(I) CHARACTERIZATION OF TYPE 2 CYTOKINE RESPONSE TO ALLERGENS IN A MOUSE MODEL OF FOOD ALLERGY AND (II) THE EFFECT OF SELECTED HERBAL SUPPLEMENTS ON ALLERGEN DRIVEN TYPE 2 CYTOKINE RESPONSE BY SPLEEN CELLS FROM FOOD ALLERGIC MICE

BY

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ABSTRACT

CHARACTERIZATION OF TYPE-2 CYTOKINE RESPONSE TO ALLERGENS IN A MOUSE MODEL OF FOOD ALLERGY AND THE EFFECT OF SELECTED HERBAL SUPPLEMENTS ON ALLERGEN DRIVEN TYPE-2 CYTOKINE RESPONSE BY SPLEEN CELLS FROM FOOD ALLERGIC MICE

By

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Food allergy is a growing problem with potential for fatality. However, mechanisms underlying food allergy are not completely understood. The present study was conducted using a mouse model of food allergy. In the first part, studies were conducted to characterize hazelnut and sesame allergen driven Type-2 cytokine response by spleen cells. We found a CD4-dependent, recall IL-4, IL-5 and IL-13 responses in food allergic mice. These Type-2 cytokine responses were observed in mice strains with different H-2 genetics. Further studies using *Stat6* and *Il4* knockout mice suggested that allergic and anaphylactic response was dependent on IL-4 but not IL-5 or IL-13. In the second part of the study, the impact of selected herbal supplements on type-2 cytokine responses was studied. Among the herbal supplements tested, only licorice (from three different sources) consistently inhibited Type-2 cytokine responses. In summary: (1) these results have advanced the knowledge on the mechanism of food allergy in this mouse model; and (2) licorice was identified as an inhibitor of Type-2 cytokine response in this model.

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INTRODUCTION

Allergic reactions to food (commonly called food allergy) are due to abnormal responses of the immune system to food proteins (1). The food allergies are classified as immediate hypersensitivity reactions because they occur within minutes after exposure to the food to which an individual has been sensitized (2).

Significance of Food Allergy

Food allergy is a growing public health problem of serious proportions in many Westernized countries including the United States of America (3). Recent epidemiological data suggests that nearly 4% of American adults are afflicted with food allergies; the disease is more prevalent in children with nearly 6-8% of them below the age of 3 years affected (4, 5). Food allergy has both psychological (6) and economic (7) impact on the society by causing nearly 200 deaths per year and 30,000 emergency room visits (8, 9).

Similar to other chronic diseases such as asthma, diabetes, and psychiatric diseases, food allergy can impact the quality of life. For example, people with food allergies, constantly worry about the possibility of exposure to food allergens in a variety of places such as restaurants, schools and airplanes (10, 11).

Food allergens pose a significant challenge to the food industry. Food allergens continue to be the major cause of Class-1 food recalls and negatively impact the food industry (12). According to Dick Dahl (2006), the restaurant industry might face an increasing number of food allergen related lawsuits in coming days, even though McDonald's is the only corporation sued for the wheat and milk contents in their French fries (13). The agricultural biotech industry faces the challenge of potential allergenicity of novel foods produced by genetic engineering. For example, a recent episode of contamination of the human food chain by a genetically engineered corn (Star link) led to scores of food recalls; the total cost of cleanup has been estimated to be ~100 million dollars (14-18).

Mechanism of Food Allergy

The precise mechanism of food allergy is not completely understood at present (19). It is generally believed to be similar to other allergic disorders such as allergic rhinitis and allergic asthma (20). According to one proposed mechanism, peanut allergy develops as follows: first exposure of susceptible individuals to peanut results in the production of peanut-specific IgE antibodies. These antibodies bind to high affinity IgE receptor present on the mast cells and basophils. Once this occurs, this individual is considered 'sensitized' to peanuts. When this individual is re-exposed to peanuts, the IgE antibodies bind to the peanut protein and initiate a series of biochemical reactions leading to the clinical symptoms of food allergy such as hives,

vomiting, diarrhea, respiratory distress or in rare cases, systemic anaphylaxis and death (21).

As alluded earlier, specific mechanisms of food allergy are not completely understood at present. Mouse models of food allergy have been developed to facilitate investigation into the mechanisms. For example, an adjuvant-free mouse model has been recently developed in our laboratory of Food Allergy and Immunology at the Michigan State University (19, 22). During the first part of my thesis work, I used this model to study the role of Type-2 cytokines in tree nut and sesame allergy.

Prevention and Therapy of Food Allergy

Despite the potential for a fatal outcome, no effective preventive methods (e.g., a vaccine) are available for food allergy. Currently, avoiding the offending food is the only proven way to prevent food allergic reaction. Therefore, researchers have been examining a number of novel methods for prevention and therapy of food allergy (23-32). Many of these studies use mouse models of food allergy for testing.

There is growing interest in the potential of herbal supplements in the prevention and therapy of allergies and asthma including food allergies. A recent study demonstrated that a mixture of Chinese herbal supplements was effective in preventing systemic anaphylaxis in a mouse model of peanut

allergy (23, 29). Thus, the potential of herbal supplements in food allergy remains to be examined in detail. Mouse models of food allergy provide a valuable tool for such studies. In the second part of my thesis work, I tested a number of herbal supplements for their potential utility using the mouse model of food allergy developed in our laboratory.

An Adjuvant-free Mouse Model of Food Allergy

Our laboratory has previously developed an adjuvant-free mouse model of food allergy using hazelnut and sesame as model allergenic foods (19, 22). The major features of this model are: (1) induction of food-specific IgE antibody response following transdermal exposure to food allergens without any adjuvant; and (2) induction of clinical signs of systemic anaphylaxis following oral challenge with a food allergen.

Using this mouse model, the present study was conducted in two parts. In Part-I, allergen-driven Type-2 cytokine responses were characterized in hazelnut and sesame allergy mouse models. In Part-II, the impact of selected herbal dietary supplements on allergen-driven Type-2 cytokine responses by spleen cells from food allergic mice was studied.

In the next section, I have presented an extensive review of the literature relevant to my studies. In Chapters 1 and 2 I have presented findings from part-I and part-II of my studies in a manuscript style.

REVIEW OF THE LITERATURE

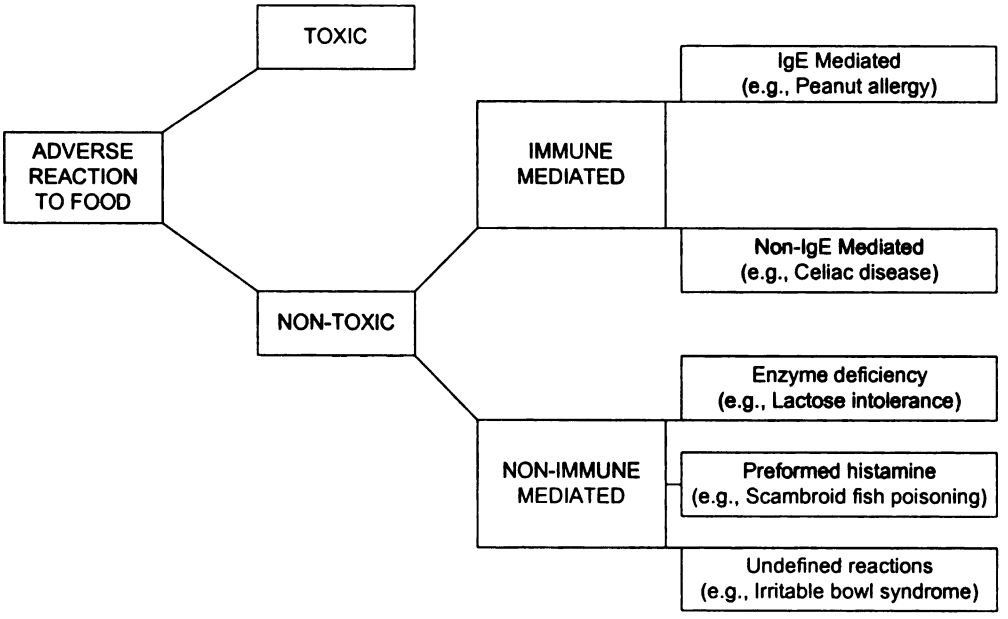
In this chapter I have reviewed the relevant published work available on the following topics in food allergy: food induced adverse reactions and food allergy; allergenic foods vs. food allergens; food allergy - relevance to food industry; prevalence of food allergy; mechanism of food allergy; prevention and treatment of food allergy - potential role for herbal supplements; mouse models of food allergy - valuable tools to elucidate disease mechanisms and to develop new preventive and therapeutic approaches.

Food Induced Adverse Reactions and Food Allergy

Food induced adverse reactions are broadly classified into two types: 1) toxic and 2) non-toxic reactions **(Fig. 1)**. Whereas, toxic adverse reactions are mediated by biologically active microbial toxins, non-toxic reactions are due to specific food constituents such as allergens or lactose. Non-toxic reactions are further classified as immune mediated and non-immune mediated. In this system of classification, food allergies are classified as non-toxic, immune-mediated adverse reactions (33).

The mechanism underlying food intolerance such as lactose intolerance is different from that of food allergy. Thus, as opposed to food allergy, food intolerance is not caused by the immune system. For example, lactose intolerance is due to a precise metabolic defect such as absence and/or deficiency of lactase (34).

Fig. 1: Classification of food induced adverse reactions Food induced adverse reactions are classified primarily into two types: toxic and non-toxic reactions. Non-toxic reactions are further classified as immune mediated and non-immune mediated reactions. Immune mediated reactions are subdivided as IgE mediated and non-IgE mediated reactions. Most food allergies are examples of IgE mediated immune reactions, where as celiac disease and some milk allergies are examples of non-IgE mediated reactions. Lactose intolerance is an example of non-immune mediated adverse reaction due to enzyme deficiency. Scambroid fish poisoning is an example of adverse reaction due to preformed chemicals (such as histamine) in


ADVERSE REACTION TO FOOD
TOXIC
NON-TOXIC
IMMUNE MEDIATED
IgE Mediated (e.g., Peanut allergy)
Non-IgE Mediated (e.g., Celiac disease)
NON-IMMUNE MEDIATED
Enzyme deficiency (e.g., Lactose intolerance)
Preformed histamine (e.g., Scambroid fish poisoning)
Undefined reactions (e.g., Irritable bowl syndrome)

Allergenic Foods vs. Food Allergens

Any food that triggers an allergic reaction in a sensitized individual is called an allergenic food. More than 160 different types of foods have been documented to trigger allergic reaction in sensitized individuals (35). However, according to the Food and Agriculture Organization (FAO), the Food Allergy Task Force of the International Life Sciences Institute (ILSI), and the United States Food and Drug Administration (US FDA), nearly 90% of all food allergies are caused by eight main food types called "red flag" allergenic foods. They are: Chicken egg, cow's milk, peanut, soybean, wheat, tree-nut, fish, and shell-fish (36-39). However, in addition to these eight foods, the Canadian Food Inspection Agency (CFIA) has added sesame seeds and sulphites to their list of allergenic foods (40). The European Food safety Authority (EFSA) considers cow's milk, eggs, fish, peanuts, tree nuts, soy, celery, cereals containing gluten, crustacean (crab, crayfish, lobster and shrimps), sesame, mustard, and sulphites as major allergenic foods (41).

Tree nuts have been identified as a major group of red flag allergenic foods by the US FDA, the CFIA as well as the EFSA (39-41). Tree nuts, along with peanuts and sesame, are the leading cause of life-threatening allergic reactions such as systemic anaphylaxis (42, 43). Accordingly, US FDA has identified the following tree nuts as "red-flag" tree nuts for regulatory purpose: Almond; beech nut; Brazil nut; butternut; cashew; chestnut (Chinese, American, European, Seguin); chinquapin; coconut; filbert/hazelnut; ginko nut; hickory

nut; lichee nut; macadamia nut/bush nut; pecan; pine nut/pinon nut; pili nut; pistachio; sheanut; and walnut (English, Persian, black, Japanese, California) (44).

The active ingredient present in the allergenic food that is responsible for triggering an allergic reaction is called a food allergen. Typical food allergens are either proteins or glyco-proteins (45, 46). These allergens elicit IgE antibodies and bind to them specifically. Food allergens are usually: 1) proteins of 10-70 kDa; 2) heat resistant (90°C, 5 minutes); 3) acid stable (pH $\leq$ 2.0); 4) resistant to pepsin digestion; and 5) water soluble (21). However, these properties have little value in predicting allergenic potential of a given protein (47). Following are some examples of major food allergens: *Ara h1*, *Ara h2*, and *Ara h3* are major peanut allergens; casein, α lactalbumin, and β lactoglobulin are major milk allergens (48, 49).

Food Allergy: Relevance to Food Industry

Food industry is facing several challenges because of food allergy. Some of these challenges are: 1) food sensitized consumers are at risk of potentially fatal allergic reactions; 2) compensation to food allergy victims, lawsuits; 3) product recalls due to food allergen contamination; 4) allergen labeling issue; 5) potential allergenicity of exotic foods and genetically engineered foods; 6) lack of consensus on the threshold for allergenic foods to elicit a clinical reaction; and 7) the effect of processing on food allergenicity (21).

According to the United States Food and Drug Administration (US FDA), presence of "red flag" allergenic foods in a product should be identified on the label (50). Failure to include such information on the label is the main reason for class I food recalls in the United States of America (12).

To protect food allergic consumers, a new law (Food Allergen Labeling and Consumer Protection Act) came into effect in January 2006. It is intended to make the food allergen labeling much more consumer friendly than it was before. Thus, all food products containing the major allergenic foods must identify them with common names on the label (e.g., 'milk' instead of 'casein'; 'peanut protein extract' instead of 'protein extract' etc). Furthermore, in the case of tree nuts, fish and crustacean shellfish, more specific information on the type of nut and species of fish/shellfish must be identified on the label (50).

Availability of commercial rapid immunoassays (such as enzyme linked immunosorbent assay, often called ELISA) to detect allergenic foods in field samples has helped the identification of allergen contamination. However, a major issue is about acceptable levels of food allergen contamination. According to the US FDA there are no recommended threshold levels for allergenic food contamination of food products. Consequently, zero tolerance is the current policy of US FDA for food allergen contamination (21, 51, 52).

Potential impact of food processing on food allergenicity is another area of concern for the food industry (53, 54). However the specific impact of different processing methods on specific allergens in different food types is not well studied (21).

Potential allergenicity of novel foods such as genetically engineered foods is another significant problem for the food biotechnology industry. Since genetically engineered foods express new non-indigenous proteins, potential allergenicity of the newly introduced protein is an issue. During 2000-2001, contamination of food with a genetically engineered corn (StarLink) occurred. This corn was originally approved for use as animal feed. Subsequent consumer complains of potential allergies to this corn led to food recalls. Total cost of the cleanup from this episode of contamination has been estimated be ~100 million dollars (14-18).

Prevalence of Food Allergy

Overall prevalence of food allergy in the United States of America is ~ 4% in adults and ~6-8 % among children (55). Among children, cow's milk with a prevalence of 2.5%, is the leading cause of food allergy. Prevalence rate of other food allergies among children are as follows: egg at 1.3%; peanut at 0.8%; wheat and soy at 0.4%; tree nuts at 0.2%; and fish as well as shell fish at 0.1% (55).

Children usually outgrow milk, egg, wheat and soy allergies by the age of 3 years. Other food allergies such as peanut, tree nut, fish and shell fish

allergies are rarely outgrown. Among adults, the prevalence rate of the later four food allergies is as follows: shell fish at 2%; peanut at 0.6%; tree nuts at 0.5%; and fish at 0.4% (55).

The overall prevalence of tree nut allergy in the United States of America is ~0.5% (55, 56). According to the peanut and tree nut registry, which is the largest source of self-reported allergic reactions in the USA, walnut is the leading cause of tree nut allergies (at 34%). It is followed by cashew (at 20%), almond (at 15%), pecan (at 9%), pistachio (at 7%), and other nuts (at 5%) (57). In another report, where 54 tree nut allergic pediatric patients were followed at Johns Hopkins, walnut was again the leading allergenic nut at 26% followed by almond (13%), pecan (13%), cashew (11%), hazelnut (7%), pine nut (7%), pistachio (7%), and Brazil nut (4%)(58). Notably, hazelnut allergy has been reported to be more common in Europe than in the USA. It is thought to be due to increased exposure to hazel pollen in Europe (59, 60).

Currently the US FDA, as opposed to CFIA and EFSA, does not consider sesame as a major allergenic food (39-41). However, a previous study from our laboratory found that the reporting of sesame allergy is growing world-wide (61). An Australian study found that sesame was the fourth (0.42%) leading cause of food allergy among children after egg (3.2%), milk (2%), and peanut (1.9%). This study also found that sensitivity to sesame was greater than any single tree nut studied (62). Prevalence of severe allergic reactions to sesame

was estimated at 0.05% in the general population of the United Kingdom (63). A recent study on food allergy among Israeli children concluded that sesame was the third most common food causing sensitization at (0.18%) after egg (0.5%) and cow's milk (0.3%) (64). Thus, there is considerable evidence to suggest that the sesame allergy may be a growing food allergy with serious life-threatening consequences such as systemic anaphylaxis (43).

Mechanism of Food Allergy

Coombs and Gel Classification of Immune Mediated Adverse Reactions

Immune mediated adverse reactions in general were originally classified by Coombs and Gel as hypersensitivity reactions (21, 65). Based on the type of immune component involved, the time of onset of the reaction after exposure to the antigen, and the type of antigen involved, they classified hypersensitivity reactions into four groups: Type-I; Type-II; Type-III; and Type-IV reactions. Major features of these reactions are briefly reviewed below.

Type-I Hypersensitivity Reaction

Type I hypersensitivity is also called as the immediate hypersensitivity reaction because it occurs within minutes after exposure to a type of antigens called allergens. It is mediated by IgE antibodies produced in response to allergens. Allergen binding to the IgE antibody fixed onto mast cell leads to cellular activation and the release of chemical mediators such as histamine—a

major mediator of clinical symptoms of allergy such as anaphylaxis. Most food allergies, and allergic asthma are examples of Type-I hypersensitivity reactions.

Type-II Hypersensitivity Reaction

Type II hypersensitive reaction, also known as cytotoxic reaction, is mediated by the IgG antibody. Here antibodies bind to the insoluble antigen (e.g. antigen present on cells and/or bound to cells) and activate the complement system leading to tissue damage. Role for this mechanism in food allergy is unclear at present. Examples include autoimmune hemolytic anemia and some drug allergies.

Type-III Hypersensitivity Reaction

In type III hypersensitivity, IgG and IgM antibodies bind to the circulating antigen and form immune-complexes. Deposition of these immune-complexes leads to tissue damage. It is not known at present whether this mechanism may play a role in food allergy. Lupus—an autoimmune disease, is a common example of type-III hypersensitivity reaction.

Type-IV Hypersensitivity Reaction

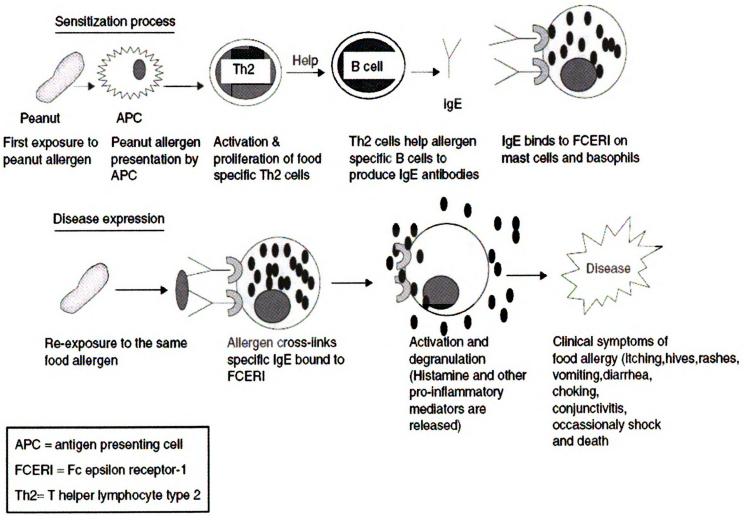
Type-IV hypersensitivity reaction is also called as delayed hypersensitivity reaction. This is because, after exposure to the antigen, it takes at least 24 hrs for the reaction to manifest. It is mediated by antigen specific T lymphocytes. Although food allergies in general are considered as immediate

hypersensitivity reactions, it is proposed that milk allergy may involve both Type-I and Type-IV mechanisms (66). Furthermore, Type-IV mechanism is considered to be a major player in gluten enteropathy (or the Celiac disease).

Mechanism of Food Allergy: An Immune Response Model

The mechanism underlying food allergy is not completely understood at present. It is generally thought to be due to, Type-I hypersensitivity reactions similar to other allergic diseases such as airways allergies (20). Accordingly, an immune response model has been proposed to explain how foods such as peanuts trigger allergic reactions. The major feature of this model is illustrated **(Fig. 2)** (21). According to this proposed mechanism, there are two general stages in the development of food allergy: (1) sensitization phase; and (2) disease expression phase. When a genetically susceptible individual is exposed to peanut for the first time, peanut proteins are phagocytosed by antigen presenting cells (such as dendritic cells/macrophages) and peanut allergens are presented along with MHC class II molecules to the CD4 positive T cells. The latter undergo differentiation into T helper (Th)-2 subset and help the allergen specific B cells to produce IgE antibodies. Most of these antibodies are taken-up and displayed by mast cells and basophils that express the high affinity receptor for IgE (called Fc epsilon receptor-I). Once an individual has produced IgE antibodies and displayed them on their mast cells, he/she is considered to be 'sensitized' to that food. At this point there are no clinical symptoms. However, following re-exposure to the same food (in this case, the peanut),

Fig. 2: Mechanism of peanut allergy: Immune response model. Exposure of a genetically susceptible individual to peanuts results in presentation of peanut allergens by antigen presenting cells (APC) to T helper (Th) lymphocytes. A consequence of this is the proliferation and activation of peanut specific Th-2 lymphocytes that help peanut specific B cells to produce peanut specific IgE antibodies. IgE antibodies bind to the high affinity IgE receptor present on cells such as mast cells that are rich in histamine and other proinflammatory mediator containing granules. Subsequent exposure to the same allergen (peanut in this case) results in cross linking of surface IgE receptors on mast cells results in cellular activation, degranulation. Host response to histamine and other mediators results in the expression of clinical symptoms of food allergy within minutes of exposure to allergens (Source: Ref. No (21)).


Sensitization process
Peanut
APC
Th2
Help
B cell
IgE
First exposure to peanut allergen
Peanut allergen presentation by APC
Activation & proliferation of food specific Th2 cells
Th2 cells help allergen specific B cells to produce IgE antibodies
IgE binds to FCERI on mast cells and basophils
Disease expression
Disease
Re-exposure to the same food allergen
Allergen cross-links specific IgE bound to FCERI
Activation and degranulation (Histamine and other pro-inflammatory mediators are released)
Clinical symptoms of food allergy (itching,hives,rashes, vomiting,diarrhea, choking, conjunctivitis, occassionaly shock and death
APC = antigen presenting cell
FCERI = Fc epsilon receptor-1
Th2= T helper lymphocyte type 2

peanut allergens can cross-link the IgE antibodies displayed on mast cells and cause cellular activation and release of histamine and other mediators of allergic reactions. This results in clinical symptoms such as hives, anaphylaxis etc.

Thus, allergen presentation via MHC class II molecules, activation of CD4 positive Th2 cells, and production of IgE antibodies are thought to be critical mechanisms underlying food allergy development. Therefore, I have reviewed these events in detail in the next section.

The Major Histocompatibility Complex Class II and The Allergic Response

The major histocompatibility complex (MHC) in humans is called the human leukocyte antigen (HLA) and in mice it is called the H-2 complex (67-69). It is located on the human chromosome 21q and the mouse chromosome 17 (69). The *H-2* complex consists of several major genes including MHC class II encoding genes (70).

As discussed earlier, MHC class II molecules are involved in presenting antigen/allergen peptides to the CD4 positive T cells. The CD4 positive T cells are able to recognize only those antigens that are presented with MHC class II molecules **(Fig. 3)**.

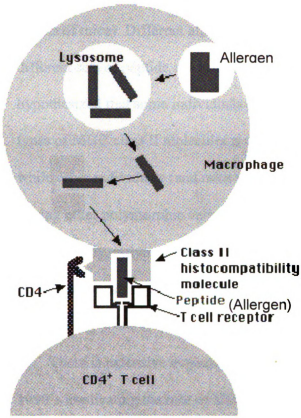


Fig. 3: Allergen presentation to the T lymphocyte: Role of MHC class II and CD4 molecules Antigen presenting cells (such as macrophages) present the processed allergen-derived peptides along with MHC class II molecules to T lymphocytes. The T cell receptor can recognize the allergen only when it is presented with MHC class II molecules. The CD4 molecule expressed on the surface of $CD4^+$ T cells act as co-stimulatory molecules in the allergen presentation (Modified from http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/AntigenPresentation.html).

Polymorphism is a major feature of the MHC class II molecules. Thus, different allelic forms are inherited by different subjects (and by different strains of mice). Different allelic forms of MHC class II molecules present different sets of peptide antigens/allergens to T cells (2). Consequently, it is hypothesized that some individuals (and some mice strains) with particular types of MHC class II molecules may be able to present allergens much better while other individuals (and mice strains) may be unable to do so due to their having other polymorphic variants of MHC class II molecules (71). Consequently, polymorphisms in MHC class II is considered as an important genetic factor that may render susceptibility for allergic disorders (72).

There is extensive evidence from studies conducted during 1980 and 1990's implicating the role of MHC class II molecules in the production of specific IgE antibodies to pollen and house dust mite allergens (73). Similarly, it is well established that whereas, some strains of mice with certain MHC class II haplotypes (e.g., Balb/c, H-2 ^{d}) are 'high IgE responders' to allergens, other strains with different MHC class II variants (e.g., C57BL/6, H-2 ^{b}) are intermediate responders, and some other strains (e.g., SJL, H-2^{S}) are low/non-IgE responders (74-76).

As opposed to airways allergies and asthma, role of MHC class II polymorphism in food allergy has not been well studied. A recent study suggested the possible role of HLA class II genetic polymorphism in

determining susceptibility to peanut allergy (77). Similar to human disease, role of MHC class II genes in mouse models of food allergy has not been examined in-depth.

Role of CD4 Positive T helper (Th) - 2 Cells in Allergic Responses

The CD4 positive T cells play an important role in allergic immune responses by helping B cells to produce the IgE antibody (2). The CD4 molecules expressed on the surface of CD4 positive T cells enable them to bind to antigen/allergen presenting cells. Once activated, CD4 positive T cells proliferate and differentiate into two main subsets of cells--T helper Type 1 (Th1) and T helper Type 2 (Th2) cells. Whereas Th1 cells play a major role in cell mediated immunity (e.g., delayed type hypersensitive reaction), Th2 cells are key players in allergic responses.

Mossman et al (1986) originally named the mouse T helper cells into Th1 and Th2 based upon cytokines they secrete. Whereas, secretion of IFN γ defines a Th1 cell, Th2 cells secrete mainly IL-4, IL-5 and IL-13 (78). A similar profile was also recognized later in humans (79). Other studies found that in addition to CD4 positive Th2 cells, other immune cells can also produce Th2 cytokines. For example, IL-4 can be produced by mast cells (80), Natural Killer (NK) cells (81) , basophils (82) and eosinophils (83); IL-5 can be produced by eosinophils (83) and NK cells (84); IL-13 can be produced by B cells (85), mast cells (86), NK cells (87), dendritic cells (88) and basophils

(89). Recognizing that diverse types of immune cells can produce these cytokines, a Th2 cytokine is also sometimes referred to as a Type-2 cytokine when the cellular source is not clear.

It is well established that there is excessive production Type-2 cytokines such as IL-4, IL-5 and IL-13 in allergic disorders such as allergic rhinitis and asthma (90). Several studies have demonstrated that the transcription factor, STAT6, plays a major role in the regulation of Th2 cytokine responses (91, 92). Some studies indicate that Type-2 cytokines are important in food allergies such as peanut allergy (20, 93), although other studies do not support this (94). Furthermore, relative contribution of these cytokines to disease expression and the mechanism of their regulation are not completely understood in food allergy. It is also unclear whether different types of food allergies may exhibit different profile of Type-2 cytokine responses. Thus, because of their importance in allergic disease in general and their potential role in food allergy, I have briefly reviewed each individual Type-2 cytokine followed by a discussion on the role of STAT6 in food allergy in the next section.

Interleukin-4 (IL-4)

Interleukin (IL)-4 is a highly pleiotropic cytokine produced primarily by activated CD4 positive Th2 cells (95, 96). It is also produced by mast cells (78), NK cells (79), basophils (80) and eosinophils (81). Consistent with its pleiotropic nature IL-4 receptor is present on T cells, other hematopoietic cells

(e.g., basophil, eosinophil) as well as non-hematopoietic cells (e.g., fibroblasts and epithelial cells) (95).

Molecular and Structural Characteristics

Human IL-4 is a 129 amino acid long, 18 kD protein. The IL-4 gene spans about 10 kb and is located on chromosome 5 (5q31.1-31.2) (95). Mouse IL-4 is a 120 amino acid long, 18 kD protein and its gene is located on chromosome 11 (95, 97). The crystallographic structure of recombinant human IL-4 revealed that, it is a highly compact globular protein with a predominantly hydrophobic core (98).

Major Functions of IL-4

Interleukin-4 was first described as a B cell growth factor in the mouse system. It is vital for B cell growth, immunoglobulin class switch to IgE as well as IgG1 classes (96). IL-4 induces the expression of MHC class-II molecules and the high affinity IgE receptor (FcεRI) on B cells (95). It acts as a T cell growth factor; it induces proliferation of both human and murine T lymphocytes (99). It also differentiates T cells into Th2 cells (100, 101). It increases the growth of mast cells (102). It also increases the vascular responsiveness to mediators of allergic inflammation (103). The phenotype of mice deficient in IL-4 gene demonstrates the major role of this cytokine in IgG1 and IgE production (104). However specific role of IL-4 in food allergy is unknown.

Interleukin-5 (IL-5)

Interleukin (IL)-5 is produced mainly by activated Th2 cells, as well as mast cells, NK cells, and eosinophils (83, 84). The major target cells for IL-5 are eosinophils which express IL-5 receptor (105, 106). Consequently, IL-5 is a major player in chronic inflammation associated with eosinophil infiltration such as asthma (107). Studies using IL-5 gene knockout mouse model have demonstrated a key role for IL-5 in allergic eosinophlic esophagitis --an inflammatory condition often associated with food allergy in some individuals (108).

Molecular and Structural Characteristics

Human IL-5 is a 117 amino acid long protein. The IL-5 gene is located on chromosome 5 (5q31.1) (109). Mouse IL-5 is a 111 amino acid long protein and its gene is located on chromosome 11 (109). Compared to IL-4, IL-5 is a bigger protein with a molecular weight of 45 kDa. IL-5 exists as a dimer with two identical polypeptide chains wound together to achieve functional confirmation (110).

Major Functions of IL-5

The major target cell for IL-5 is an eosinophil. Stimulation of eosinophils with IL-5 leads to their activation and degranulation (111). It also inhibits the apoptosis of eosinophils (112, 113). IL-5 is the key cytokine in the production

and survival of eosinophils. Importance of IL-5 in the production of eosinophils is evidenced by the dramatically reduced number of eosinophils in IL-5 knockout mice (114). IL-5 also facilitates entry of eosinophils from the circulation into the tissue (115). Since eosinophilia is a characteristic feature of chronic allergic inflammation, IL-5 is considered as a major player in allergic disorders (107). Notably, the specific role of IL-5 in food induced systemic anaphylaxis is unknown.

Interleukin-13 (IL-13)

Interleukin (IL)-13 is a pleiotropic cytokine produced by activated Th2 cells, B cells (83), mast cells (84), NK cells (85), dendritic cells (86) and basophils (87). It plays a critical role in allergic inflammation of the airways associated with asthma (116, 117). IL-13 shares many functions similar to that of IL-4 because these two cytokines share a common component in their receptor called IL-4Rα (118). The shared actions of IL-4 and IL-13 include: 1) induction of MHC class-II expression; 2) helping B cells to produce IgE in humans (but not in mice) (119).

Molecular and Structural Characteristics of IL-13

Human IL-13 gene is located on chromosome 5 along with genes of IL-3, IL-4, IL-5, IL-9, and GM-CSF (120). The mouse IL-13 gene is located on chromosome 11 (121). The high resolution multidimensional NMR structure of

human IL-13 found that it has a short chain left handed four-helix bundled structure (120).

Major Functions of IL-13

Along with the common functions it shares with IL-4, researchers found that IL-13 has an important role in pathophysiology of asthma (116, 117). For example blocking of IL-13 can inhibit pathophysiological changes in asthma such as excess mucus secretion (122). The phenotype of mice deficient in IL-13 gene revealed its importance in expulsion of helminth parasites from the gastrointestinal tract (123). In addition to IL-4, IL-13 is important in IgE production in humans; but its role in IgE production in mice is controversial. Notably, specific role of IL-13 in food allergy and anaphylaxis is unknown.

STAT6: Potential Role in Food Allergy

Signal transducers and activators of transcription (STATs) are the members of a family of transcription factors that activate gene transcription in response to a signal from cytokines (124). STATs are cytoplasmic proteins that are activated by tyrosine phosphorylation by the cytokine receptor associated Janus kinases after the binding of cytokine to its receptor (125). Phosphorylation leads to dimerization of individual STAT proteins. The resulting dimer then migrates to the nucleus and activates the transcription by binding to the target DNA sequence (125).

STAT6 is a member of this group of transcription factors. It is especially important in the regulation of IL-4 gene transcription (126). Studies using *Stat6* knockout mice found that STAT6 was necessary for the gene activation signal in response to IL-4 stimulation. Lymphocytes from *Stat6* deficient mice are unable to express MHC Class II expression in response to IL-4 (91). *Stat6* deficient B lymphocytes were unable to switch to the immunoglobulin isotypes IgG1 and IgE in response to IL-4 (127). *Stat6* deficient T helper cells were also unable to differentiate into Th2 cells *in vitro* or *in vivo* (91). Studies using mouse models have shown that STAT6 also plays an important role in IL-13 responses to helminth infection in mice (128, 129).

Thus, it is well established that the signaling molecule, STAT6 plays a key role in the Type-2 immune responses in general. However, specific role of STAT6 in food allergy is not well studied. There is one study suggesting a potential role for STAT6 in nut allergy in particular in humans. Thus, Amoli et al (2000) initially identified a single nucleotide polymorphism (SNP) in 3' UTR of the STAT6 gene in a cohort in the United Kingdom. Subsequently they studied the frequency of this SNP in 71 nut allergic patients and compared it with 45 atopic patients without nut allergy and 184 healthy controls. They found that a polymorphic variant of STAT6 (called as G allele) frequency was significantly increased in nut allergic patients when compared to atopic individuals without nut allergy and healthy controls. Based on these data, they concluded that STAT6 3' UTR polymorphism might play an important role in

determining susceptibility and severity among nut allergic patients (130). Notably, the role of STAT6 in mouse models of food allergy has not been examined previously.

IgE Antibody: A Central Role in Most Food Allergies

Prausnitz and Kustner (1921) first demonstrated the presence of a serum component responsible for an allergic reaction (131). Later, Ishizaka and Ishizaka (1966) discovered that IgE antibody was the serum component responsible for an allergic reaction (132). This antibody was later named as 'IgE ('E' for erythema or a rash)'.

Immunoglobulin (Ig) E (also known as the reaginic antibody), is one of the five isotypes of immunoglobulins (2). IgE plays a central role in allergic reactions (2). However, it is thought to have evolved to protect hosts against helminth parasite infestations (133).

Among immunoglobulins, IgE is the least abundant isotype in the blood, normally present in nanogram per mL of blood. These levels are significantly lower than other isotype such as IgG that is present in milligram per mL of blood. Among immunoglobulins, IgE has shortest serum half-life (2 days) (2). After its production, IgE binds to the high affinity IgE receptor (FcεR-I) present on the surface of mast cells and basophils (134). Even though it has very short serum half-life, IgE remains for weeks or months when bound to FcεR-I on the

surface of mast cells and basophils. Binding of allergen to the IgE-IgE receptor complex leads to the cellular activation and release of inflammatory mediators such as histamine. These reactions are important in the clearance of parasites as well in triggering allergic reactions as described earlier (**Fig. 2**) (133).

Although central role of IgE in airways allergies is well known, the key role of IgE antibodies in food allergy was demonstrated only recently in peanut allergic subjects (28). Thus, neutralization of circulating IgE using an anti-IgE vaccine in peanut allergic children was found to confer clinical protection against peanut allergy (28, 135). Furthermore, other studies demonstrated that circulating levels of food specific IgE correlated with food induced clinical reactions and higher IgE levels were predictive (~95% chances) of food allergy (136). The predictive IgE level for egg allergies at ≤ 2 years age is 2 kIU/L and at age 5 is 7 kIU/L. In case of milk allergies, the predictive levels are 5 and 15 kIU/L at ages 2 and 5 respectively (136). Predictive IgE level for peanut allergy is 14 kIU/L at mean age of 5 years (136). Notably, predictive values for other food allergies such as tree nut allergies have not been established as yet.

It is noteworthy that there are reports of a subset of food allergies independent of IgE antibodies. For example some milk allergic patients do not have demonstrable milk specific IgE antibodies in the blood, but continue to react to oral exposure to milk (66). However, mechanisms underlying such IgE independent food allergies are not completely clear at present (21).

Prevention and Treatment of Food Allergy

Currently effective preventive methods (such as a vaccine) are not available for food allergy, except an experimental peanut allergy vaccine that was reported to confer partial protection (28). Although exclusive breast feeding has been suggested to provide some protection against food allergy, it continues to be an intense research area to clarify doubts raised by others (137, 138). Thus, the only recommended and effective preventive method for food allergy is to avoid exposure to the causal food. Although this is a very useful method, many food allergy cases occur due to accidental exposure to the offending food (e.g., in a restaurant or in a processed/packaged food without adequate labeling). Thus, novel methods for prevention of food allergy are highly desirable.

Currently available treatment methods for food allergies include: 1) use of epinephrine injections to save life in case of systemic anaphylaxis; 2) use of anti-histamines to control symptoms such as hives; and 3) corticosteroids to control inflammation (55).

Several novel approaches to treat and prevent food allergies are under investigation. These include: 1) the reduction of IgE by the infusion of anti-IgE antibodies; 2) the use of probiotics; (3) the use of gene therapy; 4) use of cytokines such as IL-12; and 5) immunotherapy with mutated allergen derived proteins and peptides; and 6) herbal supplements (24-30, 32, 139-142).

Since the second part of my thesis work involved the testing of herbal supplements, I have briefly reviewed the potential of herbal supplements for application in food allergy in the next section.

Herbal Supplements: Potential Utility in The Prevention and/or Therapy of Food Allergy

A large body of evidence in the literature suggests that herbal supplements can modulate immune responses (**Table. 1**). Consequently, it is possible that some herbal supplements may be useful in the prevention and/or therapy of immune mediated disorders such as allergies and autoimmune disorders (143).

Many studies demonstrate that several herbal supplements can modulate immune responses especially Th1/Th2 cytokine production in mouse models of diseases (**Table 1**). Consequently, there is potential for application of herbal supplements in immune mediate disorders characterized by Th1 or Th2 polarized cytokine responses.

There is a growing interest in the potential utility of herbal supplements in the prevention and treatment of allergies and asthma (143). However, the potential utility of herbal supplements for food allergy has not been explored in-depth. There are few recent studies testing the potential of Chinese herbal supplements in a mouse model of peanut allergy (23, 29). Thus, researchers at the Mount Sinai school of medicine (New York), using a cholera-toxin based peanut

Table 1: Impact of herbal supplements on Th1/Th2 cytokine production in various experimental model systems

Herbal supplement	Experimental model system	Results	Reference
Garlic/Alliin	Leishmania infected BALB/c mice	Increase in IFN gamma and decrease in IL-4 and IL-10 in spleen cell cultures	(145)
Echinacea	Rat macrophages	Increase in IFN gamma	(146)
Licorice/ Glycyrrhizin	Peritoneal macrophages	Increase in IL-12 production	(147)
Milk thistle/Silymarin	Murine mixed lymphocyte culture	Increase in IFN gamma production	(148)
Neem leaf extract	Rat/ Oral feeding	Increase in IFN gamma production in lymph node cultures	(149)
Rumex japonicus	NC/Nga mice/oral administration	Decrease in IL-4 and no change in the IFN gamma levels in serum	(142)
Ailanthus altissima	BALB/c mice/ Oral feeding	Decrease in IL-4 and IL-13 mRNA expression in lung tissue	(150)

allergy mouse model, have been testing the utility of Chinese herbal supplements in their model. They found that a Chinese herbal formula—that they named Food Allergy Herbal Formula (FAHF), was effective in completely blocking the peanut induced systemic anaphylaxis in their mouse model (23, 29).

Thus, these studies using a peanut allergy mouse model, in principle, demonstrates the potential utility of herbal supplements in food allergy prevention and therapy. However, in addition to these few herbs, there are a large number of other herbal supplements available and their potential remains to be tested. Furthermore, effectiveness of herbal supplements against different subsets of food allergy (e.g., tree nut allergy etc) also remains to be tested. This was the focus of the second part of my thesis work.

Mouse Models of Food Allergy: Valuable Tools to Elucidate Disease Mechanisms and to Develop New Preventive and Therapeutic Approaches

A number of animal species have been used to develop food allergy models including rats, mice, dog and swine (150-152). However, mouse model of food allergy has been identified as a model of choice by regulatory agencies such as United States Environmental Protection Agency (US EPA) (153). This is because, mouse models offer several advantages including: 1) availability of inbred strains, large litter size and short generation time facilitate studies on the role of genetics in disease susceptibility; 2) availability of gene knockout mice

assist in elucidating the role of a specific gene in disease pathogenesis; 3) relatively lower cost; and 4) the availability of immunological reagents such as antibodies to study disease markers and mechanisms.

A significant obstacle in the development of a mouse model of food allergy is the development of oral tolerance to ingested food antigens. Researchers have used two major strategies to overcome induction of oral tolerance: 1) use of adjuvant (e.g., oral adjuvant, cholera toxin; or systemic adjuvant, alum); and 2) the use of non-oral route (e.g., transdermal route) for sensitization without the use of any adjuvant. I have reviewed these two approaches in the next section below.

Adjuvant-based Food Allergy Mouse Models

A group at the Mount Sinai school of medicine developed a mouse model of cow's milk and peanut allergy using cholera toxin as an adjuvant (154, 155). They found that C3H/Hej mice developed peanut and milk allergy only when orally exposed to peanut or milk protein along with cholera toxin but not peanut or milk protein alone (154, 155). They demonstrated that cholera toxin facilitated allergic response to peanut and milk by overcoming the innate oral tolerance.

Subsequently, a number of other researchers have followed the same principle of using cholera toxin to develop mouse models for other food allergies such as

soybean allergy, buckwheat allergy, and shrimp allergy (156-158). Roy et al (1999) used a complex approach of using a combination of oral cholera toxin and intra-peritoneal injection with alum to develop a mouse model of peanut allergy (140).

Thus, adjuvant based model of food allergy has been very popular among researchers. They are useful to understand food allergy development in the presence of adjuvant such as cholera toxin. However, it is very difficult to separate the effects of adjuvant from that of the food allergen in such models. Furthermore, there is a concern that the use of cholera toxin may enhance sensitivity of the model at the cost of specificity (159). That is, any protein—food allergen or not, administered with cholera toxin may lead to an allergic response. Consequently, it is reported that adjuvant based mouse models may not be useful to assess the allergenic potential of novel food such as genetically modified foods (159).

An adjuvant-free Mouse Model of Food Allergy Using Hazelnut and Sesame as Model Allergenic Foods

Although the adjuvant based models may be useful in studying mucosal immune mechanisms induced by dietary proteins in the context of adjuvant as a co-factor, their relevance to human disease is difficult to assess. Consequently, our laboratory has focused on developing an adjuvant-free method for food protein induced allergic and anaphylactic reactions.

The mechanisms of sensitization to dietary proteins in humans are not yet completely understood. Although the original route of exposure to food proteins leading to initial sensitization in humans is largely suspected to be oral, it is not clear whether all human food allergies results from oral route of sensitization. However, as noted earlier, in most animal models, oral exposure results in tolerance rather than sensitization (160-162). Since it is very common to see infants and children getting exposed to food proteins *via* skin (hands, face, neck, chest, etc) while eating, it is plausible that Langerhan's cells present in the skin function as potent antigen presenting cells and initiate food allergen sensitization (163, 164).

Investigating factors associated with the development of peanut allergy in childhood, Lack et al (2003) concluded that that "sensitization to peanut protein may occur in children through the application of peanut oil (containing small amounts of peanut protein) to inflamed skin" (165). Furthermore, there are reports of sensitization to food proteins *via* occupational exposure to food in food industries (166, 167). Consequently, we and others have proposed that some food allergies may actually develop following initial sensitization to food proteins *via* skin exposure (19, 22, 168).

Based on the rationale presented above, previous workers in our laboratory tested whether transdermal exposure to dietary proteins (in the absence of

adjuvant) in mice leads to allergenic sensitization. They found that found that transdermal exposure to Lipo Poly Saccharide (LPS)-free allergenic food proteins (sesame and hazelnut) but not a non-allergenic food protein (vanilla), in the absence of adjuvant, elicits specific IgE antibody response in BALB/c mice (**Fig. 4 and 5**) (19, 22).

To test whether transdermal exposure to food protein sensitizes mice for a clinical reaction, they examined clinical responses of sesame and hazelnut sensitized mice to oral food protein challenge. As evident, oral challenge with sesame and hazelnut elicited clinical symptoms of systemic anaphylaxis (**Figs. 6 and 7**). In addition, mice undergoing systemic anaphylaxis also exhibited a significant hypothermia (i.e., drop in rectal temperature) at 30 minutes following oral food challenge (**Figs. 6 and 7**) (19, 22).

Using IgE-Western blot and protein sequencing techniques, they studied sesame and hazelnut allergens in this model and found them to be identical to human allergens (19, 22). These data further add validity to the approach and the model that has been established to study food allergy in our laboratory.

Taken together, our laboratory has established an adjuvant-free transdermal allergic sensitization method for food proteins that yield three readouts highly consistent with human allergic sensitization: (1) food protein specific IgE antibody response (an immune marker); (2) systemic anaphylaxis to oral food allergen challenge (a clinical marker); and (3) hypothermia to oral food

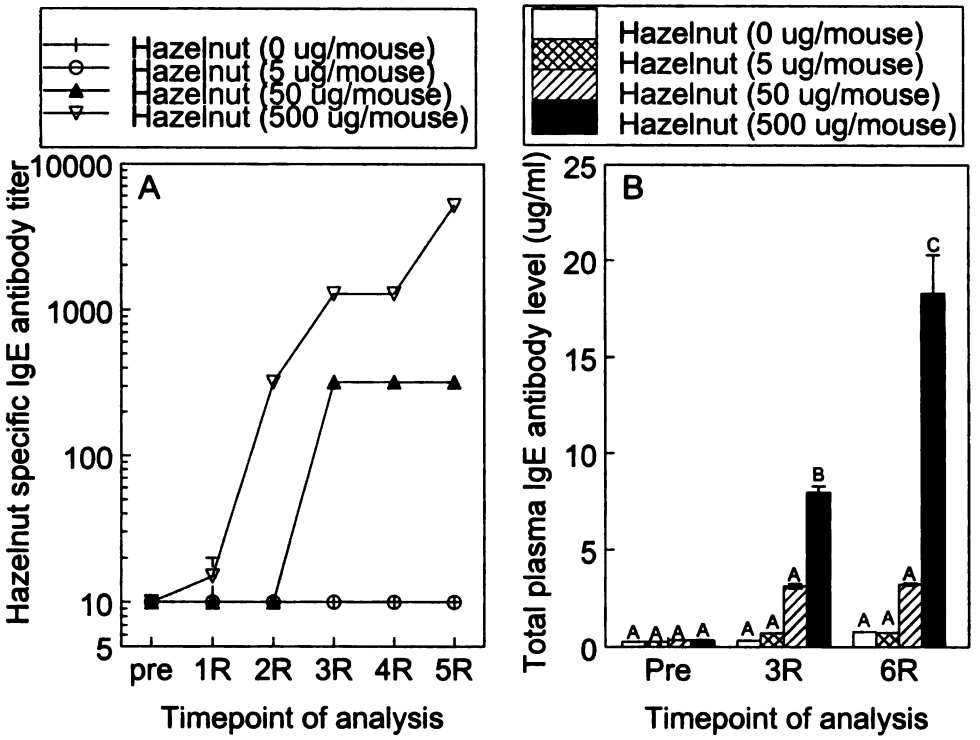


Fig. 4: (A & B): Dose-response and time-course analyses of IgE antibody response to transdermal hazelnut exposure in BALB/c mice. Groups of mice (n=4/group) were transdermally exposed to indicated dose of hazelnut protein extract (0, 5, 50 or 500 μg/mouse). A. Specific IgE antibody levels were measured before (pre) and after 1st, 2nd, 3rd, 4th, 5th and 6th exposure (1R-6R). Data are shown as geometric means ± SE. B. Total IgE levels were measured before (3 days before 1st exposure, Pre) and 3 days after 3rd (3R) and 6th (6R) exposure. Data shown as means ± SE). ANOVA test: bars with different letters are significantly different ($P<0.05$) (Ref. No. (19)).

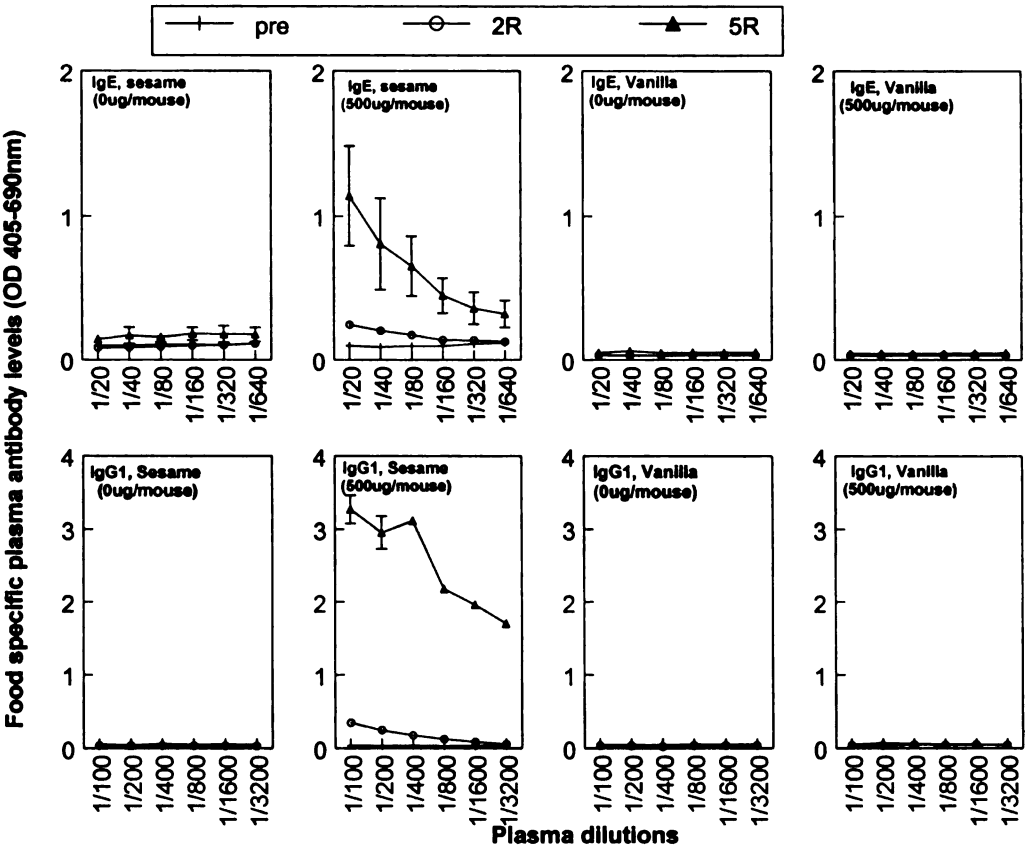


Fig. 5: Immune response to sesame seed protein following transdermal exposure in BALB/c mice Groups of mice (n=4 per group) were exposed to saline or sesame seed extract or control food (vanilla) extract by transdermal applications. Food specific IgE (upper panel) and IgG1 (lower panel) antibodies were measured using pre-immune plasma and following five weeks of exposure. Data is shown from pre treatment, secondary response (2R) and fifth response (5R) (Ref. No. (22)).

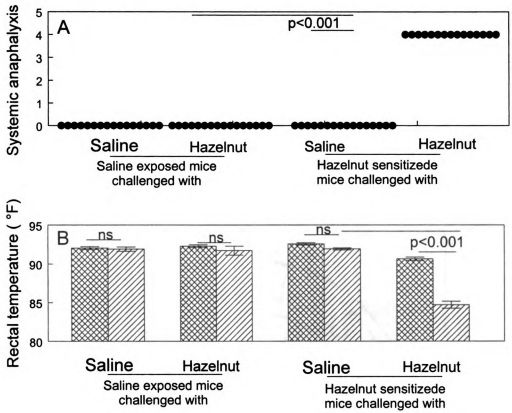


Fig. 6. Transdermal exposure to hazelnut is sufficient to sensitize BALB/c mice for systemic anaphylaxis following oral challenge with hazelnut.

Groups of BALB/c mice (16 mice/group) were sensitized with hazelnut (500 μg/ mouse) or saline by transdermal exposure as described in Materials and Methods. After 6 cycles of exposure, IgE induction was confirmed and then orally challenged with hazelnut (13 mg/mouse) or saline and examined for indicators of systemic anaphylaxis. Data are shown as means ± SE. A. Clinical symptoms are shown as scatter plot with each symbol representing 1 mouse. Kruskal-Wallis test results: hazelnut sensitized mice with hazelnut challenge, $p<0.05$ vs. all other groups. B. Changes in rectal temperature ANOVA test results: bars with different letters are significantly different ($P<0.01$) (Ref. No. (19)).

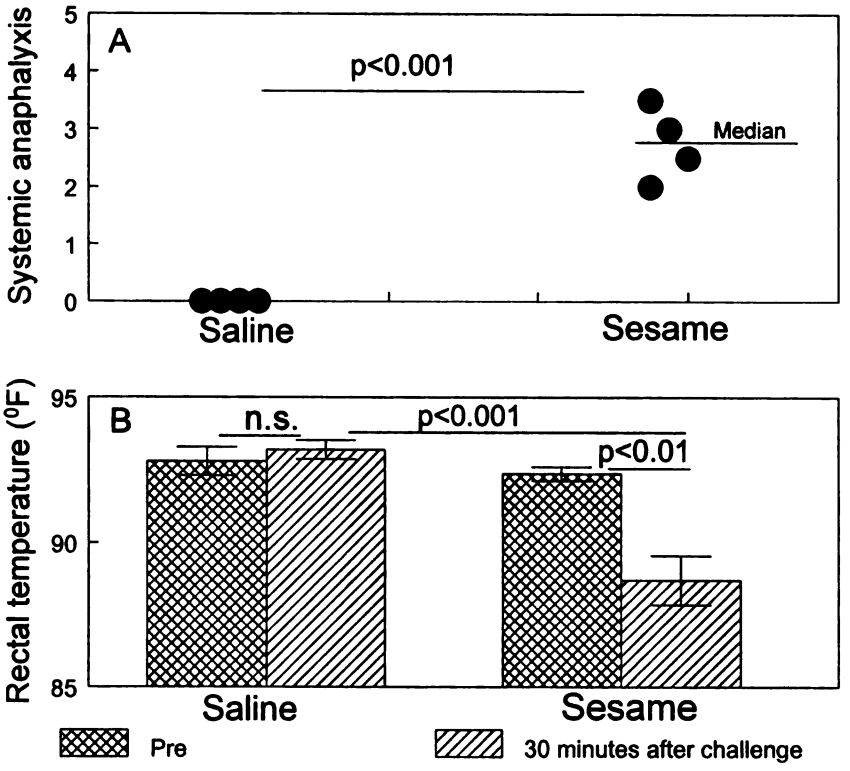


Fig. 7: Systemic anaphylaxis to sesame seed protein in transdermally sensitized mice. Groups of BALB/c mice (n=4 per group) were sensitized with sesame seed protein (500 ug per mouse) or saline via transdermal exposure for 6 weeks and confirmed for IgE response. **A.** Clinical scores of systemic anaphylaxis. **B.** The profile of rectal temperature before and at 30 min following oral challenge with saline or sesame protein (13mg/ mouse). Differences were compared using Student's t test (Ref. No. (22)).

challenge (a physiological marker). This model was used in the present study as discussed in the next section.

Statement of the Problem

As alluded earlier, specific mechanisms of food allergy are incompletely understood at present. Also, the potential of herbal supplements for treating or preventing food allergy has not been completely explored. The work reported in this thesis addressed these two specific problems using an adjuvant-free mouse model developed in our laboratory (19, 22).

This work was conducted in two parts. In the first part, allergen-driven Type-2 cytokine responses were studied in hazelnut and sesame allergy mouse models. In the second part, impact of selected herbal supplements on allergen-driven Type-2 cytokine responses was studied. Specific hypotheses and objectives of this study were as follows:

Part-I

Hypothesis and Objectives

"Transdermal exposure to food allergens leads to activation of allergen-driven CD4 dependent, Type-2 cytokine responses in hazelnut and sesame allergy mouse models"

This hypothesis was tested with the following objectives: 1) to study allergen-driven recall Type-2 cytokine responses in hazelnut and sesame allergy mouse models; 2) to test the role of CD4 in Type-2 cytokine responses; 3) to test the role of H-2 complex in Type-2 responses using mouse strains with different H-2 genetics. Additional follow-up studies were conducted using *Stat6* and *Il4* knockout mice to further characterize the Type-2 cytokine responses in this model.

Part-II

Hypothesis and Objectives

"Selected herbal supplements will inhibit allergen-driven Type-2 cytokine responses by spleen cells from food allergic mice"

This hypothesis was tested with the following objectives: 1) to study the impact of selected herbal dietary supplements on spleen cell viability; 2) to study the impact of above supplements on allergen-driven recall Type-2 cytokine responses by spleen cells from food allergic mice.

CHAPTER 1

CHARACTERIZATION OF TYPE-2 CYTOKINE RESPONSE TO ALLERGENS IN A MOUSE MODEL OF FOOD ALLERGY

Abstract

Background

Food allergy is a growing problem of serious proportions with potential for fatality. The specific role of Type-2 cytokines in food allergy is not completely understood. It is critical to understand such mechanisms as this may lead to better methods for prevention and treatment of food allergies. Our laboratory has developed an adjuvat-free mouse model to study food allergy.

Hypothesis

We hypothesized that, transdermal exposure to food allergens leads to activation of allergen-driven CD4-dependent, Type-2 cytokine responses in this mouse model.

Approach and Results

Initially, the allergen-driven recall Type-2 cytokine responses using spleen cells from hazelnut and sesame-sensitized BALB/c mice were studied. In both models robust allergen-driven, dose-dependent, recall IL-4, IL-5 and IL-13 responses were observed. Using anti-CD4 antibodies in the culture, it was found that, Type-2 cytokine response to allergens were largely CD4 dependent. To test the role of H-2 genetics in Type-2 response, mice strains

with different H-2 locus (BALB/c, H-2^d; ASW, H-2^s, and SJL, H-2^s) were used. Whereas BALB/c and ASW exhibited significant allergen-driven IL-4 and IL-13 responses, SJL did not. However, all three strains produced significant IL-5 responses. Furthermore, follow-up studies using *Stat6* and *Il4* gene knockout mice provided evidence that allergic and anaphylactic responses in this model are dependent on IL-4 but not IL-5 or IL-13.

Conclusion

These data argue that transdermal exposure to food allergen tested results in activation of IL-4, IL-5 and IL-13 responses by positive spleen cells. However, only IL-4 response, that is STAT6 dependent, is required for allergic response and systemic anaphylaxis.

Introduction

Specific role of Type-2 cytokines in food allergy is not completely understood. It is critical to understand such mechanisms as this may lead to better methods for prevention and treatment of food allergies.

Mossman et al (1986) originally divided and named the mouse T helper cells into Th1 and Th2 based upon cytokines they secrete (79). Thus, Th1 cells in general secrete IL-2, Interferon gamma (IFN γ) and Tumor Necrosis Factor alpha (TNF α), whereas Th2 cells secrete mainly IL-4, IL-5 and IL-13. Later studies identified that some of these cytokines can also be produced by non-T

cells that lead to classification of cytokines as Type-1 and Type-2 instead of Th1 and Th2 respectively. Type-1 cytokines have main role in delayed type of hypersensitivity reactions (DTH) and class switch factor for IgG2a antibodies in mice, where as Type-2 cytokines IL-4, IL-5 and IL-13 have role in immediate hypersensitive reactions. Importance of these Type-2 cytokines in allergy relevant phenotypes is evident from various gene knock out studies. Several researchers showed the importance of Type-2 cytokines such as IL-4, IL-5 and IL-13 in allergic diseases. These cytokines play many different roles and participate in allergic reactions as summarized in review of the literature section. Because of their crucial role in IgE class switching IL-4 and IL-13 have gained special significance.

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To understand the patho- physiology of any disease good animal models are desirable. Several efforts were made in the past few years for developing animal models which allow the researchers to study the disease mechanism and also predict the allergencity of new compounds. These include using dogs, pigs, and mice. While each model offers various advantages it also suffers from several limitations.

The hypothesis that transdermal exposure to food allergens leads to activation of systemic allergen-driven CD4-dependent, Type-2 cytokine responses was tested in the mouse model. There were three specific aims: (1) To study allergen-driven recall Type-2 cytokine response in hazelnut and

sesame allergy mouse models; (2) To test the role of CD4 in Type-2 cytokine response; and (3) To test the role of H-2 complex in Type-2 response using mice strains with different H-2 genetics. Additional follow-up studies were conducted using *Stat6* and *Il4* knockout mice (KO) to further characterize the Type-2 cytokine response in this model

Material and Methods

Hazelnut protein extract, Sesame seed extract (Greer Labs, Lenoir, NC, USA); Protein content of these three protein extracts was measured by Lowry's method (170). Normal saline was prepared in our lab (0.85 % W/V NaCl solution); Bovine serum albumin, L-glutamine, Penicillin, Streptomycin and neomycin liquid, Inomycin, Phorbol myristate acetate (PMA), *p*-nitro-phenyl phosphate and MTT assay kit (Sigma, St Louis, MO, USA); 2 Mercapto-ethanol (Gibco BRL Grand Island NY USA); Anti-CD4 antibody, Isotype control antibody and Paired antibodies, recombinant standards for mouse IL-4 and IL-5 (BD PharMingen, San Diego, CA, USA); Paired antibodies and recombinant standards for mouse IL-13 (R&D Minneapolis MN); Streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); 96-well cell culture plates (FALCON, Franklin Lake NJ USA); 96-well ELISA plates (Costar, Corning NY USA). Reagents prepared for ELISA were10X PBS ($NaH_2PO_4.H_2O$, Na_2HPO_4, NaCl), Wash buffer (10XPBS, Tween 20, 2%NaN_3), Dilution buffer (10XPBS, Tween 20, 2%NaN_3, BSA), Blocking buffer (10XPBS, 2%NaN_3, BSA), 5% gelatin

blocking buffer (Gelatin), Coating buffer (Na_2CO_3, $NaHCO_3$, 2%NaN_3, BSA), Substrate buffer (2M $MgCl_2.6H_2O$, Di ethnolamine, pH- 9.8)

Mice

All mice (Table 2) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All animals were females and used at 7 weeks of age. All procedures involving mice were in accordance with Michigan State University animal use policies. Mice were acclimated for one week to their new environment before starting the experiment.

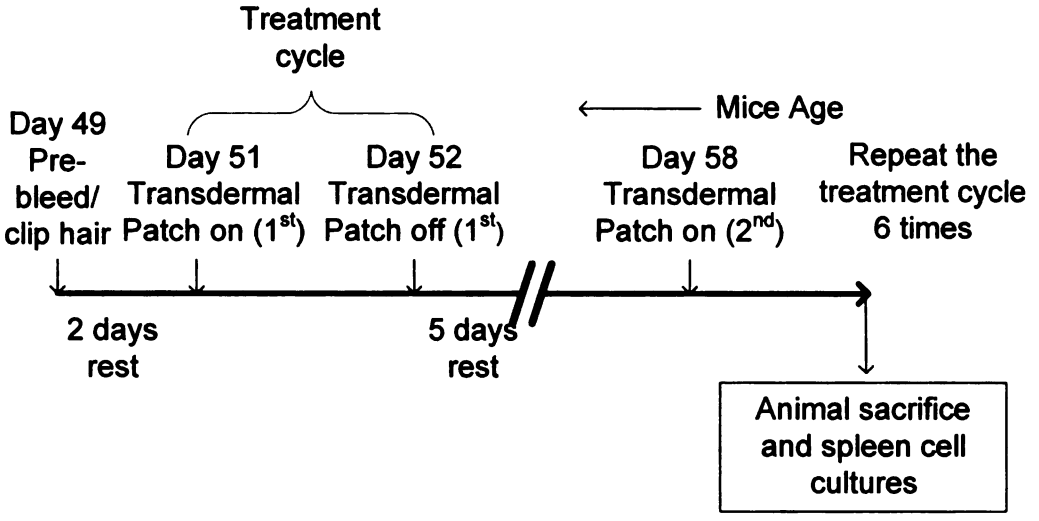
Table 2: Different strains of mice used in the study

Mice strain	MHC Background	Age at arrival (In weeks)	Age at the start (In weeks)
BALB/c	H-2^d	5	7
BALB/c *Stat6* KO	H-2^d	5	7
BALB/c *Il4* KO	H-2^d	5	7
ASW	H-2^s	5	7
SJL	H-2^s	5	7

Transdermal Sensitization

Transdermal exposure experiments were performed using the method described previously (22). Groups of mice were exposed to saline, hazelnut protein or sesame protein extract (500 µg/100 µl in saline per mouse per application); each mouse had the reagent applied to the skin of the back that had the hair clipped-off and covered with non-latex bandage for 1 day. Mice were kept idle for 5 days. Then the cycle of exposure to saline or Food allergen was continued for a total of six times (**Fig.** 8).

Figure 8: Transdermal sensitization protocol



Spleen Cell Culture

Spleen cells were harvested and standard cell cultures were set up essentially as described. Briefly, pooled spleen cells from either saline-exposed mice or food allergen sensitized mice were cultured ($7.5X10^6$ cells/ml) in duplicates in the absence and presence of food allergen (100 and 500 μg/ml). Cells cultured in presence of culture medium alone served as a negative control whereas cells cultured with PMA (1 μg/ml) plus ionomycin (1 μg/ml) served as a positive control. Cells that were cultured for 3 days, the duplicate cultures were removed from the 37^0C incubator at the end of day 3 and pooled in appendorf tube after through mixing, whereas cells that were cultured for 5 days the duplicate cultures were pooled at the end of 5 days. Later the supernatants were separated and stored separately from the mixtures by spinning the tubes at 2000 rpm for 5 minutes.

In the experiments where role of CD4 molecule was tested the pooled splenocytes from both hazelnut and sesame-sensitized mice were cultured in duplicates in the presence of 500 ug of food allergen, with or without Anti-CD4 antibody (20 ug/ml) and Isotype control antibody (20 ug/ml). Kinetics of cytokine production was studied by culturing the cells for 3, 4 and 5 days. Cell culture supernatants were collected in similar fashion described above.

Composition of culture medium: Fetal bovine serum -10%, Antibiotic - 01%, L-Glutanine- 1%, Beta mercapto ethanol- 0.1% make the volume to 100% with RPMI 1640.

Induction of Systemic Anaphylaxis, Clinical Symptoms Scoring, and Measurement of Rectal Temperature

Ten days after the last transdermal application mice were orally gavaged with either hazelnut for hazelnut-sensitized and saline-exposed mice (15 mg/mouse; 0.5 ml volume) sesame for sesame-sensitized mice (13 mg/mouse; 0.5 ml volume) and saline for saline exposed and hazelnut-sensitized mice (0.5 ml/mouse) and observed for clinical signs of systemic anaphylaxis during the next 60 minutes by 2 individuals in a blinded manner according to the method described previously (156) (Table 3). Rectal temperature was measured by using temperature probe (Yellow Springs Instrument, Yellow Springs, Ohio, USA) before and 30 minutes after oral challenge.

Table 3: Systemic anaphylaxis symptom score

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around nose and head
2	Puffiness around the eyes and mouth, pilar erecti, decreased activity with increased respiration
3	Wheezing, labored respiration, cyanosis around mouth and tail
4	Slight or no activity after prodding, tremor and convulsion
5	Death

ELISA Method of Cytokine Estimation

All cytokine measurements were done using sandwich ELISA method. The sandwich ELISA protocol is a 3 day protocol starting with coating the 96-well plate with 50 ul/well of antibody against the protein of interest in coating buffer on day one. After 12-16 hours of keeping the coated plate in the refrigerator, blocked with blocking buffer (75ul/well) and kept them in the 37^0C incubator. After 3 hours of incubation wash the plates thrice with wash buffer and add samples and standard in duplicates with a final volume of 50 ul/well. After adding the samples keep the plate in the refrigerator ($+4^0$C) over night. On day 3 remove the plate from refrigerator and add 50 ul/well biotin labeled secondary antibody against protein of interest after wash the plate three times. Incubate the plate for 90 minutes in 37^0C incubator. After 90 minutes remove the plate from the incubator and add 50 ul/well enzyme (Streptavidin conjugated alkaline phosphatase) after washing the plate for three times. Incubate the plate for 30 minutes in 37^0C incubator before washing and adding

50 ul/well pnp substrate. After adding the substrate read the absorbance at 405-690 nm.

Statistical Analysis

ANOVA and Student's t test were used to evaluate significance using GraphPad Instat ^{TM} software program (GraphPad software, San Diego, USA). The statistical significance level was set at 0.05.

Results

Transdermal Exposure to Hazelnut and Sesame Proteins Results in Activation of Type-2 Cytokine Responses in BALB/c Mice:

Groups of BALB/c mice were exposed to either saline or hazelnut protein extract transdermally for 6 times as mentioned in materials and methods section. Splenocytes from saline-exposed mice and hazelnut-sensitized mice were cultured in the presence or absence of hazelnut protein for 5 days. Supernatants from the cultures were collected on day 3 and day 5. There was significant production of IL-4, IL-5 as well as IL-13 in hazelnut-sensitized mice when compared with saline-exposed mice (**Fig. 9**). Within the hazelnut-exposed group there was significant difference ($p<0.05$) between culture medium alone and those that were cultured with hazelnut protein extract (**Fig. 9**).

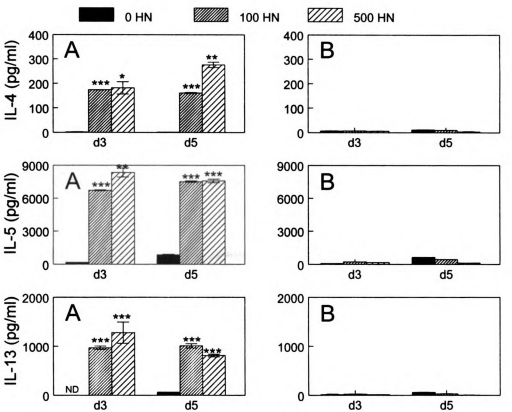


Fig. 9: Hazelnut-driven IL-4, IL-5 and IL-13 responses of hazelnut-sensitized mice vs saline exposed mice Groups of BALB/c (n=5 to 6 per group) were sensitized with hazelnut protein (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured with the indicated dose of hazelnut protein. Cell culture supernatants were collected on days 3 and 5; samples were analyzed for IL-4 (top panel), IL-5 (middle panel) and IL-13 (bottom panel) using ELISA. Data shown are average +/- SE of duplicate analyses from hazelnut-sensitized mice (n=6) (A) and saline-exposed mice (n=5) (B). All the differences were compared using ANOVA and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Groups of mice were evaluated for antigen driven memory Type-2 cytokine responses to sesame proteins in sesame-sensitized vs saline-exposed mice. Protocol for this experiment was similar to the one used for hazelnut proteins. The only difference was sesame protein extract was used in place of hazelnut protein extract. Sesame allergic mice but not control mice exhibited sesame-driven recall Type-2 cytokine production (**Fig. 10**).

Food Allergen-driven Type-2 Cytokine Responses in this Mouse Model is CD4-Dependent

Splenocytes were cultured as described above along with anti-CD4 antibody or an isotype-matched control antibody. Hazelnut and sesame proteins were used at 500 ug/ml and with and without anti-CD4 antibody and a control antibody. Supernatants were collected on days 3, 4 and 5 and then stored at -80°C till they were analyzed for cytokines IL-4, IL-5 and IL-13 levels by ELISA. The production of IL-4 (**Fig. 11**), IL-5 (**Fig. 12**) and IL-13 (**Fig. 13**) were significantly inhibited by the anti- CD4 antibody in both hazelnut and sesame allergy mouse models.

Type-2 Cytokine Responses to Hazelnut Allergens Mouse with Different Major histocompatibility (H-2) Genetic Locus

The protocol used for transdermal sensitization followed by spleen cell culture was identical to those described in previous experiments. There was a significant difference between all three different strains in the production of

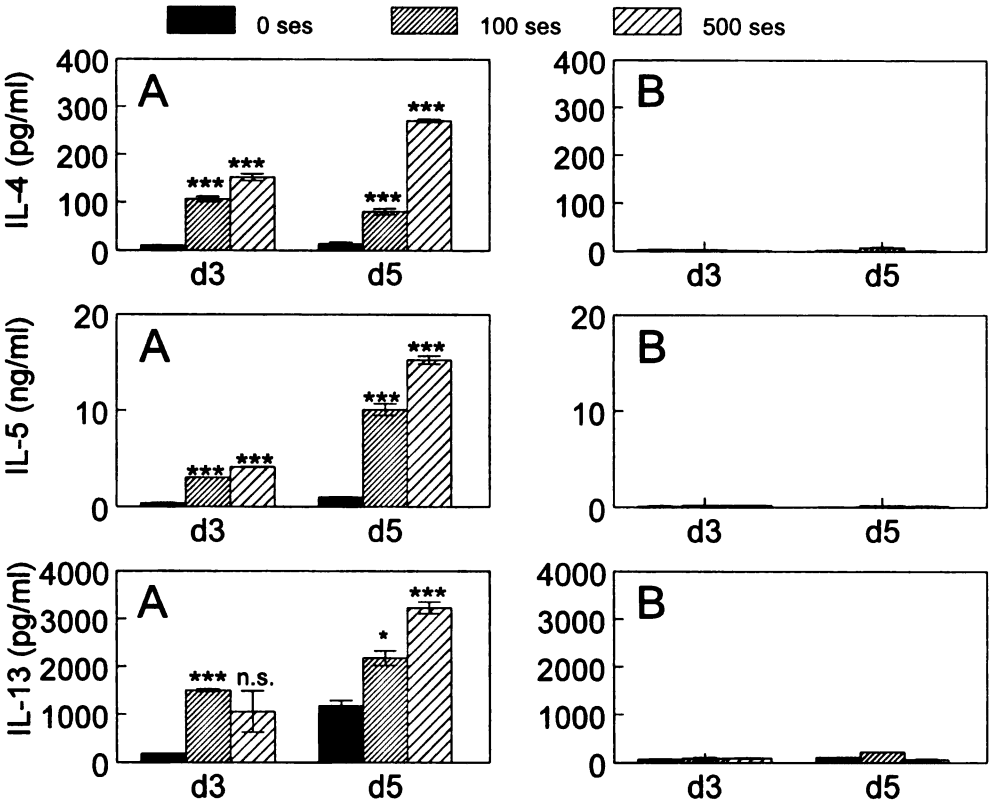


Fig. 10: Sesame-driven IL-4, IL-5 and IL-13 responses of sesame-sensitized mice vs saline exposed mice Groups of BALB/c (n=7 to 8 per group) were sensitized with sesame protein extract (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured with the indicated dose of sesame protein extract. Cell culture supernatants were collected on days 3 and 5; samples were analyzed for IL-4 (top panel), IL-5 (middle panel) and IL-13 (bottom panel) using ELISA. Data shown are average +/- SE of duplicate analyses from sesame -sensitized mice (n=8) (A) and saline-exposed mice (n=7) (B). All the differences were compared using ANOVA and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$

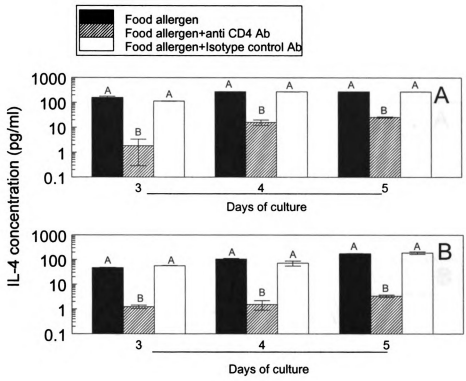


Fig. 11: Hazelnut and sesame-driven IL-4 levels are CD4 dependent

Groups of BALB/c mice were sensitized with sesame seed extract (A) and hazelnut protein extract (B) (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured with indicated dose of sesame, hazelnut proteins or isotype control antibody and anti-CD4 antibodies. Cell culture supernatants were collected on days 3, 4 and 5 and analyzed for IL-4 using ELISA. Data shown are average +/- SE of duplicate analysis from sesame-sensitized mice (n=8) and hazelnut-sensitized mice (n=6). The differences between different groups on same day of collection were compared using ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

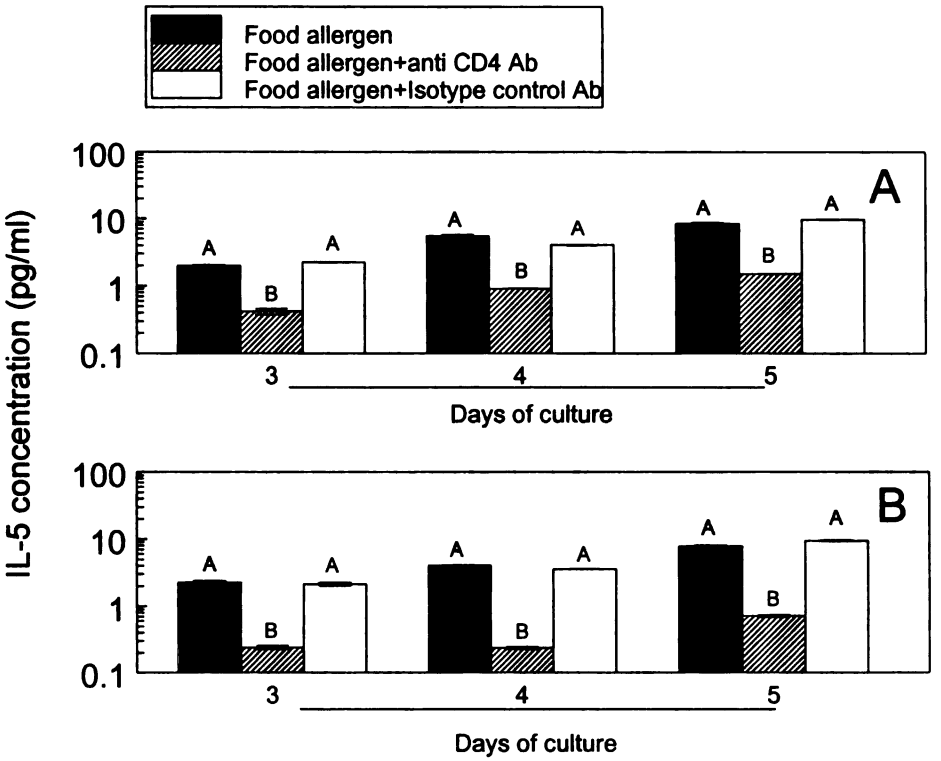


Fig. 12: Hazelnut and sesame-driven IL-5 levels are CD4 dependent

Groups of BALB/c mice were sensitized with sesame seed extract (A) and hazelnut protein extract (B) (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured with indicated dose of sesame, hazelnut proteins or isotype control antibody and anti-CD4 antibodies. Cell culture supernatants were collected on days 3, 4 and 5 and analyzed for IL-5 using ELISA. Data shown are average +/- SE of duplicate analysis from sesame-sensitized mice (n=8) and hazelnut-sensitized mice (n=6). The differences between different groups on same day of collection were compared using ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

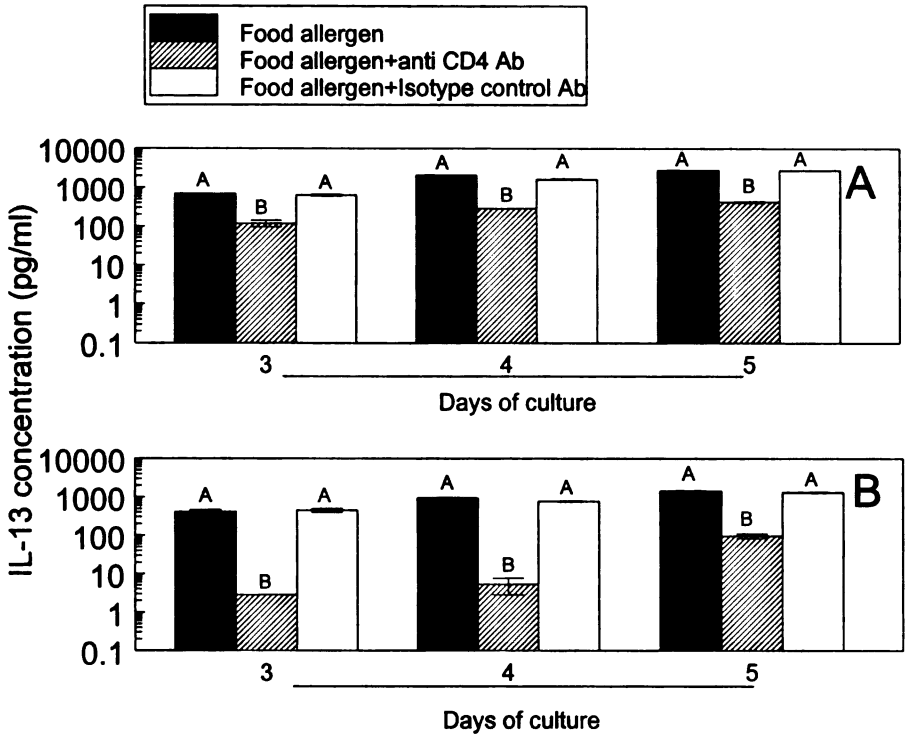


Fig. 13: Hazelnut and sesame-driven IL-13 levels are CD4 dependent

Groups of BALB/c mice were sensitized with sesame seed extract (A) and hazelnut protein extract (B) (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured with indicated dose of sesame, hazelnut proteins or isotype control antibody and anti-CD4 antibodies. Cell culture supernatants were collected on days 3, 4 and 5 and analyzed for IL-13 using ELISA. Data shown are average +/- SE of duplicate analysis from sesame-sensitized mice (n=8) and hazelnut-sensitized mice (n=6). The differences between different groups on same day of collection were compared using ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

IL-4, IL-5 and IL-13. In the case of IL-4 production, there was a significant difference between saline group and hazelnut group in BALB/c and ASW strains but that difference was not present in SJL mice implying that IL-4 production has genetic factor in its etiology (**Fig. 14**). In the case of IL-5 production there was no significant difference between BALB/c and SJL strains where as ASW strain produced significantly higher amount of IL-5 when compared with the other 2 strains (**Fig. 15**). In the case of IL-13 production in SJL mice there was no significant allergen-driven production and it was not significantly different from ASW mice, where as BALB/c produced significantly higher amount of IL-13 when compared to ASW and SJL strains (**Fig. 16**).

IL-4 but not IL-5/ IL-13 Responses to Hazelnut Allergen is STAT 6 Dependent in our Mouse Model

The protocol used for transdermal sensitization, followed by spleen cell culture was identical to what has been described earlier. There was a 90% reduction in the production of IL-4 in *Stat6* Knock out mice was observed, where as that reduction was only 40% in the case of IL-5 and 15% in the case of IL-13. Interestingly the difference in IL-13 production was not significantly different between wild type and knock out mice. Thus, only hazelnut-driven IL-4 but not IL-5/ IL-13 responses was found to be largely STAT6 dependent in this model (**Fig. 17**).

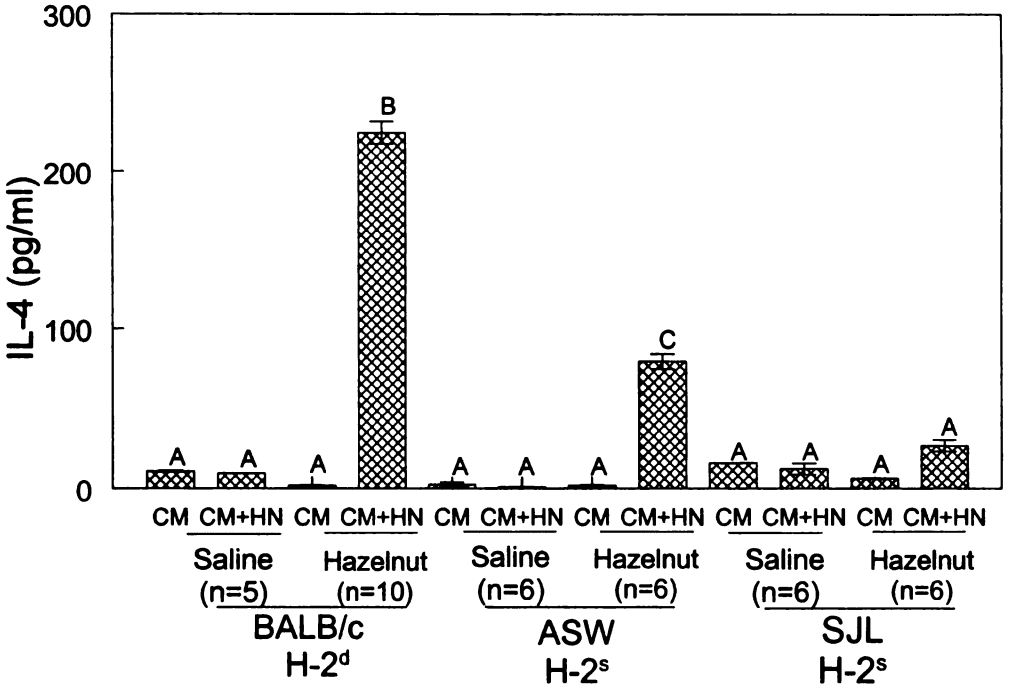


Fig. 14: Hazelnut-driven IL-4 response from three different mouse strains; BALB/c, ASW and SJL Groups of BALB/c, ASW and SJL mice (n=5-10 per group) were sensitized with hazelnut protein extracts (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure, mice were intraperitoneally challenged with hazelnut protein and 1 hour later spleen cells were harvested and cultured with 0, 100 or 500 ug/ml dose of hazelnut proteins. Cell culture supernatants were collected on days 1, 3 and 5. Samples were analyzed for IL-4 using optimized ELISA. Data shown is average +/- SE of duplicate analysis from peak response. All differences were compared using ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

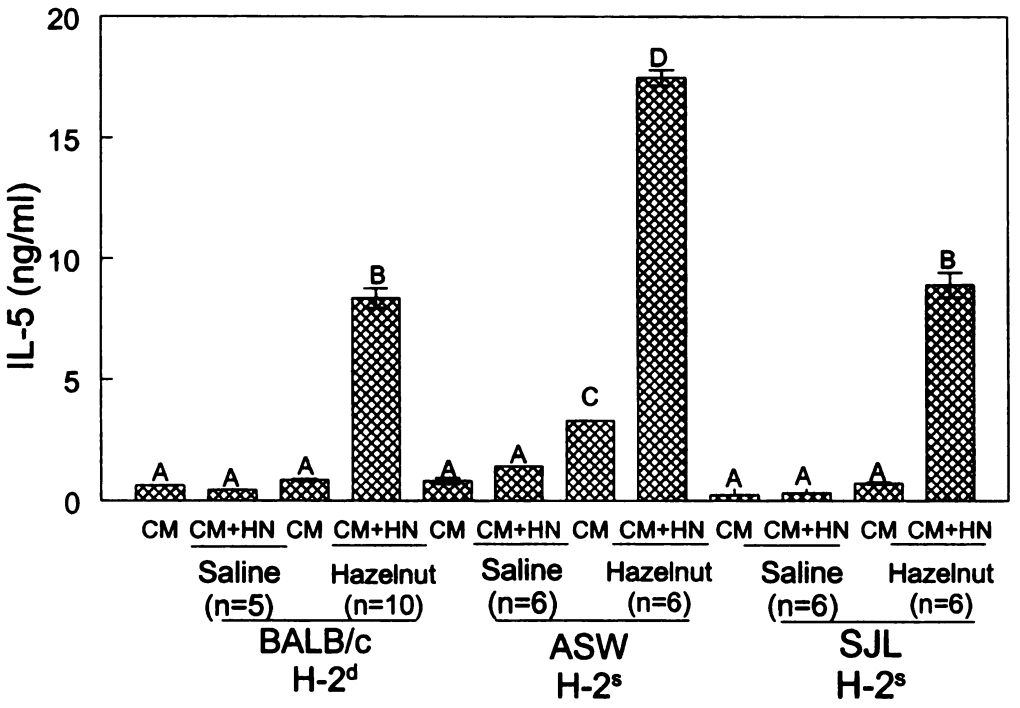


Fig. 15: Hazelnut-driven IL-5 response from three different mouse strains; BALB/c, ASW and SJL Groups of BALB/c, ASW and SJL mice (n=5-10 per group) were sensitized with hazelnut protein extracts (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure, mice were intraperitoneally challenged with hazelnut protein and 1 hour later spleen cells were harvested and cultured with 0, 100 or 500 ug/ml dose of hazelnut proteins. Cell culture supernatants were collected on days 1, 3 and 5. Samples were analyzed for IL-5 using optimized ELISA. Data shown is average +/- SE of duplicate analysis from peak response. All differences were compared using ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

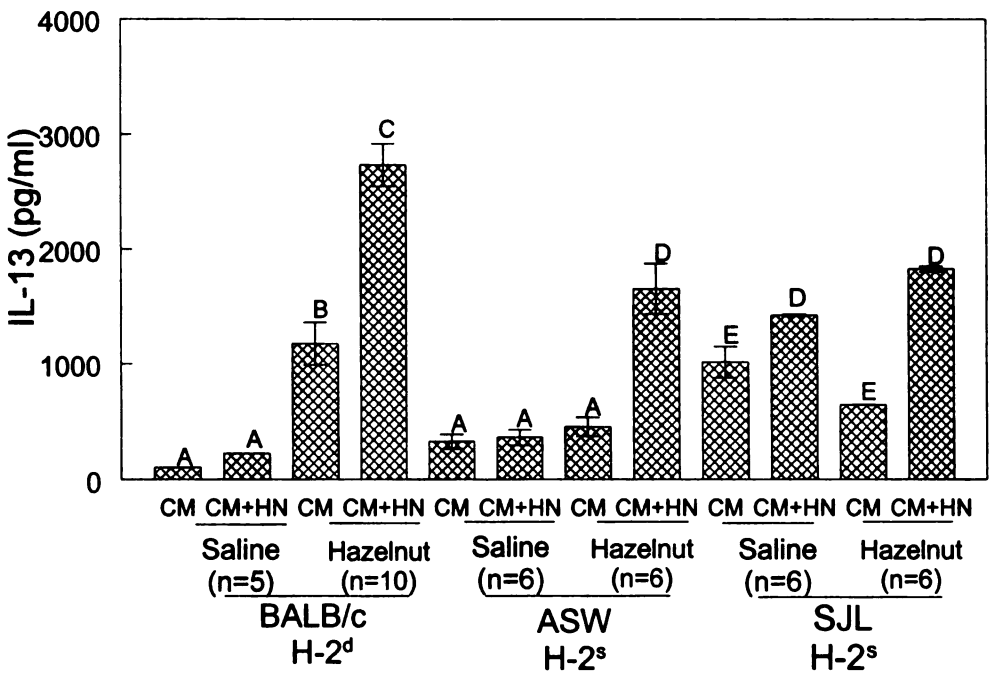


Fig. 16: Hazelnut-driven IL-13 response from three different mouse strains; BALB/c, ASW and SJL Groups of BALB/c, ASW and SJL mice (n=5-10 per group) were sensitized with hazelnut protein extracts (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure, mice were intraperitoneally challenged with hazelnut protein and 1 hour later spleen cells were harvested and cultured with 0, 100 or 500 ug/ml dose of hazelnut proteins. Cell culture supernatants were collected on days 1, 3 and 5. Samples were analyzed for IL-13 using optimized ELISA. Data shown is average +/- SE of duplicate analysis from peak response. All differences were compared using ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

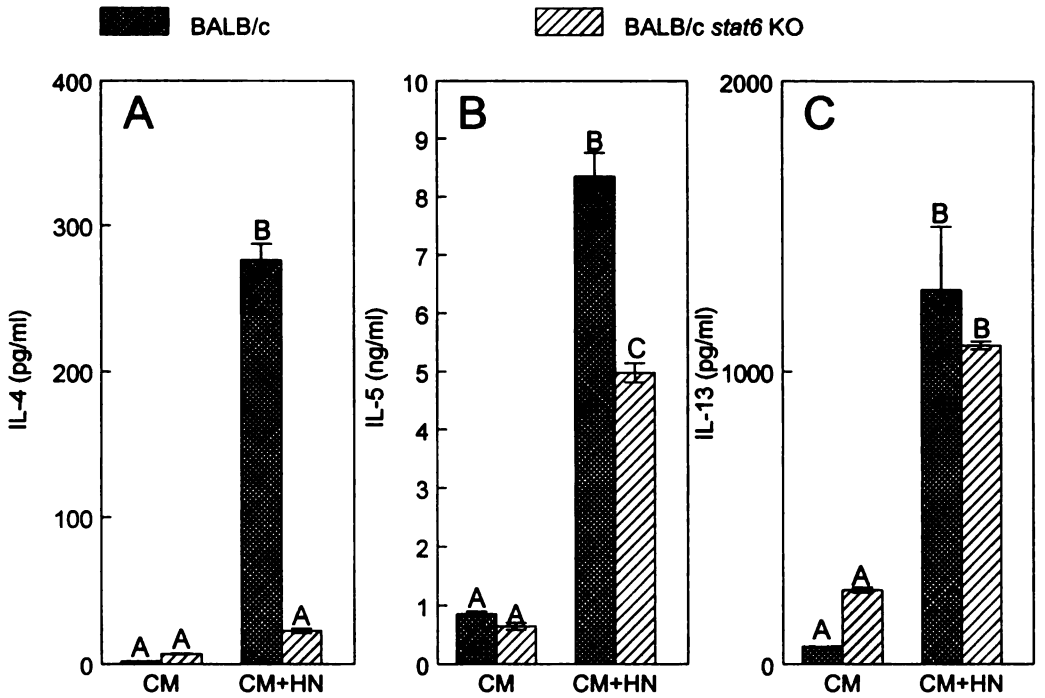


Fig. 17: Hazelnut-driven IL-4, IL-5 and IL-13 response in *Stat6* knockout and wild type animals Groups of BALB/c (n=6 per group), BALB/c stat6 KO (n=4 to 6 per group) mice were sensitized with hazelnut protein extracts (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure mice were intraperitoneally challenged with hazelnut and one hour later spleen cells were harvested and cultured with two doses of hazelnut proteins for three different days. Cell culture supernatants which collected on days 1, 3 and 5 were analyzed for levels of IL-4 (A), IL-5 (B) and IL-13 (C) using ELISA. Data shown are average +/- SE of duplicate analysis of peak response. All the differences between wild type and knockout mice were compared by ANOVA. Bars labeled with different letters are significantly different ($p<0.05$)

Allergic Response and Systemic Anaphylaxis to Hazelnut is IL-4 Dependent in this Mouse Model

The protocol used for transdermal sensitization followed by spleen cell culture was identical to what has been described earlier. *Il4* KO mice failed to exhibit significant allergic (IgE) response to transdermal hazelnut exposure (**Fig. 18**) or systemic anaphylaxis following oral challenge with hazelnut (**Fig. 19**). These data suggest that allergic and anaphylactic response to hazelnut in this model is dependent on IL-4.

Discussion

This study was undertaken with the aim of characterizing the Type-2 cytokine responses in an adjuvant-free mouse model that our laboratory has developed. There are five novel and important findings from this study: (1) transdermal exposure to hazelnut and sesame allergens was sufficient to activate recall Type-2 cytokine responses by spleen cells; (2) recall allergen-driven Type-2 cytokine responses was largely CD4-dependent in both hazelnut and sesame allergy models; (3) Type-2 cytokine responses to hazelnut was H-2^s independent; (4) hazelnut allergen-driven IL-4 but not IL-5/IL-13 is STAT6 dependent; and (5) IL-4 is required for allergic and systemic anaphylaxis to hazelnut in this mouse model.

Initially this study looked at the Type-2 cytokine responses using two different food allergens to determine if there will be any major differences

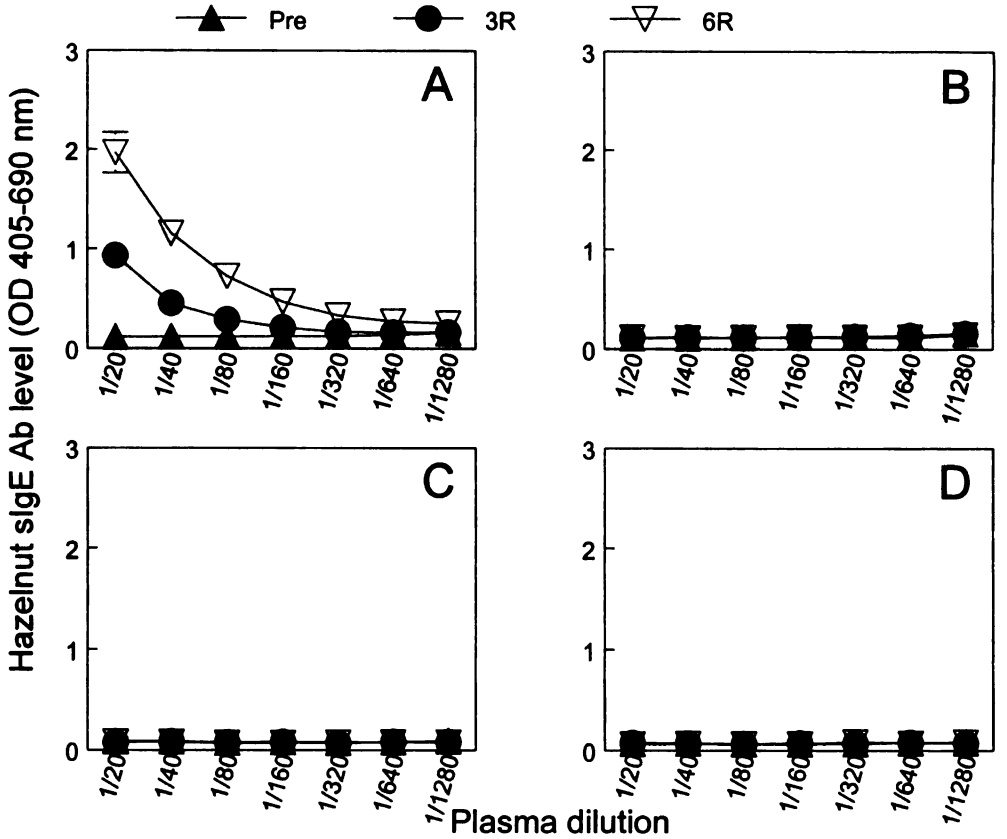


Fig. 18: Hazelnut-specific IgE response in BALB/c wild type animals and *Il4* knockout animals Groups of mice (n=6 per group) were sensitized with saline (B&D) (100 ul) or hazelnut protein extract (A&C) (500 ug/mouse in 100 ul) via transdermal exposure for 6 times. Plasma samples were collected before transdermal application (Pre), after 3 applications (3R) and after 6 applications (6R). Hazelnut-specific IgE levels were evaluated using ELISA. Data shown are hazelnut-specific IgE values (OD 450-690) average +/- SE of duplicate analysis from wild type BALB/c mice (A&B) in comparison to *Il4* Knockout mice (C&D)

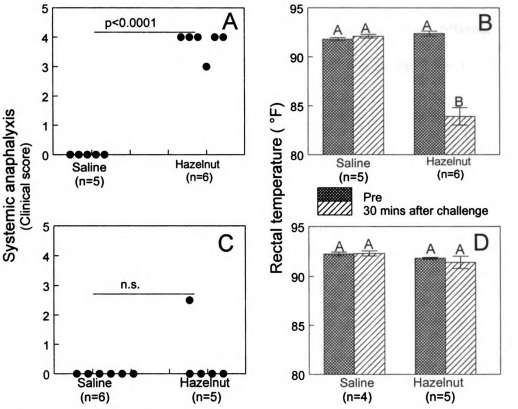


Fig. 19: Systemic anaphylaxis and rectal temperature before and after oral challenge with hazelnut protein in wild type and *Il4* knock out mice BALB/c-Wild type (A&B)(n=5-6 per group) and BALB/c-*Il4* Knockout (C&D) (n=4-5 per group) were sensitized with saline and hazelnut (500 ug/mouse) via transdermal exposure for 6 times. One week following the last exposure hazelnut-sensitized mice were orally challenged with hazelnut (15 mg/mouse in 500 ul) and saline-exposed mice were challenged with saline (500 ul). Rectal temperatures were recorded before and 30 minutes after oral challenge by using rectal probe (B&D). Data shown are average +/- SE. Differences were compared using ANOVA in B&D and Student's *t* test in A&C. Bars labeled with different letters are significantly different ($p<0.05$)

between food allergens in the way they activate the immune system. However, the data suggest that, although hazelnut and sesame belong to two different food groups (tree nuts and seeds respectively), they activate the Type-2 cytokine responses in a similar fashion.

We are not aware of previous studies examining hazelnut or sesame allergen-driven cytokine responses in mice or other animals or in humans. There are previous studies examining Type-2 cytokines in mouse models of other allergens such as ovalbumin and peanut (23, 151, 152, 157, 158, 168, 170-172). These models either used tape-stripping skin exposure method or cholera toxin to elicit of allergen sensitization. Nevertheless, they reported that ovalbumin and peanut activated IL-4 and IL-4, IL-5 and IL-13 cytokine responses by spleen cells respectively. However, *Il4* or *Stat6* gene knockout studies were not conducted.

There are several cell types that were present in spleen cell suspension such as basophils, eosinophils, NK cells and mast cells that are able to produce Type-2 cytokines. Therefore, the role of CD4 positive cells was tested in this model. Type-2 cytokine responses were found to be largely CD4-dependent. We are not aware of previous food allergy model studies characterizing Type-2 cytokines in this manner (15, 150-152, 157, 158, 168, 170-175).

In order to examine the role of host genetics in Type-2 cytokine responses to hazelnut allergens, the immune response in mouse strains with different MHC genetics was studied. The classical major histocompatibility complex (MHC) molecules have a unique function in the complex immunological dialogue that must occur between T cells and other cells of the body. T cells can able to recognize only those antigens that were presented along with MHC molecules. Researchers showed that MHC molecules has critical role in antigen presentation and cytokine production in different systems. *H-2* is a complex of genetic loci on chromosome 17 that define the major histocompatibility complex (MHC) of the mouse (67). *H-2* is homologous to *HLA* in man (68). The *H-2* complex consists of several major genes whose products are important in immune system, auto immunity and graft rejection (70). As evident, in contrast to SJL, only BALB/c and ASW exhibited significant allergen-driven IL-4 and IL-13 responses. However, all three strains produced significant IL-5 responses. These data argue that Type-2 cytokine responses to hazelnut allergens is dependent on mice strain and are H-2^s independent.

The reason why SJL did not produce robust IL-4 response is not known at this point although previous reports demonstrate that SJL lack NK 1.1 cells that are required for robust IL-4 (176). Furthermore, SJL mice are also unable to produce IgE response to allergens(177). The difference between ASW and BALB/c might be due to generally more Th2 response by the BALB/c mice

compared to the ASW. However, both produce robust IL-4 and allergic response.

Since previous studies in allergy mouse models and in humans implicated a critical role for STAT6 in Type-2 cytokine responses, this study examined *Stat6* gene knockout mice (91, 125, 127, 130, 178). Hazelnut-driven IL-4 but not IL-5 and IL-13 responses was almost completely abrogated in *Stat6* KO mice. Furthermore, these mice were unable to develop allergic (IgE) response to transdermal hazelnut exposure or systemic anaphylaxis to i.p. challenge with hazelnut (179). These data together implicated STAT6 is a critical player in this mouse model. It also suggested that IL-4 may be critical in this model that led to the follow-up study using *Il4* KO mice.

To directly test the role of IL-4 in our model, allergic response to transdermal hazelnut exposure and systemic anaphylaxis following oral hazelnut allergen challenge was studied. As evident, *Il4* KO mice were resistant to both allergic response as well as systemic anaphylaxis arguing the critical role of this prototypic Type-2 cytokine in our adjuvant-free mouse model of food allergy.

Taking all these data into consideration, a working model was developed to explain how transdermal exposure to food allergens might shape the immune response leading to food allergy disease in this model (**Fig. 20**).

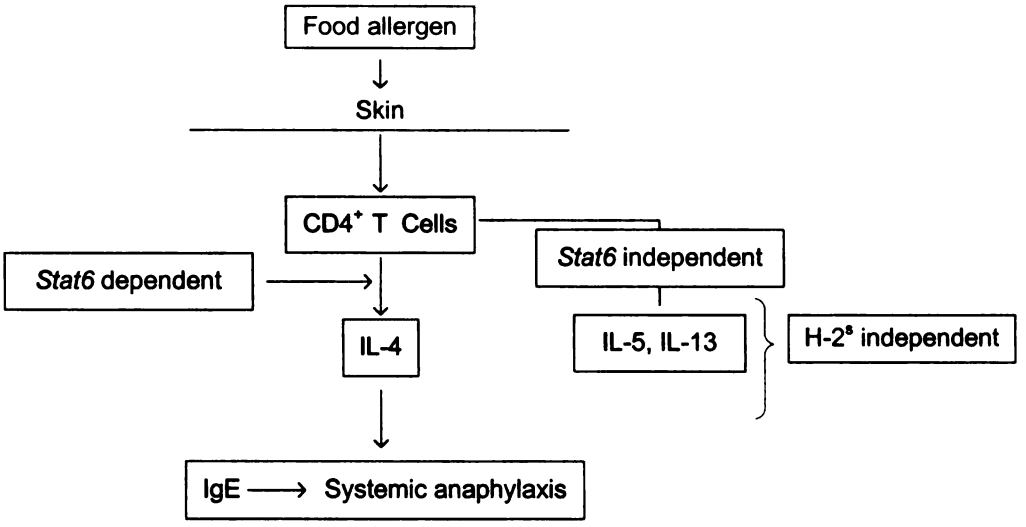


Fig. 20: Working model of Type-2 cytokine responses in our adjuvant-free mouse model of transdermal sensitization Exposure to food allergens via skin leads to activation of Type-2 cytokine (IL-4, IL-5 and IL-13) responses by CD4 positive spleen cells. However, in contrast to IL-5 and IL-13, only IL-4 response is STAT6 dependent. Furthermore, both IL-4 and STAT6 are required for allergic response and systemic anaphylaxis in this model.

According to this model after expose the animal through skin, allergen is activating the CD4 positive T cells. After activation CD4 positive T cells producing allergen-driven IL-4, IL-5 and IL-13 cytokines. Out of these three cytokines

IL-4 production is dependent on STAT6 and IL-4 is crucial cytokine for IgE production and systemic anaphylaxis.

CHAPTER 2

THE EFFECT OF SELECTED HERBAL SUPPLEMENTS ON ALLERGEN-DRIVEN TYPE-2 CYTOKINE RESPONSE BY SPLEEN CELLS FROM FOOD ALLERGIC MICE

Abstract

Background

Food allergies afflict nearly 4% adults and 6-8% children in most developed countries including USA. Despite the potential fatal outcome, no specific therapies except symptomatic treatment are available for food allergy. Currently avoiding the food is the only sure way to prevent food allergy. Therefore, improved methods are needed for prevention and/or treatment of food allergies. Using a food allergy mouse model, here attempts were made to identify potential anti-allergy herbal supplements using a novel approach.

Hypothesis and approach

Here the hypothesis that selected herbal supplements will inhibit allergen-driven Type-2 cytokine response by spleen cells from food allergic mice was tested. There were two specific objectives for this study: (1) To study impact of selected herbal dietary supplements on spleen cell viability; (2) To study the impact of above supplements on allergen-driven recall Type-2 cytokine response using spleen cells from food allergic mice.

Results

Except neem leaf extract, none of the herbal supplements studies exhibited cytotoxic effect at dilutions of 1/1000 or 1/5000 on spleen cells from hazelnut allergic mice. Among the herbal supplements tested only licorice consistently inhibited allergen-driven IL-4, IL-5 and IL-13 responses by spleen cells from hazelnut and sesame allergic mice. Furthermore, licorice from two additional sources also exhibited inhibition of all three Type-2 cytokine responses in both models.

Conclusion

These studies demonstrate a novel approach for identifying herbal supplements and phyto chemicals that inhibit allergen-driven recall Type-2 cytokine responses in mice. Furthermore, these data suggest the need for future studies to test the potential of licorice to inhibit Type-2 cytokine responses in vivo and its impact on allergic (IgE) and anaphylactic responses in this mouse model.

Introduction

Food allergies afflict nearly 4% adults and 6-8% children in most developed countries including USA. Despite the potential fatal outcome, no therapies except symptomatic treatment are available for food allergy. Currently avoiding the food is the only sure way to prevent food allergy. But

the major problem in food avoidance is the hidden nature of food allergens in several processed foods.

Researchers are trying to find newer methods for treatment or prevention of food allergies. Recent clinical trail showed increase in the threshold amount of peanut required to induce systemic anaphylaxis after monthly injections of humanized recombinant anti-IgE antibodies, however taking monthly injections is laborious, time consuming, expensive and not fool-proof .Therefore there is need to develop alternative therapeutic strategies (e.g. use of herbal supplements).

There is growing interest in the potential utility of herbal dietary supplements in the prevention and treatment of several human disorders including allergies and asthma. Many studies demonstrate that several herbal supplements can modulate immune response especially Th1/Th2 cytokine production. However, the potential of individual herbal supplements in modulating food allergen-driven Type-2 cytokine response has not been adequately studied.

This part of the study examined the possibility of identifying herbal supplements that might be potentially useful for food allergy. The hypothesis that selected herbal supplements will inhibit allergen-driven Type-2 cytokine response by spleen cells from food allergic mice was tested. There were two

specific objectives for this study: (1) To study impact of selected herbal dietary supplements on spleen cell viability; (2) To study the impact of above supplements on allergen-driven recall Type-2 cytokine response using spleen cells from food allergic mice.

Material and methods

Herbal supplements and their sources were as listed in table 4. This study used both whole extracts of herbal supplements and active ingredients. The whole extracts used in this study were from Echinacea, Garlic, Licorice (three different sources), and Milk Thistle. All these supplements were supplied in capsule form and they were added to the culture by making extract from these supplements. The extracts were made by mixing each capsule in 2.5 ml of culture media and after through mixing the whole mixture was centrifuged at 2500 rpm for 10 minutes. Supernatant was collected and filter sterilized before use it in the culture as stock solution. This supernatant was used in the culture at 1 in 1000 and 1 in 5000 dilutions. The active ingredients were dissolved as per the instructions given by the manufacturers (**Table 4**). All the stock solutions were made at 10 mg/ml concentration and used in the culture at 1 in 1000 (10 ug/ml) and 1 in 5000 (2 ug/ml) dilutions. Neem leaf extract and Licorice from Herbs Pharma which were provided by the manufacturer as liquid preparation and they were considered as stock solutions and used in the culture after filter sterilization at 1 in 1000 and 1 in 5000 dilutions.

Spleen cell culture

Spleen cells were harvested and standard cell cultures were set up essentially as described. Briefly the pooled spleen cells from food allergen sensitized mice were cultured in quadruplicates in the presence of 100 ug of food allergen, with and without herbal supplements at two different dilutions that is at 1 in 1000 and 1 in 5000. Cell culture supernatants were harvested as described above at the end of 4 days. Cell culture supernatants from all the cell culture experiments were stored at -70^{0}C till they were analyzed for levels of different cytokines.

Table 4: Herbal supplements used in this study

Herbal supplement	Extract prepared with	Extract made At	Stock concentartion	Source
Echinacea	Culture Medium	160 mg/ml	Supernatant	solaray®, Foods for Living store, East Lansing, MI, USA
Garlic	Culture Medium	128 mg/ml	Supernatant	solaray®, Foods for Living store, East Lansing, MI, USA
Genistein	Ethyl alcohol	10 mg/ml	10 mg/ml	Sigma, St Louis, MO, USA

Herbal supplement	Extract prepared with	Extract made At	Stock concentartion	Source
Milk Thistle	Culture Medium	240 mg/ml	Supernatant	solaray®, Foods for Living store, East Lansing, MI, USA
Alliin	Water	10 mg/ml	10 mg/ml	Sigma, St Louis, MO, USA
Glycyrrhizin	Aluminum Hydroxide	10 mg/ml	10 mg/ml	Sigma, St Louis, MO, USA
Licorice	Culture Medium	180 mg/ml	Supernatant	solaray®, Foods for Living store, East Lansing, MI, USA
Licorice	Culture Medium	Liquid preparation	Neat preparation	Herbs Pharma®, Foods for Living store, East Lansing, MI, USA
Neem Leaf Extract		Liquid preparation	Neat preparation	NATURAL S®, Foods for Living store, East Lansing, MI, USA
Silymarin	DMSO	10 mg/ml	10 mg/ml	Sigma, St Louis, MO, USA

MTT assay for cell viability

Cell viability was measured by MTT based assay following manufacturers' instructions: Initially cultures which were in quadruplicates were removed from incubator and placed in laminar flow hood. At the same time MTT to be used was reconstituted with 3 ml of balanced salt solution without phenol red and serum, and then add reconstituted MTT in an amount equal to 10% of the culture volume. Cultures along with MTT solution were incubated for 3 hours in the 37^0C incubator. After the incubation period, cultures were removed from incubator and dissolve the resulting formazan crystals by adding an amount of MTT solubilization solution equal to the original culture volume. While mixing make sure formazen crystals were completely dissolved. After mixing measure the absorbance Spectrophotometrically at a wavelength of 570 nm. Measure the background absorbance of multi well plates at 690 nm and subtract from the 570 nm measurement.

ELISA method of cytokine estimation

All cytokine measurements were done using sandwich ELISA method. The sandwich ELISA protocol is a 3 day protocol starting with coating the 96-well plate with 50 ul/well of antibody against the protein of interest in coating buffer on day one. After 12-16 hours of keeping the coated plate in the refrigerator, blocked with blocking buffer (75ul/well) and kept them in the 37^0C incubator. After 3 hours of incubation wash the plates thrice with wash

buffer and add samples and standard in duplicates with a final volume of 50 ul/well. After adding the samples keep the plate in the refrigerator (+4^{0}C) over night. On day 3 remove the plate from refrigerator and add 50 ul/well biotin labeled secondary antibody against protein of interest after wash the plate three times. Incubate the plate for 90 minutes in 37^{0}C incubator. After 90 minutes remove the plate from the incubator and add 50 ul/well enzyme (Streptavidin conjugated alkaline phosphatase) after washing the plate for three times. Incubate the plate for 30 minutes in 37^{0}C incubator before washing and adding 50 ul/well PNP substrate. After adding the substrate read the absorbance at 405-690 nm.

Statistical Analysis

ANOVA was used to evaluate significance using GraphPad Instat ™ software program (GraphPad software, San Diego, USA). The statistical significance level was set at 0.05.

Results

Impact of herbal supplements on spleen cell viability

In the case of saline-exposed control mice, there was a significant decrease in cell viability when co-cultured with neem leaf extract at 1in 1000 dilution. In contrast, there was a significant increase in cell viability when co-cultured with licorice at both dilutions (**Figs 21 and 22**). In the case of hazelnut-sensitized mice there was no significant decrease in cell viability for all the herbal supplements at both dilutions (**Fig. 24**). But there was a

significant increase in the cell viability in case of echinacea and milk thistle at both dilutions tested (**Figs 23 and 24**).

Impact of herbal supplements on hazelnut-driven Type-2 cytokine (IL-4, IL-5 and IL-13) responses by spleen cells from hazelnut allergic mice

There was no significant decrease or increase in IL-4 production at both dilutions in the case of Echinacea, Genistein, Glycyrrhizin, Neem Leaf Extract and Silymarin (**Figs. 25 and 26**). There was a significant increase in the production of IL-4 when co-cultured with Alliin at both dilutions (**Fig. 26**). There was a significant decrease in the production of IL-4 at 1 in 5000 dilution for garlic and milk thistle, but these herbs didn't show any decrease at 1 in 1000 dilution (**Fig. 25 and 26**). Only licorice exhibited dose-dependent decrease in the production of allergen-driven IL-4 (**Fig. 26**)

Results showed that there was no significant effect on the production of IL-5 by Milk thistle, Alliin, Glycyrrhizin and Silymarin at both dilutions (**Figs. 27 and 28**). There was a significant increase in the production of IL-5 at both dilutions by herbal supplement Echinacea. There was a decrease in the production of IL-5 by garlic at 1 in 1000 dilution but not at 1 in 5000 dilution. There was a significant decrease in the production of Interleukin-5 by Genistein and Neem Leaf Extract at 1 in 1000 dilution and Licorice at both dilutions in a dose dependent manner (**Figs. 27 and 28**).

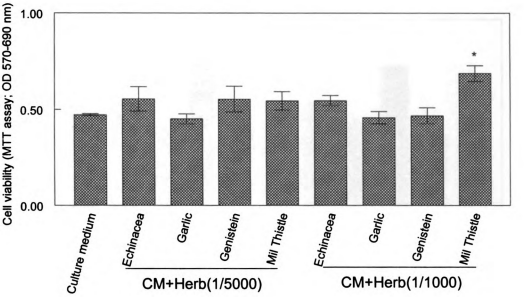


Fig. 21: Cell viability in spleen cell cultures in saline exposed mice after culture with four different herbal supplements Mice (n=6) were exposed to saline via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone and with different herbal supplements Echinacea, Garlic, Genistein and Milk Thistle at 1 in 1000 and 1 in 5000 dilutions. Cells were cultured for 4 days and cell viability at the end of 4th day was measured by MTT assay. Data shown are OD mean +/- SE of quadruplicate analysis. Differences of Culture Medium vs others were analyzed by ANOVA and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

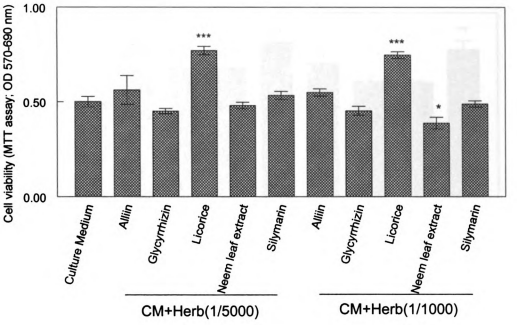


Fig. 22: Cell viability in spleen cell cultures in saline exposed mice after culture with five different herbal supplements Mice (n=8) were exposed to saline via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone and with different herbal supplements Alliin, Glycyrrhizin, Licorice, Neem Leaf Extract and Silymarin at 1 in 1000 and 1 in 5000 dilutions. Cells were cultured for 4 days and cell viability at the end of 4th day was measured by MTT assay. Data shown are OD mean +/- SE of quadruplicate analysis. Differences of Culture Medium vs others were analyzed by ANOVA and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

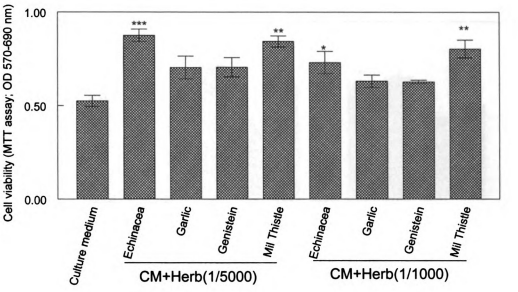


Fig. 23: Cell viability in spleen cell cultures in hazelnut sensitized mice after culture with four different herbal supplements Mice (n=8) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone and with different herbal supplements Echinacea, Garlic, Genistein and Milk Thistle at 1 in 1000 and 1 in 5000 dilutions. Cells were cultured for 4 days and cell viability at the end of 4th day was measured by MTT assay. Data shown are OD mean +/- SE of quadruplicate analysis. Differences of Culture Medium vs others were analyzed by ANOVA. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

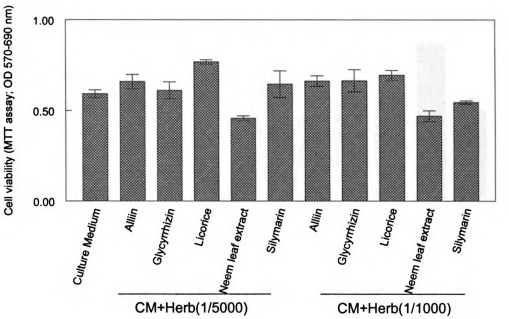


Fig. 24: Cell viability in spleen cell cultures in hazelnut sensitized mice after culture with five different herbal supplements Mice (n=8) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone and with different herbal supplements Alliin, Glycyrrhizin, Licorice, Neem Leaf Extract and Silymarin at 1 in 1000 and 1 in 5000 dilutions. Cells were cultured for 4 days and cell viability at the end of 4th day was measured by MTT assay. Data shown are OD mean +/- SE of quadruplicate analysis. Differences of Culture Medium vs others were analyzed by ANOVA and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

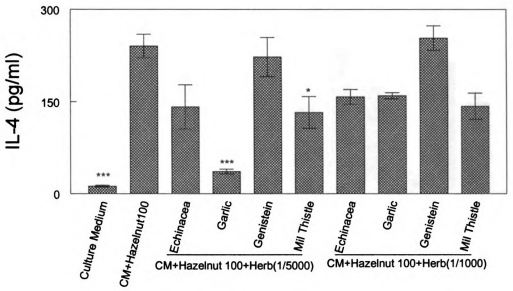


Fig. 25: Impact of herbal supplements on hazelnut-driven IL-4 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with different herbal supplements Echinacea, Garlic, Genistein and Milk Thistle at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-4 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

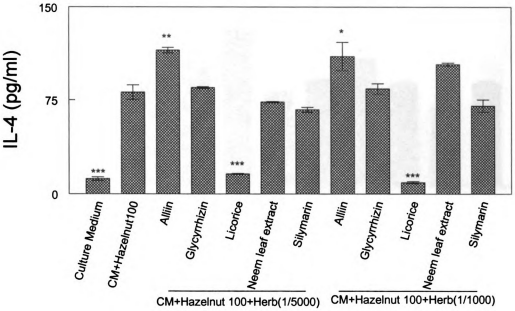


Fig. 26: Impact of herbal supplements on hazelnut-driven driven IL-4 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with different herbal supplements Alliin, Glycyrrhizin, Licorice, Neem Leaf Extract and Silymarin at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-4 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

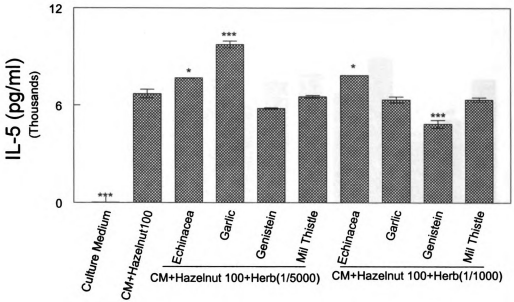


Fig. 27: Impact of herbal supplements on hazelnut-driven driven IL-5 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with different herbal supplements Echinacea, Garlic, Genistein and Milk Thistle at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-5 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

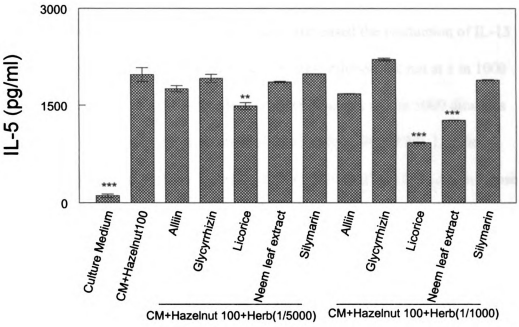


Fig. 28: Impact of herbal supplements on hazelnut-driven driven IL-5 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with different herbal supplements Alliin, Glycyrrhizin, Licorice, Neem Leaf Extract and Silymarin at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-5 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

Results showed that Echinacea, Genistein, Alliin, and Silymarin had no major effect on the production of IL-13 when tested at 1 in 1000 and 1 in 5000 (**Figs. 29 and 30**). Glycyrrhizin significantly increased the production of IL-13 at 1 in 1000 dilution (**Fig. 30**). Garlic at 1 in 5000 dilution but not at 1 in 1000 and Neem Leaf Extract, Milk thistle at 1 in 1000 but not at 1 in 5000 dilutions significantly decreased the production of IL-13 (**Figs. 29 and 30**). Licorice significantly decreased the production of IL-13 at both dilutions tested in a dose dependent manner (**Fig. 30**).

Impact of licorice from three different sources on hazelnut-driven Type-2 cytokine (IL-4, IL-5 and IL-13) responses

Licorice was the only herbal supplement tested that significantly decreased the production of all three Type-2 cytokines in a dose dependent manner. Since we had tested the licorice from only one company, we examined the effect of licorice from two other sources. Our hypothesis was that the effect of licorice on memory Tpe-2 cytokine production was independent of the source of licorice. Cytotoxic effect of licorice supplements from two other sources on splenocytes from hazelnut-sensitized mice was tested by MTT assay. As evident, there was no significant effect at either dilutions on cell viability (**Fig. 31**).

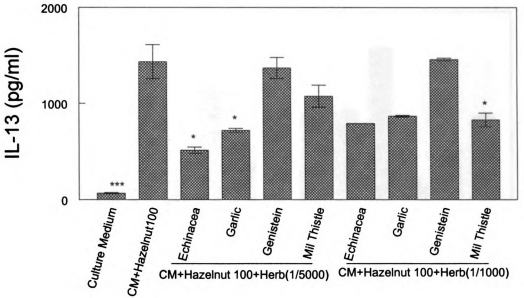


Fig. 29: Impact of herbal supplements on hazelnut-driven driven IL-13 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with different herbal supplements Echinacea, Garlic, Genistein and Milk Thistle at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-13 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

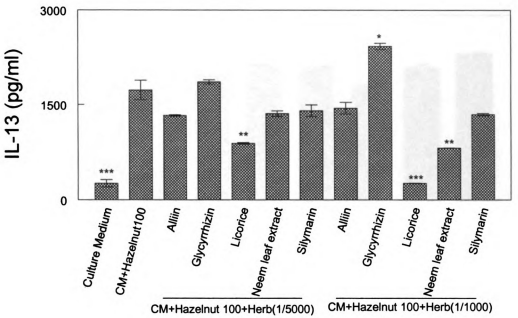


Fig. 30: Impact of herbal supplements on hazelnut-driven driven IL-13 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with different herbal supplements Alliin, Glycyrrhizin, Licorice, Neem Leaf Extract and Silymarin at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-13 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

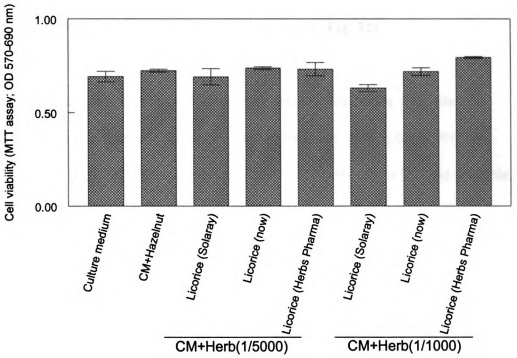


Fig. 31: Cell viability in spleen cell cultures of hazelnut sensitized mice after culturing with licorice from three different sources Mice (n=6 per group) were sensitized with hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, cells with 100 ug of hazelnut protein and with licorice from 3 different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Cell viability on day 4 was measured by optimized MTT assay. Data shown are mean +/- SE of quadruplicate analysis. ANOVA: Cell viability of culture medium was compared to other conditions and and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

Culture supernatants were screened for IL- 4 by using ELISA. Results showed that licorice from the other two sources were also able to reduce the production of IL-4 significantly at both dilutions (**Fig. 32**).

Results showed that licorice from one another source was able to significantly reduce the IL-5 production at both 1 in 1000 and 1 in 5000 dilutions (**Fig. 33**). Whereas licorice from third source Herbs Pharma was able to reduce the IL-5 production only at higher concentration (1 in 1000) (**Fig. 33**).

As evident, licorice from one source along with the licorice from the original source was able to significantly reduce the IL-13 production at both dilutions (**Fig. 34**). Licorice from the third source was able to significantly reduce the IL-13 production only at 1 in 1000 dilution, but not significantly at 1 in 5000 (**Fig. 34**).

Impact of licorice from three different sources on sesame-driven Type-2 cytokine (IL-4, IL-5 and IL-13) responses by spleen cells from sesame-sensitized mice

Cell viability was measured by using MTT assay when the splenocytes from sesame-sensitized mice were cultured with licorice from three different companies. Results showed that licorice had no significant effect on cell viability (**Fig. 35**).

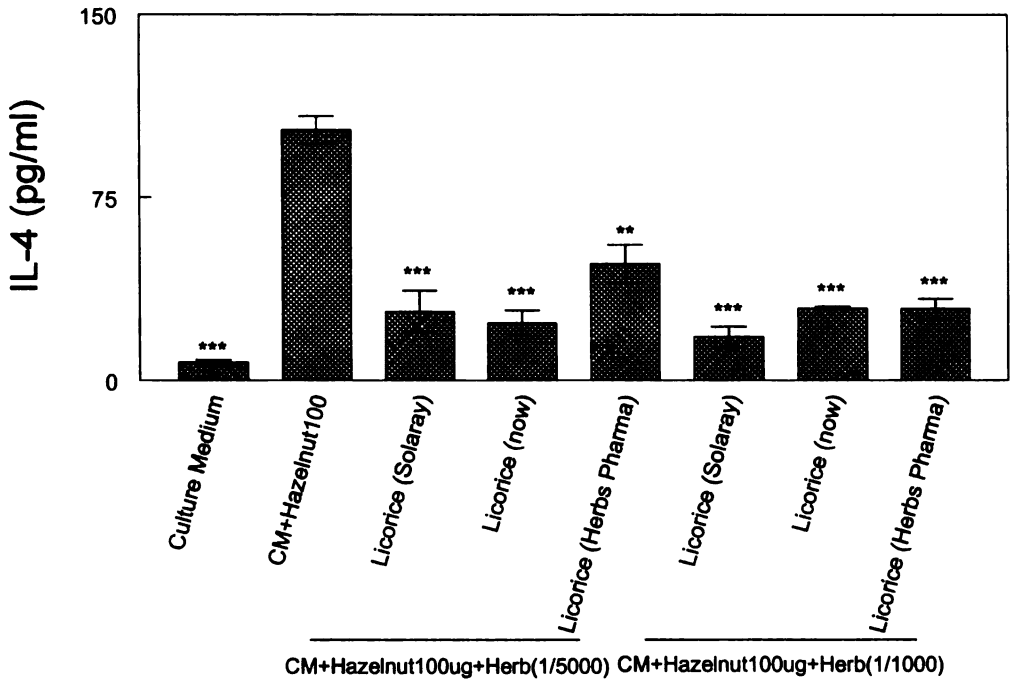


Fig. 32: Impact of licorice from three sources on hazelnut-driven driven IL-4 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with supplement licorice from three different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-4 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

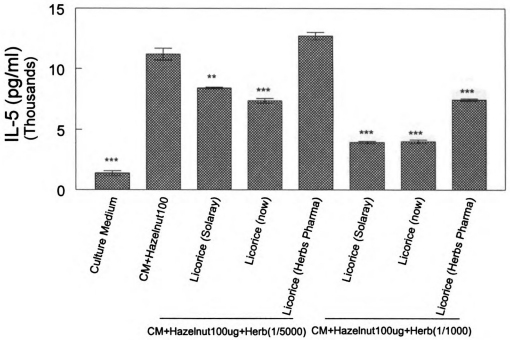


Fig. 33: Impact of licorice from three sources on hazelnut-driven driven IL-5 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with supplement licorice from three different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-5 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

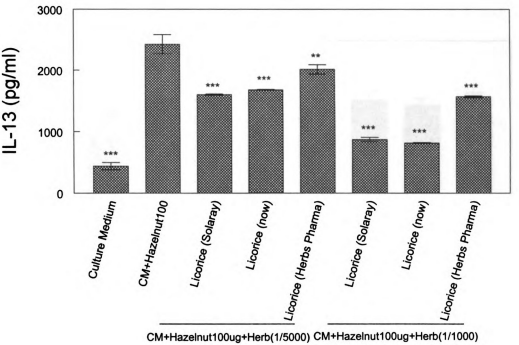


Fig. 34: Impact of licorice from three sources on hazelnut-driven driven IL-13 response by spleen cells from hazelnut allergic mice Mice (n=6) were exposed to hazelnut protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with hazelnut protein 100 ug and with supplement licorice from three different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-13 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Hazelnut was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

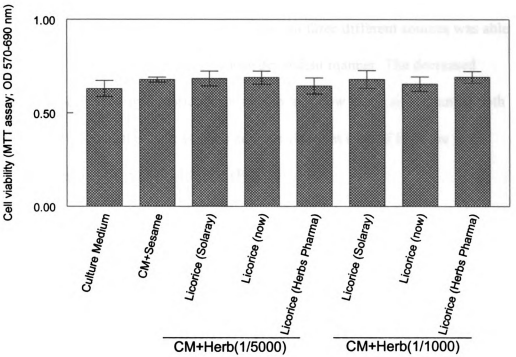


Fig. 35: Cell viability in spleen cell cultures of sesame sensitized mice after culturing with licorice from three different sources Mice (n=8 per group) were sensitized with sesame seed protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, cells with 100 ug of sesame seed protein and with licorice from 3 different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Cell viability on day 4 was measured by MTT assay. Data shown are mean +/- SE of quadruplicate analysis. ANOVA: Cell viability of culture medium was compared to other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

Culture supernatants of the spleen cell cultures from sesame-sensitized animals with licorice from three different sources were screened for IL-4 with sandwich ELISA. As evident, licorice from all three different sources was able to reduce the IL-4 production in a dose dependent manner. The decreased production of IL-4 by Licorice from solaray and now was a significant at both dilutions whereas the decrease was not significant in case of Licorice from herbs pharma at 1 in 5000 dilution (**Fig. 36**).

IL-5 levels were tested in the culture supernatants of spleen cell cultures of sesame-sensitized animals with licorice from different sources. Licorice from solaray and now were able to significantly reduce this cytokine production at both dilutions tested, whereas Licorice from herbs pharma was able to reduce only at 1 in 1000 dilution (**Fig. 37**).

IL-13 levels were screened in the supernatants of the spleen cell cultures from sesame-sensitized mice with licorice from different sources. As evident, that only Licorice from now was able to significantly reduce the IL-13 production at both dilutions tested. Licorice from solaray reduced IL-13 production at 1 in 1000 but not at 1 in 5000. Licorice from herbs pharma had no effect at either dilution (**Fig. 38**).

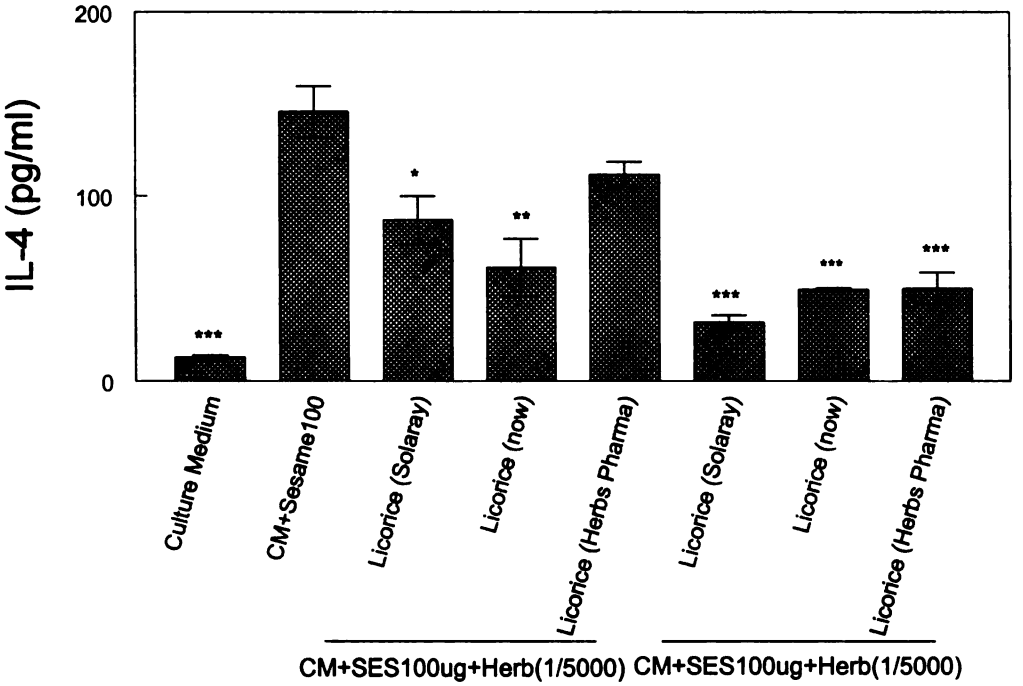


Fig. 36: Impact of licorice from three sources on sesame-driven IL-4 response by spleen cells from sesame allergic mice Mice (n=6) were exposed to sesame seed protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with sesame seed protein 100 ug and with supplement licorice from three different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-4 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Sesame was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

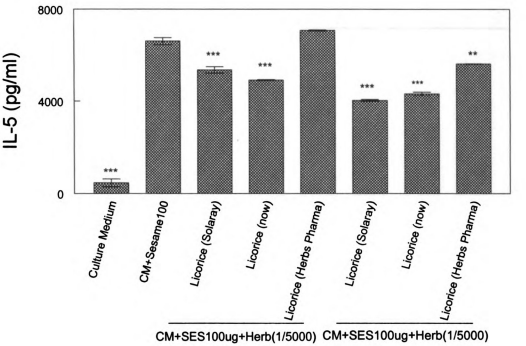


Fig. 37: Impact of licorice from three sources on sesame-driven IL-5 response by spleen cells from sesame allergic mice Mice (n=6) were exposed to sesame seed protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with sesame seed protein 100 ug and with supplement licorice from three different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-5 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Sesame was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

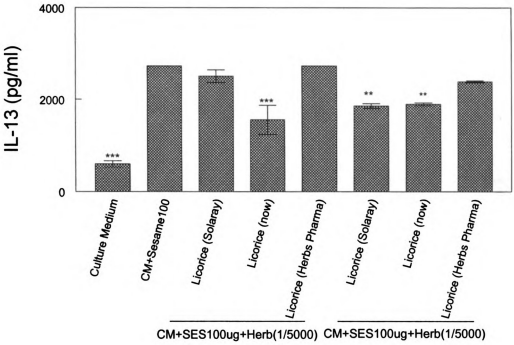


Fig. 38: Impact of licorice from three sources on sesame-driven IL-13 response by spleen cells from sesame allergic mice Mice (n=6) were exposed to sesame seed protein via transdermal exposure for 6 times. One week following the last exposure spleen cells were harvested and cultured the cells alone, with sesame seed protein 100 ug and with supplement licorice from three different sources (solaray, now and herbas pharma) at 1 in 1000 and 1 in 5000 dilutions. Supernatants were collected at the end of day 4 and screened for IL-13 levels by ELISA. Data shown are mean +/- SE of duplicate analysis. ANOVA: Culture medium+ Sesame was compared with other conditions and the statistical significances are expressed as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; others non significant.

Discussion

As aim two this study looked at the possibility of identifying potential anti-allergy herbal supplements using a novel approach was tested. Thus herbal supplements that inhibit the allergen-driven Type-2 cytokine responses in hazelnut and sesame allergy models were tested.

Out of 9 herbal supplements tested only licorice consistently exhibited a dose dependent inhibition of the production of all three Type-2 cytokines (IL-4, IL-5 and IL-13) tested. Since licorice from one source tested, whether licorice from different sources might exhibit similar activity and found that to be the case.

Later the question whether licorice might inhibit Type-2 cytokine responses to another food allergen using sesame allergy mouse model was addressed. Interestingly licorice from all three sources showed similar activity in sesame allergy model as well.

This study used extracts of herbal supplements from commercial sources (**Table 4**). Therefore, identities of component responsible for the observed inhibition of Type-2 cytokine responses by licorice remain to be determined. However, glycyrrhizin, one active component present in licorice was not able to inhibit Type-2 cytokine responses (**Fig. 25, 27 and 29**). This

data suggest that active ingredient of licorice responsible for inhibition of Tytpe-2 cytokine responses is other than glycyrrhizin.

This study examined the Type-2 cytokines as target cytokines. Because these cytokines exhibit several pro-allergic functions and participate in allergic disorders. As discussed, IL-4 was identified as critical Type-2 cytokine in this model. Since licorice inhibits IL-4 response to allergens in both hazelnut and sesame allergy model, one hypothesis to test would be that licorice prevents de novo allergic response and/or established allergic response in our model.

There are two published papers (Hugh Samson's lab Mount Sinai School of Medicine, NY, USA) that discussed the role of herbal supplements in an animal model of food allergy. They tested the efficacy of Chinese herbal formula, Food Allergy Herbal Formula (FAHF) in their adjuvant based mouse model of peanut allergy. They found the original Chinese formula (FAHF-1) and modified Chinese formula (FAHF-2) were efficient in reducing the all three Type-2 cytokines; IgE levels and there by reduce the anaphylactic symptoms in this model. This study however, used cholera toxin based food allergy model and they looked at a cocktail herbal formula (23, 29). A number of previous studies demonstrate that licorice inhibits IL-4 response in mouse models of other disorders. Some studies also suggest that licorice might be useful in alleviating disease symptoms of allergies as summarized (**Table 5**).

Table 5: Immunomodulatory effects of licorice

Form of Licorice used	Model system	Result	Reference
Glycyrrhizin	Mouse model of asthma	Decrease in IL-4 and 5 in BAL fluid	(181)
Glycyrrhizin	Mouse model of pulmonary carcinoma	Induces CD4 positive CD28 positive TCR alpha/beta positive T cells (which counteracts the production of Type-2 cytokines)	(182)
Glycyrrhizin	Mouse model of Candida albicans infection	Decreases all Type-2 cytokine production.	(183)
Glycyrrhizin	Mouse model of Candida albicans infection	Decreases all Type-2 cytokine levels in serum.	(184)
Glycyrrhizin	In-vitro mouse spleen cell cultures	Decrease in IL-4 and IL-10 and increase in IFN	(185)
Glycyrrhizin	Peritoneal macrophages	Increase in IL-12	(147)
Glycyrrhizin	Thermally injured mice	Increase in the IL-12 levels in both serum and spleen cell cultures	(186)
Glycyrrhizin derivative - hetero-30-OH-GL	Human lung fibro blasts	Decrease in EOTAXIN-1 production	(187)
Licorice roasted vs un roasted	Passive sensitization model	Inhibition of ear swelling by both roasted and un roasted licorice	(188)
Licorice gel	Human clinical trial	Effective agent in treating atopic dermatitis	(189)
Glycyrrhizin, Glycyrrhetinic acid	Contact hypersensitivity mouse model	Inhibited the passive cutaneous anaphylaxis and skin contact inflammation	(190)
Glycyrrhizin	Patient with eosinophilic peritonitis	Resolved with eosinophilic peritonitis problem	(191)

Even though this study identified an herbal supplement that inhibited all Type-2 cytokines it can not be extrapolated to humans. The dose recommended in human beings for the herbal supplements and the estimated doses for mouse based on body weight were summarized in table 6.

Table 6: Dose calculations of herbal supplements

S.No	Name	Recommended human dose	Estimated Mouse dose
1	Echinacea	1600 mg/day	4.5 ug/day
2	Garlic	320 mg/day	1.0 ug/day
3	Milk Thistle	1800 mg/day	5 ug/day
4	Licorice- solaray	900 mg/day	2.5 ug/day
5	Licorice- now	900 mg/day	2.5 ug/day
6	Licorice- Herbs Pharma	15 to 30 drops/day	
7	Neem Leaf Extract	15 to 30 drops/day	

Since this study was done in ex-vivo system and whole extracts were used the data can not be extrapolated to human studies.

There are no previous studies that examined the potential of licorice in food allergy. Data from this work along with the literature discussed above strongly supporting the idea of conducting further in vivo studies using our mouse model to evaluate the potential of licorice as an anti-food allergy herbal supplement.

SUMMARY AND FUTURE DIRECTIONS

This study was conducted in two parts using an adjuvant-free mouse model that had been developed in our laboratory. In the first part, Type-2 cytokine responses in hazelnut and sesame allergy models were characterized. There are four novel and important findings from this study: (1) transdermal exposure to hazelnut and sesame allergens was sufficient to activate Type-2 cytokine response by spleen cells; (2) recall allergen-driven Type-2 cytokine response was largely CD4 dependent in both hazelnut and sesame allergy models; (3) hazelnut allergen-driven IL-4 but not IL-5/IL-13 was STAT6 dependent; and (4) IL-4 is required for allergic and systemic anaphylaxis to hazelnut in this mouse model.

In the second part, a novel approach of examining the impact of herbal supplements on ex vivo allergen-driven Type-2 cytokine responses by spleen cells from hazelnut and sesame allergy mouse models was identified. This study found that among the herbal supplements tested, only licorice from all three sources, inhibited allergen-driven ex vivo Type-2 cytokine responses in both hazelnut and sesame allergy models.

Future directions

Part-I

- Further characterization of Type-2 cytokine response need to be done in this model. Mainly one has to study the upstream regulation of this response by

studying the role of transcription factors such as GATA-3 and c-MAF that control IL-4 gene expression. This can be tested either by using gene knock out mouse or by using techniques like RNA interference.

- One has to study the heritability of IL-4 control of food allergy in this model. One way to test this hypothesis was using the crossing and back crossing between the strains that produces IL-4 (ASW) and the strains that don't produce IL-4 (SJL).

Part-II

- This study used the ex-vivo model to study the effect of different herbal supplements on Type-2 cytokine responses in hazelnut and sesame allergy models. To further validate the effects that were shown by the licorice in ex-vivo model one need to do the in-vivo studies such as feeding licorice and study the IL-4 responses and on disease readouts (study both preventive and therapeutic approaches).
- This study found that the whole licorice extract was responsible for inhibiting the Type-2 cytokine responses in ex-vivo model. This study also tested glycyrrhizin one of the active ingredient is not responsible for the effects observed in the ex-vivo model. One need to isolate and identify the active ingredient of licorice responsible for decreasing the Type-2 cytokine responses in this model.

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