THE ROLE OF NATURAL LIPIDS IN AN *IN VIVO* MODEL OF SENSITISATION TO BER e 1

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ABSTRACT

The prevalence of food allergy is increasing in westernized countries, affecting 5-8% of children and 1-3% of adults. Although innumerous proteins are encountered in normal diets, only few proteins are commonly implicated as food allergens. In this vein, the major focus in allergy studies falls into intrinsic features of allergens; however, it is known that extrinsic factors can play a role in allergic processes. The allergenicity of Ber e 1, the major allergen from Brazil nuts, is well established and it has been shown that natural lipids from Brazil nuts are essential for the development of an immune response towards Ber e 1. The present study aimed to characterize the humoral response induced by recombinant (r)Ber e 1 alone or in the presence of lipids, and to investigate the mechanism(s) by which natural lipids influence the development of an immune response. BALB/c mice were sensitised intraperitoneally with rBer e 1 alone or in the presence of different lipid fractions. It was found that rBer e 1 alone did not induce an immune response and only one specific fraction of Brazil nut lipids (SPC fraction C), composed of a mixture of lipid classes, was able to induce a Th2-type humoral response, with the presence of Ber-specific anaphylactic antibodies, high levels of Ber-specific IgG1, and low levels of Ber-specific IgG2a. CD1-restricted natural killer (NK)T cells recognize lipids and therefore to test the hypothesis that NKT cells may be involved in the response, the sensitisation protocol with rBer e 1 and SPC lipid fraction C was tested in mice lacking these cells (Jα18 KO mice). These animals presented significantly lower titers of Ber-specific anaphylactic antibodies, Ber-specific IgG1, and total IgE than sensitised wild type mice, indicating that one of the pathways by which lipids triggered an immune response involved NKT cells. In conclusion, the present work found

that lipids from Brazil nuts were essential for the development of a Th2-type humoral response to rBer e 1 and that the immune response induced by lipids involved NKT cells.

PUBLICATIONS

Mirotti L, Mucida D, de Sá-Rocha LC, Costa-Pinto FA, Russo M (2010) "Food aversion: a critical balance between allergen-specific IgE levels and taste preference." Brain Behav Immun. 2010 Mar; 24(3):370-5.

Mirotti L, Leite-de-Moraes M, Cochrane S, Alcocer M (2010) "NKT are involved in the production of anaphylactic antibodies induced by natural lipids from Brazil nuts" (in preparation).

Renault NK, Gaddipati S, Wulfert F, **Mirotti L**, et al (2010) "Multiple food protein microarray for profiling human food-specific Immunoglobulins A, M, G and E" (in preparation).

Renault N K, **Mirotti L**, Alcocer M J. (2007) "Biotechnologies in new throughput food allergy tests: Why do we need them?" *Biotechnology Letters* 29: 333-339

COMMUNICATIONS IN CONGRESSES

Mirotti L, Gaddipati S, Renault N, McSharry C, Karamitri A, Alcocer M. Natural lipids are essential for Brazil nut allergy in BALB/c mice. *8th Macedonian Congress of Nutrition and Dietetics. Thessaloniki, Greece. October 2008*. Oral Presentation.

Mirotti L, Renault N, Alcocer M et al. Natural lipids are essential for Brazil nut allergy in BALB/c mice. *British Society of Immunology, Glasgow, 2008*

Renault N, Alcocer M, **Mirotti L** et al. Microarray profiling to quantify multiple immunoglobulins against a complete plethora of dietary ingredients – quantification meets clinical promises? *Allergy 63 (Supplem 88): 158-61, 2008*

Mirotti L, Renault N, Alcocer M "The role of natural lipids in the sensitisation to Brazil nut proteins" *XXXIII Annual Meeting of the Portuguese Society of Immunology, Lisbon Portugal, November 2007*

Alcocer M, **Mirotti L**, Renault N. The all diet array. A novel diagnostic tool for the detection of sensitisation to food proteins. *Geneva-Giract. March 2007*. Oral Presentation

Renault N, Lin J, **Mirotti L**, Haas H, Schramm G, Falcone F and Alcocer M "All Diet on a Chip": A Novel Diagnostic Tool for the Detection of Food Allergy Combining Protein Microarrays with human Basophils" *1st Joint*

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LIST OF CONTENTS

ABBREVIATIONS

AHR: Airway hyper-reactivity

AP-1: Activating protein-1

APC: Antigen presenting cells

ASG: acylsterylglycosides

BAL: Bronchoalveolar lavage

BHT: Butylated hydroxytoluene

BSA: Bovine serum albumin

CER: Cerebroside

DC: Dendritic cell

DGDG: digalactosyldiacylglycerol

DPG: diphosphatidylglycerol

dsRNA: double strand RNA

ESG: Esterified steryl-glucoside

ELISA: Enzyme-linked immune sorbent assay

GALT: Gut associated lymphoid tissue

IEC: Intestinal epithelial cells

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

ITAM: Immunoreceptor tyrosine-based activation motifs

ITIM: Immunoreceptor tyrosine-based inhibitory motifs

LBP: LPS-binding protein

LPE: Lyso-phosphatidylethanolamine

LPI: Lyso-phosphatidylinositol

LPS: lipopolysaccharide

Lps: Lyso-phosphtidylserine

M: Microfold cells

MAL: MyD88-adaptor-like

MD: myeloid differentiation

MGDG: Monogalactosyldiacylglycerols

MHC: Major histocompatibility complex

MUFA: Monounsaturated fatty acids

MyD88: Myeloid differentiation primary response gene 88

NF: Nuclear factor

NKT: Natural killer T cells

NMR: Nuclear magnetic resonance

PA: Phosphatidic acid

PBS: Phosphate buffered saline

PC: Phosphatidylcholine

PCA: Passive cutaneous anaphylaxis

PE: Phosphatidylethanolamine

PG: Phosphatidylglycerol

PI: Phosphatidylinositol

PS: Phosphatidylserine

PUFA: Polyunsaturated fatty acid

S: Sterols

SFA: Saturated fatty acid

SE: Sterol esters

SG: Sterylglycosides

SL: Sulpholipid

SPC: Solid-phase chromatography

SQDG: Sulfoquinovosyldiacylglycerol

ssRNA: single strand RNA

TICAM-1: TIR-domain containing adaptor molecule

TIR: Toll/interleukin-1 receptor

TIRAP: TIR domain-containing adaptor protein

TG: Triacylglycerol or triglycerides

TLC: Thin layer chromatography

TLR: Toll-like receptors

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain containing adaptor inducing IFN-β

TSFA: total saturated fatty acids

WE: Wax esters

α-GalCer: alpha-galactosylceramide

MAIN HYPOTHESIS

The presence of natural lipids from Brazil nuts is essential for the *in vivo* sensitisation to Ber e 1, the major allergen in Brazil nuts.

OBJECTIVES

1. To extract and fractionate lipids from Brazil nuts;

2. To identify fraction(s) of Brazil nut lipids able to induce anaphylactic antibody synthesis in an animal model of sensitisation;

3. To investigate mechanism(s) involved in the lipid-protein induction of a Th2-humoral response.

CHAPTER 1

GENERAL INTRODUCTION

1. Allergy

The term allergy was created by Clemens von Pirquet in 1906 from the Greek *allos* meaning "other" and *ergon* meaning "reaction" (Silverstein, 2000). Allergy is a significant health problem in developed countries and its prevalence is continually on the increase. Today, it is estimated that 10-25% of the population is affected by allergic diseases (Nouri-Aria e Durham, 2008; Niederberger, 2009).

Allergy, referring to type-I hypersensitivity reaction, occurs when an immunologically sensitised individual has a further contact with the antigen, known as allergen, leading to a secondary boosting of the immune system (Roitt, 1998). This reaction is usually mediated by Immunoglobulin E (IgE) and involves diverse cells and molecules from the immune system.

Allergens are usually harmless proteins and the most common allergens that drive an IgE-mediated type-I response originate from food, pollens, mites, drugs, latex and insect venom. The development of allergic reactions by allergens depends on numerous factors such as environment, genetic predisposition, age, dose and route of sensitisation, structure and physicochemical properties of the allergen (Dearman e Kimber, 2001; Breiteneder, H. e Mills, E. N., 2005; Cochrane, Beyer *et al.*, 2009).

Different types of allergies are described depending upon the organ system affected, such as allergic rhinitis, conjunctivitis, asthma, dermatitis and food allergies. The symptoms observed range from skin, gastrointestinal and respiratory reactions to life threatening anaphylactic reactions.

1.1. Mechanisms of allergy

The elucidation of mechanisms involved in the pathogenesis of allergic responses initiated in 1879, when Ehrlich described two new cellular types: mast cells and eosinophils (Larche, Akdis *et al.*, 2006). In 1921, Prausnitz and Kustner first described the passive transfer of antigen-specific hypersensitivity by means of serum from an atopic donor to a normal individual; the factor responsible for this reaction was named reagin (Prausnitz e Kustner, 1921; Mittal, Saha *et al.*, 1971). This factor was later identified as IgE by two independent research groups (Ishizaka, Ishizaka *et al.*, 1966; Johansson e Bennich, 1967). Based on these findings, allergic responses started to be associated with the inflammation caused by the degranulation of mast cells which, in turn, is caused by the linking of the antigen to IgE molecules attached to the membrane of mast cells.

An allergic process is initiated when antigen presenting cells (APCs) capture the allergen, migrate to the draining lymph nodes and, in the context of class II MHC (Major Histocompatibility Complex) molecules, present peptides from the allergen to naive T lymphocytes. These T lymphocytes then differentiate into CD4+ Th2 cells and produce specific cytokines, such as interleukin (IL)-4, IL-5, and IL-13 (Gould e Sutton, 2008). In this scenario, antigen-specific B cells are also activated and, in the presence of Th2 cytokines, produce antigen-specific IgE. This antibody binds to high-affinity (FcεRI) and low affinity (FcεRII) receptors expressed on the surface of mast cells and basophils. These events characterize the sensitisation phase of the type-I-hypersensitivity (Bischoff, 2007; Gould e Sutton, 2008). In a subsequent contact with the allergen, the allergen binds to specific-IgE on the surface of immune cells and the consequent release of potent biologically active mediators and their combined effects on various target organs are responsible for the symptoms

observed in the acute elicitation phase of an allergic process (Sampson, 2000; Cianferoni e Spergel, 2009).

High-affinity FcεRI receptors are expressed mainly on the surface of mast cells, basophils, dendritic cells, Langerhans cells and monocytes (Sihra, Kon *et al.*, 1997). Binding of antigens to specific IgE molecules occupying FcεRI on circulating basophils and tissue-resident mast cells is an initial step in the process of an allergic reaction. On these cells, FcεRI has a heterotetrameric form and consists of one α subunit, one β subunit and two disulphide-linked γ chains (Kinet, 1999; Gould, Sutton *et al.*, 2003). FcεRIα is the receptor subunit responsible for the IgE binding and the remaining FcεRI subunits are responsible for signal transduction across the cell membrane. FcεRIα has two extracellular immunoglobulin-like domains, a transmembrane hydrophobic region, and a positively-charged cytoplasmic tail; FcεRIβ has four transmembrane domains and two cytoplasmic tails; FcεRIγ has a single transmembrane domain and a cytoplasmatic tail. Intracellular components of FcεRI undergo protein phosphorylation upon signalling at the immunoreceptor tyrosine-based activation motifs (ITAM), culminating with cell activation (Kinet, 1999; Gould, Sutton *et al.*, 2003). IgE-activated mast cells secret preformed granular mediators such as histamine and proteases; lipid mediators synthesized from membrane phospholipids, and cytokines. Basophils are also activated by IgE-FcεRI cross-linking, releasing preformed granule mediators such as proteases and histamine but also secrete large quantities of Th2 cytokines such as IL-4. The role of these cells is gaining attention in allergic diseases (Falcone, Haas *et al.*, 2000; Mukai, Obata *et al.*, 2009).

Low affinity FcεRII receptors (CD23) are present on the surface of macrophages, monocytes, lymphocytes and platelets. The exact function of FcεRII remains poorly understood (Rosenwasser e Meng, 2005; Zhang, Murphy *et al.*, 2007); however, the role of these receptors has been reported to be involved in allergic diseases by enhancing IgE-antigen complex presentation, regulating IgE synthesis, influencing cell differentiation and growth of both B- and T-cells, and stimulating production of pro-inflammatory mediators from immune cells, such as monocytes, macrophages and eosinophils (Rosenwasser e Meng, 2005).

Moreover, it is important to mention that the involvement of IgG and its receptors is also well established in murine allergic processes (Hazenbos, Gessner *et al.*, 1996; Miyajima, Dombrowicz *et al.*, 1997; Ravetch e Bolland, 2001; Strait, Morris *et al.*, 2006). The role of IgG, particularly IgG1, is critical for the induction of systemic anaphylaxis (Dombrowicz, Flamand *et al.*, 1997; Miyajima, Dombrowicz *et al.*, 1997) and this event occurs through the binding of IgG1 to its activation FcγRIII receptors present in mast cells and basophils and the consequent release of inflammatory mediators (Miyajima, Dombrowicz *et al.*, 1997; Nimmerjahn, Bruhns *et al.*, 2005; Mukai, Obata *et al.*, 2009).

The humoral response is essential for the manifestation of type 1 allergic reactions; however, they are also largely dependent upon preceding cellular mechanisms. The description of the different population of T lymphocytes, Th1 and Th2 significantly elucidated mechanisms of allergy (Del Prete, Maggi *et al.*, 1988; Mosmann e Coffman, 1989). Activated allergen-specific Th2 lymphocytes play an essential role in the induction and maintenance of allergic processes, producing cytokines such as IL-4, IL-5, and IL-13 (so called

Th2 cytokines) (Leung, 1998; Romagnani, 2000; Woodfolk, 2007). IL-4 is involved with the production of IgE and IgG1 by B cells (Del Prete, Maggi *et al.*, 1988; Bacharier e Geha, 2000; Faquim-Mauro e Macedo, 2000); IL-5 is essential for the development, differentiation, recruitment and activation of eosinophils (Lopez, Sanderson *et al.*, 1988; Sanderson, 1990); and IL-13 regulates mucus secretion (Zhu, Homer *et al.*, 1999; Mattes, Yang *et al.*, 2001). Therefore, the differentiation of naive T lymphocytes into the Th2 phenotype is a critical step for the development of allergic responses.

2. Food allergy

The occurrence of food allergies is on the rise in westernized countries and the condition affects approximately 6% of young children and 3% of adults (Sicherer e Sampson, 2009). The individual food allergy varies by culture and population but globally the most common food allergies are milk, egg, peanut, soy, wheat, tree nuts, fish, shellfish and fruits (Eriksson, Moller *et al.*, 2004; Vierk, Koehler *et al.*, 2007; Imamura, Kanagawa *et al.*, 2008; Fernandez Rivas, 2009).

Food allergy is an immunological reaction against a food that occurs in susceptible individuals. Based on immunological mechanisms, food allergies can be classified in IgE-mediated, which are mediated by immunoglobulin E (IgE); cell-mediated, in which immune cells are responsible for the allergic process and, overlap, when both IgE and immune cells are involved in the reaction (Lee e Burks, 2006; Nowak-Wegrzyn e Sampson, 2006). The different symptoms observed in each of these immune-mediated food reactions are described in the table below (Metcalfe, 2005; Lee e Burks, 2006; Nowak-Wegrzyn e Sampson, 2006; Cianferoni e Spergel, 2009).

Table 1.1.: Immune-mediated adverse reaction to foods

IgE-mediated (immediate)	• Urticaria and angioedema • Rhinoconjunctivitis, rhinitis, asthma • Oral allergy syndrome • Nausea, colic, vomiting, diarrhea • Anaphylactic shock
Non-IgE-mediated (delayed)	• Food induced enterocolitic syndromes • Celiac disease
Overlap	• Atopic dermatitis • Allergic eosinophilic gastroenteritis

The best-characterized food allergy reactions are IgE mediated and the symptoms involved with this pathology include urticaria, asthma, oral allergy syndrome, digestive system symptoms and anaphylactic shock. These responses occur after the release of chemical mediators from mast cells and basophils as a result of interactions between food proteins and specific IgE molecules on the surfaces of these effector cells (Metcalfe, 2005; Lee e Burks, 2006). Under normal circumstances in most individuals, ingested proteins are not allergenic and; thus, do not elicit allergic immune responses. However, genetic alterations in the mechanisms that regulate the immune recognition of foreign proteins can predispose certain individuals to develop allergy (Metcalfe, 2005).

Mucosal surfaces represent the intersection between the organism and the environment. The gut associated lymphoid tissue (GALT) in mammals is the largest lymphoid organ in the body. The intestinal mucosa of an adult presents 300 m^2 of area (Moog, 1981) and it is estimated that adults ingest in average 30 kg

of proteins per year (Mestecky, 1987). Despite the extent of protein exposure, very few individuals develop food allergies due to the induction of oral tolerance. Different immune cells participate in oral tolerance, with regulatory T cells being the most important. Disturbances at different steps in the path to oral tolerance, antigen- or host-related, can trigger an allergic response (Chehade e Mayer, 2005).

Once proteins contact the intestinal surface, they are sampled by different cells and, depending on their characteristics, result in different responses. Antigens might be taken up by epithelial cells, Microfold (M) cells overlying Peyer's patches or DCs (Chehade e Mayer, 2005). Intestinal epithelial cells (IEC) express CD23 (FcεRII) following allergic stimulation and it was demonstrated that CD23 is involved in the rapid transepithelial transport of intact allergens in sensitised animals, increasing the uptake of IgE/allergen complexes (Bevilacqua, Montagnac *et al.*, 2004). It was recently shown that digested peanuts allergens are exclusively transported across the epithelium by specialized antigen-sampling M cells and delivered to the lymphoid tissue of Peyer's patch. These conditions favour strongly the induction of immune responses rather than oral tolerance (Chambers, Wickham *et al.*, 2004). The role of intestinal DCs in allergic processes is not entirely understood, but it was shown that DCs open the tight junctions between epithelial cells, send dendrites outside the epithelium and can directly sample antigens in the intestinal lumen (Rescigno, Urbano *et al.*, 2001).

3. Animal models of allergy

Animal models play a valuable role in increasing our understanding of the complex immunological and pathophysiological mechanisms involved in the

development of food allergic responses (Finkelman, 2007). Such systems can also help on the identification of truly novel proteins, which have not been experienced previously in the diet, being useful to estimate the allergenic potential of proteins.

Food allergy is a complex disease, influenced by genetic predisposition, allergen properties, environmental factors and exposure conditions. Therefore, the ideal animal model should mimic all aspects of a clinical situation in humans, including sensitisation and challenge using the oral route, lack of requirement for adjuvants, selected responses for known allergens, induction of IgE antibody, identification of similar IgE epitopes to those observed in human sera, production of clinically relevant symptoms on challenge and presenting reproducible results. Although it is unlikely that a single method using experimental animals will be able to assess all aspects of food allergy, the main objective of animal experimental models is to provide reliable information to be used conjunctly with relevant results obtained from other techniques.

Several animal models of food allergy are described in the literature using mice, rats, dogs or neonatal swine (Knippels, Houben *et al.*, 1999; Li, Serebrisky *et al.*, 2000; Teuber, Del Val *et al.*, 2002; Rupa, Hamilton *et al.*, 2008). The most commonly used species is mouse, due to the accessibility of a great range of antibodies and reagents as well as transgenic animals in which genes of immune interest are deleted or over expressed. It is also generally accepted that man and mouse share similar mechanisms of immune regulation (Griffiths, Dearman *et al.*, 2005; Maizels, 2005).

The main drawback of animal models of food allergy is the difficulty of sensitizing animals by the oral route, as would reflect the process of allergen sensitisation in humans. The oral administration of allergens in the absence of

adjuvants promotes tolerance rather than sensitisation (Keller, Mucida *et al.*, 2006; Sun, Czerkinsky *et al.*, 2010). The few models able to achieve appropriate oral sensitisation imply long protocols, in the presence of mucosal adjuvants, such as cholera toxin (Van Wijk, Nierkens *et al.*, 2007; Perrier, Thierry *et al.*, 2009). In order to circumvent this aspect, numerous models of food allergy animal apply different routes of sensitisation such as intra-peritoneal and transdermal (Birmingham, Parvataneni *et al.*, 2007; Dearman, Alcocer *et al.*, 2007). There are several mouse strains used to study IgE-mediated processes, BALB/c mice, for instance, are more susceptible to develop allergic diseases than other strains, such as C57BL/6, which is considered more resistant (Drazen, Arm *et al.*, 1996; Alcorn, Ckless *et al.*, 2010). There are also transgenic strains of mice that develop exacerbated Th2/IgE responses. These animals bear monoclonal populations of T and B lymphocytes towards one specific antigen (e.g. DO11.10, specific for OVA) and are useful for IgE-mediated studies (Curotto De Lafaille, Muriglan *et al.*, 2001; Mucida, Kutchukhidze *et al.*, 2005).

In animal models of food allergy, the parameters usually evaluated to characterize the molecular and cellular mechanisms involved in the allergic process are mainly: levels of IgE and IgG1, anaphylactic activity of antibodies, splenocytes proliferation and cytokine secretion profile, histological aspects of gastro-intestinal mucosa, and clinical symptoms of anaphylaxis (Li, Serebrisky *et al.*, 2000; Morafo, Srivastava *et al.*, 2003; Adel-Patient, Bernard *et al.*, 2005; Cardoso, Teixeira *et al.*, 2008; Proust, Astier *et al.*, 2008).

4. Anaphylaxis in animal models of allergy

Anaphylactic reactions have been widely used in the last few decades for experimental investigations of allergy (Inagaki, Goto *et al.*, 1986; Fung-Leung, De

Sousa-Hitzler *et al.*, 1996; Kennedy Norton, Barnstein *et al.*, 2008; Inagaki e Nagai, 2009; Pushparaj, Tay *et al.*, 2009). In animal models, anaphylaxis can be actively or passively induced. Active anaphylactic reactions can be systemic or cutaneous, both being provoked in sensitised animals, by challenging them with the antigen. Active systemic anaphylaxis uses intravenous challenge and the parameters evaluated are body temperature, heart rate, mortality rate and other (Miyajima, Dombrowicz *et al.*, 1997; Strait, Morris *et al.*, 2002). Active cutaneous anaphylaxis challenges the animal via intra-dermal, evaluating the vascular permeability in the skin by dye extravasation (Woo, Kim *et al.*, 2006; Nonaka, Izumo *et al.*, 2008). Passive anaphylactic reactions can also be systemically or cutaneously performed. For passive systemic anaphylaxis, animals are passively transferred with serum or antibodies intravenously and then challenged with the antigen (Miyajima, Dombrowicz *et al.*, 1997). The parameters evaluated are the same as those used for active systemic anaphylaxis. In passive cutaneous anaphylactic reactions, serum or antibodies are intradermally injected and the animals are challenged via intravenous with the antigen (Ovary, 1982; Inagaki, Goto *et al.*, 1986).

Due to is reliability and simplicity, a widely used protocol for an anaphylactic response is the passive cutaneous anaphylactic reaction for which the term PCA (Passive Cutaneous Anaphylaxis) was created (Ovary, 1950). PCA is a simple *in vivo* model to identify antigen-specific anaphylactic antibodies in experimental allergy in animals. Although other i*n vitro* techniques for determining antibody concentration such as RAST (radioallergosorbent immunoassay test), ELISA (Enzyme-linked immuno sorbent assay) and microarrays are regarded as more sensitive (Eriksson e Ahlstedt, 1977; Wiese, Belosludtsev *et al.*, 2001; Lebrun, Petchpud *et al.*, 2005; Harwanegg e Hiller,

2006), PCA is still utilized because, by partially mimicking an immediate allergic process, it is able to assess the biological function of antibodies (Miyajima, Dombrowicz et al. 1997; Woo, Kim et al. 2006; Nonaka, Izumo et al. 2008).

PCA relies on one of the fundamental characteristics of an immediate allergic reaction, namely the liberation of vasoactive substances and their action on increasing the permeability of capillaries in the skin. The most well known mechanism responsible for this process involves mast cell activation. Mast cells present multiple cell-surface receptors (Hallgren e Gurish, 2007; Hakim-Rad, Metz *et al.*, 2009), but the main players in the mast cell activation for anaphylactic processes are the immunoglobulin receptors.

4.1. Mast cell activation

Tissue mast cells are central players in the initiation of PCA reactions. Mast cells are widely distributed in the connective tissues of mammals and other vertebrates, being located around blood vessels in mucosal and epithelial interfaces that are exposed to the external environment, such as those of the respiratory and gastrointestinal tract and skin (Galli, Zsebo *et al.*, 1994; Galli, Maurer *et al.*, 1999). Mast cell activation leads to the release of a plethora of inflammatory mediators. These include preformed granular mediators, lipid mediators synthesized from membrane phospholipids, and cytokines (Bochner e Lichtenstein, 1991; Ryan, Kashyap *et al.*, 2007).

The widely accepted concept for mast cell activation involves the cross-linking activation of the high-affinity FcεRI receptors by IgE at the mast cell surface (Bochner e Lichtenstein, 1991). Stimulation of IgE-sensitised mast cells with antigen induces FcεRI receptor cross-linking and this procedure leads to the

activation of β subunit-associated LYN, a SRC family protein tyrosine kinase. Activated LYN phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs), present in the cytoplasmic regions of FcεRI β and γ subunits. This tyrosine phosphorylation happens within the first 15 seconds following FcεRI aggregation by antigen on mast cells (Paolini, Jouvin *et al.*, 1991). Phosphorylated β and γ ITAMs recruit LYN and SYK, respectively (Benhamou e Siraganian, 1992). The tethering of SYK allows phosphorilation of its catalytic domain, as well as phosphorylation by LYN, thereby increasing its catalytic activity. SYK binds to phosphorylated ITAMS and is activated on binding. The subsequent SYK- and/or LYN-mediated tyrosine phosphorylation of the transmembrane adaptor molecule LAT (linker for activation of T cells) is crucial for coordination of the downstream signaling pathways that are required for the degranulation of pro-inflammatory mediators such as histamine, serotonin (5-hydroxytryptamine), leukotrienes, prostaglandins, nucleotides as well as the for the production of cytokine and chemokine molecules (Gilfillan e Tkaczyk, 2006; Rivera e Gilfillan, 2006). These biologically active compounds released from activated mast cells are responsible for the immediate increasing on vascular permeability observed in anaphylactic reactions as well as for the modulation of innate and adaptive immune responses (Galli, Nakae *et al.*, 2005).

The high affinity receptor for IgE, FcεRI, is the most thoroughly studied mediator of mast cell function (Bochner e Lichtenstein, 1991; Gould, Sutton *et al.*, 2003; Chang e Shiung, 2006; Prussin e Metcalfe, 2006; Matsuda, Okayama *et al.*, 2008; Carroll-Portillo, Spendier *et al.*, 2009). However, recent studies have emphasized the importance of the IgG Fc receptors present not only in mast cells, but also on monocytes, macrophages and neutrophils (Oettgen, Martin *et al.*, 1994; Hazenbos, Gessner *et al.*, 1996; Miyajima,

Dombrowicz *et al.*, 1997). There are two distinct classes of murine IgG FcRs, one for activation and an other for inhibitory activity. Activation receptors include the high affinity FcγRI, and the low affinity FcγRIII. Aggregation of FcγRI or FcγRIII can mediate a variety of inflammatory processes, amongst them mast cell degranulation (Daeron, 1997; Ravetch e Bolland, 2001). Both high- and low-affinity constant region binding receptors (FcR) trigger cell responses with equal efficiency. The difference bears on the order of the events which, in both cases, result in FcR aggregation. In general, high-affinity FcRs bind non-complexed monomeric immunoglobulins before these antibodies are complexed by multivalent antigens and low-affinity FcRs bind aggregated immunoglobulins already complexed to multivalent antigens (Daeron, 1997). Similar to Fcε receptors, signal transduction through these receptors is mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the Fc receptor γ chain, through recruitment and activation of tyrosine kinases such as LYN and SYK (Daeron, 1997; Ravetch e Bolland, 2001). The inhibitory receptor is named FcγRIIb and it mediates inhibitory IgG signaling through immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which preferentially interact with tyrosine and inositol phosphatases. Crosslinkage of FcγRIIb with an activation receptor such as the FcεRI, FcγRI, or FcγRIII can inhibit pro-inflammatory signalling (Daeron, Latour *et al.*, 1995). The contrasting functions of FcγRIIb and FcγRIII, which present identical ligand binding properties and co-expression on mast cells allow control of IgG-mediated signalling (Daeron, 1997; Ravetch e Bolland, 2001; Chong, Andrew Bouton *et al.*, 2003).

The involvement of IgG and Fcγ receptors in mouse mast cell degranulation was demonstrated several decades ago using an *in vitro* model of

anaphylaxis (Vaz, Vaz *et al.*, 1970). Some years later, it was shown that *in vivo* anaphylaxis (PCA reactions) could be induced by defined IgG antibody subclasses and that the response was species specific. Mouse mast cells, for instance, were activated and degranulated through IgG1, but not through IgG2 or IgG3 (Ovary, 1982). Differently, studies in rats showed that FcγR does not trigger degranulation on rat mast cells (Bocek, Draberova *et al.*, 1995). This study also demonstrated that FcεRI saturation by IgE on rat mast cells completely blocks their response to IgG immune complexes (Bocek, Draberova *et al.*, 1995). Other studies demonstrated that IgG1 antibodies are exclusively dependent on FcγRIII, a low-affinity activation receptor (Nimmerjahn, Bruhns *et al.*, 2005). Currently, it is well established in rodent models that antigen cross-linking of IgG1 bound to FcγRIII on mast cells and basophils is able to elicit anaphylactic reactions in a similar way to those induced through IgE-FcεRI (Hazenbos, Gessner *et al.*, 1996; Dombrowicz, Flamand *et al.*, 1997; Miyajima, Dombrowicz *et al.*, 1997; Wakayama, Hasegawa *et al.*, 1998; Strait, Morris *et al.*, 2002). Passive systemic anaphylaxis induced by IgG1 was attenuated in FcγRIII-deficient mice (Miyajima, Dombrowicz *et al.*, 1997), and, moreover, it was demonstrated that IgE is not essential for anaphylaxis since IgE null mice expressed fatal active anaphylaxis responses (Oettgen, Martin *et al.*, 1994).

Further, the existence of two biologically different mouse IgG1 was suggested, differing in their affinity for protein A and in their PCA activity (Mota e Perini, 1982). More recent studies better described these two functionally distinct IgG1 types, demonstrating that one type of IgG1 (anaphylactic IgG1) has anaphylactic activity and is synthesised in an IL-4-dependent environment, being inhibited by IL-12 or IFN-γ. The non-anaphylactic IgG1 lacks this activity and its

synthesis is stimulated by IL-12 or IFN-gamma (Faquim-Mauro, Coffman *et al.*, 1999). It was also shown that the production of these two types of antibodies, anaphylactic- and non-anaphylactic-type IgG1, can be modulated by different adjuvants (Faquim-Mauro e Macedo, 2000). As expected, the ability of anaphylactic IgG1 to mediate anaphylaxis is directly related to their binding to FcγRIII expressed on mast cells and that this ability is related with its *N*-linked oligosaccharide chain (Faquim-Mauro, Jacysyn *et al.*, 2003). Several studies in mice have confirmed that anaphylaxis can be induced by IgG1 immunocomplexes (Alber, Kent *et al.*, 1992; Hazenbos, Gessner *et al.*, 1996; Hochreiter, Ferreira *et al.*, 2003; Tsujimura, Obata *et al.*, 2008). Recently, the glycan composition of IgG1 antibodies was investigated and it was demonstrated that the anaphylactic IgG1 presents more sialic acid and fucose residues than the non-anaphylactic type. Moreover, the removal of the terminal sialic acid residues in anaphylactic IgG1 resulted in loss of the ability to trigger mast cell degranulation and *in vivo* anaphylactic reaction (Faquim-Mauro, Coffman *et al.*, 1999; Faquim-Mauro, Jacysyn *et al.*, 2003; Silva, Casabuono *et al.*, 2008).

The differentiation between IgG1 or IgE-mediated PCA reactions is usually performed by heating-inactivation, since IgE is described as a heat-labile immunoglobulin whereas IgG1s are heat-stable (Lehrer e Vaughan, 1976; Faquim-Mauro e Macedo, 2000). An additional property used to differentiate these two classes of immunoglobulins is the reaction duration, short intervals between sensitisation and antigen-challenge (2h) are used to identify IgG1 anaphylaxis and longer intervals are used to identify IgE anaphylaxis (48h) (Lehrer e Vaughan, 1976; Faquim-Mauro e Macedo, 2000). Furthermore, a comparative study of IgE and IgG1 eliciting PCA reactions in mice showed that the alteration of skin mast cells is smaller in IgG1 antibody than in IgE antibody

(Inagaki, Nagai *et al.*, 1988) and it has also been demonstrated that the amount of antibodies required to elicit PCA reactions is higher for IgG1 (500 ng/mL) than for IgE (15 ng/mL) (Ovary, 1982).

Therefore, since IgE and IgG1 are the only known immunoglobulin isotypes that can elicit active and passive anaphylactic reactions in mice (Mota, Wong *et al.*, 1968; Becker, 1971; Lehrer e Vaughan, 1976; Dombrowicz, Flamand *et al.*, 1997; Blaikie e Basketter, 1999; Ryan, Kashyap *et al.*, 2007), all considerations regarding IgE and IgG1 are essential for investigations on mouse models of anaphylaxis.

4.2. PCA technique

One way of performing PCA technique is by injecting serum from sensitised or non-sensitised mice into the dermis of naïve recipient mice dermis. A period of 48 hours is allowed for the possible antibodies present in the donor serum samples to bind to the receptors located on the mast cell surfaces of the recipient. Then, recipient animals are intravenously challenged with the relevant protein (which the experimental donor mice were sensitised to), with the addition of Evans blue dye. If the serum injected into the recipient mouse dermis contains protein-specific anaphylactic antibodies which bind to their receptors on mast cells, the contact with the antigen (the relevant protein injected intra-venously) will provoke a cross-linking reaction (antigen-antibody-mast cell receptor) causing mast cells degranulation. The liberation of vasoactive substances increases the vascular permeability in the skin and the extravasation of Evans blue dye from blood vessels is visible to the naked eye. This process starts within

seconds after the injection of protein and after thirty minutes following the challenge the diameter of the cutaneous reaction is measured.

A brief description of the technique is represented in the Figure 1.1. and examples of PCA results are showed in the Figure 1.2. (Ovary, 1950; Mota e Perini, 1982).

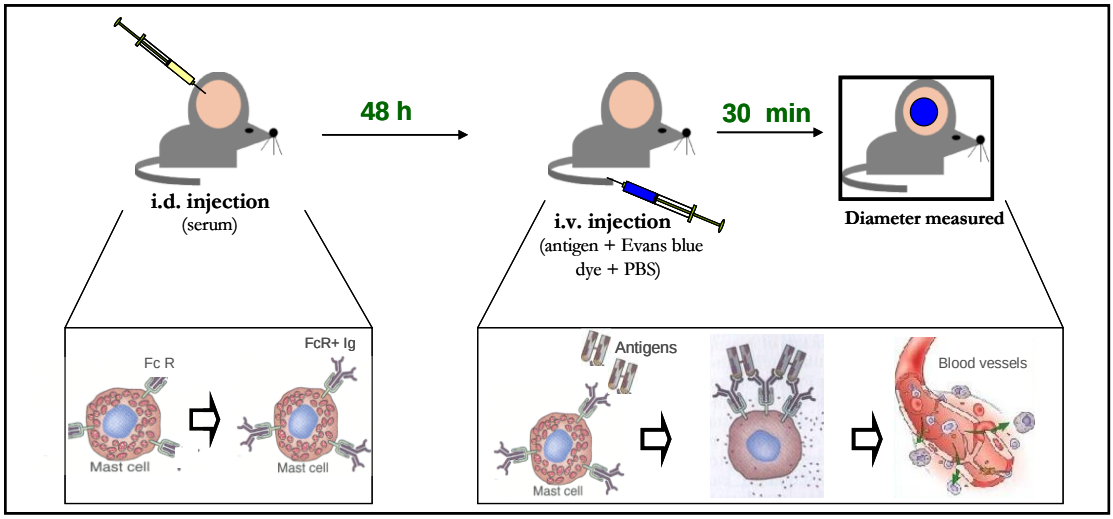


Figure 1.1.: The serum to be tested is injected via intra-dermal (i.d.) in the ears of naive recipient mice. 48 hours later an intra-venous (i.v.) injection containing the allergen and Evans blue dye is performed. The local extravasation of Evans blue dye in the tissue is visible within minutes after antigen injection and the diameter of the blue circle represents the PCA results (expressed in mm).

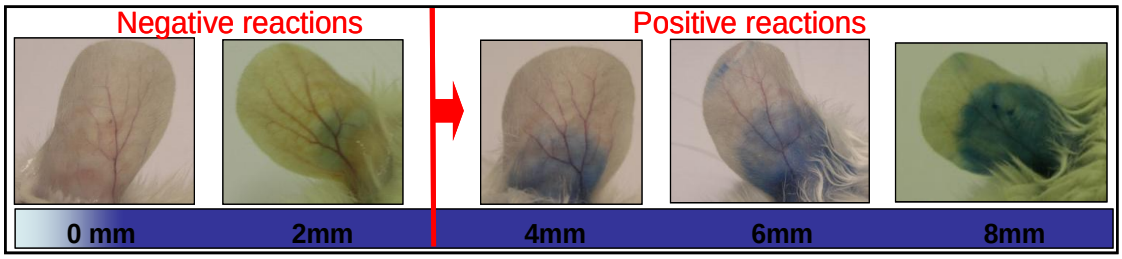


Figure 1.2.: Examples of PCA results obtained 30 minutes following the i.v. challenge. Reactions resulting in diameters smaller than 3 mm are considered negative and diameters measuring 3 mm or more are considered positive PCA reactions.

5. Food allergens

Despite the fact that innumerous proteins are encountered in normal diets, only a few specific protein families are commonly implicated as food

allergens (Bush e Hefle, 1996). The majority of plant allergens, for instance, are grouped into just 4 families (Radauer e Breiteneder, 2007), indicating that certain conserved characteristcs are determinants for the development of allergic responses. The classification of plant food allergens into families and superfamilies is based on their structural and functional properties (Breiteneder e Radauer, 2004).These families are the prolamin superfamily, the cupin superfamily, the profilins and proteins related to the major birch pollen allergen Bet v 1. The prolamin superfamily contains the 2S albumin seed storage proteins, the nonspecific lipid transfer proteins, protease inhibitors and cereal prolamins present in legumes, tree nuts, cereals, fruits, and vegetables. The cupin superfamily contains the vicilin and legumin-type seed storage globulins present in soybeans, peanuts and nut trees. The allergenic profilins are present in flowering plants, although non-allergenic profilins are present in all eukaryotic cells. Bet v 1 superfamily contains several types of proteins but the only allergenic are the pathogenesis-related proteins 10 family members (Radauer e Breiteneder, 2007).

The prolamin superfamily is the most prominent of all protein families that contain allergenic members. Prolamins are proline- and glutamine-rich α-helical proteins with a conserved skeleton of 8 cysteine residues that serve several biologic functions. The allergenic prolamins include members of the nonspecific lipid transfer protein family (Breiteneder, H. e Mills, C., 2005), the 2S albumin storage proteins (Pastorello, Pompei *et al.*, 2001), the cereal α-amylase/trypsin inhibitors (Pastorello, Farioli *et al.*, 2007), and the soybean hydrophobic protein (Baud, Pebay-Peyroula *et al.*, 1993). These protein families have little sequence homology to each other apart from the cysteine skeleton, but they have highly similar α-helical structures. This structure is highly stable to both thermal and

proteolytic denaturation, which might contribute to the allergenicity of these proteins (Breiteneder, H. e Mills, E. N., 2005).

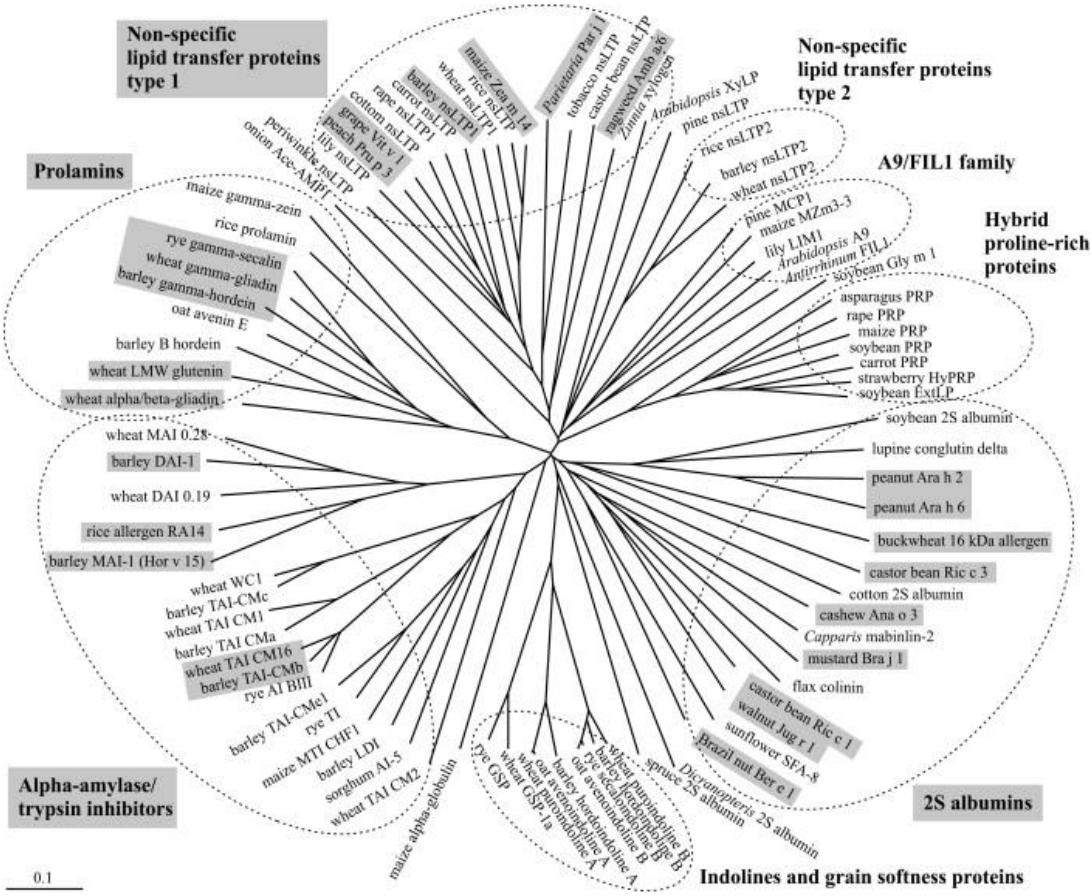


Figure 1.3. Phylogenetic tree showing the relationship of members from the prolamin superfamily. Allergens and families that contain allergens are *shaded*. *AI*, α-Amylase inhibitor;*Ace-AMP1*, *Allium cepa* antimicrobial protein 1; *DAI*, dimeric amylase inhibitor; *ExtLP*, extensin-like protein; *GSP*, grain softness protein; *HyPRP*, hybrid proline–rich protein;*LDI*, limit dextrinase inhibitor; *LMW*, low molecular weight; *MAI*, monomeric amylase inhibitor; *MTI*, monomeric trypsin inhibitor; *nsLTP*, nonspecific lipid transfer protein; *PRP*, proline-rich protein; *SFA-8*, sunflower albumin 8; *TAI-CM*, chloroform/methanol soluble tetrameric amylase inhibitor; *TI*, trypsin inhibitor; *WCI*, wheat chymotrypsin inhibitor;*XyLP*, xylogen-like protein. Diagram extracted from (Radauer e Breiteneder, 2007).

Therefore, it is clear that some proteins are intrinsically more allergenic than others. However, the membership in one of a limited number of protein families in itself is not sufficient to determine allergenic activity. Amongst the intrinsic protein features that have been reported to contribute to the

phenomenon of allergenicity are: size, solubility, glycosylation status, resistance to proteolytic digestion, heat stability, ligand bindings, disulfide bonds, number of IgE-binding sites, potential interaction with cell membranes and lipid structures, and biological function such as enzymatic activity and storage proteins (Breiteneder, H. e Mills, E. N., 2005; Thomas, Macintosh *et al.*, 2009; Traidl-Hoffmann, Jakob *et al.*, 2009).

The more common food allergens are generally water-soluble, with a reasonable degree of glycosylation and have molecular weight varying from 10 to 70 kDa (Taylor e Lehrer, 1996). Food allergens also tend to be stable and resistant to heat and digestive processes (Wal, 1998), i.e. they are able to retain their original, native, 3-dimension structure after these chemical or physical treatments. This stability is important particularly for oral allergens as it implies on their survival of cooking and gastric digestion; increasing the probability of being uptaken in the gut and recognised by the immune system. The biological function of protein is also reported to be related to protein allergenicity. Proteins with enzymatic activity, such as might enhance sensitisation by disrupting tight junctions in mucosal tissues. A well studied example is the house dust mite allergen, Der p 1, able to disrupt bronchial epithelium tight junctions (Herbert, King *et al.*, 1995). Moreover, many of the known food allergens are classified as pathogenesis-related proteins. This class of proteins is more abundant in situations of environmental stress and they exhibit functions such as antifungal activity, antibacterial activity, and proteinase inhibition (Breiteneder e Ebner, 2000; Metcalfe, 2005).

Despite these general commonalities of known allergens, it is important to highlight that not all stable proteins are allergens. Allergens from apples and milk, for example, have been shown to be labile (Wal, 1998; Huby, Dearman *et*

al., 2000). Hundreds of proteins have been identified as allergens, having their amino acid sequences characterized and incorporated into comprehensive allergen databases (Goodman, Hefle *et al.*, 2005); however, the precise features that make a protein allergenic remain elusive. The potential allergenicity of novel foods is still assessed by comparison to that of existing allergens (Esch, 2006). Therefore, there is considerable interest in defining the characteristics that confer sensitizing potential on the proteins that can provoke the quality of immune response necessary for the acquisition of allergic sensitisation. This has assumed increased importance with the need to identify and characterize potential food allergens as an essential part of food safety assessment (Goodman, Hefle *et al.*, 2005; Thomas, Macintosh *et al.*, 2009; Traidl-Hoffmann, Jakob *et al.*, 2009).

6. Brazil nuts

The Brazil nut (*Bertholletia excelsa* or *Bertholletia excels*) is a South American tree from the family Lecythidaceae and also the name of the tree's commercially harvested edible seed. The Brazil nut tree is native to the Guianas, Venezuela, Colombia, Peru, Bolivia, and Brazil. The Brazil nut tree is a large tree, reaching 30–50 metres of height and 1–2 metres of trunk diameter. It may live for 500 years or more. *Betholletia excelsa* is distinguished from all other species of Lecythidaceae by the oblong leaves with a whitish cast on the abaxial surface caused by microscopic cuticular papillae; a two-lobed calyx at anthesis; woody fruits that fall to the ground with the seeds inside; and seeds with a boney testa and no apparent cotyledons (Mori, 2006 onward-b). Brazil nuts, the seeds of the Brazil nut tree, are one of the most widely exploited tropical nuts. They are harvested almost entirely from wild trees during a five to six month period in the

rainy season. There is an average of 200 fruits per tree and a mean seed number per fruit of 15-20 (Mori, 2006 onward-b).

Tree of *Bertholletia excelsa.*

Fruit and seeds of *Betholletia excelsa.*

Seeds of *Betholletia excelsa.*

Figure 1.4.: Photographs of the tree, fruit and seeds of *Bertholletia excelsa (Mori, 2006 onward-a).*

The protein content of Brazil nuts is approximately 14.3% of dried weight (USDA, 2009). Brazil nuts are probably one of the richest food sources of the sulphur-containing amino acids; the total seed protein is reported to contain about 8.3% methionine and cysteine by weight (Sun, Altenbach *et al.*, 1987). The total protein of Brazil nuts can be fractionated into three size classes of proteins, the 11 S, 7 S, and 2 S proteins. The water-soluble 2S albumin comprises about 30% of the total protein and is exceptionally rich in the sulfur amino acids; about 30% methionine and cysteine (Sun, Altenbach *et al.*, 1987). The detailed composition of Brazil nuts is presented in the Appendix 1.

The 2S albumin family is part of the prolamin superfamily and are present in seeds of several dicotyledonous and monocotyledonous species. The 2 S albumins are generally α-helical proteins (10 to 15 kDa) composed of one small and one large subunit linked by two conserved disulphide bridges in a distinctive right-handed fold (Breiteneder e Radauer, 2004; Mills, Jenkins *et al.*, 2004). 2S albumins include several important food allergens, such as Ber e 1 from Brazil nuts, Jug r 1 from the English walnuts, Ses i 2 from sesame seeds, Sin a 1 from yellow mustard and others (Teuber, Dandekar *et al.*, 1998; Pastorello, Pompei *et al.*, 2001; Pastorello, Varin *et al.*, 2001).

6.1. Brazil nut allergy

Allergies to nuts are amongst the most common food allergies, affecting approximately 1% of the general population in the UK and the USA (Crespo, James *et al.*, 2006). Allergy to Brazil nuts was first reported in 1983, in a study describing 4 children with allergic reactions to Brazil nuts (Hide, 1983), and a further study reported 12 cases of Brazil nut allergy (Arshad, Malmberg *et al.*, 1991). However, the allergenic potential of Brazil nut became notable when its 2 S albumin proteins, rich in cysteine and methionine, were used for genetic engineering in order to improve the nutritional value of soybean. This approach was unsuccessful due to the potential passage of allergenic properties of the 2 S albumin protein of Brazil nuts to the transgenic soybean (Nordlee, Taylor *et al.*, 1996).

The first direct correlation of the sensitisation to the Brazil nut major allergen, Ber e 1, with the clinical expression of allergy to this seed was shown in a study in which sera from Brazil nut allergic and non-allergic individuals were

compared. All sera from symptomatic patients recognized Ber e 1, whereas no sera from asymptomatic subjects showed IgE binding to Ber e 1 (Pastorello, Farioli *et al.*, 1998).

The structural IgE epitope of Ber e 1 has been mapped to a single helix-loop-helix region, through the use of a chimaeric protein microarray system together with sera from well-characterised Brazil nut allergic subjects (Alcocer, Murtagh *et al.*, 2004). Corroborating with these findings, the same structural region has been reported as the immunodominant region in related food allergens by different techniques (Karisola, Alenius *et al.*, 2002).

6.2. Ber e 1

Allergens are named using the first 3 letters of the genus, followed by a single letter for the species and a number indicating the chronologic order of allergen purification. Therefore, Ber e 1 was the first purified allergen from ***Ber****tholletia* ***e****xcelsa.* As a member of the 2 S albumin protein family, the major allergen form Brazil nuts (Ber e 1) is a water-soluble heterodimeric protein, consisting of 2 polypeptide chains of approximately 4 and 9 kDa that are held together by four intra-chain disulfide bonds in a distinctive right-handed fold (Alcocer, Murtagh *et al.*, 2002).

Ber e 1 is a very stable protein, highly resistant to temperature, digestion, guanidinium chloride and acidic pH. It was shown that this protein maintains its folding properties up to 75ºC and retains most of its secondary structure at 95ºC. Importantly, the original protein structure was largely regained upon cooling. Ber e 1 was shown to be the protein from Brazil nuts least susceptible to pepsin digestion, being highly resistant to acidic pH. The resistance

to unfolding was also displayed when Ber e 1 was exposed to the chemical denaturant guanidinium chloride (Murtagh, Archer *et al.*, 2003).

A model of tridimensional structure of Ber e 1 was constructed based on the conserved 2 S albumin disulphide connectivity of this protein, together with sequence and structural alignments with other 2 S albumin proteins (Alcocer, Murtagh *et al.*, 2002).

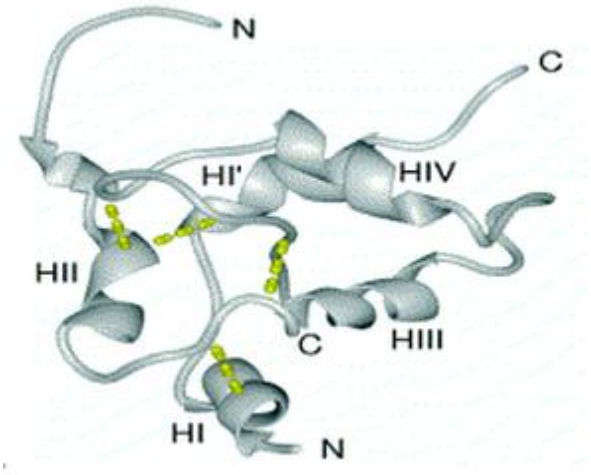


Figure 1.5.: Molecular modelling of *Ber e* 1 protein. Ribbon representation of the backbone of the model *Ber e* 1 structure. The disulphide bonds are represented as yellow broken lines (Alcocer, Murtagh *et al.*, 2002).

Although the allergenic potential of Ber e 1 is well established (Nordlee, Taylor *et al.*, 1996; Pastorello, Farioli *et al.*, 1998), it has been recently demonstrated that recombinant Ber e 1 is not able to induce anaphylactic antibodies production in mice when it is used alone, i.e., in the absence of the Brazil nuts natural lipids. In contrast, the production of anaphylactic antibodies was observed when the natural lipids from Brazil nuts were added to the protein (Dearman, Alcocer *et al.*, 2007). These results corroborate with the fact that, not only intrinsic features, but also extrinsic factors such as the presence of lipids might be determinants for the initiation of an allergic process.

6.3. Nuts composition

Throughout history, nuts have been part of the diet around the world. Records of eating pistachios date back to 7000 BC. Early descriptions of food intakes indicate that the Romans, Persians, and Arabs used nuts to cook and that this practice later extended to Europe (Brufau, Boatella *et al.*, 2006; King, Blumberg *et al.*, 2008). Nowadays, edible nuts are globally popular, and are valued for their sensory, nutritional, and health attributes (Venkatachalam e Sathe, 2006). The most popular and commercially important edible nuts are: almonds (*Prunus dulcis*), Brazil nuts (*Bertholetia excelsa),* cashew nuts (*Anacardium occidentale*), hazelnuts (*Corylus avellana*), macadamias (*Macadamia integrifolia*), pecans (*Carya illinoinensis*), pine nuts (*Pinus pinea*), pistachios (*Pistachia* V*era*), and walnuts (*Juglans regia*) (Venkatachalam e Sathe, 2006).

Nuts are rich in all major macronutrients: protein, carbohydrate, and lipid. The chemical composition of common edible nuts varies: water (1.47-9.51%), protein (7.50-21.56%), lipid (42.88-66.71%), and ash (1.16-3.28%) (Venkatachalam e Sathe, 2006). Table 1.2. shows a summarized composition of common nuts (USDA, 2009).

	Energy (kcal)	Water (g)	Protein (g)	Total lipid (g)	Ash (g)	Carbohydrate (g)
Almond	575	4.7	21.22	49.42	2.99	21.67
Brazil nut	656	3.48	14.32	66.43	3.51	12.27
Hazelnut	628	5.31	14.95	60.75	2.29	16.7
Macadamia	718	1.36	7.91	75.77	1.14	13.82
Pecan	691	3.52	9.17	71.97	1.49	13.96
Pine nut	673	2.28	13.69	68.37	2.59	13.08
Pistachio	562	3.91	20.27	45.39	2.91	27.51
Walnut	654	4.07	15.23	65.21	1.78	13.71

Table 1.2: Chemical composition of raw nuts (USDA, 2009).

The total protein content of nuts is relatively high, which makes nuts an important source of protein, especially for vegetarian diets. However, the biological protein value of nuts is not high since some essential amino acids are limiting (Brufau, Boatella *et al.*, 2006). In general, threonine is the limiting amino acid for all nuts (25–40 mg/g of protein), being Brazil nuts the poorest (3.62 mg/g) and cashews the richest in threonine (6.88 mg/g) (Brufau, Boatella *et al.*, 2006; Usda, 2009). Nuts are low in isoleucine, with a content ranging between 32 and 40 mg/g of protein. Nuts are also deficient in the dibasic amino acid lysine. Methionine and cysteine (sulphur amino acids), are found in low amounts in most nut proteins with the exception of Brazil nut, which contains high amounts of sulphur amino acids (96 mg/g of protein) (Brufau, Boatella *et al.*, 2006). Other amino acids such as phenylalanine, tyrosine, histidine and valine are present in significant amounts in the protein of all nuts (Brufau, Boatella *et al.*, 2006).

The total carbohydrate content in common edible nuts vary from 12.7 to 27.5% (Usda, 2009). The lowest amounts are found in Brazil nuts, and

progressively increasing amounts occur in walnuts, Macadamia, hazelnuts, almonds, and pistachios (Table 1.2.).

Nuts are also good sources of several other important nutrients, such as phytosterols and other phytochemicals such as phenolic compounds, flavonoids, luteolin and tocotrienols. A study showed that walnuts and pecans contained the highest phenolic content (1.5% of raw nut weight) followed by pistachios and Macadamia (0.5% of raw nut weight), cashews and hazelnuts (0.3% of raw nut weight), almonds and Brazil nuts (0.2% of raw nut weight). The flavonoid content was higher in walnuts and pecans (0.7% of raw nut weight) and lower in pistachios, macadamia, hazelnuts, Brazil nuts and almonds (approximately 0.1% of raw nut weight) (Yang, 2009). Other micronutrients present in notable quantities in most nuts include thiamine, niacin, riboflavin, selenium, potassium and iron (Brufau, Boatella *et al.*, 2006).

6.3.1. Nut lipids

The main constituent of nuts is lipid, and as shown in Table 1.2., the total lipid content per 100 g ranges from 45.4% in pistachios to 75.8% in Macadamia nuts (USDA, 2009). The extraction of total lipids from nuts is usually performed by traditional methods such as "Folch" extraction (Folch, Lees *et al.*, 1957) that is still used nowadays (Parcerisa, Codony *et al.*, 1999; Moodley, Kindness *et al.*, 2007); "Soxhlet" (1879), a common method for general triglyceride extraction, using petroleum ether (Amaral, Casal *et al.*, 2003; Venkatachalam, Kshirsagar *et al.*, 2007) or hexane (Chunhieng 2008); and also hexane extraction (Ryan, Galvin *et al.*, 2006; Miraliakbari e Shahidi, 2008).

The lipid composition of nuts is low in saturated fatty acids (SFA) (4–16%) and high in mono- and poly-unsaturated fatty acids (MUFA and PUFA, respectively). For instance, similar proportions of MUFA and PUFA are present in Brazil nuts, a predominance of PUFA over MUFA is observed in pine nuts, and in walnuts PUFA is mostly present (Ros e Mataix, 2006). Together, MUFA and PUFA contribute around 91% of the energy from fat in nuts (Kris-Etherton, Yu-Poth *et al.*, 1999). These values may vary according to the method used to analyse fatty acids as well as to climate and soil conditions where the nuts are grown (Venkatachalam e Sathe, 2006; Chunhieng 2008).

	Total lipids (g)	TSFA (g)	MUFA (g)	PUFA (g)
Almond	49.42	3.73	30.89	12.07
Brazil nut	66.43	15.14	24.55	20.60
Hazelnut	60.75	4.46	45.65	7.92
Macadamia	75.77	12.06	58.88	1.5
Pecan	71.97	6.18	40.80	21.61
Pine nut	68.40	4.90	18.80	34.10
Pistachio	45.39	5.56	23.8	13.74
Walnut	65.21	6.13	8.93	47.17

Table 1.3.: Lipid composition of several edible nuts. Data for raw nuts. TSFA, total saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids (USDA, 2009).

Lipids can be divided into two broad classes, simple and complex lipids. This classification is based on the products yielded on hydrolysis processes; simple lipids yield at most two types of primary product per mole on hydrolysis and complex lipids yield three or more primary hydrolysis products per mole. Simple lipids include mono-, di-, and triacylglycerol (also called triglycerides - TG), which are made by fatty acids and glycerol; sterol; sterol ester; waxes;

tocopherol; and free fatty acids. The complex lipids include the glycerophospholipids (or phospholipids), which contain a glycerol backbone, one or more fatty acids, a polar phosphorus moiety and an alcohol; and the glycolipids (both glycoglycerolipids and glycosphingolipids), which contain a polar carbohydrate moiety. Amongst the glycerophospholipids are phosphatidic acid (PA), cardiolipin (diphosphatidylglycerol), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylinositol (PI). The polar base is the major factor distinguishing different phospholipids. The glycerolipids include monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol or plant sulfolipid (SQDG) (Fahy, Subramaniam *et al.*, 2009).

Phospholipids isolation and analyses are usually performed by thin-layer chromatography (TLC) systems (Zlatanov M., Ivanov S. *et al.*, 1999; Chunhieng 2008) and high-performance liquid chromatography (HPLC) (Becard, Chevalier *et al.*, 1999; Parcerisa, Codony *et al.*, 1999). The total quantity of phospholipids can also be measured by the estimation of the phosphorous amount in the sample (Chunhieng 2008).

Whilst data about the fatty acid composition of nuts is greatly available in the literature, information regarding their phospholipid content is scarce (Zlatanov M., Ivanov S. *et al.*, 1999; Amaral, Casal *et al.*, 2003; Ros e Mataix, 2006; Ryan, Galvin *et al.*, 2006; Venkatachalam e Sathe, 2006; Usda, 2009).

A study with Brazil nuts showed that the main phospholipids present in these seeds were PI, PC, PE, and PA. The fatty acids extracted from these phospholipids were also analysed and the main fatty acids found were 18:1, 16:0,

18:2 and 18:0 (Chunhieng 2008). Similar results were found in hazelnuts, that were demonstrated to be composed mostly by PC, PE, and PI (Parcerisa, Codony *et al.*, 1999).

7. Lipids and immune system

Lipids are common activators of the innate immune system (Podrez, Poliakov *et al.*, 2002; Konger, Marathe *et al.*, 2008). The interaction of lipids with immune cells leads to the formation and release of a large spectrum of inflammatory mediators which are essential for the early innate and subsequent adaptive immune reaction (Freudenberg, Tchaptchet *et al.*, 2008; Galli e Calder, 2009). In this vein, lipids have traditionally been thought of as adjuvants for Th1 responses, not being usually associated with allergic processes. The Th-1 stimulating activity is attributed to the activation of immune responses by the activation of naive T cells via the Toll-like receptor (TLR)4 pathway that traditionally activates APCs to produce the Th1 cytokines (Mazzoni e Segal, 2004) or by natural killer (NK) T cells and CD1 presentation (Faveeuw, Angeli *et al.*, 2002; Hansen, Siomos *et al.*, 2003). However, there are now solid evidences for the involvement of lipids in Th2-type responses, either induced by TLR or CD1-NKT pathways (Eisenbarth, Piggott *et al.*, 2002; Faveeuw, Angeli *et al.*, 2002; Delayre-Orthez, De Blay *et al.*, 2004; Agea, Russano *et al.*, 2005; Kim, Oh *et al.*, 2007).

7.1. Toll-like receptors (TLRs)

TLRs are evolutionarily conserved innate receptors expressed in various immune and non-immune cells of the mammalian host, able to recognize

molecular patterns, that when associated with pathogens play an important role in the innate immune responses to microbial pathogens (Akira, Yamamoto *et al.*, 2003; Kawai e Akira, 2010); and that when associated with endogenous molecules play a role in inflammatory and autoimmune diseases (Kawai e Akira, 2010). TLRs are also related with humoral and cellular adaptive immune responses (Iwasaki e Medzhitov, 2004).

Historically, the first evidence that TLRs were specific receptors participating in the development of immune responses was reported when a mutant *Drosophila* carrying loss-of-function mutations in the Toll receptor resulted in high susceptibility to fungi infection and the defective induction of an antifungal peptide (Lemaitre, Nicolas *et al.*, 1996; Hoffmann, 2003). The human homolog of the *Drosophila* Toll protein was subsequently described (Medzhitov, Preston-Hurlburt *et al.*, 1997).

TLRs1-9 are conserved between humans and mice. TLR10 is expressed in humans but not in mice, whereas TLRs11-13 are present in mice but not in humans. It is known that TLRs 1, 2, 4 and 6 are primarily located on the cell surface and recognize bacterial membrane lipids. TLRs 3, 7, 8 and 9 are generally located in the intracellular endossomes and primarily recognize pathogen-derived nucleotides. Specifically, TLR4 recognizes lipopolysaccharide (LPS), TLR2 binds lipoproteins and peptidoglycans (Takeuchi, Hoshino *et al.*, 1999), TLR1/2 and TLR1/6 heterodimers bind with lipopeptides and lipoproteins, TLR3 and TLR9 recognize double strand RNA (dsRNA), and TLR7 and TLR8 recognize single strand RNA (ssRNA) (Akira e Takeda, 2004).

TLRs contain extracellular leucine-rich repeats involved with pattern recognition, and the transmembrane and cytoplasmic Toll/interleukin-1 receptor (TIR) domains required for initiating intracellular signalling. The TLR-mediated

intracellular signalling is initiated by TIR-domain-dependent interactions with TIR-domain-containing cytosolic adapters such as myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (MAL), TIR domain containing adaptor inducing IFN-β (TRIF)/TIR-domain containing adaptor molecule (TICAM)-1 and TRIF-related adaptor molecule (TRAM), also known as TICAM2. A sequence of events will culminate in the activation of nuclear factor (NF)-kB and activating protein-1 (AP-1) and in the production of pro-inflammatory cytokines (Akira e Takeda, 2004).

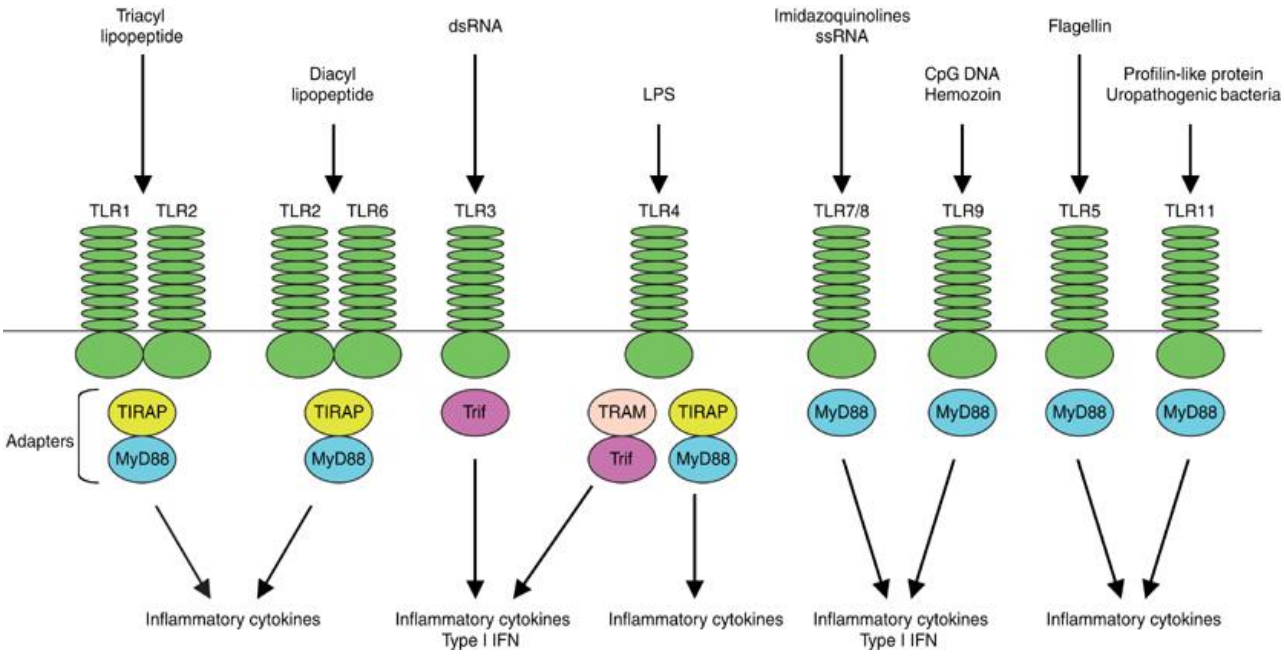


Figure 1.6. Representation of TLRs and their ligands (Kawai e Akira, 2006).

7.2. TLR and lipids

The TLRs reported to bind lipids are TLR1, TLR2, TLR4 and TLR6; however, the underlying mechanisms by which lipids activate the immune system through TLR is not completely understood. Crystallographic data for the structure of a synthetic triacylated lipopetide (Pam_3CSK_4) bound to TLR2 and

TLR1 has been published (Jin, Kim *et al.*, 2007); however, it is not clear how TLR2 and its co-receptors recognize the diverse set of physiological ligands with TLR2 agonist activity. For natural lipoproteins, it remains unknown how the peptide component or glycan component may influence signalling by TLR1, TLR2 and TLR6 (Jin, Kim *et al.*, 2007). TLR2 agonist activity can modulate APC functions, including cytokine production and antigen presentation; therefore, its activation influences the direction of an immune response.

TLR4 was the first described TLR (Lemaitre, Nicolas *et al.*, 1996; Medzhitov, Preston-Hurlburt *et al.*, 1997) and there is a plethora of studies available in this respect. TLR4 is essential for LPS recognition, which requires the formation of a protein complex containing accessory molecules. LPS is generally bound to LPS-binding protein (LBP) present in the serum and this complex is firstly recognized by CD14 receptor expressed in peripheral blood monocytes and macrophages. Once bound to CD14, LPS comes in close proximity with TLR4 and the secretion of myeloid differentiation (MD)-2 protein enhances the TLR4 signalling and triggers the inflammatory response (Hailman, Lichenstein *et al.*, 1994). TLR4 also recognizes other molecules from different origins, including some agonists derived from plants such as taxol, a strong antitumor agent in humans derived from *Taxus brevifolia* (Kawasaki, Akashi et al., 2000) as well as synthetic glycolipids (Johnson, 2008).

LPS is well-known for generating Th1 responses. However, under appropriate conditions it can also support differentiation into other T-helper lineages, demonstrating its pleiotropic nature (Mcaleer e Vella, 2008).

7.3. TLR4 and allergy

Evidence for the role of LPS in modulating Th2 immunity is robust, but the results obtained by different groups of investigators are still conflicting (Williams, Ownby *et al.*, 2005). While some epidemiological studies claim that endotoxin exposure during childhood protects against the development of asthma later in life (Von Mutius, Braun-Fahrlander *et al.*, 2000), other studies indicate that endotoxin exposure is a risk factor for asthma (Rizzo, Naspitz *et al.*, 1997; Thorne, Kulhankova *et al.*, 2005). Experimental data are also controversial, exposure to LPS in different animal models could attenuate (Hollingsworth, Whitehead *et al.*, 2006) or enhance allergic inflammation (Eisenbarth, Piggott *et al.*, 2002). It is speculated that the ability of LPS to down- or up-regulate Th2-mediated allergic responses depends on the route, concentration, timing and duration of LPS exposure (Tulic, Knight *et al.*, 2001; Eisenbarth, Piggott *et al.*, 2002; Delayre-Orthez, De Blay *et al.*, 2004; Hollingsworth, Whitehead *et al.*, 2006).

An interesting relationship between TLR4 and allergy has been recently reported using the house-dust-mite allergen, Der p 2. It was demonstrated that Der p 2 has structural and functional homology with MD-2, the LPS-binding component of the TLR4 signalling complex, facilitating signalling through direct interactions with the TLR4 complex and reconstituting LPS-driven TLR4 signalling in the absence of MD-2. Experimental allergic asthma was observed in MD-2-deficient, but not TLR4-deficient, mice sensitised to Der p 2 (Trompette, Divanovic *et al.*, 2009). MD-2 belongs to the MD-like superfamily of lipid-binding proteins and other members of this family are reported allergens (Ichikawa, Takai *et al.*, 2009). Furthermore, the most defined major allergens are thought to be lipid-binding proteins. Thus, it can be speculated that the intrinsic

adjuvant activity expressed by lipid-binding proteins may have some generality as a mechanism underlying the phenomenon of allergenicity.

In this vein, a phosporylcholine-containing glycoprotein secreted by filarial nematodes, called ES-62, is reported to directly interact with a number of cells of the immune system and this interaction appears to be dependent on complexing with TLR4 (Harnett e Harnett, 2009). It has been shown that ES-62 can inhibit pro-inflammatory/Th1 immune responses and to actively support Th2 development (Harnett e Harnett, 2006). In mice, ES-62 induces the production of IgG1 rather than IgG2a antibodies, promotes the production of IL-10, and reduces levels of IL-12, IFN-γ and pro-inflammatory cytokines (Houston, Wilson *et al.*, 2000). The immunomodulatory activity of ES-62 appears to be largely due to the presence of phosphorylcholine moieties covalently attached to N-type glycans (Harnett e Harnett, 2009). Moreover, it was shown that in schistosome infections, schistosomal but not synthetic lyso-phosphatidylserine activated TLR2, and induced IL-10-producing regulatory T cells (Van Der Kleij, Latz *et al.*, 2002).

Therefore, although the underlying mechanisms by which lipid molecules can interact with the innate and adaptive immune system are still unknown, it is clear they are important in the development of distinct immune responses.

7.4. CD1 and allergy

An additional pathway by which lipids can be recognized by the immune system is given by means of CD1. The CD1 proteins share sequence homology and overall domain structure with MHC class I molecules, being

comprised of a heavy chain with three extracellular domains that are non-covalently associated with β2-microglobulin. Similar to MHC class I molecules, CD1 heavy chains consist of α1 and α2 domains that form the antigen-binding region, contained within two antiparallel α-helical structures that are situated on a β-folded sheet (Bauer, Huttinger *et al.*, 1997; Zeng, Castano *et al.*, 1997; Porcelli e Modlin, 1999). The α1 and α2 antigen-binding region is linked to an immunoglobulin-like α3 domain, which is attached to the membrane by a transmembrane segment, followed by a short cytoplasmic tail. However, differently from MHC class I molecules, CD1 proteins bind alkyl chains in hydrophobic channels that reside beneath the surface of CD1 molecules, whereas the hydrophilic head groups of lipid antigens protrude where the hydrophobic channels open to the membrane distal surface of the CD1 molecule. These head moieties are stabilized by hydrogen bonds, which also contribute to the correct positioning of the lipid antigens. CD1 proteins have deeper and more voluminous antigen-bind compartments than MHC class I molecules (Zeng, Castano *et al.*, 1997; Porcelli e Modlin, 1999).

Five isoforms of CD1 molecules have been described: CD1a, CD1b, CD1c, CD1d and CD1e. Humans express all CD1 isoforms whereas rodents express only CD1d. CD1a, CD1b and CD1c present lipid antigens to clonally diverse T cells while CD1d molecules present lipid antigens to natural killer (NK) T cells. NKT cells belong to a novel lymphoid lineage distinct from T cells, B cells or NK cells, and they are characterized by the expression of a single invariant antigen receptor encoded by Vα14 and Jα281 segments paired with a diverse set of TCR Vβ-chains, mainly Vβ8.2 (Godfrey, Macdonald *et al.*, 2004; Kronenberg, 2005). Therefore, Vα14 NKT cells are a distinct lineage of αβ T

cells. The invariant chain in humans is Vα24-Jα18 and in mouse is Vα14-Jα18 (Godfrey, Macdonald *et al.*, 2004; Borg, Wun *et al.*, 2007). The invariant Vα14/Vβ8.2 receptor is not expressed on conventional T cells and its expression is essential for the development of Vα14 NKT cells. In fact, the deletion of the Jα281 gene segment results in the selective loss of NKT cell development (NKT-deficient mice) (Cui, Shin *et al.*, 1997).

Lipid stimulants of CD1-restricted T cells can be derived from mammalian cells (self lipids) or foreign sources, presenting considerable structural diversity. Examples of foreign lipids able to be recognized by NKT cells are diacylglycerols, sphingolipids, phospholipids and lipoproteins. Self lipids recognizable by NKT are gangliosides, phosphatidylinositols, phosphatidylethanolamines, phosphatidylcholine and others (Moody, 2006). A-galactosylceramide (α-GalCer) is the most potent specific agent for activating $V\alpha14^+$ CD1d-dependent NK T cells. A-GalCer is obtained from the marine sponge *Agelas mauritanius* and it was initially identified in a screen for agents that would prevent metastases of tumors to the livers of mice (Morita, Motoki *et al.*, 1995). The crystal structure of the TCR-α-GalCer-CD1d ternary complex was elucidated and the TCR of the NKT cells contact the protruding head group of α-GalCer and the CD1 α-helices. The footprint of the NKT-cell TCR on the surface of the α-GalCer-CD1d complex is parallel to the long axis of the Cd1 binding groove and positioned in the extreme end of CD1d (Borg, Wun *et al.*, 2007).

CD1d-restricted invariant (*i*) NKT cells represent about 1-3% of the normal splenic population and they comprise 10-20% more of the total T cell population in the liver (Matsuda, Gapin *et al.*, 2002). Resting NKT cells have a

memory or partially activated phenotype and respond rapidly following TCR stimulation to produce cytokines, such as interferon-γ (IFN-γ) and IL-4. Once activated *i*NKT cells rapidly stimulate DCs, macrophages and NK cells, and recruit neutrophils to expand the immediate innate immune response and have an impact on the subsequent adaptive T-cell and B-cell responses (Barral e Brenner, 2007).

Vα14i NKT cells have been implicated in both the progression and the resolution of diverse animal models of disease. This depends on the cytokines produced by NKT cells and by other cell types stimulated downstream of NKT activation. Th2 cytokines, such as IL-4 and IL-13, have a palliative effect on several models of autoimmune diseases, but they are essential for the pathogenesis of allergic disease. Th1 cytokines, such as IFN-γ, aggravate autoimmunity and inflammatory disease models, but they are necessary for the clearance of infections and the prevention of tumour metastases. Possible mechanisms involved in the polarization of NKT cells in order to produce preferentially Th1 (IFN-γ) or Th2 (IL-4) cytokines are still unknown. Signals such as antigen doses, IL-4, or IL12, involved in the polarization of Th cells did not affect the production of IL-4 or IFN-γ by NK T cells stimulated with α-GalCer (Matsuda, Gapin *et al.*, 2003).

However, it has been reported that structural variants of α-GalCer can induce a systemic polarization of cytokine production initiated by NKT cells. OCH, an analogue of α-GalCer with a sphingosine base reduced from 18 to 9 carbons and an acyl chain reduced by two carbons, was reported to be Th2 polarizing (Miyamoto, Miyake *et al.*, 2001). Contrastingly, the C-glycoside analog of α-GalCer has been reported to have a Th1 polarizing effect (Grajewski,

Hansen *et al.*, 2008). There are indications that the cytokine polarization of NK T is related with the binding stability of the lipid with CD1d as well as with the TCR affinity. Induction of IFN-γ secretion by NKT cells would require a longer period of stimulation and/or a higher antigen concentration while Th2-cytokines would be produced under decreased duration of glycolipid stimulation or decreased TCR affinity (Stanic, Shashidharamurthy *et al.*, 2003; Oki, Chiba *et al.*, 2004). It has been suggested that the pharmacokinetic properties of glycolipids are a major determinant of cytokine skewing (Sullivan, Nagarajan *et al.*, 2010).

The secretion of high concentrations of IL-4 by lipid-stimulated NKT cells has been implicated with the participation of these cells in the differentiation of Th2 cells (Yoshimoto, Bendelac *et al.*, 1995; Leite-De-Moraes, Moreau *et al.*, 1998; Lisbonne, Diem *et al.*, 2003). In an experimental model of allergic asthma using OVA as antigen, it has been demonstrated that the invariant Vα14 NKT cell subset is required for airway eosinophilia, hyperresponsiveness, Th2 cytokine production, and elevated levels of IgE antibodies (Lisbonne, Diem *et al.*, 2003). Other authors have reported that pollen allergic, but not normal subjects displayed circulating specific IgE and cutaneous reactions to phospholipids present in pollen (Agea, Russano *et al.*, 2005). In addition, confocal microscopy revealed that the whole pollen is ingested within a few minutes, and outer membranes are progressively concentrated and digested within DCs cytoplasmic vesicles (Agea, Russano *et al.*, 2005). These evidences support the participation of CD1 molecules in the presentation of lipid-antigens and its relation with allergic processes.

CD1 is prominently expressed on cells involved in antigen presentation (APCs), binding distinct lipid-based antigens. The presentation of antigens by CD1 proteins usually requires uptake and intracellular processing by APCs.

Different mechanisms are described for the uptake of lipids, such as phagocytosis, C-type lectins that can bind mannose residues on glycolipids and internalization through scavenger receptors that can bind modified forms of low-density lipoproteins (Barral e Brenner, 2007).

The present work aimed to investigate the role that natural lipids from Brazil nuts play in the sensitisation to Ber e 1, the major allergen in Brazil nuts. Lipids were extracted, fractionated, and tested in an *in vivo* model of sensitisation to rBer e1. The mechanism(s) involved in the immune response induced by the lipid-protein complex was also investigated. The results of the experiments performed to address these objectives are presented and discussed in the following chapters.

CHAPTER 2

MATERIAL AND METHODS

1. Nuts/seeds

Brazil nuts were purchased from a commercial supplier (Holland and Barrett, origin from Colombia). Sunflower seeds were purchased at local supermarket (Sainsbury's, UK, under the commercial name of 'CrazyJack').

2. Total lipid extraction

In order to extract lipids from seeds/nuts, a "Folch" standard protocol for total lipid extraction was performed (Folch, Lees *et al.*, 1957). Essentially, samples of seeds (70 g for Brazil nuts or 84 g for sunflower seeds) were macerated in 150 mL of chloroform : methanol (2:1) (Analytical grade, Fisher, UK). Seeds were ground in solvent solution using porcelain pestle and mortar. The paste obtained was filtered in filter paper (Whatman, UK) and dissolved in (100 mL) chloroform. This solution was again filtered and the dry paste obtained was discarded. The (250 mL) solution of chloroform and methanol (4:1) containing the total fraction of lipids present in the seeds was named fraction T and it was stored at -20ºC for further lipid fractionations and analyses.

3. Lipid fractionation

Once the total lipids were extracted, 4 procedures for fractionation were attempted:

3.1. Fractionation by Thin-layer Chromatography (TLC)

TLC was performed for preparative and analytical purposes following standard procedures (Christie, 2003). For the one dimensional preparative scale,

samples of total lipids (sample T above) were evaporated under vacuum in a rotator evaporator (Rotavapor R-3000, Buchi, UK) to eliminate the excess of solvent. The concentrated lipid samples were re-suspended in Chloroform : Methanol (2:1) and applied (2 cm from the border) to several silica gel TLC plates (150Å, 20cm x 20 cm glass plate and 250 μm thickness, Whatman, UK). The plates were firstly run all the way to the top in a saturated chamber containing chloroform only. The plates were then removed from the chloroform chamber, air dried, and placed in the same orientation in a second chamber containing chloroform, methanol, acetic acid and water in the proportion 50 : 37.2 : 3.5 : 2 (v/v) (Christie, 2003) and run until the top. After air dried, the plates were exposed to iodine vapour for the general detection of fatty acid containing products (Christie, 2003). The yellowish bands were identified and individually removed from the plates by scraping the silica gel. The mixtures containing different bands of lipids and silica gel extracted from the plates were individually dissolved in chloroform and methanol (2:1) and the silica gel was removed by paper filtration (Whatman, UK). Final lipid fractions dissolved in chloroform and methanol solution were evaporated under vacuum in a rotator evaporator to eliminate the excess of solvent and they were stored in clean and glass tubes containing small volumes of methanol, at -20°C.

3.2. Fractionation by Solid phase chromatography (SPC)

The SPC column was packed using slurry of silica gel (35 g) (230-400 mesh, Fluka, Germany) in chloroform onto a clean glass column (3 x 20 cm). Avoiding the introduction of air bubbles, the solid phase (silica gel) was successively washed with (150 mL) chloroform : methanol (2:1), (150 mL)

methanol and (300 mL) of chloroform under gravity. The washed column was loaded with the fraction T, obtained by "Folch" extraction (250 mL), dissolved in chloroform and methanol, 4:1, and the flow through fraction was named SPC fraction L. The subsequent fractions were:

(1) (150 mL) chloroform and methanol (2:1) (SPC fraction C);

(2) (500 mL) methanol (SPC fraction M);

(3) (800 mL) chloroform and acetic acid (9:1) (SPC fraction C:A);

(4) (100 mL) water and acetic acid (9:1) (SPC fraction W:A);

(5) (400 mL) methanol and acetic acid (9:1) (SPC fraction M:A).

The samples were concentrated under vacuum conditions in a rota evaporator and in order to minimize oxidation, a solution containing 1 mL of chloroform:methanol (2:1) and 200 μL of anti-oxidant Butylated hydroxytoluene (BHT) 0.01% (m/v) were added to the lipids extracted. These samples were stored protected from light at -20°C.

3.3. Fractionation by High-Performance Liquid Chromatography (HPLC)

A Perklin Elmer Series HPLC was used with a Diode Array detector (UV-detector). The flow-rate nebulizer gas was 0.8 mL/min. A column of YMC-Pack PVA-SIL-NP, S-5um, 12nm (100 x 4.9mm id) was obtained from TMC Europe GmbH. A ternary gradient elution scheme was required consisting of *iso*hexane : methyltertbutyl ether (49 : 1, v/v; solvent A) and propa*n-2*-ol : acetonitrile : butan-2-one (7 : 2 : 1, v/v/v; solvent B). The column temperature was maintained at 30°C (Christie Ww, 1998). The following gradient elution program was utilized (Table 2.1.):

Step	Time (min)	Flow (mL/min)	Solvent A	Solvent B
0	5	0.8	100	0
1	5	0.8	100	0
2	10	0.8	80	20
3	5	0.8	70	30
4	10	0.8	0	100
5	5	0.8	0	100
6	5	0.8	100	0
7	5	0.8	100	0

Table 2.1.: Gradient elution used for HPLC.

The SPC lipid fraction C described above extracted from Brazil nuts was run in the PE-HPLC system and samples were collected every 2 min for 45 min; thus, 22 samples were obtained (HPLC fraction C 1 to HPLC fraction C 22).Several runs of HPLC were performed in order to collect enough material of sub-fractions. The 22 sub-fractions were individually collected and the excess solvent was evaporated under vacuum conditions in a rota evaporator. The lipid samples were then stored in glass tubes containing (1 mL) chloroform and methanol (2:1) and (200 μL) of BHT (0.01%, m/v). The samples were stored protected from light at -20ºC.

3.4. Fractionation by acetone precipitation

Aliquots of (600 μL) of lipid samples were dissolved in (13 mL) acetone, mixed and centrifuged at 1,300 rpm (Mistral, UK) for 4 min at room temperature (Kates e Kushwaha, 1995). The supernatant, containing simple lipids (neutral and non-polar lipids), was evaporated under vacuum conditions in a rota-evaporator. The pellet, containing mainly complex lipids (polar lipids), and the concentrated supernatant, containing mainly simple lipids (neutral and non-polar lipids), were re-suspended in (900 μL) chloroform : methanol (2:1), and (200 μL), BHT (0.01%, m/v) and stored in glass tube protected from light at -20°C.

4. Lipid analysis

4.1. TLC developing systems

4.1.1. One-dimension TLC: single run

Samples of different lipid fractions were applied to TLC plates containing silica gel (aluminum 60 Å plates, thickness of 200 μm, Alumgram Sil G, Whatman, UK). The mobile phase was chloroform, methanol, acetic acid and water (50 : 37.2 : 3.5 : 2) (v/v) (Christie, 2003).

4.1.2. One-dimension TLC: multiple runs

Four multiple run systems were employed using silica gel TLC plates (aluminum 60 Å plates, thickness of 200 μm, Alumgram Sil G, Whatman, UK). (a) Two runs in a chamber containing chloroform, methanol, acetic acid and water (50 : 37.2 : 3.5 : 2) (v/v). (b) One run in a chamber containing chloroform and a second run in chloroform, methanol, acetic acid and water (50 : 37.2 : 3.5 : 2). (c) The plates run 6 times in chloroform, 1 time in chloroform, methanol, and water (75 : 25 : 25), and 2 times in chloroform, methanol, acetic acid and water (80 : 9 : 12 : 2) (Christie, 2003), (d) Two runs in the same direction, until the solvent reached the first half of the plate, in a chamber containing chloroform, methanol and water (60 : 30 : 5). And a third run in the complete plate in a chamber containing hexane, diethyl ether and acetic acid (80 : 20 : 1.5) (Kupke e Zeugner, 1978).

4.1.3. Two-dimension TLC

For identification and quantification purposes, a sample of the total lipids from Brazil nuts, fraction T was spotted in TLC plate (aluminum 60 Å plates, thickness of 200 μm, Whatman, UK). The plate was developed firstly with six runs in the same direction, in a chamber containing chloroform to remove nonpolar lipids. The next run was again in the same direction, but in a chamber containing chloroform, methanol and water (75 : 25 : 2.5). Once air dried, the plate was turned 90º to the right and two runs were performed in a chamber containing chloroform, methanol, acetic acid and water (80 : 9 : 12 : 2) (Christie, 2003).

4.1.4. Detection of lipid bands

In order to identify lipid samples separated by TLC systems, the plates were submitted to different treatments: a) exposure to iodine vapour (Iodine, Sigma, UK), which is a non-destructive technique that identify mainly unsaturated fatty acids; b) sprayed with Ninhydrin (Nynhidrin fixer spray reagent, Sigma, UK), kept at 100°C and 100% humidity; which stains lipids by binding to the amino groups; c) sprayed with Molybdenum (1.3% molybdenum blue and 4.2M sulphuric acid – Sigma, UK) for detection of phosphate groups, or d) charred in oven at 200°C, for 15 minutes in order to identify any organic compounds present in the plate, turning them brown.

4.1.5. Quantification of lipid classes identified by TLC

Lipid classes detected by TLC were quantified by scanning the TLC plate in a densitometer (Bio-Rad, UK). The density, given by the optical density (OD)/mm2, and the area (mm2) occupied by each spot were quantified by a

dedicated software (Fluor S-Multimager). The results expressed the estimated percentage of different lipid classes in the sample used in the TLC plate.

5. Phospholipids estimation

The concentration of phospholipids present in different lipid fractions from Brazil nuts was estimated by determining the amount of phosphorus present in the fraction (Christie, 2003). Samples of the different fractions of lipids were dried under nitrogen gas (BOC, UK). Samples were placed in glass tubes and digested with concentrated perchloric acid (98% purity, Sigma-Aldrich, UK) by gentle refluxing in an air condenser for 2-3 hours on a heating block at 200-300° C in a fume cupboard. When the solutions were clear and cooled, (400 μL) ammonium molybdate reagent (4.4 g/L of 1.4% sulphuric acid (v/v)), (200 μL) reducing reagent (sodium bisulphate (2.5 g), sodium sulphate (0.5 g) and amino-naphtol sulphonic acid (0.042 g) dissolved in 250 mL of water); and water (4 mL) were added. The samples were read in spectrophotometer at 700 nm every 30 minutes for 2 hours. The amount of phosphorus in the samples was read from a calibration curve, prepared at the same time by performing the reaction on known amounts (4 to 0.015 mM) of a standard solution of NaH_2PO_4.

6. Animals

6.1. Animals at the University of Nottingham

Animals kept in our facilities were used for sensitisation protocols (Chapters 4 and 5) and PCA technique (Chapters 4, 5 and 6). Young adult (8–12 weeks old) female BALB/c strain mice used throughout these studies were obtained from Harlan Seralab, Oxfordshire, UK or bred in our animal facilities.

Food and water were available *ad libitum*. Rodent maintenance diet was used (2018 Tecklad Global, 18% protein rodent diet, Harlan, UK). The diet composition includes wheat, corn, soybean, corn gluten, soybean oil, brewer yeast, vitamins and minerals. Importantly, the diet does not contain any traces of Brazil nuts or sunflower seeds. The ambient temperature was maintained at 21±2°C and relative humidity was 55 ± 10% with a 12 h light/dark cycle. All experiments were carried out under a Home Office Licence, following the guidelines of the UK Animals (Scientific Procedures) Act, 1986.

6.2. Animals at the Hospital Necker, Paris

The experiments with animals to study NKT activation were performed in the Centre National de la Recherche Scientifique (CNRS), Unite´ Mixte de Recherche 8147, Hospital Necker, Paris, France, under supervision of Prof. Maria Leite-de-Moraes.

6.2.1. BALB/c wild type mice

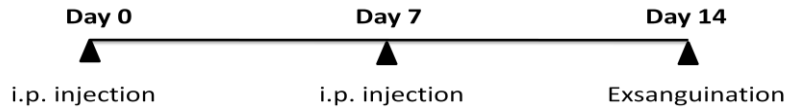
Young adult (8–12 weeks old) male BALB/c mice were bred in the facilities of Centre National de la Recherche Scientifique (CNRS), Unite´ Mixte de Recherche 8147, Hospital Necker, Paris, France. Animals were kept in well-controlled animal housing facilities and had free access to tap water and pellet food throughout the experimental period. The animal diet did not contain Brazil nuts.

6.2.2. C57BL/6 wild type and Jα18 knockout mice

Young adult (8–12 weeks old) male C57BL/6 wild-type (WT) and Jα18 -/- were bred in the facilities of Centre National de la Recherche Scientifique

(CNRS), Unite´ Mixte de Recherche 8147, Hospital Necker, Paris, France. Animals were kept in well-controlled animal housing facilities and had free access to tap water and pellet food throughout the experimental period. The animal diet did not contain Brazil nuts.

7. Sensitisation protocols



Sensitizing protocols consisted in two subsequent intra-peritoneal (i.p.) injections of protein solutions on days 0 and 7. Protein solutions' details are exposed in the following sections (Items 7.1. and 7.2). Animals were bled fourteen days after the first sensitisation injection (day 14). The exsanguinations were performed by intra-cardiac route under lethal doses of pentobarbital sodium, approximately 50 µL per animal (Dolethal® injection, 200 mg/mL, Vetoquinol, UK). An average volume of 600 µL of blood was collected in 1.5 mL plastic vials (Standard Micro Test Tube, Eppendorf, UK), stored at 4°C overnight and centrifuged at 8000 rpm for 4 min (Sanyo/MSE Hawk 15/05, UK). An average of 100 µL of serum was recovered from each blood sample and 25 µL aliquots were stored in 0.5 mL plastic vials (Standard Micro Test Tube, Eppendorf, UK) at -80°C.

7.1. OVA sensitisation

BALB/c mice received two subsequent intra-peritoneal (i.p.) injections of solutions containing 2.5 mg of Grade V chicken egg OVA (Sigma, UK) in 200 µL of phosphate buffered saline (PBS; composed by 8 g of NaCl, 0.2 g of KCl,

1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 1 L of distilled water) on days 0 and 7. Animals were bled fourteen days after the first sensitisation injection (day 14). Serum collection and storage were performed as described above (Item 7).

7.2. BER-lipid -sensitisation

BALB/c mice received two subsequent intra-peritoneal (i.p.) injections of solutions containing 2.5 mg of rBer e 1 and 600 μg of different lipid fractions in 200 μL of PBS, on days 0 and 7. Details of BER-lipid solution preparation are given below (Item 7.2.1). Animals were bled fourteen days after the first sensitisation injection (day 14). Serum collection and storage were performed as described above (Item 7).

7.2.1. Preparation of BER-lipid sensitisation solution

Ber experimental groups were sensitised with solutions containing 2.5 mg of protein and 600 μg lipid fraction C. Protein solution was prepared dissolving lyophilised rBer e 1 in deionized water to a concentration of 16 mg/mL. The appropriate amount of lipids stored in chloroform and methanol (2:1) solution was evaporated under nitrogen gas (Nitrogen compressed gas, BOC, UK) and ressuspended in methanol. The volume of methanol used to dissolve the lipids was equivalent to 20% of the protein solution used. Lipid solution was dropwise added to the protein solution and mixed thoroughly in order to avoid protein precipitation. All procedures were performed in cleaned and defatted glass tubes to avoid lipid contamination and lipid losses. The final lipid-protein solution was freeze-dried and ressuspended in PBS adjusted to a final volume of 200 μL/animal. For control groups, the solution was prepared as

described before, but no lipids were used, methanol alone was added to the protein solution.

7.3. Ber-LPS sensitisation

Lipopolysaccharide (LPS) from *Escherichia coli* (0111:B4; Sigma, UK) diluted in PBS was added to Ber e 1. The dose of protein used was 2.5 mg and the doses of LPS ranged from 0.06 to 150 μg per animal, per sensitisation. The doses used are specified in each experiment, Results and Discussion section, Chapter 5.

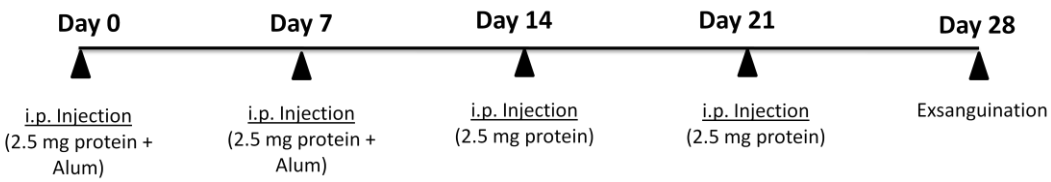
7.4. Sensitisation using Alum

Two different sensitisation protocols were performed using alum (AlOH3) (Imject alum, Thermo Scientific Pierce, UK) as adjuvant.

7.4.1. Alum protocol 1

BALB/c mice received two i.p. injections containing 2.5 mg of protein (rBer e 1 or OVA) and 2 mg of alum, diluted in 200 μL of PBS on days 0 and 7. Animals were bled on day 14. Serum collection and storage were performed as described above (Item 7).

7.4.2. Alum protocol 2



BALB/c mice received two i.p. injections containing 2.5 mg of protein (rBer e 1 or OVA) and 2 mg of Alum in 200 μL of PBS on days 0 and 7. Then, animals received two additional i.p. injections containing of 2.5 mg of protein (Ber e 1 or OVA) on days 14 and 21. Animals were bled on day 28. Serum collection and storage were performed as described above (Item 7).

7.5. SFA-sensitisation

BALB/c mice received two subsequent intra-peritoneal (i.p.) injections of solutions containing 2.5 mg of SFA-8 and 600 μg of different lipid fractions in 200 μL of PBS, on days 0 and 7. Animals were bled fourteen days after the first sensitisation injection (day 14). Serum collection and storage were performed as described above (Item 7).

8. PCA

Individual and/or pooled serum samples (25 μL) were injected into the dermis of the ears of naïve recipient mice using 30 gauge needles (Microlance needles, BD, UK) and 50 μL glass syringes (Microlitre glass syringe, Luer tip, Hamilton, UK). To facilitate the intra-dermal injection and avoid any animal suffering, animals were anesthetised with a mixture containing Ketamine (100 mg/kg, Ketaset® Injection, Fort Dodge, UK) and Acepromazine (2.5 mg/kg) intra-peritoneally. The anesthetic solution was prepared mixing 1 mL of ketamine, 1.25 mL of acepromazine and 3.875 mL of distilled water. The final volume injected was 0.1 mL/20 g of animal. The intra-dermal injection was performed as soon as the animal showed signs of adequate sedation. Animals were kept in warm environment (around 30°C) until the total recover from the

anesthesia). Two days later a protein solution containing dye was injected intravenously by tail veins. The protein solution was composed by 0.25 mg of relevant protein (OVA or BER) + Evans blue dye (1.25 mg) diluted in 0.25 mL of physiological saline solution (0.9% m/v, NaCl). The intravenous injection was also conducted under anesthesia using a mixture containing Ketamine (100 mg/kg) and Medetomidine (1 mg/kg, Dormitor®, Pfizer, UK). The anesthetic solution was prepared mixing 0.75 mL of ketamine, 1 mL of medetomidine and 3.825 mL of distilled water. The final volume injected was 0.1 mL/20 g of animal. The intravenous challenge injection was performed when the animal presented signs of deep anesthesia, i.e. absence of reflexes, absence of motor response to noxious stimuli, increase in depth and decrease in respiration rate. Animals were kept in a heating box at 39°C for thirty minutes following the challenge and then culled. The diameter of cutaneous reactions present on the ear lobe was measured in millimeters with a Vernier caliper (Absolute Digimatic caliper, Mitutoyo, USA). Diameters higher than 3 mm were considered as positive reactions.

8.1. Heating treatment

Specific anti-OVA IgE (Serotec, UK) was added to serum from naïve animals at 25,600 ng/mL and serially diluted in serum from naïve animals until reaching the concentration of 10 ng/mL. Pooled sera from OVA-sensitised animals were serially diluted in serum from naïve animals ranging from neat to 1/64. Aliquots of 50 µL of all serum samples were incubated in 0.2 mL PCR tubes (PCR tubes, Eppendorf, UK) and heated at 56ºC in a PCR machine (GeneAmp 2400 Applied Biosystem, UK) for 1 hour; following the protocol

previously described (Faquim-Mauro, Coffman *et al.*, 1999). Control serum samples were kept at 4ºC. These samples were tested in mice for PCA reactions (described in Item 8).

8.2. Protein G adsorption

For removal of IgG, equal volumes of protein G-agarose (Calbiochem®, Merck Biosciences, UK) and mouse serum were employed. This procedure was adapted from the literature (Lehrer, Reish *et al.*, 2004) and performed with pooled serum from OVA-sensitised mice and with serum from naive mice containing 6,400 ng/mL of commercial anti-OVA IgE (Serotec, UK). For this, 300 μL of neat OVA-sensitised mice serum was mixed with 300 μL of protein G in 1.5 mL vial (Standard Micro Test Tube, Eppendorf, UK). In another vial, 300 μL of serum containing commercial anti-OVA IgE (6.4 μg/mL) was mixed with 300 μL of protein G. The sample vials were incubated for 30 min at room temperature, centrifuged at 3000 g (Sanyo/MSE Hawk 15/05, UK) for 2 min and the supernatants were collected. The supernatant from OVA-sensitised mice serum sample was further diluted 8 times in serum from naive animals (1/8 diluted serum). The supernatant from serum containing commercial anti-OVA IgE was diluted in serum from naive mice to reach the concentrations of 1,600 and 400 ng/mL. These samples were tested in mice for PCA reactions (described in Item 8).

8.3. PCA time course

PCA procedure described above (Item 8) was performed in a group of 10 naïve recipient mice with serum from OVA-sensitised animals and a different

group of 10 animals with serum containing only commercial anti-OVA IgE (3.2 μg/mL). The challenge, i.e., the intra venous injection of OVA and Evans' blue was performed 2, 3, 5, 7 or 12 days after the i.d. serum injection, using 2 animals for each challenge.

9. NKT activation protocols

9.1. NKT activation protocol 1

9.1.1. Animal procedure

At T=0h, BALB/c mice received (i.p.) 2.5 mg of rBer e 1 and 600 μg of SPC fraction C (n=3) or SPC fraction M (n=3) extracted from Brazil nuts lipids. A positive control group received 2 μg of α-GalCer (Kirin Brewery Co., Japan) (n=2) and two naive mice were used as negative control. Two hours later (T=2h) animals were euthanized and the spleens were removed for flow cytometry analyses (FACs).

9.1.2. Splenocytes preparation

Spleens promptly removed from mice were kept in individual tubes (50 mL Falcon Test Tubes, BD, France) containing 15 mL of 2% of foetal calf serum (FCS) (Sterile FCS, BD) in PBS. Spleens were smashed in cell strainers (Cell strainer, 70 μm, BD). And the cell suspensions obtained after straining were centrifuged at 1,200 rpm, at 4ºC for 5 min. The supernatant was discarded and

500 μL of FCS was added to each sample and mixed thoroughly. 2 mL of red blood cells lysing solution (Pharm Lyse lysing buffer, BD, France) was added to the samples, 2 min later 15 mL of PBS containing 2% of FCS was added to the tubes and they were centrifuged at 1,200 rpm, at 4ºC for 5 min. The supernatant was discarded and the cells resuspended with 2 mL of PBS, 2% FCS. Samples of cells suspensions were diluted 1/10 and 1/100 in Trypan Blue solution (Trypan Blue solution, 0.4%, Sigma, France) and the viable cells (not blue) were counted in a haemocytometer glass chamber. Two aliquots of 4 x 10^6 cells from each animal were prepared for the extracellular staining and two aliquots of 6 x 10^6 cells from each animal were prepared for intracellular staining. Individual cell aliquots were stored in 1.5 mL plastic vials (Standard Micro Test Tube, Eppendorf, France) at 4ºC.

9.1.3. Cell staining

9.1.3.1. Surface staining

TCR, CD8, CD4, CD69 labelling

One aliquot of 4 x 10^6 cells from each animal was centrifuged at 1,200 rpm, at 4ºC for 5 min and the supernatant was discarded. Cells suspensions were incubated for 30 min at 4ºC with tetrameter CD1d-α-GalCer-biotinylated (NHI Tetramer facilities, France) and anti-biotin-APC (Allophycocyanin) antibodies (BD, Pharmingen, France). Cells suspensions were washed in cold staining buffer (2% FCS, 0.1% Sodium Azide in PBS), centrifuged at 1,200 rpm, at 4ºC for 5 min and the supernatant was discarded. Cells suspensions were then incubated for 30 min at 4ºC with a mixture containing anti-TCR-FITC (Fluorescein isothiocyanate) antibodies (BD, Pharmingen, France), anti-CD8-PB (Pacific Blue)

antibodies (BD, Pharmingen), anti-CD4-APC H7 antibodies (eBiosciences, France) and anti-CD69-PE (Phycoerythrin) antibodies (BD, Pharmingen, France) diluted in staining buffer. The cells were washed with cold staining buffer, centrifuged, and the supernatant was discarded. Cells were then fixed with 600 μL of 4% paraformaldehyde PBS solution. Cells were again washed with staining buffer, centrifuged, and the supernatant was discarded. Cells were re-suspended in 800 μL of FACSCanto II flow cytometry buffer (BD, Pharmingen, France).

TCR, Dx5, and CD24 labelling

Another aliquot of 4 x 10^6 cells from each animal was centrifuged at 1,200 rpm, at 4ºC for 5 min and the supernatant was discarded. Cells suspensions were incubated for 30 min at 4ºC with a mixture containing anti-Dx5-FITC antibodies (BD, Pharmingen, France), anti-CD24-PB (BD, Pharmingen, France) and anti-TCR-APC (Pharmingen, France). Cells suspensions were washed with cold staining buffer and again centrifuged. The supernatant was discarded and the cells were fixed with 600 μL of 4% paraformaldeid PBS solution. Cells were washed with cold staining buffer, centrifuged and the supernatant was discarded. Cells were re-suspended in 800 μL of FACSCanto II flow cytometry buffer (BD, Pharmingen, France).

9.1.3.2. Intracellular staining

Two aliquots of 6 x 10^6 cells from each animal were centrifuged at 1,200 rpm, at 4ºC for 5 min and the supernatant was discarded. Cells suspensions were first incubated for 30 min at 4ºC with surface tetrameter CD1d-α-GalCer-biotinylated (NHI Tetramer facilities, France) and anti-biotin-APC antibody (BD, Pharmingen, France). Cells were washed in cold staining buffer, centrifuged and the supernatant was discarded. Cells suspensions were then incubated for 30 min

at 4ºC with a mixture containing anti-TCR-FITC (BD, Pharmingen, France) and anti-CD4-PB (eBiosciences, France).

IL-4 and IFN-γ labelling

To intracellular staining, one aliquot of cells suspension was washed with cold staining buffer and again centrifuged. The supernatant was discarded and cells were permeabilized and fixed with 900 μL of 4% paraformaldehyde + 0.5% saponin PBS solution (Sigma-Aldrich, France) for 30 min at room temperature. Cells suspensions were centrifuged, the supernatant was discarded and the cells were washed. The cells were then incubated for 45 min at 4ºC with a mixture of anti-IL-4-PECy7 antibodies (eBiosciences, France) and anti-IFNγ-PE antibodies (BD, Pharmingen, France) in PBS 0.5% saponin buffer (Sigma-Aldrich, France). Cells suspensions were washed with PBS 0.5% saponin buffer (Sigma-Aldrich, France) and again centrifuged. The supernatant was discarded and cells were fixed with 900 μL of 4% paraformaldeid PBS solution. Cells were washed with cold staining buffer, centrifuged, and the supernatant was discarded. Cells were re-suspended in 1,200 μL of FACSCanto II flow cytometry buffer (BD, Pharmingen, France).

Control antibodies (Ig) labelling

To cellular staining, another aliquot of cells suspension was washed with cold staining buffer and again centrifuged. The supernatant was discarded and cells were permeabilized and fixed with 900 μL of 4% paraformaldehyde + 0.5% saponin PBS solution. Cells suspensions were centrifuged, the supernatant was discarded and the cells were washed. The cells were then incubated for 45 min at 4ºC with isotype control antibodies: anti-Ig-PECy7 (BD, Pharmingen, France) and anti-Ig-PE (BD, Pharmingen, France) in PBS 0.5% saponin buffer (Sigma-Aldrich, France). Cells suspensions were washed with PBS 0.5% saponin buffer

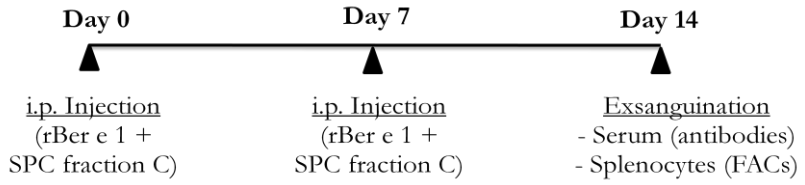
(Sigma-Aldrich, France) and again centrifuged. The supernatant was discarded and cells were fixed with 900 μL of 4% paraformaldeid PBS solution. Cells were washed with cold staining buffer, centrifuged, and the supernatant was discarded. Cells were re-suspended in 1,200 μL of FACSCanto II flow cytometry buffer (BD, Pharmingen, France).

9.1.4. Flow cytometry analyses

Stained cells were acquired in a flow cytometric FACSCanto II (Becton Dickinson, BD, Pharmingen, France) and data analyzed using FlowJo software (Flow Cytometry Analysis Software, USA).

9.2. NKT activation protocol 2

9.2.1. Animal procedure



C57BL/6 WT (n=5) and Jα18 -/- (n=5) received two subsequent i.p. injections of solutions containing 2.5 mg of rBer e 1 and 600 μg of SPC lipid fraction C in 200 μL of PBS, on days 0 and 7 (Protein-lipid solution preparation described in the Item 7.2.1. Animals were bled fourteen days after the first sensitisation injection (day 14). The exsanguination was performed by intra-cardiac route under lethal doses of pentobarbital sodium (Dolethal® injection, 200 mg/mL, Vetoquinol, UK). An average volume of 600 μL of blood was stored at 4°C in 1.5 mL vials (Standard Micro Test Tube, Eppendorf, UK) overnight, centrifuged at 8000 g for 4 min (Sanyo/MSE Hawk 15/05, UK). An

average of 100 μL of serum was recovered from each blood sample and 25 μL aliquots were stored in 0.5 mL vials (Standard Micro Test Tube, Eppendorf, UK) at -80°C. The spleen was collected for further analyses.

9.2.2. Splenocytes preparation

The same as described above (Item 9.1.2.).

9.2.3. Cell staining

9.2.3.1. Surface staining

One aliquot of 4 x 10^6 cells from each animal was centrifuged at 1,200 rpm, at 4ºC for 5 min and the supernatant was discarded. Cells suspensions were incubated for 30 min at 4ºC with tetrameter CD1d-α-GalCer-biotinylated (NHI Tetramer facilities, France) and anti-biotin-APC antibody (BD, Pharmingen, France) diluted in staining buffer (2% FCS, 0.1% Sodium Azide in PBS). Cells suspensions were washed in cold staining buffer, centrifuged at 1,200 rpm, at 4ºC for 5 min and the supernatant was discarded. Cells suspensions were then incubated for 30 min at 4ºC with a mixture containing anti-TCR-FITC antibodies (BD, Pharmigen, France), anti-CD8-PB antibodies (BD, Pharmingen), anti-CD4-APC H7 antibodies (eBiosciences, France) and anti-CD69-PE antibodies (BD, Pharmingen, France) diluted in staining buffer. Cells were washed with cold staining buffer, centrifuged, and the supernatant was discarded. Cells were then fixed with 600 μL of 4% paraformaldehyde PBS solution. Cells were again washed with staining buffer, centrifuged, and the supernatant was discarded. Cells were re-suspended in 800 μL of FACSCanto II flow cytometry buffer (BD, Pharmingen, France).

9.2.4. Flow cytometry

Cells were analyzed in a FACSCanto II (Becton Dickinson) by using FlowJo software (Flow Cytometry Analysis Software, USA).

10. ELISA (Enzyme-linked immuno sorbent assay)

10.1. Total Immunoglobulins

Total mouse IgG1/IgG2a and IgE content were determined by ELISA using commercial kits (Bethyl Laboratories®, USA) following the manufacturer's instructions and adapted to a 384 well-plate scale. In brief, 384 well-Maxisorp plates (Nunc, Copenhagen, Denmark) were coated with purified goat anti-mouse IgG1/IgG2a or IgE in carbonate buffer (0.05 M carbonate-bicarbonate pH 9.6) at room temperature (RT) for one hour (25 μL/well). After three consecutive washes with washing buffer - 50 mM Tris, 0.14 M NaCl and 0.05% (w/v) Tween-20 at pH 8 - plates were blocked with 50 mM Tris, 0.14 M NaCl and 1% bovine serum albumin (BSA) at pH 8.0 for 30 minutes at RT (50 μL /well). Then incubated with either animal sera or standard antibody at appropriate dilutions with sample dilution buffer (50 mM Tris, 0.14 M NaCl, 0.05% (w/v), 1% BSA, pH 8.0), for one hour at RT. Detection was carried out using a goat anti-mouse IgG1/G2a/E – horseradish peroxidase antibody diluted in sample dilution buffer, for one hour at RT (25 μL /well). For visualization, a ready-to-use substrate containing 3,3´,5,5´- tetramethylbenzidine (Tebu-Bio laboraties) was added to each well and incubated for 30 minutes at RT in the dark (25 μL/well). The reaction was stopped with the addition of 2M H_2SO_4 (25 μL /well) and the absorbance measured at 450 nm using FLUOstar Galaxy microplate reader (BMG Labtech, Germany). Standard curves (optical density, OD, versus

concentration) detecting minimum and maximum saturation points presented a sigmoidal shape and were analyzed using Weibull Distribution Model (CurveExpert 1.4 Software). The equation generated was equivalent to: $y = a - be^{-cx^d}$. The concentration (ng/mL) correspondent to the OD values obtained from each sample dilution was calculated using the equation above. The results represent the average of the concentrations from all dilutions referent to the same sample.

10.2. Specific Immunoglobulins

Specific IgG1/IgG2a content was determined by ELISA using commercial kits (Bethyl Laboratories, USA) following the manufacturer's instructions and adapted to a 384 well-plate scale. Basically, 384 well-Maxisorp plates (Nunc, Copenhagen, Denmark) were coated with protein solution at 400 μg/mL (rBer e 1) in carbonate buffer (0.05 M carbonate-bicarbonate pH 9.6) at room temperature for one hour (25 μL/well). After three consecutive washes with washing buffer - 50 mM Tris, 0.14 M NaCl and 0.05% (w/v) Tween-20 at pH 8 - plates were blocked with 50 mM Tris, 0.14 M NaCl and 1% bovine serum albumin (BSA) at pH 8.0 for 30 minutes at RT (50 μL/well). Then incubated with either animal sera or standard antibody at appropriate dilutions with sample dilution buffer (50 mM Tris, 0.14 M NaCl, 0.05% (w/v), 1% BSA, pH 8.0), for one hour at RT. Detection was carried out using a goat anti-mouse IgG1/G2a – horseradish peroxidase antibody diluted in sample dilution buffer, for one hour at RT (25 μL/well). For visualization, a ready-to-use substrate containing 3,3´,5,5´-tetramethylbenzidine (Tebu-Bio laboraties), was added to each well and incubated for 30 minutes at RT in the dark (25 μL/well). The reaction was

stopped with the addition of 2M H_2SO_4 (25 μL/well) and the absorbance measured at 450nm using FLUOstar Galaxy microplate reader (BMG Labtech, Germany). Standard curves were performed as described in the previous item (10.1.)

11. TLR activation

Samples of SPC fraction C were sent to a commercial laboratory (Invivogen, Toulouse, France) for an *in vitro* cell screening of human (h)TLR activation. The samples were tested on recombinant HEK-293 cell line that functionally expresses a given TLR protein as well as a reporter gene driven by NFkB promoter. The TLR activation results were given as optical density values (OD). Each 293-TLR cell line was induced with a known specific ligand as a positive control. Samples were tested in parallel. The positive control ligands used were: PAM2 (100 ng/mL) for the 293-hTLR2 cell line; Poly I:C (100 ng/mL) for the 293-hTLR3 cell line; LPS K12 (1 μg/mL) for the 293-hTLR4 cell line; Flagellin (1μg/mL) for the 293-hTLR5 cell lines; R848 (10 μg/mL) for the 293-hTLR7 and 293-hTLR8 and ODN 2006 (10 μg/mL) for the 293-hTLR9. A recombinant HEK-293 cell line for the reporter gene driven by NFkB promoter only was used as a negative control for the TLR cell lines. The negative control value for each clone was the background signal of these non induced clones. 20 μL of each sample to be tested (SPC lipid fraction C from Brazil nuts) was used to stimulate all the cell lines in a 200 μL of reaction volume.

12. Endotoxin detection

Freeze-dried samples of SPC lipid fraction L (Loading) and SPC lipid fraction C (C:M) were sent to a commercial laboratory (Profos AG, Regensburg, Germany) for detection of endotoxins by limulus amebocyte lysate (LAL) assay (Lonza, BioSciences, Lot GL017U). Detailed information is available in Appendix 2.

CHAPTER 3

LIPIDS FROM BRAZIL NUTS:

EXTRACTION AND FRACTIONATION.

INTRODUCTION

This chapter describes a series of experiments undertaken to address objective 1 of the present project: to extract and fractionate lipids from Brazil nuts. This chapter therefore provides a description of the lipid content of Brazil nuts, an overview of the methodology used in its fractionation and the main lipid analytical tools utilised in this project; thus, focusing on the isolation, putative identification and characterization of Brazil nuts lipid fractions that would be tested in *in vivo* and *in vitro* systems.

RESULTS and DISCUSSION

1. Total lipid fraction of Brazil nuts

In order to characterize the total lipid fraction from Brazil nuts, total lipids were extracted by "Folch" procedure (as described in Chapter 2, Item 2) and analysed by two-dimension TLC.

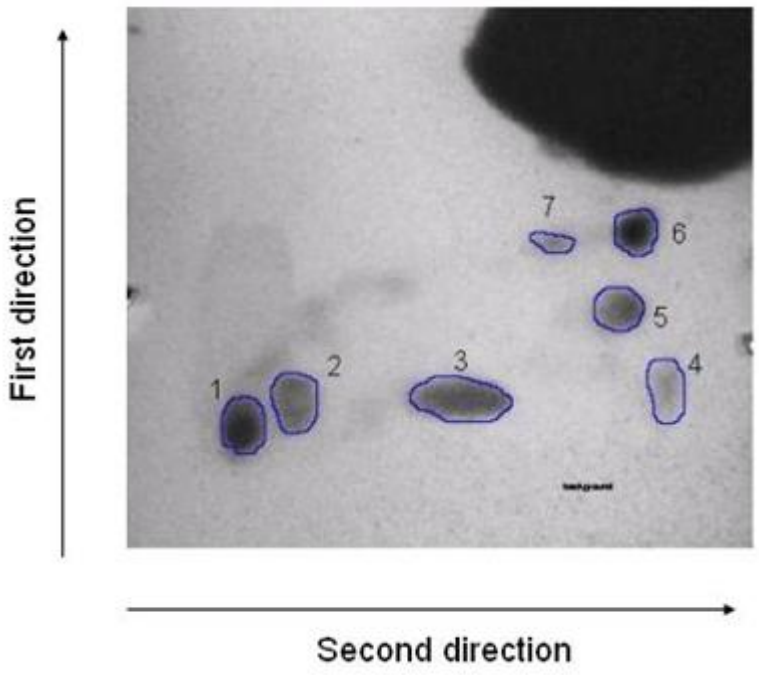


Figure 3.1.: A sample of total lipid fraction (fraction T) developed in a two-dimension TLC system, as described in Chapter 2 (Item 4.1.3.). The plate was sprayed with molybdenum and then burned at 200°C for 1 hour.

As shown in Figure 3.1., the total lipid fraction from Brazil nuts was fractionated into several sub-fractions, represented by dark spots in the TLC plate. The lipid classes present in these sub-fractions were putatively identified based on standard two-dimension TLC results in the literature, shown in Figure 3.2.. (Kupke e Zeugner, 1978). The most abundant spot was observed in the upper-right side of the figure and it is composed by simple lipids The spots

identified by numbers (1 to 7) represent different classes of complex lipids and the identification is shown in Table 3.1.

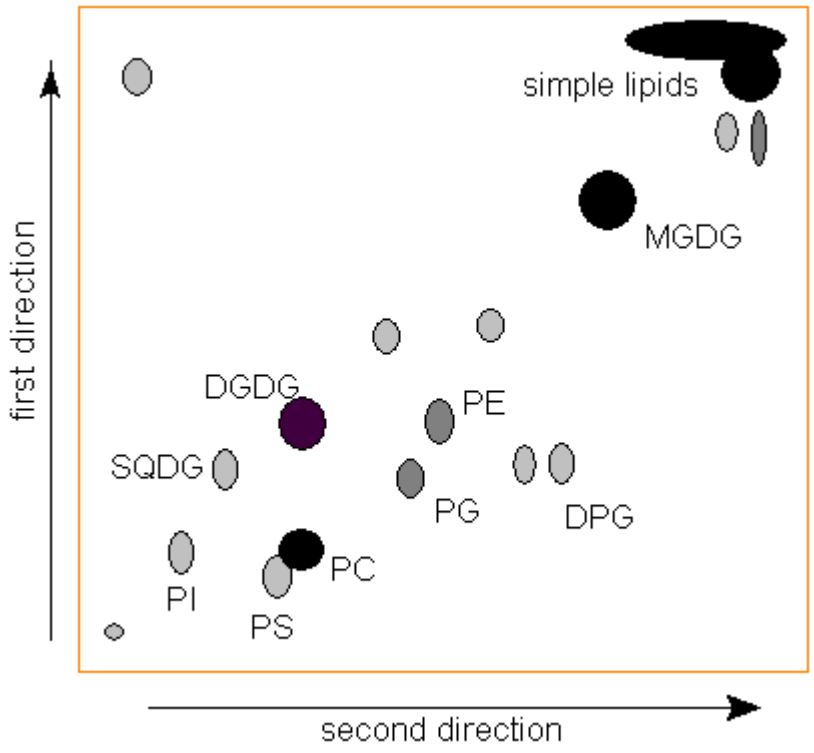


Figure 3.2. Schematic representation of two-dimensional TLC separation of complex lipids from *A. thaliana*. TLC system (Kupke e Zeugner, 1978). Lipid classes identified: monogalactosyldiacylglycerols (MGDG); digalactosyldiacylglycerol (DGDG); sulfoquinovosyldiacylglycerol (SQDG); diphosphatidylglycerol (DPG); phosphatidylglycerol (PG); phosphatidylethanolamine (PE); phosphatidylinositol (PI); phosphatidylserine(PS); and phosphatidylcholine (PC).

In order to estimate the contribution of different lipid classes present in the total lipid fraction, four replicates of the two-dimensional TLC plate described above (Figure 3.1.) were scanned and, based on the density, the estimated percentage of the lipid classes were measured (Table 3.1.). Since the quantification was based on the optical density (OD) divided by the occupied area, the fraction of simple lipids could not be quantified by this method. Due to its high concentration, the optical density of this fraction was probably over the

limit of detection and the values would be mistakenly underestimated. Therefore, only identified classes of complex lipids were quantified by this method.

The total amount of phosphorus present in the fraction T of Brazil nuts was calculated as 100.6 mM (Method described in Chapter 2, Item 5). Assuming a mean molecular weight (MW) of phospholipids in the range of 800, the concentration of phospholipids in the fraction T can be estimated as approximately 301.7 mg/L. Based on this value, the concentrations of the different classes of phospholipids were estimated and are also shown in Table 3.1. This result was comparable to the total phospholipid content of Brazil nuts found in the literature, in which the phosphorus concentration of Brazil nut oil was 377 mg/L (Chunhieng 2008).

Spot	Lipid	Area (mm^2)	Density (OD/mm^2)	Estimated percentage	Estimated concentration
1	SQDG	17.2 ± 10.7	135.5 ± 20.9	19 %	57.2 mg/L
2	PI	20.1 ± 14.1	95.9 ± 3.6	14 %	42.2 mg/L
3	PC	27.3 ± 19.5	113.8 ± 14	16 %	48.3 mg/L
4	PG	12.1 ± 5.9	72.9 ± 10.3	10 %	30.2 mg/L
5	PE	13.3 ± 9.1	107.7 ± 18.8	15 %	45.3 mg/L
6	MGDG	11.5 ± 10.5	115 ± 10.9	16 %	48.3 mg/L
7	PA	5.4 ± 3.2	70.7 ± 8.5	10 %	30.2 mg/L

Table 3.1.: Seven lipid classes present in the total lipid fraction from Brazil nuts, observed in a two-dimension TLC (Figure 3.1.). Results are in means of quadruplicate. Legend: Sulfoquinovosyldiacylglycerol (SQDG); phosphatidylinositol (PI); phosphatidylcholine (PC), phosphatidylglycerol (PG); phosphatidylethanolamine (PE); monogalactosyldiacylglycerols (MGDG) and phosphatidic acid (PA). OD: optical density.

The estimated percentage of lipid classes identified in the complex lipid fraction of Brazil nuts varied from 10% to 19% (Table 3.1.). Unexpectedly, the putative sulfoquinovosyldiacylglycerol (SQDG) was more abundant than the other classes, and the least abundant fractions were PG and PA. However, the phospholipid profile of Brazil nuts demonstrated here was similar to the data

found in the literature, in which the main phospholipids found in Brazil nuts were PI (30%), PC (24%), PE (21%), and PA (24%) (Chunhieng 2008). Similar results were also found in a study with hazelnut, in which the main phospholipids found in hazelnuts were PC, PE, and PI (Parcerisa, Codony *et al.*, 1999).

In order to estimate and compare the global contribution of total phospholipids, the total phospholipid content in oils from different nuts was shown to be at levels of 0.8% in almond oil, 2.8% in hazelnut oil, and 0.9% in walnut oil. The major components, identified by TLC systems, were PC (18–50%), PI (18–45%), and PE (8–16%). Small amounts of phosphatidylserine (PS), phosphatidic acids (PA), phosphatidylglycerols (PG), lysophosphatidylcholine, and lysophosphatidylethanolamine were also detected (Zlatanov M., Ivanov S. et al., 1999).

2. Fractionation of lipids from Brazil nuts

In order to identify potential biologically active lipids present in Brazil nuts, several lipid fractions were produced by four independent fractionation procedures: thin-layer chromatography (TLC), solid phase chromatography (SPC), and high-performance liquid chromatography (HPLC), and acetone preciptation. The fractions obtained by these different methods were analysed and then tested in an animal model of sensitisation to rBer e 1. The *in vivo* results are discussed in Chapter 5.

2.1. Lipid fractionation by acetone precipitation and TLC

Acetone precipitation is a simple and efficient procedure for separating complex lipids from neutral lipids (Kates e Kushwaha, 1995). The total lipid

fraction was extracted from Brazil nuts and then fractionated by acetone precipitation (as described in Chapter 2, Item 3.4.). Two fractions were obtained: pellet (rich in complex lipids) and supernatant (rich in simple lipids). The complex lipids-rich fraction was further fractionated by preparative TLC system.

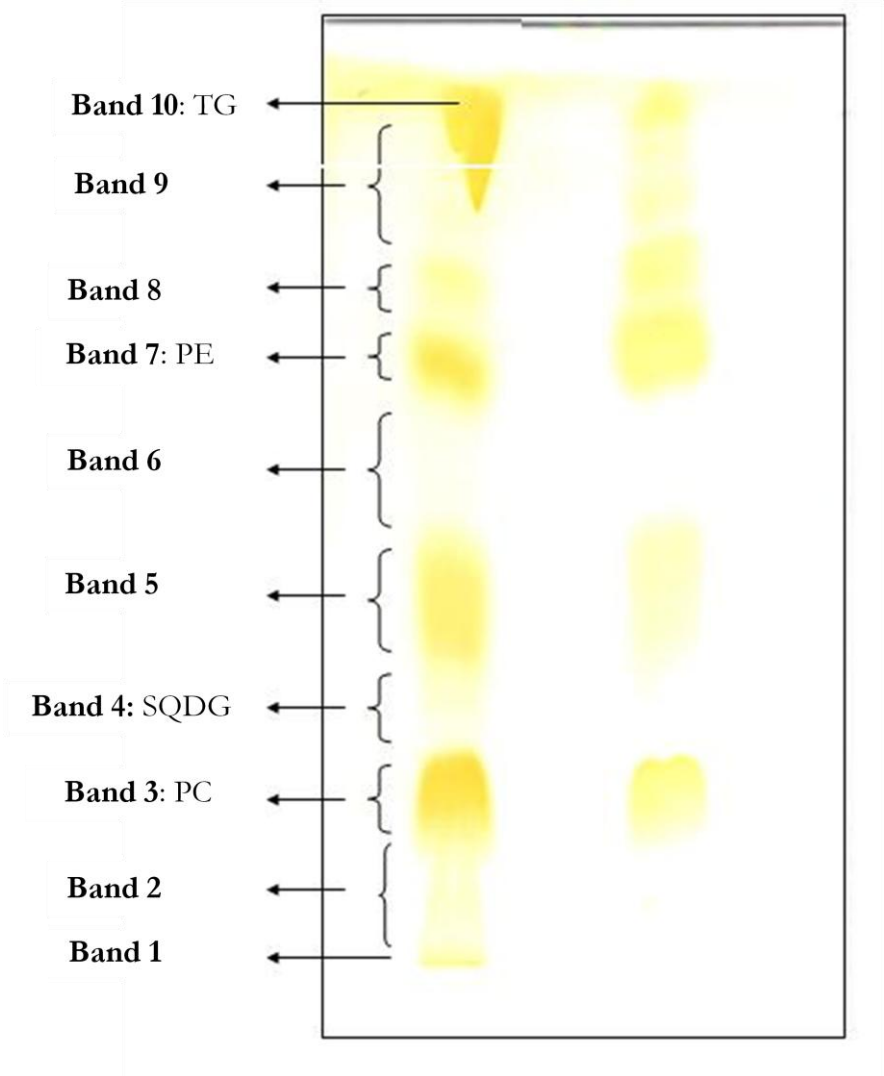


Figure 3.3.: One-dimension TLC plate developing system (as described in Chapter 2, item 4.1.2.a) was performed with a sample of complex lipids extracted from the total fraction of Brazil nuts. The plate was exposed to iodine vapour and ten yellow bands (Bands 1 - 10) were observed. Legend: triglycerides (TG); phosphatidylethanolamine (PE); Sulfoquinovosyldiacylglycerol (SQDG); phosphatidylcholine (PC).

Figure 3.3. shows the ten bands (yellow) observed after iodine vapour exposure, which is a non-destructive method of detection. In order to identify

the lipid classes detected, samples of these 10 lipid fractions (Bands 1 to 10) were extracted from the TLC plate and individually analysed by one-dimensional TLC-developing system (data not shown). The putatively identified lipid classes are showed in the Figure 3.3.: PC (Band 3), SQDG (Band 4), PE (Band 7), and TG (Band 10). These lipid classes had already been identified in the total lipid fraction from Brazil nuts (shown in Figure 3.1. and Table 3.1.).

Fractions of complex lipids containing PC, SQDG, and PE (extracted by TLC) as well as the simple lipid-rich fraction (supernatant extracted by acetone precipitation) were tested in the animal model of sensitisation carried out in the present work and the *in vivo* results are discussed in Chapter 5 (Figure 5.2.).

The extraction of lipids using the TLC system involved several steps: total lipid extraction by "Folch" method, complex lipids extraction by acetone precipitation, and several preparative TLC systems to obtain enough amounts of lipids to be tested in animals. This multi-step protocol facilitates the lipid oxidation as well as the production of undesirable artefacts. Therefore, in order to circumvent these limitations, a different method was performed to obtain lipid fractions from Brazil nuts, solid phase chromatography (SPC).

2.2. Lipid fractionation by SPC

SPC lipid extraction presents some advantages when compared to preparative TLC systems. Firstly, one single run of SPC can produce sufficient amounts of lipids to be used in lipid analyses and to be tested in animal protocols of sensitisation. Moreover, the extraction of lipids by SPC was not time-consuming, minimising the probability of lipid oxidation and the production of artefacts. Although the resolution in the fractionations is inferior to the one with

TLC or even HPLC, SPC extracted lipid fractions contain consistent groups of lipids and can be reliably reproduced (Christie, 1992).

Therefore, the total lipid fraction from Brazil nuts was fractionated by SPC (as described in Chapter 2, Item 3.2.). Two of the collected fractions, named as SPC fraction C and SPC fraction M, were analyzed by one-dimensional TLC along with known standard classes of commercial lipids.

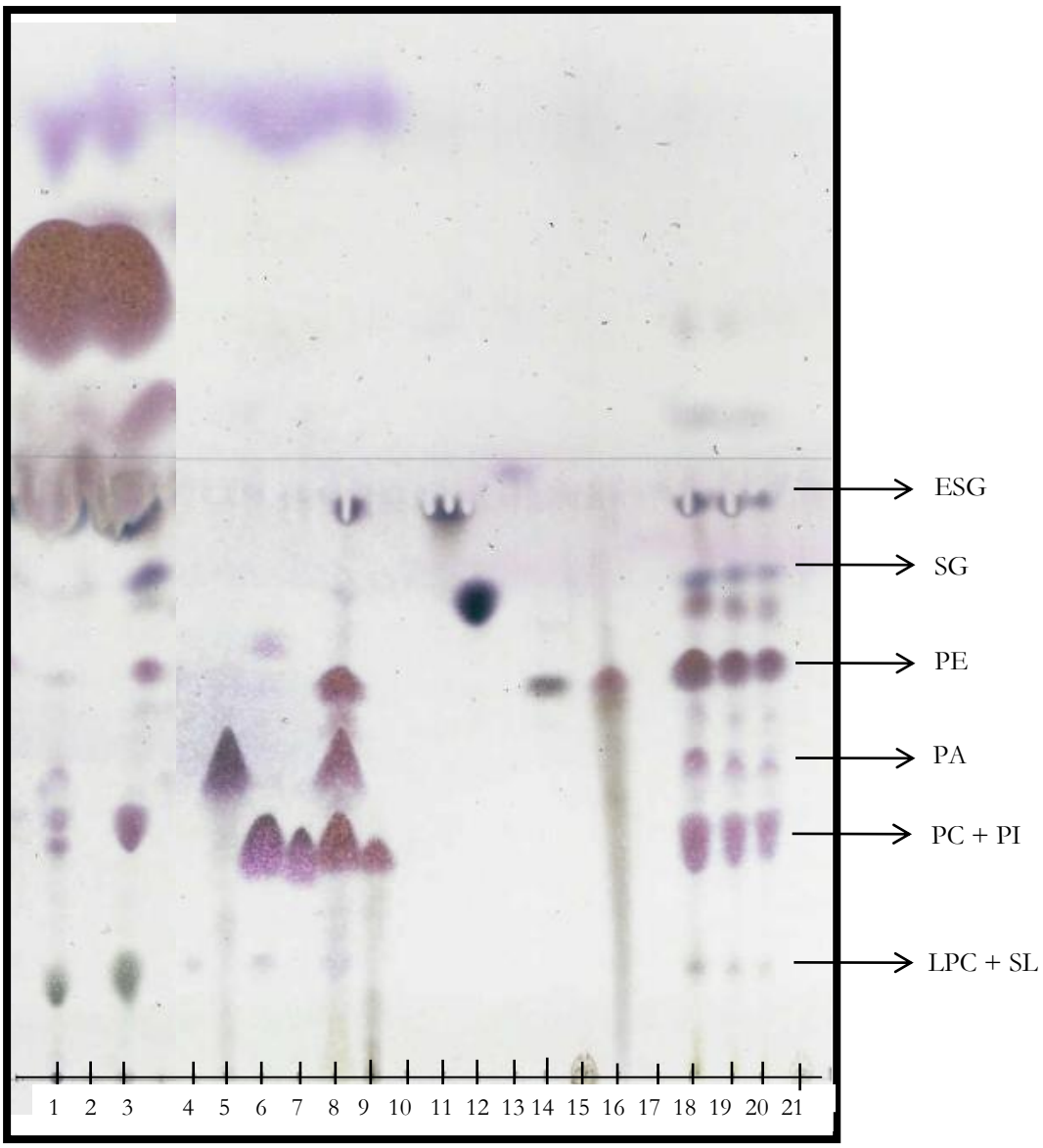


Figure 3.4.: One-dimensional TLC-developing system (as described in Chapter 2, Item 4.1.2.d). The plate was sprayed with molybdenum and then burned at 200°C for 1 hour. The samples used were: **1.** Total lipid fraction of Brazil nut (fraction T); **2.** Brazil nut SPC fraction C; **3.** Brazil nut SPC fraction M; **4-21, Commercial samples of: 4.** lyso-phosphatidylinositol (LPI); **5.** Phosphatidic acid (PA); **6.** Phosphatidylserine (PS); **7.** Lyso-phosphtidylserine (Lps); **8.** Phosphatidylinositol (PI-laboratory standard); **9.** Phosphatidylinositol (PI-Fluka); **10**. Lyso-phosphatidylethanolamine (LPE); **11.** Esterified steryl-glucoside (ESG); **12.** Steryl glucoside (SG); **13.** β-Sinositol; **14.** Digalactosyldiglyceride (DGDG); **15.** Lipopolissacharide from E. *coli* (LPS); **16.** Phospatidyletanolamine (PE); **17.** Stearic acid (SA); **18, 19, 20:** Soy lecithin; **21.** LPS from E. *coli*. Legend: LPC (Lyso-phosphatidylcholine), SL (sulfolipids), PC (Phosphatidylcholine), PI (Phosphatidylinositol), PA (Phosphatidic acid), PE (Phospatidyletanolamine), SG (Steryl glucoside), ESG (Esterified steryl-glucoside).

Figure 3.4. shows the bands detected in the fractions SPC C and SPC M from Brazil nuts and in the standard commercial lipids. The putative

identification of the lipid classes were carried out by comparison with the detected bands of standard lipids, as well as based on data from the literature (Figure 3.5.). The detected lipid bands in the SPC fraction C were mainly TG and traces of SG. The SPC fraction M presented simple lipids (TG, SG and ESG), but complex lipids were also identified: sulfolipids (SL), PC, and PE. Since the same amount of fractions were used for the TLC, it can be concluded that the concentration of complex lipids is higher in the fraction M that in the fraction C.

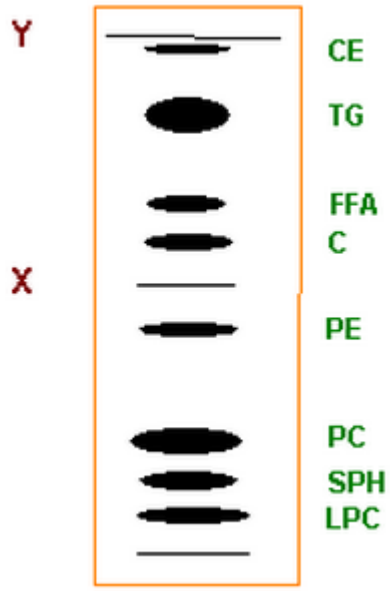


Figure 3.5.: Schematic separation of simple lipids and phospholipids of plasma by TLC (as described in Chapter 2, Item 4.1.2.d). Abbreviations: CE, cholesterol esters; TG, triacylglycerols; FFA, free fatty acids; C, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine (Kupke e Zeugner, 1978).

SPC fractions C and M were tested in the animal model of sensitisation to rBer e 1 carried out in the present work, and, as discussed in Chapter 5 (Figure 5.3.), SPC fraction C was the only fraction able to induce the production of Ber-specific anaphylactic antibodies. Since SPC fraction C is composed by different classes of lipids, this fraction was further fractionated by HPLC in order to identify possible specific lipid classes involved in the immune activation observed in animals.

2.3. Fractionation of SPC lipid fraction C by HPLC

HPLC is widely used to analyse phospholipids (Jungalwala, 1976; Samet, Friedman *et al.*, 1989; Christie, 1998). This technique provides a higher resolution system for lipid fractionation than SPC and TLC systems. Moreover, HPLC has the advantage of delivering clean fractions, i.e. without trace contaminants such as silica and dyes. For this experiment, SPC lipid fraction C was applied in a HPLC column (as described in Chapter 2, Item 3.3.), resulting in several lipid sub-fractions.

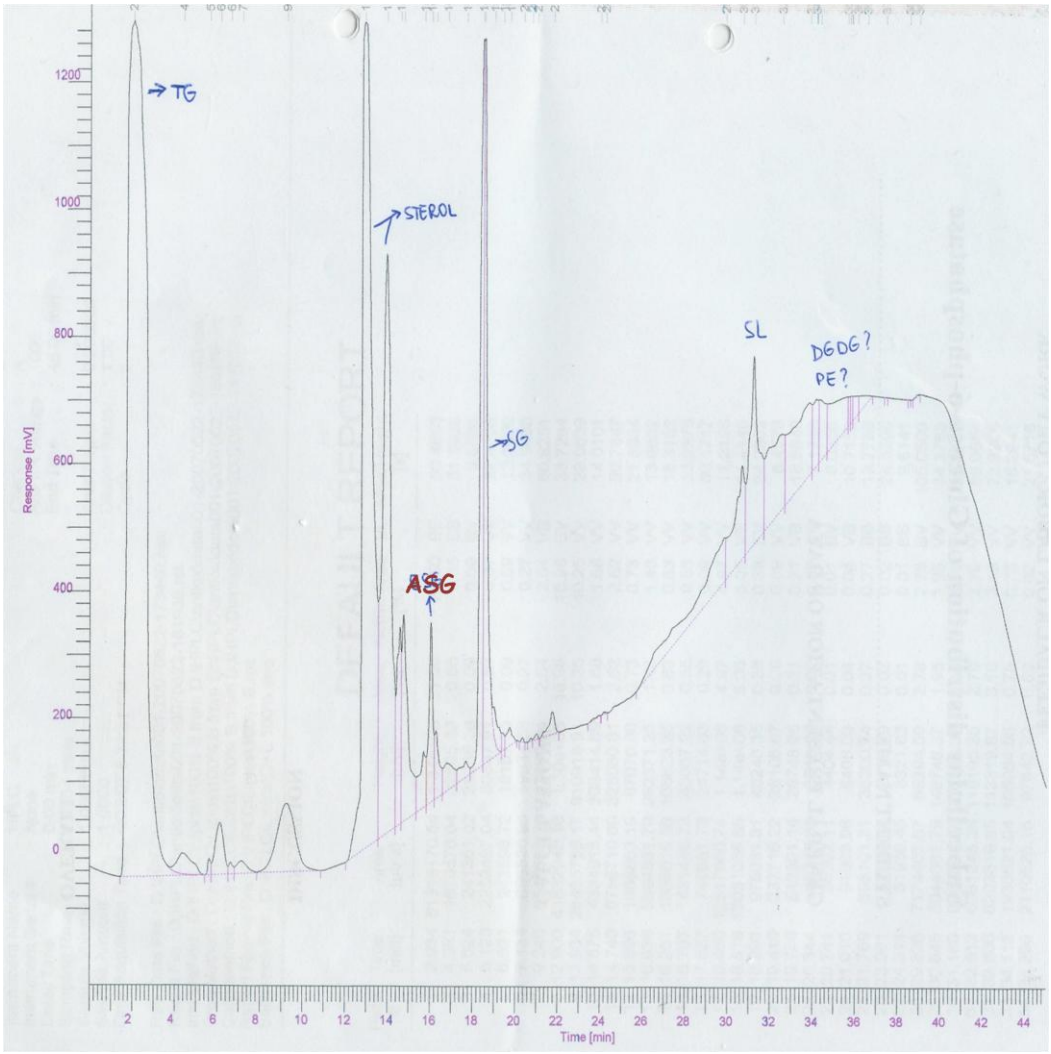


Figure 3.6.: Separation of Brazil nut SPC lipid fraction C by high performance liquid chromatography (HLPC), as described in Chapter 2 (Item 3.3.). Legend: triacylglycerols (TG); sterols; acylsterylglycosides (ASG); sterylglycosides (SG); sulpholipids (SL); digalactosyldiacylglycerol (DGDG) and phosphatidylethanolamine (PE). Samples were collected every 2 min, therefore 22 samples were obtained by HPLC.

Figure 3.6. shows the HPLC profile obtained with SPC lipid fraction C. The large base line drift and lack of detection for more complex lipids was due mainly to the type of detector used in this work. The specific light-scattering detector (ACS 750/14 Mass Detector, Applied Chromatography Systems Ltd., Luton, Beds., UK) (Christie, 1998) able to detect phospholipids was not available for this equipment. Therefore, the fractions were collected and separated by retention time only. The putative identification of lipid classes present in the SPC

lipid fraction C were performed by comparing the HPLC results (Figure 3.6.) with data in the literature (Figure 3.7). The main lipid fractions identified were: triacylglycerols (TG); sterols; acylsterylglycosides (ASG); sterylglycosides (SG); sulfolipids (SL); and digalactosyldiacylglycerol (DGDG) or PE.

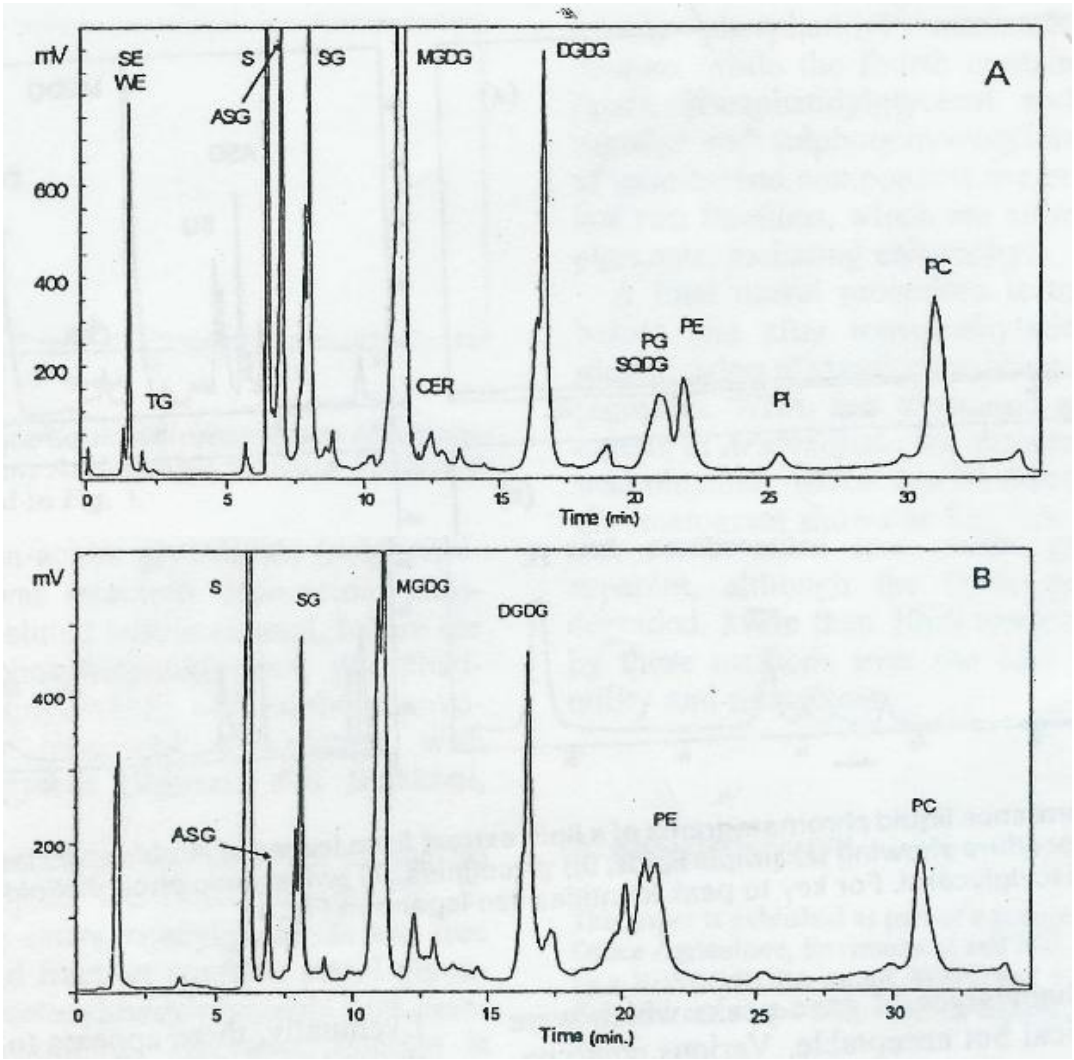


Figure 3.7.: Separation by high performance liquid chromatography on a YMC-PVA-SIL (Hichrom) column of A) Lipid classes from leaves of a single plant of *Arapidopsis thaliana*; and B) Lipids from a uncharacterised *Arapdopsis* mutant deficient in acylsterylglycosides. Key to peak identities: wax esters (WE); sterol esters (SE); triacylglycerols (TG); sterols (S); acylsterylglycosides (ASG); sterylglycosides (SG); monogalactosyldiacylglycerol (MGDG); digalactosyldiacylglycerol (DGDG); cereboside (CER); phosphatidylglycerol (PG); sulphoquinovosyldiacylglycerol (SQDG); phosphatidylethanolamine (PE); phosphatidylinositol (PI); phosphatidylserine (PS), and phosphatidylcholine (PC). Graphs extracted from (Christie, 1998).

Samples of each lipid sub-fraction were grouped accordingly to their classification, representing the main classes of identified lipids: HPLC sub-fractions C 1-5 (TG), 6-8 (ASG), 9-11 (SG), 12-14 (not identified), 15-17 (SL), and 18-22 (DGDG or PE). These grouped lipid sub-fractions were tested in the animal model of sensitisation used in the present work (Chapter 5, Figure 5.5.) in order to identify the biologically active lipid fractions present in the SPC fraction C. However, as discussed in Chapter 5, none of the grouped sub-fractions was able to induce the *in vivo* immune activation observed when the whole SPC fraction C was tested.

As previously mentioned, HPLC provides more accurately fractionated and cleaner lipid fractions than SPC or TLC obtained fractions (Christie, 1998). However, only small fractions are obtained and several runs are necessary to produce minute quantities for an *in vivo* work. Thus, possible explanations for the negative results observed in animals related to the technique could be the occurrence of undesired contaminations during the HPLC process, selective binding to the HPLC column or lipid oxidation during the process. Other possibilities, related to the animal response, are discussed in Chapter 5 (Figure 5.5). Based on the *in vivo* results, a different method (acetone precipitation) was used to fractionate the SPC fraction C into two sub-fractions: complex lipids-rich fraction and simple-lipids-rich fraction.

2.4. Fractionation of SPC lipid fraction C by acetone precipitation

The present experiment used acetone precipitation to fractionate SPC fraction C into two sub-fractions: pellet and supernatant, as described in Chapter

2 (Item 3.4.). SPC fraction C consisted in 99.9% of neutral lipids (supernatant) and 0.1% of complex lipids (pellet). A TLC system was performed in order to analyse the sub-fractions of SPC fraction C.

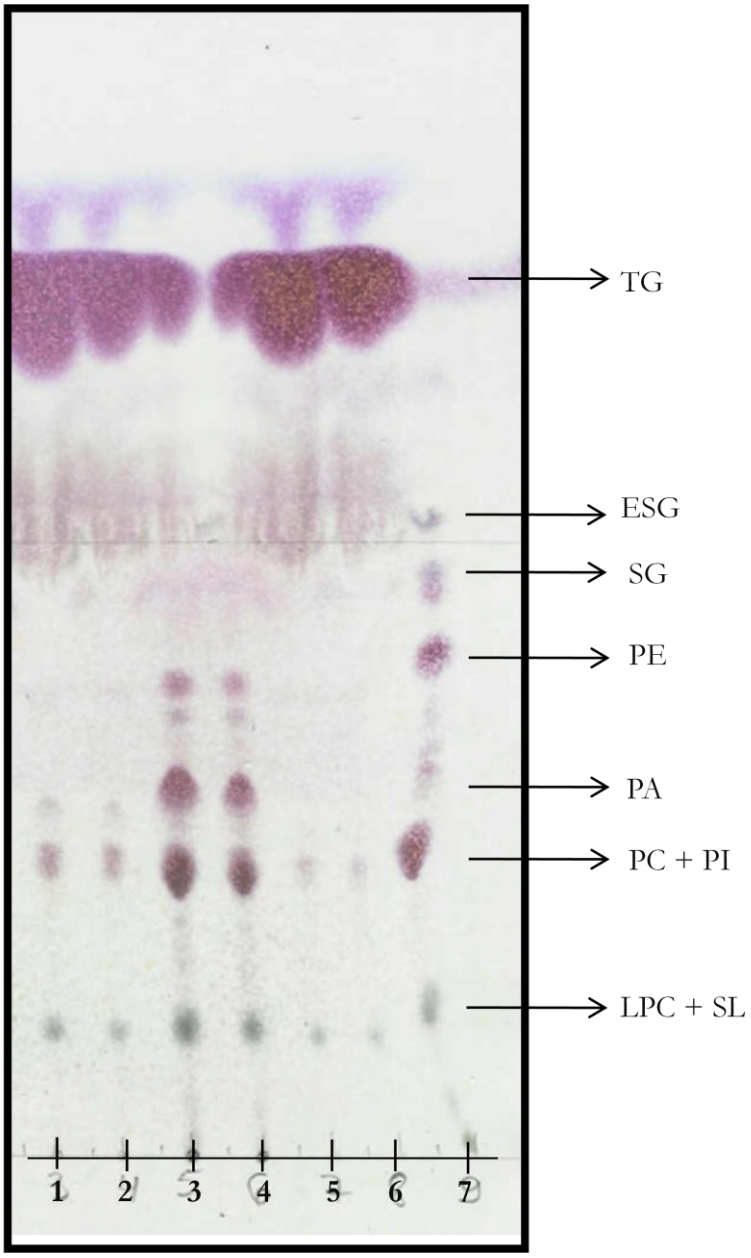


Figure 3.8.: One-dimensional TLC-developing system (as described in Chapter 2, Item 4.1.2.d) of sub-fractions from SPC fraction C. The plate was sprayed with molybdenum and then burned at 200°C for 1 hour. The samples used were: **Spots 1** and **2.** SPC fraction C; **3** and **4.** Pellet from SPC fraction C; **5** and **6.** Supernatant from SPC fraction C; **7.** Soy lecithin.

Figure 3.8. shows the detected bands from SPC fraction C and from its sub-fractions, pellet and supernatant. The classes of lipids present in these fractions were putatively identified by comparison with standard soy lecithin (spot 7) as well as with literature data, showed in Figure 3.5. (Kupke e Zeugner, 1978).

The lipid band equivalent to simple lipids (TG) was more evident in the supernatant sub-fraction and less evident in the pellet sub-fraction. The lipid bands representing complex lipids (LPC, PC, PI, SL, PA and PE) were more concentrated in the pellet sub-fraction. These results confirm the separation of simple and complex lipids by acetone precipitation. The reason why the complex lipids observed in the pellet fraction have not been identified in the original SPC fraction C (Figures 3.4. and 3.8.) is probably due to the concentration, which was much higher in the pellet fraction.

The concentration of phosphorus was measured in these three samples (SPC fraction C, pellet and supernatant), and the results obtained are shown on Table 3.2..

	Phosphorus (mM)	Molecular weight	Phosphorus (mg/L)
SPC fraction C	0.23	~800	184.00
Pellet from SPC fraction C	1.79	~800	1,432.00
Supernatant from SPC fraction C	0.15	~800	120.00

Table 3.2.: Estimation of phosphorus content in different lipid fractions from Brazil nuts.

As expected (Table 3.2), the concentration of phosphorus was approximately 10 times higher in the complex lipids-rich fraction (pellet), comparing to the neutral lipids-rich fraction (supernatant) and to the original SPC fraction C. These data confirm the results obtained by TLC (Figure 3.8.), in which the phospholipids bands were more evident in the pellet fraction.

The pellet and supernatant sub-fractions were individually tested in the animal model of sensitisation and the results are discussed in Chapter 5 (Figure 5.6.).

In summary, the total fraction of lipids from Brazil nuts, which represents approximately 66% of the seed's dry weight (Usda, 2009), was extracted by "Folch" extraction (Folch, Lees *et al.*, 1957) and fractionated by various methods, such as TLC, SPC, HPLC and acetone precipitation. The obtained lipid sub-fractions were analysed mainly by TLC and the identification of lipid classes present in each of the sub-fractions was performed in comparison with standard lipids and literature data. The total lipid fraction was found to contain mostly simple lipids, but also SQDG, MGDG, PC, PE, PI, PG, and PA, in agreement with recent literature in Brazil nuts (Chunhieng 2008). The sub-fraction C, extracted by SPC, contained mainly simple lipids (99.9%), such as TG sterols, SG, and ASG; and the identified lipid classes present in the complex lipids-rich fraction (0.01%) were LPC, PC, PI, SL, PA, and PE. The sub-fraction M, extracted by SPC, was composed mainly by simple lipids, such as TG, SG, ESG, and the other classes of lipids identified were, SL, PC, and PE.

Therefore, the methodologies used to extract, fractionate and identify Brazil nut lipids were here described. Considering the advantages and disadvantages associated with each techinique used, the results obtained in the present chapter aimed to base the investigations on the role that Brazil nut natural lipids play in *in vivo* and *in vitro* immune reactions, which is discussed in the next chapters.

CHAPTER 4

PCA:

OPTIMIZATION AND

CHARACTERIZATION

INTRODUCTION

As discussed in Chapter 1, passive cutaneous anaphylaxis (PCA) is a reliable and simple technique, widely used to identify anaphylactic antibodies (Ovary, 1950; Inagaki, Nagai *et al.*, 1988; Wakayama, Hasegawa *et al.*, 1998; Faquim-Mauro, Coffman *et al.*, 1999). Several techniques can be performed to quantify antibodies, such as RAST, ELISA, and microarrays (Eriksson e Ahlstedt, 1977; Wiese, Belosludtsev *et al.*, 2001; Lebrun, Petchpud *et al.*, 2005; Harwanegg e Hiller, 2006); however, PCA is able to assess the biological function of antibodies by partially mimicking an immediate allergic process (Miyajima, Dombrowicz et al. 1997; Woo, Kim et al. 2006; Nonaka, Izumo et al. 2008).

Immunoglobulin isotypes are described to have anaphylactic activity, IgE and IgG1 (Mota, Wong *et al.*, 1968; Becker, 1971; Lehrer e Vaughan, 1976; Dombrowicz, Flamand *et al.*, 1997; Blaikie e Basketter, 1999; Ryan, Kashyap *et al.*, 2007). Different protocols of PCA are performed in order to identify anaphylactic responses (Nishikawa e Hong, 1987; Dearman, Alcocer *et al.*, 2007) as well as to differentiate the immunoglobulin isotypes involved in the reaction (Inagaki, Nagai *et al.*, 1988; Silva, Casabuono *et al.*, 2008). However, PCA is an *in vivo* procedure and it involves a sequence of events, being evaluated only the final blood extravasation, which is consequence of the increased vascular permeability caused by mediators secreted by activated mast cells. Therefore, a definite differentiation of immunoglobulins isotypes using PCA might not be straightforward.

Therefore, since the anaphylactic activity of antibodies was the main indication of allergy in animals sensitised to rBer e 1 in the present work, this chapter describes and discusses the results from a series of experiments aimed at

optimizing and characterizing PCA reactions, focusing mainly on sensitivity, and immunoglobulin isotype specificity of this technique.

RESULTS AND DISCUSSION

1. PCA sensitivity to immunoglobulin class E (IgE)

In order to evaluate the sensitivity of the PCA technique to detect immunoglobulin class E, increasing concentrations of commercial anti-OVA IgE were tested in naïve recipient BALB/c mice (Figure 4.1).

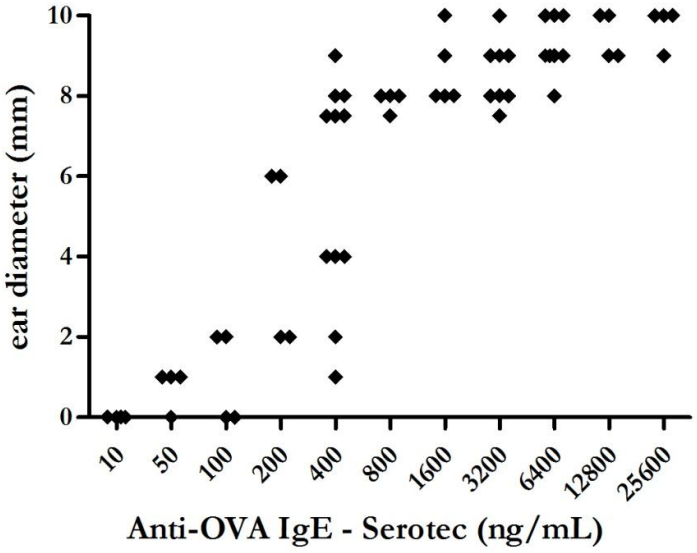


Figure 4.1. Serum samples containing different concentrations (10, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12.800, 25.600 ng/mL) of commercial anti-OVA IgE (Serotec, UK) were tested. Samples were injected into the ears of naive recipient mice and 2 days later these animals were challenged with OVA, Evans blue dye and PBS, according to the procedures described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge.

Figure 4.1. showed that the optimized PCA procedure run in our facilities was able to detect commercial anti-OVA IgE. The dose response curve was of sigmoidal shape, with the lowest significant dose detectable at 200 ng/mL and the highest quantified concentration around 800 ng/mL.

Although 100 and 50 ng/mL doses resulted in positive reactions, smaller diameters than the 3 mm published cut off (Dearman, Alcocer *et al.*, 2007) were observed. At 400 ng/mL, the diameters of reactions were variable (from 1 to 9 mm). From 800 ng/mL onwards, the dose response reached a saturation plateau and all reactions were strongly positive, the diameters varied between 8 and 10 mm. Therefore, PCA technique was able to detect concentration as low as 200 ng/mL of IgE and there was no difference in the results of doses higher than 800 ng/mL. The latter could be explained since, due to size limitation of mice ears, the maximum measurable diameter of cutaneous reactions was 8-10 mm.

Assuming that commercial anti-OVA IgE presented the same avidity and affinity as IgE produced *in vivo* sensitisation in our facilities, the lowest concentration of IgE able to be measured by cutaneous anaphylaxis using our protocol of PCA was 200 ng/mL (i.e., 5 ng of IgE in 25 μL of serum dermally injected). This result is in agreement with the literature, in which 15 ng was the amount of IgE required to elicit PCA reactions (Dearman, Skinner *et al.*, 2005). In order to expand the dynamic range of detected concentrations by PCA (measuring higher concentrations than 800 ng/mL), a sample dilution would be needed. The same procedure is commonly performed in ELISA assays, in which diluted samples are used in order to detect accurate antibody concentrations (Birmingham, Payankaulam *et al.*, 2003). However, the main aim here was to

evaluate the PCA sensitivity, evaluating the minimum concentrations of IgE that could be detected by PCA.

2. Discriminating between IgE and IgG1 activity in PCA reactions

PCA positive reactions in mice can be elicited by IgE and/or IgG1 anaphylactic antibodies (Ovary, 1982; Hazenbos, Gessner *et al.*, 1996; Miyajima, Dombrowicz *et al.*, 1997). In an attempt to identify the classes of immunoglobulins involved in the PCA reactions performed in the present work, a series of experiments were carried out exploring the heat stability of IgE and their adsorption to affinity supports. IgE antibodies are described as heat-labile immunoglobulins whereas IgG1s are heat-stable, maintaining their anaphylactic property after being heated at 56°C for one hour (Lehrer e Vaughan, 1976; Faquim-Mauro e Macedo, 2000; Bortolatto, Borducchi *et al.*, 2008). Additionally, IgG1 can be adsorbed by protein G while IgE anaphylactic activity is not affected by protein G adsorption (Peng, Becker *et al.*, 1994).

2.1. Characterizing commercial anti-OVA IgE anaphylactic activity

In order to verify the reliability of the protocols used to differentiate IgE and IgG1 involved in PCA (heating and protein G), they were performed using serum containing only anti-OVA IgE.

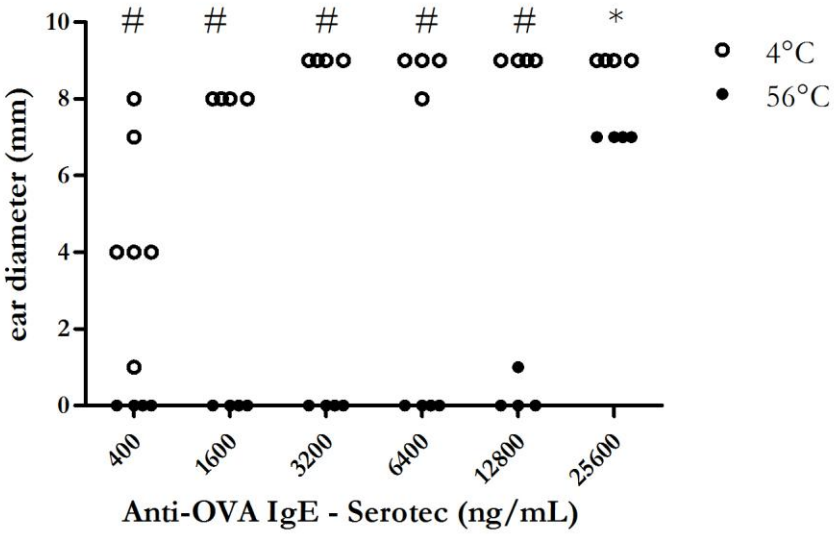


Figure 4.2. Serum samples containing different concentrations (400, 1600, 3200, 6400, 12.800, 25.600 ng/mL) of commercial anti-OVA IgE (Serotec, UK) were heated at 56°C for 1 hour. Control samples were kept at 4°C. Samples were injected into the ears of naive recipient mice and 2 days later these animals were challenged with OVA, Evans blue dye and PBS, according to the procedures described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge. Two-way ANOVA and Bonferroni post-test, significant difference between heated and non-heated sera: *$p<0.05$ and #$p<0.001$.

Figure 4.2. shows that sera containing 400 to 25,600 ng/mL of anti-OVA IgE not submitted to high temperature presented positive PCA reactions. By heating up these sera, the anaphylactic property of IgE was totally lost in sera containing 400 to 12,800 ng/mL of IgE ($p<0.001$). Thus, these results corroborate the literature, high temperature treatment was able to inactivate the anaphylactic property of IgE (Ishizaka, Ishizaka *et al.*, 1967; Kulczycki, Isersky *et al.*, 1974; Lehrer e Vaughan, 1976; Faquim-Mauro e Macedo, 2000; Bortolatto, Borducchi *et al.*, 2008). However, this effect was concentration dependent; heated serum containing high concentrations of IgE (25,600 ng/mL) had the anaphylactic property significantly diminished, but not lost. It could be speculated that the heat efficiency of the system used here was not uniform or

that 1 hour at high temperature is not enough to denaturate IgE. In some protocols, the serum is heated up for 4 hours in order to reach a complete loss of IgE anaphylactic activity (Kulczycki, Isersky *et al.*, 1974). One other possibility is that the thermal denaturation is a reversible process that can be only detected at high concentration. Therefore, in our protocol, the anaphylactic property of IgE was totally abolished by heating treatment only when IgE concentration in the sample was lower than 12,800 ng/mL.

The following experiment (Figure 4.3.) was carried out in order to verify whether mouse IgE is able to be adsorbed with protein G.

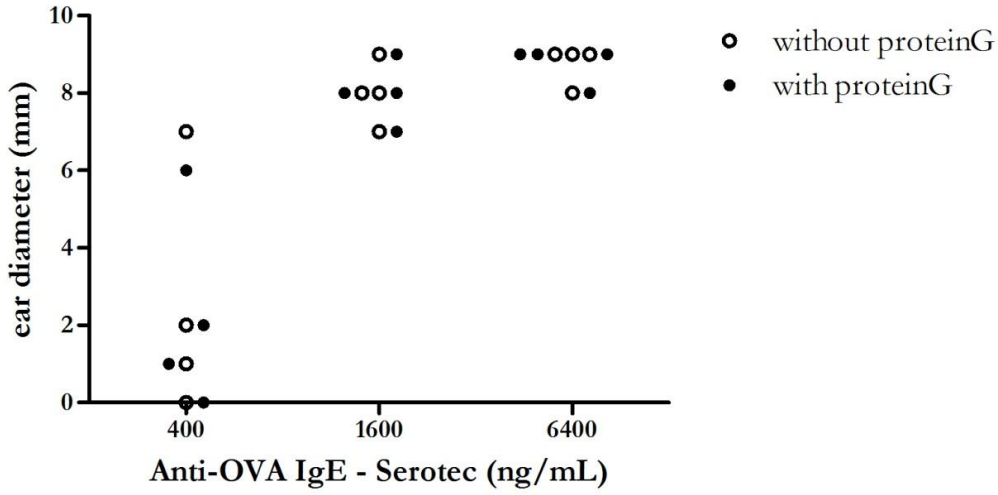


Figure 4.3. Serum samples containing different concentrations (400, 1600, 6400 ng/mL) of commercial anti-OVA IgE (Serotec, UK) were absorbed with Protein G. Control (without protein G) and experimental (with protein G) samples were injected into the ears of naive recipient mice and 2 days later these animals were challenged with OVA, Evans blue dye and PBS, according to the procedures described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge.

Figure 4.3. shows that the anaphylactic property of commercial anti-OVA IgE in the concentrations tested (400, 1,600 and 6,400 ng/mL) was not affected by adsorption with protein G. PCA reactions performed with IgE serum

treated with protein G were not statistically different from PCA reactions performed with non-treated IgE. The variability observed at the concentration 400 ng/mL was probably due to individual animal differences. The two ears of the same mouse were used to test treated and non-treated samples and the values obtained for each animal were consistently similar.

Protein G was shown to bind to the Fc portion of IgG, but not to IgE in serum from human patients (Peng, Becker *et al.*, 1994). This property is also reported for mouse immunoglobulins, with protein G being used to isolate IgG antibodies as well as to remove this class of immunoglobulin from serum samples (Kadooka, Idota *et al.*, 2000; Lehrer, Reish *et al.*, 2004). Unfortunately, certified IgG1 specific for OVA was not commercially available. The protein G used in our protocol binds very well to mouse IgG1 and IgG2a and less efficiently to IgG2b and IgG3 (technical information informally given by the manufacturer, Dave Walker, Merck Biosciences, UK). Therefore, the results obtained in this experiments confirm the data from the literature, assuring that IgE activity is maintained after been adsorbed with protein G (Peng, Becker *et al.*, 1994; Kadooka, Idota *et al.*, 2000; Lehrer, Reish *et al.*, 2004).

2.2. Anaphylactic antibodies in serum of OVA-sensitised animals in our facilities

In order to investigate the participation of OVA-specific IgE and OVA-specific IgG1 in the passive cutaneous anaphylaxis protocol performed in the present work, pooled sera obtained from 5 OVA-sensitised mice were investigated. Animals were sensitised according to described in Chapter 2 (Item 7.1). The presence of OVA-specific IgE as well as OVA-specific IgG1 in the

serum sample was first confirmed by ELISA. The sample of pooled OVA-sera were tested in triplicates in an ELISA plate and the results exposed in the following table (Table 4.1.) represent the average (AV) and standard deviation (SD) of the three replicates tested.

Immunoglobulin	**AV ± SD (ng/mL)**
Anti-OVA IgE	585.8 ± 89.08
Anti-OVA IgG1	147,386.5 ± 8,151

Table 4.1.: Concentration of anti-OVA IgE and anti-OVA IgG1 present in pooled serum from OVA-sensitised mice. Antibodies measurements performed by ELISA according to the described in Chapter 2 (Item 10.2).

These pooled sera were then tested in PCA reactions. In order to investigate the participation of IgE in the anaphylaxis induced by OVA-serum, the serum were submitted to heating treatment, since IgE but not IgG1 is reported to heat-labile (Faquim-Mauro e Macedo, 2000).

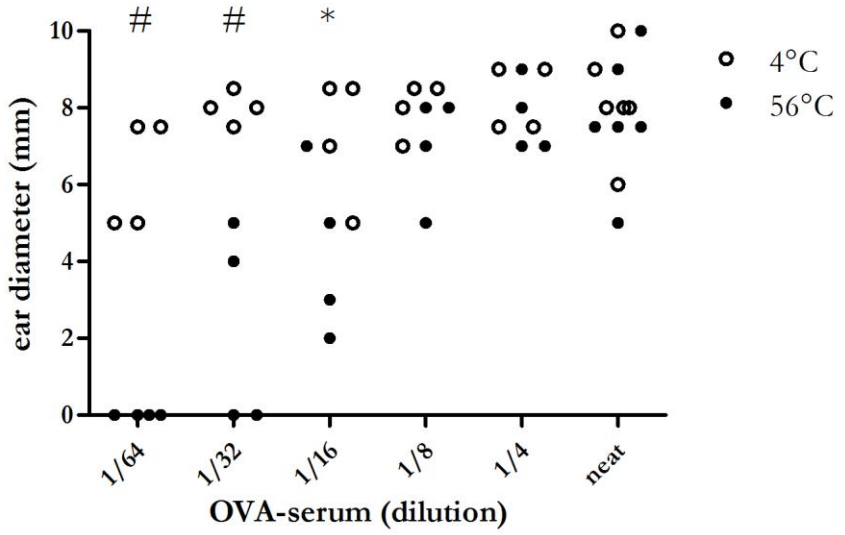


Figure 4.4. Different dilutions (1/64, 1/32, 1/16, 1/8, 1/4 and neat) of serum from OVA-sensitized mice (OVA-serum) were heated at 56°C for 1 hour. Control samples were kept at 4°C. Samples were injected into the ears of naive recipient mice and 2 days later these animals were challenged with OVA, Evans blue dye and PBS, according to the procedures described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge. Two-way ANOVA and Bonferroni post-test, significant difference between heated and non-heated sera: *$p<0.05$ and #$p<0.001$.

Figure 4.4. shows that the heating procedure influenced the PCA activity in a concentration dependent way. PCA reaction was totally abolished in heated serum diluted 64 times (1/64), while PCA reactions performed with non-heated 1/64 serum showed diameters of 5, 5, 7.5 and 7.5 mm ($p<0.001$). In 1/32 diluted sera, heated samples showed two negative and two positive reactions (0, 0, 4 and 5 mm), significantly different from non-heated serum (7.5, 8, 8 and 8.5 mm; $p<0.001$). At 1/16, the heating treatment did not abolish the anaphylactic property, but the difference between heated and non-heated serum was statistically different ($p<0.05$). At 1/8 dilution, the PCA reactions were slightly weaker in the heated than non-heated serum samples, but no statistical difference was found. More concentrated samples (1/4 and neat serum) were not affected by the heating procedure.

Considering that the concentration of anti-OVA IgE in neat serum samples was 18,627 ng/mL, positive PCA was totally abolished by heating in serum containing approximately 300 ng/mL (1/64 dilution), it was diminished in serum containing 600 or 1200 ng/mL (1/32 and 1/16 dilutions, respectively) and PCA was not affected by heating in serum containing around 2,400; 4,800 and 18,627 ng/mL (1/8, 1/4 and neat serum, respectively). Bearing in mind the results within Figure 4.2., in which PCA activity was completely abolished in serum samples containing up to 12,800 ng/mL of anti-OVA IgE; it is possible to speculate that besides IgE, other class(es) of immunoglobulin is(are) influencing PCA reactions performed with serum from OVA-sensitised animals.

In addition to IgE, the class of immunoglobulin reported to have anaphylactic activity in mice is IgG1. Considering that two types of mouse IgG1 are described in the literature, anaphylactic and non-anaphylactic IgG1 (Mota e Perini, 1982; Faquim-Mauro, Coffman *et al.*, 1999), the class of immunoglobulin that was also being detected by PCA technique in our facilities is likely to be anaphylactic IgG1. ELISA results showed that OVA-sensitised animals indeed present anti-OVA IgG1; however, this *in vitro* technique is not able to discern the two biologically distinct IgG1 fractions.

In order to further investigate the presence of anaphylactic IgG1 in serum of OVA-sensitised animals in our facilities, sera from these animals were treated with protein G and PCA was performed (Figure 4.5).

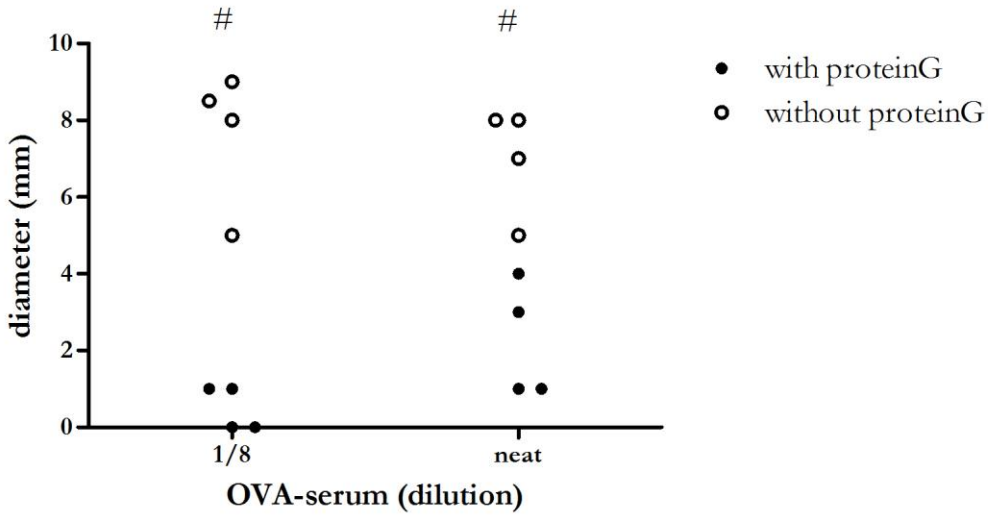


Figure 4.5. Samples of serum from OVA-sensitized mice (OVA-serum) diluted 1/8 or neat were absorbed with Protein G. Control (without protein G) and experimental (with protein G) samples were injected into the ears of naive recipient mice and 2 days later these animals were challenged with OVA, Evans blue dye and PBS, according to the procedures described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge. Two-way ANOVA and Bonferroni post-test, significant difference between protein G-treated and -non treated sera: #$p<0.001$.

Figure 4.5. shows that at 1/8 dilution the absorption with protein G totally abolished passive anaphylactic reactions ($p<0.001$). In neat serum, absorption with protein G significantly diminished ($p<0.001$) but did not abolish PCA reactions.

Protein G does not bind to polyclonal or monoclonal IgE (Peng, Becker *et al.*, 1994). This fact was confirmed in our previous experiment (Figure 4.3.), in which IgE anaphylactic activity was not affected by protein G adsorption. Therefore, the diminished PCA activity observed in OVA-serum adsorbed with protein G (Figure 4.5) corroborates previous findings (Figure 4.4.), indicating that IgG1 might be involved in the OVA-anaphylaxis model used.

The following experiment was performed in order to verify the duration of contact between different classes of anaphylactic immunoglobulins and their respective receptors on mast cells. For this, a modification was made on the PCA technique, allowing longer periods between sensitisation (serum injection on day 0) and challenge (antigen injection). Serum from OVA-sensitised animals and serum containing commercial anti-OVA IgE were tested.

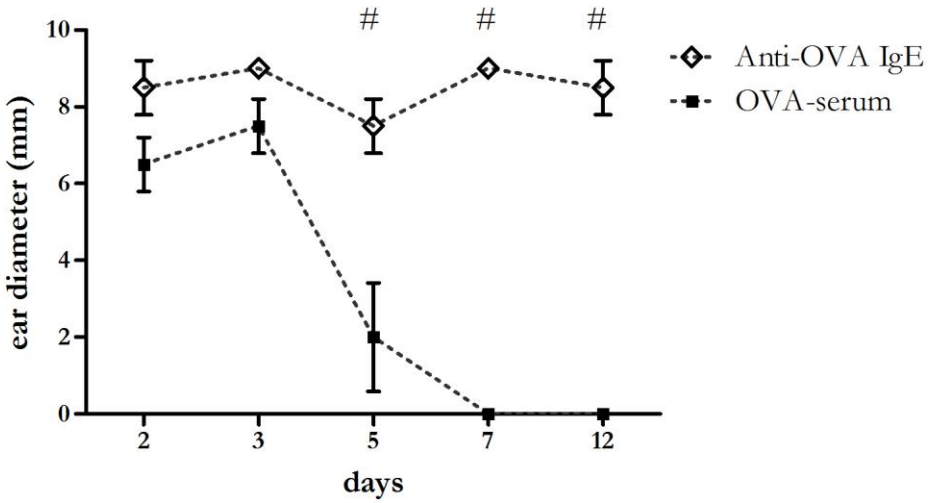


Figure 4.6. Samples of serum conatining 3.2 μg/mL of commercial anti-OVA IgE (Anti-OVA IgE) or neat serum from OVA-sensitized mice (OVA-serum) were injected into the ears of naive recipient mice (n=2). The challenge was performed on day 2, 3, 5 7 or 12, according to the protocols described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge. Two-way ANOVA and Bonferroni post-test, significant difference between anti-OVA IgE and OVA-serum: #$p<0.001$.

Figure 4.6. shows that commercial anti-OVA IgE maintained its anaphylactic activity up to 12 days after been injected on mice ears (day 0). PCA carried out with sera from OVA-sensitised mice were positive only up to 3 days following the serum injection, being all PCA reactions negative after that. These results indicate that the binding of IgE and its high affinity FcεRI lasts for at least 12 days, the longest period tested in the present experiment. This data is in agreement with other studies that showed that the half life of IgE bound to mast

cells was equivalent to 2 weeks (Ishizaka e Ishizaka, 1971). It has also been reported that sensitised skin can remain active for PCA for several weeks (Kulczycki e Metzger, 1974). However, a quantitative study of Fc-IgE showed that the half life of FcR and IgE dissociation is 12 hours. Considering that IgE binds to FcεRI in a reversible manner (Segal, Taurog *et al.*, 1977), it is possible that dissociation and rebinding of IgE to their receptors on mast cells take place during an extensive period of time.

Although anti-OVA IgE antibodies were present in the serum of OVA-allergic animals, the concentration of this class of antibody (585.8 ng/mL, Table 4.1.) was just above the limit of PCA detection (200 ng/mL, Figure 4.1.). Moreover, since OVA-sensitised mice presented other classes of immunoglobulins specific to OVA, this fact could interfere in the anaphylaxis induced by IgE. Evidence that allergen-specific IgG can block IgE-mediated anaphylaxis *in vivo* corroborates with this hypothesis (Strait, Morris *et al.*, 2006). It was shown that cross-linkage of inhibitory FcγRIIb with an activation receptors such as the FcεRI, FcγRI, or FcγRIII can inhibit pro-inflammatory signalling (Daeron, Latour *et al.*, 1995). FcγRIIb and FcγRIII present identical ligand binding properties and co-expression on mast cells (Daeron, 1997; Ravetch e Bolland, 2001; Chong, Andrew Bouton *et al.*, 2003); corroborating the idea that other immunoglobulins present in the serum of OVA-allergic mice interfere in the anaphylactic responses.

Altogether, the findings obtained so far provided a better understanding about classes of immunoglobulins involved in the PCA reactions performed in our facilities. It was clearly shown that passive anaphylaxis can be elicited by IgE as well as by IgG1 antibodies. This information is important to understand the mechanisms involved in the sensitisation protocols performed in our facilities

(discussed in Chapter 5). The differentiation between the two fractions of IgG1, anaphylactic and non-anaphylactic, is important because these immunoglobulins are modulated by different cytokines. Anaphylactic IgG1 is positively regulated by IL-4 and negatively regulated by IL-12 and/or IFN-g, indicating it is involved in a Th2-pattern response. In contrast, the synthesis of IgG1 antibodies that lack anaphylactic activity is IL-4-independent and it is enhanced in the presence of IL-12 or IFN-γ (Faquim-Mauro e Macedo, 2000). The synthesis of the two fractions of IgG1 is regulated by adjuvants. In a model of allergy using OVA, it was shown that animals sensitised with Alum (AlOH3), a Th2 adjuvant, produced mostly the anaphylactic type. When OVA was injected in complete Freund's adjuvant (CFA), it induced mainly the non-anaphylactic type production (Faquim-Mauro, Jacysyn *et al.*, 2003).

It is important to emphasize that anaphylaxis resultant of mast cells activated by IgG1 bound to its Fcγ receptors or activated by IgE bound to FcεR are of similar magnitude. It was shown that FcεRI α chain knock-out mice expressed active systemic anaphylactic responses, associated with extensive mast cell degranulation and a high mortality rate (Miyajima, Dombrowicz *et al.*, 1997). An asthma model using mutant mice that produce all Ig isotypes except IgE, showed that these animals became anaphylactic on antigen challenge and displayed tachycardia and pulmonary function changes similarly to the wild type animals (Oettgen, Martin *et al.*, 1994). Therefore; as the final parameters used to analyse PCA results are mast cell degranulation and the consequent increased blood vessels permeability; it is not possible to separately evaluate the participation of IgG1 or IgE in the passive anaphylaxis model used in the present work. Nevertheless, the most important advantage of passive cutaneous anaphylaxis is that, by evaluating the biological activity of antibodies, it

determinates exclusively anaphylactic immunoglobulins, a very important information in allergy investigations.

2.3. Investigating anaphylactic antibodies in serum of BER-sensitised animals

Previous investigation showed that serum from mice sensitised with rBer e 1 alone does not induce positive PCA reactions and does not contain titers of Ber-specific IgG or IgG1. Conversely, animals sensitised with rBer e 1 in the presence of natural lipids presented positive PCA reactions and increased titers of Ber-specific IgG and IgG1 (Dearman, Alcocer *et al.*, 2007).

The present work aimed to further investigate the role of Brazil nut lipids in a similar protocol of sensitisation to rBer e 1. The main indication of a Th2-driven response was given by the type of humoral response developed (results discussed in Chapter 5), considering mainly the titers of IgE and IgG1, typical Th2 classes of antibodies. If present in detectable levels in the serum, these immunoglobulins can be measured by ELISA; however, as previously mentioned, this techinique does not distinguish between anaphylactic and non-anaphylactic IgG1.

In order to investigate the classes of immunoglobulins associated with positive PCA reactions observed in serum from Ber-sensitised animals, animals were sensitised with Ber e 1 and an immune active fraction of natural lipids (named SPC fraction C, described in Chapters 3 and 6). For the following experiments, these animals were named BER-allergic animals. Sera from these animals were collected, pooled and investigated. The quantity of Ber-specific IgE as well as of Ber-specific IgG1 was first measured by ELISA. The sample of

pooled BER-sera was tested in triplicates in an ELISA plate and the results exposed in the following table (Table 4.2.) represent the average (AV) and standard deviation (SD) of the three replicates tested.

Immunoglobulin	AV ± SD (ng/mL)
Anti-Ber IgE	Not detected
Anti-Ber IgG1	21,050 ± 10,990

Table 4.2.: Concentration of anti-Ber IgE and anti-Ber IgG1 present in pooled serum from 5 BER-allergic mice. Antibodies measurements performed by ELISA according to the described in Chapter 2 (Item 10.2).

Surprinsingly, only titers of Ber-specific IgG1 could be detected in the serum of BER-allergic animals. ELISA was repeated several times, with the same sample of sera as well as with sera from different batches of BER-allergic animals, but no Ber-specific IgE could be detected. ELISA is the most used technique for antibody determination and can detect very low concentrations of antibodies, in the order of nanograms/mL. The sensitivity (nanograms/mL) of our tests is comparable to mouse IgE ELISAs described in the literature. (Morafo, Srivastava *et al.*, 2003; Boehme, Chen *et al.*, 2009). To detect specific titers of IgE, for instance, the antibody capture is usually made by the specific antigen pre-attached to the ELISA plate, followed by the enzyme-linked anti-IgE antibody and substrate (Yamaguchi, Sugita *et al.*, 2006; Wang, Mei *et al.*, 2007; Hashizume, Togawa *et al.*, 2008; Van Ginkel, Iwamoto *et al.*, 2008). Thus, different classes of immunoglobulins that are specific for the same antigen can be also captured, and this competition interferes with detection of allergen-specific IgE, reducing test sensitivity. It was shown that the removal of murine serum IgG antibodies, which may be in more than 100-fold excess to IgE antibodies, enhanced the IgE detection by ELISA (Kadooka, Idota *et al.*, 2000; Lehrer, Reish *et al.*, 2004). Therefore, one possibility for the negative anti-Ber IgE results obtained by ELISA could be the interference of high concentration of anti-Ber IgG1 in the serum. The serum was then tested in a sandwich-type ELISA, in which the plate was coated with anti-IgE antibodies to avoid cross-ligation with

other classes of immunoglobulins. Biotinylated-Ber, enzyme-labeled streptavidin and substrate were used to finalize the reaction, but no IgE detection was obtained. Attempts to measure anti-Ber IgE were also made by microarray technique using high power lasers but the results were unsuccessful.

Moreover, serum from OVA-sensitised animals presented approximately 150,000 ng/mL of OVA-specific IgG1 and 600 ng/mL of OVA-specific IgE (Table 4.1.), detected by ELISA. Therefore, the high concentration of antigen-specific IgG1 (around 8 times higher than in BER-allergic animals) did not impair the detection of antigen-specific IgE. This corroborates the hypothesis that the cutaneous anaphylaxis induced by serum from BER-allergic animals was elicited mainly by anaphylactic IgG1 and not necessarily IgE.

Serum from BER-allergic animals, i.e. animals sensitised with rBer e 1 and lipids from Brazil nuts, was then tested for PCA reactions (Figure 4.7.)

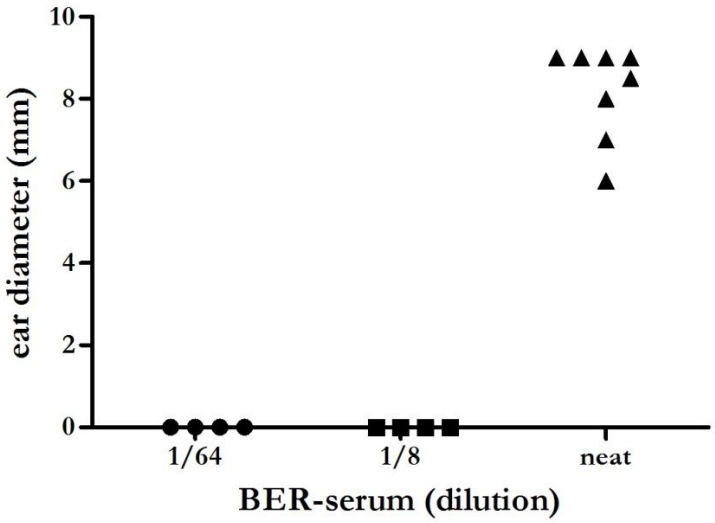


Figure 4.7. Samples of diluted (1/64 or 1/8) or neat serum from BER-allergic animals (BER-serum) were tested. Samples were injected into the ears of naive recipient mice and 2 days later these animals were challenged with BER, Evans blue dye and PBS, according to the procedures described in Material and Methods. The results express the diameter of the cutaneous reactions measured in mm 30 min after the challenge.

Figure 4.7. shows that serum from BER-allergic animals elicited strong positive PCA reactions when it was tested neat. However, after being diluted 8 or 64 times no anaphylaxis was observed. Therefore, serum from animals sensitised with rBer e 1 and SPC lipid fraction C did produce anaphylactic antibodies; however, the concentrations detected in the sera of these animals are not as high as in animals sensitised with OVA (Figure 4.4.). This is in agreement with the levels of antigen-specific IgG1 and IgE obtained by ELISA (Tables 4.1. and 4.2.) and with previous published results (Dearman, Alcocer *et al.*, 2007). However, in order to further check the presence of anaphylactic IgG1 and IgE in the serum of BER-allergic animals, a PCA investigation with heated or protein G-adsorpted serum would have to be attempted; however, these procedures required amounts

of BER-allergic serum unavailable at the time, and, for this reason, these experiments were not carried out.

Altogether, the results obtained in this chapter indicated that IgE as well as IgG1 can elicit positive PCA reactions performed in our facilities. These findings are in agreement with literature data, in which a sub-class of IgG1 is reported to have anaphylactic activities (Inagaki, Nagai *et al.*, 1988; Faquim-Mauro, Jacysyn *et al.*, 2003). Since the present PhD project aimed to evaluate the role of lipids in the development of a Th2 response towards rBer e 1 based mainly on the humoral response presented by sensitised animals, the information obtained in the present Chapter are of great importance. Furthermore, considering that Ber-specific IgE was not detectable by ELISA, a widely used technique to measure antibodies, the PCA results provide the major indication of a Th2-skewed response. In general, as no validated animal model is available to study food allergy, PCA results reveal important information regarding the immune profile presented by sensitised animals.

CHAPTER 5

HUMORAL RESPONSE INDUCED BY rBER e 1 AND LIPIDS

INTRODUCTION

The present chapter describes and discusses the results of a series of experiments, aiming to investigate the humoral response induced by rBer e 1 alone or in the presence of lipids. Different fractions and doses of lipids from Brazil nuts were tested as well as synthetic lipids or lipids extracted from sunflower seeds. rBer e 1 was also tested in the presence of two well established adjuvants: alum ($AlOH_3$), a Th2 adjuvant (Brewer, Conacher *et al.*, 1999; Bortolatto, Borducchi *et al.*, 2008), and LPS, a lipid compound known to interfere in the development of adaptive immune responses (Delayre-Orthez, De Blay *et al.*, 2004; Piggott, Eisenbarth *et al.*, 2005; Tan, Chen *et al.*, 2010).

RESULTS AND DISCUSSION

1. A comparison of 'wild type' Ber e 1 and recombinant Ber e 1 activity in an *in vivo* model of allergy

As previously reported, 'wild type' Ber e 1, which is a mixture of proteins and natural lipids from Brazil nuts, elicited anaphylactic antibody responses in mice; whereas no passive anaphylactic reactions were observed when animals were sensitised with the delipidated wild type or recombinant Ber e 1 (rBer e 1), which is free of lipids. The capacity of inducing anaphylaxis was shown to be related with the presence of natural lipids from Brazil nuts, since the anaphylaxis was re-established when rBer e 1 was used in the presence of Brazil nut lipids (Dearman, Alcocer *et al.*, 2007). The 'wild type' Ber e 1 was composed of 50% protein (Ber e 1) and 50% lipids. The procedure used to isolate and purify 'wild type' Ber e 1 from Brazil nus was previously described (Sun, Altenbach *et al.*, 1987). Recombinant Ber e 1 was produced in *Pichia pastoris* and prepared as previously published (Alcocer, Murtagh *et al.*, 2002). This preparation contained no lipids and it was shown to be biophysically equivalent to the native form of this protein (Alcocer, Murtagh *et al.*, 2002).

For the following experiment, the same extracts as those used by Dearman *et al* 2007 ('wild type" and recombinant Ber e 1) were used in the mouse model selected for this PhD in order to assess both sensitisation protocol and *in vivo* model of anaphylaxis (PCA). Animals were sensitised with 2.5 mg of protein ('wild type" Ber e 1 or rBer e 1) on days 0 and 7, as described in Chapter 2 (Item 7.2.).

The groups used are specified below:

Groups	Sensitisation
wild Ber	'wild type' Ber e 1 (2.5 mg)
rBer	rBer e 1 (2.5 mg)

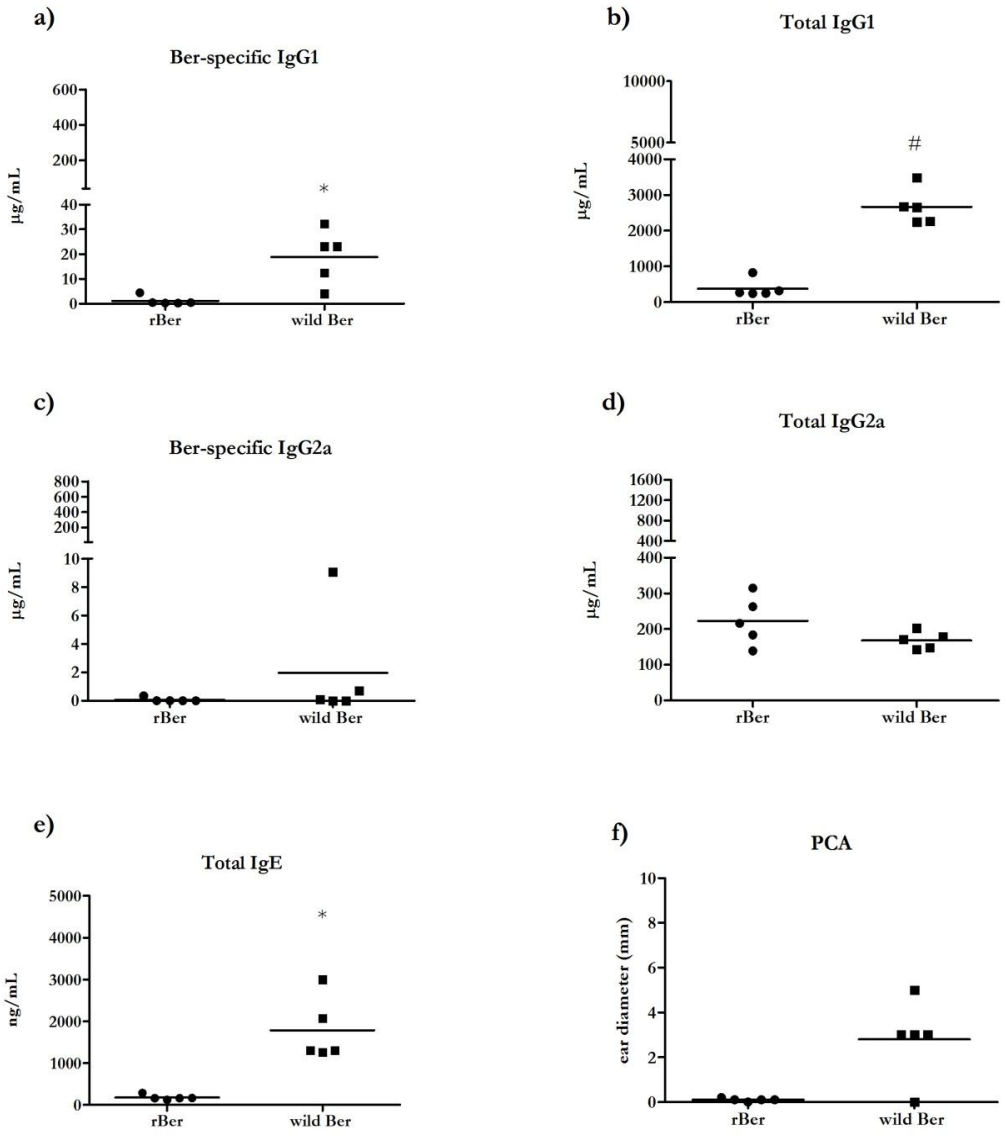


Figure 5.1. Antibody determination. **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in serum from sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols (circles and squares) represent individual animals (n=5). Bars represent groups' means. T-test, significant difference between groups: *$p<0.05$ and #$p<0.001$.

The results expressed on Figures 5.1. (a-f) indicated that animals sensitised with rBer e 1 alone did no present increase in titers of antibodies and negative PCA reactions were observed. An immune activation was observed in the group sensitised with 'wild type' Ber e 1. These animals presented significant higher levels of Ber-specific and total IgG1 (Figure 5.1.a and b), and total IgE (Figure 5.1.e) compared to animals sensitised with rBer e 1. Low titers of Ber-specific IgG2a (Figure 5.1.c) were observed in both groups. Positive cutaneous anaphylaxis was observed only in the 'wild type' Ber e 1 group (Figure 5.1.f).

The first conclusion to be taken is that the protocols used are working accordingly, since the sensitisation protocol was able to induce anaphylactic antibodies production and these antibodies could be detected by PCA. Secondly, no response was detected in the absence of lipids. These results confirm the findings previously published (Dearman, Alcocer *et al.*, 2007), in which serum from BALB/c mice sensitised with rBer e 1 failed to induce PCA reactions and did not present detectable titers of BER-specific IgG and IgG1; while animals sensitised with 'wild type' Ber e 1 presented positive PCA reactions and significant increased titers of BER-specific IgG and IgG1 (Dearman, Alcocer *et al.*, 2007).

Allergic processes are chacterized as Th2-type responses, involving increased production of IgE, IgG1, low IgG2a, and the secretion of cytokines, such as IL-4, IL-5, and IL-13 (Del Prete, 1992; Raz e Spiegelberg, 1999; Maezawa, Nakajima *et al.*, 2003; Lee e Flavell, 2004; Mishra, Rir-Sima-Ah *et al.*, 2008). Therefore, the results presented here indicated that, besides Ber e 1, lipid compounds present in the food matrix of Brazil nuts are necessary for the development of a Th2-type response, characterized by increased levels of IgG1 and IgE, low IgG2a, and positive PCA reactions. Since the lipid fraction present

in the 'wild type' Ber e 1 was composed by a mixture of different classes of lipids, the following experiments aimed to investigate the role of specific classes of lipids in the sensitisation to rBer e 1.

2. Activities of different fractions of natural lipids extracted by TLC compared to synthetic lipids

As discussed in Chapter 3, several lipid classes are present in the the total lipid fraction of Brazil nuts. This experiment aimed to investigate the role of different lipid classes in the development of a Th2-type immune response. For this, the total lipid fraction was fractionated by acetone precipitation and two sub-fractions were obtained. The first sub-fraction, rich in tryglicerides, was attached to rBer e 1 and tested in animals. The second sub-fraction, rich in complex lipids, was re-fractionated by TLC and the new sub-fractions were individually attached to rBer e 1 and tested in animals.

The identified lipid classes present in the TLC sub-fractions tested in this experiment were PC, PE and SL. The activities of equivalent synthetic PC and PE lipid fractions were also tested in order to compare the activity of natural and synthetic lipids.

As the lipid extraction and the protein-lipid attachment were performed in our facilities, this experiment also aimed to evaluate whether the manipulation of lipids and proteins would interfere in the ability of the lipid-protein complexes to induce an immune response.

For the sensitisation, the different fractions of natural and synthetic lipids (600 μg) were individually added to rBer e 1 (2.5 mg), as described in Chapter 2 (Item 7.2.1.). A PCA positive control group was performed, sensitizing

mice with 2.5 mg of rBer e 1 and 150 μg of LPS. The dose of LPS used was lower dose than the dose of lipids due to its endotoxic properties. It is reported as lethal dose of LPS 35 mg/kg body weight, approximately 700 μg per mouse (Jones, Mansell *et al.*, 2007).

The groups used are specified below:

Groups	Sensitisation
rBer	rBer e 1 (2.5 mg)
rBer + nat TG	rBer e 1 (2.5 mg) + 600 μg of natural TG
rBer + nat PC	rBer e 1 (2.5 mg) + 600 μg of natural PC
rBer + synt PC	rBer e 1 (2.5 mg) + 600 μg of synthetic PC
rBer + nat PE	rBer e 1 (2.5 mg) + 600 μg of natural PE
rBer + synt PE	rBer e 1 (2.5 mg) + 600 μg of synthetic PE
rBer + nat SL	rBer e 1 (2.5 mg) + 600 μg of natural SL
Ber + LPS	rBer e 1 (2.5 mg) + 150 μg of LPS

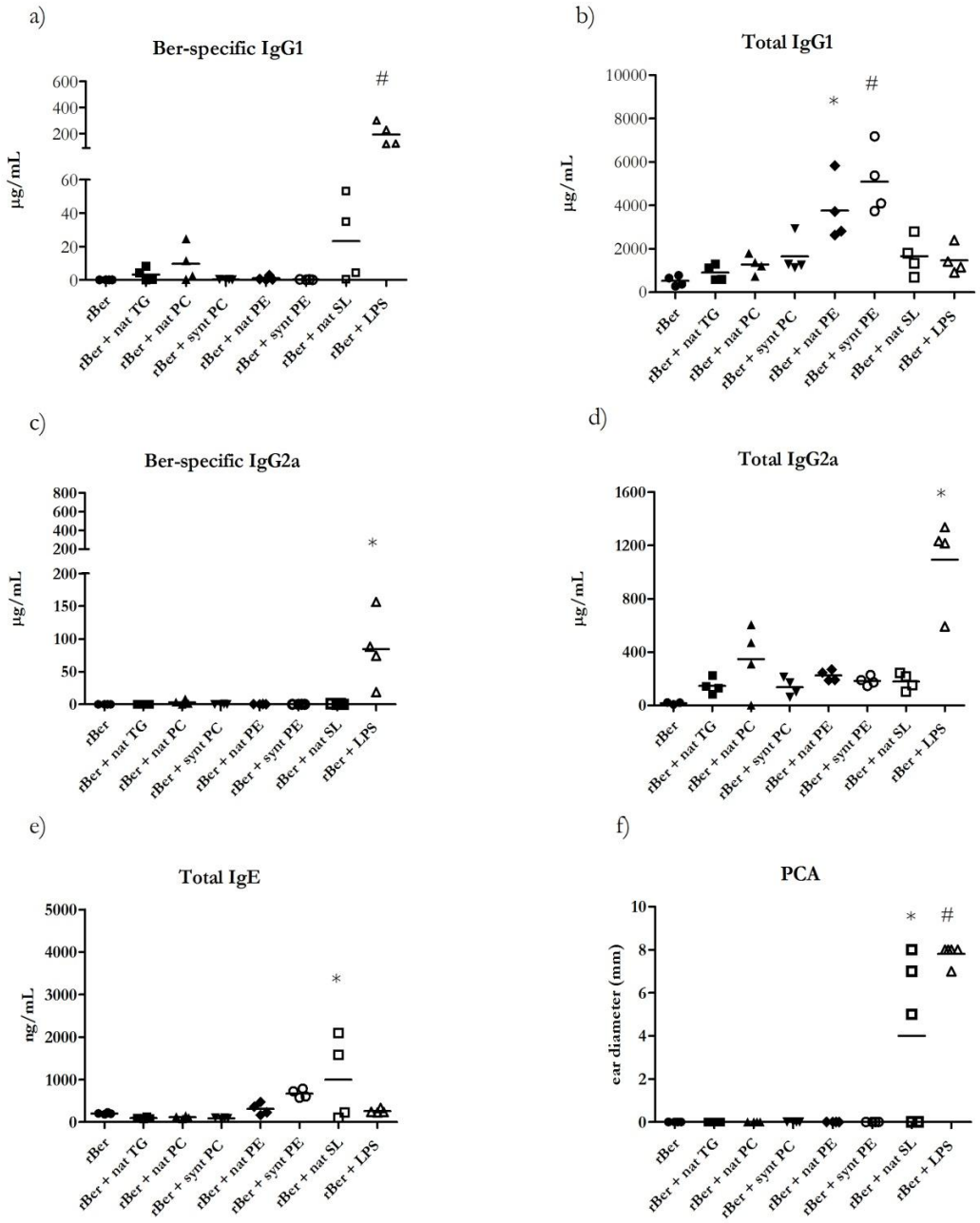


Figure 5.2. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=4). Bars represent groups' means. One-Way ANOVA and Tukey's post test. Significant difference compared to control (rBer): *$p<0.05$ and #$p<0.001$.

As shown in Figure 5.2. (a-f), animals sensitised with rBer e 1 alone did not show increase in antibodies titers or positive PCA reactions, confirming, once again, previous findings (Dearman, Alcocer *et al.*, 2007).

The fraction rich in TG did not induce immune activation, since animals sensitised with rBer e 1 and TG presented a humoral response similar to animals sensitised with the protein alone.

The other lipid fractions tested, natural or synthetic, produced different patterns of immune activation. The group sensitised with rBer e 1 and natural PC presented significantly increased titers of total IgG2a (Figure 5.2.d) and slight, but not significant, increased titers of Ber-specific IgG1 (Figure 5.2.a) when compared to the control group, treated with rBer e 1 alone. Animals sensitised with rBer e 1 and the synthetic PC did not present these differences. No differences were found in the titers of other antibodies tested (Figure 5.2.b, c and e), and no positive PCA reactions were observed in both natural and synthetic PC groups (Figure 5.2.f).

The groups sensitised with natural or synthetic PE presented significantly increased titers of total IgG1 (Figure 5.2.a and b) and slight, but not significant, increased titers of IgE (Figure 5.2.e). No differences were found in the titers of other antibodies tested (Figure 5.2.a, c, d and e), and no positive PCA reactions were observed (Figure 5.2.f) for both natural and synthetic PE groups.

The group sensitised with rBer e 1 and natural SL presented significantly increased titers of total IgE (Figure 5.2.e), and increased, but not significant, titers of specific IgG1 (Figure 5.2.a). This group presented positive PCA reactions, indicating the presence of anaphylactic antibodies in the serum of these animals

(Figure 5.2.f). No differences were found in the titers of others antibodies tested (Figure 5.2.b, c and d).

In the positive control group, rBer+LPS, it was observed a strong immune activation, with significant increase in titers of all classes of antibodies tested, except total IgG1 (Figure 5.2. a-e). Although the total IgE titer was not increased in this group, PCA reactions were strongly positive (Figure 5.2.f). As discussed in Chapter 4, these results are in agreement with the literature, in which not only IgE, but also anaphylactic IgG1 are able to induce anaphylactic reactions (Inagaki, Nagai *et al.*, 1988; Hazenbos, Gessner *et al.*, 1996; Faquim-Mauro, Coffman *et al.*, 1999; Faquim-Mauro e Macedo, 2000; Silva, Casabuono *et al.*, 2008). Several studies report the role of LPS in the development of allergy (Eisenbarth, Piggott *et al.*, 2002; Hollingsworth, Whitehead *et al.*, 2006; Bortolatto, Borducchi *et al.*, 2008; Dong, Li *et al.*, 2009); however, the role of LPS in the sensitisation to rBer e 1 will be discussed in more details in the following sections.

Comparing natural and synthetic lipids, their activities appeared to be different for PC but no differences were found between natural and synthetic PE. As the immune activation induced by these classes of lipids was not clearly evident, no important conclusions can be taken in this respect so far. In the literature, there is indication that natural and synthetic lipids play different roles in the immune system. In schistosome infections, schistosomal but not synthetic lyso-PS induced the development of IL-10-producing regulatory T cells (Van Der Kleij, Latz *et al.*, 2002). Differently, other study showed that synthetic PC-containing molecules had *in vitro* immunomodulatory effects similar to those induced by the native molecule, extracted from the parasite *Ascaris suum* (Kean, Ohtsuka et al., 2006).

Of the TLC prepared Brazil nut fractions, the SL-rich fraction was the only natural lipid that induced positive PCA reactions. Thus, amongst the tested fractions, SL appears to be the only involved with the development of allergic responses. Results previously published in Brazil nuts showed that a natural lipid fraction containing SL, PC, PE and PI induced anaphylactic antibodies productions (Dearman, Alcocer *et al.*, 2007), corroborating the immune activity of SL. However, since other classes of lipids were present in this fraction, more investigations would be necessary to conclude that the anaphylactic activity was definitely induced by SL. Here, no evident immune activation was observed when natural fractions containing PC or PE were tested. However, these results could suggest that natural PC and PE lack immune activity or that the TLC extraction altered lipid properties. Considering that TLC is a multi-step technique, it can result in lipid oxidation as well as in the production of undesirable artefacts. Since different lipid classes present distinct physical and chemical properties, the procedures such as lipid extraction and lipid-protein binding can work differently for particular classes of lipids.

Another disadvantage of using TLC for lipid extraction is that only very small quantities of lipids could be prepared. In order to circumvent these limitations, a different procedure was identified to more efficiently obtain separated classes of lipids and therefore, for the following experiments, lipid fractions were obtained by solid phase chromatography (SPC).

3. Activities of different fractions of Brazil nut natural lipids extracted by SPC

SPC is more efficient than TLC because higher quantities of lipids are obtained in a shorter period of time, avoiding lipid oxidation and the production of artefacts (Christie, 1992). As considerable amounts of lipids were necessary to perform animal experiments and lipid analyses, this technique was used to obtain lipid fractions from Brazil nuts for the following experiments.

The total fraction of Brazil nuts (fraction T) was fractionated by SPC, and from the several sub-fractions obtained; three were chosen to be tested in this experiment. The three SPC fractions used were: SPC fractions L, C and M.

Therefore, animals were sensitised with rBer e 1 (2.5 mg) and 600 μg of different lipid fractions (Fraction T, SPC fractions L, C and M), following the sensitisation protocol described in Material and Methods (Chapter 2, Item 7.2.). For this experiment a group of naive, i.e. not manipulated animals, was included.

The groups used are specified below:

Groups	Sensitisation
Naive	None
rBer	rBer e 1 (2.5 mg)
rBer + T	rBer e 1 (2.5 mg) + 600 μg of fraction T
rBer + L	rBer e 1 (2.5 mg) + 600 μg of SPC fraction L
rBer + C	rBer e 1 (2.5 mg) + 600 μg of SPC fraction C
rBer + M	rBer e 1 (2.5 mg) + 600 μg of SPC fraction M

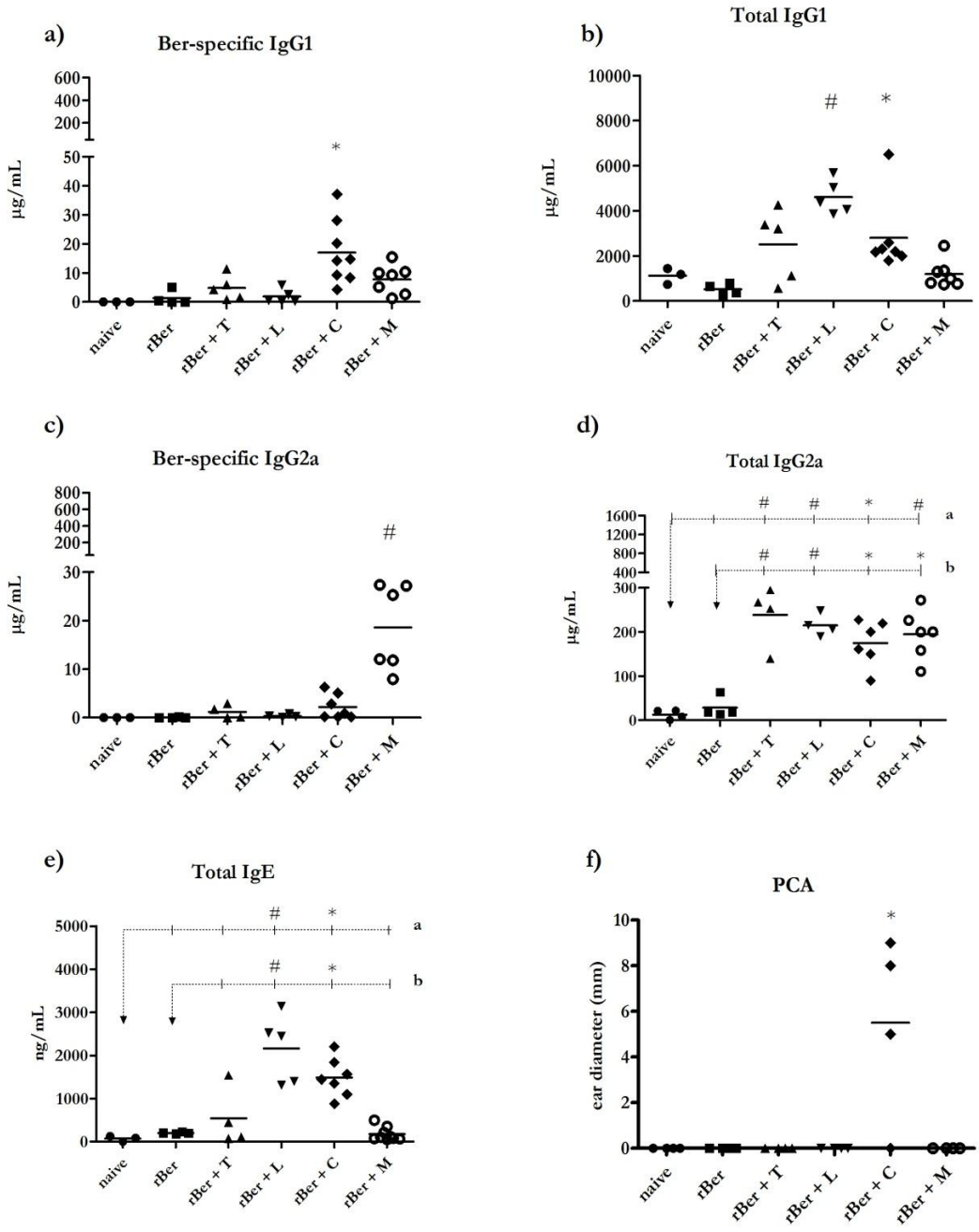


Figure 5.3. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of naive or sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of naive or sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=3 to 9). Bars represent groups' means. One-way ANOVA and Turkey's post test. Significant difference compared to naive or rBer groups: *$p<0.05$ and #$p<0.001$. Line a: comparison with naive group; Line b: comparison with rBer group.

The results presented in Figure 5.3. (a-f) showed that animals sensitised with the protein alone (rBer e 1) had the same pattern of responses to those observed in naïve animals, i.e., there were no significant changes in levels of antibodies produced. Once again, these data confirmed the previous observations that natural lipids are necessary for the development of an immune reaction to rBer e 1 (Dearman, Alcocer *et al.*, 2007).

Lipid fraction T, which represented the total lipid fraction of Brazil nuts, induced significantly increased titers only of total IgG2a (Figure 5.3.d). No differences were found in titers of the other antibodies tested and no positive PCA was observed (Figure 5.3.f). This fraction was shown to contain mainly simple lipids, such as tryglicerides, but other classes of lipids were also identified, such as PA, PC, PI, PG, PE, SQDG, and MGDG (Chapter 3, Figure 3.1. and Table 3.1). These results differ from previous findings, in which the total lipid fraction of Brazil nuts was able to induce the production of anaphylactic antibodies in 7 out of 9 sensitised animals (Dearman, Alcocer *et al.*, 2007). However, the total lipid fraction used by those investigators presented reduced levels of triglycerides, altering, therefore, the final concentration of other lipid classes present in the fraction. This difference could be the explanation for the different results observed here.

SPC lipid fraction L presented increased titers of total IgG1, total IgG2a and total IgE (Figure 5.3. b, d and e), but no differences were found in the titers of Ber-specific IgG1 or IgG2a (Figure 5.3.a and c) when compared to rBer e 1 group. Despite the increased titers of IgE, negative PCA reactions were observed (Figure 5.3.f), indicating that these immunoglobulins were not Ber-specific.

Therefore, this fraction induced a non-specific immune activation, with increased titers of total antibodies but no differences in Ber-specific antibodies.

SPC fraction M induced the production of Ber-specific and total IgG1, presenting significant high titers of these immunoglobulins (Figure 5.3.c and d). Total IgG2a was also significantly increased (Figure 5.3.d), but no other differences were observed.

SPC lipid fraction C induced significantly higher titers of Ber-specific and total IgG1 than other lipid fractions (Figure 4.3.a and b). Animals did not present increased Ber-specific IgG2a, although the titers of total IgG2a were significantly augmented (Figure 5.3.c and d). SPC fraction C also induced a significant increase in the titers of IgE (Figure 5.3.e), and most importantly, this was the only fraction able to induce positive PCA reactions (Figure 5.3.f)

Altogether, these results indicate that different fractions of lipids induced distinct patterns of immune activation. Although nearly all lipid fractions induced increased levels of total immunoglobulins, such as IgG1 or IgG2a; only fractions C and M induced a more specific immune activation. Fraction C favoured a Th2-type immune response, with high titers of specific IgG1 and total IgE, low titers of specific IgG2a and the presence of anaphylactic antibodies confirmed by the positive PCA reactions. This pattern of humoral response is observed in murine models of allergy, in which animals present high levels of IgE and IgG1, and low levels of IgG2a (Keller, Mucida *et al.*, 2006; Barbas, Downing *et al.*, 2008). In contrast, lipid fraction M induced low titers of IgG1 and IgE, high titers of IgG2a and absence of anaphylactic antibodies (negative PCA), a Th1-skewed response (Hochreiter, Ferreira *et al.*, 2003; Takeno, Yoshikawa *et al.*, 2004). Therefore, the lipid fraction most likely to be involved in the sensitisation to rBer e 1 was the SPC fraction C.

The contents of SPC lipid fractions C and M were analysed by TLC (as described in Chapter 3) and, although triglycerides (TG) was the most abundant lipid class present in both fractions, they differed in other components. The lipid classes SL, LPC, PC, PI, PA, and SG were putatively identified in the fraction C, whereas SL, PC, PE, SG, and ESG were the putative fractions present in SPC fraction M. Therefore, we could speculate that the active lipid(s) were the ones present exclusively in the SPC fraction C. In the literature, different lipid classes of complex lipids are shown to interact with adaptive immune responses. Lyso-PS from schistosome, for instance, was shown to affect immune polarization through TLR-2 (Van Der Kleij, Latz *et al.*, 2002); and a phosphorylcholine-containing molecule (ES-62) present in nematodes was reported tosupport Th2 immunological phenotype though TLR-4 (Harnett e Harnett, 2009). LPC was shown to promote mature dendritic cell generation (Coutant, Perrin-Cocon *et al.*, 2002) and act as adjuvant triggering cellular and specific humoral immunity (Perrin-Cocon, Agaugue *et al.*, 2006; Bach, Perrin-Cocon *et al.*, 2010). However, SPC fraction C was extracted from the total fraction of Brazil nuts lipids (fraction T) and the pattern of immune response induced by these fractions was different. Suggesting, therefore, that not only the class, but also the concentration of lipids may be important in determining the course of the immune response generated.

4. Activity of SPC lipid fraction C in different concentrations

As the results from the previous experiment indicated that the doses of lipids used to sensitize animals in the presence of rBer e 1 might interfere in the type of immune response developed, the present experiment aimed to evaluate the immune response induced by different doses of SPC lipid fraction C.

For this, animals were sensitised with rBer e 1 (2.5 mg) and different doses of the SPC lipid fraction C, according to the protocol described in Chapter 2 (Item 7.2.). The doses of lipids used were: 15 μg, 150 μg, 600 μg, 1,500 μg or 3,750 μg.

The groups used are specified below:

Groups	**Sensitisation**
rBer	rBer e 1 (2.5 mg)
rBer + 15C	rBer e 1 (2.5 mg) + 15 μg of SPC fraction C
rBer + 150C	rBer e 1 (2.5 mg) + 150 μg of SPC fraction C
rBer + 600C	rBer e 1 (2.5 mg) + 600 μg of SPC fraction C
rBer + 1500C	rBer e 1 (2.5 mg) + 1,500 μg of SPC fraction C
rBer + 3750C	rBer e 1 (2.5 mg) + 3,750 μg of SPC fraction C

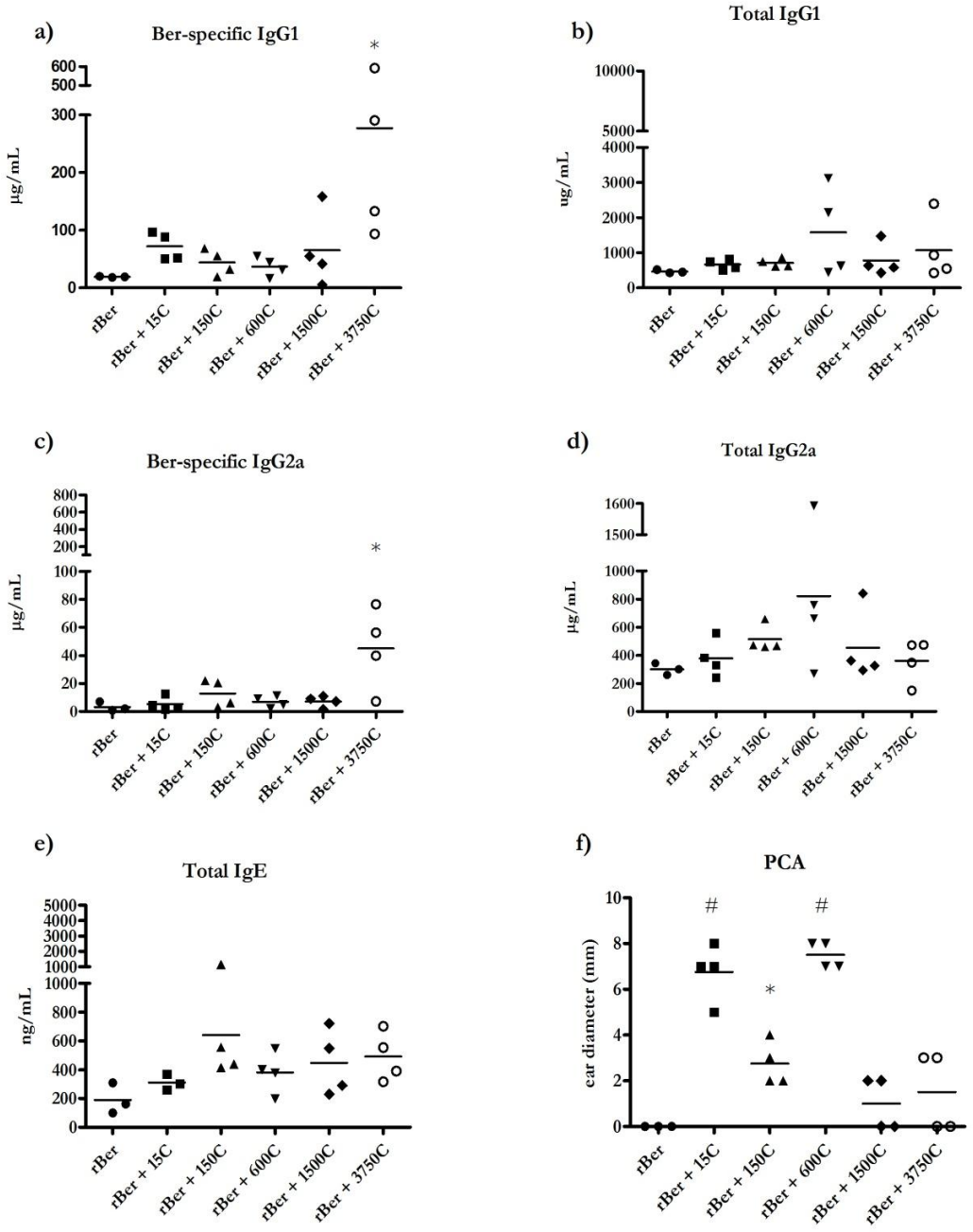


Figure 5.4. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=3 or 4). Bars represent groups' means. One-way ANOVA and Tukey's post test. Significant difference compared to rBer group: *$p<0.05$ and #$p<0.001$.

Figure 5.4. (a-f) showed that the lipid doses varying from 15 μg to 1,500 μg induced similar immune activation. The concentration of Ber-specific IgG1 in these groups was slightly higher than the control group (rBer e 1 alone), but this difference was not significant (Figure 5.4.a). No significant differences were observed in the titers of total IgG1, Ber-specific and total IgG2a compared to the control group. The total IgE was slightly, but not significantly, augmented compared to the control group. However, different concentrations of lipids induced different PCA results (Figure 5.4.f). While lower doses of SPC fraction C (15 and 600 μg) induced clear positive passive anaphylactic reactions, this was not observed when higher dose of lipids were used (1,500 μg).

The group immunized with rBer e 1 and high dose of SPC fraction C (3,750 μg) presented an evident immune activation. Significant increases in the levels of Ber-specific IgG1 and IgG2a, and slight increase in the total IgE were observed in these animals (Figure 5.7.a and c). However, this group presented weak PCA results (Figure 5.7.f): two negative reactions (0 mm) and two weakly positive reactions (3 mm). Considering PCA is an *in vivo* techinique, the animal variation has always to be considered. However, as discussed in Chapter 4, IgG2a can directly inhibit mast cell and basophil degranulation by triggering inhibitory signal transduction by means of inhibitory FcγRII receptors (Daeron, Latour *et al.*, 1995; Takai, Ono *et al.*, 1996), suggesting why these animals presented weak PCA reactions. Furthermore, since PCA reactions can be triggered by antigen-specific IgE as well as by antigen-specific IgG1 (Inagaki, Nagai *et al.*, 1988), positive PCA could be expected in this group. However, the weakly positive PCA could be explained if the IgE antibodies detected were not Ber-specific and the Ber-specific IgG1antibodies were non-anaphylactic (Faquim-Mauro, Jacysyn *et al.*, 2003). Thus, the weak PCA reactions observed in mice sensitised with high dose

of lipids (3,750 μg) could be interpreted as the presence of low concentration of specific anaphylactic antibodies, or as the competition with the high concentration of Ber-specific IgG2a.

Altogether, these results indicate that the concentration of lipids was important for the development of the immune reaction. While doses varying from 15 to 600 μg of SPC fraction C induced similar humoral response, characterized by increased levels of Ber-specific IgG1, low levels of Ber-specific IgG2a and presence of anaphylactic antibodies; higher lipid concentrations induced strong immune activation, with increased titers of Ber-specific IgG1 and IgG2a, but negative PCA. However, based on these data, it is not possible to conclude whether the different patterns of immune response were consequence of different concentrations of one particular lipid (the putative immune active lipid fraction) or due to the increasing amounts of lipids to which animals were exposed to.

In order to investigate possible classes of lipids present in the SPC fraction C involved immune activation observed, this fraction was further fractionated by HPLC and the obtained sub-fractions were tested in the animal protocol of sensitisation used in the present work.

5. Activities of sub-fractions of lipids extracted from SPC fraction C by HPLC

Of the three SPC fractions tested, fraction C was shown to be the only fraction able to induce the production of anaphylactic antibodies (Figure 5.3.). It was shown that the lipid concentration was important to direct the immue response (Figure 5.4.). This experiment aimed to identify whether specific lipid

classes present in the SPC fraction C were responsible for the Th2-type response observed.

For this, the SPC fraction C was fractionated into 22 sub-fractions by HPLC (HPLC sub-fractions C). The putative identification of the lipid classes present in the 22 sub-fractions was performed by comparison with literature data and the sub-fractions were grouped according to it (as described in Chapter 3, Item 2.3.). HPLC sub-fractions C 1-5 contained mainly TG, 6-8 contained ASG, 9-11 contained SG, 12-14 could not be identified, 15-17 contained SL, and 18-22 contained DGDG and/or PE. Therefore, six groups of lipids were tested: HPLC sub-fractions C 1-5, 6-8, 9-11, 12-14, 15-17 and 18-22.

Animals were sensitised with rBer e 1 (2.5 mg) and 600 μg of pooled HPLC sub-fractions C, following the sensitisation protocol described in Chapter 2 (Item 7.2.).

The groups used are specified below:

Groups	**Sensitisation:**
rBer	rBer e 1 (2.5 mg)
rBer + C(1-5)	rBer e 1 (2.5 mg) + 600 μg of pooled HPLC sub-fractions C 1-5
rBer + C(6-8)	rBer e 1 (2.5 mg) + 600 μg of pooled HPLC sub-fractions C 6-8
rBer + C(9-11)	rBer e 1 (2.5 mg) + 600 μg of pooled HPLC sub-fractions C 9-11
rBer + C(12-14)	rBer e 1 (2.5 mg) + 600 μg of pooled HPLC sub-fractions C 12-14
rBer + C(15-17)	rBer e 1 (2.5 mg) + 600 μg of pooled HPLC sub-fractions C 15-17
rBer + C(18-22)	rBer e 1 (2.5 mg) + 600 μg of pooled HPLC sub-fractions C 18-22

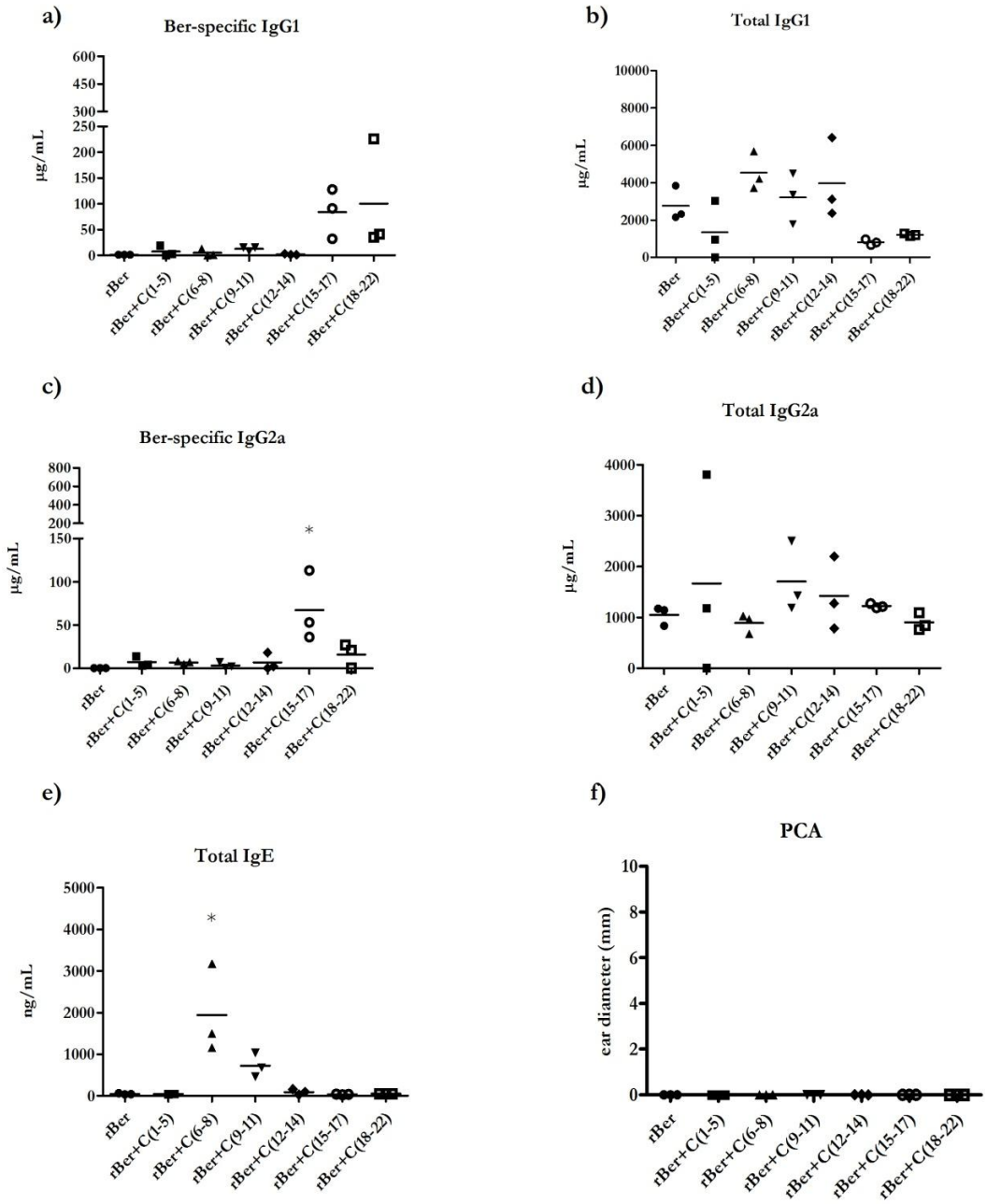


Figure 5.5. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=3). Bars represent groups' means. One-way ANOVA and Tukey's post test. Significant difference compared to rBer group: *$p<0.05$.

The results presented in Figure 5.5. (a-f) showed that animals sensitised with rBer e 1 alone did not present detectable titers of Ber-specific antibodies and no positive PCA reactions were observed, confirming previous results (Figures 5.1. to 5.4.) and literature data (Dearman, Alcocer *et al.*, 2007).

The titers of antibodies induced by HPLC sub-fractions C 1-5 (TG) were similar to the observed in the rBer e 1 group. Therefore, no detected immune activation was induced by these sub-fractions. The HPLC sub-fractions C 6-8 (ASG) induced significantly increased titers of total IgE (Figure 5.5.e), but no differences were observed in the titers of other immunoglobulins. HPLC sub-fractions C 9-11 (SG) did not induce increase in the titers of antibodies, except a slight increase observed in the titers of total IgE (Figure 5.5.e). HPLC sub-fractions C 12-14 did not induce any relevant increase in antibodies titers. HPLC sub-fractions C 15-17 (SL) induced increased titers of Ber-specific IgG1 and Ber-specific IgG2a (Figure 5.5.a and c) but the difference was significant only for IgG2a. No differences were found in the titers of other antibodies. Finally, the HPLC sub-fractions C 18-22 (DGDG and/or PE) did not induce increase in the titers of antibodies, except a slight increase observed in the titers of Ber-specific IgG1 (Figure 5.5.a). None of the groups presented anaphylactic antibodies, since the PCA reactions were all negative.

Therefore, only two fractions induced significantly high levels of antibodies. The fraction containing ASG (6-8) induced increased IgE and the fraction containing SL (15-17) induced increased Ber-specific IgG1 and IgG2a. There are no previous results on the activity of ASG classs of lipid; however, a TLC-extracted lipid fraction containing SL induced increased titers of IgG1, IgE and positive PCA reactions (Figure 5.2.). Therefore, although the HPLC sub-fractions 15-17 (SL) did not induce positive PCA, the increased concentration of

antibodies observed could indicate that this class of lipid might be involved with immune activation.

Altogether, these results showed that although some sub-fractions were able to induce high levels of particular classes of antibodies, no clear pattern of immune activation could be identified. Furthermore, despite the fact SPC fraction C was able to induce the production of anaphylactic antibodies, causing positive PCA reactions (Figure 5.3.f); none of its sub-fractions was able to produce a similar effect. The frst possibility to be considered is that the lipids were affected by the HPLC procedure, as discussed in Chapter3 (Figure 3.7.). Since several HPLC runs had to be performed in order to obtain enough amounts of lipids for this experiment, this could have facilitated the occurrence of undesired contaminations, selective binding to the HPLC column, or lipid oxidation. In order to eliminate this possibility, a group receiveing all HPLC-sub-fractions in the same proportion as present in the original SPC fraction C would be necessary; however, due to the limited amount of lipids extracted by HPLC, this group could not be performed. Other explanation for these results could be a possible synergistic effect between two or more sub-fractions of lipids. Finally, as previously mentioned, the concentration of specific lipid classes might play a role in the development of immune reactions. This possibility is in agreement with the results obtained from animals sensitised with rBer e 1 and the total lipid fraction from Brazil nuts (fraction T, Figure 5.3.), in which all lipid classes were present and no anaphylactic antibodies were produced. And also in agreement with the previous experiment, in which animals receiving different concentrations of SPC fractio C presented different humoral response towards rBer e 1.

In order to further investigate the presence of potential biologically active lipid classes in the SPC fraction C, a different method was used to its fractionation, named acetone precipitation.

6. Activities of sub-fractions of lipids extracted from SPC lipid fraction C by acetone precipitation

Since none of the HPLC sub-fractions extracted from SPC fraction C induced the production of anaphylactic antibodies, SPC fraction C was fractionated by acetone precipitation, resulting in two sub-fractions: pellet (phospholipid-rich fraction) and supernatant (triglycerides-rich fraction). It is important to mention that SPC fraction C consisted of 0.1% of pellet and 99.9% of supernatant.

Using these two sub-fractions (pellet and supernatant), animals were sensitised with rBer e 1 (2.5 mg) and 600 μg of lipids. Two control groups were added for this experiment. One group was sensitised with the rBer e 1 and the whole lipid fraction C, working as a positive control group. The other group was included as a control for the acetone precipitation technique, with both fractions extracted from fraction C (pellet and supernatant) being reconstituted together in a similar proportion as present in the original fraction C. Animals were sensitised as described in Chapter 2 (item 7.2.).

The groups used are specified below:

Groups	Sensitisation
rBer	rBer e 1 (2.5 mg)
rBer + C	rBer e 1 (2.5 mg) + 600 μg of SPC fraction C
rBer + Cpell	rBer e 1 (2.5 mg) + 600 μg of pellet from SPC fraction C
rBer + Csup	rBer e 1 (2.5 mg) + 600 μg of supernatant from SPC fraction C
rBer + Cpell + Csup	rBer e 1 (2.5 mg) + 0.6 μg of pellet + 599.4 μg of supernatant from SPC fraction C

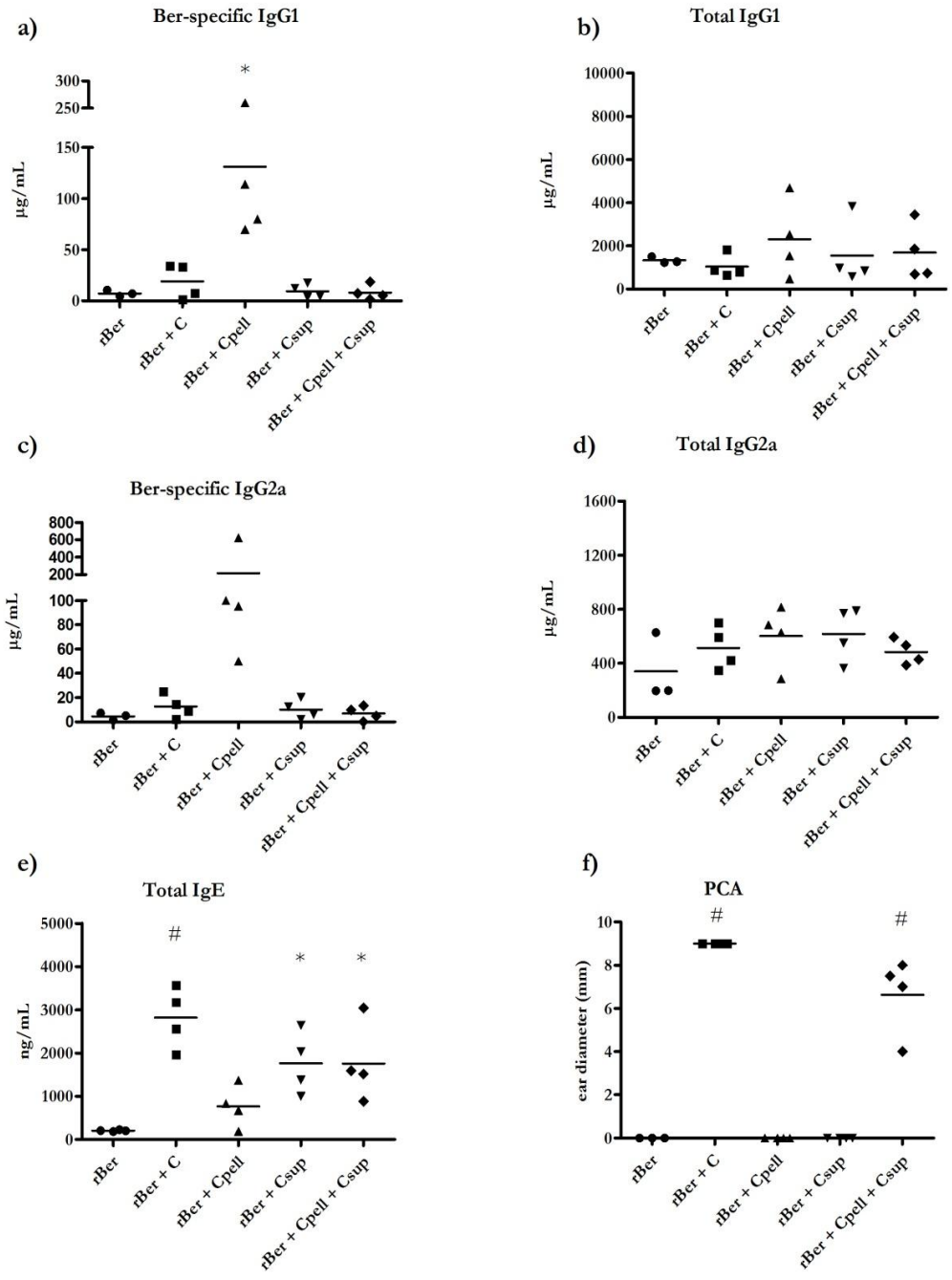


Figure 5.6. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=3 or 4). Bars represent groups' means. One-way ANOVA and Tukey's post test. Significant difference compared to rBer group: *$p<0.05$ and #$p<0.001$.

The first observation in Figure 5.6. was that, once again, the presence of natural lipids was essential for an immune activation. Animals sensitised with rBer e 1 alone did not present increased titers of antibodies and negative PCA reactions were observed.

Secondly, it was observed that the positive control group (rBer + C) worked well. Animals sensitised with rBer e 1 and SPC lipid fraction C induced similar immune activation as found in the previous experiment (Figure 5.3.). This fraction induced a slight but not significant increase in the levels of Ber-specific IgG1 (Figure 5.6.a), reaching values similar to the observed in the previous experiment, an average of 18.9 $\pm$ 16.9 μg/mL. Low titers of Ber-specific IgG2a (Figure 5.6.c) and significantly increased titers of total IgE (Figure 5.6.e) were observed. SPC fraction C also induced positive PCA reactions (Figure 5.6.f).

The group used to control the process of lipid fractionation (rBer+Cpell+Csup) showed similar immune activation to the positive control (group rBer+C). This group presented significant increased titers of total IgE (Figure 5.6.e), low titers of Ber-specific IgG2a (Figure 5.6.c), and positive PCA reactions (Figure 5.6.f). Therefore, indicating that the procedure used to extract the two sub-fractions from SPC fraction C did not alter the main features of the lipids.

The pellet fraction extracted from SPC fraction C induced an evident immune activation. Animals from this group (rBer+Cpell) presented significantly increased titers of Ber-specific IgG1 and Ber-specific IgG2a (Figure 5.6.a and c). The total titers of IgE were also increased when compared to rBer e 1 group (Figure 5.6.e), but no statistical difference was found. Yet despite the high titers of Ber-specific IgG1 and total IgE present in this group, no positive anaphylactic response was observed (Figure 5.6.f). As mentioned before, this could be due the

inhibitory function that IgG2a antibodies play in the mast cell degranulation (Daeron, Latour *et al.*, 1995; Takai, Ono *et al.*, 1996), or due to the lack of anaphylactic IgG1 (Faquim-Mauro, Coffman *et al.*, 1999)and Ber-specific IgE.

In the animals sensitised with rBer e 1 and the supernatant extracted from SPC fraction C (rBer+Csup), the only class of antibody significantly increased was total IgE (Figure 5.6.e). However, PCA reactions were negative (Figure 5.6.f), indicating that the detected total IgE antibodies were not Ber-specific. Therefore, triglycerides, the main component of the SPC fraction C (99.9%), were not able to induce a detectable immune activation. This confirms previous results, in which triglycerides extracted from the total lipid fraction from Brazil nuts did not induce immune activation (Figure 5.2.).

The main conclusions from this experiment were that the SPC fraction C was confirmed to be able to induce anaphylactic antibodies and that the two sub-fractions from SPC fraction C (pellet and supernatant) used separately did not present this property. However, when these two sub-fractions were reconstituted and added to rBer e 1, the PCA reactions were strongly positive, showing that the fractionation process did not affect the immune potential of the lipids tested. Two hypotheses could explain this phenomenon: (i) there might be a synergistic effect and certain classes of lipids present in the SPC fraction C need to interact to be able to stimulate the immune system; or (ii) there is a dose response effect. Since SPC fraction C is composed by 99.9% of supernatant and 0.1% of pellet, animals sensitised with 600 μg of fraction C receive 599.4 μg of supernatant and 0.6 μg of pellet. When performing this experiment, the rBer+Cpell group received 600 μg of pellet, a thousand times more than the quantity in the SPC fraction C. The second hypothesis would also explain the pronounced immune activation observed in the rBer+Cpell group, since these

animals received very high concentrations of the phospholipid-rich fraction. In addition, if the second hypothesis is true, the lipids responsible for the immune activation observed in the SPC fraction C are likely to be present in the pellet fraction, which is rich in phospholipids. This is because the dose of supernatant used for the rBer+Csup group was similar to the concentration present in the original SPC fraction C and no anaphylactic antibodies were observed in this group.

7. rBer e 1 in the presence of LPS, Alum or sunflower lipids

The following experiments were performed in order to investigate the humoral response induced by rBer e 1 in the presence of different compounds and then compare with the effects induced by natural lipids from Brazil nuts. The compounds chosen to be tested were LPS, which has been reported to be involved with Th1 as well as Th2 responses (Eisenbarth, Piggott *et al.*, 2002; Williams, Ownby *et al.*, 2005; Hollingsworth, Whitehead *et al.*, 2006), alum, which is a Th2-adjuvant (Brewer, Conacher *et al.*, 1999; Bortolatto, Borducchi *et al.*, 2008), and lipids from sunflower seeds, edible seeds whose overall incidence of allergy is very low (Alcocer, Murtagh *et al.*, 2002).

7.1. Activity of different concentrations of LPS in the sensitisation to rBer e 1

Evidences of the role of LPS in modulating Th2 immunity is robust, although the data in this area is still controversial (Williams, Ownby *et al.*, 2005). It has been reported that the pattern of immune response in the presence of LPS

is dose-dependent (Eisenbarth, Piggott *et al.*, 2002; Delayre-Orthez, De Blay *et al.*, 2004; Dong, Li *et al.*, 2009).

This experiment was performed in order to observe the effect of different doses of LPS in the antibody production of rBer sensitised animals. For this, animals were sensitised with rBer e 1 in the presence of different doses of LPS (0.06 μg, 0.6 μg, 6 μg, 60 μg or 150 μg), according to the protocol described in Chapter 2 (Item 7.3.)

The groups used are specified below:

Groups	**Sensitisation**
rBer	rBer e 1 (2.5 mg)
rBer + LPS 0.06	rBer e 1 (2.5 mg) + 0.06 μg of LPS
rBer + LPS 0.6	rBer e 1 (2.5 mg) + 0.6 μg of LPS
rBer + LPS 6	rBer e 1 (2.5 mg) + 6 μg of LPS
rBer + LPS 60	rBer e 1 (2.5 mg) + 60 μg of LPS
rBer + LPS 150	rBer e 1 (2.5 mg) + 150 μg of LPS

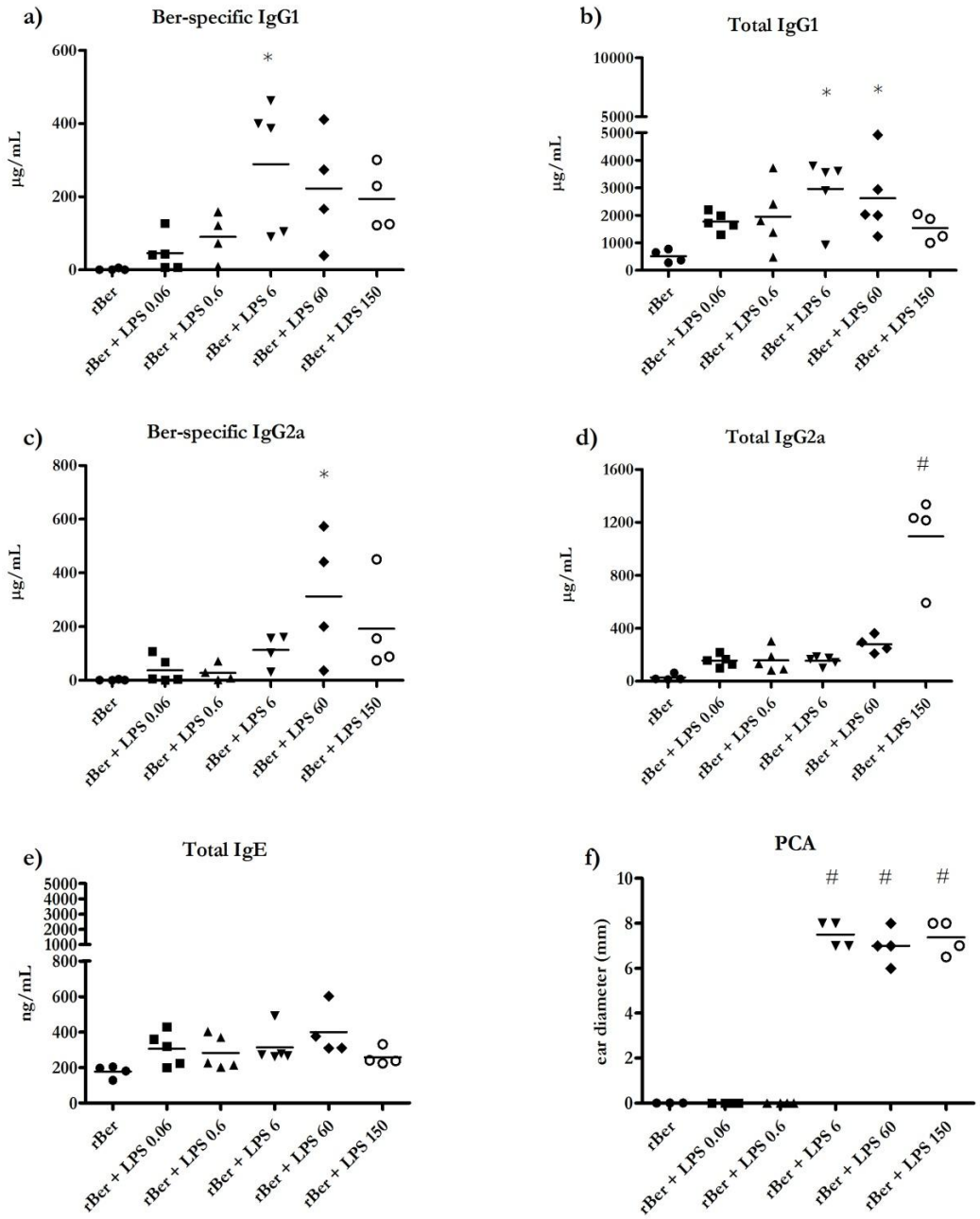


Figure 5.7. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=4). Bars represent groups' means. One-way ANOVA and Tukey's post test. Significant difference compared to rBer group: *$p<0.05$ and #$p<0.001$.

Figure 5.7. (a-f) shows that the groups sensitised with rBer e 1 and low doses of LPS (0.06 μg and 0.6 μg) did not present significant differences in antibody production compared to the control group (rBer e 1 alone). No anaphylactic antibodies were detected by PCA.

The group rBer+LPS6 (6 μg of LPS) presented significant increase in the titers of Ber-specific and total IgG1 (Figure 5.7.a), but no significant differences were found in the titers of IgG2a or IgE. Positive PCA reactions were observed (Figure 5.7.f).

The groups sensitised with rBer e 1 and higher doses of LPS (60 μg or 150 μg) presented increased titers of Ber-specific IgG1 (Figure 5.7.a) but this increase was not significantly different from the control group (rBer). The titers of Ber-specific IgG2a were also increased in both groups (60 μg or 150 μg), but the difference was significant only for the rBer+LPS60 (Figure 5.7.c). The total levels of IgG2a were significantly increased in the group rBer+ LPS150 (Figure 5.7.d). Both groups presented positive PCA reactions (Figure 5.7.f).

The first conclusion to be taken is that LPS had an adjuvant effect in the sensitisation model used in the present work. The antibody response observed in animals sensitised with rBer e 1 and different doses of LPS was different from the response observed in animals immunized with rBer e 1 alone. The dose of LPS also had an impact in the development of the immune response to rBer e 1, since only higher doses of LPS were able to induce the production of anaphylactic antibodies.

Although it is known that signalling through TLRs is required for adaptive T helper cell type 1 (Th1) responses, the mechanisms by which TLRs may participate in the Th2 priming are still unclear (Williams, Ownby *et al.*, 2005).

A report showing the effect of different doses of LPS in a model of allergic asthma showed that low doses of LPS (0.01 μg) induced Th2-type responses, with increased titers of antigen-specific IgE and IgG1, low titers of antigen-specific IgG2a, type-2 cytokines and eosinophils; whereas high levels of LPS (100 μg) induced a Th1-patterned response, with increased titers of antigen-specific IgG1 and IgG2a, low titers of antigen-specific IgE, increased number of neutrophils, and increased production of IFN-γ (Eisenbarth, Piggott *et al.*, 2002). Another study in mouse model of asthma has also demonstrated that low doses of LPS favour a Th2-type response while higher doses favour a Th1-type response (Dong, Li *et al.*, 2009). In contrast, a study using a C57BL/6 murine model of AHR (Airway hyper-reactivity) showed that LPS-free antigens were more efficient to induce an allergic reaction than antigens contaminated with low doses of LPS (Watanabe, Miyazaki *et al.*, 2003). In our model, only doses of LPS higher than 6 μg were able to induce increased titers of IgG1 and IgG2a as well as the production of anaphylactic antibodies. Therefore, the role played by different doses of LPS in murine models of allergy is indeed controversial. However, it is relevant to emphasize that different animal models are used, with different sensitisation routes, exposure time, and LPS doses.

Comparing the effects induced by increasing concentrations of LPS (Figure 5.7.) with the effects induced by increasing concentrations of the natural SPC lipid fraction C (Figure 5.5.), it is observed that they were contrasting. While high doses of LPS were necessary to induce the production of anaphylactic antibodies, only low doses of SPC lipid fraction C were able to induce positive PCA reactions. However, it is important to emphasize that the doses of LPS and natural lipids used were of different magnitude. LPS doses varied from 0.06 to

150 μg and the doses SPC lipid fraction C varied from 15 to 3,750 μg. Higher doses of LPS could not be tested due to its endotoxic properties, being the lethal dose of LPS in mice 750 μg (Jones, Mansell *et al.*, 2007). The lowest dose of SPC fraction C able to induce anaphylactic antibodies was not evaluated.

Lipids can be recognized by the immune system by different mechanisms. LPS, for instance, is recognized TLR4 and the mechanisms underlying its recognition and the consequent intracellular signalling events are well described (Lu, Yeh *et al.*, 2008; Kawai e Akira, 2010). Although TLRs are usually related with innate responses, it is now well established that LPS participates in Th1 as well as in Th2 responses. Other mechanisms by which lipids can be recognized by the immune system, not involving TLRs, will be discussed later in the present study.

Hence, the different pattern of humoral response induced by rBer e 1 in the presence of LPS or natural lipids are probably related not only to different doses used, but also to the different mechanisms by which lipids are being recognized by the immune system. The possible mechanism(s) by which lipids from Brazil nuts may activate the immune system will be discussed in Chapter 6.

7.2. Activity of Alum in the sensitisation to rBer e 1 and OVA

rBer e 1 was able to induce the production of anaphylactic antibodies in the presence of SPC fraction C from Brazil nuts and in the presence of different doses of LPS, which is reported to participate of allergic processes (Eisenbarth, Piggott *et al.*, 2002; Kim, Oh *et al.*, 2007; Dong, Li *et al.*, 2009). However, rBer e 1 sensitisation has not been tested in the presence of alum ($AlOH_3$), a robust Th2 adjuvant (Brewer, Conacher *et al.*, 1999).

Ber e 1 is the major allergen in Brazil nuts and no studies have investigated its activity in the presence of a well-established Th2-adjuvant. The following experiment was performed in order to evaluate the activity of rBer e 1 in the presence of alum and to compare the results with the same protocol performed using OVA as allergen.

Two different sensitisation protocols were used, as described in Chapter 2 (Item 7.4.). In order to facilitate the visualization, the groups used are specified below:

Groups	**Sensitisation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	
	Protein+ Alum	Protein+ Alum	Protein	Protein	**Exsanguination**
rBer	√	√			**Day 14**
rBer+Al (14d)	√	√			**Day 14**
rBer+Al (28d)	√	√	√	√	**Day 28**
OVA	√	√			**Day 14**
OVA+Al (14d)	√	√			**Day 14**
OVA+Al (28d)	√	√	√	√	**Day 28**

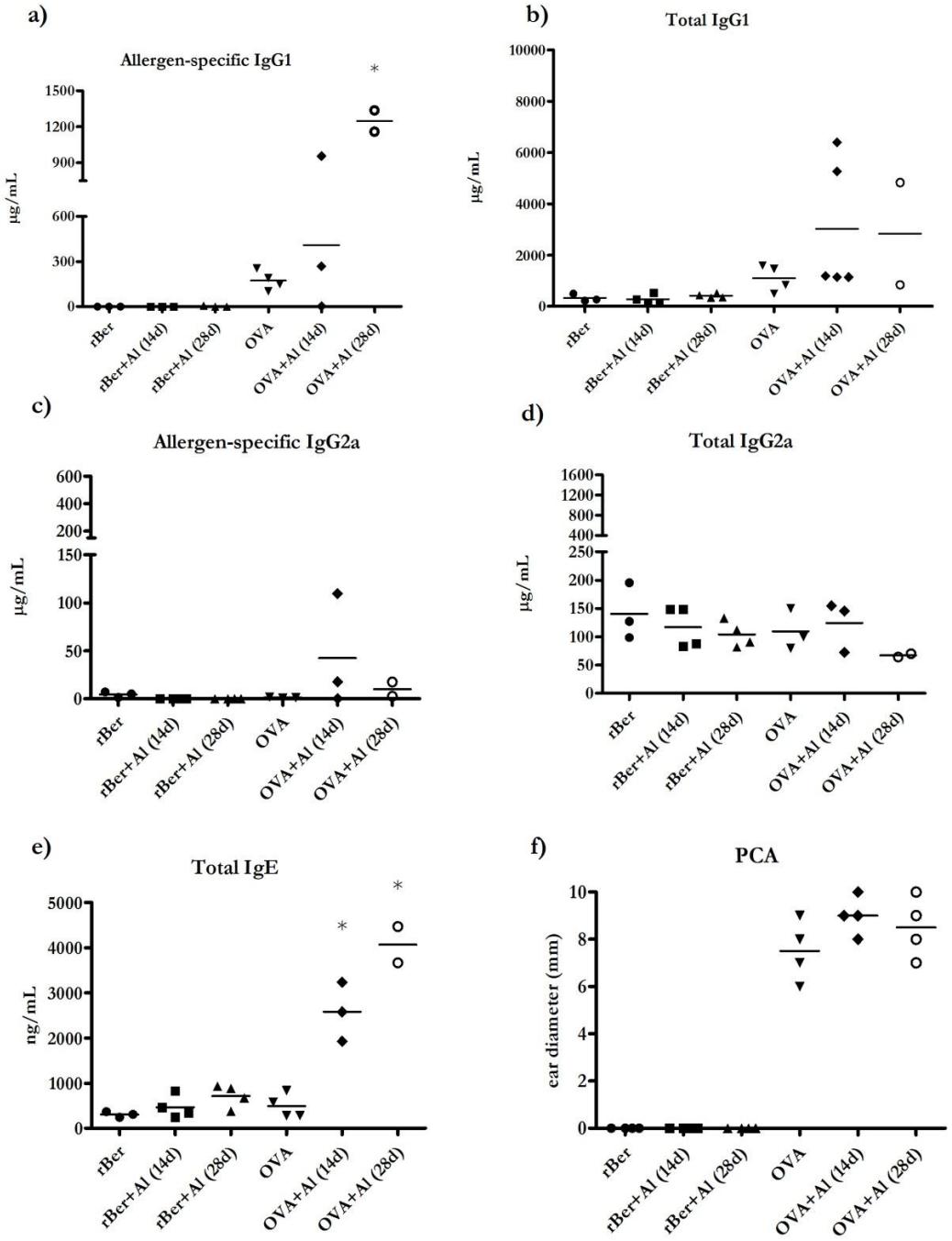


Figure 5.8. Antibody determination; **(a to e)** Total and allergen-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** allergen-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=2, n=3 or n=4). Bars represent groups' means. One-way ANOVA and Tukey's post test, significant difference compared to control group (OVA alone): *$p<0.05$.

The results (Figure 5.8. a-f) indicated that rBer e 1 and alum did not induce immune activation in both sensitisation protocols tested. No statistical

differences were found comparing levels of antibodies in the presence or absence of alum.

In contrast, animals sensitised with OVA and alum presented a clear Th-2 pattern of antibody production, with significantly increased titers of anti-OVA IgG1 (Figure 5.8.a) and total IgE (Figure 5.8.e), and low titers of anti-OVA IgG2a (Figure 5.8.c). These animals also presented positive PCA reactions (Figure 5.8.f), indicating that the adjuvant used was able to induce Th2-type responses.

Mice sensitised with OVA alone (with no adjuvant) presented low levels of antibodies but positive PCA reactions were observed, indicating that OVA alone is able to induce the production of anaphylactic antibodies.

Comparing the two sensitisation protocols used, the longer protocol (with challenges on days 14 and 21) induced a more pronounced Th2 antibody response in OVA-sensitised mice, inducing higher levels of IgG1 and IgE than mice sensitised on days 0 and 7 with no further challenges. It is important to note that the longer the titers of OVA-specific IgG1 and total IgE were much higher compared with previous results on rBer e 1-sensitised animals. Contrastingly, when rBer e 1 was used in the presence of alum, not even the longer protocol induced detectable levels of antibodies.

Therefore, alum did show potent Th2-adjuvant properties when used in the presence of OVA, but this was not observed when in the presence of rBer e 1. Alum is a Th2 adjuvant widely used in animal models of allergy, inducing the secretion of Th2-type immunoglobulins cytokines and (Bortolatto, Borducchi *et al.*, 2008; Darcan-Nicolaisen, Meinicke *et al.*, 2009; Fujita, Teng *et al.*, 2009; Raymond, Rubio *et al.*, 2009; Suzuki, Zheng *et al.*, 2009). Animal models of asthma using OVA and alum show increased levels of IgE and IgG1 in the

serum, IL-4, IL-5 and IL-13 in the broncho-alveolar lavage (BAL), increased airway hyper-activity (AHR) and eosinophil infiltration (Keller, Mucida *et al.*, 2006; Nakajima e Takatsu, 2007). Animal models of food allergy also use alum as adjuvant in the presence of several antigens, such as OVA, peanut and cow milk, and a Th2-pattern response is observed, with high levels of IgE, IgG1, IL-4 and IL-5 (Brandt, Strait *et al.*, 2003; Adel-Patient, Bernard *et al.*, 2005).

The major allergen from Brazil nuts induces Th2-type responses in the presence of a particular fraction of natural lipids (SPC fraction C) and in the presence of LPS; however, no immune activation was observed when rBer e 1 was administrated with alum. Although SPC fraction C and LPS have distinct compositions, both products contain lipids while this is not present in Al(OH)3. Therefore, it is possible to speculate that the mechanisms by which the immune system builds a Th2-type response towards rBer e 1 involve lipid recognition. As mentioned before, LPS acts through TLR4 and lipids can be recognized by the immune system in the context of TLR or CD1 molecules (Miyamoto, Miyake *et al.*, 2001; Moody, 2006). These mechanisms will be investigated and discussed in Chapter 6. Here, the main conclusion is that alum does not induce a Th2-response in the presence of rBer e 1, whereas this response is achieved when rBer e 1 is attached to natural lipid fractions (SPC fraction C) or to LPS.

It is also relevant to consider that the capacity of rBer e 1 to bind lipids could interfere in the course of the immune response. It is been reported that the allergenic 2S albumin Sin a 1 from mustard seeds is able to interact with phospholipid vesicles and this could be involved with its allergenicity (Onaderra, Monsalve *et al.*, 1994). A different study showed that melittin, an allergen from bee venom, binds to cell membranes and it is correlated with an enhancement of its immunogenicity (Fehlner, Kochoumian *et al.*, 1991). Therefore, it is possible

that the immune system recognizes the whole complex, protein-lipid, in order to develop an immune response towards rBer e 1.

7.3. Activity of lipids from sun flower seeds in the sensitisation to rBer e 1.

rBer e 1 was demonstrated to induce Th2-skewed responses in the presence of natural lipids from Brazil nuts and in the presence of LPS. However, it was not observed in the presence of alum, a Th2-adjuvant. In order to investigate whether lipids from other seeds could also induce an allergic response to rBer e 1, lipid fractions were extracted from sunflower seeds by SPC and tested in the presence of rBer e 1. The fractions obtained from sunflower were: SPC fraction C(SF) and SPC fraction M(SF).

Therefore, animals were sensitised with rBer e 1 in the presence of 600 μg of sunflower lipids (SPC fraction C(SF) or SPC fraction M(SF)), according to the protocol described in Chapter 2 (Item 7.2.)

The groups used are specified below:

Groups	Sensitisation
rBer	rBer e 1 (2.5 mg)
rBer + C(SF)	rBer e 1 (2.5 mg) + 600 μg of SPC lipid fraction C from sunflower
rBer + M(SF)	rBer e 1 (2.5 mg) + 600 μg of SPC lipid fraction M from sunflower

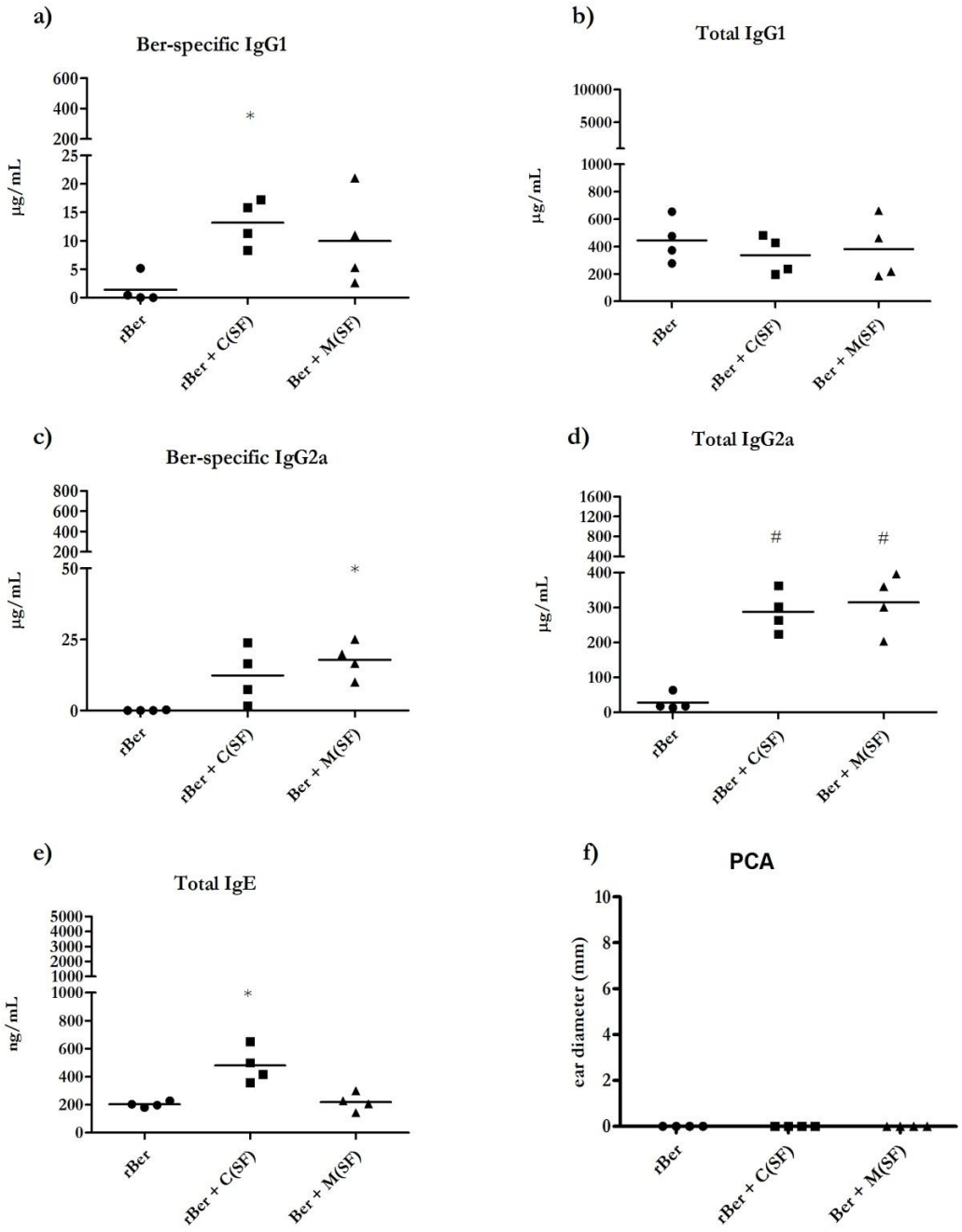


Figure 5.9. Antibody determination; **(a to e)** Total and Ber-specific immunoglobulins in the serum of sensitized animals measured by ELISA; **(f)** Ber-specific anaphylactic antibodies in the serum of sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=4). Bars represent groups' means. One-way ANOVA and Tukey's post test. Significant difference compared to rBer group: *$p<0.05$ and #$p<0.001$.

Figure 5.9. (a-f) shows that lipid fractions from sunflower seeds induced a slight immune activation in animals sensitised with rBer e 1. SPC fraction C(SF) induced significant increased titers of Ber-specific IgG1 and total IgE (Figure 5.10.a and e), and SPC fraction M(SF) induced significantly increased titers of Ber-specific IgG2a (Figure 5.10.c). However, none of the lipid fractions from sunflower were able to induce the production of anaphylactic antibodies (Figure 5.10.f).

It is important to note that the levels of Ber-specific antibodies presented by animals sensitised with rBer e 1 and SPC C and M from sunflower lipids (Figure 5.9.) were similar to the levels observed in animals sensitised with SPC fractions C and M from Brazil nuts (Figure 5.3. and 5.6.). However, the levels of total IgG1 and total IgE were lower in animals sensitised with lipids from sunflower (Figure 5.9.) than in animals sensitised with lipids from Brazil nuts (Figure 5.3.and 5.6).

Therefore, lipids from sunflower seemed to have an immunogenic effect but no indications of a Th2-patterned response was observed. It is important to highlight that, although extracted by the same technique, lipid fractions extracted from different sources differ in composition, and some special classes of lipids may be present in one seed and absent in the other. This experiment was one more indication that SPC fraction C from Brazil nuts has a distinct adjuvant property in the model of allergy used in the present work.

8. Activity of natural lipid fraction C attached to SFA-8 protein

The results obtained so far indicated that SPC fraction C from Brazil nus are involved with the Th2-patterned responsed observed in Ber-sensitised

animals. This phenomenon was not observed when alum or lipids from sunflower seeds were used.

In order to further investigate the role of Brazil nut SPC fraction C and check whether its Th2-immune activity was also present when attached to different proteins, similarly to Th2 adjuvants like alum, it was tested in the presence of SFA-8, a protein from sunflower.

Therefore, for this experiment, Brazil nut SPC fraction C was added to SFA-8 and tested in our protocol of sensitisation. SPC fraction L, which did not induce anaphylactic antibodies in Ber-sensitisation protocol, was used as a negative control; and LPS, which induced anaphylactic antibodies in the presence of rBer e 1, was used as a positive control.

Animals were sensitised according to the protocol described in Chapter 2 (Item 7.2.). The groups used are specified below:

Groups	**Sensitisation**
Naive	None
SFA	SFA-8 (2.5 mg)
SFA + L	SFA-8 (2.5 mg) + 600 μg of SPC fraction L
SFA + C	SFA-8 (2.5 mg) + 600 μg of SPC fraction C
SFA + LPS	SFA-8 (2.5 mg) + 150 μg of LPS

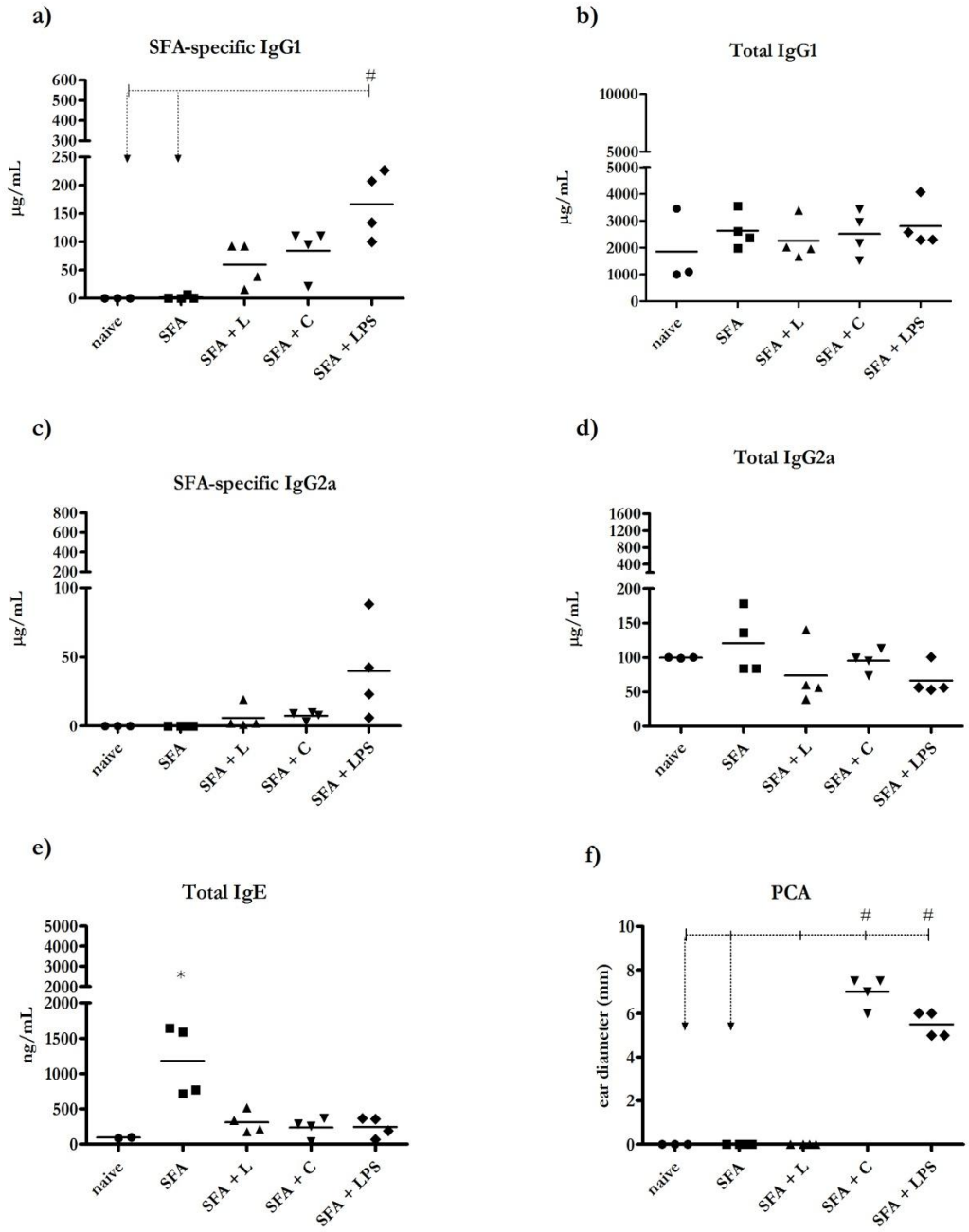


Figure 5.10. Antibody determination; **(a to e)** Total and SFA-specific immunoglobulins in the serum of naive and sensitized animals measured by ELISA; **(f)** SFA-specific anaphylactic antibodies in the serum of naive and sensitized animals evaluated by PCA. Procedures performed as described in Material and Methods. Symbols represent individual animals (n=3 or 4). Bars represent groups' means.One-way ANOVA and Tukey's post test. Significant difference compared to naive and/or SFA groups (indicated by narrows): *$p<0.05$ and #$p<0.001$.

Figure 5.10. (a-f) shows that SFA-8 alone was not able to induce a specific immune activation. Mice sensitised with SFA-8 (group SFA) presented levels of Ber-specific IgG1 and Ber-specific IgG2a similar to naive animals. The titers of total IgE were significantly increased (Figure 5.10.e), but no positive PCA reactions were observed (Figure 5.10.f). PCA reactions can be elicited by anaphylactic IgG1 or IgE, since these animals did not present detectable titers of Ber-specific IgG1, it can be speculated that the high levels of total IgE levels presented by animals sensitised with the protein alone were composed mainly by non-specific immunoglobulins.

Mice sensitised with SFA-8 and SPC fraction L did not present important immune activation, all antibody titers tested were low. A slight increase was observed in the titers of SFA-specific IgG1 (Figure 5.10.a) but it was not significantly different from the controls (naive and SFA groups).

The group sensitised with SFA-8 and SPC lipid fraction C presented increased SFA-specific IgG1 (Figure 5.10.a), but not statistically different from the control. Low IgG2a and IgE were observed (Figure 5.10.c-e). However, strongly positive PCA reactions were observed (Figure 5.10.f), indicating that these animals presented anaphylactic antibodies.

Finally, the group SFA+LPS presented significantly increased levels of SFA-specific IgG1 (Figure 5.10.a), slight increase in the titers of SFA-specific IgG2a (Figure 5.10.c) and low titers of IgE (Figure 5.10.e). The PCA reactions were strongly positive (Figure 5.10.f).

These results indicated that the Th2-adjuvant property of SPC fraction C from Brazil nuts was not Ber e 1 specific. The humoral response induced in SFA-8 sensitised animals was similar to the response observed in Ber-sensitised

animals, characterized by increased titers of antigen-specific IgG1, low titers of IgG2a and positive PCA reactions.

SFA-8 and Ber e 1 are proteins of the same 2 S albumin seed storage family that share structural and biological similarities (Murtagh, Archer *et al.*, 2003). However, whereas the allergenicity of Ber e 1 is well established (Nordlee, Taylor *et al.*, 1996; Pastorello, Farioli *et al.*, 1998), SFA-8 appears to be a weaker allergen. The incidence of sunflower allergy is very low even in countries where this seed is highly consumed, such as Spain, Germany and Greece (Alcocer, Murtagh *et al.*, 2002; Kean, Goodridge *et al.*, 2006). Confirming epidemiological data, an *in vitro* work showed that Ber e 1 but not SFA-8 was able to polarize dendritic cells in a Th2 direction (Kean, Goodridge *et al.*, 2006). Considering Ber e 1 and SFA-8 originate from different seeds and differ on their allergenic potential, the fact that the fraction SPC C from Brazil nuts was able to induce Th2-skewed humoral responses in the presence of Ber e 1 as well as in the presence of SFA-8 could indicate that these lipids present a Th2-adjuvant like effect, being able to play a non-specific role. However, considering the structural similarities between these two proteins, the possible lipid-protein binding prior to the immune recognition cannot be discarded.

In summary, the results presented in this Chapter confirmed previous findings (Dearman, Alcocer *et al.*, 2007) evidencing the essential role that natural lipids play in the sensitisation to rBer e 1, since this protein alone did not induce the production of anaphylactic antibodies. Several fractions of lipids from Brazil nuts were tested in the *in vivo* model of sensitisation to rBer e 1 and only one fraction (SPC C) was able to induce a Th2-skewed humoral response. The pattern of immune activation induced by this fraction was shown to be dose-dependent, since lower doses induced anaphylactic antibodies and higher doses

did not. Several lipid classes were putatively identified in the SPC fraction C, but when further fractionated into sub-fractions containing identified classes of lipids, SPC lipid fraction C lost its ability to induce the production of anaphylactic antibodies, indicating then, that the doses and/or the presence of particular classes of lipids (possibly synergistically) are important for determining the course of the Th2 immune reaction observed. The immune effect of rBer e 1 was also tested in the presence of different compounds, such as LPS, alum and natural lipids from sunflower. Amongst them, LPS was the only molecule that, similarly to SPC fraction C, induced the production of anaphylactic antibodies in Ber-sensitised animals also in a dose-dependent way. However, the pattern of humoral response induced by LPS and SPC fraction C was different in the sense that LPS induced significant increase in titers of antigen-specific IgG1 and IgG2a and SPC fraction C induced only increase titers if IgG1. Similar humoral pattern was observed when a LPS or SPC fraction C were tested in animals sensitised to a different protein, SFA-8, from sunflower.

Therefore, the *in vivo* findings described so far evidenced an immune phenomenon in which natural lipids play an essential role. The mechanisms by which these lipids might be interacting with the immune system and directing the course of the response will be investigated in the next Chapter.

CHAPTER 6

MECHANISMS OF IMMUNE ACTIVATION BY LIPIDS FROM BRAZIL NUTS

INTRODUCTION

As discussed in depth in Chapter 1, the role of plant and animal lipids has been investigated in different aspects of the immune system and two main ways of lipid immune activation have been reported, involving TLR or CD1 molecules. This chapter describes and discusses the results of experiments carried out to investigate the potential mechanism(s) by which natural lipids interact with the immune system, particularly focusing upon TLR and CD1-NKT sytems.

RESULTS AND DISCUSSION

1. The ability of the SPC lipid fraction C to bind and activate TLRs

In the present work, we have shown that animals sensitised with rBer e 1 and different doses of LPS produced anti-Ber anaphylactic antibodies. The same results were observed in animals sensitised with rBer e 1 and SPC lipid fraction C from Brazil nuts. Animals sensitised with rBer e 1 alone did not produce anaphylactic antibodies (Results in Chapter 5).

In order to investigate whether the immune activation induced by SPC lipid fraction C from Brazil nuts involved TLRs, samples of this fraction of lipids were sent to a commercial company and tested in an *in vitro* model of TLRs activation (assay details are provided in Chapter 2, Item11).

Within the context of immune regulation involving TLRs, it is relevant to mention that SPC lipid fraction C was also tested for endotoxin testing in a LAL system and no LPS or measured endotoxin contamination was found. This information is shown in the Appendix 2 (fraction C:M refers to SPC fraction C).

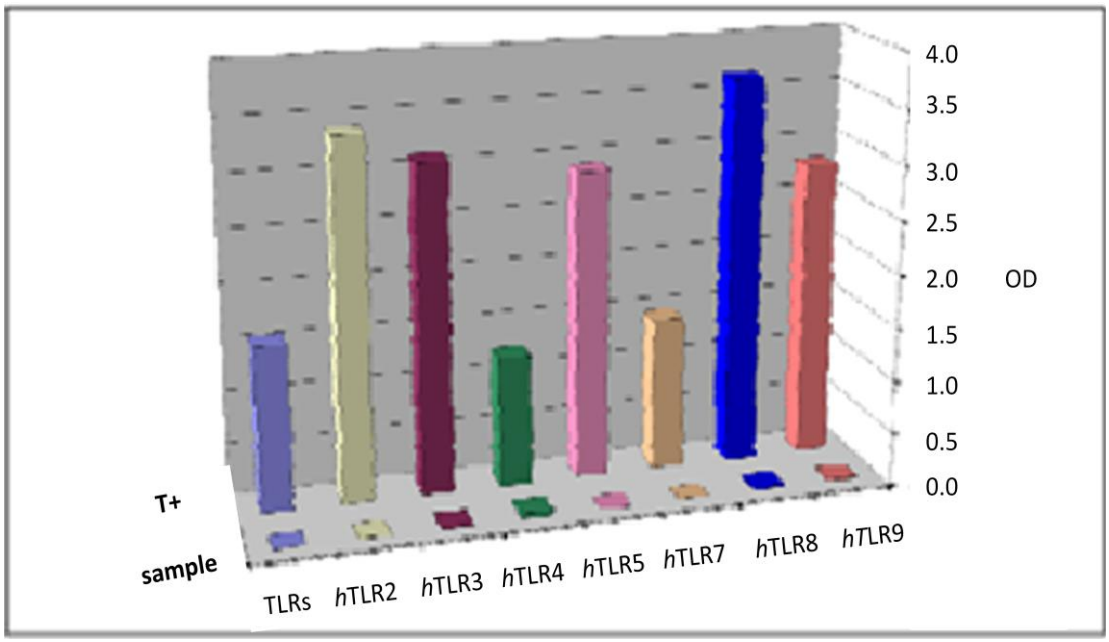


Figure 6.1.: Activation of different human (h) TLRs (2, 3, 4, 5, 7, 8 and 9) by positive controls (T+) and by SPC lipid fraction C (sample). Results are given in OD.

Figure 6.1. shows that the SPC lipid fraction C (sample) was not able to activate any of the human TLRs tested. This result and the endotoxin analysis strongly suggest that the immune activation induced by SPC lipid fraction C is not through TLRs.

Therefore, although lipid molecules are reported to direct the adaptive response via TLRs, such as LPS via TLR4 (Mcaleer e Vella, 2008), ES-62 from nematodes via TLR4 (Harnett e Harnett, 2009), and lyso-PS from schistosome via TLR2 (Van Der Kleij, Latz *et al.*, 2002); this does not seem to be the case for lipids from Brazil nuts.

Nevertheless, the possible interaction between SPC fraction C and TLRs cannot be completely excluded. The first consideration in this respect is that the *in vitro* system used might not have reproduced properly the events happening *in vivo*. The presence of lipids was shown to be essential for the development of an *in vivo* Th2-biased response (Dearman, Alcocer *et al.*, 2007)

and these results were confirmed in the present work (Chapter 5). Conversely, when the same rBer e 1 proteins were tested in an *in vitro* model using bone marrow DCs (Kean, Goodridge *et al.*, 2006), the presence of natural lipids did not change the profile of cytokines expressed by DCs. In the absence of other regulatory mechanisms, rBer e 1 alone was able to induce a Th2 polarization of DCs (Kean, Goodridge *et al.*, 2006). This observation corroborates the absence of *in vitro* TLR activation observed in the present experiment. It is known that DCs express TLR and these cells are also able to efficiently present antigens to T cells (antigen presenting cells, APC). DCs bridge the native and adaptive immune system playing a key role in driving the development of an immune response, and therefore, *in vitro* systems lack other regulatory factors present *in vivo* experiments.

It is also important to highlight that the role of the natural lipids from Brazil nuts in the development of an allergic response was observed in mice (Dearman, Alcocer *et al.*, 2007). No evidence of this activity is reported in humans. The TLRs present in the cell lines tested *in vitro* were human TLRs and this fact could interfere in the results, not reproducing the events observed *in vivo*. Therefore, although the results indicate that the activation of TLRs is not the main mechanism of activation of SPC lipid fraction C, this can not be categorically proved due to limitations on the *in vitro* system.

In order to circumvent the limitations of *in vitro* systems, the activity of SPC lipid fraction C could be tested in animal models using different lineages of mice, which can be knocked-out for one or more TLRs (Calcaterra, Sfondrini *et al.*, 2008; Chandran, Verhoeven *et al.*, 2009; Hammad, Chieppa *et al.*, 2009; Stowell, Seideman *et al.*, 2009) as well as for molecules involved in TLRs signalling pathways (Cai, Batra *et al.*, 2009), such as MyD88 (Chen, Lei *et al.*, 2010), TRIF and TIRAP (Horng, Barton *et al.*, 2002). Therefore, further studies

using knockout mice would be necessary to confirm that the activity of SPC fraction C is not TLR-dependent.

2. Activity of SPC fraction C via NKT-CD1

The following experiments were performed in order to investigate whether the role of natural lipids from Brazil nuts in developing a Th2 response could involve the activation of NKT cells, and therefore, CD1 molecules.

2.1. NKT activation by SPC lipid fractions in BALB/c mice

As previously shown (Chapter 5, Figure 5.3.), SPC lipid fractions C and M extracted from Brazil nuts by SPC differed in the ability to induce the production of anaphylactic antibodies in the sensitisation protocol performed in the present work. Animals sensitised with rBer e 1 and SPC fraction C presented anaphylactic antibodies whereas it was not observed in animals sensitised with rBer e 1 and SPC fraction M (Chapter 5, Figure 5.3.).

It has been demonstrated that the injection of α-GalCer, a potent inducer of NKT cells, leads to the production of both IFN-γ and IL-4 by nearly all NK T cells in the liver and in the spleen within 2 h. These cells mostly disappear by 5 h, and they do not reappear after 1 week (Matsuda, Naidenko *et al.*, 2000). Therefore, in experiments carried out in Paris with the supervision of Dr. Leite-de-Moraes, the SPC lipid fraction C and SPC lipid fraction M were tested in a 2h-long protocol of NKT activation and compared to the activity of α-GalCer, used as a positive control (as described in Chapter 2, Item 9.1.).

The groups used are specified below:

Groups	I.p. injection:
Naive	-
SPC fraction C	rBer e 1 (2.5 mg) + 600 μg of SPC fraction C
SPC fraction M	rBer e 1 (2.5 mg) + 600 μg of SPC fraction C
α-GalCer	2 μg of α-GalCer

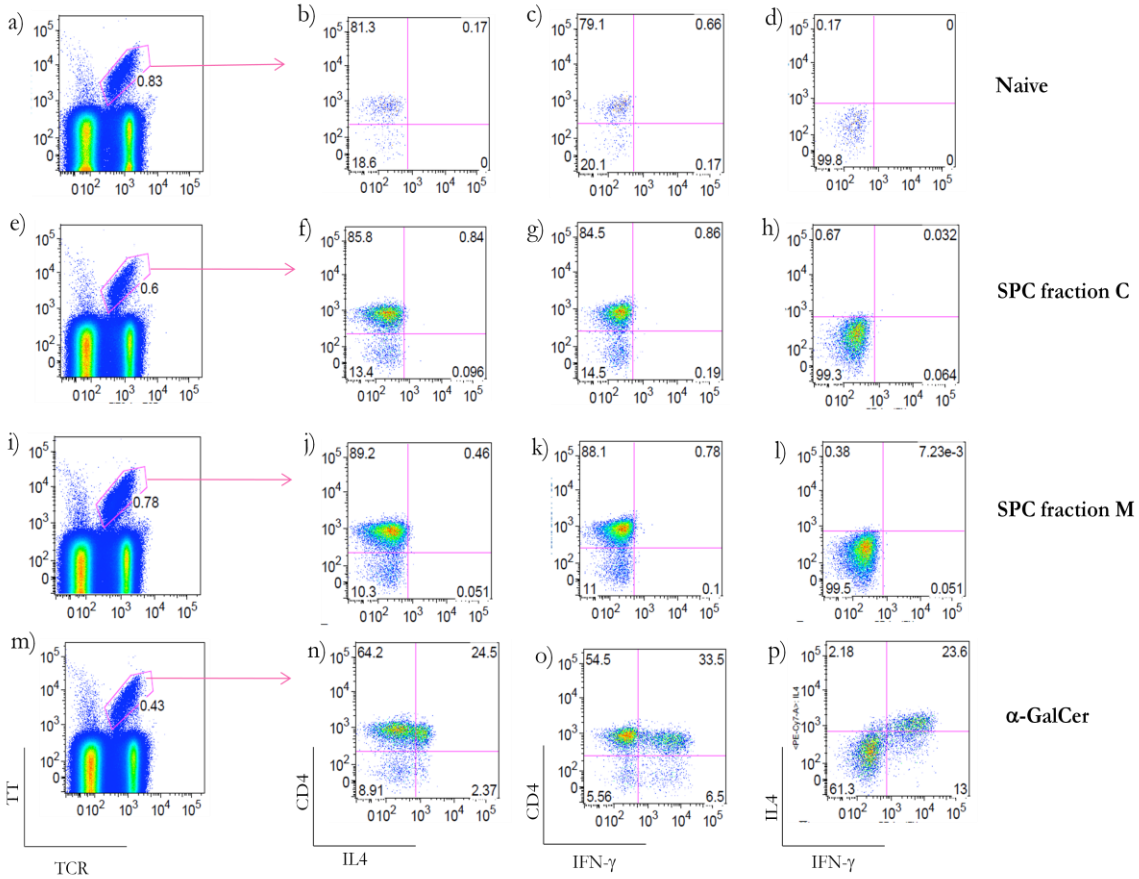


Figure 6.2. Flow cytometry analysis of spleen cells from BALB/c mice. a-d) Cells from naive mice; e-h) Cells from mice injected with rBer e 1 and SPC lipid fraction C from Brazil nuts; i-l) Cells from mice injected with rBer e 1 and SPC lipid fraction M from Brazil nuts; m-p) Cells from mice injected with α-GalCer. Plots in the first column represent gates of SSC^{low} and FSC^{low} cells – lymphocytes (TT x TCR). Plots in the second, third and forth columns represent gates of TT+ and TCR+ cells. Values in the plots indicate the percentage of marked cells. Lipid injection and cells staining protocols are described in Material and Methods. Data is representative of one animal from each group.

Figure 6.2. shows that all animals present NKT cells, which are TCR+ and TT+, and are represented in the gate underlined in pink (Figure 6.2.a, e, i and m). The other plots represent the IL-4 and/or IFN-γ production by NKT cells. It was observed that animals that received rBer e 1 with SPC fraction C or with SPC fraction M did not show NKT cells producing detectable levels of IL-4 or

IFN-γ. The upper-right quadrants (Figure 6.2.f-h and j-l) presented similar amount of marked cells compared to not-manipulated animals (Naive group, Figure 6.2.b-d). Differently, 24.5% of NKT cells from animals that received α-GalCer produced IL-4 (Figure 6.2.n), 33% of NKT cells produced IFN-γ, and 23.6% of NKT cells produced both IL-4 and IFN-γ.

A-GalCer is the most potent ligand for the invariant Vα14 NKT antigen receptor, which is exclusively presented by CD1d. When activated by α-GalCer, NKT cells promptly produce large amounts of IFN-γ and IL-4 (Kawano, Cui *et al.*, 1997). The activation of NKT cells by different lipids might differ in timing or other mechanisms involving their presentation by DCs, altering the results observed. Therefore, the fact that the NKT cells from animals injected with natural lipids from Brazil nuts did not show as evident production of IL-4 and IFN-γ as observed with α-GalCer does not necessarily mean that these lipids were not recognizable by NKT.

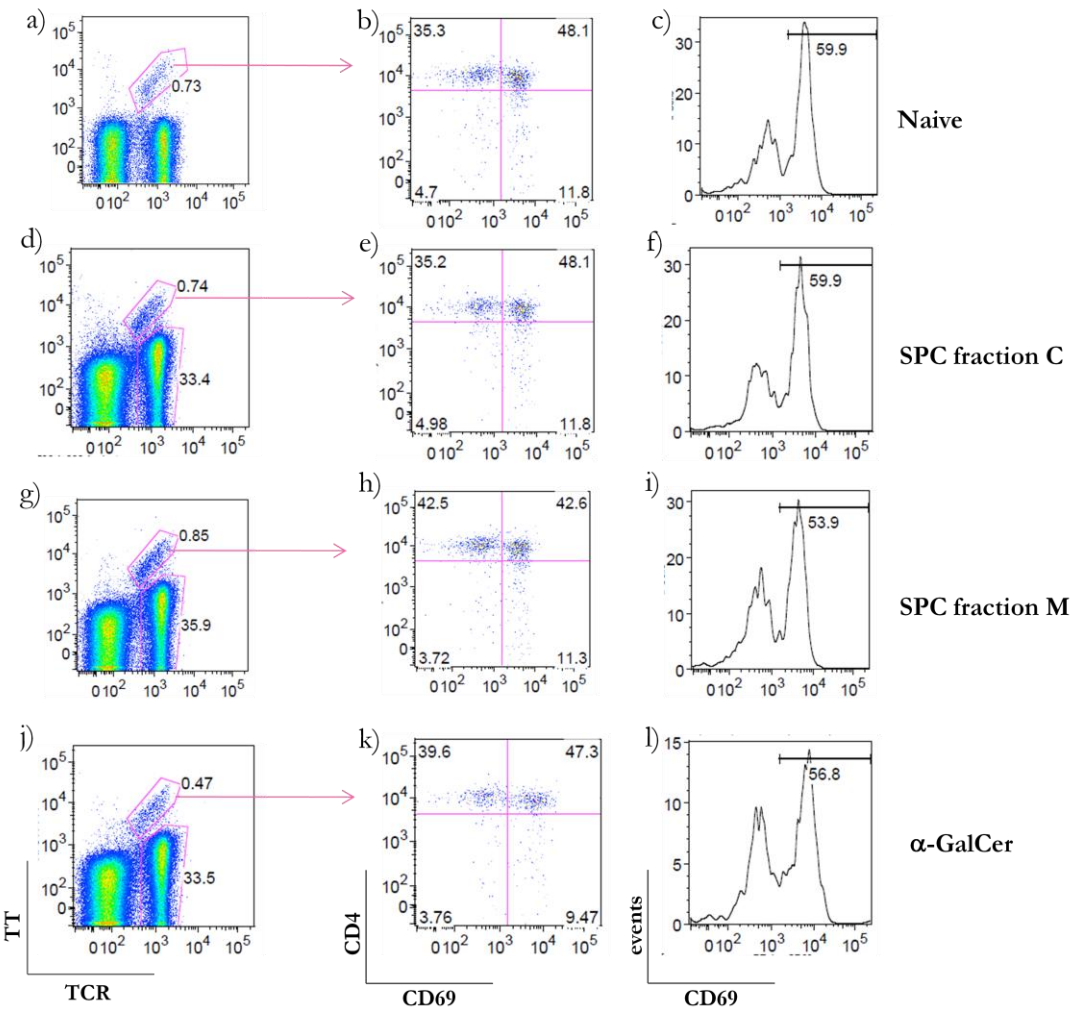


Figure 6.3. Flow cytometry analysis of spleen cells from BALB/c mice. a-c) Cells from naive mice; d-f) Cells from mice injected with rBer e 1 and SPC lipid fraction C from Brazil nuts; g-i) Cells from mice injected with rBer e 1 and SPC lipid fraction M from Brazil nuts; j-l) Cells from mice injected with α-GalCer. Plots in the first column represent gates of SSC^{low} and FSC^{low} cells - lymphocytes (TT x TCR); plots in the second and third columns represent gates on TT+ and TCR+ cells. Values in the plots and histograms indicate the percentage of marked cells. Lipid injection and cells staining protocols are described in Material and Methods. Data is representative of one animal from each group.

The expression of CD69 indicates the activation of lymphocytes. Figure 6.3. shows the activated NKT cells in plots (second column of graphs) and histograms (third column of graphs). No significant differences in NKT activation were observed amongst all groups of animals studied. However, by overlaying the histograms of individual groups it was possible to observe that the α-GalCer group presented higher activation than the naive group (Figure 6.4.). No differences were observed overlaying the graphs from other groups.

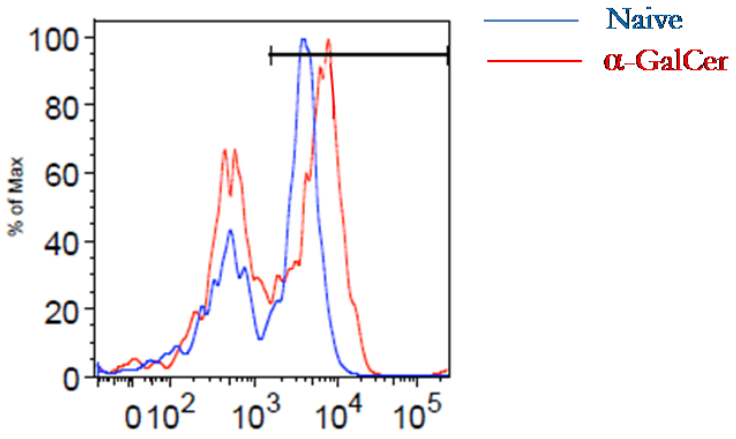


Figure 6.4. Overlaying histograms from naive and α-GalCer groups (Histograms presented in Figure 6.3.c and l)

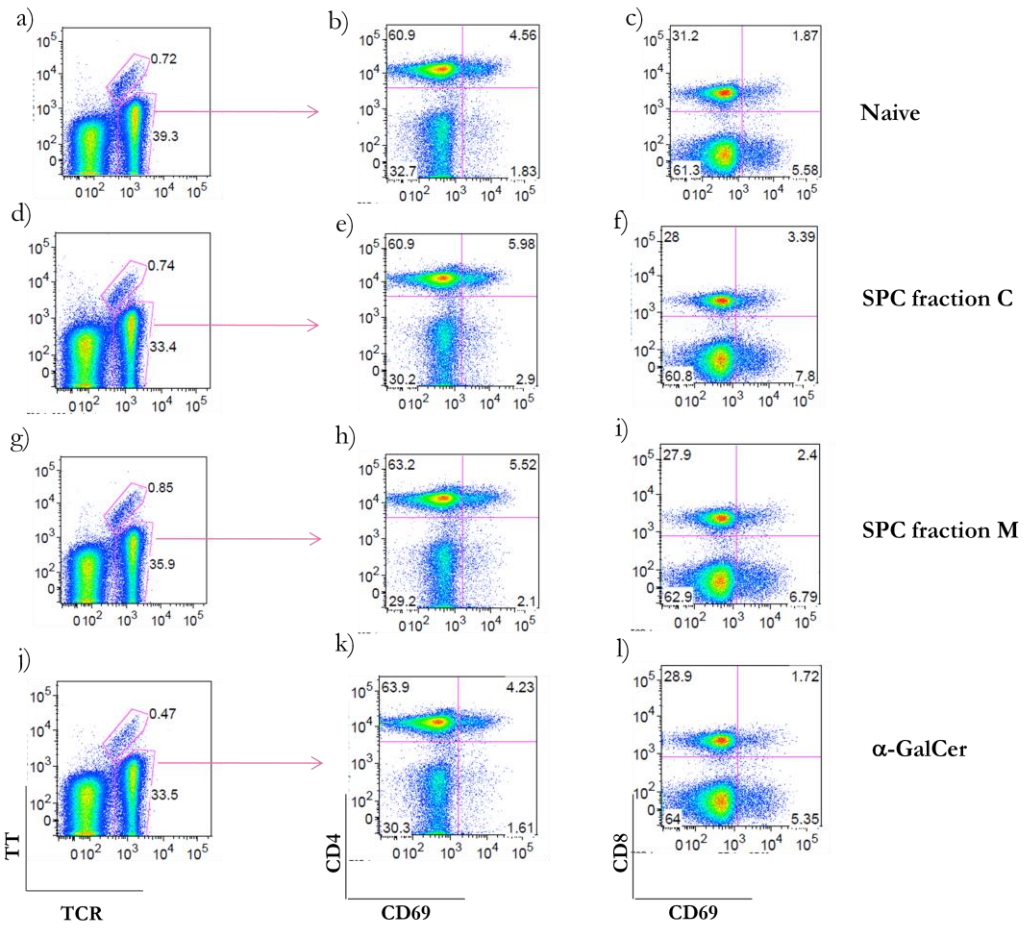


Figure 6.5. Flow cytometry analysis of spleen cells from BALB/c mice. a-c) Cells from naive mice; d-f) Cells from mice i.p. injected with rBer e 1 and SPC lipid fraction C from Brazil nuts; g-i) Cells from mice i.p. injected with rBer e 1 and SPC lipid fraction M from Brazil nuts; j-l) Cells from mice i.p. injected with α-GalCer. Plots in the first column represent gate of SSC^{low} and FSC^{low} cells – lymphocytes (TT x TCR); plots in the second and third columns represent gates of TT- TCR+ cells. Values in the plots indicate the percentage of marked cells. Lipid injection and cells staining protocols are described in Material and Methods. Data is representative of one animal from each group.

Figure 6.5. showed the expression of CD69 by CD4 and CD8 T cells. Second and third columns of plots were gated in T cells (TT- and TCR+), showed in first column of plots (Figure 6.5.a, d, g and j). No statistically significant differences were observed amongst the groups, indicating that under the conditions of the present protocol the lipids did not activate T cells.

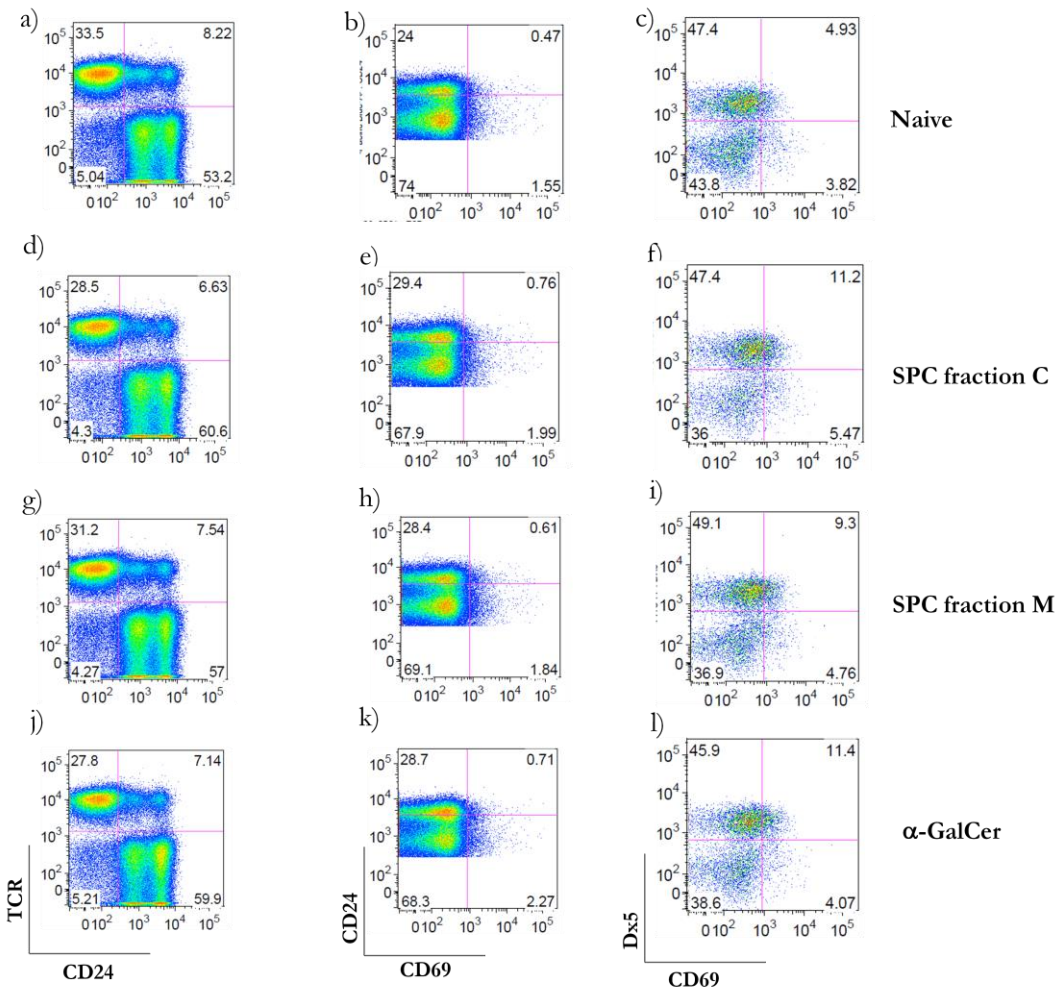


Figure 6.6. Flow cytometry analysis of spleen cells from BALB/c. a-c) Cells from naive mice; d-f) Cells from mice i.p. injected with rBer e 1 and SPC lipid fraction C from Brazil nuts; g-i) Cells from mice i.p. injected with rBer e 1 and SPC lipid fraction M from Brazil nuts; j-l) Cells from mice i.p. injected with α-GalCer. Plots in the first column represent gates of SSC^{low} and FSC^{low} cells – lymphocytes (TCR x CD24). Plots in the second column represent gate of TCR- CD24+ cells. Plots in the third column represent gate of TCR- CD24-. Values in the plots indicate the percentage of marked cells. Lipid injection and cells staining protocols are described in Material and Methods. Data is representative of one animal from each group.

Figure 6.6. shows the expression of CD69 by B cells (CD24high+) and NK cells (Dx5+). No statistic differences ($p>0.05$) were found in the activation of B cells comparing all groups (Figure 6.6.b, e, h and k). However, there was an indication that both lipid fractions SPC C (Figure 6.6.f) and SPC M (Figure 6.6.i) presented more activated NK cells compared to naive animals (Figure 6.6.c). This difference was not statistically different ($p>0.05$) from the control (naive), probably due to the low number of animals tested (n=2 or n=3). A similar

increase was observed in the cells from animals that received α-GalCer (Figure 6.6.l), but it was not statistically different from the control either ($p>0.05$).

Altogether, the results showed that α-GalCer clearly activated NKT cells and that this activation was not observed when SPC fraction C or SPC fraction M was used. Therefore, using the present protocol, natural lipids from Brazil nuts did not induce evident NKT cell activation. There was an indication however, that the SPC fractions C and M of lipids from Brazil nuts induced an activation of NK cells, similarly to the observed with α-GalCer, indicating that the conjugates lipid+rBer e 1 injected in the animals are activating the innate immune system. It is important to consider; however, that different classes of lipids present different levels of affinity to bind CD1 molecules (Stanic, Shashidharamurthy *et al.*, 2003; Oki, Chiba *et al.*, 2004). This fact as well as the dose of antigen and the affinity to NKT TCR determinate the duration of TCR signalling (Sullivan, Nagarajan *et al.*, 2010); therefore, in order to further investigate the role of natural lipids in the activation of NKT cells using the present model of NKT activation, different protocols testing different doses of lipids and time duration would need to be carried out.

2.2. Activity of SPC lipid fraction C in wild type and Jα18 -/- mice

The activity of SPC fraction C, the only lipid fraction from Brazil nuts able to induce the production of anaphylactic antibodies *in vivo* (Chapter 5), was then studied in animals lacking NKT cells in order to rule out the immune activation involving the participation of NKT cells.

Therefore, the same sensitisation protocol used in the present work (Chapter 5) was performed in knockout mice (Jα18-/-), which are exclusively deficient in the invariant Vα14 (iVα14), CD1d-restricted NKT cells. Therefore, Jα18 -/- and wild type C57BL/6 mice were sensitised with rBer e 1 and SPC fraction C and several parameters were observed.

The groups used are specified below:

Groups	**Animals**	**Sensitisation:**
WT	Wild type C57BL/6	rBer e 1 (2.5 mg) + 600 µg of SPC fraction C
Jα18-/-	Jα18 knockout C57BL/6	rBer e 1 (2.5 mg) + 600 µg of SPC fraction C

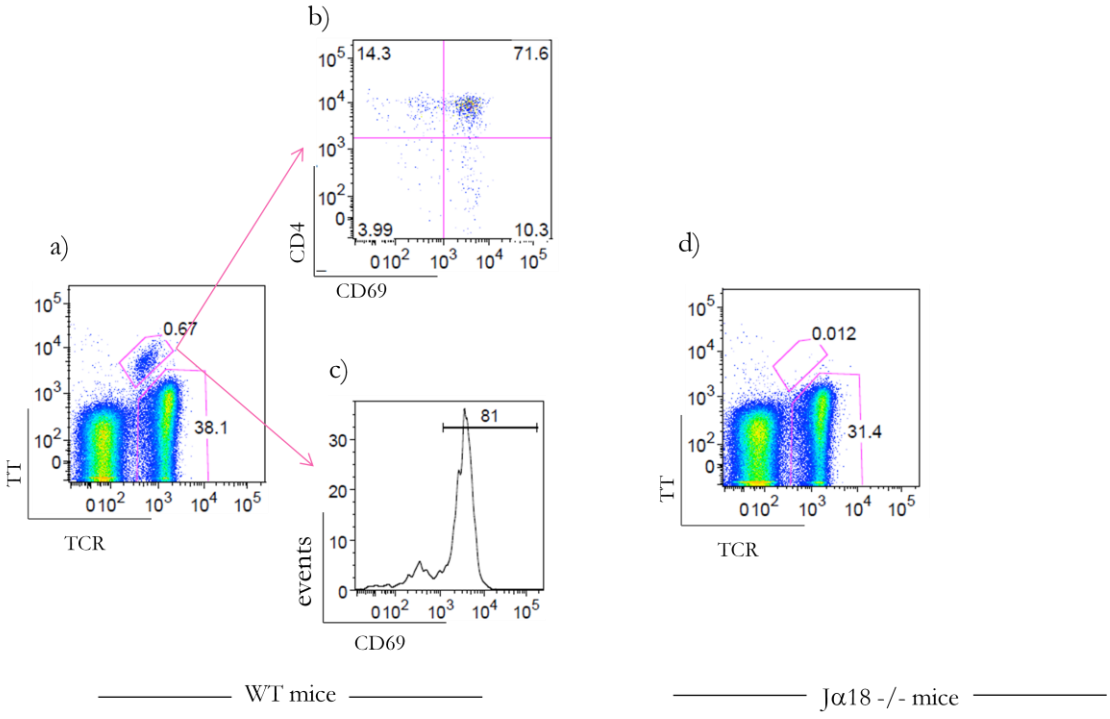


Figure 6.7. Flow cytometry analysis of spleen cells from C57BL/6 mice. a-c) Cells from wild type (WT) mice sensitized with rBer e 1 and SPC lipid fraction C. a) Plot represents gate of SSC^{low} and FSC^{low} cells – lymphocytes (TT x TCR); b) Histogram of TT+ TCR+ cells (NKT cells); c) Histogram of TT+ TCR+ cells (NKT cells); d) Cells from Jα18 -/- mice sensitized with rBer e 1 and SPC lipid fraction C. Plot represents gate of SSC^{low} and FSC^{low} cells – lymphocytes(TT x TCR). Values in the plots and histograms indicate the percentage of marked cells. Sensitization and cells staining protocols are described in Material and Methods. Data is representative of one animal per group (n=5).

Figure 6.7. (a-d) shows that NKT cells (TT+ and TCR+) were detected in WT animals (Figure 6.5.a), but not in Jα18-/- mice (Figure 6.4.d). The plot (Figure 6.4.b) and histogram (Figure 6.5.c) showed the expression of CD69 by NKT cells from wild type animals, indicating the activation of these cells. Since these animals were injected with rBer e 1 and SPC lipid fraction C, it would be necessary a group of wild type animals that had not been injected with lipids to evaluate whether the NKT cell activation observed was due to the lipids activity. However, due to the low availability of animals, the control groups necessary to evaluate the activation of NKT cells in WT animals were not carried out.

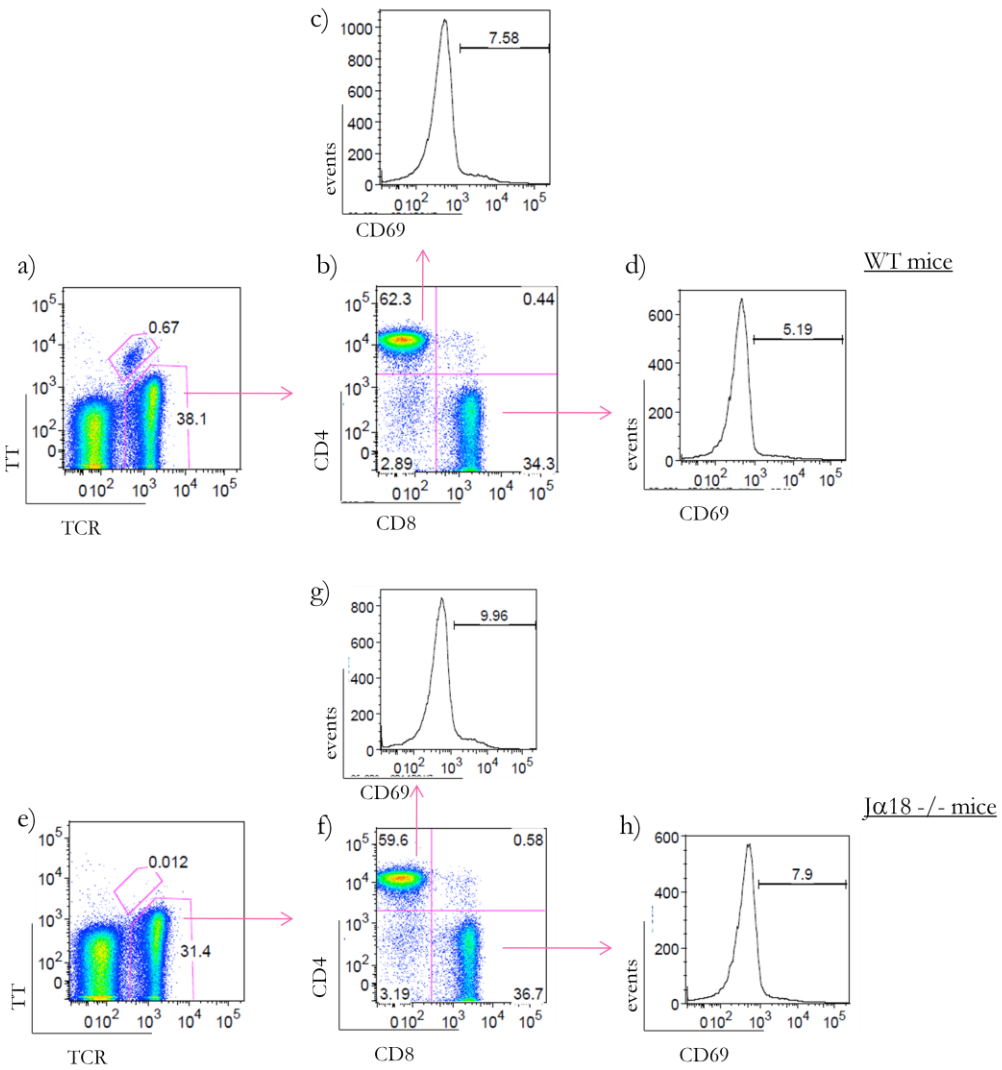


Figure 6.8. Flow cytometry analysis of spleen cells from C57BL/6 mice. a-d) Cells from WT mice sensitized with rBer e 1 and SPC lipid fraction C. a) Plot represents gate of SSC^{low} and FSC^{low} cells – lymphocytes (TT x TCR); b) Plot represents gate of TT- TCR+ cells (T cells); c) Histogram of CD4+ CD8- cells (T CD4 cells); d) Histogram of CD4- CD8+ cells (T CD8 cells); e-h) Cells from Jα18 -/- mice sensitized with rBer e 1 and SPC lipid fraction C. e) Plot represents gate of SSC^{low} and FSC^{low} cells – lymphocytes (TTx TCR); f) Plot represents gate of TT- TCR+ cells (T cells); g) Histogram of CD4+ CD8- (T CD4 cells); h) Histogram of CD4- CD8+ cells (T CD8 cells). Values in the plots and histograms indicate the percentage of marked cells. Sensitization and cells staining protocols are described in Material and Methods. Data is representative of one animal per group (n=5).

In order to study the activation of CD4+ and CD8+ T cells in WT and Jα18-/- mice, these markers as well as CD69 were evaluated. Figure 6.8. shows the activation of CD4+ and CD8+ T cells; i.e. CD69+ T cells. The gate for the analyses of T cells is shown in the Figure 6.8.a for WT animals and in the Figure 6.8.e for Jα18-/- animals. The histograms show the expression of CD69 by CD4

T cells (Figure 6.8.c and g) and by CD8 T cells (Figure 6.8.d and h). There is a slight increase in the expression of C69 in CD4 and CD8 T cells of Jα18-/- animals when compared to WT mice, however no statistical differences were found comparing the two groups of animals.

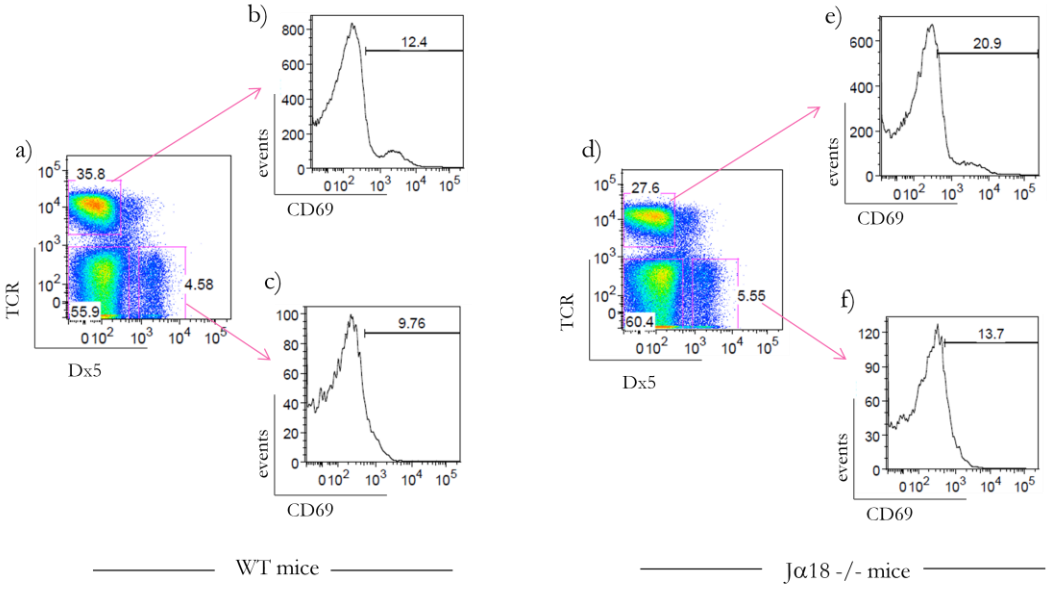


Figure 6.9. Flow cytometry analysis of spleen cells from C57BL/6 mice. a-c) Cells from WT mice sensitized with rBer e 1 and SPC lipid fraction C. a) Plot represents gate of SSC^{low} FSC^{low} cells – lymphocytes (TCR x Dx5); b) Histogram of TCR+ Dx5- cells (T cells); c) Histogram of TCR- Dx5+ cells (NK cells); d-f) Cells from Jα18 -/- mice sensitized with rBer e 1 and SPC lipid fraction C. d) Plot represents gate of SSC^{low} FSC^{low} cells – lymphocytes (TCR x Dx5); e) Histogram of TCR+ Dx5- cells (T cells); f) Histogram of TCR- Dx5+ cells (NK cells). Values in the plots and histograms indicate the percentage of marked cells. Sensitization and cells staining protocols are described in Material and Methods. Data is representative of one animal from each group (n=5).

The activation of TCR+ cells (T cells) and Dx5+ cells (NK cells) was studied through the expression of CD69. Figure 6.9. (a-f) indicates that the expression of CD69 is increased in Jα18-/- T cells (Figure 6.9.e) and NK cells (Figure 6.9.f) when compared to WT T cells and NK cells (Figure 6.9.b. and 6.9.c, respectively). However, no statistic differences ($p>0.05$) were found comparing animals form both groups. Therefore, although there is an indication that NK and T cells from Jα18 knockout animals are more activated than NK and T cells from WT animals, this fact could not be statistically confirmed.

It is known that NKT cells, when activated, produce large amounts of IL-4 and IFN-γ, activate DCs, macrophages and NK cells, and recruit neutrophils, expanding the immediate innate immune response. These events interfere in the subsequent adaptive T-cell and B-cell responses (Barral e Brenner, 2007). Therefore, considering this complex network of interactions, the absence of NKT cells (in Jα18-/- mice) could interfere in the behaviour of other cells of the immune system, explaining, for instance, the different activation of T cells and NK cells observed in NKT knockout animals.

In order to compare the results that originated from NKT experiments and the role that lipids from Brazil nuts play in the immune response to rBer e 1, the next series of experiments aimed to evaluate the antibody profile of WT and NKT knockout animals sensitised to rBer e 1.

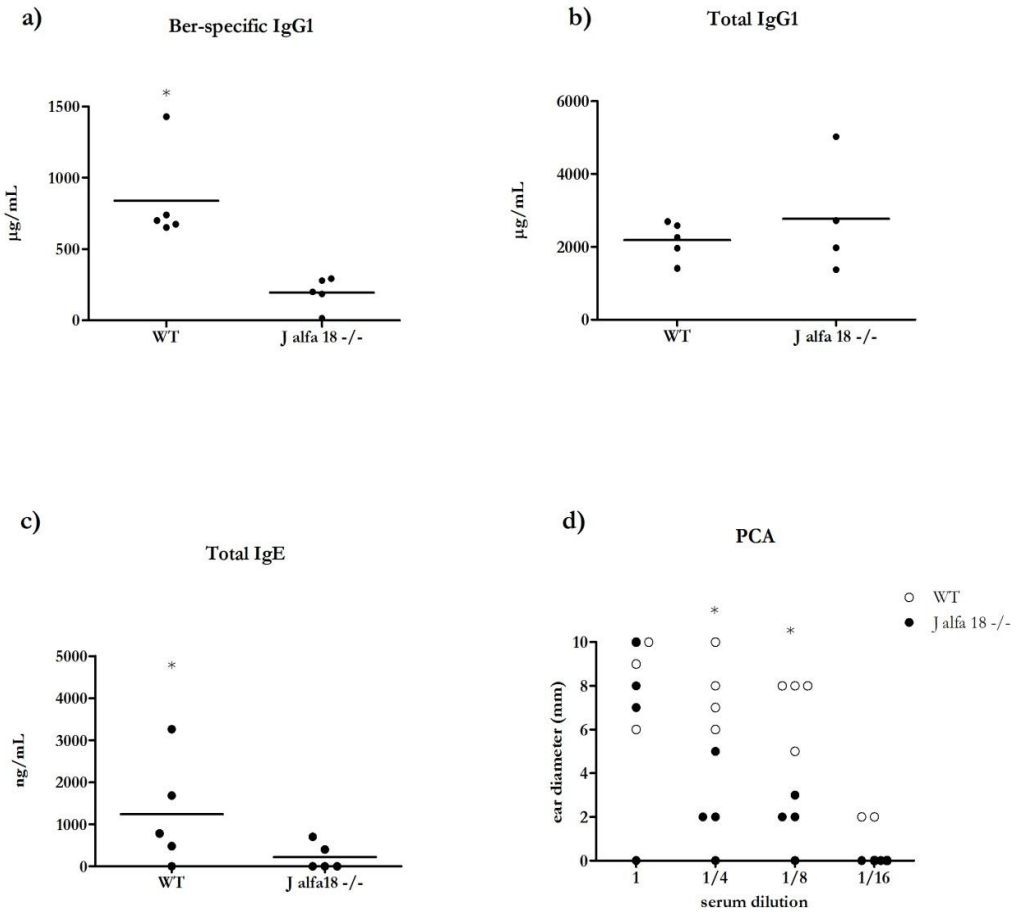


Figure 6.10. Antibody determination; **(a to c)** Total and Ber-specific immunoglobulins in the serum of Ber-sensitized wild type (WT) and Jα18 knockout (J alfa 18-/-) C57BL/6 mice measured by ELISA; **(d)** Ber-specific anaphylactic antibodies in different dilutions of serum from Ber-sensitized WT and Jα18 -/- C57BL/6 mice evaluated by PCA. Procedures performed as described in Material and Methods, Chaper 4. Symbols represent individual animals (n=5). Bars represent groups' means. T-student test or Two-way ANOVA with Bonferroni post test. Significant difference between groups: *$p<0.05$.

Figure 6.10. shows the immunoglobulins produced by WT and Jα18 -/- C57BL/6 mice sensitised with rBer e 1 and SPC lipid fraction C. It was possible to observe that WT animals produced significantly ($p<0.05$) increased titers of Ber-specific IgG1 (Figure 6.10.a) and total IgE (Figure 6.10.c) when compared to Jα18 -/- mice. The titers of anaphylactic immunoglobulins detected by PCA were also increased in WT mice (Figure 6.10.d). This difference was detected in the serum dilutions 1/4 and 1/8, in which serum from WT animals induced

significantly stronger PCA reactions than the serum from Jα18 -/- mice. These data clearly demonstrated that, although NKT cells were not essential for the immune activation induced by SPC lipid fraction C, the presence of these cells interfered in the development of the allergic response observed.

After stimulated by lipid ligands, NKT cells rapidly produce IL-4 and IFN-γ. IL-4 induces the differentiation of Th2 cells while IFN-γ is related with the development of Th1 cells. In animal models of asthma, direct activation of NKT cells by glycolipids results in the secretion of extensive amounts of cytokines and triggers the development of airway hyperreactivity. Moreover, in patients with chronic asthma, significant amounts of NKT cells can be found in their bronchoalveolar lavage fluids, suggesting that NKT plays an important role in allergy pathogenesis (Stock e Akbari, 2008; Umetsu e Dekruyff, 2010). Therefore, the less evident Th2-pattern of antibody production observed in Jα18-/- animals was probably due to the absence of NKT cells presented by these animals.

These results also confirmed the data obtained in Chapter 5, in which rBer e 1 in the presence of SPC lipid fraction C induced a Th2 humoral response, with increased titers of Ber-specific IgG1, total IgE, and the production of anaphylactic antibodies (Figure 5.3.). The average titers of Ber-specific IgG1 were higher in rBer+SPC fraction C sensitised C57BL/6, approximately 800 μg/mL (Figure 6.10.a), compared to rBer+SPC fraction C sensitised BALB/c, approximately 30 μg/mL (Figures 5.3., 5.4. and 5.6.a). However, this could be due to strain differences in the production of antibodies. BALB/c and C57BL/6 mice are known to differ in some aspects related to allergy. In BALB/c mice, for

instance, allergic responses are more dominated by IL-4 than IL-5 comparing to C57BL/6 mice (Drazen, Arm *et al.*, 1996).

Altogether, the results presented in this chapter corroborate the hypothesis that the recognition of lipids from Brazil nuts by the immune system might involve the activation of NKT cells. Firstly, lipid fractions from Brazil nuts were shown not to be able to activate humans TLRs. Furthermore, although no NKT activation was found using the 2h-protocol performed in BALB/c mice, the sensitisation protocol performed in Jα18-/- animals showed that these animals presented significantly decreased titers of Ber-specific IgG1, total IgE and anaphylactic antibodies compared to WT mice. These data indicate that the role of natural lipids from Brazil nuts might involve the activation of NKT cells and consequently CD1 presentation. Considering the complexity of the immune system, which is composed by numerous types of cells and compounds that interact with each other as well as with cells and compounds from other systems, such as nervous and endocrine systems, it is very likely that other cells participate in the role that natural lipids from Brazil nuts play in the development of an allergic response to rBer e 1. Moreover, it is important to consider that the fraction of lipids that induced the Th2-mediated response (SPC fraction C) is composed by a mixture of different classes of lipids and that, the individual minor components of this mixture were not able to induce the same pattern of immune response. This could be due to a synergistic effect amongst different classes of lipids or, alternatively, that the lipid concentration of the active compound is important for the development of the immune response. Further studies would be then necessary to find out the lipid aspects of immune activation, the network of cells and mechanisms involved with the recognition of

these active lipids by the immune system and the consequences of it in the development of allergy.

FINAL DISCUSSION

The prevalence of allergic diseases has been increased in recent years, and the reasons underlying this augmented incidence are still unclear (Niederberger, 2009). Extensive research using *in vivo* and *in vitro* models has been performed, and the general mechanisms, immune cells and molecules involved in allergic processes are well described in the literature, although the details are far from complete (Gould, Sutton *et al.*, 2003; Prussin e Metcalfe, 2006; Finkelman, 2007). Questions such as why some people are more prone to develop allergy and why only few proteins act as allergens remain unanswered. In fact, several factors are suggested to influence the development of allergy as, for instance, the structure and functional properties of a protein (Breiteneder, H. e Mills, E. N., 2005; Cochrane, Beyer *et al.*, 2009; Togias, Fenton *et al.*, 2010). However, not all proteins with 'typical allergen' features are involved in allergy cases and, moreover, not everybody develops allergy towards well-established allergens. Therefore, it is clear that there is still information missing in order to fully understand the networked events involved in allergic processes.

It has been recently shown that natural lipids play an essential role in the development of a Th2-type humoral response towards Ber e 1 (Dearman, Alcocer *et al.*, 2007). The present project further investigated the role of natural lipids in the sensitisation to Ber e 1, focusing in the Brazil nut lipids characterization (Chapter 3), humoral responses observed in animals sensitised with Ber e 1 in the presence of different fractions of lipids (Chapter 4 and 5), and mechanisms by which natural lipids modulate the sensitisation process (Chapter 6).

Firstly, no immune reaction was observed in animals sensitised with rBer e 1 alone, confirming previous findings (Dearman, Alcocer *et al.*, 2007). Although the allergenicity of Ber e 1, the major allergen from Brazil nuts, is well established (Arshad, Malmberg *et al.*, 1991; Pastorello, Farioli *et al.*, 1998; Bansal, Chee *et al.*, 2007) and this 2S albumin protein possesses several allergen-related features (Murtagh, Archer *et al.*, 2003; Alcocer, Murtagh *et al.*, 2004), this protein alone was not able to induce Th2-biased response in the animal model of sensitisation used in the present study.

Of the several fractions of Brazil nut lipids tested, only one fraction (SPC fraction C) consistently induced the production of increased titers of Ber-specific IgG1 and anaphylactic antibodies (found by PCA) in Ber-sensitised animals. These results indicated that particular classes of lipids present in this fraction, which is a complex mixture of several lipids, might be involved in the role that natural lipids play in the development of Brazil nut allergy. Other allergens, such as those from peanuts, were also reported to need the presence of the whole food extract in order to induce an immune response in mice (Van Wijk, Nierkens *et al.*, 2005).

As discussed in Chapter 3, both IgE and IgG1 antibody isotypes possess anaphylactic properties in mice (Mota, Wong *et al.*, 1968; Becker, 1971; Lehrer e Vaughan, 1976; Dombrowicz, Flamand *et al.*, 1997; Blaikie e Basketter, 1999; Ryan, Kashyap *et al.*, 2007). Although no titers of specific-IgE could be detected by ELISA in our protocol of sensitisation using rBer e 1 and the immune active lipid fraction (SPC fraction C), the consistent positive passive anaphylaxis reactions induced by serum from lipid-Ber sensitised animals indicate that the phenomenon observed can indeed be related to an allergic process. IgG1 is a Th2-type immunoglobulin usually present in murine allergic protocols

(Faquim-Mauro, Coffman *et al.*, 1999; Adel-Patient, Bernard *et al.*, 2005; Fujita, Teng *et al.*, 2009), and the isotypes equivalent to mouse IgG1 found in humans are IgG4 and IgG2 (Unkeless, Scigliano *et al.*, 1988). However, in humans the focus in allergic diseases is strongly related to IgE and not to other immunoglobulin subclasses (Wang, Lin *et al.*, 2010).

Sub-fractions extracted from the SPC fraction C containing different classes of lipids were tested *in vivo* in order to find specific lipid classes responsible for the immune effect observed, but although one of them induced evident immunogenic effect (phospholipid-rich sub-fraction), with increased titers of Ber-specific IgG1 and IgG2a, none of them induced anaphylactic antibodies. These results indicated that the dose of particular lipid classes might be important to modulate the immune respone to rBer e 1. The fact that in the present study the whole lipid extract from Brazil nuts was not able to induce the production of anaphylactic antibodies also corroborates this hypothesis. Dearman *et al* showed that the total Brazil nut lipid fraction induced anaphylactic antibodies when the level of triglycerides, the most abundant lipid class present in Brazil nuts, was reduced, altering therefore the proportion of other lipid constituents (Dearman, Alcocer *et al.*, 2007).

Indeed, the immune effect of SPC fraction C was shown to be concentration-dependent. Whereas lower doses induced a Th2-type response, with the presence of anaphylactic antibodies; higher doses induced evident immunogenic effect, with a vigorous increase in the titers of Ber-apecific IgG1 and IgG2a, but no anaphylactic antibodies. Doses of antigens as well as adjuvants are reported to play a role in the development of allergic processes; however, the underlying mechanisms are still unknown (Cochrane, Beyer *et al.*, 2009). In this vein, the level of LPS exposure is reported to determine the type of inflammatory

response towards the antigen, generating Th1- or Th2-skewed responses (Eisenbarth, Piggott *et al.*, 2002; Watanabe, Miyazaki *et al.*, 2003; Dong, Li *et al.*, 2009); although there are controversies regarding the levels necessary to modulate the direction of the immune response. The immune response induced by rBer e 1 in the presence of different doses of LPS was tested in the present study and, consistent with this, the LPS effect was demonstrated to be dose-dependent. Only higher doses of LPS induced the production of anaphylactic antibodies. Here it is relevant to mention that although LPS as well as natural lipids were able to induce anaphylactic antibodies in Ber sensitised animals, the humoral response induced by these lipids was different. Whereas natural lipids induced a more defined Th2 response, with increased IgG1 and low IgG2a, LPS induced robust increase in both antibodies isotypes, IgG1 and IgG2a (a Th1 isotype). Therefore, the immune response developed towards rBer e 1 was shown to be dependent not only on the presence of adjuvant compounds, such as natural lipids or LPS, but also dependent on the concentrarion of the adjuvants used.

The allergenicity of rBer e 1 was investigated in the presence of other lipids such as different commercial classes of phospholipids, and lipid fractions from sunflower seeds; as well as in the presence of alum, a well-established Th2 adjuvant. Interestingly, none of these compounds were able to induce detectable titers of Ber-specific antibodies in sensitised mice. These results suggest that Brazil nut natural lipids present exclusive features that are crucial for the development of a specifc immune response. Similar data are observed in the immune response induced by schistosome lipids, in which parasite but not synthetic lyso-phosphatidylserine presented immunomodulatory funtions (Van Der Kleij, Latz *et al.*, 2002). These results and the absence of immune response observed when rBer e 1 was injected in the presence of alum, a potent Th2

response inducer, bring out the possibility that the immunomodulatory effect is dependent on a protein-lipid complex. The fact that lipid binding is a common characteristic of major allergens (Thomas, Hales *et al.*, 2005) corroborates this hypothesis. For instance, the 2S albumin Sin a 1, the major allergen from mustard seeds, was shown to interact with phospholipid vesicles, binding to dimyristoylglycerophosphoglycerol vesicles with a dissociation constant of approximately 2.4 pM. The number of phospholipid molecules affected by one protein molecule was approximately 20 (Onaderra, Monsalve *et al.*, 1994).

Consistent with this, preliminary results investigating the lipid binding properties of rBer e 1 using NMR (nuclear magnetic resonance) spectroscopy indicated that the binding of lipid to Ber e 1 did push the protein to populate a conformational state that was different from the lipid-free form. Two types of lipid interactions were identified, one interaction in fast exchange, probably related to weak unspecific interactions with one or more lipid classes; and other interaction in slow exchange, probably related to a specific lipid interaction. Based on the volume of cross-peaks observed, the NMR results also suggested that Ber e 1 specifically interacts with one of the less abundant lipids in the SPC lipid fraction C. This study is being carried out in collaboration with Dr. G. Larsson at the University of Umea (Sweden), and the final data have not yet been published. Other evidences show that Ber e 1 is intrinsically a lipid binding protein. Non-specific binding results carried out in our lab and repeated at the University of Umea have shown that the hydrophobic site of Ber e 1 can accommodate a lipid molecule and the stoichiometric of this interaction is 1:1. It is relevant to highlight that the immune effect observed in the present study was induced at very low stoichiometry levels, approximately one part of protein to four parts of lipids. This fact is important to rule out other potential adjuvant or

micelle forming effects. Therefore, the lipid binding properties of Ber e 1 and the conformation state assumed by this protein after the lipid binding may be determinant factors for its allergenicity.

The SPC fraction C from Brazil nuts was composed by a mixture of different classes of lipids, mainly triglycerides, but also acylsterylglycosides, sterylglycosides, sulfolipids, lyso-phosphatidylcholine, phosphatidylcholine, phosphatisylinositol, and phosphatidic acid. In the data previously published, the lipid fractions able to induce anaphylactic antibodies and increased IgG1 and IgG contained (i) sterol, sterol ester and free fatty acids; (ii) all lipids with reduced triglyceride level; or (iii) sulfolipid, phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine. However, the lipid fractions used here and in the previous study (Dearman, Alcocer *et al.*, 2007) have been extracted by different techniques (SPC and TLC, respectively) and, furthermore, since the methods available to identify lipid classes are relative, precise identification of all lipid classes present in the fractions might not have been achieved. Therefore, in spite of lipid analyses have been useful for the development of the present study; the direct comparison with published data should be treated with caution.

In the literature, particular classes of lipids are reported to modulate adaptive immune responses. Specifically in Th2 responses, phosphorycholine was reported to be responsible for the immunomodulatory effects of ES-62, a glycoprotein present in nematodes (Harnett e Harnett, 2006; Harnett, Melendez *et al.*, 2009). ES-62 promoted the differentiation of naïve CD4+ T cells with a Th2 phenotype (Whelan, Harnett *et al.*, 2000; Goodridge, Mcguiness *et al.*, 2007) and suppressed the production IL-12, IL-6 and TNF-α. Its activity was shown to be TLR-4 and Myd88-dependent (Goodridge, Marshall *et al.*, 2005). Similarly to our results, in which Brazil nut lipids had immune effects when attached to SFA-

8 (protein from sunflower), the immunomodulatory properties of phosphorycholine was also shown when conjugated to other proteins, such as bovine serum albumin or ovalbumin (Goodridge, Mcguiness *et al.*, 2007; Harnett, Kean *et al.*, 2008). Lyso-phosphatidylserine is another example in the parasitology area; this lipid extracted from schistosomes contains specific acyl-chains, and it was able to polarize the maturation of dendritic cells into Th2-direction. The immunomodulatory function of this lipid was via TLR2 activation (Van Der Kleij, Latz *et al.*, 2002). The role of LPS, a lipid component of Gram-negative bacteria, has also been investigated in Th2-processes. Although controversial results are found concerning its participation in allergic processes (Williams, Ownby *et al.*, 2005), convincing data show that LPS, signalling through TLR4, can down- or up-regulate Th2-mediated allergic responses (Tulic, Knight *et al.*, 2001; Eisenbarth, Piggott *et al.*, 2002; Delayre-Orthez, De Blay *et al.*, 2004; Hollingsworth, Whitehead *et al.*, 2006).

Therefore, evidences demonstrating the participation of different classes of lipids in modulating Th2 immune responses are robust. Since the described underlying mechanisms by which the lipids cited above are recognized by the immune system involved TLRs (Medzhitov, 2001), we investigated whether the Th2 activation induced by Brazil nut lipids also involved TLRs. However, no human TLRs (2, 3, 4, 5, 7, 8 and 9) were activated by the SPC fraction C from Brazil nuts in the *in vitro* model used. Based on the possibility that a structural attachment of natural lipids and rBer e 1 is necessary for its immune recognition and consequent immunomodulation, it can be speculated that this is also a pre-requisite to TLR activation. And in the *in vitro* model used herein, lipids were tested in the absence of protein. In this vein, the immune activation induced by LPS, classically via TLR-4 (Medzhitov, 2001 (Poltorak, He *et al.*, 1998), is

preceded by its interaction with plasma lipid binding proteins (Schumann, Leong *et al.*, 1990). LPS-lipid binding protein complexes are subsequently recognized by CD14 (Wright, Ramos *et al.*, 1990) and cellular signaling is transmitted through TLR4 (Poltorak, He *et al.*, 1998). Therefore, although the results of the *in vitro* test indicated that the immune activation induced by lipids from Brazil nuts does not involve TLR activation, this possibility can not be completely ruled out.

Another way by which lipids can be recognized by the immune system involves CD1-NKT pathways. CD1 is a MHC class I-like molecule expressed in antigen presenting cells that are capable of presenting lipid antigens to NKT cells. Stimulated NKT cells rapidly produce both Th1 (IFN-γ) and Th2 (IL-4) cytokines, and also activate several immune cells (Porcelli e Modlin, 1999; Barral e Brenner, 2007). Due to its pronounced secretion of IL-4, NKT cells have been implicated in Th2 processes (Yoshimoto, Bendelac *et al.*, 1995; Leite-De-Moraes, Moreau *et al.*, 1998; Lisbonne, Diem *et al.*, 2003). It has been shown that NKT cells are required for airway eosinophilia, Th2 cytokine production, and elevated levels of IgE in an animal model of allergic asthma to ovalbumin (Lisbonne, Diem *et al.*, 2003). CD1 molecules are also involved in pollen allergy. Phospholipids present in the pollen membrane, particularly phosphatidylserine, cardiolipin, phosphatidylethanolamine and phosphatidylcholine, were reported to be implicated in early pollen grain capture and recognition by CD1 (Russano, Agea *et al.*, 2008). The involvement of NKT cells in modulating the Th2-type humoral response observed in animals sensitised to rBer e 1 and SPC fraction C from Brazil nuts was demonstrated in the present study, since sensitised NKT-knockout mice presented significantly less IgE, IgG1 and anaphylactic antibodies than wild type animals. No significant differences were observed in the cytokine production or activation of NKT cells stimulated by Brazil nut lipids; however,

this could have been due to the very limited number of animals used for this experiment. Therefore, the participation of CD1-NKT pathways was demonstrated not be essential for the sensitisation to rBer e 1, but these results clearly showed that the immune activity of Brazil nut lipids does involve this mechanism. In addition to the hypothesis that the lipid-binding features of rBer e 1 are essential for its immune recognition, it may be speculated that the CD1 positive dendritic cells recognize natural lipids from Brazil nuts in the early stages of the allergic response and present them to CD1-restricted T cells. As antigen presenting cells can express CD1 and MHC class II molecules, two different kinds of antigen recognition may coexist. This was observed in pollen, intact grain was degraded in the lysosomal compartment where both CD1 and MHC class II molecules were present (Van Rhijn, Zajonc *et al.*, 2005).

In summary, the data presented in this study demonstrated that natural lipids from Brazil nuts do play a crucial role in the sensitisation to rBer e 1, the major allergen in these nuts. Lipid concentration as well as lipid classes was important for the development of the humoral response towards rBer e 1, which was characterized by increased titers of Ber-specific IgG1 and Ber-specific anaphylactic antibodies. The mechanisms by which natural lipids orchestrate the immunomodulation observed herein might involve CD1-NKT related pathways. Therefore, although further studies are necessary to fully understand the role that natural lipids play in Brazil allergy, this study provided insights into interaction of this class of molecules with the immune system and the role they may play in immunopathologies.

APPENDIX 1

Brazil nuts, dried, unblanched

Refuse: 49% (Shells)
Scientific name: *Bertholletia excelsa*

Nutrient	Units	Value per 100 grams	Number of Data Points	Std. Error
Proximates				
Water	g	3.48	9	0.170
Energy	kcal	656	0	
Energy	kJ	2743	0	
Protein	g	14.32	7	0.146
Total lipid (fat)	g	66.43	7	0.237
Ash	g	3.51	7	0.033
Carbohydrate, by difference	g	12.27	0	
Fiber, total dietary	g	7.5	3	0.232
Sugars, total	g	2.33	7	0.078
Sucrose	g	2.33	7	0.078
Glucose (dextrose)	g	0.00	7	0.000
Fructose	g	0.00	7	0.000
Lactose	g	0.00	7	0.000
Maltose	g	0.00	7	0.000
Galactose	g	0.00	7	0.000
Starch	g	0.25	7	0.000
Minerals				
Calcium, Ca	mg	160	7	5.660
Iron, Fe	mg	2.43	7	0.030
Magnesium, Mg	mg	376	7	3.746
Phosphorus, P	mg	725	7	5.614
Potassium, K	mg	659	7	8.853
Sodium, Na	mg	3	7	0.252
Zinc, Zn	mg	4.06	7	0.043
Copper, Cu	mg	1.743	7	0.021
Manganese, Mn	mg	1.223	7	0.052
Selenium, Se	mcg	1917.0	15	231.798

Vitamins				
Vitamin C, total ascorbic acid	mg	0.7	3	0.000
Thiamin	mg	0.617	7	0.053
Riboflavin	mg	0.035	7	0.001
Niacin	mg	0.295	7	0.019
Pantothenic acid	mg	0.184	7	0.012
Vitamin B-6	mg	0.101	7	0.004
Folate, total	mcg	22	4	2.316
Folic acid	mcg	0	0	
Folate, food	mcg	22	4	2.316
Folate, DFE	mcg_DFE	22	0	
Choline, total	mg	28.8	0	
Betaine	mg	0.4	2	
Vitamin B-12	mcg	0.00	0	
Vitamin B-12, added	mcg	0.00	0	
Vitamin A, RAE	mcg_RAE	0	1	
Retinol	mcg	0	0	
Carotene, beta	mcg	0	1	
Carotene, alpha	mcg	0	1	
Cryptoxanthin, beta	mcg	0	1	
Vitamin A, IU	IU	0	1	
Lycopene	mcg	0	1	
Lutein + zeaxanthin	mcg	0	1	
Vitamin E (alpha-tocopherol)	mg	5.73	6	1.540
Vitamin E, added	mg	0.00	0	
Tocopherol, beta	mg	0.00	6	0.000
Tocopherol, gamma	mg	7.87	6	2.148
Tocopherol, delta	mg	0.77	6	0.657
Vitamin D (D2 + D3)	mcg	0.0	0	
Vitamin D	IU	0	0	
Vitamin K (phylloquinone)	mcg	0.0	7	0.000
Lipids				
Fatty acids, total saturated	g	15.137	0	
4:0	g	0.000	0	
6:0	g	0.000	0	

8:0	g	0.000	3	0.000
10:0	g	0.000	3	0.000
12:0	g	0.000	3	0.000
14:0	g	0.052	3	0.000
15:0	g	0.000	3	0.000
16:0	g	9.085	3	0.200
17:0	g	0.047	3	0.012
18:0	g	5.794	3	0.145
20:0	g	0.160	3	0.007
22:0	g	0.000	3	0.000
Fatty acids, total monounsaturated	g	24.548	0	
14:1	g	0.000	3	0.000
15:1	g	0.000	3	0.000
16:1 undifferentiated	g	0.229	3	0.004
17:1	g	0.044	3	0.009
18:1 undifferentiated	g	24.223	3	0.835
20:1	g	0.052	3	0.000
22:1 undifferentiated	g	0.000	0	
Fatty acids, total polyunsaturated	g	20.577	0	
18:2 undifferentiated	g	20.543	3	0.492
18:3 undifferentiated	g	0.035	0	
18:3 n-3 c,c,c (ALA)	g	0.017	3	0.000
18:3 n-6 c,c,c	g	0.017	3	0.000
18:4	g	0.000	0	
20:2 n-6 c,c	g	0.000	3	0.000
20:3 undifferentiated	g	0.000	3	0.000
20:4 undifferentiated	g	0.000	3	0.000
Amino acids				
Tryptophan	g	0.141	0	
Threonine	g	0.362	0	
Isoleucine	g	0.516	0	
Leucine	g	1.155	0	
Lysine	g	0.492	0	
Methionine	g	1.008	0	
Cystine	g	0.367	0	
Phenylalanine	g	0.630	0	

Tyrosine	g	0.420	0	
Valine	g	0.756	0	
Arginine	g	2.148	0	
Histidine	g	0.386	0	
Alanine	g	0.577	0	
Aspartic acid	g	1.346	0	
Glutamic acid	g	3.147	0	
Glycine	g	0.718	0	
Proline	g	0.657	0	
Serine	g	0.683	0	
Other				
Alcohol, ethyl	g	0.0	0	
Caffeine	mg	0	0	
Theobromine	mg	0	0	

USDA National Nutrient Database for Standard Reference, Release 22 (2009)

APPENDIX 2

profos
creative bioscience solutions

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Regensburg, 04.07.2008

ENDOTOXIN Detection: Limulus Amoebocyte Lysate (LAL) Assay

Endotoxin Detection Service for order: n.a. (sample arrived: 20.06.2008)
Method: kinetic, chromogenic Limulus Amoebocyte Lysate Test
(LAL-Assay, Lonza BioScience, Lot GL017U)

Sample 1 Loading (extract from plant seeds)
Dilution Buffer: Aqueous 0.5 % detergent solution

Sample 2 C:M (extract from plant seeds)
Dilution Buffer: Aqueous 0.5 % detergent solution

Dilutions for LAL-Test: Both samples had to be diluted before running the LAL assay:
1)12 µl of sample diluted in 988 µl dilution buffer
2) 200 µl of the first dilution diluted in 800 µl dilution buffer
3) 200 µl of the second dilution diluted in 800 µl dilution buffer
The samples were heated to 65 °C for 20 minutes after each dilution step to achieve complete dissolution.

Measured values:

Sample 1 (2nd Dilution, 2.4 µl sample/ml)

dilution	measured value	EU/ml	positive control, spike recovery, % (Variance: 50 - 200 %) according to EP guideline 2001	
1/100	min	< 0.5	64 %	OK
Value		< 0.5		

With a sample concentration of 2.4 µl/ml, the average value of <0.5 EU/ml corresponds to approximately < 0.2 EU/µl sample. The LPS content is below the detection limit of the assay.

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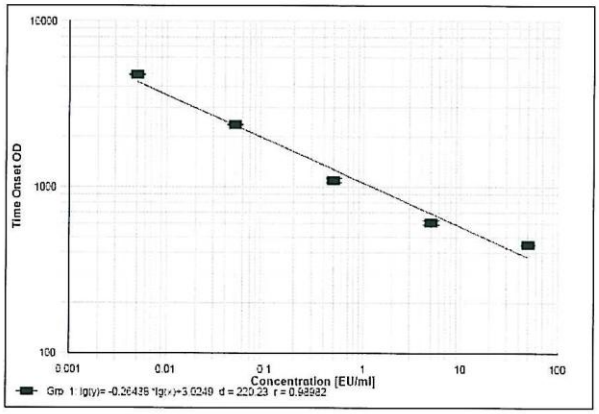
Sample 2 (3rd Dilution, 0.48 µl sample/ml)

dilution	measured value	EU/ml	positive control, spike recovery, % (Variance: 50 - 200 %) according to EP guideline 2001
1/100	min	< 0.5	54 % OK
Value		< 0.5	

With a sample concentration of 0.48 µl /ml, the average value of 0.5 EU/ml corresponds to approximately < 1 EU/µl sample. The LPS content is below the detection limit of the assay.

Standardcurve
(Standard Endotoxin from Lonza BioScience, Lot FL GL0009)



Regression coefficient (target: 0.98982 [has to be > 0.98]): OK

Controls
Standard inhibition/enhancement assay
Negative control: endotoxin free water
Positive control: sample spiked with Standard Endotoxin (5 EU/ml)

Summary
Special treatment of the samples was necessary to ensure complete dissolution. Therefore the samples were diluted with an aqueous solution containing an appropriate detergent and heat treated before every dilution step. As an inhibition of the LAL assay at lower dilutions was observed the only valid results were obtained at higher dilutions (see results).

Date 04.07.2008

Christian Arzt, Separations & Services
Profos AG

REFERENCES

Adel-Patient, K., H. Bernard*, et al.* Peanut- and cow's milk-specific IgE, Th2 cells and local anaphylactic reaction are induced in Balb/c mice orally sensitized with cholera toxin. Allergy, v.60, n.5, May, p.658-64. 2005.

Agea, E., A. Russano*, et al.* Human CD1-restricted T cell recognition of lipids from pollens. J Exp Med, v.202, n.2, Jul 18, p.295-308. 2005.

Akira, S. e K. Takeda. Toll-like receptor signalling. Nat Rev Immunol, v.4, n.7, Jul, p.499-511. 2004.

Akira, S., M. Yamamoto*, et al.* Role of adapters in Toll-like receptor signalling. Biochem Soc Trans, v.31, n.Pt 3, Jun, p.637-42. 2003.

Alber, G., U. M. Kent*, et al.* Functional comparison of Fc epsilon RI, Fc gamma RII, and Fc gamma RIII in mast cells. J Immunol, v.149, n.7, Oct 1, p.2428-36. 1992.

Alcocer, M. J., G. J. Murtagh*, et al.* The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins. J Mol Biol, v.324, n.1, Nov 15, p.165-75. 2002.

Alcocer, M.J.. The major human structural IgE epitope of the Brazil nut allergen Ber e 1: a chimaeric and protein microarray approach. J Mol Biol, v.343, n.3, Oct 22, p.759-69. 2004.

Alcorn, J. F., K. Ckless*, et al.* Strain-dependent activation of NF-kappaB in the airway epithelium and its role in allergic airway inflammation. Am J Physiol Lung Cell Mol Physiol, v.298, n.1, Jan, p.L57-66. 2010.

Amaral, J. S., S. Casal*, et al.* Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (Juglans regia L.) cultivars grown in Portugal. J Agric Food Chem, v.51, n.26, Dec 17, p.7698-702. 2003.

Arshad, S. H., E. Malmberg*, et al.* Clinical and immunological characteristics of Brazil nut allergy. Clin Exp Allergy, v.21, n.3, May, p.373-6. 1991.

Bach, G., L. Perrin-Cocon*, et al.* Single lysophosphatidylcholine components exhibit adjuvant activities in vitro and in vivo. Clin Vaccine Immunol, v.17, n.3, Mar, p.429-38. 2010.

Bacharier, L. B. e R. S. Geha. Molecular mechanisms of IgE regulation. J Allergy Clin Immunol, v.105, n.2 Pt 2, Feb, p.S547-58. 2000.

Bansal, A. S., R. Chee*, et al.* Dangerous liaison: sexually transmitted allergic reaction to Brazil nuts. J Investig Allergol Clin Immunol, v.17, n.3, p.189-91. 2007.

Barbas, A. S., T. E. Downing*, et al.* Chronic aspiration shifts the immune response from Th1 to Th2 in a murine model of asthma. Eur J Clin Invest, v.38, n.8, Aug, p.596-602. 2008.

Barral, D. C. e M. B. Brenner. CD1 antigen presentation: how it works. Nat Rev Immunol, v.7, n.12, Dec, p.929-41. 2007.

Baud, F., E. Pebay-Peyroula*, et al.* Crystal structure of hydrophobic protein from soybean; a member of a new cysteine-rich family. J Mol Biol, v.231, n.3, Jun 5, p.877-87. 1993.

Bauer, A., R. Huttinger*, et al.* Analysis of the requirement for beta 2-microglobulin for expression and formation of human CD1 antigens. Eur J Immunol, v.27, n.6, Jun, p.1366-73. 1997.

Becard, J. C., C. Chevalier*, et al.* Quantitative Analysis of Phospholipids by HPLC with a Light Scattering Evaporating Detector. Application to Raw Materials for Cosmetic Use. . *J. High Resolut. Chromatog.* , v.13, p.126-129. 1999.

Becker, E. L. Nature and classification of immediate-type allergic reactions. Adv Immunol, v.13, p.267-313. 1971.

Benhamou, M. e R. P. Siraganian. Protein-tyrosine phosphorylation: an essential component of Fc epsilon RI signaling. Immunol Today, v.13, n.6, Jun, p.195-7. 1992.

Bevilacqua, C., G. Montagnac*, et al.* Food allergens are protected from degradation during CD23-mediated transepithelial transport. Int Arch Allergy Immunol, v.135, n.2, Oct, p.108-16. 2004.

Birmingham, N., S. Payankaulam*, et al.* An ELISA-based method for measurement of food-specific IgE antibody in mouse serum: an alternative to the passive cutaneous anaphylaxis assay. J Immunol Methods, v.275, n.1-2, Apr 1, p.89-98. 2003.

Birmingham, N. P., S. Parvataneni*, et al.* An adjuvant-free mouse model of tree nut allergy using hazelnut as a model tree nut. Int Arch Allergy Immunol, v.144, n.3, p.203-10. 2007.

Bischoff, S. C. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. Nat Rev Immunol, v.7, n.2, Feb, p.93-104. 2007.

Blaikie, L. e D. A. Basketter. Experience with a mouse intranasal test for the predictive identification of respiratory sensitization potential of proteins. Food Chem Toxicol, v.37, n.8, Aug, p.889-96. 1999.

Bocek, P., Jr., L. Draberova*, et al.* Characterization of Fc gamma receptors on rat mucosal mast cells using a mutant Fc epsilon RI-deficient rat basophilic leukemia line. Eur J Immunol, v.25, n.10, Oct, p.2948-55. 1995.

Bochner, B. S. e L. M. Lichtenstein. Anaphylaxis. N Engl J Med, v.324, n.25, Jun 20, p.1785-90. 1991.

Boehme, S. A., E. P. Chen*, et al.* Antagonism of CRTH2 ameliorates chronic epicutaneous sensitization-induced inflammation by multiple mechanisms. Int Immunol, v.21, n.1, Jan, p.1-17. 2009.

Borg, N. A., K. S. Wun*, et al.* CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature, v.448, n.7149, Jul 5, p.44-9. 2007.

Bortolatto, J., E. Borducchi*, et al.* Toll-like receptor 4 agonists adsorbed to aluminium hydroxide adjuvant attenuate ovalbumin-specific allergic airway disease: role of MyD88 adaptor molecule and interleukin-12/interferon-gamma axis. Clin Exp Allergy, v.38, n.10, Oct, p.1668-79. 2008.

Brandt, E. B., R. T. Strait*, et al.* Mast cells are required for experimental oral allergen-induced diarrhea. J Clin Invest, v.112, n.11, Dec, p.1666-77. 2003.

Breiteneder, H. e C. Ebner. Molecular and biochemical classification of plant-derived food allergens. J Allergy Clin Immunol, v.106, n.1 Pt 1, Jul, p.27-36. 2000.

Breiteneder, H. e C. Mills. Nonspecific lipid-transfer proteins in plant foods and pollens: an important allergen class. Curr Opin Allergy Clin Immunol, v.5, n.3, Jun, p.275-9. 2005.

Breiteneder, H. e E. N. Mills. Molecular properties of food allergens. J Allergy Clin Immunol, v.115, n.1, Jan, p.14-23; quiz 24. 2005.

Breiteneder, H. e C. Radauer. A classification of plant food allergens. J Allergy Clin Immunol, v.113, n.5, May, p.821-30; quiz 831. 2004.

Brewer, J. M., M. Conacher*, et al.* Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. J Immunol, v.163, n.12, Dec 15, p.6448-54. 1999.

Brufau, G., J. Boatella*, et al.* Nuts: source of energy and macronutrients. Br J Nutr, v.96 Suppl 2, Nov, p.S24-8. 2006.

Bush, R. K. e S. L. Hefle. Food allergens. Crit Rev Food Sci Nutr, v.36 Suppl, p.S119-63. 1996.

Cai, S., S. Batra*, et al.* Both TRIF- and MyD88-dependent signaling contribute to host defense against pulmonary Klebsiella infection. J Immunol, v.183, n.10, Nov 15, p.6629-38. 2009.

Calcaterra, C., L. Sfondrini*, et al.* Critical role of TLR9 in acute graft-versus-host disease. J Immunol, v.181, n.9, Nov 1, p.6132-9. 2008.

Cardoso, C. R., G. Teixeira, *et al.* Modulation of mucosal immunity in a murine model of food-induced intestinal inflammation. Clin Exp Allergy, v.38, n.2, Feb, p.338-49. 2008.

Carroll-Portillo, A., K. Spendier, *et al.* Formation of a Mast Cell Synapse: Fc{varepsilon}RI Membrane Dynamics upon Binding Mobile or Immobilized Ligands on Surfaces. J Immunol, Dec 30. 2009.

Chambers, S. J., M. S. Wickham, *et al.* Rapid in vivo transport of proteins from digested allergen across pre-sensitized gut. Biochem Biophys Res Commun, v.325, n.4, Dec 24, p.1258-63. 2004.

Chandran, S. S., D. Verhoeven, *et al.* TLR2 engagement on dendritic cells promotes high frequency effector and memory CD4 T cell responses. J Immunol, v.183, n.12, Dec 15, p.7832-41. 2009.

Chang, T. W. e Y. Y. Shiung. Anti-IgE as a mast cell-stabilizing therapeutic agent. J Allergy Clin Immunol, v.117, n.6, Jun, p.1203-12; quiz 1213. 2006.

Chehade, M. e L. Mayer. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol, v.115, n.1, Jan, p.3-12; quiz 13. 2005.

Chen, L., L. Lei, *et al.* Mice Deficient in MyD88 Develop a Th2-Dominant Response and Severe Pathology in the Upper Genital Tract following Chlamydia muridarum Infection. J Immunol, Feb 1. 2010.

Chong, H. J., L. Andrew Bouton, *et al.* IL-4 selectively enhances FcgammaRIII expression and signaling on mouse mast cells. Cell Immunol, v.224, n.2, Aug, p.65-73. 2003.

Christie, W. W. Advances in Lipid Methodology: Ed. W.W. Christie, Oily Press, Ayr, v.One. 1992. 1-17 p.

Chrisite, W. W.. *Lipid Analysis* The Oily Press, Bridgwater 2003

Christie, W. W., Gill, S., Nordbäck, J., Itabashi, Y., Sanda, S. And Slabas, A.R. . New procedures for rapid screening of leaf lipid components from Arabidopsis. . Phytochemical Anal., v.9, p.53-57. 1998.

Chunhieng , H. A., Pioch D, Brochier J and Montet D. Detailed Study of Brazil Nut (*Bertholletia excelsa*) Oil Micro-Compounds: Phospholipids, Tocopherols and Sterols. J. Braz. Chem. Soc., v.19, n.7, p.1374-1380. 2008.

Cianferoni, A. e J. M. Spergel. Food allergy: review, classification and diagnosis. Allergol Int, v.58, n.4, Dec, p.457-66. 2009.

Cochrane, S., K. Beyer, *et al.* Factors influencing the incidence and prevalence of food allergy. Allergy, v.64, n.9, Sep, p.1246-55. 2009.

Coutant, F., L. Perrin-Cocon*, et al.* Mature dendritic cell generation promoted by lysophosphatidylcholine. J Immunol, v.169, n.4, Aug 15, p.1688-95. 2002.

Crespo, J. F., J. M. James*, et al.* Food allergy: nuts and tree nuts. Br J Nutr, v.96 Suppl 2, Nov, p.S95-102. 2006.

Cui, J., T. Shin*, et al.* Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science, v.278, n.5343, Nov 28, p.1623-6. 1997.

Curotto De Lafaille, M. A., S. Muriglan*, et al.* Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. J Exp Med, v.194, n.9, Nov 5, p.1349-59. 2001.

Daeron, M. Fc receptor biology. Annu Rev Immunol, v.15, p.203-34. 1997.

Daeron, M., S. Latour*, et al.* The same tyrosine-based inhibition motif, in the intracytoplasmic domain of Fc gamma RIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. Immunity, v.3, n.5, Nov, p.635-46. 1995.

Darcan-Nicolaisen, Y., H. Meinicke*, et al.* Small interfering RNA against transcription factor STAT6 inhibits allergic airway inflammation and hyperreactivity in mice. J Immunol, v.182, n.12, Jun 15, p.7501-8. 2009.

Dearman, R. J., M. J. Alcocer*, et al.* Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. Clin Exp Allergy, v.37, n.4, Apr, p.582-91. 2007.

Dearman, R. J. e I. Kimber. Determination of protein allergenicity: studies in mice. Toxicol Lett, v.120, n.1-3, Mar 31, p.181-6. 2001.

Dearman, R. J., R. A. Skinner*, et al.* Evaluation of an in vitro method for the measurement of specific IgE antibody responses: the rat basophilic leukemia (RBL) cell assay. Toxicology, v.206, n.2, Jan 15, p.195-205. 2005.

Del Prete, G. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. Allergy, v.47, n.5, Oct, p.450-5. 1992.

Del Prete, G., E. Maggi*, et al.* IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. J Immunol, v.140, n.12, Jun 15, p.4193-8. 1988.

Delayre-Orthez, C., F. De Blay*, et al.* Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. Clin Exp Allergy, v.34, n.11, Nov, p.1789-95. 2004.

Dombrowicz, D., V. Flamand*, et al.* Absence of Fc epsilonRI alpha chain results in upregulation of Fc gammaRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between Fc epsilonRI and Fc gammaRIII for limiting amounts of FcR beta and gamma chains. J Clin Invest, v.99, n.5, Mar 1, p.915-25. 1997.

Dong, L., H. Li*, et al.* Different doses of lipopolysaccharides regulate the lung inflammation of asthmatic mice via TLR4 pathway in alveolar macrophages. J Asthma, v.46, n.3, Apr, p.229-33. 2009.

Drazen, J. M., J. P. Arm*, et al.* Sorting out the cytokines of asthma. J Exp Med, v.183, n.1, Jan 1, p.1-5. 1996.

Eisenbarth, S. C., D. A. Piggott*, et al.* Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med, v.196, n.12, Dec 16, p.1645-51. 2002.

Eriksson, N. E. e S. Ahlstedt. Diagnosis of reaginic allergy with house dust, animal dander and pollen allergens in adult patients. V. A comparison between the enzyme-linked immunosorbent assay (ELISA), provocation tests, skin tests and RAST. Int Arch Allergy Appl Immunol, v.54, n.1, p.88-95. 1977.

Eriksson, N. E., C. Moller*, et al.* Self-reported food hypersensitivity in Sweden, Denmark, Estonia, Lithuania, and Russia. J Investig Allergol Clin Immunol, v.14, n.1, p.70-9. 2004.

Esch, R. E. Allergens. Clin Rev Allergy Immunol, v.30, n.2, Apr, p.71-2. 2006.

Fahy, E., S. Subramaniam*, et al.* Update of the LIPID MAPS comprehensive classification system for lipids. J Lipid Res, v.50 Suppl, Apr, p.S9-14. 2009.

Falcone, F. H., H. Haas*, et al.* The human basophil: a new appreciation of its role in immune responses. Blood, v.96, n.13, Dec 15, p.4028-38. 2000.

Faquim-Mauro, E. L., R. L. Coffman*, et al.* Cutting edge: mouse IgG1 antibodies comprise two functionally distinct types that are differentially regulated by IL-4 and IL-12. J Immunol, v.163, n.7, Oct 1, p.3572-6. 1999.

Faquim-Mauro, E. L., J. F. Jacysyn*, et al.* Anaphylactic and non-anaphylactic murine IgG1 differ in their ability to bind to mast cells: relevance of proper glycosylation of the molecule. Immunobiology, v.207, n.3, p.169-77. 2003.

Faquim-Mauro, E. L. e M. S. Macedo. Induction of IL-4-dependent, anaphylactic-type and IL-4-independent, non-anaphylactic-type IgG1 antibodies is modulated by adjuvants. Int Immunol, v.12, n.12, Dec, p.1733-40. 2000.

Faveeuw, C., V. Angeli*, et al.* Antigen presentation by CD1d contributes to the amplification of Th2 responses to Schistosoma mansoni glycoconjugates in mice. J Immunol, v.169, n.2, Jul 15, p.906-12. 2002.

Fehlner, P. F., L. Kochoumian*, et al.* Murine IgE and IgG responses to melittin and its analogs. J Immunol, v.146, n.8, Apr 15, p.2664-70. 1991.

Fernandez Rivas, M. Food allergy in Alergologica-2005. J Investig Allergol Clin Immunol, v.19 Suppl 2, p.37-44. 2009.

Finkelman, F. D. Anaphylaxis: lessons from mouse models. J Allergy Clin Immunol, v.120, n.3, Sep, p.506-15; quiz 516-7. 2007.

Folch, J., M. Lees*, et al.* A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem, v.226, n.1, May, p.497-509. 1957.

Freudenberg, M. A., S. Tchaptchet*, et al.* Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. Immunobiology, v.213, n.3-4, p.193-203. 2008.

Fujita, H., A. Teng*, et al.* Production of both IL-27 and IFN-gamma after the treatment with a ligand for invariant NK T cells is responsible for the suppression of Th2 response and allergic inflammation in a mouse experimental asthma model. J Immunol, v.183, n.1, Jul 1, p.254-60. 2009.

Fung-Leung, W. P., J. De Sousa-Hitzler*, et al.* Transgenic mice expressing the human high-affinity immunoglobulin (Ig) E receptor alpha chain respond to human IgE in mast cell degranulation and in allergic reactions. J Exp Med, v.183, n.1, Jan 1, p.49-56. 1996.

Galli, C. e P. C. Calder. Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. Ann Nutr Metab, v.55, n.1-3, p.123-39. 2009.

Galli, S. J., M. Maurer*, et al.* Mast cells as sentinels of innate immunity. Curr Opin Immunol, v.11, n.1, Feb, p.53-9. 1999.

Galli, S. J., S. Nakae*, et al.* Mast cells in the development of adaptive immune responses. Nat Immunol, v.6, n.2, Feb, p.135-42. 2005.

Galli, S. J., K. M. Zsebo*, et al.* The kit ligand, stem cell factor. Adv Immunol, v.55, p.1-96. 1994.

Gilfillan, A. M. e C. Tkaczyk. Integrated signalling pathways for mast-cell activation. Nat Rev Immunol, v.6, n.3, Mar, p.218-30. 2006.

Godfrey, D. I., H. R. Macdonald*, et al.* NKT cells: what's in a name? Nat Rev Immunol, v.4, n.3, Mar, p.231-7. 2004.

Goodman, R. E., S. L. Hefle*, et al.* Assessing genetically modified crops to minimize the risk of increased food allergy: a review. Int Arch Allergy Immunol, v.137, n.2, Jun, p.153-66. 2005.

Goodridge, H. S., F. A. Marshall*, et al.* Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. J Immunol, v.174, n.1, Jan 1, p.284-93. 2005.

Goodridge, H. S., S. Mcguiness*, et al.* Phosphorylcholine mimics the effects of ES-62 on macrophages and dendritic cells. Parasite Immunol, v.29, n.3, Mar, p.127-37. 2007.

Gould, H. J. e B. J. Sutton. IgE in allergy and asthma today. Nat Rev Immunol, v.8, n.3, Mar, p.205-17. 2008.

Gould, H. J., B. J. Sutton, *et al.* The biology of IGE and the basis of allergic disease. Annu Rev Immunol, v.21, p.579-628. 2003.

Grajewski, R. S., A. M. Hansen, *et al.* Activation of invariant NKT cells ameliorates experimental ocular autoimmunity by a mechanism involving innate IFN-gamma production and dampening of the adaptive Th1 and Th17 responses. J Immunol, v.181, n.7, Oct 1, p.4791-7. 2008.

Griffiths, C. E., R. J. Dearman, *et al.* Cytokines and Langerhans cell mobilisation in mouse and man. Cytokine, v.32, n.2, Oct 21, p.67-70. 2005.

Hailman, E., H. S. Lichenstein, *et al.* Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med, v.179, n.1, Jan 1, p.269-77. 1994.

Hakim-Rad, K., M. Metz, *et al.* Mast cells: makers and breakers of allergic inflammation. Curr Opin Allergy Clin Immunol, v.9, n.5, Oct, p.427-30. 2009.

Hallgren, J. e M. F. Gurish. Pathways of murine mast cell development and trafficking: tracking the roots and routes of the mast cell. Immunol Rev, v.217, Jun, p.8-18. 2007.

Hammad, H., M. Chieppa, *et al.* House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med, v.15, n.4, Apr, p.410-6. 2009.

Hansen, D. S., M. A. Siomos, *et al.* Regulation of murine cerebral malaria pathogenesis by CD1d-restricted NKT cells and the natural killer complex. Immunity, v.18, n.3, Mar, p.391-402. 2003.

Harnett, M. M., D. E. Kean, *et al.* The phosphorycholine moiety of the filarial nematode immunomodulator ES-62 is responsible for its anti-inflammatory action in arthritis. Ann Rheum Dis, v.67, n.4, Apr, p.518-23. 2008.

Harnett, M. M., A. J. Melendez, *et al.* The therapeutic potential of the filarial nematode-derived immunodulator, ES-62 in inflammatory disease. Clin Exp Immunol, v.159, n.3, Mar, p.256-67. 2009.

Harnett, W. e M. M. Harnett. Filarial nematode secreted product ES-62 is an anti-inflammatory agent: therapeutic potential of small molecule derivatives and ES-62 peptide mimetics. Clin Exp Pharmacol Physiol, v.33, n.5-6, May-Jun, p.511-8. 2006.

Harnett, W. Immunomodulatory activity and therapeutic potential of the filarial nematode secreted product, ES-62. Adv Exp Med Biol, v.666, p.88-94. 2009.

Harwanegg, C. e R. Hiller. Protein microarrays for the diagnosis of allergic diseases: state-of-the-art and future development. Eur Ann Allergy Clin Immunol, v.38, n.7, Sep, p.232-6. 2006.

Hashizume, T., A. Togawa*, et al.* Peyer's patches are required for intestinal immunoglobulin A responses to Salmonella spp. Infect Immun, v.76, n.3, Mar, p.927-34. 2008.

Hazenbos, W. L., J. E. Gessner*, et al.* Impaired IgG-dependent anaphylaxis and Arthus reaction in Fc gamma RIII (CD16) deficient mice. Immunity, v.5, n.2, Aug, p.181-8. 1996.

Herbert, C. A., C. M. King*, et al.* Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. Am J Respir Cell Mol Biol, v.12, n.4, Apr, p.369-78. 1995.

Hide, D. W. Clinical curio: allergy to Brazil nut. Br Med J (Clin Res Ed), v.287, n.6396, Sep 24, p.900. 1983.

Hochreiter, R., F. Ferreira*, et al.* TH1-promoting DNA immunization against allergens modulates the ratio of IgG1/IgG2a but does not affect the anaphylactic potential of IgG1 antibodies: no evidence for the synthesis of nonanaphylactic IgG1. J Allergy Clin Immunol, v.112, n.3, Sep, p.579-84. 2003.

Hoffmann, J. A. The immune response of Drosophila. Nature, v.426, n.6962, Nov 6, p.33-8. 2003.

Hollingsworth, J. W., G. S. Whitehead*, et al.* TLR4 signaling attenuates ongoing allergic inflammation. J Immunol, v.176, n.10, May 15, p.5856-62. 2006.

Horng, T., G. M. Barton*, et al.* The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature, v.420, n.6913, Nov 21, p.329-33. 2002.

Houston, K. M., E. H. Wilson*, et al.* Presence of phosphorylcholine on a filarial nematode protein influences immunoglobulin G subclass response to the molecule by an interleukin-10-dependent mechanism. Infect Immun, v.68, n.9, Sep, p.5466-8. 2000.

Huby, R. D., R. J. Dearman*, et al.* Why are some proteins allergens? Toxicol Sci, v.55, n.2, Jun, p.235-46. 2000.

Ichikawa, S., T. Takai*, et al.* Lipopolysaccharide binding of the mite allergen Der f 2. Genes Cells, v.14, n.9, Sep, p.1055-65. 2009.

Imamura, T., Y. Kanagawa*, et al.* A survey of patients with self-reported severe food allergies in Japan. Pediatr Allergy Immunol, v.19, n.3, May, p.270-4. 2008.

Inagaki, N., S. Goto*, et al.* Studies on vascular permeability increasing factors involved in 48-hour homologous PCA in the mouse ear. Int Arch Allergy Appl Immunol, v.80, n.3, p.285-90. 1986.

Inagaki, N. e H. Nagai. Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment:mouse models for the development of remedies for human allergic dermatitis. J Pharmacol Sci, v.110, n.3, Jul, p.251-9. 2009.

Inagaki, N., H. Nagai, *et al.* Comparative study of IgG1 and IgE antibody mediated homologous PCA in the mouse ear. Lack of cross-desensitization of IgG1 antibody mediated PCA to IgE antibody mediated PCA. Int Arch Allergy Appl Immunol, v.86, n.3, p.325-30. 1988.

Ishizaka, K. e T. Ishizaka. IgE and reaginic hypersensitivity. Ann N Y Acad Sci, v.190, Dec 31, p.443-56. 1971.

Ishizaka, K., T. Ishizaka, *et al.* Physico-chemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. J Immunol, v.97, n.1, Jul, p.75-85. 1966.

Ishizaka, K.. Physicochemical properties of reaginic antibody. VI. Effect of heat on gamma-E-, gamma-G- and gamma-A-antibodies in the sera of ragweed sensitive patients. J Immunol, v.99, n.3, Sep, p.610-8. 1967.

Iwasaki, A. e R. Medzhitov. Toll-like receptor control of the adaptive immune responses. Nat Immunol, v.5, n.10, Oct, p.987-95. 2004.

Jin, M. S., S. E. Kim, *et al.* Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell, v.130, n.6, Sep 21, p.1071-82. 2007.

Johansson, S. G. e H. Bennich. Immunological studies of an atypical (myeloma) immunoglobulin. Immunology, v.13, n.4, Oct, p.381-94. 1967.

Johnson, D. A. Synthetic TLR4-active glycolipids as vaccine adjuvants and stand-alone immunotherapeutics. Curr Top Med Chem, v.8, n.2, p.64-79. 2008.

Jones, K. L., A. Mansell, *et al.* Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia. Proc Natl Acad Sci U S A, v.104, n.41, Oct 9, p.16239-44. 2007.

Jungalwala, F. B., Evans, J.E. And Mccluer, R.H. High-performance liquid-chromatography of phosphatidylcholine and sphingomyelin with detection in the range of 200 nm. Biochem. J., v.155, p.55-60. 1976.

Kadooka, Y., T. Idota, *et al.* A method for measuring specific IgE in sera by direct ELISA without interference by IgG competition or IgG autoantibodies to IgE. Int Arch Allergy Immunol, v.122, n.4, Aug, p.264-9. 2000.

Karisola, P., H. Alenius, *et al.* The major conformational IgE-binding epitopes of hevein (Hev b6.02) are identified by a novel chimera-based allergen epitope mapping strategy. J Biol Chem, v.277, n.25, Jun 21, p.22656-61. 2002.

Kates, M. e S. Kushwaha. Archaea: A laboratory manual, halophiles: Cold Spring Harbor Laboratory Press. 1995 (Isoprenoids and polar lipids of extreme halophiles)

Kawai, T. e S. Akira. TLR signaling. Cell Death Differ, v.13, n.5, May, p.816-25. 2006.

Kawai, T.. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol, v.11, n.5, May, p.373-84. 2010.

Kawano, T., J. Cui*, et al.* CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science, v.278, n.5343, Nov 28, p.1626-9. 1997.

Kawasaki, K., S. Akashi*, et al.* Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem, v.275, n.4, Jan 28, p.2251-4. 2000.

Kean, D. E., H. S. Goodridge*, et al.* Differential polarization of immune responses by plant 2S seed albumins, Ber e 1, and SFA8. J Immunol, v.177, n.3, Aug 1, p.1561-6. 2006.

Kean, D. E., I. Ohtsuka*, et al.* Dissecting Ascaris glycosphingolipids for immunomodulatory moieties--the use of synthetic structural glycosphingolipid analogues. Parasite Immunol, v.28, n.3, Mar, p.69-76. 2006.

Keller, A. C., D. Mucida*, et al.* Hierarchical suppression of asthma-like responses by mucosal tolerance. J Allergy Clin Immunol, v.117, n.2, Feb, p.283-90. 2006.

Kennedy Norton, S., B. Barnstein*, et al.* IL-10 suppresses mast cell IgE receptor expression and signaling in vitro and in vivo. J Immunol, v.180, n.5, Mar 1, p.2848-54. 2008.

Kim, Y. K., S. Y. Oh*, et al.* Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. J Immunol, v.178, n.8, Apr 15, p.5375-82. 2007.

Kinet, J. P. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. Annu Rev Immunol, v.17, p.931-72. 1999.

King, J. C., J. Blumberg*, et al.* Tree nuts and peanuts as components of a healthy diet. J Nutr, v.138, n.9, Sep, p.1736S-1740S. 2008.

Knippels, L. M., G. F. Houben*, et al.* An oral sensitization model in Brown Norway rats to screen for potential allergenicity of food proteins. Methods, v.19, n.1, Sep, p.78-82. 1999.

Konger, R. L., G. K. Marathe*, et al.* Oxidized glycerophosphocholines as biologically active mediators for ultraviolet radiation-mediated effects. Prostaglandins Other Lipid Mediat, v.87, n.1-4, Dec, p.1-8. 2008.

Kris-Etherton, P. M., S. Yu-Poth, *et al.* Nuts and their bioactive constituents: effects on serum lipids and other factors that affect disease risk. Am J Clin Nutr, v.70, n.3 Suppl, Sep, p.504S-511S. 1999.

Kronenberg, M. Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol, v.23, p.877-900. 2005.

Kulczycki, A., Jr., C. Isersky, *et al.* The interaction of IgE with rat basophilic leukemia cells. I. Evidence for specific binding of IgE. J Exp Med, v.139, n.3, Mar 1, p.600-16. 1974.

Kulczycki, A., Jr. e H. Metzger. The interaction of IgE with rat basophilic leukemia cells. II. Quantitative aspects of the binding reaction. J Exp Med, v.140, n.6, Dec 1, p.1676-95. 1974.

Kupke, I. R. e S. Zeugner. Quantitative high-performance thin-layer chromatography of lipids in plasma and liver homogenates after direct application of 0.5-microliter samples to the silica-gel layer. J Chromatogr, v.146, n.2, Sep 1, p.261-71. 1978.

Larche, M., C. A. Akdis, *et al.* Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol, v.6, n.10, Oct, p.761-71. 2006.

Lebrun, S. J., W. N. Petchpud, *et al.* Development of a sensitive, colorometric microarray assay for allergen-responsive human IgE. J Immunol Methods, v.300, n.1-2, May, p.24-31. 2005.

Lee, G. R. e R. A. Flavell. Transgenic mice which overproduce Th2 cytokines develop spontaneous atopic dermatitis and asthma. Int Immunol, v.16, n.8, Aug, p.1155-60. 2004.

Lee, L. A. e A. W. Burks. Food allergies: prevalence, molecular characterization, and treatment/prevention strategies. Annu Rev Nutr, v.26, p.539-65. 2006.

Lehrer, S. B., R. Reish, *et al.* Enhancement of murine IgE antibody detection by IgG removal. J Immunol Methods, v.284, n.1-2, Jan, p.1-6. 2004.

Lehrer, S. B. e J. H. Vaughan. Properties of mouse homocytotropic and heterocytotropic antibodies. J Allergy Clin Immunol, v.57, n.5, May, p.422-9. 1976.

Leite-De-Moraes, M. C., G. Moreau, *et al.* IL-4-producing NK T cells are biased towards IFN-gamma production by IL-12. Influence of the microenvironment on the functional capacities of NK T cells. Eur J Immunol, v.28, n.5, May, p.1507-15. 1998.

Lemaitre, B., E. Nicolas, *et al.* The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell, v.86, n.6, Sep 20, p.973-83. 1996.

Leung, D. Y. Molecular basis of allergic diseases. Mol Genet Metab, v.63, n.3, Mar, p.157-67. 1998.

Li, X. M., D. Serebrisky, *et al.* A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol, v.106, n.1 Pt 1, Jul, p.150-8. 2000.

Lisbonne, M., S. Diem, *et al.* Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. J Immunol, v.171, n.4, Aug 15, p.1637-41. 2003.

Lopez, A. F., C. J. Sanderson, *et al.* Recombinant human interleukin 5 is a selective activator of human eosinophil function. J Exp Med, v.167, n.1, Jan 1, p.219-24. 1988.

Lu, Y. C., W. C. Yeh, *et al.* LPS/TLR4 signal transduction pathway. Cytokine, v.42, n.2, May, p.145-51. 2008.

Maezawa, Y., H. Nakajima, *et al.* Role of IgE in Th2 cell-mediated allergic airway inflammation. Int Arch Allergy Immunol, v.131 Suppl 1, Jun, p.2-6. 2003.

Maizels, R. M. Infections and allergy - helminths, hygiene and host immune regulation. Curr Opin Immunol, v.17, n.6, Dec, p.656-61. 2005.

Matsuda, A., Y. Okayama, *et al.* High-affinity IgE receptor-beta chain expression in human mast cells. J Immunol Methods, v.336, n.2, Jul 31, p.229-34. 2008.

Matsuda, J. L., L. Gapin, *et al.* Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. Proc Natl Acad Sci U S A, v.100, n.14, Jul 8, p.8395-400. 2003.

Matsuda, J. L.. Homeostasis of V alpha 14i NKT cells. Nat Immunol, v.3, n.10, Oct, p.966-74. 2002.

Matsuda, J. L., O. V. Naidenko, *et al.* Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med, v.192, n.5, Sep 4, p.741-54. 2000.

Mattes, J., M. Yang, *et al.* IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. J Immunol, v.167, n.3, Aug 1, p.1683-92. 2001.

Mazzoni, A. e D. M. Segal. Controlling the Toll road to dendritic cell polarization. J Leukoc Biol, v.75, n.5, May, p.721-30. 2004.

Mcaleer, J. P. e A. T. Vella. Understanding how lipopolysaccharide impacts CD4 T-cell immunity. Crit Rev Immunol, v.28, n.4, p.281-99. 2008.

Medzhitov, R. Toll-like receptors and innate immunity. Nat Rev Immunol, v.1, n.2, Nov, p.135-45. 2001.

Medzhitov, R., P. Preston-Hurlburt*, et al.* A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature, v.388, n.6640, Jul 24, p.394-7. 1997.

Medzhitov, R.. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell, v.2, n.2, Aug, p.253-8. 1998.

Mestecky, J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol, v.7, n.4, Jul, p.265-76. 1987.

Metcalfe, D. D. Genetically modified crops and allergenicity. Nat Immunol, v.6, n.9, Sep, p.857-60. 2005.

Mills, E. N., J. A. Jenkins*, et al.* Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. Crit Rev Food Sci Nutr, v.44, n.5, p.379-407. 2004.

Miraliakbari, H. e F. Shahidi. Oxidative stability of tree nut oils. J Agric Food Chem, v.56, n.12, Jun 25, p.4751-9. 2008.

Mishra, N. C., J. Rir-Sima-Ah*, et al.* Nicotine primarily suppresses lung Th2 but not goblet cell and muscle cell responses to allergens. J Immunol, v.180, n.11, Jun 1, p.7655-63. 2008.

Mittal, M. M., K. Saha*, et al.* A study of Prausnitz-Kustner reaction in leprosy. Clin Exp Immunol, v.8, n.4, Apr, p.657-61. 1971.

Miyajima, I., D. Dombrowicz*, et al.* Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. J Clin Invest, v.99, n.5, Mar 1, p.901-14. 1997.

Miyamoto, K., S. Miyake*, et al.* A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature, v.413, n.6855, Oct 4, p.531-4. 2001.

Moodley, R., A. Kindness*, et al.* Elemental composition and chemical characteristics of five edible nuts (almond, Brazil, pecan, macadamia and walnut) consumed in Southern Africa. J Environ Sci Health B, v.42, n.5, Jun-Jul, p.585-91. 2007.

Moody, D. B. TLR gateways to CD1 function. Nat Immunol, v.7, n.8, Aug, p.811-7. 2006.

Moog, F. The lining of the small intestine. Sci Am, v.245, n.5, Nov, p.154-8, 160, 162 et passiom. 1981.

Morafo, V., K. Srivastava, *et al.* Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. J Allergy Clin Immunol, v.111, n.5, May, p.1122-8. 2003.

Mori, S. A. G. T. P. The Lecythidaceae Pages. http://sweetgum.nybg.org/lp/index.html. The New York Botanical Garden, Bronx, New York. 2006 onward-a.

Morita, M., K. Motoki, *et al.* Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. J Med Chem, v.38, n.12, Jun 9, p.2176-87. 1995.

Mosmann, T. R. e R. L. Coffman. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol, v.7, p.145-73. 1989.

Mota, I. e A. Perini. Two biologically different mouse IgG1 antibodies. Immunol Lett, v.5, n.2, Aug, p.107-10. 1982.

Mota, I., D. Wong, *et al.* Mouse homocytotropic antibodies. I. Specific differentiation between mouse 7S gamma 1 and mouse reagin-like antibodies. Life Sci, v.7, n.24, Dec 15, p.1289-93. 1968.

Mucida, D., N. Kutchukhidze, *et al.* Oral tolerance in the absence of naturally occurring Tregs. J Clin Invest, v.115, n.7, Jul, p.1923-33. 2005.

Mukai, K., K. Obata, *et al.* New insights into the roles for basophils in acute and chronic allergy. Allergol Int, v.58, n.1, Mar, p.11-9. 2009.

Murtagh, G. J., D. B. Archer, *et al.* In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. Clin Exp Allergy, v.33, n.8, Aug, p.1147-52. 2003.

Nakajima, H. e K. Takatsu. Role of cytokines in allergic airway inflammation. Int Arch Allergy Immunol, v.142, n.4, p.265-73. 2007.

Niederberger, V. Allergen-specific immunotherapy. Immunol Lett, v.122, n.2, Feb 21, p.131-3. 2009.

Nimmerjahn, F., P. Bruhns, *et al.* FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity, v.23, n.1, Jul, p.41-51. 2005.

Nishikawa, M. e R. Hong. Effect on IgE production of transplanted cultured thymic fragments. Immunology, v.60, n.1, Jan, p.91-5. 1987.

Nonaka, Y., T. Izumo, *et al.* Antiallergic effects of Lactobacillus pentosus strain S-PT84 mediated by modulation of Th1/Th2 immunobalance and induction of IL-10 production. Int Arch Allergy Immunol, v.145, n.3, p.249-57. 2008.

Nordlee, J. A., S. L. Taylor*, et al.* Identification of a Brazil-nut allergen in transgenic soybeans. N Engl J Med, v.334, n.11, Mar 14, p.688-92. 1996.

Nouri-Aria, K. T. e S. R. Durham. Regulatory T cells and allergic disease. Inflamm Allergy Drug Targets, v.7, n.4, Dec, p.237-52. 2008.

Nowak-Wegrzyn, A. e H. A. Sampson. Adverse reactions to foods. Med Clin North Am, v.90, n.1, Jan, p.97-127. 2006.

Oettgen, H. C., T. R. Martin*, et al.* Active anaphylaxis in IgE-deficient mice. Nature, v.370, n.6488, Aug 4, p.367-70. 1994.

Oki, S., A. Chiba*, et al.* The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. J Clin Invest, v.113, n.11, Jun, p.1631-40. 2004.

Onaderra, M., R. I. Monsalve*, et al.* Food mustard allergen interaction with phospholipid vesicles. Eur J Biochem, v.225, n.2, Oct 15, p.609-15. 1994.

Ovary, Z. Recherches sur l'anaphylaxie passive cutanee locale du cobaye. Influence de la procaine et de l'adrenaline en infiltrations locales dans l'anaphylaxie passive cutanee lcale du cobaye. . Revue Immunol., v.15, p.375. 1950.

Ovary, Z. Recent insight into class-specific properties of murine immunoglobulins obtained with the help of monoclonal antibodies. Int Arch Allergy Appl Immunol, v.69, n.4, p.385-92. 1982.

Paolini, R., M. H. Jouvin*, et al.* Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement. Nature, v.353, n.6347, Oct 31, p.855-8. 1991.

Parcerisa, J., R. Codony*, et al.* Triacylglycerol and phospholipid composition of hazelnut (Corylus avellana L.) lipid fraction during fruit development. J Agric Food Chem, v.47, n.4, Apr, p.1410-5. 1999.

Pastorello, E. A., L. Farioli*, et al.* Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. Int Arch Allergy Immunol, v.144, n.1, p.10-22. 2007.

Pastorello, E. A.. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. J Allergy Clin Immunol, v.102, n.6 Pt 1, Dec, p.1021-7. 1998.

Pastorello, E. A., C. Pompei*, et al.* Lipid transfer proteins and 2S albumins as allergens. Allergy, v.56 Suppl 67, p.45-7. 2001.

Pastorello, E. A., E. Varin*, et al.* The major allergen of sesame seeds (Sesamum indicum) is a 2S albumin. J Chromatogr B Biomed Sci Appl, v.756, n.1-2, May 25, p.85-93. 2001.

Peng, Z., A. B. Becker*, et al.* Binding properties of protein A and protein G for human IgE. Int Arch Allergy Immunol, v.104, n.2, p.204-6. 1994.

Perrier, C., A. C. Thierry*, et al.* Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. Clin Exp Allergy, Aug 18. 2009.

Perrin-Cocon, L., S. Agaugue*, et al.* Lysophosphatidylcholine is a natural adjuvant that initiates cellular immune responses. Vaccine, v.24, n.9, Feb 27, p.1254-63. 2006.

Piggott, D. A., S. C. Eisenbarth*, et al.* MyD88-dependent induction of allergic Th2 responses to intranasal antigen. J Clin Invest, v.115, n.2, Feb, p.459-67. 2005.

Podrez, E. A., E. Poliakov*, et al.* Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. J Biol Chem, v.277, n.41, Oct 11, p.38503-16. 2002.

Poltorak, A., X. He*, et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science, v.282, n.5396, Dec 11, p.2085-8. 1998.

Porcelli, S. A. e R. L. Modlin. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu Rev Immunol, v.17, p.297-329. 1999.

Prausnitz, C. e H. Kustner. Studien uber die Uberempfindlichkeit. Zentrabl. Bakt. 86, 160. Zentrabl. Bakt., v.86
p.160. 1921.

Proust, B., C. Astier*, et al.* A single oral sensitization to peanut without adjuvant leads to anaphylaxis in mice. Int Arch Allergy Immunol, v.146, n.3, p.212-8. 2008.

Prussin, C. e D. D. Metcalfe. 5. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol, v.117, n.2 Suppl Mini-Primer, Feb, p.S450-6. 2006.

Pushparaj, P. N., H. K. Tay*, et al.* The cytokine interleukin-33 mediates anaphylactic shock. Proc Natl Acad Sci U S A, v.106, n.24, Jun 16, p.9773-8. 2009.

Radauer, C. e H. Breiteneder. Evolutionary biology of plant food allergens. J Allergy Clin Immunol, v.120, n.3, Sep, p.518-25. 2007.

Ravetch, J. V. e S. Bolland. IgG Fc receptors. Annu Rev Immunol, v.19, p.275-90. 2001.

Raymond, M., M. Rubio*, et al.* Selective control of SIRP-alpha-positive airway dendritic cell trafficking through CD47 is critical for the development of T(H)2-

mediated allergic inflammation. J Allergy Clin Immunol, v.124, n.6, Dec, p.1333-42 e1. 2009.

Raz, E. e H. L. Spiegelberg. Deviation of the allergic IgE to an IgG response by gene immunotherapy. Int Rev Immunol, v.18, n.3, p.271-89. 1999.

Rescigno, M., M. Urbano*, et al.* Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol, v.2, n.4, Apr, p.361-7. 2001.

Rivera, J. e A. M. Gilfillan. Molecular regulation of mast cell activation. J Allergy Clin Immunol, v.117, n.6, Jun, p.1214-25; quiz 1226. 2006.

Rizzo, M. C., C. K. Naspitz*, et al.* Endotoxin exposure and symptoms in asthmatic children. Pediatr Allergy Immunol, v.8, n.3, Aug, p.121-6. 1997.

Roitt, I. Essential Immunology. United Kingdom: Blachwell Science. 1998. 476 p.

Romagnani, S. The role of lymphocytes in allergic disease. J Allergy Clin Immunol, v.105, n.3, Mar, p.399-408. 2000.

Ros, E. e J. Mataix. Fatty acid composition of nuts--implications for cardiovascular health. Br J Nutr, v.96 Suppl 2, Nov, p.S29-35. 2006.

Rosenwasser, L. J. e J. Meng. Anti-CD23. Clin Rev Allergy Immunol, v.29, n.1, Aug, p.61-72. 2005.

Rupa, P., K. Hamilton*, et al.* A neonatal swine model of allergy induced by the major food allergen chicken ovomucoid (Gal d 1). Int Arch Allergy Immunol, v.146, n.1, p.11-8. 2008.

Russano, A. M., E. Agea*, et al.* Complementary roles for lipid and protein allergens in triggering innate and adaptive immune systems. Allergy, v.63, n.11, Nov, p.1428-37. 2008.

Russano, A. M.. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted gamma delta T cells. J Allergy Clin Immunol, v.117, n.5, May, p.1178-84. 2006.

Ryan, E., K. Galvin*, et al.* Fatty acid profile, tocopherol, squalene and phytosterol content of brazil, pecan, pine, pistachio and cashew nuts. Int J Food Sci Nutr, v.57, n.3-4, May-Jun, p.219-28. 2006.

Ryan, J. J., M. Kashyap*, et al.* Mast cell homeostasis: a fundamental aspect of allergic disease. Crit Rev Immunol, v.27, n.1, p.15-32. 2007.

Samet, J. M., M. Friedman*, et al.* High-performance liquid chromatography separation of phospholipid classes and arachidonic acid on cyanopropyl columns. Anal Biochem, v.182, n.1, Oct, p.32-6. 1989.

Sampson, H. A. Food anaphylaxis. Br Med Bull, v.56, n.4, p.925-35. 2000.

Sanderson, C. J. The biological role of interleukin 5. Int J Cell Cloning, v.8 Suppl 1, Jan, p.147-53; discussion 153-4. 1990.

Schumann, R. R., S. R. Leong*, et al.* Structure and function of lipopolysaccharide binding protein. Science, v.249, n.4975, Sep 21, p.1429-31. 1990.

Segal, D. M., J. D. Taurog*, et al.* Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. Proc Natl Acad Sci U S A, v.74, n.7, Jul, p.2993-7. 1977.

Sicherer, S. H. e H. A. Sampson. 10. Food allergy. J Allergy Clin Immunol, Dec 28. 2009.

Sihra, B. S., O. M. Kon*, et al.* Expression of high-affinity IgE receptors (Fc epsilon RI) on peripheral blood basophils, monocytes, and eosinophils in atopic and nonatopic subjects: relationship to total serum IgE concentrations. J Allergy Clin Immunol, v.99, n.5, May, p.699-706. 1997.

Silva, S. R., A. Casabuono*, et al.* Sialic acid residues are essential for the anaphylactic activity of murine IgG1 antibodies. J Immunol, v.181, n.12, Dec 15, p.8308-14. 2008.

Silverstein, A. M. Clemens Freiherr von Pirquet: explaining immune complex disease in 1906. Nat Immunol, v.1, n.6, Dec, p.453-5. 2000.

Singh, N., S. Hong*, et al.* Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. J Immunol, v.163, n.5, Sep 1, p.2373-7. 1999.

Stanic, A. K., R. Shashidharamurthy*, et al.* Another view of T cell antigen recognition: cooperative engagement of glycolipid antigens by Va14Ja18 natural T(iNKT) cell receptor [corrected]. J Immunol, v.171, n.9, Nov 1, p.4539-51. 2003.

Stock, P. e O. Akbari. Recent advances in the role of NKT cells in allergic diseases and asthma. Curr Allergy Asthma Rep, v.8, n.2, Apr, p.165-70. 2008.

Stowell, N. C., J. Seideman*, et al.* Long-term activation of TLR3 by poly(I:C) induces inflammation and impairs lung function in mice. Respir Res, v.10, p.43. 2009.

Strait, R. T., S. C. Morris*, et al.* IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc gamma RIIb cross-linking. J Clin Invest, v.116, n.3, Mar, p.833-41. 2006.

Strait, R. T.. Pathways of anaphylaxis in the mouse. J Allergy Clin Immunol, v.109, n.4, Apr, p.658-68. 2002.

Sullivan, B. A., N. A. Nagarajan*, et al.* Mechanisms for glycolipid antigen-driven cytokine polarization by Valpha14i NKT cells. J Immunol, v.184, n.1, Jan 1, p.141-53. 2010.

Sun, J. B., C. Czerkinsky*, et al.* Mucosally induced immunological tolerance, regulatory T cells and the adjuvant effect by cholera toxin B subunit. Scand J Immunol, v.71, n.1, Jan, p.1-11. 2010.

Sun, S. S., S. B. Altenbach*, et al.* Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (Bertholletia excelsa H.B.K.). Eur J Biochem, v.162, n.3, Feb 2, p.477-83. 1987.

Suzuki, M., X. Zheng*, et al.* Inhibition of allergic responses by CD40 gene silencing. Allergy, v.64, n.3, Mar, p.387-97. 2009.

Takai, T., M. Ono*, et al.* Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. Nature, v.379, n.6563, Jan 25, p.346-9. 1996.

Takeno, M., H. Yoshikawa*, et al.* Th1-dominant shift of T cell cytokine production, and subsequent reduction of serum immunoglobulin E response by administration in vivo of plasmid expressing Txk/Rlk, a member of Tec family tyrosine kinases, in a mouse model. Clin Exp Allergy, v.34, n.6, Jun, p.965-70. 2004.

Takeuchi, O., K. Hoshino*, et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity, v.11, n.4, Oct, p.443-51. 1999.

Tan, A. M., H. C. Chen*, et al.* TLR4 Signaling in Stromal Cells Is Critical for the Initiation of Allergic Th2 Responses to Inhaled Antigen. J Immunol, Mar 1. 2010.

Taylor, S. L. e S. B. Lehrer. Principles and characteristics of food allergens. Crit Rev Food Sci Nutr, v.36 Suppl, p.S91-118. 1996.

Teuber, S. S., A. M. Dandekar*, et al.* Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (Juglans regia), a major food allergen. J Allergy Clin Immunol, v.101, n.6 Pt 1, Jun, p.807-14. 1998.

Teuber, S. S., G. Del Val*, et al.* The atopic dog as a model of peanut and tree nut food allergy. J Allergy Clin Immunol, v.110, n.6, Dec, p.921-7. 2002.

Thomas, K., S. Macintosh*, et al.* Scientific advancement of novel protein allergenicity evaluation: an overview of work from the hesi protein allergenicity technical committee (2000-2008). Food Chem Toxicol, Feb 10. 2009.

Thomas, W. R., B. J. Hales*, et al.* Structural biology of allergens. Curr Allergy Asthma Rep, v.5, n.5, Sep, p.388-93. 2005.

Thorne, P. S., K. Kulhankova, *et al.* Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. Am J Respir Crit Care Med, v.172, n.11, Dec 1, p.1371-7. 2005.

Togias, A., M. J. Fenton, *et al.* Asthma in the inner city: the perspective of the National Institute of Allergy and Infectious Diseases. J Allergy Clin Immunol, v.125, n.3, Mar, p.540-4. 2010.

Traidl-Hoffmann, C., T. Jakob, *et al.* Determinants of allergenicity. J Allergy Clin Immunol, v.123, n.3, Mar, p.558-66. 2009.

Trompette, A., S. Divanovic, *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature, v.457, n.7229, Jan 29, p.585-8. 2009.

Tsujimura, Y., K. Obata, *et al.* Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. Immunity, v.28, n.4, Apr, p.581-9. 2008.

Tulic, M. K., D. A. Knight, *et al.* Lipopolysaccharide inhibits the late-phase response to allergen by altering nitric oxide synthase activity and interleukin-10. Am J Respir Cell Mol Biol, v.24, n.5, May, p.640-6. 2001.

Umetsu, D. T. e R. H. Dekruyff. Natural killer T cells are important in the pathogenesis of asthma: The many pathways to asthma. J Allergy Clin Immunol, Mar 23. 2010.

Unkeless, J. C., E. Scigliano, *et al.* Structure and function of human and murine receptors for IgG. Annu Rev Immunol, v.6, p.251-81. 1988.

USDA National Nutrient Database for Standard Reference: United States Department of Agriculture - Agricultural Research Service. Release 22**:** www.nal.usda.gov p. 2009.

Van Der Kleij, D., E. Latz, *et al.* A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J Biol Chem, v.277, n.50, Dec 13, p.48122-9. 2002.

Van Ginkel, F. W., T. Iwamoto, *et al.* Immunity to a self-derived, channel-forming peptide in the respiratory tract. Clin Vaccine Immunol, v.15, n.2, Feb, p.260-6. 2008.

Van Rhijn, I., D. M. Zajonc, *et al.* T-cell activation by lipopeptide antigens. Curr Opin Immunol, v.17, n.3, Jun, p.222-9. 2005.

Van Wijk, F., S. Nierkens, *et al.* The CD28/CTLA-4-B7 signaling pathway is involved in both allergic sensitization and tolerance induction to orally administered peanut proteins. J Immunol, v.178, n.11, Jun 1, p.6894-900. 2007.

Van Wijk, F.. The effect of the food matrix on in vivo immune responses to purified peanut allergens. Toxicol Sci, v.86, n.2, Aug, p.333-41. 2005.

Vaz, N. M., E. M. Vaz*, et al.* Relationship between histocompatibility (H-2) genotype and immune responsiveness to low doses of ovalbumin in the mouse. J Immunol, v.104, n.6, Jun, p.1572-4. 1970.

Venkatachalam, M., H. H. Kshirsagar*, et al.* Biochemical composition and immunological comparison of select pecan [Carya illinoinensis (Wangenh.) K. Koch] cultivars. J Agric Food Chem, v.55, n.24, Nov 28, p.9899-907. 2007.

Venkatachalam, M. e S. K. Sathe. Chemical composition of selected edible nut seeds. J Agric Food Chem, v.54, n.13, Jun 28, p.4705-14. 2006.

Vierk, K. A., K. M. Koehler*, et al.* Prevalence of self-reported food allergy in American adults and use of food labels. J Allergy Clin Immunol, v.119, n.6, Jun, p.1504-10. 2007.

Von Mutius, E., C. Braun-Fahrlander*, et al.* Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp Allergy, v.30, n.9, Sep, p.1230-4. 2000.

Wakayama, H., Y. Hasegawa*, et al.* IgG-mediated anaphylaxis via Fc gamma receptor in CD40-deficient mice. Clin Exp Immunol, v.114, n.2, Nov, p.154-60. 1998.

Wal, J. M. Cow's milk allergens. Allergy, v.53, n.11, Nov, p.1013-22. 1998.

Wang, J., J. Lin*, et al.* Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. J Allergy Clin Immunol, v.125, n.3, Mar, p.695-702, 702 e1-702 e6. 2010.

Wang, Y., Y. Mei*, et al.* Vasoactive intestinal polypeptide enhances oral tolerance by regulating both cellular and humoral immune responses. Clin Exp Immunol, v.148, n.1, Apr, p.178-87. 2007.

Watanabe, J., Y. Miyazaki*, et al.* Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. J Biol Chem, v.278, n.43, Oct 24, p.42361-8. 2003.

Whelan, M., M. M. Harnett*, et al.* A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. J Immunol, v.164, n.12, Jun 15, p.6453-60. 2000.

Wiese, R., Y. Belosludtsev*, et al.* Simultaneous multianalyte ELISA performed on a microarray platform. Clin Chem, v.47, n.8, Aug, p.1451-7. 2001.

Williams, L. K., D. R. Ownby*, et al.* The role of endotoxin and its receptors in allergic disease. Ann Allergy Asthma Immunol, v.94, n.3, Mar, p.323-32. 2005.

Woo, H. Y., Y. S. Kim*, et al.* Mechanism for acute oral desensitization to antibiotics. Allergy, v.61, n.8, Aug, p.954-8. 2006.

Woodfolk, J. A. T-cell responses to allergens. J Allergy Clin Immunol, v.119, n.2, Feb, p.280-94; quiz 295-6. 2007.

Wright, S. D., R. A. Ramos*, et al.* CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science, v.249, n.4975, Sep 21, p.1431-3. 1990.

Yamaguchi, N., R. Sugita*, et al.* Gastrointestinal Candida colonisation promotes sensitisation against food antigens by affecting the mucosal barrier in mice. Gut, v.55, n.7, Jul, p.954-60. 2006.

Yang, J. Brazil nuts and associated health benefits: A review. LWT - Food Science and Technology v.42, p.1573-1580. 2009.

Yoshimoto, T., A. Bendelac*, et al.* Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. Science, v.270, n.5243, Dec 15, p.1845-7. 1995.

Zeng, Z., A. R. Castano*, et al.* Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. Science, v.277, n.5324, Jul 18, p.339-45. 1997.

Zhang, M., R. F. Murphy*, et al.* Decoding IgE Fc receptors. Immunol Res, v.37, n.1, p.1-16. 2007.

Zhu, Z., R. J. Homer*, et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest, v.103, n.6, Mar, p.779-88. 1999.

Zlatanov M., Ivanov S.*, et al.* Phospholipid and fatty acid composition of Bulgarian nut oils. Fat, v.101, n.11, p.S437-439. 1999.