels, family size and medication (Gronlund et al., 1999). Due to the presence of excess oxygen in neonate's gut, majority of neonate's gut microbiota

compose of aerobic or facultative anaerobic bacteria such as enterococci (*E. faecalis and E. faecium*) and *E.coli, Klebsiella, Enterobacter*, *Pseudomonas* and *Acinetobacter*. After a certain period, growing facultative anaerobes consume oxygen and create anaerobic environment. As a result, obligatory anaerobes population is steadily increased in the gut. Establishment of gut microbiota is an ongoing process and it may take several years (Ellis-Pegler et al., 1975). It is estimated that the number of obligatory anaerobes is 100 to 1000 folds higher than facultative bacteria in adults. Study in mice revealed that functional maturation of intestinal tract occurs during weaning while a majority of aerobic bacteria population is replaced with anaerobes. In an individual with established gut microbiota, the concentration and type of colonized bacteria along the gastrointestinal tract vary due to different environmental and physiological conditions, such as acid, bile and pancreatic secretions, peristalsis, throughout the gut. In general, the bacteria concentration is lowest in stomach and proximal small intestine (about 10^3 CFU/mL), increasing concentrations in jejunum and ileum (10^4–10^8 CFU/mL), and the highest concentration in the colon (10^9–10^{12} CFU/mL) (Blaut and Clavel, 2007). Microbial diversity seems to play an important role in healthy immune maturation, since a comparative study revealed that infants with eczema have a less diverse microbial population than that of healthy infants. DNA-based techniques revealed that over 1000 species of bacteria, an estimated total of 10^{14} microorganisms, are colonized in an adult's gut and it creates a new name of "superorganisms" for human beings (Nicholson et al., 2005).

1.4.2 The role of intestinal epithelium

One of the essential requirements for survival is to get continuous and sufficient supply of harmless nutrients to the body. This task is efficiently carried out routinely by gastrointestinal tract (approximately $300m^2$ of mucosal surface) (Moog, 1981) and associated tissues. Precisely, a single layer of intestinal epithelium cells (IECs) separates the internal body from external world which were introduced in a form of ingested foods, antigens and microorganisms. The IECs maintain intestinal homeostasis by forming a physical barrier, providing the first line of innate defense against microorganisms and

influencing the functions of surrounding antigen presenting cells and lymphocytes (Artis, 2008).

Intestinal epithelial cells include goblet cells, enteroendocrine cells, enterocytes, Paneth cells and M cells (microfold cells). Each type of IEC has specialized functions. Intercellular tight junctions prevent cross-passing between the cells. The brush border formed by the actin-rich microvilli on the apical surface of IECs interfere the attachment of microorganisms. Goblet cells secrete glycosylated mucin along with trefoil factors (TFFs), which form a thick viscous mucus layer (about 150μm). The combined effects of tight junctions, brush border and mucus layer create an efficient physical barrier on the intestinal mucosa (McAuley et al., 2007). Intestinal epithelial cells reinforce the physical barrier by secreting antimicrobial compounds, that include antimicrobial peptides (defensins and lectins) secreted by Paneth cells; antimicrobial protease inhibitors and hydrophobic phospholipids produced by epithelial cells, to the luminal mucus layer (McGuckin et al., 2009).

Intestinal epithelial cells also provide the first line of innate immune responses through M cells. M cells have a unique ability to stimulate mucosal immunity by allowing the entry of microorganisms and antigens and delivering them to induction sites, Peyer's patches, where antigen presenting cells (dendritic cells and macrophages) and lymphocytes are densely populated. Furthermore, like immune cells, IECs also express Toll-like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Philpott and Girardin, 2004). Recognition of pathogen-associated molecular patterns (PAMPs) by these receptors results in activation of innate immune responses and subsequently expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides (Fritz et al., 2006).

IECs regulate antigen presenting cells (macrophages and dendritic cells) and lymphocytes to response to commensal bacteria in a hyposensitive manner. This concept is based on the finding that IECs secrete immunoregulatory molecules, such as thymic stromal lymphopoietin (TSLP), transforming growth factor-β (TGF-β), and prostaglandin E2, that can inhibit the NF-κB dependent gene expression and limit expression of pro-inflammatory cytokines by macrophages (Smythies et al., 2005). TSLP and TGF-β

prevent DC's ability to produce pro-inflammatory cytokines and enhance differentiation towards regulatory T cells (Rimoldi et al., 2005; Watanabe et al., 2004). Most of the lymphocytes from GALT react to the commensal bacteria with immunosuppressive nature. In vitro studies revealed that IECs have cathepsins, proteolytic molecules, to process antigens and express MHC class II molecules to present antigens (Telega et al., 2000). However, IECs do not express conventional costimulatory molecules such as CD80 and CD86. Instead, IECs express new B7 family members, such as B7h and B7-H1 (programmed death ligand) (Nakazawa et al., 2004). Immunosuppression effects rather than immune response are, therefore, achieved upon the antigen presentation done by IECs to the intraepithelial lymphocytes.

1.4.3 Host-microbes cross-talk

Symbiotic relationship between commensal bacteria and mammalian intestine has been well-established millions of years ago. The host provides the commensal bacteria with abundant supply of nutrients and comfort environment for successful colonization. In return, the commensal bacteria benefit the host in many ways which include assisting the intestinal epithelium development, promoting angiogenesis, facilitating digestion, absorption and storage of nutrients, protection from colonization of pathogenic microorganisms by nutrient competition and antimicrobial peptides secretion (Backhed et al., 2005; Hooper et al., 2001; Sonnenburg et al., 2004).

Considering the magnitude of the antigen exposed (estimated 10^{14} commensal bacteria and 130g of proteins absorbed daily) (Brandtzaeg, 1998), IECs and gut associated lymphoid tissue (GALT) respond exceptionally well to ingested antigens and commensal flora by hyposensitive reactions and allowing digestion and absorption with little or no inflammatory responses. The gut associated lymphoid tissue (GALT), the largest immune organ in the body, includes intraepithelial lymphocytes, scattered immune cells in the lamina propria, lymphoid follicles and Peyer's patches and mesenteric lymph nodes. Continuous exposure of microbial load and ingested antigens to intestinal epithelial cells (IECs) in the gastrointestinal tract may explain the need of such a large and efficient

GALT. Recent evidence suggests that intestinal lumen contents are constantly sampled by the GALT to discriminate self-antigens from harmful antigens.

Antigens sampling is initiated with recognition of pathogen-associated molecular patterns (PAMPs) from antigens by pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), expressed on or within the IECs and immune cells. Specific PRRs recognize different components of microorganisms for examples; peptidoglycan, cell wall component of Gram-positive bacteria, is recognized by TLR2, lipopolysaccharide (LPS) from Gram-negative bacteria is recognized by TLR4 and bacterial flagella are recognized by TLR5 (Uematsu and Akira, 2008). Upon stimulation of TLRs, sequestered NF-κB, transcription factor, become activated and turns on the gene expression of specific genes that have DNA-binding sites for NF-κB. Transcription of expressed genes leads to synthesis of immunomodulatory molecules, such as cytokines and chemokines, and exhibition of relevant immunological responses. Since inhibitor KappaB (IκB) expression is limited in foetal intestinal cells, it is very common to see severe inflammatory responses in newborns and children, regardless of having commensal bacteria dominated gut micribiota (Claud et al., 2004).

Under normal physiological condition, rapid sampling of luminal contents, across the fine physical barrier of intestinal epithelial cells, can be achieved by three pathways. First, most of the antigens and microorganisms are sampled by specialized M cells and delivered to professional antigen-presenting cells by transcytosis (Neutra, 1999). Second, intestinal dendritic cells (DCs), distributed in the lamina propria of small intestine, can directly sample antigens and microorganisms by extending their dendrites between tight junctions of IECs (Rescigno et al., 2001). However, sampling efficiency via this channel is influenced by the composition of gut microbiota and expression of CX3C-chemokine receptor 1 (CX3CR1) (Niess et al., 2005). Finally, the IECs were also reported to be capable of sampling antigens through the TLRs and nucleotide-binding oligomerization domain (NOD) - like receptors (NLRs) (Philpott and Girardin, 2004), and inducing pro-inflammatory cytokines and antimicrobial peptides (Fritz et al., 2006). In general, sampled antigens are processed and presented to naïve T cells, which lead to release of appropriate pro-inflammatory cytokines and eventually differentiation of T cells subsets. Surprisingly, the gut immune system responds differently to friend and foe; selectively

discriminates pathogenic bacteria by activating innate immune responses and suppresses immune responses upon the encounter with commensal bacteria. Conventional explanation for pathogenesis of pathogens is based on its ability to survive in host's tissue, for example *Listeria monocytogenes* and *Shigella* species, due to the presence of pathogenicity genes. Recent studies, however, revealed that commensal bacteria prevent activation of innate immune responses by inhibiting the NF-κB pathway in IECs, via blocking the ubiquitylation of IκB or hijacking the peroxisomeproliferation- activated receptor-γ (PPARγ) pathway (Artis, 2008).

1.5 Effects of Probiotics in allergy development

1.5.1 General overview of probiotic

Probiotics are defined as live microorganisms, isolated from the gastrointestinal tract of human or animals, which offer beneficial effects to the host when administered orally (FAO/WHO, 2002). *Lactobacilli* and *Bifidobacteria* are the most commonly used probiotics while the growing uses of other strains, such as *Streptococcus thermophiles* and *Propionibacterium*, as probiotics have been reported. Currently, probiotics are readily accessible for human consumption in the form of pure probiotics or through varieties of probiotics added foods. Although many desirable contributions from probiotics on the host have been reported, it is generally agreed that, these effects are genera, species and strains specific. Research findings favour the use of multiple strains of probiotics derived from different genera or species rather than using a single strain (Timmerman et al., 2004). Furthermore, the dose, timing and duration of administration; and age of the host play important roles for the success of probiotics treatment. Sazawal et al. (2006) reported that short term administration of high doses of probiotics is more effective than low doses for treating acute infectious diarrhoea. It is clear that, to maximize the beneficial preventive potential of probiotics, it would be a good practise to administer a mixture of probiotics from early life while gut microbiota is not yet completely established. By doing so, the host will have a gut microbiota composed with more diverse strains of bacteria which are dominated by beneficial microorganisms.

1.5.2 Beneficial role of probiotics on hosts

Decades of research reveal that probiotics contribute several beneficial effects to the host by readjusting the intestinal microbiota composition, interacting with the intestinal epithelial cells and modulating host's immune systems. These beneficial effects can be summarized into immune related and non-immune related effects.

Probiotics elicit non-immune related beneficial effects in the intestinal lumen. In the intestinal lumen dominated with the potentially harmful bacteria, such as *E. coli* and *Clostridia*, high concentrations of ammonia, amines and indoles may be released after degradation of dietary proteins by these bacteria and can cause harmful outcome to the host (Cummings and Bingham, 1987). Probiotics bacteria, that have little proteolytic ability, however, will ferment carbohydrates and remove or reduce the quantity and efficiency of pathogenic bacteria via releasing antimicrobial substances including hydrogen peroxide, diacetyl and bacteriocins (Servin, 2004), reducing luminal pH and competing with adhesion sites (Bernet et al., 1994; Collins and Gibson, 1999). Moreover, probiotics strengthen the gut barrier function by interactions with intestinal epithelial cells. These interactions lead to reduce mucosal permeability, increase mucus production and integrity of intestinal tight junctions, and inhibit bacterial translocation (Kennedy et al., 2002; Stratiki et al., 2007). Recently, Pagnini et al. (2010) demonstrated that multi-strains probiotics bacteria (VSL#3) prevented the onset of intestinal inflammation via direct activation to the NF-κB located in the intestinal epithelial cells that led to epithelial production of TNF-α.

Probiotics induce immune related beneficial effects through modulation of both intestinal and systemic immune systems. Probiotics are known for providing increased luminal secretory IgA and phagocytes, and stimulating release of cytokines that promote differentiation of naïve T cells toward Th_1 or Treg cells (Fujii et al., 2006; Takeda et al., 2006).

1.5.3 Rationale for interest of probiotic in allergic disease

Like in parasite infestation, most allergic diseases progress through Th_2 cell mediated immune responses. The interest to use probiotics in prevention and treatment of allergic

diseases was conceived in the research community when so called “hygiene hypothesis” was discovered through an epidemiological study (Strachan, 1989). This hypothesis claimed that children exposed to a less variety of microorganisms, due to modernized and more hygienic life style, were more susceptible to allergic diseases. One of the approaches used to create a broader spectrum of gut microbiota was oral administration of probiotics. Gradually, interest in probiotics has become a major focus for clinicians and researchers, who specialized on allergy related discipline, due to the findings of different microbiota composition in allergic children in comparison with healthy ones. Unlike in healthy children, the gut microbiotas of allergic infants, generally, have higher levels of clostridia but lower level of *lactobacilli* and *bifidobacteria* (Bjorksten et al., 2001; Sepp et al., 2005). This has drawn attention of scientific community to use probiotics, preferentially *lactobacilli* and *bifidobacteria*, in prevention or treatment of allergic diseases among infants.

1.5.4 Antiallergic mechanisms of probiotics in animal models

Although many mechanisms have been proposed to explain antiallergic roles of probiotics, through in vitro and animals model studies, their entire modes of action are not yet completely understood. Up to now, studies have revealed that probiotics contribute antiallergic properties against dietary antigens by improving mucosal barrier functions, degrading food antigens, modulating intestinal microbiota composition and activity, stimulating production of secretory IgA (Thang et al., 2013), influencing mucus production and direct immune modulation. Recently, Kasakura et al. (2009) proposed that probiotics can prevent allergic reactions by inhibiting degranulation of IgE-sensitized mast cells in the presence of antigens. The authors explained that this suppressive effect of mast cells results from TLR2 activation of mast cells by TLR2 ligands from probiotics, which are usually gram positive bacteria.

The probiotics therapy is aimed to modulate gut microbiota to get ideal composition which can then initiate the host to react with harmless immunological responses whenever the antigens are exposed. At the moment, although probiotics supplementation seems to be promising, quite a few questions, such as timing, dosage, strains and duration

of probiotics administration, still need to be answered in order to create balanced gut microbiota composition.

1.6 Oral tolerance development

1.6.1 General mechanism of oral tolerance

Under normal physiological condition, our innate and adaptive immune systems will effectively respond to entering exogenous antigens while inducing immunological tolerance to self-antigens. In fact, the immune system has another kind of unique tolerance, known as oral tolerance, to certain foreign antigens including dietary nutrients and commensal microorganisms.

Oral tolerance can be defined as specific suppression of local and systemic immunological responses to an antigen which is achieved by prior ingestion of that antigen.

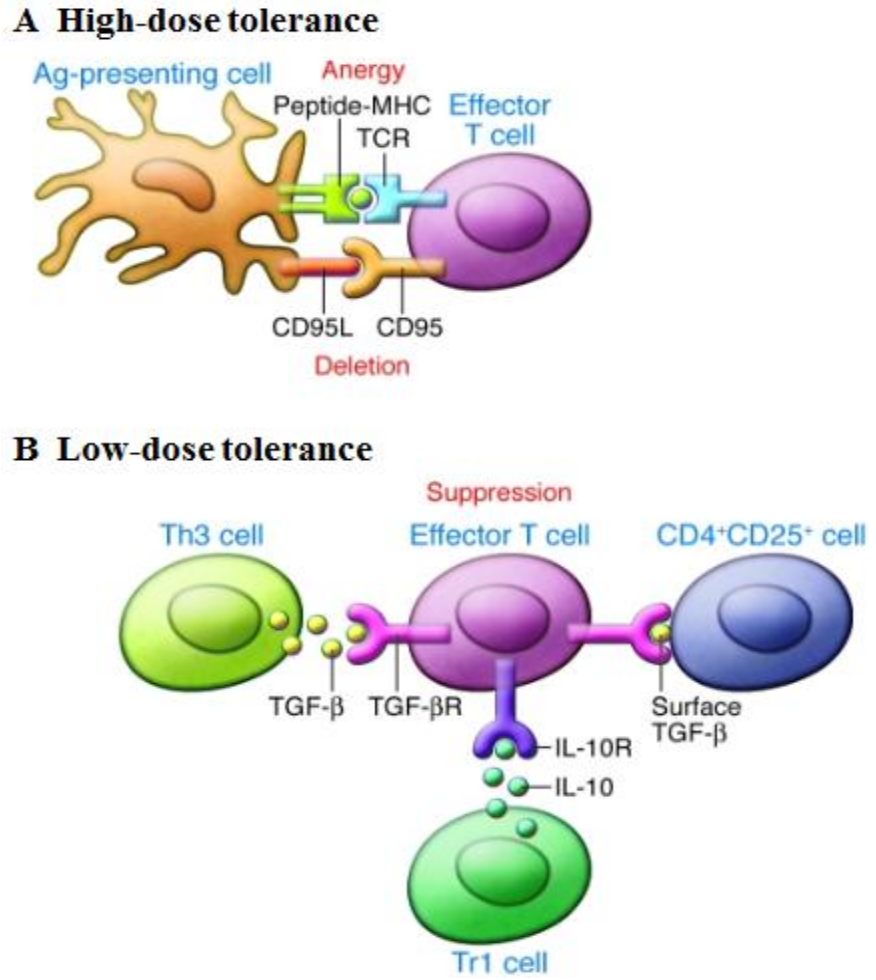
The role of digestive enzymes in oral tolerance development has been studied during the past two decades. When food is ingested, it goes through a series of mechanical and biochemical reactions, such as gastric acid and digestive enzymes; and is exposed to commensal gut microbiota in gastrointestinal (GI) tract until they are being absorbed. Michael (1989) demonstrated in a mouse model that either ingestion of bovine serum albumin (BSA) or injection of pepsin treated BSA to ileum was tolerogenic. However, injection of untreated BSA to ileum was found to be immunogenic.

In general, the intestinal immune system separates the ingested food (antigens) into offending and non-offending antigens; and responds accordingly. When non-offending antigens, such as dietary nutrients and commensal bacteria, are ingested, oral tolerance will be developed. But, local (secretory IgA) and systemic (cellular or humoral) immune responses will be induced when offending antigens, such as pathogens and their toxins, are ingested. Currently, studies on development of oral tolerance mechanism have gained much attention, due to the expectation that understanding such mechanism will give new insight to reduce or treat allergic diseases. Although the complete mechanism of oral tolerance development is not yet fully understood, some antigen related factors, such as dosage, frequency and nature of antigen; and some host related factors, such as maturity,

genetic background and gut microbiota composition of the host; have been identified as significant determinants for induction of oral tolerance (Chehade and Mayer, 2005).

Recent research advances on antigen dosage studies have suggested that oral tolerance can be induced by administering either low or high dose of allergens (Figure 1.2). Administration of low doses of antigens will favour naïve T cells (Th_0) to be differentiated into regulatory T cells (Treg) which secrete tolerogenic cytokines, such as transforming growth factor- β (TGF-β) and interleukin-10 (IL-10) (Miller et al., 1992). High dose allergens exposure can induce oral tolerance by lymphocyte anergy (absence of costimulation or interactions between CD28 on T cells and CD80/86 on APCs) and clonal deletion by FAS-mediated apoptosis (CD95) (Chen et al., 1995).

Figure 1.2 Mechanisms of oral tolerance[1]



[1]Adopted from Wang and Sampson (2011). (**A**) During high-doses of allergen exposure, allergen presentation to effector T cell can occur in the absence of costimulation or interactions between CD28 on T cells and CD80/86 on APCs or in the presence of inhibitory ligands (CD95 and CD95 ligand). (**B**) Low doses of oral allergen supplementation activate regulatory T cells (Treg), which in turn suppress the immune responses through IL-10, and TGF-β.

1.6.2 Development in oral tolerance induction

Although Foxp3+ Treg cells have gained great attention in oral tolerance development, recent findings suggest that other cell types, such as γδ T cells, and NKT cells, may also involve in oral tolerance (Wang and Sampson, 2011). Furthermore, gut mucosal

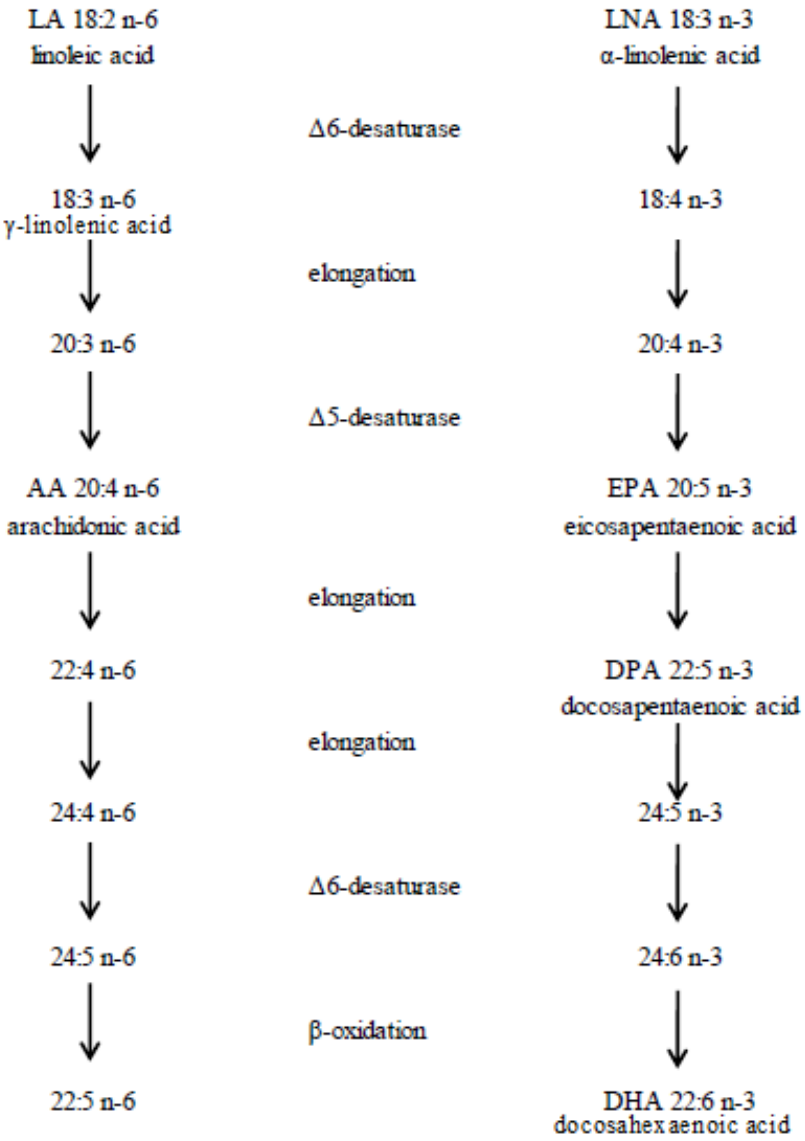
epithelium may, in addition to barrier function, be involved in oral tolerance induction. Because these epithelial cells, as well as stromal cells and basophils, express thymic stromal lymphopoietin (TSLP) which is known to enhance allergic Th2 responses in the gut without the need of primary sensitization or tolerance (Blazquez et al., 2010).

1.7 Dietary fatty acids

The dietary fats are readily available from animal and vegetable sources. They are usually present in the form of triacylglycerols (TG), the main storage form of body fat, in which three fatty acid moieties are bound to a glycerol backbone. Fatty acids serve as an important energy source for the body and are major components of all cell membranes, precursors to signalling molecules and inflammatory mediators production, and regulators of cellular responses (Calder, 2012). Fatty acids consist of unbranched hydrocarbon chains with a terminal carboxylic acid and can be further categorized into saturated fatty acids (SFAs) that contain no carbon double bonds, monounsaturated fatty acids (MUFAs) that have one double bond, and polyunsaturated fatty acids (PUFAs) that have more than one double bond. PUFAs can be classified into two main families namely, omega-3 and omega-6. The name omega (ω) is gained from the nomenclature system that counts the double bond from the methyl end of the fatty acid molecule. In general, naturally occurring fatty acids have an even number of carbon atoms and the orientation of double bonds is usually *cis,* rather than *trans* (Ratnayake and Galli, 2009). Most of the fatty acids, except linoleic acids (LA, 18:2 ω-6) and α-linolenic acids (ALA, 18:3 ω-3), are regarded as non-essential since humans can synthesize SFAs, mainly in the cytosol of liver and lactating mammary glands, and a smaller extent in the adipose tissue. The SFAs can be further converted to longer SFAs through elongation and to MUFAs by desaturation (Ratnayake and Galli, 2009). Major dietary sources of LA includes cereals, eggs, poultry, whole-grain breads, baked goods, margarine and most vegetable oils such as sunflower, saffola, and corn oils. For ALA, the major dietary sources are canola oil, flaxseed oil, linseed and rapeseed oils, walnuts, and leafy green vegetables such as purslane. The body can convert a certain amount of dietary LA and ALA to longer ω-6 and ω-3 PUFAs using common enzymes, Δ6- and Δ5-desaturases, in endoplasmic reticulum membranes. Therefore, these ω-6 and ω-3 PUFAs are competing for enzymes

in their metabolic pathways (Figure 1.3). On the other hand, longer chain ω-3 PUFAs, such as eicosapentaenoic acid (EPA, C20:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3) can also be directly acquired through the consumption of oily fish and seafood.

Figure 1.3 The metabolic pathway for polyunsaturated fatty acids linoleic and α-linoleic acids[1]



[1]Adapted from Johansson (2011).

1.7.1 Physiological roles of dietary fatty acids

Fatty acids have various structural and functional roles, and biological activities.

1.7.1.1 Energy reservoir

Excess fatty acids are stored as triacylglycerols in adipocytes and released to the blood as free fatty acids and glycerol when the body requires energy. These fatty acids bind to albumin and are then released from albumin and enter to the cells by passive diffusion. Activated fatty acids become fatty acyl-CoAs in cytosol and are transported to mitochondria to initiate the β-oxidation pathway. Through β-oxidation fatty acids chains are transformed into acetyl CoA molecules which subsequently enter the citric acid cycle and release energy, carbon dioxide and water (Mathews et al., 2000).

1.7.1.2 Vital components for cell membranes

Fatty acids are essential components of the phospholipid bilayer of all cell membranes. The composition of fatty acids in the phospholipid layers vary with the dietary intake and greatly influence the membrane fluidity. When high levels of saturated fatty acids and cholesterol integrated into the cell membrane phospholipids, more rigid membranes are formed. Membrane fluidity increases with higher proportion of PUFAs such as DHA because, unlike SFAs, they provide very flexible structure that allows instant conformational changes (Feller and Gawrisch, 2005). Cell membranes also have small areas of lipid complexes known as lipid rafts. These rafts are normally made up of SAFs, sphingolipids, cholesterol and glycosyl phosphatidyl inositol(GPI)- anchored proteins (Yaqoob, 2003).

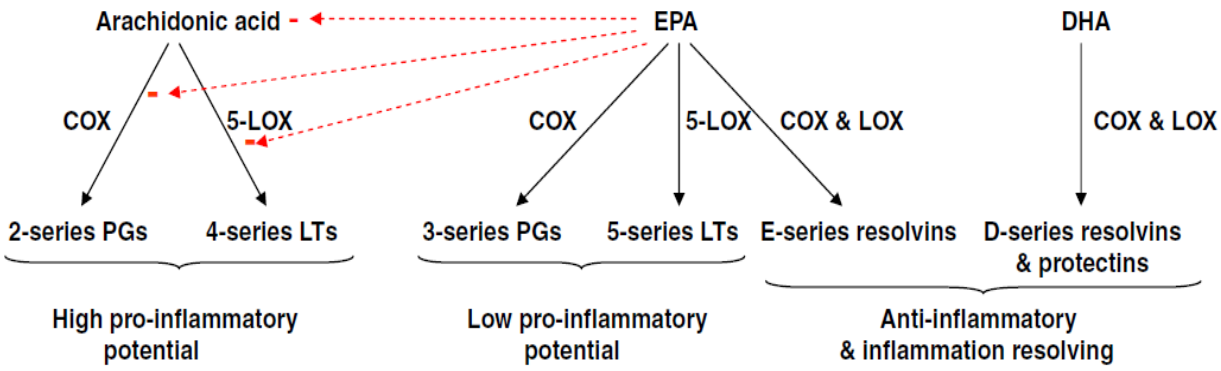
1.7.1.3 Precursor for signaling molecules and inflammatory mediators

The lipid rafts of the cell membrane regulate receptors and membrane protein trafficking and also serve as depot for intracellular signaling molecules (Yaqoob, 2003). Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that belong to nuclear receptor superfamily. Cell membrane bound PUFAs and their derivatives are endogenous ligands for PPARs. Recently, activation of PPARγ by DHA, ω-3 PUFA, was reported to induce lower pro-inflammatory cytokines (TNFα and IL-6)

production after endotoxin stimulation (Kong et al., 2010) suggesting that changes in PUFA intake can influence inflammatory process. Similarly, ω-3 PUFAs, but not ω-6 PUFAs, has been demonstrated, via binding of cell surface G-protein coupled receptor (GPR120) in macrophages, to inhibit LPS-induced IkB phosphorylation and subsequently inhibit the production of TNFa, IL-6, and monocyte chemotactic protein-1(Oh et al., 2010).

Moreover, PUFAs in the phospholipid layer and lipid rafts serve as precursors for inflammatory mediators. These mediators are formed from C_{20} PUFAs, usually AA, and thus called eicosanoids, which include prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT). When a cell receives an inflammatory signal, phospholipase A2 (PLA2) enzyme bind to the cell membranes and cause release of AA. This AA is further converted via cyclooxygenases (COX) and 5- lipoxygenases (5-LOX) to 2-series of PG and TX, and 4-series of LT. Cell membranes with higher ω-3 PUFAs content will release EPA, instead of AA, which will be converted via the same COX or lipoxygenases to 3-series PG and TX, and 5-series LT (Figure 1.4). In fact, EPA-derived 5-series LT, such as LTB_5 is (10-100) fold less potent as a neutrophil chemoattractant in comparison with AA-derived LTB_4 (Calder, 2012) . A recent study also reported that neutrophil-derived LTB_4 (metabolites of AA) is important for the development of allergic skin inflammation (Oyoshi et al., 2012). Lipid mediators derived from EPA and DHA via COX and lipoxygenase enzymes, such as E- and D-series resolvins, and protectins have inflammation resolving as well as anti-inflammatory properties (Calder, 2012). In addition, a recent murine model study demonstrated that DHA-derived D-series resolvins have anti-inflammatory and promote resolution of allergic airway responses (Figure 1.4) (Rogerio et al., 2012).

Figure 1.4 General overview of inflammatory potential of ω-6 and ω-3 PUFAs derived lipid mediators.[1]



[1]Adapted from (Calder, 2011)). COX, cyclooxygenase; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin.

1.7.2 Changes in Dietary fatty acids intake and allergy development

Allergy and other inflammatory diseases such as Crohn's disease, rheumatoid arthritis, type-1 diabetes, and multiple sclerosis, share a common characteristic of producing excessive or inappropriate inflammatory mediators including eicosanoids and cytokines (Calder, 2011). Continuously increasing allergy and asthma incidence, particularly in the industrialized nations, has been proposed to link largely with the changes in composition and quality of dietary intake, fatty acids in particular, within the past few decades. Total energy contribution of current western diet was carbohydrate- 51.8%, fat- 32.8%, and protein- 15.4% respectively (Cordain et al., 2005), while energy contribution from the diet of hunter gatherers was estimated at 46% from carbohydrate, 33% from protein, and 21% from fat (Cordain et al., 2002; Simopoulos, 2008). Application of recent technology development in farming (such as grain fed, feedlot farms that produce marbled meat), and food processing (e.g. introduction of novel *trans* fatty acids from hydrogenated vegetable oils), and changing of the eating habit (e.g. eating more ready-made and fast foods) have altered the dietary fatty acids profiles. Most of the SFAs rich diets of developed countries, such as fatty meats, baked goods, cheese, milk, margarine, and butter, would not have been available in the diet of people who lived prior to the development of animal husbandry and Industrial Revolution. Currently, it is estimated that 72.1% of total daily energy intake of the people in United States is so called "novel foods" that were

obtained via advanced technology in agriculture and food processing (Table 1.8) (Cordain et al., 2005).

Table 1.8 Food types found in diets of developed countries, which were unavailable to pre-agricultural hominins[1]

Food	Value (energy %)
Dairy products	
Whole milk	1.6
Low-fat milk	2.1
Cheese	3.2
Butter	1.1
Other	2.6
Total	**10.6**
Cereal grains	
Whole grains	3.5
Refined grains	20.4
Total	**23.9**
Refined sugars	
Sucrose	8
High-fructose corn syrup	7.8
Glucose	2.6
Syrups	0.1
Other	0.1
Total	**18.6**
Refined vegetable oils	
Salad, cooking oils	8.8
Shortening	6.6
Margarine	2.2
Total	**17.6**
Alcohol	1.4
Total energy	**72.1**

[1]Adapted from Cordain et al., (2005).

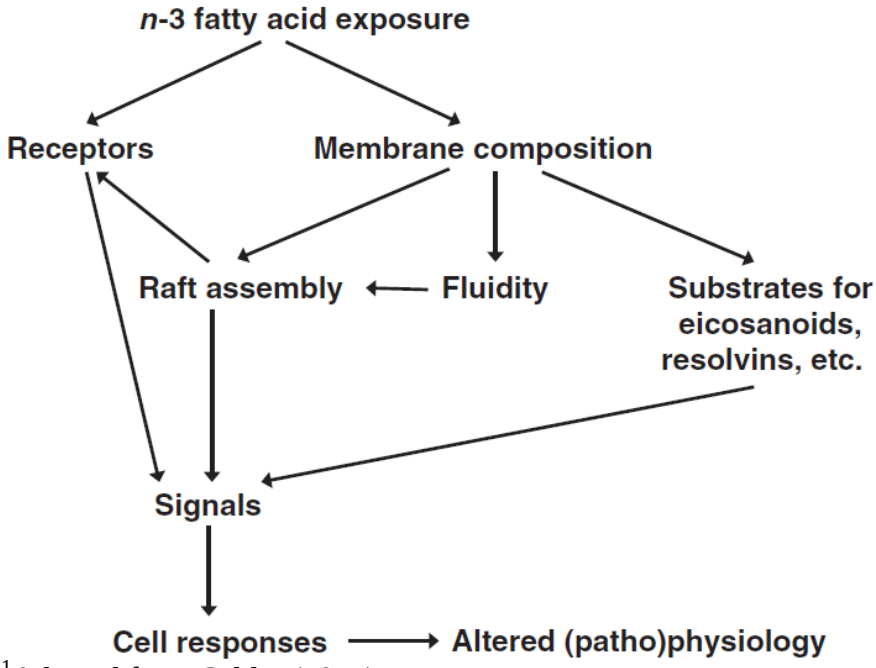
Emergence of the oil-seed processing industry at the beginning of the 20th century has also significantly increased vegetable oils consumption that creates a dietary fatty acid profile with high in ω-6 PUFAs but low in ω-3 PUFAs because most vegetable oils have high concentrations of ω-6 PUFAs and low concentrations of ω-3 PUFAs (Cordain,

2002). Because of the recent change of dietary fatty acid composition, present typical Western diet contains excessive *trans* fatty acids and SFAs and very high ratio of ω-6/ ω-3 PUFAs (Cordain et al., 2005). Currently, the ω-6/ ω-3 ratio in western diets has been estimated as (15-17):1 (Simopoulos, 2008), while estimation of ω-6/ ω-3 ratio for hunter-gatherers diet was no more than 3:1 (Cordain et al., 2002). The current recommended ratio is 4:1 (Wall et al., 2010).

1.7.3 Potential immunomodulatory effects of ω-3 PUFAs on allergy

The focus of ω-3 PUFAs specifically on allergy is initiated with the findings of the anti-inflammatory properties of these fatty acids and epidemiological relationship between diminishing ω-3 PUFAs intakes and growing allergy incidence (Calder, 2001, 2003). On the other hand, the fact of increased ω-6 intakes (approximately 15 times higher than ω-3) in western societies along with the findings of its pro-inflammatory property have suggested increased intakes of ω-6 PUFAs as an active allergy promoter. Currently, there are three potential mechanisms by which ω-3 PUFAs may modulate the function of inflammatory cells and thus alleviate allergic disease (Figure 1.5) (Calder, 2012).

Figure 1.5 Overview of the mechanisms by which ω -3 PUFAs can influence inflammatory cell function[1]



[1]Adapted from Calder (2012).

1.7.3.1 By incorporating into the phospholipids of inflammatory cell membranes

Inflammatory cells usually have a relatively high proportion of ω-6 PUFAs in their cell membranes. Bulk phospholipids of immune cells, such as lymphocytes, neutrophils, and macrophages, collected from rodents fed with standard chow contain 15-20% of fatty acids as ω-6 PUFA, AA, with very little ω-3 PUFAs (Calder et al., 1990; Calder et al., 1994) . Similarly, immune cells of humans consuming typical Western diets contain approximately 20% AA, 1% EPA, and 2.5% DHA in their bulk phospholipids (Calder, 2007). These findings suggest that cells involved in inflammatory response are typically rich in ω-6 PUFAs, particularly AA. However, increased consumption of ω-3 PUFAs can modify the fatty acid composition of cell membranes at the expense of ω-6 PUFAs, AA. The cell membranes with rich ω-3 PUFAs in phospholipids will increase membrane fluidity, influence lipid raft formation, create the proper environment for membrane protein functions, and serve as precursors for anti-inflammatory and less-potent inflammatory mediators (Yaqoob, 2009). It has been reported that PUFAs promote cell

membrane fluidity which is associated with higher expression of cell membrane receptors, and elevate their affinity to the corresponding proteins, e.g. increases in number of insulin receptors expression and reduces in insulin resistance (Coetzer et al., 1994; Das, 2005). In contrast, increased cell membrane rigidity is known to link with reduction of the number and affinity of cell membrane bound receptors.

1.7.3.2 By acting directly to cell surface or intracellular receptors

Bacterial lipopolysaccharides (LPS), through the recognition of cell surface receptor TLR4, activate NF-κB, a key transcription factor involved in upregulation of inflammatory cytokines, adhesion molecules and cyclooxygenase (COX)-2 genes, and induce inflammation. *In vitro* studies revealed that ω-3 PUFAs (EPA and DHA) inhibit the LPS-induced inflammatory cytokines (TNFα, IL-1, IL-6, IL-8, and IL-12) and COX-2 production from immune cells (Babcock et al., 2002; Khalfoun et al., 1997; Lo et al., 1999; Weatherill et al., 2005). Fish oil supplementation studies in mice reported similar finding of reduced TNFα, IL-1β, and IL-6 from LPS-stimulated macrophages (Renier et al., 1993; Yaqoob and Calder, 1995). In humans, some studies, but not all, reported that fish oil consumption lower TNFα, IL-1β, IL-6, and various growth factors from LPS-stimulated monocytes (Baumann et al., 1999; Caughey et al., 1996; Meydani et al., 1991; Trebble et al., 2003). On the other hand, lauric acids (SFA, C12:0) was capable to activate certain TLRs, particularly TLR4 and TLR2, enhance NF-κB activation, and subsequently promote inflammatory gene expression (Lee et al., 2001; Lee et al., 2004; Weatherill et al., 2005). This evidence suggest that saturated fatty acids, similar to microbial molecules, can lead to TLR-mediated inflammation, while ω-3 PUFAs inhibit TLR-mediated inflammation. Recent study had provided a new insight in understanding these mechanisms by demonstrating lauric acids induced, while DHA inhibited, dimerization and recruitment of TLR4 into lipid raft fraction of plasma membrane that lead to activation of downstream signalling pathways and target gene expression (Wong et al., 2009).

Furthermore, ω-3 PUFAs DHA can separately activate cell surface receptor GPR120 or nuclear receptor PPARγ, a transcription factor that acts in an anti-inflammatory manner,

and inhibit or reduce the production of pro-inflammatory cytokines TNFα, and IL-6 after stimulated with LPS (Kong et al., 2010; Oh et al., 2010).

1.7.3. 3 Precursors for molecules that have anti-inflammatory and resolving properties

The ω-3 PUFAs derived eicosanoids, such as 3-series of PGs and 5-series of LTs, have a low pro-inflammatory potential compared to those derived from ω-6 PUFAs, such as 2-series of PGs and 4-series of LTs. Moreover, fairly newly revealed metabolites of ω-3 PUFAs, including D- and E-series of resolvins and protectins, have inflammation resolving property and inhibition of inflammatory cytokines, such as TNFα and IL-1β, production (Serhan et al., 2008). These effects are summarized in Figure 1.4 (Calder, 2011).

1.8 The use of mouse models in the food allergy study

In theory, human beings are the most appropriate experimental subjects in the study of human food allergy. However, human subjects are no longer allowed to participate in the experimental studies that involve direct administration of allergens owing to a number of reasons, including the risk of anaphylactic shock, development of persistent allergy, and most importantly ethical reasons. Therefore, alternative animal models are necessary for the study of human food allergy. The use of rodents, dog and pig as model animals has been reported in the studies of human food allergy (Dearman and Kimber, 2009). An ideal model animal for food allergy study should reveal every aspect of clinical and immunological responses including hypersensitivity symptoms, induction of allergen-specific IgE antibody, that exhibit in humans. In reality, it is impractical to develop an animal model that possesses immune responses identical to humans because responsiveness to an allergen varies widely among the different animal strains and species (Goodman et al., 2005; McClain and Bannon, 2006). Therefore, certain strain or species of an animal that provide the most reliable and useful information is generally selected as a model animal for a particular study. There is no fixed single animal species that can be used as a model for all types of human disease studies. Dogs have been used

as a model animal to study allergenicity of the proteins since canine IgE-mediated food allergy is a common complication in veterinary clinics (Helm et al., 2003; Jackson and Hammerberg, 2002). Pigs are another large animal species used occasionally for food allergy study as their mucosal immunity development is closely resembled to that of humans (Bailey et al., 2005; Helm et al., 2003). There are many advantageous of using large animal models which include similarity of gut anatomy, physiology and nutritional requirements to humans, possibility to perform endoscopic analysis of GI tract, availability of a larger volume of blood for analysis and possibility to immunize the same animal with multiple allergens (Teuber et al., 2002). Nevertheless, the occurrence of a greater inter-animal variation in response to allergens in dogs and pigs than in rodents, lack of commercial immunological reagents and expensive maintenance cost make large animal models less attractive than rodent models. Rats and mice have, therefore, become interesting subjects to be used as model animals for food allergy research. Many investigators initially preferred to use rats, Brown Norway (BN) rat in particular, as model animals in the allergy study due to their ability to induce IgE antibody and their bigger body size than that of mice. Subsequent studies, however, reveal that rat's ability to induce IgE responses are greatly variable and depend on many factors including environmental conditions, age, sex and health status of the rat (Jia et al., 2005; Knippels et al., 2000; Pilegaard and Madsen, 2004). On the other hand, a mouse model, particularly Balb/C mouse, has become a well-established model for the study of various human allergic diseases such as atopic dermatitis (Li et al., 2001), asthma (Kuribayashi et al., 2002) and food allergy (Schouten et al., 2009). Highly resembling nature of immunological responses between mouse and human at cellular and molecular level make the mouse a popular model (Griffiths et al., 2005; Maizels, 2005). Moreover, the availability of inbred and high IgE responder mouse strain has made mouse as an extremely attractive animal model to study human food allergy (Mori et al., 1990).

In summary, food allergy is increasingly emerging as a global health problem and the impact is enormous. Food allergy affects not only susceptible individuals with severe clinical symptoms but it also demands lifestyle adjustment and causes financial and psychological damages to their families, caregivers, and the society. Continuous studies have brought new insights on food allergy development mechanism. Many promising

therapeutic approaches are currently being investigated as the result of such advancements. However, there are very little reports on mouse models of milk protein induced allergy, although CMA is the most prevalent food allergy in children of the developed nations. Furthermore, to the best of our knowledge, there is no published work using mouse models to examine the effects of promising probiotics (specifically on LGG and VSL#3), low doses of allergen supplementation, and omega-3 PUFAs rich Western-style diet on CMA. This thesis was, therefore, designed to address these issues.

WORKING HYPOTHESES AND OVERALL RESEARCH OBJECTIVES

1. Working hypotheses

- Supplementation of Probiotics such as *Lactobacillus rhamnosus GG* (LGG) would alter the composition of gut microbiota and contribute antiallergic property against dietary antigens by improving mucosal barrier functions, degrading food antigens, stimulating the secretory IgA and mucus production. Moreover, probiotics will influence the naïve CD4+ T cell differentiation and may assist in Th-shifting from Th2 to Th1 or Treg subset and prevent allergic reactions. In addition, we expect that supplementing multi-strains probiotics (VSL#3) would render more distinct beneficial outcomes.

- Repeated administration of low doses of allergen would induce oral tolerance against that particular allergen. We believe that low doses of milk proteins would activate Treg and result in active suppression of allergic responses through the released anti-inflammatory cytokines, IL-10 and TGF-β.

- Supplementing the diet with high levels of omega 3 PUFAs, through addition of EPA and DHA rich fish oil, would alter plasma fatty acid profiles, as well as cellular levels (membranes and mediators) fatty acid compositions. Since omega 3 derived mediators, such as resolvins and protectins, have anti-inflammatory property, high omega 3 supplemented animals would have reduced allergic reactions during challenge.

2. Overall research objectives

The overall objective of this thesis is to find intervention strategies to reduce development of milk protein allergy in a mouse model. Three experiments were carried out to test promising concepts. In first experiment, we studied the effects of a single strain probiotics (LGG) supplementation on the allergy development. In second experiment, we examined the effects of multi-strains probiotics (VSL#3) and/or low doses of allergen exposure on the allergy development. Lastly, we investigated the effects of high levels of omega 3 PUFAs on the milk protein allergy. For all three experiments, the effects of different treatments were determined by monitoring clinical response, for example hypersensitivity scores, local immune responses, (for example cytokines from mesenteric lymph nodes, and secretory IgA), and systemic immune responses including monitoring anaphylaxis shock (rectal temperature), allergen-specific immunoglobulins (IgE, IgG1, IgG2a and IgG) and serum cytokines.

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CHAPTER II EFFECTS OF *LACTOBACILLUS RHAMNOSUS* GG SUPPLEMENTATION ON COW'S MILK ALLERGY IN A MOUSE MODEL[¶]

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2.1 ABSTRACT

Background: Cow's milk allergy (CMA) is one of the most prevalent human food-borne allergies, particularly in infants and young children from developed countries. Our study aims to evaluate the effects of *Lactobacillus rhamnosus* GG (LGG) administration on CMA development using whole cow's milk proteins (CMP) sensitized Balb/C mice by two different sensitization methods.

Methods: LGG supplemented mice were either sensitized orally with CMP and cholera toxin B-subunit (CTB) as adjuvant, or intraperitoneally (IP) with CMP but without the adjuvant. Mice were then orally challenged with CMP and allergic responses were accessed by monitoring hypersensitivity scores, measuring the levels of CMP-specific immunoglobulins (IgG1, IgG2a and IgG) and total IgE from sera, and cytokines (IL-4 and IFN-γ) from spleen lysates.

Results: Sensitization to CMP was successful only in IP sensitized mice, but not in orally sensitized mice with CMP and CTB. Interestingly, LGG supplementation appeared to have reduced cow's milk allergy (CMA) in the IP group of mice, as indicated by lowered allergic responses.

Conclusions: Adjuvant-free IP sensitization with CMP was successful in inducing CMA in the Balb/C mice model. LGG supplementation favourably modulated immune reactions by shifting Th2-dominated trends toward Th1-dominated responses in CMP sensitized mice. Our results also suggest that oral sensitization by the co-administration of CMP and CTB, as adjuvant, might not be appropriate to induce CMA in mice.

2.2 BACKGROUND

Cow's milk allergy (CMA), an immunologically mediated reaction to cow's milk proteins (Bahna, 2002), is one of the most prevalent human food-borne allergies, particularly in infants and young children. In North America, incidence of CMA is estimated at 2.5% in children and about 1% in adults with a 75% outgrowing rate at 16 years of age (Sicherer and Sampson, 2010). Milk protein comprises a mixture of multiple proteins, including whey (such as β-lactoglobulin, α-lactalbumin and bovine serum albumin) and casein (such as α-S1-, α-S2-, β-, κ-, and γ-caseins) proteins. Hypersensitivity reactions may occur upon exposure to a single or multiple milk protein(s). Numerous attempts have been made to reduce or eliminate the allergenicity of milk proteins. Of these attempts, most have focussed on two approaches: to alter the structure and property of milk proteins through thermal treatments, biochemical processes (enzymatic digestion), irradiation (Lee et al., 2001) and high pressure treatments (Bonomi et al., 2003), and to modulate immune responses through sensitization and tolerance induction by means of controlled exposure to a specific allergen which is commonly referred to as specific immunotherapy (Vickery and Burks, 2009). Nevertheless, total avoidance of cow's milk or its associated products still remains as the best remedy for CMA. Hypersensitivity to orally ingested food usually occurs upon failure to induce oral tolerance. Research with germ-free mice has indicated that the interaction between allergens and host's gut microbiota plays a crucial role in oral tolerance development (Sudo et al., 1997) and in reducing secretions of allergen-specific antibodies (Hazebrouck et al., 2009). The gut microbiota is also reported to favour anti-allergenic reactions by mediating T-helper-1 (Th1) type of immunity (Martinez and Holt, 1999) or inducing IL-10 and transforming growth factor-β (TGF-β) that suppresses T-helper-2 (Th2) type of immunity (Kalliomaki and Isolauri, 2003). Recently, delayed microbial exposure and/or reduced diversity of the gut microbiota among children have been associated with higher allergy incidences (Adlerberth and Wold, 2009). This concept was first reported by Strachan (Strachan, 1989) and later widely known as the 'hygiene hypothesis'. Interestingly, whereas the gut microbiota of allergic infants contained higher levels of *Clostridia*, intestinal *Lactobacilli* and *Bifidobacteria* were more predominant among healthy infants (Bjorksten et al., 2001;

Sepp et al., 2005). Such findings have triggered considerable scientific interests in probiotics, particularly *Lactobacilli* and *Bifidobacteria*, for prevention or treatment of allergies among infants. The allergy reducing effects of probiotics against food allergens such as egg ovalbumin (Hougee et al., 2010; Shida et al., 2002) and whey proteins (Gonipeta et al., 2009) have been demonstrated in mouse allergy models. But, to the best of our knowledge, the probiotic effects of *Lactobacillus rhamnosus* GG (LGG) to reduce or control allergy to whole cow's milk protein (CMP) have not yet been reported in a mouse allergy model. We used the Balb/C mice model based on its similarity with the human immune system, most particularly the Th1 and Th2 responses that are profoundly involved in allergic responses (Romagnani, 1991).

Oral sensitization is well recognized as an ideal route to investigate allergic responses to food allergens. Because mice usually develop oral tolerance and fail to manifest allergic responses to ingested allergens, allergens are frequently co-administered with an adjuvant. However, recent reports indicate that commonly used adjuvants, such as cholera toxin (CT) and alum, possess immune-stimulatory properties that may falsely test non-allergenic food products as positive (Dearman and Kimber, 2009). Consequently, there is increasing interest to develop adjuvant-free systemic sensitization models for testing of food allergenicity in mice. The main objectives of this study were to evaluate the probiotic effects of LGG on CMA development in a Balb/C mouse model when using either adjuvant-assisted oral sensitization (CMP with cholera toxin B-subunit, CTB) method or adjuvant-free systemic sensitization (CMP only) method.

2.3 MATERIALS AND METHODS

Cow's Milk Proteins

Cow's milk proteins were prepared from fresh milk. Briefly, milk was defatted by centrifuging at 1,000 g for 10 min at 4 ºC and discarding the upper fat layer (Lara-Villoslada et al., 2004). After 12% trichloroacetic acid (TCA) (w⁄v) addition, milk proteins were allowed to precipitate for 2 h at 4 ºC before centrifuging at 9,300 g for 10 min at 4 ºC. The supernatant was discarded and equal volume of distilled water was added more than five times to the precipitated whole milk protein to remove excess TCA.

The concentrated CMP was then lyophilized and stored at 4 ºC. CMP's protein content (82.34 ± 0.53%) was verified by the Kjeldahl method whereas presence of major milk proteins was confirmed by 12% SDS-PAGE gel electrophoresis.

Mice

Three weeks-old female Balb/C mice were purchased from Charles River Breeding laboratories (St. Constant, Quebec, Canada). All mice were fed a diet that was free from animal proteins and microbes (Harlan Teklad, Madison, WI, USA). Feed and water were provided ad libitum. Mice were raised in individual cages, and under a 12L:12D lighting cycle, 20-24 ºC range of ambient temperature and 40-70% of relative humidity. The animal use protocol was approved by the McGill University Animal Care Committee.

Sensitization and Challenge Procedures

Intragastrically Sensitized (Gavage) Group. The experimental design for the CMP intragastrically sensitized group is shown in Figure 2.1a. At 4 weeks of age, mice were sub-divided into 5 experimental sub-groups (n=6/group) which included mice gavaged with: PBS (CTL-), PBS + CTB [0.25 µg/g BW] (CTB), and CMP [1mg/g BW] + CTB (CTL+) in a total volume of 200 µl at weekly intervals over 4 consecutive weeks. The last 2 treatments, namely LGG1 and LGG2, included mice from the CTL+ sub-group that were orally treated with a viable dose of LGG (1x 10^9 CFU/day) over 3 days per week. Mice in the LGG1 sub-group received their first LGG dose at 23 d which lasted over 5 weeks whereas LGG2 received LGG from 31 d of age over 4 weeks. The LGG dosage was adopted from a dose-response study with LGG strain HN001 in mice (Gill and Rutherfurd, 2001). Four weeks after the first sensitization, mice in all treatment groups were orally challenged with CMP as previously described (Lara-Villoslada et al., 2004) with minor modifications. Briefly, mice were fasted overnight and challenged with two doses of CMP (30 mg/mouse) at 30 min interval. Two hours after the last dose, mice were euthanized by carbon dioxide asphyxiation. Blood was collected by intracardiac puncture and then spleens were aseptically excised and cryopreserved for later cytokine analyses.

Intraperitoneally Sensitized (IP) Group. The experimental design for CMP intraperitoneally sensitized group is shown in Figure 2.1b. There were 4 treatments in the IP group of mice which included CTL-, CTL+, LGG1, and LGG2 similar to the gavage group of mice. At 6 and 7 weeks of age, each mouse in the 4 treatment groups received 2 doses of CMP (10 mg/mouse) dissolved in 250 µl of PBS intraperitoneally. One week after the second IP sensitization, mice were orally challenged with CMP, and blood and spleen samples were collected similar to mice of the gavage group.

Evaluation of Hypersensitivity Symptoms

Within an hour following the final oral CMP challenge, hypersensitivity symptoms were scored by a person blind to the study using the score system as described by Schouten et al. (2009). The scores were as follows: 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding and puffiness around the eyes and mouth; 4 = no activity after prodding, laboured respiration, and cyanosis around the mouth and the tail; and 5 = death.

Determination of Serum CMP-specific (IgG1, IgG2a and IgG) and Total IgE

Blood samples from mice in the gavage and IP groups were collected into serum separator tubes (Sarstedt, Montreal, Quebec, Canada). The serum portion was separated by centrifugation at 10,000 x g for 5 min at 20 ºC. Serum samples were then aliquoted into eppendorf tubes and stored at -20 ºC until analysis. CMP-specific serum immunoglobulins, namely IgG1, IgG2a and IgG as well as total IgE, were detected by ELISA. Briefly, 96-well plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 µg/mL of CMP in 0.1 mol/L Na-bicarbonate/carbonate coating buffer (pH 9.6). After overnight incubation at 4 °C, plates were washed 3 times with 150 µl of PBS plus 0.05% Tween-20 (PBS-T) and blocked with 100 µl of 2% fish gelatine (Sigma Aldrich, Ontario, Canada) in PBS-T for 1 h at 37 °C. Subsequently, the plates were washed 3 times and 100 µl of serially diluted serum samples (started from1:10 dilutions for IgE, IgG1 and IgG2a and 1:100 for IgG) were added to the wells and incubated at 37 °C for 90 min.

Plates were then washed 3 times, and 100 µL of horseradish peroxidase (HRP) conjugated anti-mouse antibodies (1:2000 for IgE, IgG1 and IgG2a and 1: 4000 for IgG) were added to each well. The plates were again incubated at 37 °C for another 60 min and washed 3 times. Then, 100µl of ABTS were added to each well and 15 min were allowed for the development of colorimetric reactions. Absorbance was read at a wavelength of 405 nm in a microplate reader (Bio-Tek, Winooski, VT). All analyses were performed in duplicates and the average values were used in statistical analysis. Serum titres were calculated by the intersection of least square regression of A405 versus logarithm of dilution (Kerro-Dego et al., 2006).

Cytokine Measurements from Spleen Lysates

Spleen lysates were prepared as previously described (Alkan et al., 1996). Briefly, individual spleens were placed in eppendorf tubes containing 0.5 ml of lysate buffer. The spleen cells were lysed and homogenized by sonication for 30 s on ice. Supernatants were collected after centrifugation at 17,500 g for 10 min at 4 ºC and stored at -20 ºC. Interleukin 4 (IL-4) and interferon gamma (IFN- γ) from spleen lysates were analyzed by commercially available ELISA kits following the manufacturer's protocol (R&D systems, Minneapolis, MN). Total protein content in spleen lysates was determined using the detergent compatible protein assay (Bio-Rad Laboratories Inc., Hercules, CA) and bovine serum albumin as standard. Cytokine concentrations from spleen lysates were expressed as pg/mg of total protein of spleen.

PCR Identification of Fecal Lactobacillus

For PCR analysis, 100 mg of fecal samples were aseptically weighed, placed in sterile tubes and homogenized in 1.4 mL stool lysis buffer (QIAamp DNA stool kit; Qiagen, ON, Canada). Genomic DNA extraction was performed according to the manufacturer's protocol. DNA was then amplified using the Crimson taq DNA Polymerase with Mg-free Buffer, 25 mM MgCl2, 10 mM dNTP and Crimson Taq DNA Polymerase (New England BioLabs, ON, Canada) and *Lactobacillus*-specific primers. The primer-set sequences

were as described previously (Blumer et al., 2007). PCR reactions were performed at 95 ºC for 2 min and 95 ºC for 30 s, followed by 30 cycles of 45s at 60 ºC and 68 ºC for 5 min in an Eppendorf Mastercycler EP Gradient 5341 (Fisher Scientific, ON, Canada). Finally, the presence of lactobacillus strains was detected by performing agarose gel electrophoresis 1% (w/v) with the PCR products and using genomic DNA from pure *L. rhamnosus* GG as control. The PCR amplicons were visualized under UV light (260 nm) followed by a subsequent SafeView nucleic acid staining (0.5 µg/ml; NBS Biologicals, UK).

Enumeration of Fecal Lactobacilli

Lactobacilli concentration was determined from fecal samples of IP mice at 16 and 30 d. At 12 h prior to sample collection, mice were transferred to new cages which were lined with moist paper towels instead of the standard rodent bedding. After fecal sample collection into sterile microbiology bags, mice were returned into their respective treatment cages. Sample homogenization, serial dilutions and culture on bacteria-specific agars were as previously described (Baurhoo et al., 2007). Briefly, fresh fecal pellets were diluted 10-folds by weight in buffered peptone water, homogenized, and serially diluted in 0.85% sterile saline solution. *Lactobacilli* were anaerobically cultured on *Lactobacilli* MRS agar for 24 h at 37 ºC. Bacterial colonies were counted at the end of incubation period. Microbiological analyses were performed in duplicates and the mean values were used in statistical analyses.

Statistical Analysis

Data were analyzed by a one-way ANOVA using the GLM procedure of SAS (SAS Institute, 2003) except for the hypersensitivity scores data which were analyzed using the Kruskal-Wallis test and SigmaStat software (Systat Software Inc., San Jose, CA). Differences among treatment means were tested using the Scheffe's multiple comparison test. *P*-values ≤ 0.05 were considered significantly different. Results are presented as

means ± SEM. All microbiological concentrations were subjected to base-10 logarithm transformation before analyses.

2.4 RESULTS

Hypersensitivity Responses

Hypersensitivity scores were recorded within an hour after the final challenge with CMP. In the IP group of mice, moderate level of discomfort was observed among CMP-sensitized mice in the CTL+, LGG1 and LGG2 treatment groups, while CTL- mice did not show any visible signs (Figure 2.2). The average scores for hypersensitivity symptoms were 2.5 ± 1.05 in CTL+, 1.33 ± 0.82 in LGG1, and 1.167 ± 0.75 in LGG2, respectively. There were no significant differences in hypersensitivity scores among mice in the CTL+, LGG1 and LGG2 treatment groups. But, in comparison with CTL+ mice, hypersensitivity scores were numerically lowered in LGG1 and LGG2 mice. In the gavage group, however, mice did not show any noticeable hypersensitivity responses (data not shown).

CMP-specific Immunoglobulin Levels in Serum

Serum samples were analyzed by ELISA. In the IP group of mice, CMP-specific IgG1, IgG2a and IgG levels were significantly higher in CMP-sensitized (CTL+, LGG1 and LGG2) than non-sensitized (CTL-) mice (Figure 2.3 and Table 2.1). Moreover, among CMP-sensitized mice, CMP-specific IgG2a level was higher in LGG2 than CTL+ mice. But, CMP-specific IgG1 levels were lower in both LGG1 and LGG2 in comparison to CTL+ mice. Total IgE levels were similar across all treatment groups (Table 2.1). But, in the gavage group, CMP-specific IgG1 level did not differ between mice in the CTL+, CTL- and CTB treatments (Table 2.2).

Cytokine Levels in Spleen Lysates

The IL-4 and IFN-γ concentrations from the spleen of mice in the IP group were not different across treatments (Table 2.3). In the gavage group of mice, although similar

levels of IL-4 were observed in all the treatments, IFN-γ levels were significantly lower in the CTL- mice when compared to mice in the remaining four treatment groups (Table 2.2).

Fecal Counts and PCR Analysis of Lactobacilli

In the IP group of mice, fecal *Lactobacilli* counts in LGG1 and LGG2 were similar to those in the CTL- and CTL+ groups at d 16 (Table 2.4). But, at d 30, mice in the LGG2 group had greater *Lactobacilli* counts than CTL- mice. However, *Lactobacilli* counts were similar between CTL+ and LGG1 mice groups.

PCR analysis was performed on fecal samples to determine whether probiotic-treated mice excreted higher lactobacilli. Indeed, higher concentrations of *lactobacillus*-specific products were detected in fecal samples from LGG-treated mice than mice in the control groups (data not shown). These findings were indicative of higher levels of the bacteria in the intestinal tract of LGG supplemented mice.

2.5 DISCUSSION

CMA is a global health concern that occurs more frequently among children than adults. In infants, high CMA incidence occurs upon first exposure to CMP, for example through infant formulas, while the immune system is still immature. On the other hand, the intestinal immune-modulating effects of probiotics (Savilahti et al., 2008) have been shown to reduce the risks of developing allergic diseases in both mice (Blumer et al., 2007; Hougee et al., 2010; Shida et al., 2002) and humans (Kalliomaki et al., 2001; Kukkonen et al., 2007). The present study evaluated whether oral LGG administration could help reduce or control CMA in Balb/C mice that were sensitized with CMP either via the oral (gavage) or systemic (IP) route. Moreover, to better simulate CMA in infants, we specifically used 3 week-old newly weaned Balb/C mice as an animal model and whole CMP as allergen rather than purified single CMPs.

In the IP group of mice both LGG1 and LGG2 groups, in comparison with CTL+ mice, may possibly alleviate allergy as indicated by numerically lower hypersensitivity responses (Figure 2.2), lower IL-4 levels, and lower CMP-specific IgG1 but higher IFN-γ and CMP-specific IgG2a levels (Figure 2.3 and Table 2.3). Generally, an increase in Th2

response in mice results in higher secretions of IL-4, and allergen-specific IgE and IgG1, whereas increasing the Th1 response leads to higher IFN-γ and IgG2a levels (Mosmann et al., 2005). Therefore, together with previous in vitro (Pochard et al., 2002) and mice (Kim et al., 2008; Shida et al., 2002) studies, our findings suggest that LGG supplementation may alleviate allergic reactions by supressing Th2-mediated immune responses. Similarly, allergy reducing effects by probiotics have been reported in clinical studies (Kalliomaki et al., 2001; Kukkonen et al., 2007), in which LGG or a mixture of probiotics containing LGG prevented atopic eczema in high risk infants. The anti-allergic effects of probiotics have also been demonstrated in ovalbumin-induced asthma (Hougee et al., 2010) and atopic dermatitis NC/Nga mice models (Sawada et al., 2007). However, we also observed that an additional week of oral LGG administration between the LGG1 and LGG2 groups of mice did not significantly alter total *Lactobacilli* counts, hypersensitivity scores, and serum concentrations of IL-4, CMP-specific IgG1 or CMP-specific IgG2a. Therefore, under the conditions of this study, it appears that greater probiotic supplementation beyond 4 time points had no major benefits in alleviating CMA. But, at this stage, the exact reason is unknown.

Elevated IL-4, allergen-specific IgE and IgG1 are well recognized principal immune mediators of IgE-mediated allergy. Allergen-specific IgE is not easy to be measured when using conventional methods because IgE is generally present at low levels in the serum and its measurement is more complicated by the serum's higher IgG levels. Moreover, it is reported that both CMP-specific IgE and IgG have competitive binding ability to similar epitopes regions of CMP, and that the greater serum IgG levels significantly reduced the binding capacity of serum IgE to CMP (Lehrer et al., 2004). But, in the event that specific IgE is undetectable or non-measurable, IgG1 can be used as a surrogate maker for IgE if it is accompanied by increased IL-4 (Faquim-Mauro et al., 1999; Miyajima et al., 1997). Although we were unable to detect CMP-specific IgE, our findings about elevated serum IL-4 together with significantly higher CMP-specific IgG1 in CTL+ mice in comparison with CTL- mice indicate that IgE-mediated allergy might have occurred in CMP-sensitized mice. In agreement with our results, CMP-specific IgE was also undetectable in other mice allergy studies (Lara-Villoslada et al., 2004; Lara-Villoslada et al., 2005). It appears that serum concentrations of allergen-specific IgE are

highly dependent on the type of administered allergen. For instance, when mice were orally sensitized with casein or whey, Schouten et al. (2008) successfully measured whey-specific IgE and IgG1 but could not detect casein-specific IgE. We presume that CMP-specific IgE responses could have been reduced in our study considering the fact that whole CMP contains 20% whey only, but 80% casein. In addition, we suspect that the direct ELISA method used in this study might not have been sensitive enough to detect and measure CMP-specific serum IgE.

Our results about higher hypersensitivity scores and significantly higher titres of CMP-specific antibodies (IgG1, IgG2a and IgG) in CMP-sensitized (CTL+) than CTL- mice demonstrate that adjuvant-free IP sensitization successfully stimulated CMP-specific immune responses (Figure 2.3 and Table 2.1). Therefore, adjuvant-free systemic sensitization mice models can well be adopted to study the allergenicity of low allergen-containing foods, considering that adjuvant co-administration may falsely test non-allergic food products as allergic (Dearman and Kimber, 2009).

We did not observe visible hypersensitivity symptoms and different levels of CMP-specific IgG1 and IgG between CTL+ and CTL- subgroups in orally sensitized (Gavage) mice (Table 2.2). We have chosen CTB due to its non-toxic adjuvant property and it has been used in food allergy studies (Spangler, 1992; Untersmayr and Jensen-Jarolim, 2006). However, it seems that under the conditions of this study, the oral administration of CMP and CTB mixture was not allergenic to mice. In addition to the well-known fact that oral sensitization could induce tolerance development, recent reports indicate that CTB possesses allergy suppressing rather than stimulating effects in mice through induction of secretory IgA (Smits et al., 2009). Based on our findings and the above-mentioned report, it seems that CTB may not be an appropriate oral adjuvant that can successfully induce CMP-specific allergic responses in Balb/C mice.

2.6 CONCLUSIONS

To our knowledge, we are the first to investigate the effects of LGG supplementation on CMA in mice that were sensitized with the whole CMP. We believe that the adjuvant-free systemic sensitization model may be particularly useful in the testing of food

products with low allergenicity. LGG administration seems to favour suppression of Th2 responses such as reduced hypersensitivity scores and lowered serum CMP-specific IgG1 while promoting Th1 responses by causing elevated IFN-γ and CMP-specific IgG2a levels. Although further experimental and clinical studies are required to elucidate the mechanism involved and complete beneficial effects of LGG, the current study suggests LGG as a potential preventive tool in the fight against CMA.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

CLT, JIB, and XZ designed the research; CLT and BB conducted the research; CLT analyzed the data; BB performed PCR and statistical analysis; CLT and XZ wrote the paper; JIB, BKS and XZ helped to edit the manuscript and CLT and XZ had primary responsibility for final content. All authors read and approved the final manuscript.

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2.8 TABLES AND FIRURES

Table 2.1. Immunoglobulins titres from the sera of IP mice.

Treatments	CMP-specific IgG	Total IgE*****
CTL-	71.67 ± 10.75[b]	74.67 ± 28.62
CTL+	101.0 ± 9.07[a]	43.67 ± 14.58
LGG1	102.17 ± 10.64[a]	42.67 ± 5.06
LGG2	112.17 ± 8.76[a]	79.43 ± 48.21

CTL-: PBS; **CTL+:** CMP (10mg/mouse); **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. Values are presented as Mean ± standard deviation. Values with different letters in the same column differ, $P \leq 0.05$.

*Data were not statistically different, P> 0.05.

Table 2.2. CMP-specific Immunoglobulin titres from the sera and cytokines level from spleen lysate in Gavage group.

Treatments	CMP-specific		Cytokine (pg/mg)	
	IgG*	IgG1	IL-4*	IFN- γ
CTL-	68.17± 4.14	35.33 ± 11.35[b]	9.70 ± 4.64	9.93 ± 0.37[b]
CTL+	68.0 ± 6.08	34.83 ± 5.87[b]	10.22 ± 5.65	13.65 ± 1.42[ab]
CTB	68.50 ± 3.45	59.50 ± 26.44 [b]	12.42 ± 6.41	15.52 ± 1.39[ab]
LGG1	76.83 ± 13.98	131.33 ± 51.39[a]	11.90 ± 4.60	14.47 ± 3.20[ab]
LGG2	69.67 ± 5.62	92.17 ± 37.47[ab]	8.42 ± 4.66	16.45 ± 2.26[a]

CTL-: PBS; **CTB:** PBS+ CTB (0.25µg/g BW); **CTL+:** CMP (10mg/mouse) + CTB; **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. Values with different letters in the same column differ, $P \leq 0.05$. Values are presented as Mean ± standard deviation.

*Data were not statistically different, P> 0.05.

Table 2.3. Cytokines level from spleen lysate of IP mice (pg/mg of total protein).

Treatments	IL-4 (pg/mg)*	IFN- γ (pg/mg)*
CTL-	15.33 ± 4.51	18.33 ± 3.25
CTL+	19.3 ± 6.36	21.53 ± 5.69
LGG1	17.35 ± 3.56	26.82 ± 8.02
LGG2	14.47 ± 5.07	26.3 ± 4.69

CTL-: PBS; **CTL+:** CMP (10mg/mouse); **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. Values are presented as Mean ± standard deviation.

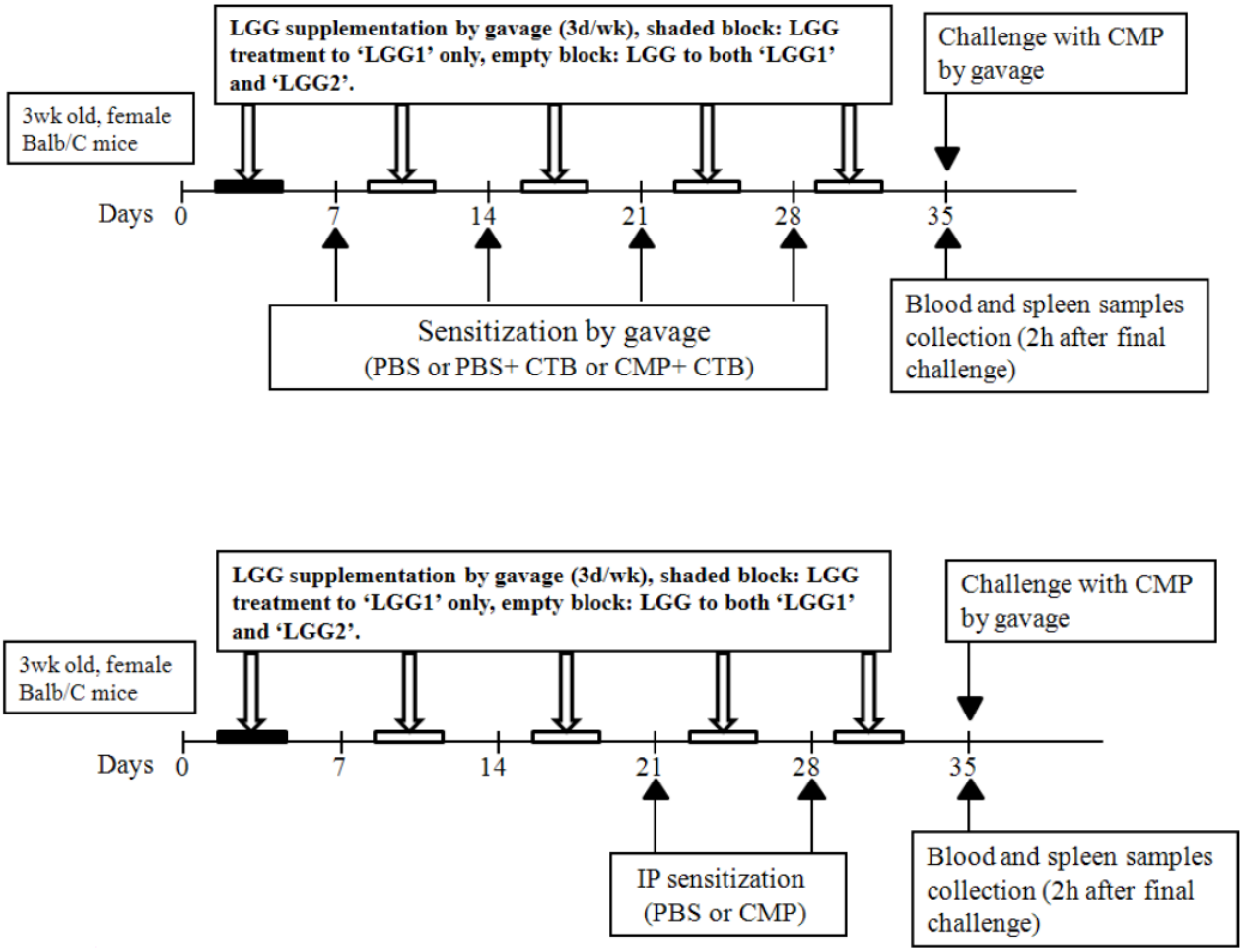
*Data were not statistically different, *P> 0.05*.

Table 2.4. Fecal counts of total *Lactobacilli* from IP group at d 16 and d 30 of age.

Treatments	*Lactobacilli*	
	d16	d30
CTL-	9.01 ± 0.08	8.70 ± 0.17[b]
CTL+	8.83 ± 0.16	8.89 ± 0.18[ab]
LGG1	9.04 ± 0.12	8.86 ± 0.10[ab]
LGG2	8.80 ± 0.28	9.03 ± 0.13[a]

CTL-: PBS; **CTL+:** CMP (10mg/mouse); **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. Data are presented as the mean log10 colony forming units/g ± standard deviation of fecal sample. For each bacterium, treatment means with different letters in the same column differ, $P \leq 0.05$.

Figure 2.1. Schematic overview of CMP sensitization and challenge protocol in Balb/C mice.



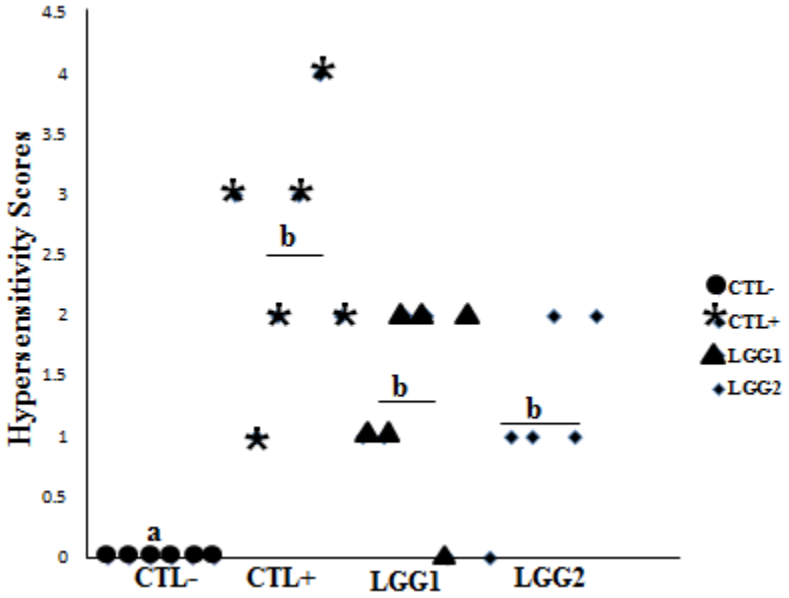
(a) Intragastrically sensitized (gavage) group

Mice were sub-divided into 5 treatments and sensitized intragastrically for 4 weeks as follows (n= 6); **CTL-:** PBS; **CTB:** PBS+ CTB (0.25µg/g BW); **CTL+:** CMP (1mg/g BW) + CTB; **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. At d 35, all sensitized mice were intragastrically challenged two times with CMP (30mg/mouse) at 30 min apart. Two hours after final challenge, mice were euthanized with CO2 inhalation and blood and spleen samples were collected.

(b) Intraperitoneally sensitized (IP) groups

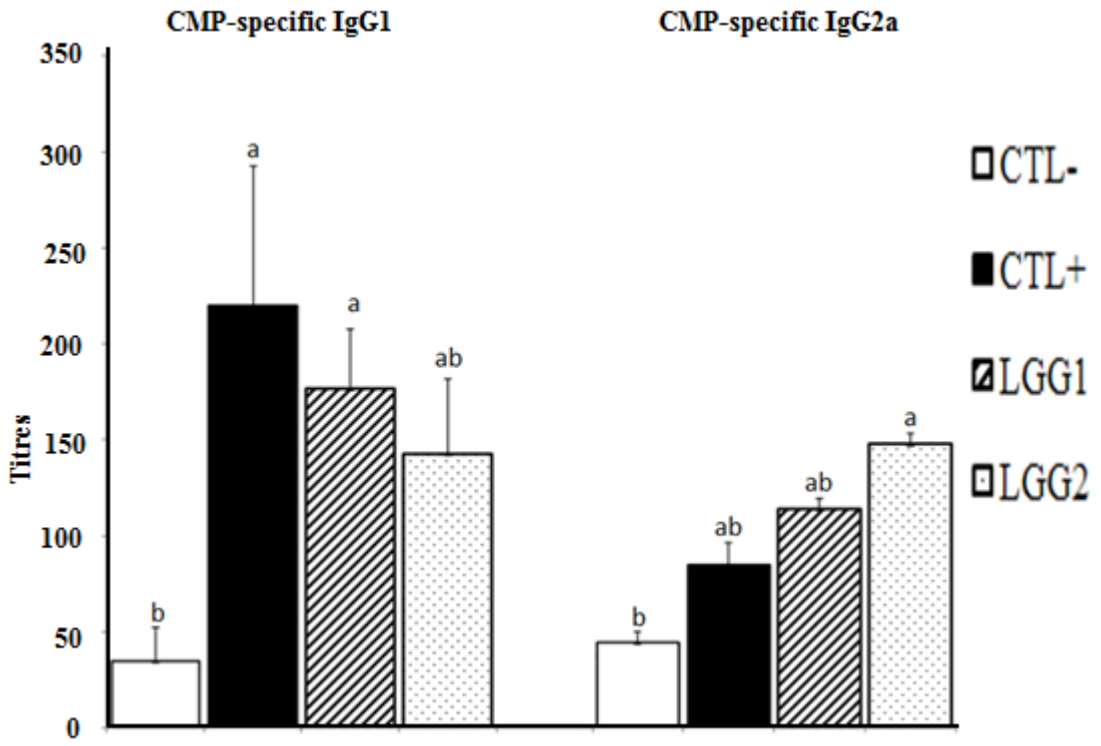
Mice were sub-divided into 4 treatments and sensitized intraperitoneally for 2 weeks as followed (n= 6); **CTL-:** PBS; **CTL+:** CMP (10mg/mouse); **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. At d 35, all sensitized mice were intragastrically challenged two times with CMP (30mg/mouse) at 30 min apart. Two hours after final challenge, mice were euthanized with CO2 inhalation and blood and spleen samples were collected.

Figure 2.2. Hypersensitivity scores of intraperitoneally sensitized (IP) mice.



Hypersensitivity symptoms were scored one hour after last challenge with CMP. Each point represents an individual mouse. Values are means, n = 6 per treatment. **CTL-:** PBS, control mice; **CTL+:** CMP sensitized mice; **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. Means with different letters differ, $P \leq 0.05$.

Figure 2.3. CMP-specific serum IgG1 and IgG2a titres of IP mice



CTL-: PBS; **CTL+:** CMP (10mg/mouse); **LGG1:** CTL+ mice supplemented with LGG for 5weeks and **LGG2:** CTL+ mice supplemented with LGG for 4weeks. Values with different letters in the same column differ, $P \leq 0.05$.

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CONNECTING STATEMENT 1

In Chapter II, we investigated the effects of LGG probiotic administration on whole cow's milk proteins (CMP) induced allergy by either oral (Gavage) or intraperitonael (i.p,) sensitization of Balb/c mice. The main objectives for this experiment were (1) to determine a better sensitization model between gavage and i.p., and (2) to determine the effects of LGG on cow's milk allergy (CMA) development.

To further explore the potential of probiotics, a multi-strains probiotics, VSL#3, was studied, using i.p. sensitization method (PROC group) in the next experiment. In addition, we also included oral tolerance induction group (via low doses of allergen supplementation) in the experiment (OT group). In order to see the combined benefits of both probiotics and oral tolerance induction, a treatment group that would receive both VSL#3 and low doses of allergen (OTP group) were also included in the same experiment (Chapter III).

CHAPTER III. LOW DOSES OF ALLERGEN AND PROBIOTIC SUPPLEMENTATION SEPARATELY OR IN COMBINATION ALLEVIATE ALLERGIC REACTIONS TO COW'S β-LACTOGLOBULIN IN MICE[¶]

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Running title: **Oral tolerance and probiotics on food allergy**

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3.1 ABSTRACT

Probiotic supplementation and oral tolerance (OT) induction can reduce certain types of food allergy. The objectives of this study were to investigate allergy reducing effects of probiotics (VSL#3) and/or OT induction via low doses of an allergen supplementation in β-lactoglobulin (BLG)-sensitized mice. Three-week-old, male BALB/c mice were divided into six groups (n=8 per group); sham-sensitized negative control (CTL-), BLG-sensitized positive control (CTL+), oral tolerance-induced and BLG-sensitized group (OT), probiotic-supplemented OT group (OTP), probiotic-supplemented CTL- (PRO), and probiotic-supplemented and BLG-sensitized (PROC) groups. Mice were intraperitoneally (i.p) sensitized with BLG and alum, and then orally challenged with BLG. Immunological responses were assessed by monitoring hypersensitivity scores and measuring levels of BLG-specific serum immunoglobulins, total serum IgE and fecal IgA, and cytokines from serum and spleen lysates. In comparison with CTL+ mice, PROC, OT, and OTP mice had significantly lower hypersensitivity scores (2.00 ± 0.53, 0.75 ± 0.46 , 1.00 ± 0.53 and 2.63 ± 0.52 for PROC, OT, OTP and CTL+, respectively) and BLG-specific serum IgE levels, (34.3±10, 0.442±0.36, 3.54±3.5 and 78.5±8.7 μg/L for PROC, OT, OTP and CTL+, respectively). Our results suggest that supplementation of VSL#3 suppressed the allergic reaction mainly through increased intestinal secretary IgA (sIgA) in PROC mice, while OT offered allergen-specific protective effects to BLG induced allergy probably through CD4+CD25+ regulatory T cells mediated active suppression. In OTP mice, probiotics did not induce further reduction of hypersensitivity score compared to OT mice but may provide additional protection to unforeseen non-specific challenges through increased intestinal sIgA.

3.2 INTRODUCTION

Food allergy is a complex disease that poses a growing threat to public health and the underlying immunological mechanisms are still not fully understood. Cow's milk protein allergy (CMA) is the most common food allergy in infants and children from developed countries with an estimated incidence rate of 2.2% (Boyce et al., 2010). Although CMA patients may be allergic to several milk proteins, caseins and β-lactoglobulin (BLG) are considered as the most allergic cow's milk proteins (Wal, 2002). Depending on the nature of immunological response, CMA may be divided into IgE-mediated (immediate reactions) and non-IgE-mediated (delayed reactions) reactions. Clinical manifestations of CMA include skin reactions (e.g. urticaria, angioedema, and contact dermatitis), respiratory reactions (e.g. wheezing, rhinitis, dry cough, laryngeal edema, and asthma with severe respiratory distress), and gastrointestinal reactions (e.g. diarrhea and vomiting, bloody stools, constipation, severe irritability colic). IgE-mediated allergic reactions are exhibited within minutes to an hour after an allergen exposure (typically within 10-20 min) with a risk of anaphylaxis, while non-IgE-mediated reactions are displayed within an hour to several days after ingestion of an allergen (Fiocchi et al., 2010; Solinas et al., 2010). In general, healthy individuals with mature immune systems have tolerance to most ingested food proteins and commensal bacteria (Burks et al., 2008). Food allergy may develop when specific oral tolerance against ingested food is impaired or failed to develop. Results from mouse models have showed promising protection effects of oral tolerance induction on food allergy (Adel-Patient et al., 2011; Yamashita et al., 2012). On the other hand, emergences of the hygiene hypothesis and the findings from a germ-free mouse study have revealed a protective role of gut microbiota in food allergy development (Sudo et al., 1997). Recently, a mouse model study on CMA reaffirmed that germ-free mice were more susceptible to CMA than conventional mice (Rodriguez et al., 2011). Another recent finding demonstrated that continuous microbial exposure at the early stage of life was necessary in establishment of a normal gut microbiota and this process was compromised under excessive hygienic conditions (Schmidt et al., 2011). Gut microbiota is essential for initiation of an infant's own IgA production and influences development of the entire IgA system (Macpherson et al.,

2000). We hypothesized that mice with prior supplementation of low doses of an oral allergen and probiotics individually or concurrently before the CMA challenge would alleviate CMA attack. Therefore, the purpose of this study was to investigate effects of oral tolerance and/or probiotics on allergic responses to bovine β-lactoglobulin.

3.3 MATERIALS AND METHODS

Animals and experimental design

Three weeks-old male BALB/c mice (Charles River laboratories, St. Constant, Quebec, Canada) were fed a diet free from animal proteins (no. 2918 Teklad Harlan Global Diet) (Theil et al., 2012) and raised as previously described (Thang et al., 2011). Our choice of just-weaned 3 week old mice was intended to achieve better reflection of human infant food allergy. Mice were used in the experiment after a week of acclimation period. Animal care use and protocols were approved by the McGill University Animal Care Committee.

A food allergy sensitization method developed recently in our lab was used in this study with minor adjustments (Thang et al., 2011). Systemic sensitization through i.p. injection of allergens with alum was used in this study, since alum has been increasingly applied in mouse models for oral tolerance and food allergy studies (Adel-Patient et al., 2011; Shin et al., 2010) due to its relative safety and strong Th2-favoured adjuvant nature.

Mice were divided into six groups: oral tolerance induced mice (OT), probiotic-supplemented mice (PROC), oral tolerance induced and probiotic-supplemented mice (OTP), control mice for probiotic supplementation that received probiotics but were sham-sensitized with PBS (PRO), BLG sensitized positive control mice (CTL+) and sham-sensitized negative control (CTL-), with 8 mice per group. The oral tolerance induction, BLG sensitization and probiotic supplementation schedules are detailed in Figure 3.1. Briefly, starting at 28d of age, oral tolerance induction to OT and OTP mice were performed by intragastric administration of 2 mg of BLG (Sigma Aldrich) in 200 µL of PBS for 5 consecutive days. VSL#3 probiotics were supplemented daily via gavage to OTP, PROC and PRO mice (15×10^9 CFU in 250 µL of PBS) from week 4 to week 9 of age. VSL#3 is a well-recognized mixture of probiotics that contain 8 species of freeze-

dried live probiotics namely: *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus delbrueckii* subsp. *Bulgaricus, Bifidobacteria breve, Bifidobacteria infantis, Bifidobacteria longum,* and *Streptococcus salivarius* subsp. *thermophilus*. VSL#3 has been shown to promote gut health and prevent chronic intestinal inflammation through stimulation of epithelial innate immunity (Pagnini et al., 2010) and also attenuate liver fibrosis (Velayudham et al., 2009). The dosages of BLG and VSL#3 were determined after careful consideration of dosages used in previously published papers (Pagnini et al., 2010; Shin et al., 2010; Wang et al., 2009; Yamashita et al., 2012). OT, OTP, PROC and CTL+ mice were sensitized i.p. with 3 weekly (at weeks 6, 7 and 8) doses of 250 µL PBS based solution that contained BLG (50µg) and 2 mg alum (Pierce Chemical, Rockford, IL). One week after the last sensitization, mice were challenged intragastrically with 2 doses of 30 mg of BLG in 250 µL PBS solutions 30 min apart. In every step of the treatment and sensitization, negative control mice were sham administered through the same route with the same volume of PBS solution devoid of BLG or probiotics.

Reagents. Horseradish peroxidase conjugated anti-mouse antibodies were obtained from Bethyl Laboratories (Montgomery, TX). Purified anti-mouse immunoglobulins were obtained from BD Pharmingen (San Diego, CA) and ABTS peroxidase substrate was purchased from Mandel Scientific (Guelph, Ontario, Canada). Bio-Plex Pro Mouse Cytokine Assay kits (Bio-Rad, Hercules, CA) were used to detect spleen lysate cytokines. VSL#3 was obtained from VSL pharmaceuticals Inc. (Fort Lauderdale, FL). All other chemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Nepean, ON, Canada).

Evaluation of Hypersensitivity Symptoms

Within an hour following the final oral BLG challenge, hypersensitivity symptoms were scored by a person blind to the study using the score system as described by Schouten et al. (2009). The scores were as follows: 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding

and puffiness around the eyes and mouth; 4 = no activity after prodding, labored respiration, and cyanosis around the mouth and the tail; and 5 = death.

Fecal, serum and spleen sample collections

Fresh fecal pellets were collected at d 51 of age by transferring mice into new cages which were lined with moist paper towels. Fecal pellets were immediately stored in -80 ºC until further analysis. Three hours after the final oral BLG challenge, mice were euthanized by CO_2 inhalation and blood samples were collected by intra-cardiac puncture. Blood samples were collected into serum separator tubes (Sarstedt, Montreal, Quebec, Canada). The serum portion was separated by centrifugation at 10,000 x g for 5 min at 20 ºC. Serum samples were then aliquoted into eppendorf tubes and stored at -20 ºC until analysis. Spleens were also aseptically collected, snap frozen in liquid nitrogen and stored in -80 ºC until further analysis.

Determination of Serum immunoglobulins and fecal IgA

BLG-specific serum IgG1, IgG2a and IgA as well as total IgE and total fecal IgA were measured by indirect ELISAs by adopting previously described procedure with minor modifications (Thang et al., 2011). Briefly, 96-well plates (Dynatech Laboratories, VA) were coated with 50 mg/L of BLG in 0.1 mol/L Na-bicarbonate/carbonate coating buffer (pH 9.6). Serum samples were diluted 1:100 for BLG-specific serum IgE, 1:200 for total IgE, and 1:1000 for other antibodies, such as IgG1, IgG2a, and IgA, before being used in the assays. All other procedures and conditions, such as washing, blocking, incubation, volume and diluent used, were exactly the same as the procedure previously described (Thang et al., 2011).

For detection of total and BLG-specific serum IgE, a Signal Boost Immunoreaction Enhancer Kit (EMD Millipore, USA) was used to dilute serum and HRP-conjugated anti-mouse IgE. For fecal IgA concentration, fecal supernatants were prepared from fecal samples as described by Adel-Patient et al. (Adel-Patient et al., 2005). Fecal pellets (100 mg) were placed in 1 mL of PBS containing 0.01% (v/v) of the protease inhibitor cocktail (Sigma-Aldrich). The mixture was vortexed and centrifuged at

16,000g for10 min. Supernatants were collected. Undiluted supernatants were used to determine fecal secretory IgA (sIgA) by ELISA. All analyses were performed in duplicates or triplicates and the mean values were used. Relative immunoglobulin concentrations were determined by extrapolation from standard curves with purified mouse immunoglobulin standards. The limit of detection for BLG-specific IgE, IgA, and IgG subclass was 3.7 µg/L.

Cytokine analyses of serum samples and spleen lysates

Spleen lysates were prepared as previously described (Alkan et al., 1996; Thang et al., 2011). Briefly, individual spleens were placed in eppendorf tubes in 0.5 mL of PBS containing 0.01% (v/v) of the protease inhibitor cocktail. Cytokines from spleen lysates and serum samples were analyzed by Mouse Cytokine 23-plex Assay kits. After following the manufacturer's protocol, beads were read with Bio-Plex Reader and data were analyzed with the Bio-Plex Manager 2.0 software (Bio-Rad Laboratories, Hercules, CA).

Statistical analyses

Data were analyzed by using PROC MIXED of SAS 9.3 (SAS Institute, 2003) except for the anaphylaxis data, which were analyzed using the Kruskal-Wallis test and SigmaStat software (Systat Software Inc., San Jose, CA). Differences among treatment means were tested using the Scheffé multiple comparison test. Data are expressed as means ± SE, and a *p*-value less than 0.05 is considered significantly different.

3.4 RESULTS

Hypersensitivity scores

Hypersensitivity scores were recorded within an hour after the final challenge with BLG. Hypersensitivity scores were significantly lower in OT, OTP, and PROC groups than that in the CTL+ group (Figure 3.2).

BLG-specific and total immunoglobulins from serum

As shown in Table 3.1, serum BLG-specific IgE and total IgE levels were significantly higher in CTL+ mice in comparison with all other groups (P <0.05). Serum BLG-specific IgE and total IgE levels in the PROC group were significantly lower than those in CTL+ mice, but were significantly higher than those in the CTL-, OT, and OTP groups (P <0.05) . There were no differences of serum BLG-specific IgE and total IgE levels among the CTL-, PRO, OT, and OTP groups. In contrast to their serum BLG-specific IgE and total IgE levels, BLG-specific serum IgG1 levels in PROC group was significantly higher than that in CTL+ group (P <0.05) (Table 3.1). BLG-specific serum IgG2a levels in the CTL+ and PROC groups were similar and significantly higher than those in the CTL-, OT, and OTP groups (P <0.05) (Table 3.1).

BLG-specific serum and total fecal IgA

Both OT and OTP groups had significantly lower BLG-specific serum IgA levels in comparison to PROC group (Table 3.1). In fecal samples, non-specific sIgA levels were detectable from all treatment groups. However, probiotic-supplemented mice, namely PRO, PROC, and OTP groups, had significantly higher total fecal sIgA levels than groups of mice that did not receive probiotic: CTL-, CTL+, and OT groups (P <0.05) (Table 3.1).

Cytokines concentrations in serum and spleen lysates

Serum and spleen lysate cytokines levels of PRO mice were lower than other groups (Tables 3.2, 3.3). IL-4 levels in spleen lysates were significantly higher in CTL+ group compared with that in the CTL- group (P <0.05). IL-4 concentrations of the other groups were between those of the CTL+ and CTL- groups and did not differ from one another (Table 3.2). Spleen lysate IFN-γ levels in OT group were higher significantly compared to CTL- and PRO groups; but did not differ from the CTL+, PROC, and OTP groups (Table 3.2). Spleen lysate IL-10 levels were significantly higher in BLG-sensitized groups, namely CTL+, OT, PROC, and OTP; in comparison with groups without BLG sensitization (PRO and CTL-) (Table 3.2). OT and OTP groups had significantly higher

levels of serum IL-12(p40) in comparison to CTL+ group. But, no differences were observed among the PROC, PRO, CTL+, and CTL- groups or between the OT and OTP groups (Table 3.3). Serum cytokine concentrations (both Th1- and Th2- mediated profile) were similar in PROC and CTL+ mice (Table 3.3).

Mouse monocyte chemoattractant protein-1 (MCP-1) from serum and spleen lysates

MCP-1 levels, in both serum and spleen lysates, were significantly higher in the OT group compared to the other groups. Serum MCP-1 levels were lower significantly in PRO group and spleen lysates MCP-1 levels were also lower significantly in CTL- and PRO groups in comparison with other groups (Table 3.4).

3.5 DISCUSSION

Probiotic supplementation and oral tolerance induction, individually or concurrently, reduced bovine β-lactoglobulin induced allergy in this study as indicated by significantly lower hypersensitivity scores and BLG-specific serum IgE levels in OT, OTP and PROC mice in comparison with CTL+. Examination of immunological parameters suggested that oral tolerance induction and/or probiotic supplementation may have distinct mechanisms to reduce allergy.

Two widely acknowledged mechanisms for oral tolerance induction are active suppression through CD4+CD25+ regulatory T cells (Treg) (Dubois et al., 2003) and clonal anergy or deletion through apoptosis (Friedman and Weiner, 1994). Active suppression could be induced by low doses of allergen supplementation and clonal anergy could be induced by one time supplementation of a high dose allergen (Faria and Weiner, 2005). However, researchers now have focused mainly on low doses of allergens to induce oral tolerance because high dose allergen supplementation is often associated with anaphylactic shock. Thus, we administered low doses of BLG (2mg of BLG for 5d) to induce active suppression in mice. Elevated food allergy markers, such as higher hypersensitivity scores along with higher BLG-specific serum IgE, IgG1 and spleen-derived IL-4, in CTL+ mice (Tables 3.1, 3.2) confirmed the validity of our model.

OT mice were capable of suppressing both allergen-specific Th1 response, indicated by reduced BLG-specific serum IgG2a and elevated IL-12(p40), and Th2 response, as indicated by lower BLG-specific serum IgE and IgG. Our results were similar to those from a mouse peanut allergy model study (Strid et al., 2004). In addition, our results were in full agreement with the observation from Adel-Patient et al. (Adel-Patient et al., 2011), who reported that oral tolerance and Tregs were induced in BALB/c mice after gavage with bovine BLG. Furthermore, recent studies have demonstrated that allergy suppression induced by oral tolerance were mediated by CD4+CD25+ Treg that express the transcription factor forkhead p3 (Foxp3) (Adel-Patient et al., 2011; Dubois et al., 2009; Rupa and Mine, 2012). All of these reports clearly support the notion that the major pathway for OT induced tolerance in our study was associated with $Foxp3^{+}$Treg mediated active suppression.

Intestinal sIgA may not be involved in OT induced tolerance, since total fecal IgA and BLG-specific serum IgA levels in OT mice were lower than those in CTL+ mice (Table 3.1). Our finding of lower allergen-specific serum IgA and fecal IgA in OT mice was also in full agreement with the result of a recently published paper (Yamashita et al., 2012) which stated that OVA-specific serum IgA was suppressed at the basal level in oral tolerance induced mice. In addition, we have chosen MCP-1 as a complementary marker for histamine release by basophils and mast cells because MCP-1 causes mediator release from these cells in a dose-dependent manner (Conti et al., 1995). Interestingly, both serum and spleen MCP-1 concentrations in OT mice were higher than those in the CTL+ group (Table 3.4) while OTP group showed a similar MCP-1 level with CTL+ group. This suggests that OT may induce higher levels of MCP-1 and probiotics may prevent OT-induced increase in MCP-1 production. In line with our result from the OT group, a recent CMA desensitization study revealed higher levels of serum MCP-1 as well as lower CMP-specific IgE in CMA subsided children (Glez et al., 2012).

Probiotics have also been used to suppress food allergy. Our results from PRO mice indicated that, under normal unchallenged condition, probiotics have tendency to suppress both Th1- (lower serum IFN- γ) and Th2- (lower serum IL-5, IL-9 and IL-13) associated immune responses (Table 3.3). However, this probiotic-induced basal immunological setting was changed when the mice were sensitized and challenged with

BLG. Examination of immunological responses in PROC mice revealed that probiotics supplementation may alleviate allergic reactions mainly through producing sIgA.

Higher fecal IgA levels in PROC in comparison with CTL+ support the notion that sIgA was responsible for allergy reducing effect of probiotics in this study. In line with our findings, a study conducted in a cohort of allergy-prone infants revealed that probiotic supplementation tended to increase fecal IgA and it is associated with reduced risk for IgE-mediated allergic diseases (Kukkonen et al., 2010). The immunological response pattern of VSL#3 supplemented mice in our study was also in complete agreement with the findings of a recent study (Schiavi et al., 2011) that reported the allergy suppression effects of VSL#3 through elevated fecal IgA in shrimp tropomyosin protein sensitized mice. Probiotics have been reported to increase total and antigen/allergen-specific sIgA (Galdeano and Perdigon, 2006). Large amounts of noninflammatory IgA can be generated in gut with or without T cells involvement. T cells-dependent intestinal IgA production is initiated by Ig class switching of IgM^+ B cells to IgA^+ B cells in Payer's patches that requires IgA class switching factors, such as B cells-activating factor of the TNF family and a proliferation-inducing ligand released by dendritic cells and other cell types (He et al., 2007; Litinskiy et al., 2002; Xu et al., 2007). On the other hand, T cells-independent sIgA are generated in lamina propria without T cells involvement. It was estimated that almost half of the intestinal sIgA were T cells-independent (Kroese et al., 1989). Taken together, these findings strongly suggested that CMA reduction in our PROC mice was achieved at least partly through high and low affinity IgA produced by activated B cells.

Lower levels of BLG-IgE and higher levels of BLG-IgG1 were seen in PROC mice in comparison with CTL+ (Table 3.1). However, similar levels of Th1-favoured IgG2a and IL-12, Th2 cytokines IL-5 and IL-9 were observed between 2 groups (Table 3.3). Further, high levels of BLG-IgG1 were observed in PROC mice (Table 3.1). These contradictory results did not strongly support the Th-shifting from Th2 to Th1. Although lower allergen-specific IgE and IgG1 are commonly accepted indicators for allergy relief, high IgG1 is not always associated with strong allergy reactions. Allergen-specific IgG1 can be produced by IL-4 dependent and IL-4 independent (via IL-12 or IFN-γ stimulation) manners (Dearman and Kimber, 2009; Faquim-Mauro et al., 1999). IgG1

synthesized by the later manner causes no allergic reactions (Dearman and Kimber, 2009). Considering the fact that IL-4 concentration in PROC was not higher than that in CTL+ (Table 3.2), the increased levels of IgG1 in PROC group may be generated through IL-4 independent manner.

There are no published studies investigating combined effects of oral tolerance and probiotics on CMA or food allergy, to our best knowledge. In OTP mice, probiotic supplementation did not further reduce hypersensitive scores but increased intestinal sIgA in comparison with OT group (Table 3.1). Similar to OT group, OTP was capable of suppressing both allergen-specific Th1 response and Th2 response. The higher intestinal sIgA may contribute an immunoregulatory role against other antigen/allergen in non-specific fashion.

Under current experimental conditions, oral supplementation of low doses of allergen would induce allergen-specific oral tolerance and suppress development of allergic reaction upon the subsequent exposure to that same allergen. VSL#3 supplementation could reduce allergic reaction to BLG mainly through generation of low and high affinity sIgA. Our findings also suggest that concurrent application of low doses of oral allergen and VSL#3 supplementation might offer both allergen-specific protection, through active suppression, and allergen-non-specific protection, through production of low affinity intestinal sIgA.

3.6 ACKNOWLEDGEMENT

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Authors Contributions

CLT designed and conducted the study, acquired the data, and drafted the manuscript. JIB was involved in planning the study and revising the manuscript. XZ designed the study, interpreted the data and revised the manuscript critically. All authors have read and approved the final manuscript.

3.7 TABLES AND FIGURES

Table 3.1. Effect of low doses of allergen and probiotic supplementation on serum and fecal antibodies in mice[1]

	CTL -	PRO	CTL+	OT	PROC	OTP
BLG-specific serum antibodies						
IgE, *µg/L*	ND	ND	78.5±8.7^{a}	0.442±0.36^{c}	34.3±10^{b}	3.54±3.5^{c}
IgG1, *mg/L*	ND	ND	40.5±4.1^{b}	11.2±2.1^{c}	58.0±3.8^{a}	11.0±1.7^{c}
IgG2a, *mg/L*	ND	ND	10.1±2.3^{a}	0.334±0.29^{b}	9.32±1.5^{a}	0.924±0.68^{b}
IgA, *mg/L*	ND	ND	4.83±0.93^{a}	0.334±0.13^{b}	4.21±0.90^{a}	0.541±0.19^{b}
Total antibodies						
Serum IgE, *µg/L*	32.1±3.9^{c}	23.7±1.7^{c}	101±7.5^{a}	26.4±2.3^{c}	62.4±6.2^{b}	30.2±3.8^{c}
Fecal IgA, *µg/L*	14.3±0.80^{b}	25.6±2.3^{a}	14.0±1.3^{b}	15.3±1.3^{b}	22.9±1.8^{a}	22.2±1.0^{a}

[1]Values are mean ± SE, *n*= 8. Means without a common letter differ, $P < 0.05$. CTL- = sham-sensitized negative control mice; CTL+ = BLG-sensitized positive control mice; ND= not detectable; OT= oral tolerance induced and BLG-sensitized group; OTP= probiotic-supplemented OT group; PRO = control group for probiotics (probiotic-supplemented sham-sensitized); PROC = probiotic-supplemented and BLG-sensitized group.

Table 3.2. Effect of low doses of allergen and probiotic supplementation on spleen lysate cytokines in mice[1]

Cytokines, *ng/L*	**CTL -**	**PRO**	**CTL+**	**OT**	**PROC**	**OTP**
IL-4	206±4.8^{c}	232±10^{bc}	342±51^{a}	278±12^{abc}	316±9.9^{ab}	265±18^{abc}
IFN-γ	89.0±24^{b}	106±27^{b}	192±18^{ab}	234±18^{a}	192±24^{ab}	179±18^{ab}
IL-10	52.1±7.8^{c}	80.2±9.8^{bc}	112±7.1^{ab}	149±7.6^{a}	134±11^{a}	118±8.7^{ab}
IL-12	633±29^{b}	787±50^{ab}	829±20^{ab}	971±51^{a}	937±42^{a}	977±63^{a}

[1]Values are mean ± SE, *n*= 8. Means without a common letter differ, P < 0.05. CTL- = sham-sensitized negative control mice; CTL+= BLG-sensitized positive control mice; OT= oral tolerance induced and BLG-sensitized group; OTP= probiotic-supplemented OT group; PRO = control group for probiotics (probiotic-supplemented sham-sensitized); PROC = probiotic-supplemented and BLG-sensitized group.

Table 3.3. Effect of low doses of allergen and probiotic supplementation on serum cytokines in mice[1]

Cytokines, *ng/L*	**CTL -**	**PRO**	**CTL +**	**OT**	**PROC**	**OTP**
Th1 cytokines						
IL-12 (p40)	130±3.3^{b}	109±6.9^{b}	139±4.7^{b}	191±14^{a}	133±9.2^{b}	190±11^{a}
IFN-γ	154±14^{a}	64.0±10^{b}	127±22^{ab}	119±14^{ab}	90.0±12^{ab}	115±9.4^{ab}
Th2 cytokines						
IL-5	16.0±1.4^{a}	5.50±1.4^{b}	17.1±1.9^{a}	18.6±1.4^{a}	12.6±1.9^{a}	14.2±1.0^{a}
IL-9	211±10^{ab}	160±6.8^{b}	207±14^{ab}	217±7.9^{a}	158±13^{b}	200±14^{ab}
IL-13	161±12^{a}	85.0±10^{b}	157±23^{a}	141±9.0^{ab}	109±17^{ab}	145±9.0^{ab}

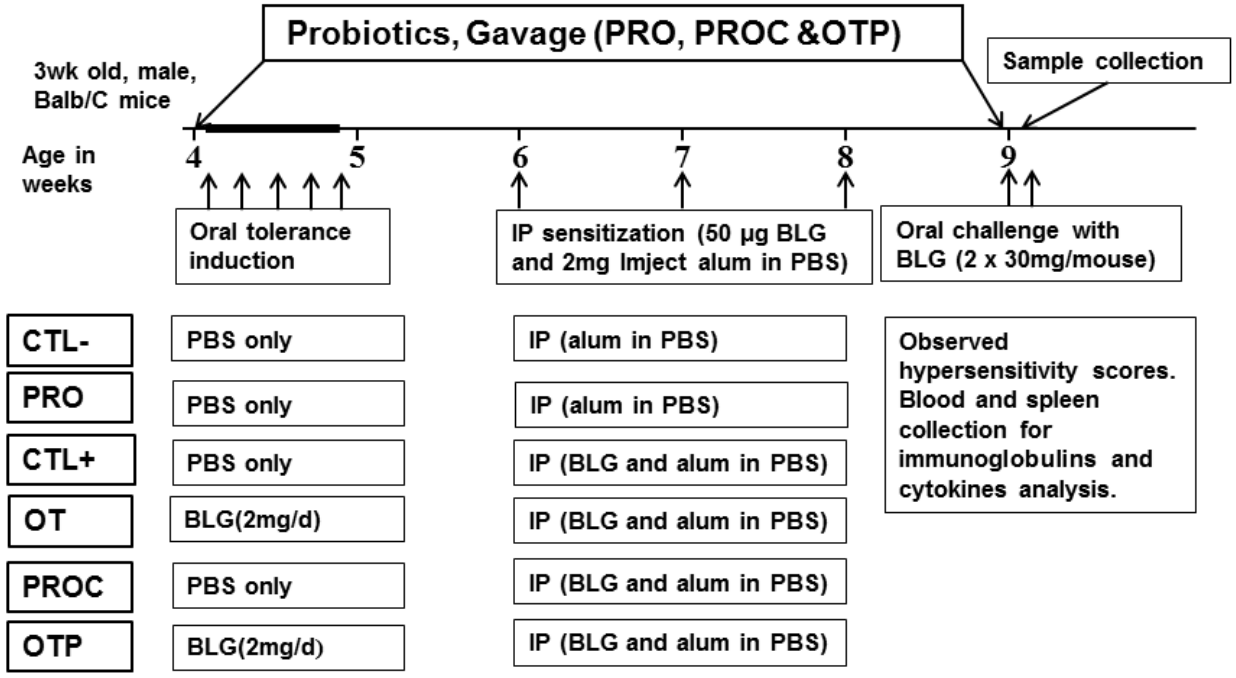
[1]Values are mean ± SE, *n*= 8. Means without a common letter differ, P < 0.05. CTL- = sham-sensitized negative control mice; CTL+= BLG-sensitized positive control mice; OT= oral tolerance induced and BLG-sensitized group; OTP= probiotic-supplemented OT group; PRO = control group for probiotics (probiotic-supplemented sham-sensitized); PROC = probiotic-supplemented and BLG-sensitized group.

Table 3.4. Effect of low doses of allergen and probiotic supplementation on serum and spleen lysate MCP-1 in mice[1]

MCP-1, *ng/L*	**CTL -**	**PRO**	**CTL+**	**OT**	**PROC**	**OTP**
Serum MCP-1	303 ± 12^{b}	175 ± 17^{c}	267 ± 28^{bc}	436 ± 32^{a}	232 ± 17^{bc}	288 ± 16^{b}
Spleen MCP-1	466.0 ± 119^{c}	520.2 ± 140^{c}	1787 ± 170^{b}	2940 ± 230^{a}	1473 ± 203^{b}	2008 ± 218^{b}

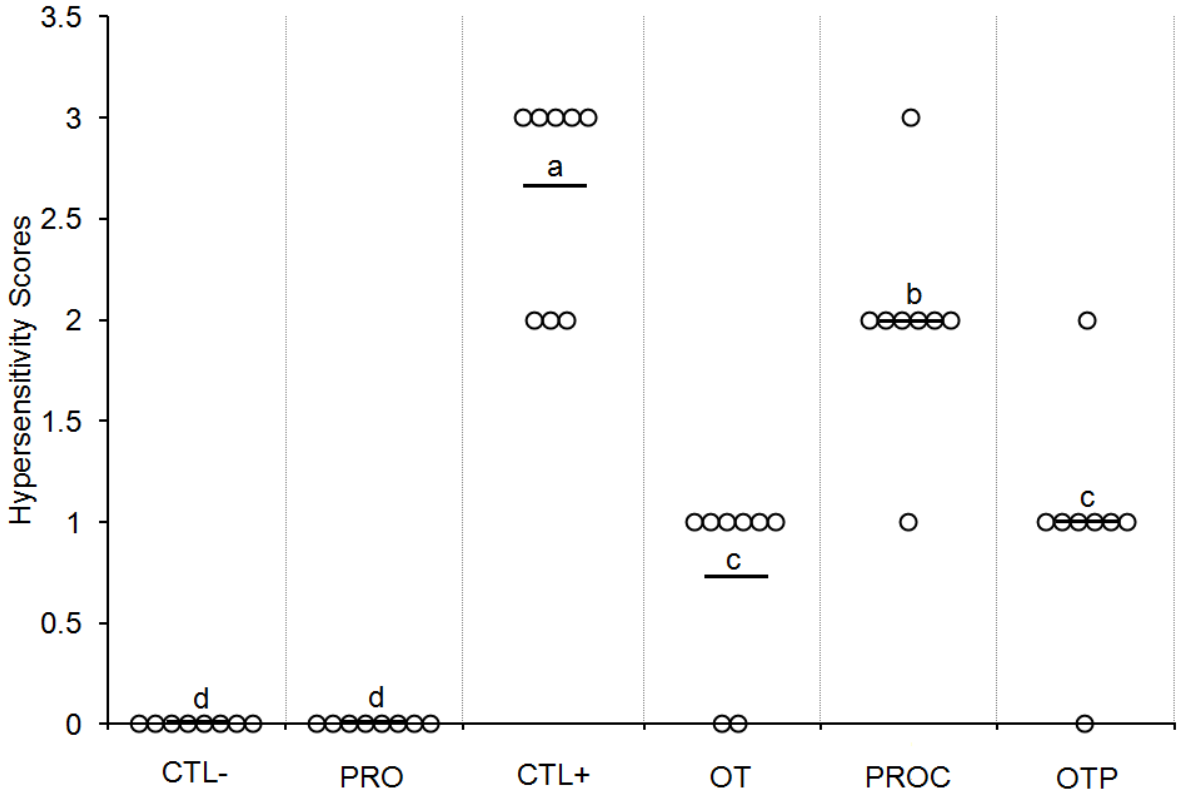
[1]Values are mean ± SE, *n*=8. Means without a common letter differ, $P < 0.05$. CTL- = sham-sensitized negative control mice; CTL+ = BLG-sensitized positive control mice; OT= oral tolerance induced and BLG-sensitized group; OTP= probiotic-supplemented OT group; PRO = control group for probiotics (probiotic-supplemented sham-sensitized); PROC = probiotic-supplemented and BLG-sensitized group.

Figure 3.1. Schematic representation of experimental protocol.



Corresponding treatments (*n*= 8 per treatment) were given to the mice as stated in the above diagram. Three hours after the final oral BLG challenge, mice were euthanized by CO_2 inhalation and samples were collected. Alum= aqueous solution of aluminum and magnesium hydroxide adjuvant; BLG= β-lactoglobulin; CTL- group= sham-sensitized negative control mice; CTL+ group= BLG sensitized positive control mice; OT= oral tolerance induced and BLG-sensitized group; OTP= probiotic-supplemented OT group; PRO = control group for probiotics (probiotic-supplemented sham-sensitized); PROC = probiotic-supplemented and BLG-sensitized group.

Figure 3.2. Hypersensitivity scores for individual mice recorded within an hour after final oral BLG challenge. Each point represents an individual mouse.



Mean scores (n=8) are shown by horizontal lines. Means without a common letter differ, $P < 0.05$**.** CTL- group= sham-sensitized negative control mice; CTL+ group= BLG sensitized positive control mice; OT= oral tolerance induced and BLG-sensitized group; OTP= probiotic-supplemented OT group; PRO = control group for probiotics (probiotic-supplemented sham-sensitized); PROC = probiotic-supplemented and BLG-sensitized group.

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CONNECTING STATEMENT 2

In Chapters II and III, we have determined the effects of single- (LGG) and multi-strains (VSL#3) probiotics on CMA development. Accordingly, we have demonstrated Th1 promoting and secretory IgA (sIgA) inducing effects of probiotics. We have also observed the effects of supplementing low doses of allergen alone or in combination with VSL#3 in Chapter III. The next Chapter was intended to investigate the role of the body's internal stimuli (precursor of inflammatory mediators) on allergic reaction by adjusting dietary levels of ω-3 and ω-6 PUFAs. Three experimental diets containing different ratios of ω-6 and ω-3 PUFAs (10:1, 4:1, and 1:4) were fed to BLG-sensitized Balb/c mice and clinical symptoms as well as local and systemic immune responses were observed.

CHAPTER IV EFFECTS OF SUPPLEMENTING DIFFERENT RATIOS OF OMEGA-3 AND OMEGA-6 FATTY ACIDS IN WESTERN-STYLE DIETS ON COW'S MILK PROTEIN ALLERGY IN A MOUSE MODEL[¶]

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Keywords: β-lactoglobulin, Food allergy, Omega 3 fatty acids, Mouse model, Western-style diet

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4.1 ABSTRACT

Scope: This study investigated the effects of supplementing different ratios of omega-6 and omega-3 fatty acids (O6H=10:1, O3O6=4:1, and O3H=1:4) to Western-style diets on cow β-lactoglobulin (BLG) induced allergic reactions in Balb/c mice.

Methods and results: Three weeks-old mice were randomly assigned to three diet groups (n= 20/group). At 9 weeks of age, half of the mice from each dietary treatment (n=10) were intraperitoneally (i.p.) sensitized with 3 weekly doses of BLG and alum while the remaining half from each group was sham sensitized (controls). One week after the final sensitization, all mice were orally challenged with BLG. Elevated BLG-specific serum Igs were observed in all sensitized and challenged mice. IFN-γ, MCP-1, and IL-12p40 concentrations from lymphocytes of mesenteric lymph nodes were highest in O3H mice, compared to O3O6 and O6H mice. O6H mice had the highest IL-4 concentrations from splenic lymphocytes and a significantly lower rectal temperature after the challenge in comparison to O3O6 and O3H mice.

Conclusions: Our results suggest that the ω-3 PUFA rich diets alleviated the severity of allergic reactions, and may modulate immune response toward Th1-favoured immune response while the ω-6 PUFA rich diet exhibited no allergy alleviation with a stronger Th2 polarized immune response.

4.2 INTRODUCTION

Food allergy is an immune-mediated hypersensitive response in susceptible individuals after ingestion of food that is considered harmless to others. Prevalence of food allergy and its outgrowing age, particularly in the children of developed nations, are significantly increased in the past three decades. Food allergy-associated menaces, starting from discomfort lifestyle to life-threatening anaphylaxis reactions as well as its growing incidence rate and current incurable status, pose an alarming risk to current patients and future generations; and cause a huge burden to families and social communities. Food allergy patients have also increased susceptibility to aeroallergens (Cianferoni and Spergel, 2009) and asthma is diagnosed in over 30% of children with food allergy (Bird and Burks, 2009). Today, cow's milk protein allergy (CMPA) still remains as the major food allergy to children and youth of developed countries. Among many possible reasons, early introduction of cow's milk protein to infants either as replacement or reinforcement for breast feeding coupled with consumption of recently widespread Western-style diets may partake a substantial role in steady increase of CMPA. The Western-style diet, also known as the standard American diet, is generally defined as a diet that contains a high level of energy (derived mainly from highly processed and refined grains, sugar, and red meat), a high level of fat (usually high in saturated and trans-fatty acids but low in monounsaturated [MUFAs] and polyunsaturated fatty acids [PUFAs]), and a high level of sodium (Odermatt, 2011). In regard to increased incidence of allergy, the composition of fatty acids from the Western-style diet, rather than its energy and sodium contents, is more concerned.

Variations of long-chain polyunsaturated fatty acid (LCPUFA) composition, particularly the rapid increase of ω-6/ ω-3 ratio in diets, have been considered as one of the major reasons for increased allergy incidence. Eaton et al. estimated that the ω-6/ ω-3 ratio in the diet of human beings in the Paleolithic period was 0.79 and remained relatively the same for millions of years (Eaton et al., 1998). However, the ω-6/ ω-3 ratio was suddenly sky rocketed within the last century due to rapid dietary changes. Currently the ω-6/ ω-3 ratio in western societies has been estimated as (15-17):1 (Simopoulos, 2008) while the suggested optimal dietary ratio of ω-6/ ω-3 is 4:1 (Wall et al., 2010). The

unbalanced ω-6/ ω-3 ratio in the diet is due to increased consumption of ω-6 rich foods, for example linoleic acid (LA, C18:2 ω-6)-rich vegetable oils and decreased consumption of ω-3 rich foods, such as fish and green vegetables, flaxseed, rapeseed oil or nuts that have a relatively high content of α-linolenic acid (ALA, C18:3 ω-3). This substantial shifting of dietary ω-6/ ω-3 ratios coincides with the recent higher food allergy incidence.

The association between a higher ω-6/ ω-3 ratio and allergy has not been fully explained. LCPUFAs serve as substrates for inflammatory mediators. It has been suggested that diets rich in ω-6 LCPUFAs generate a high level of potent inflammatory mediators, such as prostaglandin E2 and leukotriene 4, and lead to a Th2 favoured immune response. On the other hand, increased intake of ω-3 has been reported to reduce inflammatory reactions by producing less potent inflammatory mediators, such as prostaglandin E3 and leukotriene 5. Moreover, ω-3 derived resolvin molecules, particularly eicosapentaenoic acid (EPA) derived resolvin E1, is known to induce an anti-inflammatory effect by binding to Chemr23 receptor on APCs and subsequently attenuating NFκB activation (Arita et al., 2005) and reduce allergic airway responses in mice (Haworth et al., 2008). The epidemiological finding of the linkage between reduced ω-3 LCPUFA intake and increased prevalence of allergic diseases and the discovery of anti-inflammatory effects contributed by ω-3 LCPUFA derived molecules have generated enormous attention to study the role of these fatty acids in allergic diseases (Prescott and Calder, 2004). The results obtained so far are inconsistent (Furuhjelm et al., 2009; Hesselmar et al., 2010; Olsen et al., 2008; Palmer et al., 2012; Thien Francis et al., 2002). To our knowledge, the effects of dietary fatty acids on allergy have been studied selectively in a mouse model of allergic airway disease using ovalbumin. There is no report about effects of dietary fatty acids on cow's milk protein induced food allergy.

The dietary content of ω-3 and -6 LCPUFAs in the diet can be adjusted in two ways: by adding essential fatty acids, LA and ALA, (precursors of LCPUFAs) or by adding fish oils that contain preformed EPA and docosahexaenoic acids (DHA). Although mammals can synthesize EPA and DHA through its precursor ALA, its bioconversion rate, through desaturation and elongation processes, is limited since arachinonic acid (AA) is also metabolized by the same pathway (Burdge and Calder, 2005). A recent review indicated that a long term intake of ALA has increased the

proportions of EPA, but not DHA, in plasma or cell lipids (Burdge and Calder, 2005). Therefore, we have used fish oils, rather than ALA containing oils, as the source of ω-3 LCPUFAs in our experimental diets.

Studies about effects of maternal supplementation with ω-3 during pregnancy on offspring have gained attention due to the findings of its possible preventive effect on asthma and allergy from randomized, controlled trials (Dunstan et al., 2003; Olsen et al., 2008), while therapeutic approach to asthma with ω-3 (fish oil) was unsuccessful (Thien Francis et al., 2002). However, preventive potentials of ω-3 supplementation directly to new born animals, not through mothers, are less studied. We hypothesize that feeding young mice with Western-style diets that contain a high level of ω-3 LCPUFAs (EPA and DHA) may have a direct effect on Th-switching and T cell-mediated immune responses which may subsequently reduce allergic reactions to milk proteins. The purpose of this mouse model study was to evaluate whether increasing ω-3 PUFAs in Western-style diets can reduce BLG-induced food allergy.

4.3 MATERIALS AND METHODS

Animals and experimental design

Three weeks old female Balb/C mice (Charles River Breeding laboratories, St. Constant, Quebec, Canada) were housed in individual cages under a 12L:12D lighting cycle, 20-24 ºC range of ambient temperature and 40-70% of relative humidity. Mice were randomly assigned to three groups of twenty mice each that receive a ω-6 high diet (**O6H**), a ω-6 and ω-3 balanced diet (**O3O6**), and a ω-3 high diet (**O3H**). Mice were given ad libitum access to feed and water. At 9 weeks of age, half of the mice from each dietary treatment (n=10) were intraperitoneally (i.p.) sensitized with 3 weekly doses of 50 μg β-lactoglobulin (BLG) and 2 mg alum in PBS (Figure 4.1). The remaining half from each group was sham sensitized with alum in PBS and used as controls. One week after the final sensitization, all mice were orally challenged with 60 mg of BLG per mouse. Animal care use and protocols were approved by the McGill University Animal Care Committee (protocol # 5940).

Experimental Diets

Energy and fat rich Western-style rodent diets with different ratios of ω-6/ ω-3 (10:1 (**O6H**), 4:1 (**O3O6**) and 1:4 (**O3H**)) were formulated (Research diets Inc., New Brunswick, NJ) by slight modification of a previously described Western-style rodent formula (40 % kcal fat/ 15% protein/ 45% carbohydrate; 4.62 kcal/g) (Marsh et al., 2009; Medford et al., 2012). Energy contribution of each experimental diet was 45% from carbohydrate, 20% from protein and 35% from fat (Table 4.1). The total energy level of each diet was 4.24 kcal/g. Our diets are different from a standard laboratory rodent diet, which usually contains 3.0 kcal/g of energy derived from protein, fat and carbohydrate with the percentage of 32, 14 and 54 respectively (Teklad Rodent Diet-8604, Harlan Laboratories).

Menhaden oil was used for the source of ω-3 PUFAs and safflower oil was used for the source of ω-6 PUFAs. The diet manufacturer has added antioxidant tert-Butylhydroquinone (tBHQ) to the oils to prevent deterioration of fatty acids (Fritsche and Johnston, 1988). Upon arrival, the diets were packed into daily portions in zip-lock bags, sealed and stored at -80˚C. Mice were fed daily with the fresh pellets throughout the experimental period. Feed intake and body weight from all treatment groups were recorded.

Reagents

Horseradish peroxidase conjugated anti-mouse antibodies were obtained from Bethyl Laboratories (Montgomery, TX). Purified anti-mouse immunoglobulins were obtained from BD Pharmingen (San Diego, CA) and ABTS peroxidase substrate was purchased from Mandel Scientific (Guelph, Ontario, Canada). Bio-Plex Pro Mouse Cytokine Assay kits (Bio-Rad, Hercules, CA) were used to detect cytokines. All other chemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Nepean, ON, Canada).

Recording rectal temperature and hypersensitivity scores

Rectal temperatures were recorded at three time points; just before final oral challenge (0 min), 30 and 45 min post-oral challenge, using BAT-12 Microprobe Thermometer with a mouse specific rectal probe (Physitemp Instruments, Inc., Clifton, New Jersey). Within an hour following the final oral BLG challenge, hypersensitivity symptoms were scored by using the score system as described by Schouten et al. (2009). The scores were as follows: 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding and puffiness around the eyes and mouth; 4 = no activity after prodding, labored respiration, and cyanosis around the mouth and the tail; and 5 = death.

Fecal and serum samples collection

Fresh fecal pellets were collected at d 51 of age by transferring mice into new cages which were lined with moist paper towels. Fecal pellets were immediately stored in -80 ºC until further analysis. Three days after the second i.p. sensitization, blood samples were collected from tail vein. Three hours after the final oral BLG challenge, mice were euthanized by CO_2 inhalation and blood samples were collected by intra-cardiac puncture. All blood samples were collected into serum separator tubes (Sarstedt, Montreal, Quebec, Canada). The serum portion was separated by centrifugation at 10,000 x g for 5 min at 20 ºC. Serum samples were then aliquoted into Eppendorf tubes and stored at -20 ºC until analysis.

Determination of Serum BLG-specific Igs, total IgE and fecal IgA

BLG-specific serum IgG1, IgG2a and IgA as well as total IgE and total fecal IgA were detected by ELISA as previously described (C.L. Thang et al., 2011). Serum samples were diluted 1:100 for BLG-specific serum IgE, 1:200 for total IgE， and 1:1000 for others before being used in the assays. All analyses were performed in duplicates or triplicates and the mean values were used. Relative immunoglobulin concentrations were determined by extrapolation from standard curves with purified mouse immunoglobulin

standards. For detection of BLG-specific serum IgE, the SignalBoost Immunoreaction Enhancer Kit was used to dilute serum and HRP-conjugated anti-mouse IgE.

Fecal supernatants were prepared from fecal samples as described by Adel-Patient et al. (Adel‐Patient et al., 2005). Fecal pellets (100 mg) were placed in 1mL of PBS containing 0.01% (v/v) of the protease inhibitor cocktail (Sigma-Aldrich). The mixture was vortexed, centrifuged at 16,000g for 10 min and the supernatants were collected. Undiluted supernatants were used to determine fecal secretory IgA (sIgA) by ELISA.

Lymphocyte isolation and in vitro restimulation with anti-CD3

Three hours after the final oral BLG challenge, mice were sacrificed and lymphocytes from spleen and mesenteric lymph nodes (MLN) were collected as previously described (Su et al., 2011). Briefly, spleens and MLNs were aseptically removed. Lymphocyte suspensions were obtained by pressing the cells through a 70 µM nylon cell strainer (Falcon; BD Labware) in complete DMEM. After lysing red blood cells, the cells were washed and cultured (5 x 10^6cells/mL) in 24-well plates in the presence or absence of anti-CD3 monoclonal antibody (MAb; 10µg/mL) and BLG (100µg/mL) for 72 h at 37˚C. Culture supernatants were collected, labeled and stored at -20ºC until cytokines analysis.

Cytokine measurements from lymphocytes culture supernatants

Cytokines from lymphocytes culture supernatants were analyzed by Mouse Cytokine 23-plex Assay kits. After following the manufacturer's protocol, beads were read with a Bio-Plex Reader and data were analyzed with the Bio-Plex Manager 2.0 software (Bio-Rad Laboratories, Hercules, CA).

Serum fatty acid analysis

Serum fatty acid composition was analyzed by gas chromatography (GC) following a recently developed protocol with minor modifications (Glaser et al., 2010). This protocol is particularly useful for our experiment since it requires only 100 µL of

serum. Briefly, 100 µL each of serum and an internal standard, and 1.5 mL of methanolic HCl (3 N), containing 75 µg/mL of butylated hydroxytoluene (BHT), were mixed in the closed glass vial. Heptadecanoic acid, C 17:0, (Nu-Check Prep Inc., Elysian, MN) was used as the internal standard. The vials were then shaken for 30s and heated at 85˚C for 45 min. When the vials were cooled down to room temperature, 0.5 mL of hexane was added to each vial. The vials were shaken for 30s and upper hexane phase containing fatty acid methyl esters (FAME) was collected in 2 mL vials for GC analysis. Fatty acid composition of the FAME were measured by a CP-3900 GC (Varian, Palo Alto, CA) using a capillary column (CP-7489, 100 m × 0.25 mm, 0.2 µm film) with a flame ionization detector and a splitless injection. Serum fatty acids were identified by comparing the retention times of their peaks with the fatty acid methyl mix standard (Supelco 37 Comp. FAME Mix 10 mg/mL in CH2CL2) (Supleco, Bellefonte, PA).

Statistical analyses

Data were analyzed by using the PROC MIXED of SAS 9.3 (SAS Institute, 2003) except for the hypersensitivity scores data which were analyzed using the Kruskal-Wallis test and the SigmaStat software (SPSS Inc. Chicago, IL). Differences among treatment means were tested using the Scheffé multiple comparison test. Data are presented as means ± SEM. A *p*-value less than 0.05 was considered significantly different.

4.4 RESULTS

Serum fatty acid compositions

To confirm the efficiency of fatty acid supplementation in the diets, serum fatty acid compositions were analyzed (Table 4.2). Comparison of serum ω-3 and ω-6 fatty acids between control and BLG sensitized mice that received the same diet generally show similar levels of fatty acids, with the exception of C20:5 ω-3 EPA levels in O3H diet supplemented mice. In O3H mice, the C20:5 ω-3 EPA levels in BLG sensitized mice were lower than that in corresponding control mice. The serum ω-3 and ω-6 ratios reflected the ω-3 and ω-6 ratios in three diets.

Rectal temperature, hypersensitivity scores, body weights and feed intake

A sudden decline of body temperature, itchy skin and reduced activity are common signs of anaphylactic reactions. Body temperature recorded at 30 and 45 min after oral BLG challenge was compared with the initial body temperature. BLG sensitized O6H mice had a significantly lower rectal temperature, while other groups had relatively similar temperatures in comparison to control mice (Figure 4.2). After the final oral BLG challenge, all mice sensitized and challenged with BLG had slightly reduced activity with scratching behaviour while control mice showed no visible symptoms (Figure 4.3). There were no differences of hypersensitivity scores among all treatment groups.

The digestive system can also be affected by anaphylactic reactions. Body weight and feed intake were recorded for a period of 4 weeks starting from 7 weeks of age. Although no differences in body weight were observed, mice on O3H diets had a greater feed intake during week 7 and week 9 (Table 4.3).

Serum Immunoglobulins

In comparison with control mice, sera in all BLG sensitized mice had significantly higher BLG-specific serum IgE, IgG1 and IgG, which are systemic markers for allergic reaction (Table 4.4, Figure 4.4A, 4.4B and 4.4D). At the same time, mice receiving O3O6 and O3H diets had significantly higher concentrations of Th1 type antibody, BLG-IgG2a, than that in mice receiving O6H diet (Figure 4.4C).

Cytokines from lymphocyte culture supernatants

Cytokines in lymphocyte culture supernatants from spleen and mesenteric lymph nodes (MLN) were measured in order to see the effect of supplemented fatty acids on BLG-induced cytokines. When splenic lymphocytes were co-cultured with BLG, all sensitized mice showed significantly increased IL-4 levels (Figure 4.5A) in comparison to control mice. Among the three sensitized groups, mice that received O6H diet showed a higher IL-4 level than the other 2 groups. Results from MLN lymphocyte culture

revealed that IL-10 and IFN-γ levels of the mice that received O6H and O3O6 diets were significantly lowered than that in mice that received O3H diet (Figure 4.6C and 4.6D).

4.5 DISCUSSION

We investigated whether different levels of ω-3 and ω-6 LCPUFAs in high fat energy diets had any effect on BLG-induced immediate type hypersensitivity responses in a mouse model. We have used an energy rich diet (4.24 kcal/g) with a high level of fat (35% of energy from fat) as a base diet to reflect nutritional composition of typical Western-style diets. The three experimental diets contained different ratios of ω-6/ ω-3 (10:1 for the O6H diet, 4:1 for the O3O6 diet, and 1:1 for the O3H diet, respectively).

The allergy preventing effects of ω-3 LCPUFA supplementation in humans were generally obtained from observations of epidemiological studies or randomized controlled studies. The power of such studies could be compromised by many factors such as the complicated nature of allergy, inclusion criteria, or lack of synchronized allergen sensitization steps in experimental subjects. Allergy is a complex disease resulting from an interaction of multiple genetic and environmental factors (Holloway et al., 2010). Therefore, mouse models such as the one in our study, using genetically identical inbred Balb/C mice, could be valuable in mechanistic and confirmatory studies.

We sensitized the mice i.p. for 3 weeks with BLG and alum, the Th2-adjuvant (Lindblad, 2004). Observation of allergen-specific serum immunoglobulins from the sera collected three days after the second i.p. sensitization confirmed that Th2-favoured allergic responses were established in sensitized mice (Table 4.4). Immune responses to BLG challenge including allergen-specific systemic immune responses (such as elevated BLG-specific serum immunoglobulins, Figure 4.4), non-specific systemic (e.g. elevated IL-4 and IFN-γ from lymphocyte culture supernatants of spleen, Figure 4.5) and local immune responses (such as elevated IFN-γ, IL-12p40 and monocyte chemotactic protein 1 [MCP-1] from lymphocyte culture supernatants of mesenteric lymph nodes, Figure 4.6) were analysed to determine allergy reducing effects of the diets. Our results showed distinct patterns of immune responses to the BLG challenge exhibited by the mice that received three experimental diets.

The shifting of Th2 toward Th1 immune responses was observed in O3H mice. Higher concentrations of local Th1 cytokines IFN-γ and IL-12p40, chemokine MCP-1, and anti-inflammatory cytokine IL-10 from MLN of O3 mice (Figure 4.6B, 4.6C, 4.6D, 4.6E), in comparison to O6H and O3O6 mice, demonstrated the ability of ω-3 LCPUFAs to suppress Th2 and induce Th1-favoured immune response. A recent asthma mouse model study using IL-12p40$^{-/-}$ mice confirmed that IL-12p40 was essential for down-regulation of airway hyperresponsiveness (Onari et al., 2009). MCP-1 has recently received more attention in allergy research despite the fact that its mechanism is still unclear. It was reported that children with resolved CMA had significantly higher concentrations of serum MCP-1 than children with persistent CMA (Glez et al., 2012). More recently, our lab also reported the findings of higher concentrations of serum and spleen lysate MCP-1 in mice that developed protection against CMA through oral tolerance induction (Thang et al., 2013). Accordingly, higher MCP-1 concentrations from mesenteric lymph nodes of O3H mice indicated ω-3 LCPUFA's activity to induce MCP-1 and to inhibit allergic responses locally. Likewise, higher serum BLG-IgG2a (Figure 4.4C) and lower spleen IL-4 (Figure 4.5A) concentrations were observed in O3H mice, in comparison with O6H mice. These systemic immune responses lead toward Th1-favoured immune responses. However, there were no differences of the other BLG-specific serum Igs and cytokines among the 3 experimental groups. In general, these observations suggest that under current experimental conditions, ω-3 LCPUFAs have the ability to reduce the severity of allergic attack by inducing Th1-favoured immune responses, but may have little or no role in preventing allergy development. Our results are in agreement with the report of Johansson et al., which found that ω-3 LCPUFA supplemented mice produced higher allergen-specific serum Ig-E and eosinophil counts in a Th2-type airway hypersensitivity mouse model (Johansson et al., 2010). Similarly, Palmer et al. recently reported that ω-3 LCPUFA did not reduce the overall incidence of Ig-E associated allergies in the first year of life (Palmer et al., 2012). However, in contrast to our results, Yamashiro et al. (1994) revealed that mice fed with 7-9% of perilla oil (a rich source of parent ω-3 LCPUFA, ALA) had lower ovalbumin specific IgE (Watanabe et al., 1994), and attenuated intestinal mucosal damage. Also, mice fed with the diet containing 7% of fish oil had lower serum anti-ovalbumin IgE and IgG1 levels

(de Matos et al., 2012). Interestingly, Korotkova et al. (2004) have studied whether dietary ratio of ω-6 to ω-3 PUFA influences the induction of immunological tolerance to ovalbumin (OVA) in neonatal rats. The group reported that exposure to OVA via the milk in the offspring on the ω-3 diet resulted in a lower delayed type hypersensitivity reaction (DTH) and lower ovalbumin-specific and non-specific antibody responses, suggesting that ω-3 PUFAs facilitated the oral tolerance induction process (Korotkova et al., 2004). In comparison to the study (Korotkova et al., 2004), our O3H mice had a lower serum ω-6/ ω-3 ratio (1.1 for our mice vs 2.5 for their rats), and more than 2 times higher EPA and DHA levels. However, we did not observe reduction of allergen-specific antibodies (Table 4.2). Considering the fact that we added 16.4% of oils in our diets to reflect the fat and energy rich Western-style diet, instead of usual 7% of perilla or linseed oil in other studies, we tempt to speculate that the fat and energy rich Western-style diet will confer beneficial effects only when overall dietary fat content is low. This hypothesis needs to be tested. More studies are necessary to confirm the effects of ω-3 PUFAs on food allergy since recent double-blind randomized controlled trial (D'Vaz et al., 2012), and meta-analysis studies have suggested that ω-3 or ω-3 PUFAs may not involve in primary prevention of allergic disease (Anandan et al., 2009).

Mice that received the O6H diet had higher levels of local and systemic immune responses when challenged with BLG, supporting the notion that high dietary ω-6 LCPUFA promotes allergy development. Unlike O3H mice, our O6H mice revealed more pronounced Th2-mediated immune responses, such as lower concentrations of serum BLG-IgG2a (Figure 4.4C), mesenteric lymph nodes derived IL-12p40, IFN-γ, IL-10, and MCP-1 (Figure 4.6B, 4.6C, 4.6D, and 4.6E), and higher concentrations of spleen derived IL-4 (Figure 4.5A).

Mice that received the O3O6 diet expressed a mixed pattern of immune responses seen in O3H and O6H mice. As in O6H mice, significantly lower concentrations of IL-12p40, IFN-γ, IL-10, and MCP-1 from mesenteric lymph nodes of O3O6 mice (Figure 4.6B, 4.6C, 4.6D, and 4.6E), in comparison to O3H mice, suggest the development of Th2-mediated immune response at the local level in O3O6 mice. However, the findings of higher concentrations of serum BLG-IgG2a (Fig. 4C), and lower concentrations of

spleen derived IL-4 in O3O6 mice (Figure 4.5A), as in O3H mice, suggest the progress of Th1 favoured immune responses at the systemic level.

An anaphylactic reaction was observed in O6H mice (Figure 4.2). This was indicated by a lower rectal temperature and is caused by the sudden release of mast cell and basophil-derived mediators into the systemic circulation (Khan and Kemp, 2011). Interestingly, anaphylaxis development was prevented in O3O6 and O3H mice although these mice had high concentrations of serum BLG-IgE and IgG1 same as in O6H mice. Recent findings suggest that ω-3 derived lipid mediators may be responsible for anaphylaxis suppression through inhibiting degranulation of mast cells and/or reducing the potency of the released inflammatory mediators. For example, Martin et al. (2012) reported the inhibition of IgE-dependent lung mast cell degranulation from asthmatic human subjects by the lipid mediators lipoxin A4 (AA), resolvin D1 and resolvin D2 (DHA). Similarly, Rogerio et al. (2012) demonstrated the therapeutic potential of ω-3 PUFA derived D-series resolvins in allergic disorders. It is well known that typical inflammatory mediators are usually ω-6 PUFA (AA) derived eicosanoids. However, oral administration of fish oil can turn AA rich cell membrane into EPA and DHA rich cell membrane, and consequently produces 3-series of PGs and 5-series of LTs, which are less potent inflammatory mediators than 2-series of PGs and 4-series of LTs (LTB4) derived from ω-6 PUFAs. ω-6 PUFAs are essential molecules for allergic inflammation. The combination of these recent findings of ω-3 PUFAs efficacy together with the Th1-favoured immune response seen in O3O6 and O3H mice may lead to prevention of anaphylaxis development in these groups. Taken together, these results suggest that the anaphylaxis suppression effect is a potential positive effect of ω-3 supplementation.

In accordance with our experimental setting; our results suggest that ω-3 LCPUFA offered allergy alleviation and subsequently anaphylaxis suppression effects through locally elevated IL-10 and ω-3 PUFAs derived lipid mediators, but may not prevent allergy development when BLG-sensitized mice were challenged with BLG. Our study also indicates the possibility that modulation of energy rich Western-style diet by adding ω-3 LCPUFAs may not exhibit full beneficial effects of ω-3 LCPUFAs.

4.6 ACKNOWLEDGMENTS

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The authors have declared no conflict of interest.

4.7 TABLES AND FIGURES

Table 4.1. Calculated fatty acids composition (grams per 100g of total fatty acids)

Fatty acids	O6H	O3O6	O3H
C14	1.33	2.66	6.58
C16	8.04	9.68	14.5
C16:1	1.90	3.73	9.37
C16:2	0.32	0.63	1.58
C16:3	0.32	0.57	1.46
C16:4	0.32	0.57	1.46
C18	2.34	2.41	2.53
C18:1 (ω9)	11.52	11.08	9.68
C18:2 (ω6), LA	63.61	48.86	5.06
C18:3 (ω3), ALA	0.38	0.63	1.46
C18:4 (ω3)	0.57	1.20	2.97
C20:2 (ω6)	0.06	0.06	0.19
C20:3 (ω6)	0.06	0.13	0.38
C20:4 (ω6)	0.38	0.82	2.03
C20:5 (ω3), EPA	2.72	5.44	13.6
C21:5 (ω3)	0.13	0.32	0.76
C22:4 (ω6)	0.06	0.06	0.25
C22:5 (ω3)	0.57	1.08	2.72
C22:6 (ω3), DHA	1.96	3.99	9.81
SFA (g)	12.09	15.51	25.6
MUFA (g)	13.86	15.70	21.1
PUFA (g)	71.46	64.43	43.5
ω-3: ω-6	10	4	0.25

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; **O6H**: n-6 high diet (ω-6/ ω-3 ratio:10:1), **O3O6**: ω-6 and ω-3 balanced diet (ω-6/ ω-3 ratio:4:1), and **O3H**: ω-3 high diet (ω-6/ ω-3 ratio:1:4).

Table 4.2. Serum fatty acid composition in mice fed high energy diets containing different ratios of ω6 and ω3 fatty acids.

Fatty acid	O6H (c)	O6H	O3O6 (c)	O3O6	O3H (c)	O3H
C14:0	1.01±0.07^{c}	0.93±0.07^{c}	1.26± 0.07^{bc}	1.22± 0.05^{bc}	1.63±0.08^{ab}	1.79± 0.05^{a}
C16:0	21.32± 0.37^{c}	21.44± 0.37^{c}	25.07± 0.37^{b}	25.86± 0.30^{b}	29.88± 0.43^{a}	31.22± 0.30^{a}
C16:1	0.91± 0.08^{cd}	0.88± 0.08^{d}	1.32± 0.08^{c}	1.07± 0.07^{cd}	3.79± 0.10^{a}	3.31± 0.07^{b}
C18:0	12.76± 0.37	13.62± 0.37	13.47± 0.37	14.38± 0.31	12.89± 0.43	13.89± 0.31
C18:1 ω9	6.21± 0.38^{ab}	5.91± 0.38^{b}	6.71± 0.38^{ab}	5.93± 0.31^{b}	8.27± 0.44^{a}	8.12± 0.31^{a}
C18:2 ω6 LA	40.62±0.65^{a}	40.12±0.65^{a}	31.94±0.65^{b}	29.21±0.53^{b}	12.15±0.75^{c}	11.21±0.53^{c}
C20:3 ω6	0.79± 0.10^{b}	0.72± 0.10^{b}	1.33±0.10^{a}	1.39± 0.08^{a}	0.74± 0.12^{b}	0.36± 0.08^{b}
C20:4 ω6 AA	10.54± 0.46	10.35± 0.46	9.91± 0.46	11.36± 0.37	10.05± 0.53	9.76± 0.37
C20:5 ω3 EPA	1.61± 0.16^{c}	1.73± 0.16^{c}	1.88± 0.16^{c}	1.81± 0.13^{c}	8.34± 0.19^{a}	7.31± 0.13^{b}
C22:6 ω3 DHA	4.25± 0.21^{c}	4.29± 0.21^{c}	7.09± 0.21^{b}	7.77± 0.17^{b}	12.27± 0.25^{a}	13.04± 0.17^{a}
Total ω6	51.95± 0.43^{a}	51.19± 0.43^{a}	43.18± 0.43^{b}	41.96± 0.35^{b}	22.93± 0.49^{c}	21.32± 0.35^{c}
Total ω3	5.85± 0.25^{c}	6.02± 0.25^{c}	8.97± 0.25^{b}	9.57± 0.21^{b}	20.61± 0.29^{a}	20.35± 0.21^{a}
ω6/ ω3	8.88	8.51	4.81	4.38	1.11	1.05

[1]Values are least squares means ± SEM. Fatty acid content is expressed as percentage of total fatty acids. Values with different superscripts within the same row are different ($P \leq 0.05$). **O6H**: n-6 high diet (ω-6/ ω-3 ratio:10:1), **O3O6**: ω-6 and ω-3 balanced diet (ω-6/ ω-3 ratio:4:1), and **O3H**: ω-3 high diet (ω-6/ ω-3 ratio:1:4). The diet with "(c)" represents the control group without BLG sensitization for that diet.

Table 4.3. Feed intake (FI) and body weight (BW) in grams.

	Week 7		Week 8		Week 9		Week 10	
Group	FI	BW	FI	BW	FI	BW	FI	BW
O6H (c)	2.61[b]	*19.16*	2.72	*19.61*	2.73[b]	*20.69[b]*	2.61	*21.41[ab]*
O3O6 (c)	2.56[b]	*18.65*	2.69	*19.39*	2.76[b]	*20.51[b]*	2.5	*21.29ab*
O3H (c)	3.51[a]	*19.59*	2.71	*20.31*	3.19[a]	*21.89[a]*	2.55	*22.12[a]*
O6H	2.57[b]	*19.12*	2.71	*19.89*	2.69[b]	*20.43[b]*	2.33	*20.5[b]*
O3O6	2.5[b]	*19.13*	2.71	*19.8*	2.76[b]	*20.83[ab]*	2.52	*21.15[ab]*
O3H	3.58[a]	*19.71*	2.62	*19.91*	3.19[a]	*21.07[ab]*	2.6	*21.44[ab]*
SEM	0.12	*0.41*	0.25	*0.37*	0.09	*0.39*	0.22	*0.39*

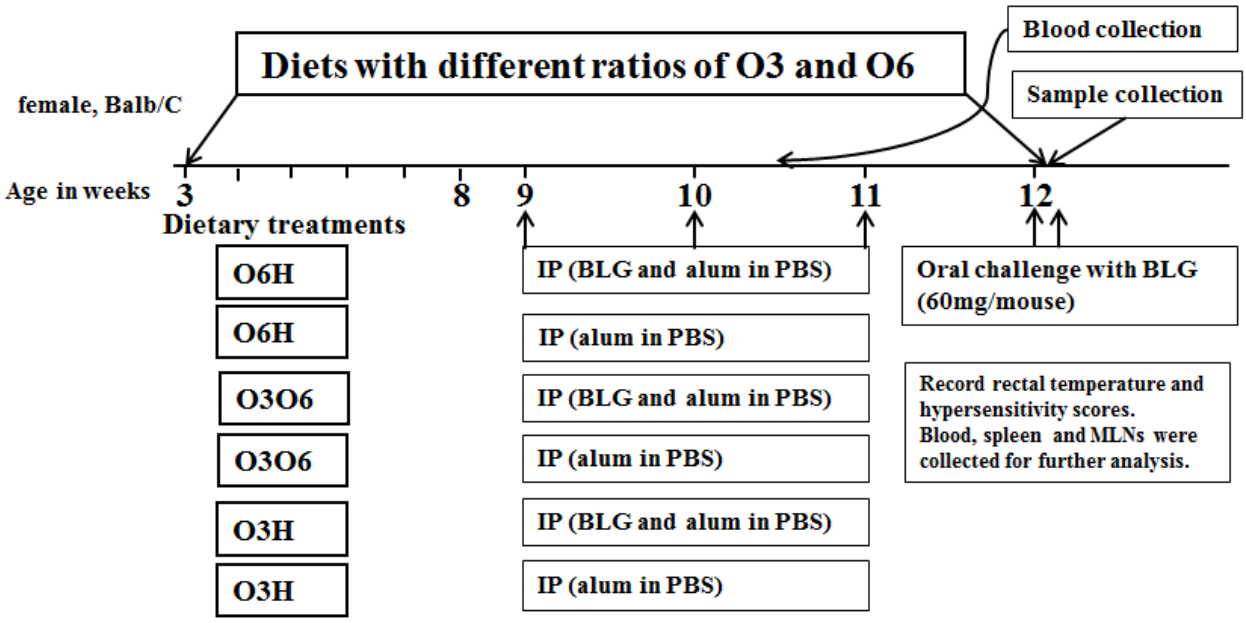
O6H: n-6 high diet (ω-6/ ω-3 ratio:10:1), **O3O6**: ω-6 and ω-3 balanced diet (ω-6/ ω-3 ratio:4:1), and **O3H**: ω-3 high diet (ω-6/ ω-3 ratio:1:4). The diet with **"(c)"** represents the control group for that diet. Different letters in the same column designate statistically significant differences ($P \leq 0.05$).

Table 4.4. BLG-specific antibodies and total IgE in sera

Group	BLG-IgE	BLG-IgG1	BLG-IgG2a	BLG-IgG	BLG-IgA	Total-IgE
O6H (c)	ND	ND	ND	ND	ND	1585.1^{d}
O3O6 (c)	ND	ND	ND	ND	ND	1639.1^{cd}
O3H (c)	ND	ND	ND	ND	ND	2126.6^{b}
O6H	183.2^{a}	17300^{a}	12825^{b}	45451^{a}	9489.7^{a}	2022.8^{bc}
O3O6	396.6^{a}	16300^{a}	24975^{a}	64439^{a}	1468.5^{b}	2124.0^{b}
O3H	412.5^{a}	21665^{a}	20524^{ab}	68435^{a}	4474.2^{b}	2796.6^{a}
SEM	82.49	3979.91	3740.08	12741	1598.82	139.24

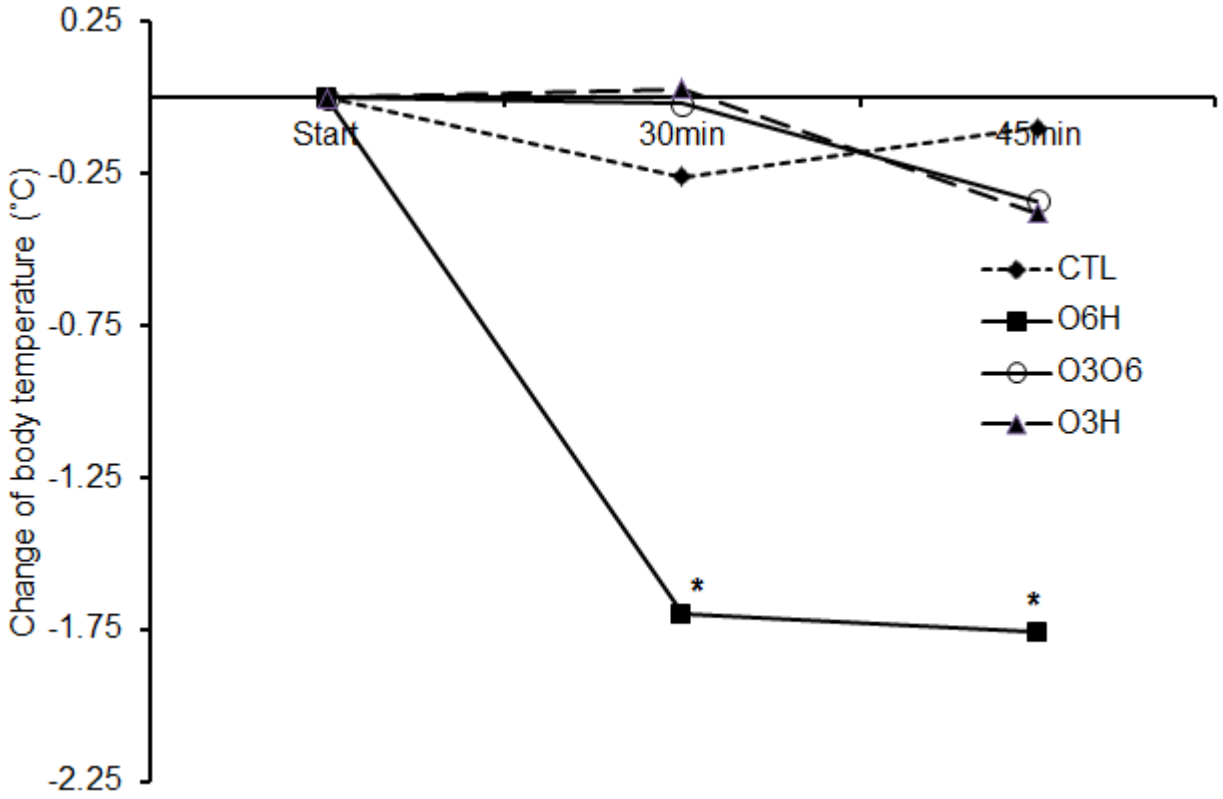
BLG-specific immunoglobulins and total IgE were determined from serum samples collected at 3d after second dose of i.p. sensitization. Relative serum antibody concentrations (ng/mL) were obtained by interpolation from the standard curves using purified mouse antibodies (BD Pharmingen). Different letters in same columns designate statistically significant differences ($P \leq 0.05$). **ND**: not detectable.

Figure 4.1. Experimental design.



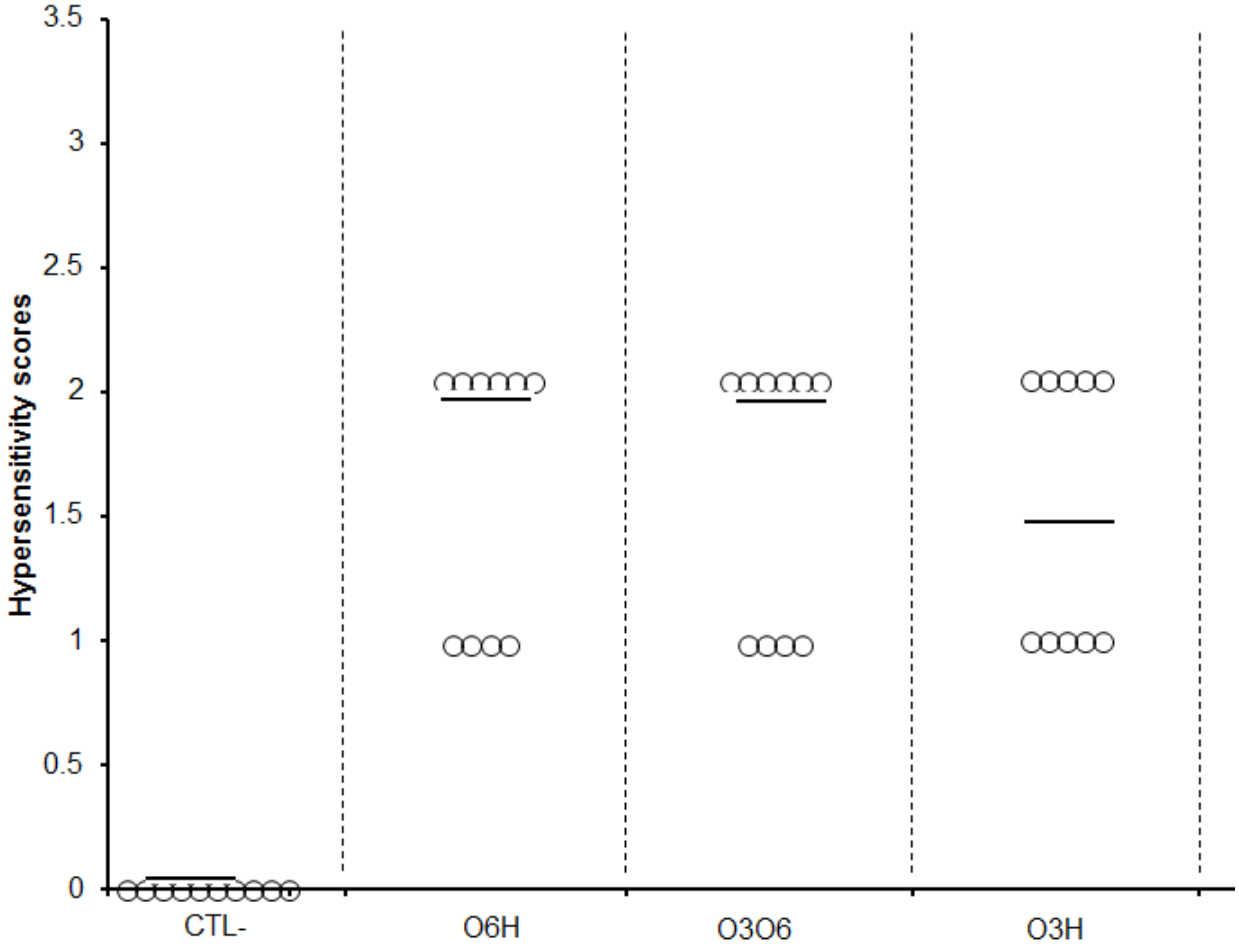
Three weeks old mice were divided into 6 groups (n= 10 per group) and fed with high energy diets containing the following three different ratios of ω-6 and ω-3 throughout the experiment. **O6H**: ω-6 high diet (ω-6/ ω-3 ratio- 10:1), **O3O6**: ω-6 and ω-3 balanced diet (ω-6/ ω-3 ratio- 4:1), and **O3H**: ω-3 high diet (ω-6/ ω-3 ratio- 1:4). After 3 doses of i.p. sensitization with BLG and alum, food allergy was induced by oral administrations of 60 mg BLG. Control groups for each diet were sham sensitized with alum in PBS. Three days after second i.p. sensitization, tail blood samples were collected. Hypersensitivity scores and rectal temperatures were recorded within 1h after final oral challenge. Three hours after final oral challenge, mice were sacrificed by CO2 inhalation and blood, tissues and organ samples were collected for further analyses.

Figure 4.2. Changes of body temperature in mice after challenge with BLG.



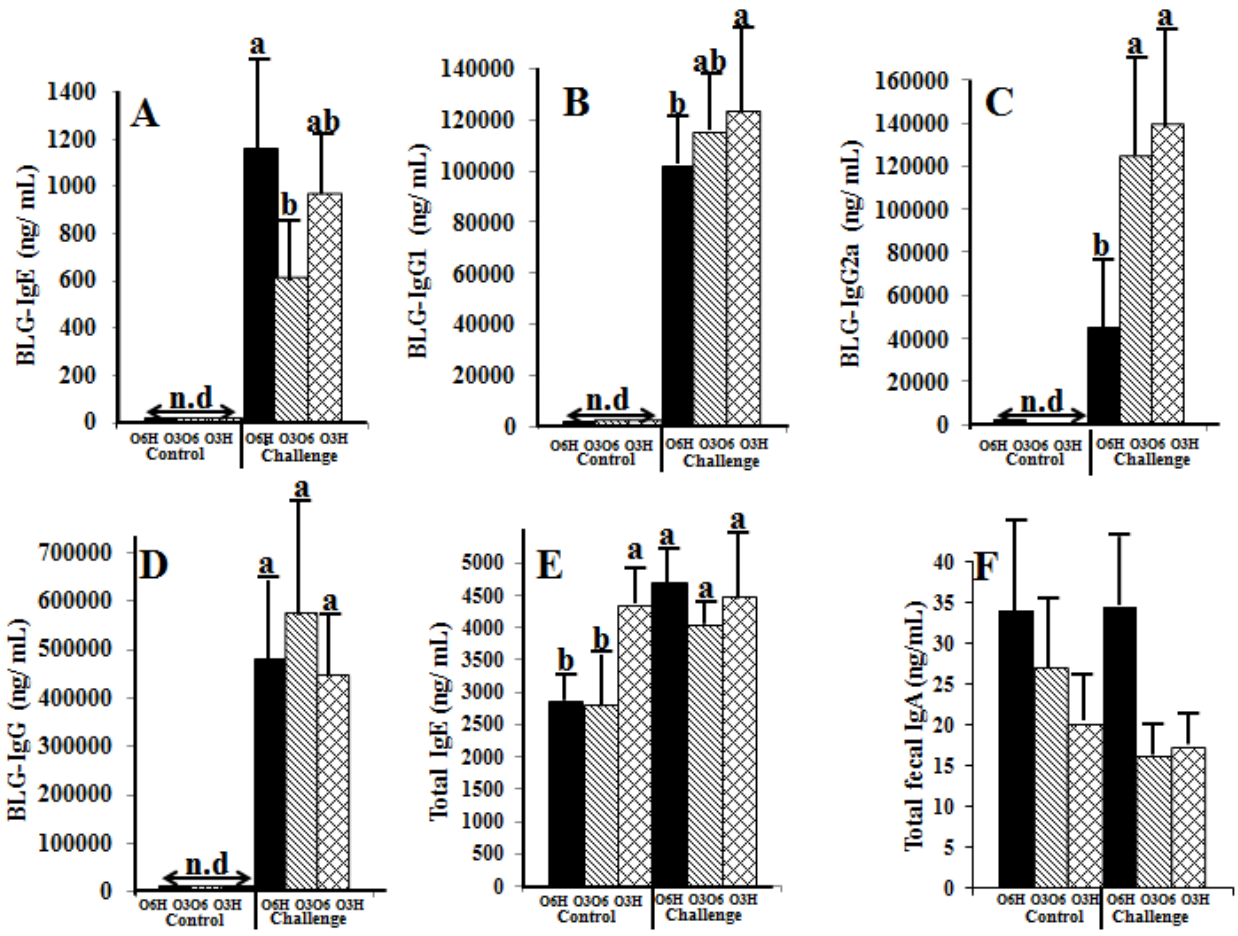
Rectal temperatures were recorded just before final oral BLG challenge (0 min), 35 min and 45 min post challenge. The asterisk mark (*) designates statistically significant differences ($P \leq 0.05$) in comparison to control mice. CTL, control mice that received sham sensitization with alum in PBS; O6H, ω-6 high diet (ω-6/ ω-3 ratio- 10:1); O3O6, ω-6 and ω-3 balanced diet (ω-6/ ω-3 ratio- 4:1); O3H, ω-3 high diet (ω-6/ ω-3 ratio- 1:4).

Figure 4.3. Hypersensitivity scores for individual mice (n=10) recorded within an hour after final oral BLG challenge.



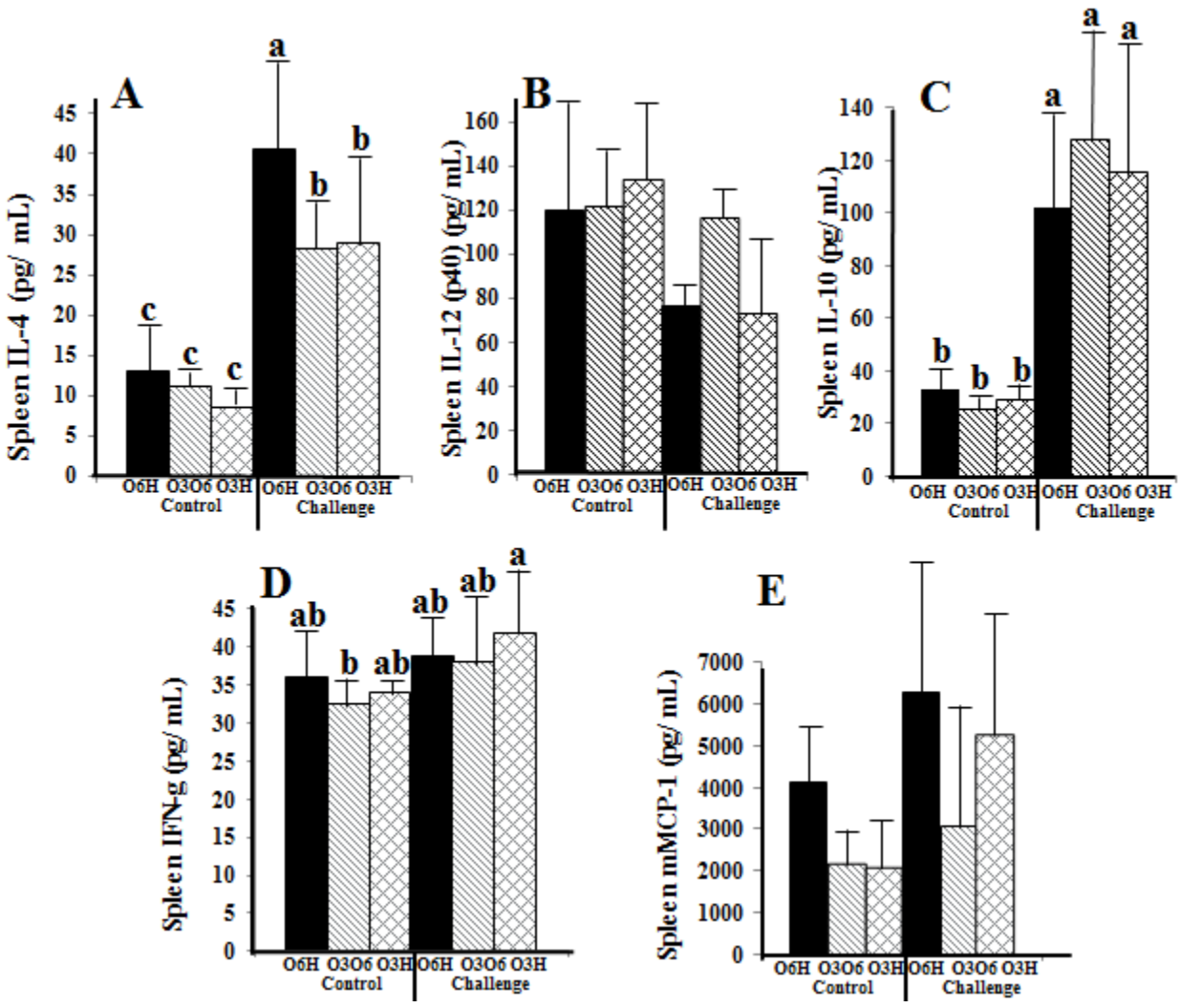
Each point represents an individual mouse. Median scores were shown by horizontal lines. CTL, control mice that received sham sensitization with PBS; O6H, ω-6 high diet (ω-6/ ω-3 ratio- 10:1); O3O6, ω-6 and ω-3 balanced diet (ω-6/ ω-3 ratio- 4:1); O3H, ω-3 high diet (ω-6/ ω-3 ratio- 1:4).

Figure 4.4. Allergen-specific and total antibodies.



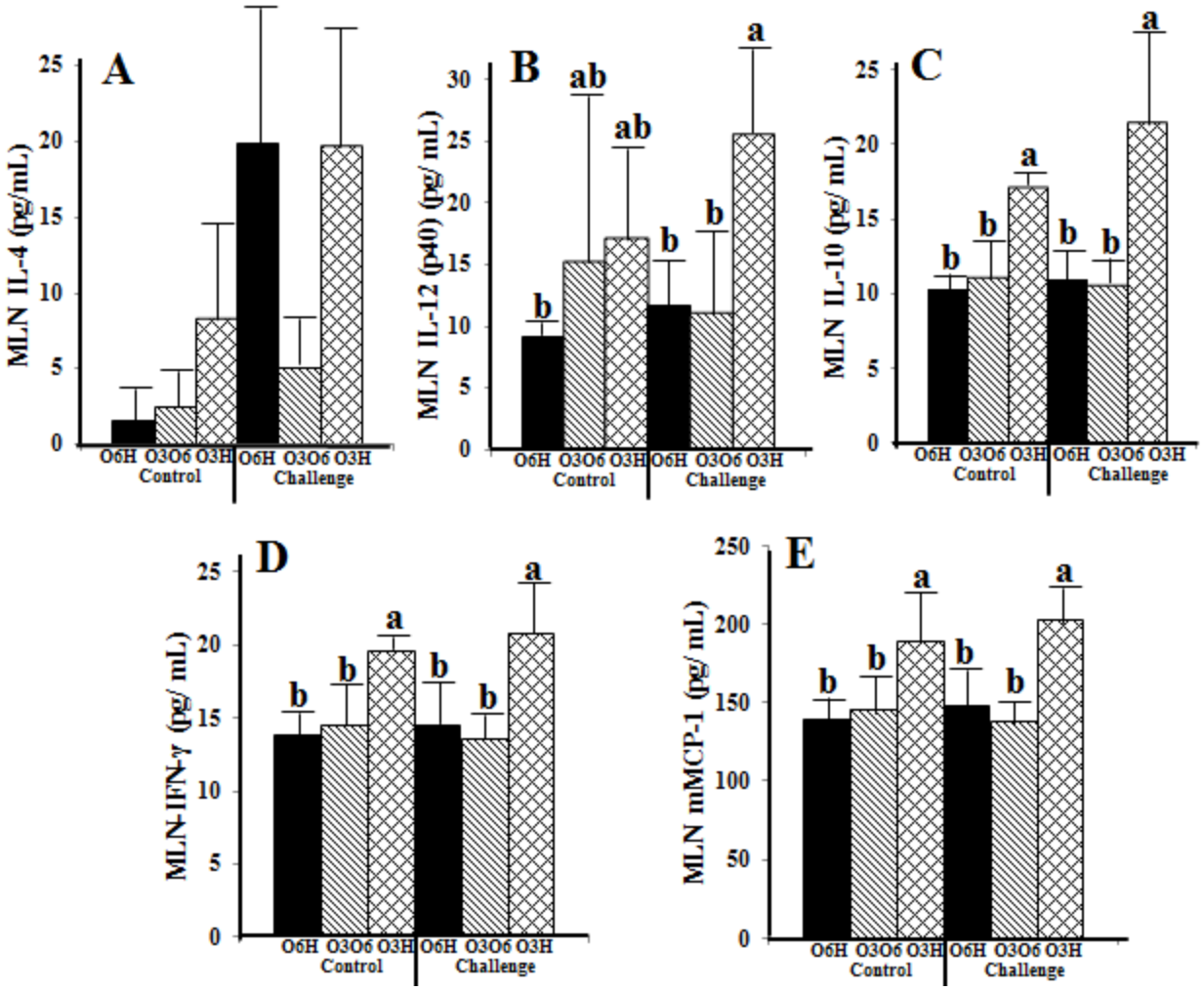
BLG-specific IgE (**A**), IgG1 (**B**), IgG2a (**C**) and IgG (**D**) as well as total IgE (**E**) and total fecal IgA (**F**) antibodies were measured by ELISA. Each value represents the mean ± SEM of ten mice and different letters denote statistically significant differences ($P < 0.05$). **nd**: not detectable).

Figure 4.5. Cytokine profiles from the culture supernatant of spleen derived lymphocytes.



Three hours after the final oral BLG challenge, mice were sacrificed and lymphocytes from spleens were aseptically removed. After 72h culture at 37˚C in the presence of BLG in complete DMEM, the supernatants were collected and analysed for IL-4 (**A**), IL-12p40 (**B**), IL-10 (**C**), IFN-γ (**D**), and MCP-1 (**E**). Each value represents the mean ± SEM of ten mice and different letters denote statistically significant differences ($P < 0.05$).

Figure 4.6. Cytokine profiles from the culture supernatant of lymphocytes derived from mesenteric lymph nodes.



Three hours after the final oral BLG challenge, mice were sacrificed and lymphocytes from mesenteric lymph nodes were aseptically removed. After 72h culture at 37˚C in the presence of BLG in complete DMEM, the supernatants were collected and analysed for IL-4 (**A**), IL-12p40 (**B**), IL-10 (**C**), IFN-γ (**D**), and MCP-1 (**E**). Each value represents the mean ± SEM and different letters denote statistically significant differences ($P < 0.05$).

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CHAPTER V. GENERAL DISCUSSION AND CONCLUSION

General Discussion

Until recently, food allergy was a disease with a relatively low profile and low prevalence that mostly affects residents of developed countries. But, today food allergy has become a major public concern due to its increased severity and worldwide prevalence. Increasing food allergy incidence in today's children population could be due to the fact that they are surrounded by a more pro-inflammatory environment (e.g. lower microbial exposure and diets with high ω-6 PUFAs), while their immune systems are still developing.

In reality, when speaking about food allergy, the burden is not simply ended with the clinical symptoms and possibility of developing life threatening anaphylaxis reaction in allergic patients. It also has a profound psychosocial impact on patients, their families, and the community, such as day care centers, because a constant vigilance to allergens and daily management to food allergy are necessary (Cummings et al., 2010). In addition, a recent mother-infant nutrition study in Finland revealed that CMA is the most expensive allergic disease (Alanne et al., 2012). In that study, the authors estimated that a child with atopic dermatitis will cost 425 Euro, food allergy will cost 4348 Euro, and asthma will cost 1858 Euro, according to 2006 prices, during the first 24 months of life. All these data indicate the growing public burden caused by food allergy.

Parallel to its growing impact, our knowledge on molecular features of food allergens, predisposing environmental factors, and host's immune response mechanisms are also expanding enormously. Progress in molecular research has expanded our understanding of structural and functional nature of allergens. We now know that major allergens share common features including negative charges and relatively small (<70 kd) size, low hydrophobicity and high stability (glycosylation or the presence of disulfide bonds) (Chapman et al., 2007; de Groot et al., 2007; Sen et al., 2002). These parameters, however, are unsuitable to use as a guideline to determine the allergenicity of unknown foods (Traidl-Hoffmann et al., 2009). Food allergy research focussed on the changes in environmental factors, for instance, hygiene hypothesis, vitamin D hypothesis, and fatty acids hypothesis, have also generated valuable information. Furthermore, experimental and clinical studies so far have broadened our understanding about food allergy

mechanism and provided new therapies. For example, monoclonal anti-IgE and Chinese herbal medicine can alleviate the clinical manifestations. Mosmann and Coffman identified 2 subsets of CD4+ T cells, Th1 and Th2 cells, in 1986. Today, less than 30 years later, researchers have isolated at least seven distinct T cell subsets (Th1, Th2, Th9, Th17, Th22, Tfh, and Treg) derived from naïve CD4+T cells (Oh and Ghosh, 2013).

Despite much progress on research and discovery of new insights, there is no cure for food allergy at present, reaffirming the complex nature of food allergy. Strict avoidance of offending allergens along with immediate access to rescue medication is the current best management (Cummings et al., 2010). As such, development of efficient preventive or therapeutic strategies for food allergy has become increasingly important than ever.

Food allergy studies conducted so far in mouse models had commonly used hen's egg protein, ovalbumin (OVA), as an experimental allergen (Inoue et al., 2007; Johansson et al., 2010; Zhang et al., 2001). On the other hand, literature on cow's milk protein (e.g. β-lactoglobulin, BLG) induced allergy in mouse models is limited. Allergens are proteins with diverse structural features and biological functions, such as proteases, lipid-transfer proteins, and calcium-binding proteins, consequently these allergens were sensed differently by type 2 inducing innate immune system using diverse mechanisms (Pulendran and Artis, 2012). Pulendran and Artis (2012) recently proposed that innate immune system can sense Th2-inducing allergens by (1) Protease activity (e.g. papain, bromelain), (2) Pattern recognition via innate receptors (e.g. certain TLR ligands), (3) Tissue damage (e.g. caused by adjuvants such as alum), and (4) Metabolic changes in environment (amino acid, oxygen changes caused by pathogen growth). We have used cow's milk proteins (whole milk protein in Chapter II and purified BLG in Chapter III and IV) as the key allergen in order to get a more comprehensive understanding of food allergy. In addition, cow's milk allergy (CMA) is usually the most common food allergy particularly in infants and children. We also used just weaned mice (21d old) in all our experiments with the goal to better reflect the more susceptible children population.

We developed a new mouse model of CMA by i.p injection of CMP in the absence of an adjuvant (Chapter II) in the first experiment. Literature has suggested that probiotics may contribute anti-allergic effects via local stimulation of epithelial innate immunity (i.e., increased production of epithelial-derived TNF-α and restoration of epithelial barrier

function) (Pagnini et al., 2010), promoting Th1 differentiation and inducing Treg population and sIgA production (Prescott and Bjorksten, 2007). Although several studies found the allergy reducing effects of probiotics, the results were inconsistent. These varying results may be due to variation in the probiotics used (e.g. strains, dose, viability, duration, methods of administration), host factors (e.g. genetic predisposition to allergic disease, polymorphisms in microbial recognition pathways, susceptibility to colonization), and environmental factors (influences on colonization such as maternal flora, delivery method, feeding practices and weaning diet, antibiotics/prebiotics treatment) (Prescott and Bjorksten, 2007). In our first and second experiments, we found that probiotics supplementation reduced CMA at least via two distinct mechanisms. First, we observed LGG supplementation modulated immune reactions by promoting Th1-dominated responses in CMP sensitized mice (Chapter II). More pronounced allergy alleviation, in comparison to Chapter II, through increased intestinal IgA, was observed in VSL#3 supplemented mice (Chapter III). Higher dose of supplemented probiotics (VSL#3 dose: 15 x 10^9 CFU, LGG dose: 1 x 10^9 CFU) and strain differences (VSL#3: 8 strains, LGG: single strain) may play a key role in contributing such different immunomodulatory effects.

Feeding of either low or high doses of an allergen can induce tolerance by a mechanism known as “oral tolerance”. A high dose allergen may lead to anergy/deletion while a low dose feeding may result in active suppression via Treg induction (Wang and Sampson, 2011; Weiner et al., 2011). Feeding low doses of BLG in our second experiment induced allergen-specific tolerance by Treg-mediated active suppression (Chapter III). Since there is no published study determining the combine effects of supplementing probiotics and low doses allergen, we also included a treatment group in our experiment to determine the combined effect. In mice that received both treatments (probiotics and low doses allergen), Treg-induced allergen-specific suppression effects, as in OT mice (low doses of allergen alone supplemented mice), as well as increased intestinal sIgA levels, as in probiotics alone supplemented mice, were observed. Although no further allergy reductions were seen in these mice, compared to OT mice, their increased sIgA levels may be beneficial for unforeseen non-specific challenges.

To answer a very interesting experimental question whether high levels of ω-3 PUFA in energy and fat rich diet has effect on food allergy, the effects of diets containing different ratios ω-3 and ω-6 PUFAs were investigated in our third experiment. The energy and particularly fat (usually high in saturated and trans-fatty acids) densely contained diet, often referred as Western-style diet, is also regarded as one of the reasons for recent rise of food allergy in developed nations. This theory was supported by epidemiological findings, high ω-6/ ω-3 ratio in current diet (15:1) while recommended ratio is 4:1, and newly discovered anti-inflammatory and resolving properties of ω-3 PUFAs. Our feeding study revealed that diets with high levels of ω-6 or ω-3 PUFAs have no effect on suppressing the development of allergen-specific immune responses. However, we have found that ω-3 PUFAs contributed allergy alleviation effects through suppression of an anaphylactic reaction, and promoting Th1-favoured immune responses. These effects were confirmed by observation of the unchanged rectal temperatures, and higher levels of Th1 markers (IFN-γ, IL-12p40, and serum BLG-IgG2a) in mice that received diets with high levels of ω-3 PUFAs (O3H and O3O6) (Chapter IV). Recent studies revealed that ω-3 PUFAs derived molecules such as 3-series PG and TX, 5-series LT, protectins, and D- and E-series resolvins (Figure 1.4) have inflammation resolving and anti-inflammatory properties. Our serum fatty acids analysis confirmed that dietary ω-3 PUFAs were transported and maintained at the systemic levels at the time of challenge with BLG (Table 4.2). Taken together, all of these findings revealed that ω-3 PUFA has allergy reducing, but not suppressing, effects. Furthermore, it also suggests that current Western-style diet may involve, at least in part, in the recent rise in food allergy prevalence.

Conclusions

In this extensive study, we have studied and demonstrated three different approaches in a mouse model that could reduce and suppress food allergy. Probiotics supplementation can reduce CMA symptoms by promoting Th1 responses and increasing sIgA. Low doses of allergen supplementation could suppress development of an allergic reaction by inducing Treg-mediated active suppression. Synergistic supplementation of probiotics and low doses of allergen provide the animal allergen-specific tolerance and increased

sIgA. Finally, ω-3 PUFA high diets provide higher systemic ω-3 PUFA in the animals and consequently offer inflammation resolving and anti-inflammatory properties when an allergic reaction occurs. We believe that these findings have collectively provided valuable scientific knowledge and may lead to applicable therapy in the near future in the fight against food allergy.

Future study

The beneficial effects, against CMP sensitization, observed from this study can be categorized into (i) allergy alleviating effects, and (ii) allergy suppression effects. Administration of probiotics; LGG (Figure 2.2), and VSL#3 (Figure 3.2, Table 3.1), and ω-3 PUFA enriched diet (Figure 4.2 and 4.3) lessen the severity of allergic reactions via different mechanisms including Th-shifting, sIgA, and ω-3 derived mediators. However, feeding of low doses of allergen completely suppressed allergen-specific immune responses in mice (Figure 3.2, Table 3.1) via Treg-mediated active suppression mechanism. These findings suggest an upcoming study that determines the combined effects of probiotics (multi-strains, high dose) and ω-3 enriched diet (lower energy and fat than Western-style diet) will be highly promising for food allergy control. More importantly, based on our low doses allergen feeding study results and recent literature, we highly anticipate that a future study by feeding immune-dominant epitopes (immunogenic peptides) from BLG, rather than feeding the whole allergen protein, will generate allergen-specific tolerance with no or minimal risk of anaphylaxis reaction. Rupa and Mine (2012) reported that feeding of immuno-dominant peptides from egg-white allergen, ovomucoid, significantly reduced allergy to egg-white protein in Balb/c mice.

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