Mouse models of peanut allergy Contribution of oral and nasal sensitization to allergic reactions to peanut and cross-reactivity with food and environmental antigens

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INSTITUT NATIONAL AGRONOMIQUE PARIS-GRIGNON

UNIVERSITY OF ALABAMA AT BIRMINGHAM

THÈSE

pour obtenir le grade de
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Discipline : Immunologie
présentée et soutenue par

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Ingénieur Agronome
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Mouse models of peanut allergy

Contribution of oral and nasal sensitization to allergic reactions to peanut and cross-reactivity with food and environmental antigens

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RESUME

Il n'est pas rare que les personnes souffrant d'une allergie alimentaire présentent des troubles gastriques ou respiratoires au contact de protéines végétales de la même famille botanique que celle responsable de l'allergie alimentaire ou appartenant à une famille botanique différente. Les réponses de type Th2 et les IgE sont reconnus depuis longtemps pour leur rôle dans ces réactions allergiques, mais des publications récentes soulignent que des interactions cellulaires et moléculaires plus nombreuses et plus complexes sont impliquées. L'objectif de ce travail a été d'étudier l'influence du site initial de sensibilisation aux protéines de l'arachide sur les réponses adverses induites par exposition respiratoire à ces mêmes protéines ou à des protéines liées ou non à la famille des légumineuses. Pour ce faire, nous avons d'abord analysé la réponse sérique en anticorps après sensibilisation aux protéines de l'arachide par voie orale ou nasale en présence de toxine cholérique. Nous avons ensuite examiné le recrutement de cellules dans les poumons ainsi que la production de cytokines induit par la ré-exposition aux protéines d'arachide. Nos résultats ont montré que les taux d'IgE étaient élevés et ceux d'IgG spécifiques étaient faibles après sensibilisation par voie orale et en comparaison à ceux induits par la voie nasale. Les deux groupes de souris ont présenté une hyper-réactivité respiratoire après ré-exposition aux protéines de l'arachide. Cependant, les poumons des souris sensibilisées par voie orale étaient caractérisés par une éosinophilie forte et la présence de taux élevés de cytokines Th2, alors que ceux du groupe nasal étaient caractérisés par l'absence d'éosinophiles, un fort recrutement de neutrophiles et de macrophages ainsi que par la prédominance de cytokines inflammatoires. Enfin, l'exposition aux protéines d'autres légumineuses ou d'antigènes environnementaux a induit une réaction pulmonaire inflammatoire chez les souris sensibilisées par voie nasale mais pas chez celles sensibilisées par voie orale. Ces résultats suggèrent que la voie de sensibilisation a une influence cruciale sur la nature de la réaction immunitaire induite et nos deux modèles de souris constituent des outils intéressants pour étudier les réactions adverses dans la

population générale, sensibilisée aux protéines alimentaires au niveau du tractus gastro-intestinal ou chez certains professionnels de l'industrie agro-alimentaire, sensibilisés au niveau respiratoire.

ABSTRACT

Food-allergic patients can develop adverse reactions to members of the same and even unrelated botanical families. IgE and Th2-type responses play an important role in allergic reactions although it is now clear that these reactions involve other cellular and molecular interactions. We investigated the role played by the mucosal sites of initial priming to peanut on subsequent immune responses to peanut and reactivity to airway antigen challenge. For this purpose, we analyzed antibody responses to peanut immunization, as well as cytokine and airway responses to nasal antigen challenge in mice orally or nasally sensitized to peanut in the presence of cholera toxin. Oral peanut sensitization induced higher levels of IgE but lower IgG responses than nasal immunization. Both mice sensitized orally and nasally to peanut experienced airway hyper-reactivity upon nasal peanut challenge. However, orally sensitized mice exhibited higher levels of lung eosinophilia and IL-4 in response to peanut challenge. In contrast, higher levels of lung MAC-1^{+} cells and inflammatory cytokines were seen in nasally sensitized mice. Finally, nasal but not oral sensitization promoted lung inflammatory responses to unrelated antigens. These findings suggest that the initial route of sensitization influence the responses of peanut allergic individuals to airborne antigens and allergens.

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ABBREVIATIONS

Ab	Antibody
AHR	Airway hyper-reactivity
APC	Antigen-presenting cell
BALF	Bronchoalveolar lavage fluids
BSA	Bovine albumine serum
CDD	Cross-reactive carbohydrate determinants
CP	Crossing point
CT	Cholera toxin
DBPCFC	Double-blind placebo-controlled food challenge
Der f	Dermatophagoides farinae
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissues
GI tract	Gastrointestinal tract
HRP	Horseradish peroxydase
IFN	Interferon
IL	Interleukine
IP	Intraperitoneal
KO	Knock-out
M cells	Microfold cells
MALT	Mucosal-associated lymphoid tissues
NALT	Nasal-associated lymphoid tissues
OAS	Oral allergy syndrome
OVA	Ovalbumine
PBS	Phosphate-buffered saline
PE	Phycoerythrin
Penh	Enhanced pause
PPE	Peanut protein extract
PP	Peyer’s patch
RAST	Radioallergosorbent assays
Th	T helper
TNF	Tumor necrosis factor
WT	Wild-type

INTRODUCTION

1. Food allergies

Mucosal surfaces broadly interface with the environment making them the initial sites of interactions between cells of the immune system and pathogens or environmental antigens. The immune system must discriminate between pathogenic and innocuous stimuli and mount the most appropriate responses. Allergy is a disease resulting from inappropriate immune responses to otherwise harmless antigens. Symptoms of food allergy generally occur upon subsequent contact with a food product in individuals previously sensitized to it. They can also develop in the context of cross-reaction upon contact with an allergen different than the primary offending food product.

After defining a food allergy and closely characterize the two classes involved in this disease, we will detail mechanisms of both primary and cross-reactive reactions.

1.1. Definition and incidence of food allergies

Food allergies are immune-mediated adverse reactions to food products. These reactions are distinct from the other types of adverse reactions to foods, related to toxins, pharmacologically active chemicals, enzymatic deficiencies or psychological reactions. Food allergies include IgE-dependent but also IgE-independent reactions such as dietary protein enterocolitis, dietary protein prostitis and celiac disease. Recent studies estimate that IgE-mediated food allergies affect 6 % of children under 3 years of age and 4 % of the general population [1], a prevalence much higher than evaluated in the past [2]. For example, the prevalence of peanut allergy has doubled among American children from 1997 to 2002 [3].

Table 1: Allergens of class 1 food allergy

from Astwood JD et al. Nat Biotechnol. 1996

True food allergens *		Resistance to digestion	Symptoms
Egg	Ovalbumin Phosvitin Ovomucoid Conalbumin	high high intermediate low	Generalized Skin Gastrointestinal Respiratory Systemic
Milk	β-lactoglobulin Casein BSA	high intermediate intermediate	
Soybean	β-conglycinin β β-conglycinin α SKTI** Soy lectin Gly m1	high low high intermediate intermediate	
Peanut	ARA h2 Peanut lectin	high high	

* Sensitizing and symptom eliciting allergens
**Soybean Kunitz trypsin inhibitor

1.2. Classes of food allergies

It is well known that food allergies develop in genetically predisposed individuals [4, 5]. Epidemiologic studies also suggest a major role of environmental factors for the development of allergic reactions [6-8]. In this regard, the "hygiene hypothesis" is based on the assumption that the high rate of infectious diseases in developing nations could counter the development of allergic disorders [9]. More important for this study, the immaturity of the gastrointestinal tract and subsequent increased internal permeability or breakdown of oral tolerance are believed to be involved in sensitization to foods in infancy [10]. The resulting type of food allergy is named class 1 food allergy. Another type of IgE-mediated food allergy or class 2 food allergy is mainly seen in adults. This type of reaction unlikely results from impaired gastrointestinal functions and is believed to develop as a consequence of sensitization to inhalant allergens [11].

1.2.1. Class 1 or true food allergy

1.2.1.1. Main allergens and clinical symptoms

In class 1 food allergy, the sensitization process occurs in the gastrointestinal tract. The most important allergens involved in class I food allergy [1] include milk, egg, seafood, nuts and legumes, which are particularly resistant to gastric digestion [12, 13] (Table 1). These allergies tend to be outgrown later in childhood, peanut being an exception [14, 15].

Symptoms of class 1 food allergy may affect different parts of the body in addition to the site of contact with the offending allergens. Gastrointestinal tract symptoms include

Table 2: Allergens of class 2 food allergy

Plant food allergens*		Resistance to digestion	Symptoms	Inhalant sensitizing allergen
Apple	Mal d1[1]	low[3]	OAS Restricted to the oral cavity	Birch pollen (Bet v1, Bet v2)
Cherry	Pru av1[1]	low[3]		
Apricot	Pru ar1[1]			
Pear	Pyr c1[1]			
Soybean	Gly m3[2]			
Peanut	Ara h5[2]			
Celery	Apig1[2]	low[4]		
Tomato Peanut[5,6]	Profilin[5,6]			Grass pollen
Hazelnut Peach Apple Peanut				Platanus pollen
Celery Carrot				Mugwort pollen
Watermelon Banana				Ragweed pollen

[1]Ebner C et al. J Allergy Clin Immunol. 1995
[2]Vieths S et al. Ann N Y Acad Sci. 2002
[3]Vieths S et al. Allergy. 1998
[4]Jankiewicz A et al. Int Arch Allergy Immunol. 1996
[5]Petersen A et al. J Allergy Clin Immunol. 1996
[6]de Martino M et al. Allergy. 1988
[7]Enrique E et al. Allergy. 2002
* Non sensitizing elicitors

abdominal pain, vomiting and diarrhea [16]. Allergic manifestations in the respiratory tract include rhinitis and asthma [17]. Skin symptoms include urticaria, angio-oedema and dermatitis [18]. Symptoms may be more severe when they affect the cardiovascular system. In fact, food allergy remains a leading cause of anaphylaxis treated in emergency departments [19-21].

1.2.1.2. Cross-reactivity between food allergens

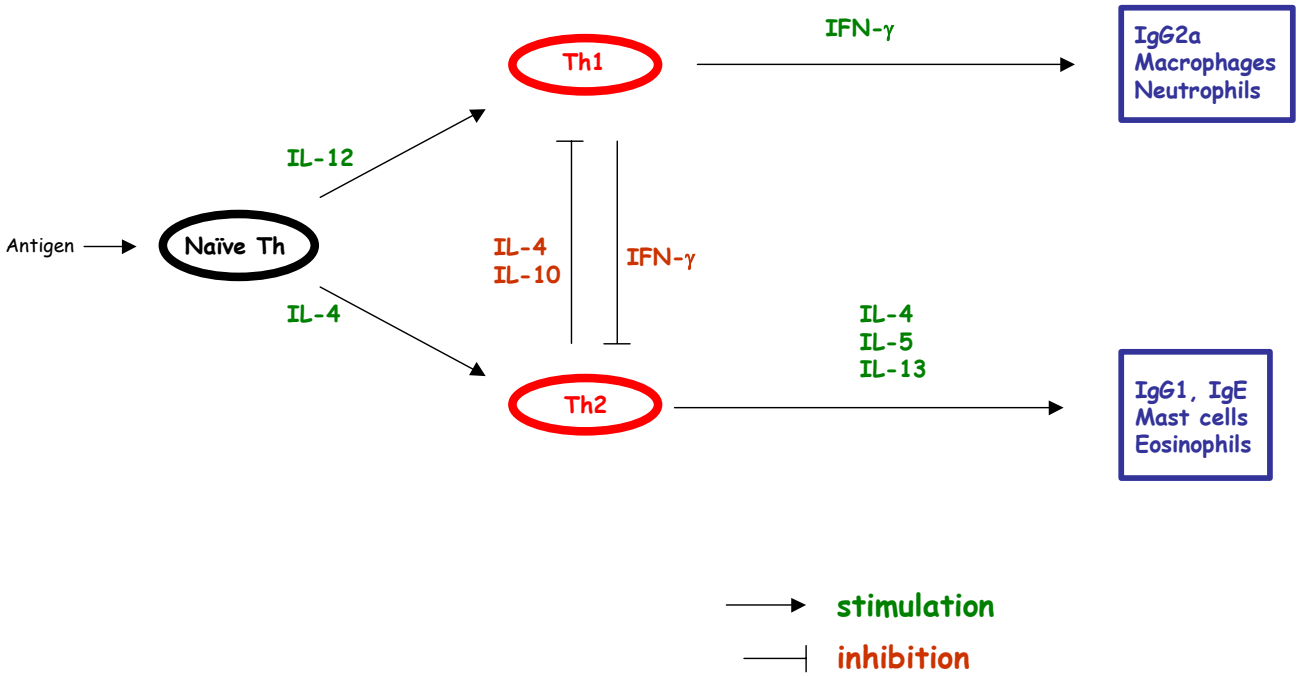
Antibody cross-reactivity has been described for many years between members of the same botanical family (i.e, legumes, tree nuts, cereals, avian food products, fish or shellfish) or between members of taxonomically unrelated families (legumes and nuts, shellfish and nuts). However, these antibody cross-reactions lead to different clinical outcomes. For example, antibody cross-reactivity in the legume family or in the cereal family is clinically irrelevant in most cases [22, 23], whereas the one among crustaceans [24] or fish [25] was reported to provoke clear clinical symptoms.

1.2.2. Class 2 or pollen-associated food allergy

Most fruit and vegetable allergens are labile to digestion (Table 2). These allergens may not effectively sensitize the organism after ingestion. However, they could contribute to food allergy via interaction with antibodies induced by primary sensitization with related inhalant allergens. Indeed, the immunologic basis for class 2 allergy is IgE cross-reactivity between common epitopes in pollen and fruit or vegetable allergens [26, 27].

Most fruit and vegetable food allergens only induce mild symptoms, restricted to the oral cavity, including itching in the mouth and throat and local edema [28]. These symptoms are

Figure 1 : Subsets of $CD4^+$ T cells



characteristic of the oral allergy syndrome (OAS) [29]. The pollen-associated food allergies best described are the parabirch [30, 31] and ragweed-banana-melon [32] syndromes.

1.3. Cellular and molecular mechanisms of food allergy

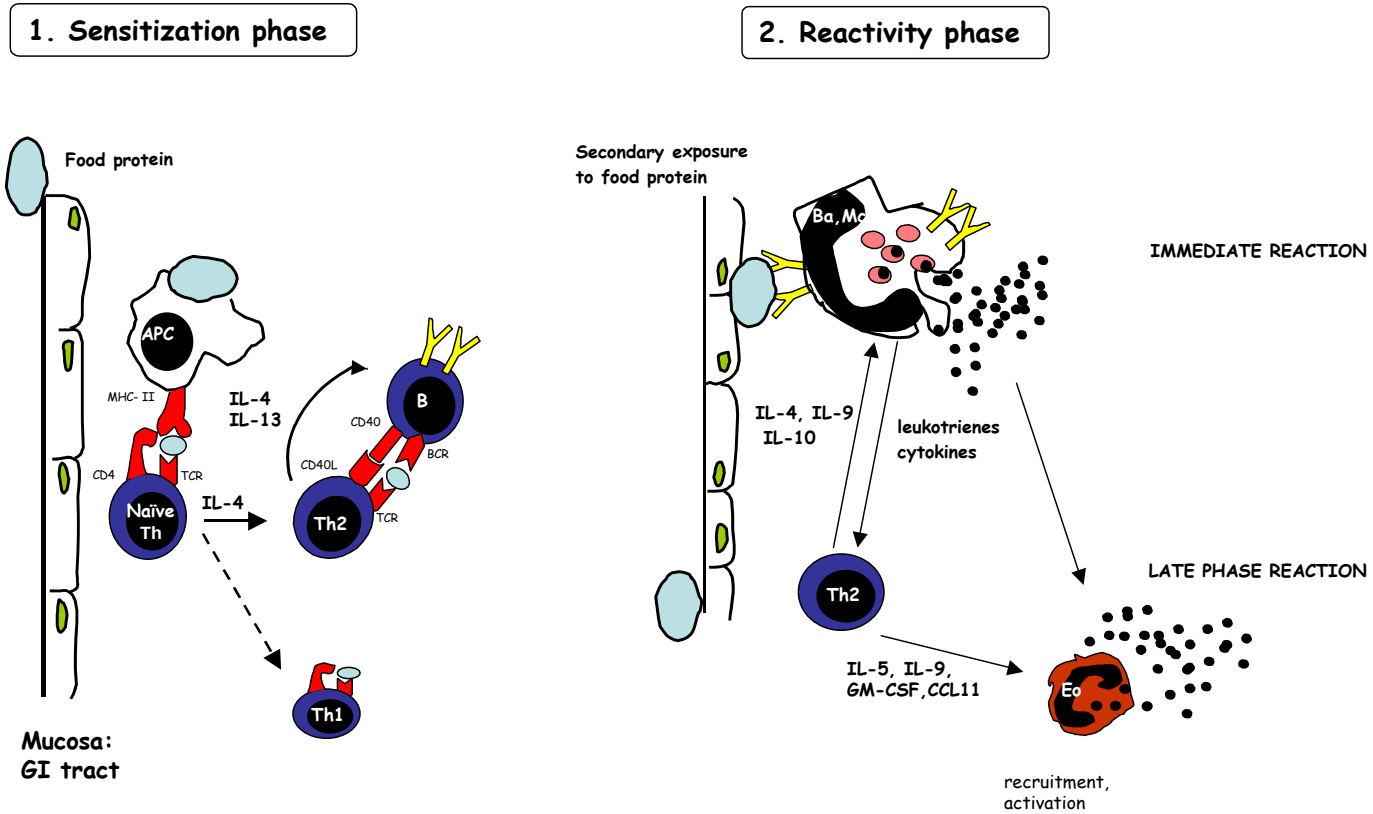
The identification in 1986 of two distinct subsets of $CD4^+$ T helper cells in mouse [33] greatly contributed to improve our understanding of mechanisms underlying allergic responses.

1.3.1. Th1 and Th2 cell subsets and responses

Th1 $CD4^+$ T cells produce a profile of cytokines, including IFN-γ, that promotes IgG2a production by B cells [33]. On the other hand, Th2 cells produce a profile of cytokines, including IL-4, IL-5 and IL-13, that promotes IgG1 and IgE production by B cells (Figure 1). Th1 and Th2 $CD4^+$ T cells also influence the innate response. Indeed, chemokines that attract monocytes and neutrophils to inflammatory sites include RANTES and MIP-1β, respectively, secreted by Th1 cells. Further, secretion of IFN-γ by Th1 cells is a potent activator of macrophages. On the other hand, chemokines that attract eosinophils to inflammatory sites include CCL11 (eotaxin), secreted by Th2 cells. Secretion of IL-5 by Th2 cells induces the activation of eosinophils.

Th1 and Th2 cells have been shown to derive from a common precursor cell, or Th0. The cytokine environment surrounding the antigen-primed Th cell determines the subset that develops. IL-12 induces the development of Th1 cells, whereas IL-4 induces the development of Th2 cells. Th1 or Th2 cytokines also exert a cross-regulatory or inhibitory effect: IL-4

Figure 2 : Molecular and cellular mechanisms of class 1 food allergy (part 1)



strikingly diminishes priming for Th1 cells whereas IFN-γ strikingly diminishes priming for Th2 cells.

Th1 and Th2 $CD4^+$ T cells were later identified in humans [34, 35], although the Th1/Th2 dichotomy is less clear-cut in human as it is in mice models.

1.3.2. Allergic reaction to a primary allergen

The pathogenesis of allergy has been traditionally characterized by excessive Th2 $CD4^+$ T cell cytokines [36] and a central role has been given to IgE Abs responses [37]. However, it is now clear that broader cellular and molecular interactions occur during both the sensitization phase, when the organism develops antibodies specific to food antigens and the effector phase when the allergic reaction occurs after a secondary contact with the allergen.

1.3.2.1. Basic mechanisms of allergy: the Th2 hypothesis

The sensitization phase of the allergic reaction is initiated by the sampling of antigens by antigen presenting cells (DCs and macrophages) and presentation to B and T cells (Figure 2). Antigens that give rise to allergic reactions are thought to preferentially elicit naïve Th0 cells to differentiate into Th2-type cells [38]. The signals provided by IL-4 [39, 40] and IL-13 [41] and the interaction between CD40 on B cells and its ligand on T cells [42] stimulate immunoglobulin class switching of IgM-bearing B cells and promote secretion of antigen-specific IgE. Secreted IgE bound to high affinity surface receptors (FcεRI) on mast cells and basophils, which are normal component of the GI tract.

The allergic reaction is triggered by a secondary exposure to the sensitizing allergen. The interaction of allergen with two or more IgE molecules cross-links FcεRI receptors on mast cells and triggers their degranulation [43]. This results in the release of preformed mast-cell inflammatory mediators, such as histamine and tryptase and in the synthesis and release of newly generated lipid mediators, such as leukotriene and prostaglandin [44]. These agents rapidly mediate the symptoms of the immediate reaction.

Mast cells release cytokines that contribute to the recruitment of T cells and eosinophils. Furthermore, T cell secretion of IL-4 is not only responsible for the IgE isotype switching but also for the rolling on and adhesion to endothelial cells of circulating eosinophils, which are attracted into the gastrointestinal wall by both IL-5 and CCL11 [45-47]. Mediator release by eosinophils, basophils, and possibly other cell types is responsible for the late phase reaction.

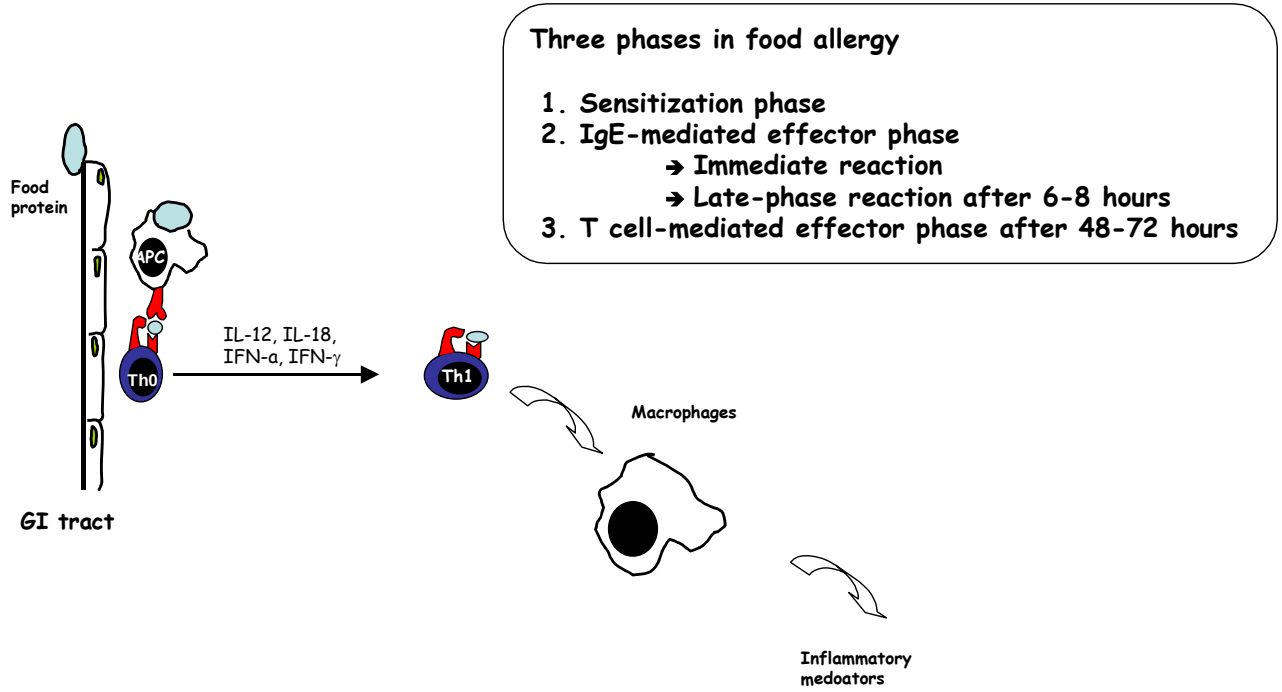
1.3.2.2. Food allergy as a polyphenotypic disease

1.3.2.2.1. Data supporting the Th2 hypothesis

The "Th2 hypothesis in allergy" [48], as detailed above, is supported by studies with peripheral blood mononuclear cells showing that the food-specific T cell response in patients with food allergy is Th2-skewed when compared to that of food-tolerant individuals [49-51]. It is also supported by genetic studies. Indeed, gene linkage and structure analysis, genome-wide screening and positional cloning have linked several genes to the potential for high IgE responses and IgE-dependent allergic reactions. One group of candidate genes is located in the 5q23-35 region in chromosome 5 and codes for IL-4, IL-5, IL-9 and IL-13, which increase IgE levels (IL-4 and IL-13) and favor eosinophil recruitment and survival (IL-5) and allergic manifestations.

<u>Figure 3</u> : Molecular and cellular mechanisms of class 1 food allergy (part 2)

from Bohle B et al. Mol Nutr Food Res. 2004



1.3.2.2.2. Role of Th1 cells in food allergy

As allergic symptoms are more readily inducible in the lung than in the GI tract, the role of Th1-type cells in the allergic process has been mostly investigated in animal models of asthma. Th1-type responses were initially believed to only counter Th2-type diseases, based upon the reciprocal regulatory effect of IFN-γ and IL-4. There is now evidence that Th1 cells could enhance rather than inhibit Th2 cell recruitment in asthma [52, 53]. In a similar way, it is possible that food-specific Th1 cells mediate the appearance of food allergy symptoms. In this regard, patients suffering from atopic dermatitis developed enhanced skin symptoms upon bovine casein oral challenge. The majority of food-specific T cells isolated from the peripheral blood of these individuals were of the Th1 phenotype. Investigators showed that the initial phase of the skin disease was predominantly associated with Th2 cells, and was followed by a second phase mostly associated with Th1 cells and IgE-independent skin lesions [54]. Other investigators proposed a role for food-specific Th1 cells in IgE-independent inflammatory reactions [55] (Figure 3) and suggest that food allergic reactions consist of three phases. During the initial sensitization phase, Th0 cells would differentiate into Th2 cells under the influence of IL-4. However, the presence of IL-12, IL-18, IFN-γ and IFN-α may induce the differentiation of Th1 cells as well. The second phase is the traditionally described IgE-mediated effector phase, which can be further divided into an immediate and a late phase that occurs after 6 to 8 hours. Th1 cells and macrophages are suggested to maintain an inflammatory response during the third phase, after 48-72 hours.

1.3.2.2.3. Regulation of the immune response to dietary antigens

The Th1/Th2 response to dietary antigens may be regulated by several factors, including Th cells involved in oral tolerance, and both pathogenic and non-pathogenic microorganisms. Another environmental factor that influences the immune response is the antigen.

The Th2 phenotype may be counterbalanced by a subset of $CD4^+$ T cells suppressing antigen-specific immune responses, designated as T regulatory cells [56]. These cells produce IL-10 and they are believed to be involved in oral tolerance, or the induction of non-responsiveness to orally administered antigens, including food [57]. Other studies link TGF-β-producing Th3 cells to oral tolerance [58].

As mentioned before, the cytokine environment plays a major role in the differentiation of allergen-primed Th0 cells into Th1 or Th2 cells. Thus, microorganisms that alter the cytokine balance may regulate allergic responses. For example, probiotics (lactobacilli or bifidobacteria) have been shown to inhibit antigen-induced IgE secretion trough induction of IL-12 secretion by macrophages [59]. Stimulation of the innate immune system by infectious agents, such as those that activate toll-like receptors (TLRs), may drive the immune system toward a Th1 phenotype and away it from the Th2 phenotype associated with food allergy. For example, Bashir et al [60] showed that oral sensitization with peanut proteins and the adjuvant cholera toxin (CT) induced peanut-specific IgE and anaphylactic symptoms in mouse strains lacking a functional TLR4 but not in their WT counterparts. When the composition of bacterial flora was reduced by antibiotic administration, TLR4 WT mice were as susceptible to the induction of peanut allergy as TLR4 mutant mice. Additional evidence for the role of TRL signaling in modulating allergic diseases is provided by

mechanistic studies in animal models of asthma [61-63] that demonstrated the protective effect of CpG DNA of bacterial or viral origin. Surprisingly in regard to the hygiene hypothesis, epidemiology studies showed that helminth infections, which induce strong Th2 and IgE responses [64] have a protective effect against allergy [65-67]. A recent model of peanut allergy in the presence of the adjuvant CT showed that IgE levels and anaphylactic symptoms were greatly diminished in helminth infected mice. The allergic symptoms were similar in peanut allergic mice and in helminth infected mice that were given neutralizing Abs to IL-10 [68]. Thus, one hypothesis is that chronic parasitic infections induce a regulatory network that significantly reduces the risk of allergy [69].

The nature and amount of allergens represent the prime factors that regulate the allergic reaction. This is consistent with the fact that dietary avoidance remains the only effective way to prevent most food allergies. Most allergens are foreign proteins or glycoproteins with a molecular mass usually ranging between 5000 and 70 000. Many attempts were made to characterize such antigens but until now, there has been no indication for common structural or biological features to all allergens. Studies of *in vitro* stimulation of $CD4^+$ T cells isolated from allergic donors showed the effect of different doses of allergen on Th1/Th2 differentiation. For example, Secrist et al. [70] found that $CD4^+$ T cells from allergic donors produced high levels of IL-4 when stimulated with low concentrations of allergens but produced low levels of IL-4 when stimulated with high concentrations. This study also found that the quality and magnitude of response to the allergens was influenced by the nature of antigen presenting cells (i.e., B cells or macrophages). In this regard, the role of the innate immunity, and in particular APCs, in shaping the differentiation pathway of a naïve Th0 cell is still matter of debate. This question is of special interest since several food and environmental allergens (i.e., Der f) or other proteins associated with them could display properties such as enzymatic activities that could trigger innate responses.

1.4. Cross-reactive allergic reaction

Caracterizing a cross-reactive allergic reaction includes investigating the cross-reactivity of IgE antibodies but also its clinical relevance. As explained below, both phenomena are distinct.

1.4.1. Antibody cross-reactivity

1.4.1.1. Molecular basis: structural identity between allergens

Two allergens are cross-reactive if there is one IgE antibody that binds to both these proteins. Thus, structural characteristics of proteins are major determinants of cross-reactivity. The IgE cross-reactions are because of shared features at the level of primary and tertiary structures of proteins. Cross-reactivity is rare below 50 % sequence identity and usually requires more than 70 % amino acid identity [71].

1.4.1.2. Cross-reactive protein epitopes

Molecular biology has allowed the identification of many cross-reactive allergens and the increasing availability of purified recombinant proteins will help characterize them further at the molecular level [72]. It turned out that most cross-reactive allergens could be grouped into a small number of protein families, fulfilling important biological functions and therefore highly conserved in their sequence and structure. They include profilin and pathogenesis-related proteins that are ubiquitous cross-reactive plant allergens involved in most pollen-associated food allergies [73-75], seed storage proteins (albumins and globulins),

responsible for cross-reactivity in the legume family [76] and tropomyosin proteins of crustaceans, house dust mites, cockroaches, and mollusks [77].

1.4.1.3. Cross-reactive carbohydrate epitopes

Antibody cross-reactivity, however, extends beyond the protein level. IgE antibodies from sera of allergic patients can be directed to carbohydrate determinants [78]. Due to the high cross-reactivity of such IgE antibodies, these epitopes were called cross-reactive carbohydrate determinants (CDD) [79]. In fact, they were shown to be extremely cross-reactive not only between plant-derived glycoproteins (IgE cross-reaction between carbohydrate pollen allergens and tomato, [80] peanut [81] or zucchini [82] has been demonstrated) but also to glycoproteins from invertebrate animals (house mite dust or fish allergens). This high degree of cross-reactivity was explained by the conserved structure of N-glycans from plants and invertebrate animals [83].

1.4.2. Induction of cross-reactive allergic symptoms by B and mast cell triggering

1.4.2.1. B cell triggering by a cross-reactive allergen

The immunologic phenomenon of cross-reactivity may induce allergic symptoms when the secondary (or cross-reactive) allergen is able to trigger B cells and mast cells. The cross-reactive allergen has to cross-link two monoclonal surface-bound antibodies in order to trigger a B cell. However, it is very unlikely that an allergen displays cross-reactive repeated epitopes. The phenomenon of B cell cross-reactive priming, hypothesized by Aalberse et al.[84] describes how the involvement of a cross-linking partner allows B cell triggering by a cross-

Figure 4 : Molecular basis of Ab cross-reactivity

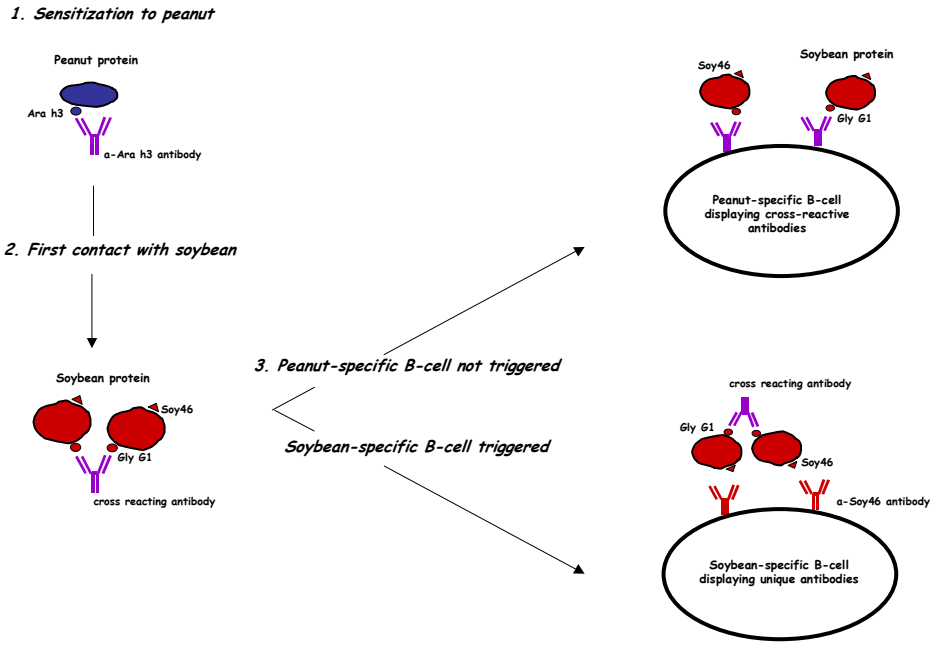
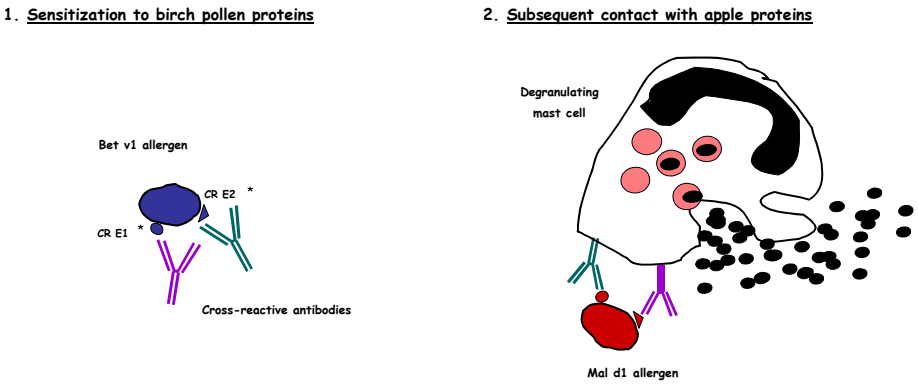


Figure 5 : Mast cell triggering by a cross-reactive protein



* CR E1, CR E2: Two cross-reactive epitopes on the allergens Bet v1 from birch proteins and Mal d1 from apple proteins (J Chromatogr B Biomed Sci Appl 2001 May 25; 756 (1-2): 307-13)

reactive protein. Figure 4 illustrates the antibody repertoire broadening to unique (non cross-reactive) soybean allergens (the 46 kd fraction - Soy46 - evidenced by Eigenmann et al. [85] for example) in peanut-sensitized patients. In this theoretical situation, sensitization to peanut induces antibodies specific to Ara h3, a peanut allergen that has been shown share IgE epitopes with soybean glycinin G1 [86]. Upon subsequent contact with soybean proteins, these antibodies might form immune complexes with glycinin G1 and present them to a naïve B cell with antibodies on it surface to the unique epitope Soy46. This may trigger the B cell to produce antibodies that are specific to soybean.

1.4.2.2. Mast cell triggering by a cross-reactive allergen

Unlike B cell antibodies, the antibodies on a mast cell are polyclonal. Thus, cross-linking of receptor bound IgE may be done be any cross-reactive molecule presenting two or more conserved epitopes (Figure 5).

1.4.3. Basis for discrepancies between *in vitro* and clinical tests

However, as mentioned before, *in vitro* cross-reactivity diagnostic tests frequently result in false-positive outcomes, which do not reflect clinical sensitivity patterns. This has especially been reported for plant foods like cereals or legumes [22, 23]. Various reasons might explain these discrepancies, including monovalent CCDs, low affinity antibodies and problems inherent to the IgE assays.

1.4.3.1. Monovalent CCDs

Anti-carbohydrate IgE antibodies may play a role in the observed differences between *in vitro* and clinical tests. In fact, monoglycosylated proteins, such as Arah1 from peanut, may explain that clinical symptoms might not appear in patients whose IgE response is restricted to CCDs [87]. Indeed, monovalent allergens are unable to induce histamine release by cross-linking IgE bound to the receptors of mast cells and basophils. However, a polyclonal IgE response to CCD and additional protein epitopes would be able to stimulate histamine release from sensitized mast cells.

1.4.3.2. Low-affinity antibodies

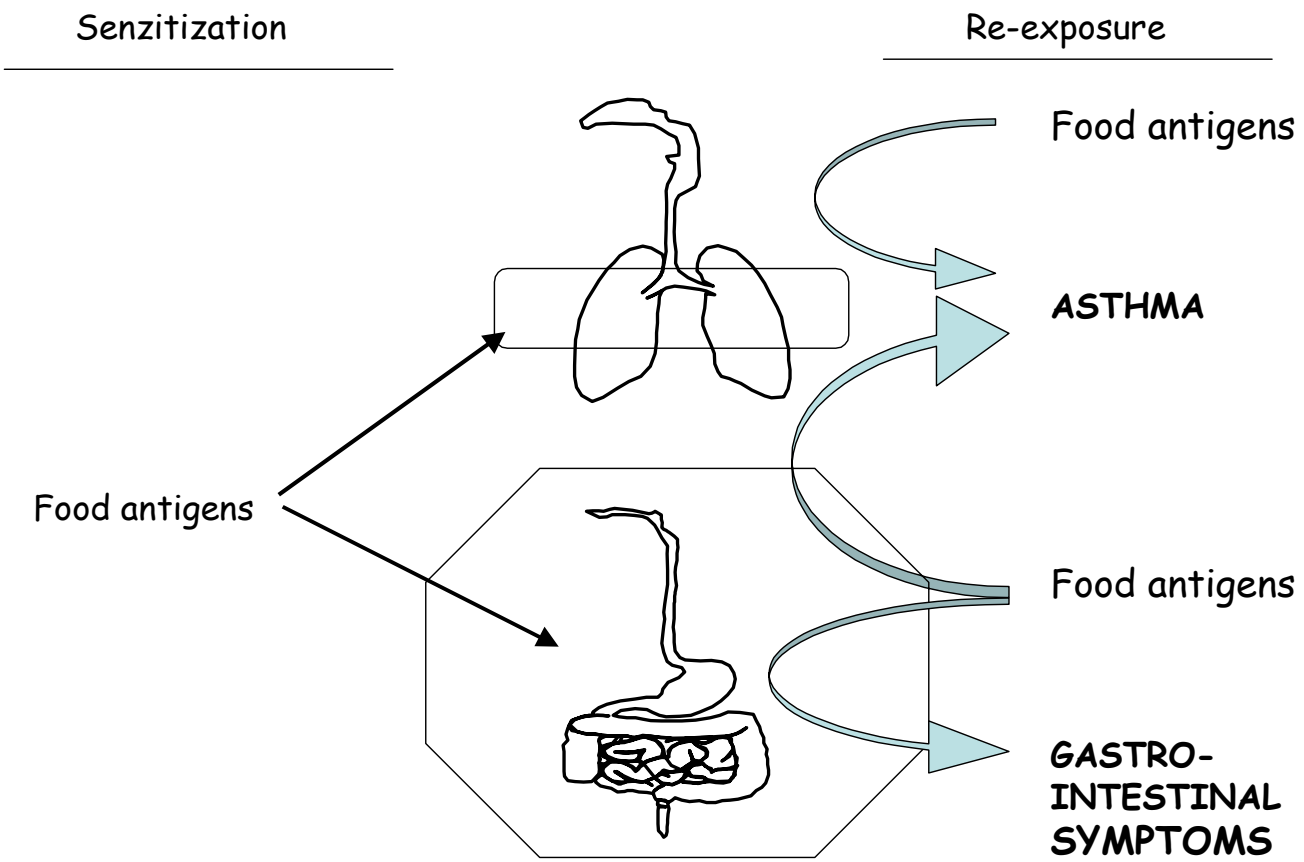
Specific IgE detection without clinical symptoms is not limited to CCDs. Low-affinity IgE antibodies could be another reason for the different outcomes of *in vitro* and clinical tests. A strong correlation was found between the affinity of the IgE for its antigen and the sensitivity of the histamine release [88]. The interaction between an antibody and the cross-reacting antigen usually has a lower affinity than that with the primary antigen [84]. However, studies are needed to determine the affinity threshold below which cross-reactivity becomes clinically insignificant.

1.4.3.3. IgE assays and detection of cross-reactive antigens

Other explanations are inherent to the IgE assay. It is obvious that the drawbacks of serologic allergy assays that already limit the diagnostic of primary allergies [89, 90], are magnified when dealing with cross-reactive proteins. Henri Malandain explains why cross-

reactions are far more frequent during serum IgE assays than in clinical challenges or skin tests [91]. During an IgE assay, serum IgE molecules are free: they can bind to all available (and compatible) epitopes. This binding is monovalent and each IgE molecule bound to one epitope generates a signal in the assay. *In vivo*, once linked to mast cells, IgE are no longer free to move. Thus, two different but cross-reactive IgE molecules must be adjacent on the mast cell and bind to adjacent epitopes on the cross-reactive allergen.

Figure 6 : Asthma and gastro-intestinal symptoms induced by food ingestion or food inhalation



2. Interplay between the GI and respiratory tracts in food allergy

Sensitization to food allergens generally occurs in the gastro-intestinal tract and primarily induces diarrhea or other gastro-intestinal symptoms. However, we will see that the respiratory tract is closely involved in both the sensitization and reactivity phases of food allergy.

2.1. Asthma induced by food ingestion or food inhalation

Asthma is an inflammatory disorder of the airways resulting in symptoms of coughing, wheezing, and shortness of breath associated with evidence of bronchial reversibility and hyperreactivity [92]. Asthma associated with exposure to aeroallergens such as pollen or house dust mite is common. However, it is now recognized that food allergens have also a significant role in the etiology of asthmatic symptoms in individuals with food allergies (Figure 6). Most commonly, asthma has been described in individuals with food allergies after ingestion of the offending food [93, 94]. Other studies reported asthma symptoms after exposure to aerosolized forms of the offending food: aerosolized fish, milk or egg proteins [17, 95], peanut airborne particles [96, 97] or legume particles [98].

Sensitization to these foods is commonly thought to occur in the gut. However, the respiratory tract may represent another site of sensitization to food allergens. In this regard, Gonzalez R et al. characterized soybean proteins responsible for respiratory allergies [99]. The allergens recognized by IgE Abs from patients suffering from respiratory allergy were different from the allergens recognized by IgE Abs from patients suffering from allergy to soybean without respiratory symptoms. Thus, it is possible that respiratory allergy to soybean resulted from a distinct sensitization process than food allergy restricted to the gut.

Furthermore, the high incidence of occupational asthma in the food industry [100] indicates that inhalation of food could be an important cause of allergic sensitization [101].

2.2. The GI and respiratory tracts as sites of sensitization and reactivity to food allergens

2.2.1. NALT and Peyer's patches: morphologic and functional similarities

Nasopharyngeal-associated lymphoreticular tissues (NALT) and Peyer's patches (PPs) in gut-associated lymphoreticular tissues (GALT) are organized secondary lymphoid tissues, part of the mucosa-associated lymphoid tissues (MALT). PPs and NALT share morphologic and functional similarities. Both are involved in local IgA production [102, 103] and are the portal of the mucosal immune system, through which most microbes and foreign substances enter the body. Generation of an immune response to food proteins partly depends on the uptake by microfold (M) cells, located in the epithelium of NALT and PPs. Antigens are transported to the underlying lymphoid tissue, which contain all of the immunocompetent cells that are required for the generation of an immune response: macrophages, DCs, B cells and T cells [104].

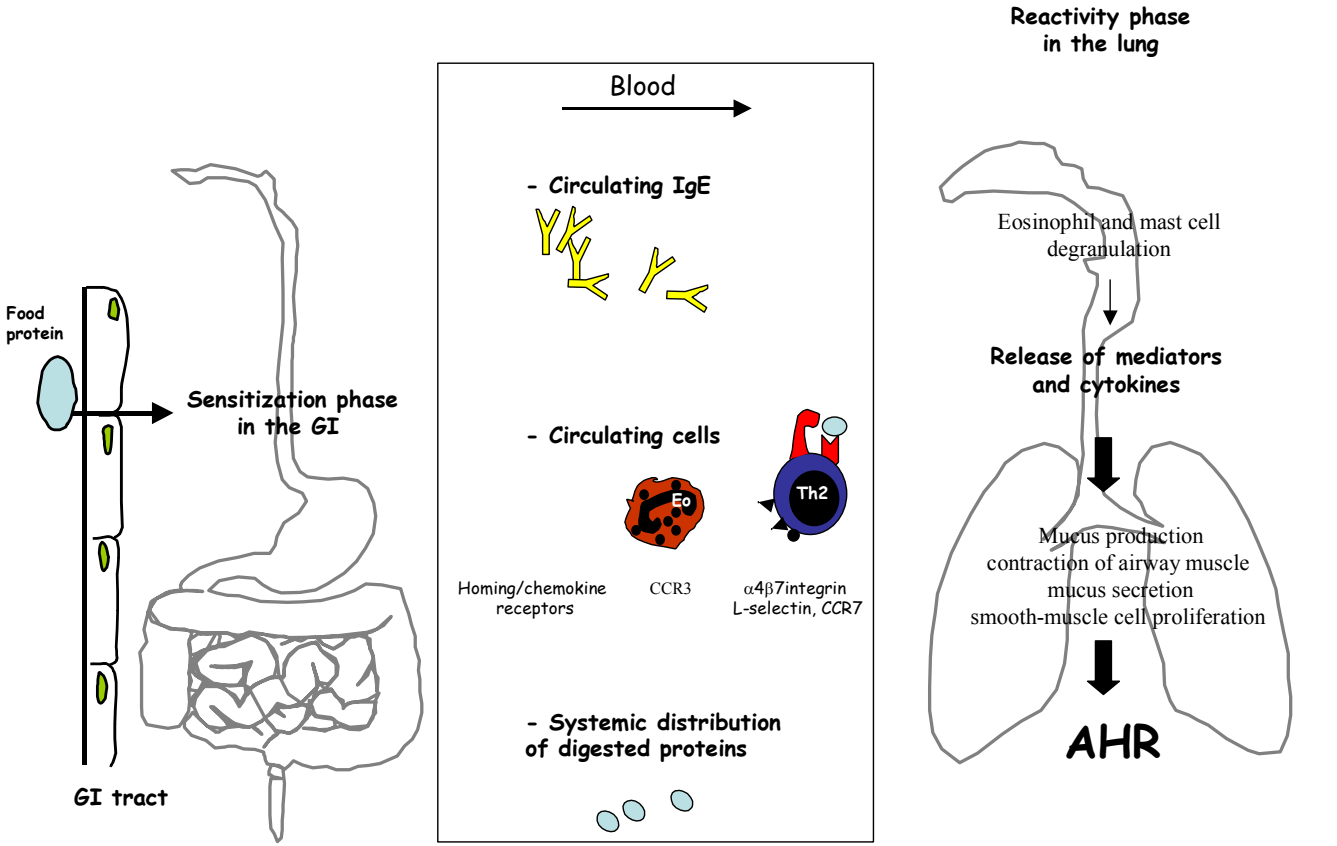
However, unique features of NALT and PPs may have a strong impact on the allergy process.

2.2.2. Distinct features of NALT and Peyer's patches

Recent studies indicate that NALT organogenesis is different from that of PPs in terms of both kinetics and cytokine requirements [105]. Furthermore, there are differences in the initiation of the immune response to food antigens in the NALT or in PPs. The GI tract is covered by a monolayer of tightly joined epithelial cells whereas respiratory tract tissues are covered by multi-layered squamous epithelial cells lacking tight junctions, making it likely that environmental antigens are more effectively sampled by the NALT than in the GALT. Accordingly, it is now well established that lower doses of antigens are needed for induction of immune responses by the nasal versus the oral route. Airway epithelial cells, more accessible to allergens than their intestinal counterparts, produce a large range of cytokines including IL-6, IL-8, IL-10, TNF-α, GM-CSF, RANTES, and CCL11, which are also secreted by classical APCs. While this could explain the higher airway responses to allergen when compared to reactions in the GI tract, contribution of immune innate factors to airway allergic responses is still poorly understood.

Additional differences in the initiation of the immune response in the NALT or in PPs are demonstrated in experimental studies using nasal or oral sensitization protocols. Nasal sensitization induces the expression of high levels of the homing and chemokine receptors CCR10 and $\alpha_4\beta_1$-integrin by B cells, allowing them to efficiently traffic to the respiratory and genito-urinary tracts [106, 107]. In contrast, orally-induced B cells express CCR9 and $\alpha_4\beta_7$-integrin and migrate to sites, such as the small intestine [108]. Few studies have addressed lymphocyte trafficking in food allergy, although this phenomenon may be of particular importance to understand mechanisms by which symptoms appear in organs distant from the sensitization site. Abernathy-Carver et al. [109] demonstrated the role of T cells expressing the cutaneous lymphocyte addressin (CLA^+) in milk-induced eczema. However, homing and

<u>Figure 7</u> : Factors contributing to food allergy-induced asthma



Eo : eosinophil

chemokine receptors expressed by T cells that were primed in PPs and migrate to the respiratory tract are currently not known. Similarly, no study has identified homing and chemokine receptors expressed by NALT T-cells sensitized to food allergens.

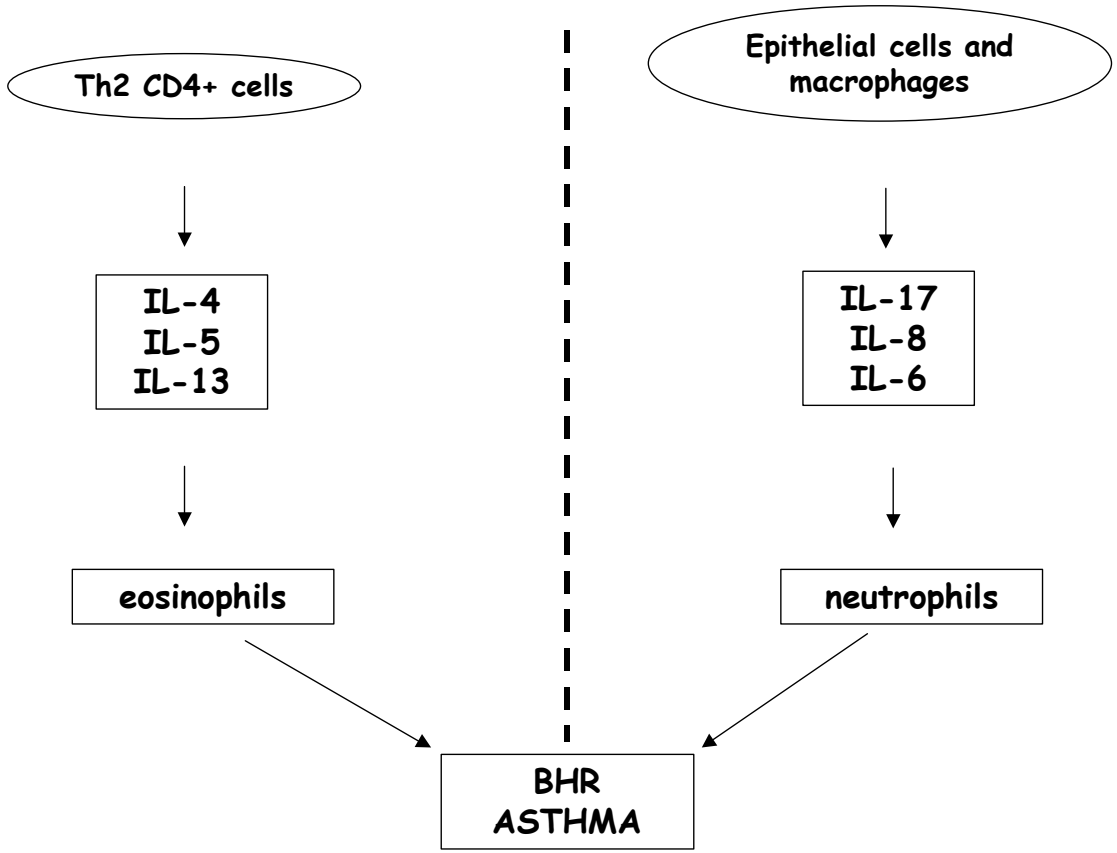
2.3. Mechanism of food-induced asthma

2.3.1. IgE-mediated asthma

Migration of immune cells and circulating antibodies play a major role in the pathogenesis of the food-induced asthma (Figure 7). As described earlier, sensitization to food allergens in the gut results in the differentiation of B cells secreting specific Abs, including IgE. These antigen-specific IgE can bind to mast cells and basophils in the GI tract, or they may reach other mucosal surfaces, including the lung, through the bloodstream.

The secondary exposure may occur by direct inhalation of food in aerosols or after systemic distribution of digested proteins [110]. As in the GI tract, Th2 cell secretion of IL-5 and CCL11 regulate eosinophilic infiltration into the respiratory tract [111]. Release of mediators such as histamine and cysteinyl leukotrienes from eosinophils and mast cells primarily causes contraction of airway muscle, increase microvascular permeability, stimulate mucus secretion, and induce smooth-muscle cell proliferation [4]. All these phenomenon lead to airway obstruction and airways hyperresponsiveness (AHR).

Figure 8 : Mechanisms of eosinophil-mediated and eosinophil-independent asthma



2.3.2. Non-IgE-mediated asthma

Until recently, asthma was exclusively regarded as an IgE-mediated atopic disease, with Th2 responses, IgE, and eosinophilic airway inflammation, resulting in enhanced bronchial reactivity [112]. There is now increasing evidence that other inflammatory mechanisms may be involved in producing airway hyperreactivity, or AHR (Figure 8). Thus, asthma could also be characterized by a massive infiltration of neutrophils in the lung [113, 114]. It has been shown that IL-1, IL-6 and IL-17 secreted by epithelial cells and macrophages play a major role in this neutrophil-associated asthma [115, 116]. Although neutrophil-induced hyperresponsiveness has been demonstrated in humans, the mechanisms involved are still not known.

Some food may have a non specific role in the induction of asthma or in the preparation of bronchi to react to other non specific stimuli. In this regard, few studies have investigated the effect of food proteins on APCs and epithelial cells and how they could affect innate and adaptive immune responses. Whereas those cells are the first to encounter the antigen, their contribution to both the sensitization phase and the reactivity phase of the allergic response are poorly understood.

3. Food allergy in the legume family

Peanut is one of the eight proteins that account for 90 % of allergic reactions to foods. Lupine, which has historically been consumed in limited areas of the world, is not recognized as a main food allergen. However, the recent generalization of its use as an additive in floor or fortified pasta makes its study of particular interest in the evaluation of the allergenicity of a novel food.

3.1. Peanut and lupine as primary allergens

3.1.1. Primary allergy to peanut

Recent studies from the United Kingdom and the United States indicate that the prevalence of peanut allergy, which affects 0.8 % of young children and 0.6 % of adults, has doubled during the last 5 years [3, 117]. This food allergy is of special importance because it has a tendency to be present early in life [118] and to induce more severe symptoms than other foods [119, 120], even after exposure to trace quantities of allergens [121, 122]. In fact, peanut allergy is the allergy most frequently associated with fatal food-induced anaphylaxis [19, 21]. Whereas clinical tolerance develops to most food allergens over time [2], peanut allergy is considered to be a lifelong threat [14] and few cases of resolution have been reported [123, 124]. A minority of patients might even redevelop clinical reactivity after a negative peanut challenge result [123]. Several studies have addressed the cellular responses to peanut allergens. As one could expect, the majority of T cells specific for peanut proteins isolated from the peripheral blood of allergic patients belonged to the Th2 subset [125, 126]. Dietary avoidance remains the only

effective way to prevent most food allergies at this time, but it is difficult to achieve in the case of peanut because of hidden traces in consumer products [14]. In addition, peanut avoidance would be of little help to prevent potential cross-reactive reactions between peanut and other members of the same botanical family or even unrelated proteins.

3.1.2. Primary allergy to lupine

3.1.2.1. Lupine in human consumption

Lupine is a legume belonging to the genius Lupinus, which includes 450 species. Sweet lupine seeds are traditionally eaten as appetizers in the Mediterranean countries. Sweet lupine is also increasingly used as additive to flour for bread, cookies and pasta [127] for both its nutritional and technological properties.

3.1.2.2. Lupine allergens

Immunoblotting using serum from lupine allergic patients has helped identify potential allergens among lupine proteins. In one study, the SDS-PAGE profile of Lupinus albus showed bands ranging from 16.5 to 175 kDa and the immunoblot analysis showed bands with molecular mass in the range of 30 to 80 kDa, the 45 kDa band appearing to be the major IgE-binding protein [98]. In another study, the most important IgE-binding lupine proteins have a molecular mass of 71, 59 and 34 kDa and the less important 24 and 17 kDa [128].

Immunoblotting of lupine antigen recognition by peanut-allergic patient sera were also performed. In Hefle's study [129], the SDS-PAGE of Lupinus albus showed numerous bands between 10 and 66 kDa. The IgE-binding proteins had a molecular mass of 21 kDa and 35 to 55 kDa. They are reported to be heat-stable. The band at 21 kDa was a major allergen in three patients but a minor one in three other patients. On the oher hand, the SDS-PAGE of Lupinus albus showed numerous bands between 14 and 75 kDa in Moneret-Vautrin and colleagues study [130]. Serum IgE bound to protein bands at 65, 43, 38 and 13 kDa. All sera bound IgE at 43 kDa, which is reported to be the major lupine flour allergen.

Hefle et al. [127] demonstrate that different extracts of the same legume vary in allergenic potency and allergen composition. Thus, one obstacle to the characterization of the lupine allergen is the composition of food extract used for SDS-PAGE. There is no standardized source of material, method of protein extraction or condition of storage of the food extract, which is currently a complex mixture of unknown substances.

3.1.2.3. Case reports of allergic reactions to lupine

Peanuts and soybeans are the major legumes involved in human food allergy [131, 132] and scarce data exist on adverse reactions to other legumes, such as lupine. Only five studies about isolated cases of primary allergy to lupine have been published until now. The first of these reports was in 1997 by Gutierrez et al [133], who described a 25-year-old patient who exhibited symptoms of immediate urticaria related to kiss of girlfriend who had previously eaten lupine seeds. Similarly, Romano et al [134] reported a case of allergic rhinitis and asthma after ingestion of lupine seeds. The first case report of respiratory symptoms after the inhalation of lupine in dust was described in a 3-year-old child [98]. Occupational allergy to

airborne lupine flour was reported in two recent studies. Moneret-Vautrin et al [128] described a patient with nasal and ocular allergic symptoms after handling of lupine flour used for prick tests and oral challenges in the diagnosis of food allergies. The symptoms disappeared after she stopped handling lupine flour. In the last study [101], three in seven subjects working in an agricultural research center reported naso-ocular symptoms immediately after being exposed to lupine.

Although these five studies describe lupine-induced allergic symptoms with tolerance to the other members of the legume family, lupine allergy is considered to be rare [134]. However, the risk of cross-reaction between lupine and other legumes should be considered.

3.2. Cross-reactivity between peanut and other legumes

In general, high amino acid sequence homologies exist among legume species for a given protein [76]. Theoretically, any identical epitope, hidden in different molecules, can lead to allergenic cross reactivity, or in other words elicite an allergic response as a result of an allergen binding to IgE antibody that has been produced through sensitization to another allergen [135]. In fact, cross-reactive reactions between peanut and other members of the legume family have been extensively studied.

3.2.1. Cross-reactivity in the legume family is clinically irrelevant in most cases

Using RAST and RAST inhibition, Barnett et al. [135] tested sera from 40 peanut allergic patients against 10 other legumes and found IgE binding to multiple legumes for 38 % of them. Extensive immunologic cross reactivity among legumes was later confirmed by Bernhisel-Broadbent et al. [22] who studied 62 children with allergy to one legume and

demonstrated by SDS-PAGE/immunoblotting that 79 % had IgE binding to more than one legume, with a band at 20 kDa in all legumes except the green bean. However, on verifying the clinical reactivity to each legume by DBPCFC, they found that less than 5 % of patients reacted to more than one legume. Results of DBPCFC in other studies, including a wide variety of legumes [136-138] demonstrated that clinical cross-reaction is rare. One study reported cross-reactions between peanut and soybean [139] but a recent review underlined the limitations of that study [140]. Clinical cross-reactivity between peanut and soya seems actually to be uncommon [14, 131] despite the recent identification of shared IgE epitopes [86].

3.2.2. Some particular legumes may favor cross-reactive reactions

Particular types of legumes, like lupine, better known in the Mediterranean countries and not included in the previous studies, may be less allergenic but more cross-reactive. Although scarce data exist on adverse reaction to lupine, several reports point out the risk of cross-reactive allergies between lupine and other legumes, especially peanut.

In 1994, Hefle et al [129] described the first case of allergy in a 5-year-old girl with peanut sensitivity to lupine, after ingestion of spaghetti-like pasta fortified with sweet lupine seed flour. Skin-prick tests and RAST with extracts of lupine fortified pasta were positive in 5 out of 7 peanut sensitive patients who had also clinical symptoms with garden pea. However, no oral challenge with lupine was performed. Three other studies linked lupine allergenicity to other legume proteins. Matheu et al [141] reported the case of a chickpea-allergic woman who was tolerant to other legumes but developed urticaria and angiodema the first time she ate lupine seeds. Clinical cross-reactivity with other legumes (lentils, white beans, and green beans) progressively developed over a 5-year period. Moneret-Vautrin et al [130] evaluated clinical reactivity to lupine by DBPCFC in 24 peanut-allergic subjects. Lupine-specific IgE

was demonstrated in 11 patients, of whom 7 had clinical reactivity to lupine. This group also reported a case of acute asthma after lupine oral challenge in a patient with allergy to peanut [142] and based on these observations, they conclude that lupine flour is a high risk-allergen in patients presenting peanut allergy.

3.3. Peanut allergy and asthma

As seen above for food allergies in general, the respiratory tract is closely involved in both the sensitization and reactivity phases of peanut allergy.

3.3.1. Aerosolized peanut as an allergic reaction-trigger

Allergic respiratory symptoms have been described in peanut-allergic patients after inhalation of peanut airborne particles in school [120] or on airline flights [96, 97]. In this regard, a study about the distribution of airborne peanut allergens in the environment has just been published [143]. In fact, the health care problem associated with peanut allergy is worsened by its link to life threatening asthma. Peanut is one of the food most commonly implicated in severe respiratory reactions [131, 144]. The reported mortality from peanut reactions has shown that many, if not all, of the known fatalities have occurred in individuals who were known to have asthma [19, 145]. Individuals with peanut allergy also present serious risks of asthma in association with inhalation of cross-reactive food allergens [142]. However, the mechanisms underlying the increased risk of life threatening asthma in the peanut-allergic population remain unclear. One possible explanation is that chronic inhalation of aerosolized food, such as that achieved in the food industry, might worsen asthma in subjects with food allergy [146].

3.3.2. Aerosolized peanut as a sensitizing agent

As indicated above, both the oral and inhalational routes of exposure can trigger severe adverse reactions in peanut allergic patients. It is also accepted that sensitization to food allergens can occur in the gastrointestinal tract or as a consequence of inhalational exposure. In this regard, most individuals who react to peanut do so, on their first known exposure. Since IgE-mediated allergic reactions require prior exposure resulting in sensitization, it has been hypothesized that the allergens are transferred in utero or via breast milk [147]. However, no study settled the question as to whether sensitization could be achieved through inhalation of peanut airborne allergens. Peanut allergy may also be associated with inhalational sensitization to other allergens. In this regard, it is frequently associated with pollen allergy [148] and in particular with birch pollen [149], grass pollen [28] and plane tree pollen [150]. It is possible that this link only reflects a co-sensitization due to an atopic background. Alternatively, there may be a causal pathophysiological mechanism [151]. For example, the role of inhalant sensitization to cross-reactive pan-allergens, like lipid transfer proteins [152] or profilins [153] in peanut allergy, has yet to be investigated [154, 155].

4. Animal models of food allergy

The double-blind placebo-controlled food challenge (DBPCFC) remains the gold standard method for identification of the offending foods in human allergies [156-158]. Unfortunately, the procedure is associated with obvious limitations such as its high cost and risk of fatal allergic reactions [159]. The less traumatic *in vivo* skin-prick test together with the *in vitro* ELISA and radioallergosorbent assays (RAST) were proven useful as alternative diagnostic tools. However, discrepancies between the *in vivo* and *in vitro* assays are common [160-162]. In addition, neither the DBPCFC nor the skin-prick test address the mechanisms implicated in primary or cross-reactive immunological reactions to food allergens. Furthermore, the use of these diagnostic tools to test on human the allergenicity of each novel food introduced in the human market raises important ethical issues. Thus, animal models are needed to better understand mechanisms of food allergies and develop more effective diagnostic and therapeutic strategies, as well as to predict the allergenicity of novel foods.

4.1. Models to investigate mechanisms of food allergies

Earlier studies used the Guinea pig model [163]. However, most recent investigations used other animal models, including rodent (i.e., rat, mouse) and nonrodent (i.e., dog, pig). Each model presented specific advantages and drawbacks.

4.1.1. Studies in mice

4.1.1.1. Advantages and limitations of the mouse models of food allergy

The advanced understanding of the immune system in mice and its similarity with the human immune system in mechanisms such as Th1 and Th2 responses [33, 34] make murine models particularly attractive for the study of mechanisms involved in food allergy. For example, mice Th2 responses result in IgG1 production, while Th1 responses lead to IgG2a production. Therefore, quantification of mouse IgG1 and IgG2a provides an insight on Th1 and Th2 responses, respectively. Furthermore, the availability of reagents and the increasing number of knock-out and transgenic mouse strains provide valuable tools to investigate the role various immune cells and their products (i.e., cytokines/chemokines) in food allergies.

Despite similarities between the immune systems in mouse and human, major differences need to be considered. For example, in human, IgE is the only immunoglobulin isotype that directly triggers degranulation of mast cells and subsequent manifestation of anaphylaxis. The role of IgG is primarily limited to potential competitors for binding of allergens. In contrast with human, mouse IgG1 also directly trigger degranulation of mast cells and together with IgE play an important role in Passive Cutaneous Anaphylaxis. [164]

4.1.1.2. Dissecting mechanisms: systemic sensitization / OVA + alum

Most studies in mice involved systemic sensitization with ovalbumin, a well-defined chicken egg allergen, in the presence of an adjuvant such as alum. This approach favors the initiation of vigorous serum IgG1 and IgE responses. Following sensitization, the mice were mucosally exposed to the antigen, in order to address the effector phase of the allergic reaction. These models have been useful in dissecting the mechanisms required for the induction of allergic gastrointestinal inflammation, including the role of CCL11 [165], IL-5 [166] and STAT6 [167].

4.1.1.3. Mimicking human food allergy: oral sensitization / food extract + CT

Other investigators attempted to develop murine models that more closely mimic the gastrointestinal sensitization, which is the prime route of sensitization to food proteins in humans. These studies used protein extracts, instead of purified allergens that were administered by the oral route. As mice have a strong innate tendency to develop tolerance to orally administered proteins [168], the Th2-inducer adjuvant cholera toxin was used in order to generate an environment that mimics the atopic background of allergic patients. In this regard, the reported murine models of cow's milk hypersensitivity [169] and peanut anaphylaxis [170], both mimic the clinical and immunological features of peanut and cow's milk allergy in humans.

The models of cow's milk and peanut allergy used by Li et al. proved useful tools to investigate the influence of genetic factors on food allergy [171] and to test new immunotherapeutic strategies. For example, the oral administration of IL-12 [172] or the

coadministration of recombinant peanut protein and Listeria monocytogenes [173] were shown to attenuate murine anaphylaxis responses.

4.1.2. Studies in nonrodent animals

Nonrodent models have also been used for investigating food allergy mechanisms. Among them, the dog and the swine models are of particular interest for studying human allergic diseases.

4.1.2.1. The atopic dog model

Food allergy is relatively common in dogs, affecting 8 % of the canine population [174]. Thus, dog models more closely mimic the situation in humans than mice, in which allergies do not occur naturally. The dog model shows clinical allergy symptoms typical of humans, since both humans and dogs experience vomiting, nausea and diarrhea. Furthermore, the large size of dogs allows gastrointestinal studies, such as sampling of mucosa, on live animals, making it possible to investigate mechanisms of sensitization and reaction on serial biopsies over days or weeks of treatment. These advantages of the atopic dog model helped mimic a proposed mechanism involved in the development of food allergies in children [175], that of a viral infection combined with sensitization to other nonviral proteins. In genetically predisposed individuals with an early bacterial infection, the immune system is suggested to be capable of responding to bystander antigens with more vigor. The inflamed gut becomes more permeable to proteins, thereby exposing the local immune system to more antigen sources.

4.1.2.2. The swine model

Swine present a number of important advantages compared with other animal models for investigating immune responses to allergens. They closely resemble humans in gastrointestinal physiology and in the development of mucosal immunity. Developing piglets have similar anatomy and nutritional requirements, a transient neonatal porosity of the gut to dietary proteins, a distribution and maturation of intestinal enzymes and an enteric absorption of antibody that are similar to what is seen in young children [176]. Thus, young piglets have been used as models for sensitization to soy [177] and peanut [178] proteins, which paralleled what is seen in young children. However, the current lack of antibodies specific for swine IgE represent a major limitation to the swine model.

4.2. Models to predict the allergenicity of novel proteins

4.2.1. Advantages of animal models compare to *in vitro* studies

The emergence of genetically modified foods and industrial proteins, as well as their potential to sensitize genetically predisposed populations, has prompted investigators to seek methodologies to screen novel proteins for allergenicity. Information concerning the amino acid sequence identity to known allergenic proteins and resistance of the novel protein to proteolic digestion in a simulated gastric fluid [179] give valuable information about the properties of the novel protein. However, none of these properties addresses directly the ability of a protein to provoke an allergic response and *in vitro* immunological assays remain to be developed. Therefore, there has been considerable interest in the possibility of developing appropriate animal models.

4.2.2. Studies in the Brown Norway rat: oral sensitization / no adjuvant

Some investigators suggested that oral sensitization is the preferred route for the evaluation of intrinsic allergenic potential of new proteins, and that the presence of an adjuvant should be avoided because it may confer on nonallergenic proteins the potential to provoke IgE production. This hypothesis was tested in the Brown Norway rat that resemble atopic humans in its genetic predisposition to readily react to food antigens with an overproduction of IgE [180]. These studies showed that Brown Norway rats could be sensitized to a purified protein (OVA), a whole food (cow's milk) or protein extracts (hen's egg white, peanut) by daily gavage without the use of adjuvant. Furthermore, the specificities of the induced antibody responses resembled those found in food allergic patients [181, 182]. Based on these studies, the Brown Norway rat may provide a suitable animal model for research on the allergenicity of food proteins. However, further testing with known allergenic and nonallergenic proteins are needed for validation of these rat models.

4.2.3. Studies in BALB/c: intraperitoneal sensitization / no adjuvant

Other investigators used BALB/c mice to evaluate the sensitizing potential of novel proteins. As oral exposure to protein without adjuvant is likely to induce tolerance, mice were intraperitoneally exposed to allergenic (OVA, peanut agglutinin) and non-allergenic proteins (BSA, potato agglutinin). While all proteins were found to be immunogenic, and induced comparable IgG responses, they displayed marked differences in their capacity to induce IgE responses [183-185]. These studies suggest that the measurement of IgE responses in this mouse

model can accurately identify allergens and distinguish them from proteins lacking allergenicity.

4.3. Conclusion

As detailed above, several animal models of food allergy are currently available, each of them having avantages and limitations. Thus, large animals present natural allergic responses but the study of the mechanisms involved is limited by the lack of reagents. On the other hand, investigators need to use non physiologic routes of sensitization and adjuvants inducing a Th2 response in order to bypass oral tolerance in rats and mice. As a consequence, no fully reliable validated model is available yet. Furthermore, several aspects of food allergies are still poorly investigated. For example, the nasal route of food sensitization and challenge has not been extensively studied, and no study on animal models explored OAS or cross-reactive allergy. Moreover, “the Th2 hypothesis” has lead investigators to carefully characterize the role of IgE and Th2 cells in food allergy whereas the of the polyisotypic antibody response to allergens and the importance of both Th1 and Th2 cells are still unclear.

MATERIALS AND METHODS

1. Peanut and legume extracts

Protein extracts of peanut were obtained as previously described by ammonium bicarbonate treatment of defatted PPEs [136]. Briefly, 200 g of dry-roasted peanuts were grounded, mixed with 600 ml of acetone and incubated overnight at 4ºC under agitation. The mixture was then subjected to 20 min centrifugation at 2000 rpm and the supernatant replaced with fresh acetone. The entire defatting procedure consisted of three incubations in acetone. The resulting pellet was allowed to dry overnight at room temperature. Proteins were then extracted by overnight incubation in 800 ml of ammonium bicarbonate 0.1 M, pH 8 with stirring. The mixture was allowed to decant overnight at 4ºC and the supernatant was centrifuged for 20 min at 2000 rpm at 4ºC. The concentration of protein was determined by using the BCA kit (Pierce Chemical Co., Rockford, IL). Flour of lupine, pea, and soybean were provided by the Cooperative Agricole de Noelle Ancenis (CANA, France). Protein extracts were prepared by overnight incubation of legume floors in 800 ml of ammonium bicarbonate 0.1 M, pH 8 as indicated above for peanut. The peanut and legume protein extract were dialyzed in 3 kDa cutoff membranes against distilled water and either lyophilyzed or concentrated and stored at - 20ºC until used.

In order to determine the pattern of protein contained in peanut and legume extracts, 30 mg of protein extracts were added to the buffer (Tris HCl 1M, glycerol, SDS and b-mercaptoethanol) and heated for 5 min at 90ºC before being loaded into 10% polyacrylamide gels (BioRad, Hercules, CA). After migration (1 h at 100 mV), the gels were stained with Coomassie blue and the pictures were analyzed by Kodak 1D 3.0 software.

2. Mice.

Female C57BL/6 and BALB/c wild-type mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Female IL-4, IL-12p40 and IFN-γ KO mice were on the C57BL/6 background. Female IL-4/IL-13 double KO mice were generated on a BALB/c background. All KO mice were obtained from the Jackson Laboratory Animal Resources (Bar Harbor, Maine). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum. Studies were performed in accordance with both NIH and the University of Alabama at Birmingham Institutional guidelines to avoid pain and distress.

3. Mucosal sensitizations.

Protein extracts of peanut were obtained as previously described by ammonium bicarbonate treatment of defatted PPEs (10). Mice 8 to 12 weeks of age were sensitized two times a week apart with whole PPE and CT as adjuvant. Anesthetized mice were nasally sensitized by administration of 100 μg of PPE and 1 μg of CT in a total volume of 10 μl with 5 μl placed into each nare. For oral sensitization, mice were deprived of food for two hrs, and then orally treated with 250 μl of sodium bicarbonate as previously described (37). Oral sensitization consisted in intragrastric administration of 1 mg of PPE plus 15 μg of CT in 250 μl of PBS. Some experiments included mice sensitized to OVA (Sigma Chemical, Saint Louis, MO) as antigen instead of peanut. Mice were then either nasally sensitized to 100 μg of OVA plus 1 μg CT or orally administered 1 mg of OVA plus 15 μg of CT. Plasma samples

were collected at day 7 and 14 for analysis of peanut-specific Ab responses. Other doses of CT (5 μg nasal or 60 μg oral) and peanut (25, 50 or 200 μg nasal or 2 μg oral) were tested in separate experiments.

4. Nasal challenge with peanut and unrelated proteins.

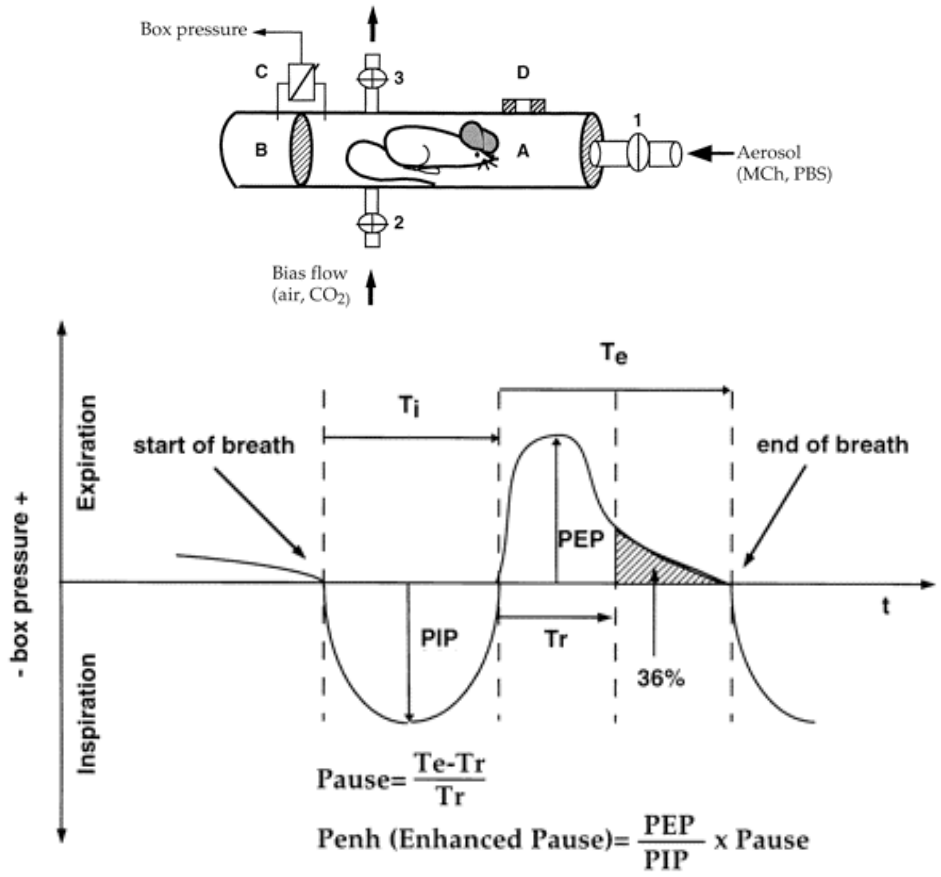
Mice nasally or orally sensitized to peanut were nasally challenged on days 15 and 16 with 200 μg of PPE in a total volume of 100 μl. More specifically, anesthetized mice were given 25 μl of PPE per nare, four times at 3-4 min intervals. For analysis of lung responses to unrelated proteins, mice were challenged with 200 μg of OVA or 40 μg of Dermatophagoides farinae (Der f) extract (Greer laboratories, Lenoir, NC) instead of PPE.

5. Plasma antibody responses.

Plasma levels of peanut-specific antibodies were measured by ELISA. Briefly, 96-well microplates (Falcon) were coated with 50 μg/ml of PPE in PBS and incubated overnight at 4ºC. After blocking with PBS-1% BSA, serial dilutions of plasma samples were added and incubated overnight at 4ºC. Peanut-specific IgG Abs were detected using 0.3 μg/ml of HRP-labeled goat anti-mouse γ-heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL). Biotin-conjugated rat anti-mouse γl (clone A85-1), γ2a (clone R19-15), γ2b (clone R12-3) or γ3 (clone R40-82) heavy chain mAbs (BD-PharMingen, San Diego, CA) were employed at 0.5 μg/ml and streptavidin-HRP (BD-PharMingen) was diluted at 1:2000 for the detection of peanut-specific IgG subclasses. The colorimetric reaction was developed by the addition of 2,2'-azino-bis (3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma) and H_2O_2. Endpoint titers were expressed as the $\log_2$ of plasma dilution

<u>Figure 9</u> : Barometric plethysmograph : parameters measured

(Hamelmann E et al. Am J Respir Crit Care Med. 1997)



giving an optical density at 415 nm of ≥ 0.1 above those obtained with control plasma. To determine the potential of plasma of peanut-sensitized mice to react with irrelevant protein antigens, plasma samples were added to ELISA plates coated with OVA (1 mg/ml) or Der f (10 μg/ml) protein extracts.

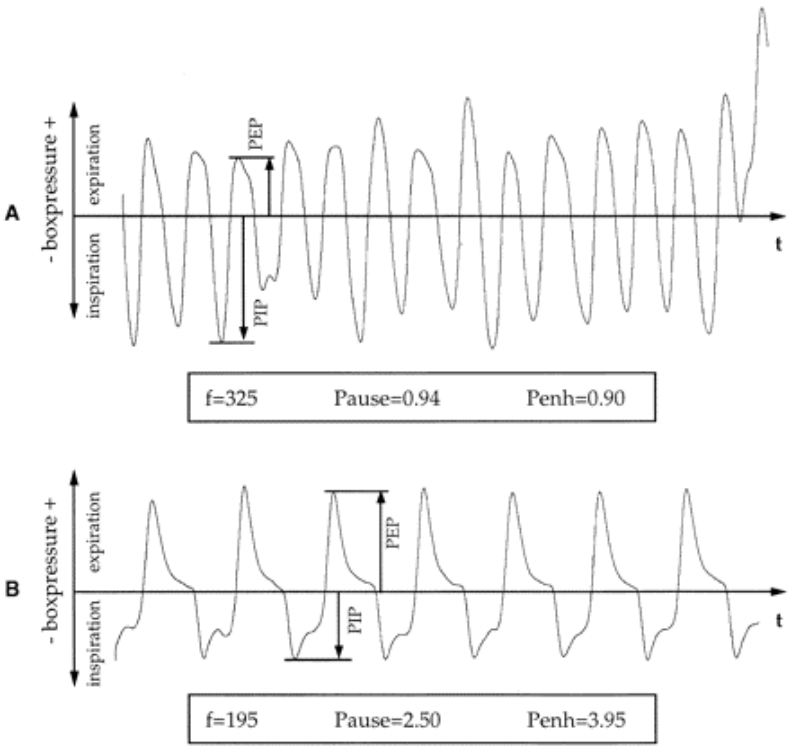
The removal of IgG has been shown to improve the detection of IgE Abs [186]. Thus, dilutions of plasma samples were first depleted in IgG by overnight incubation at 4ºC in protein G coated 96-well plates (Reacti-Bind plates, Pierce, Rockford, IL). Total and antigen-specific IgE levels were then analyzed by ELISA. For detection of antigen-specific IgE Abs, IgG-depleted samples were added to ELISA plates coated with PPE (50 μg/ml, 100 μl per well). The IgE were detected with 0.5 μg/ml biotin-conjugated rat anti-mouse IgE (clone R35-118; BD-PharMingen) followed by streptavidin-HRP (1:2000). The levels of total IgE Abs were determined using capture and detection antibodies, as well as IgE standard, from the BD OptEIA Set mouse IgE kit (BD Biosciences, San Diego, CA).

6. Airway hyper-reactivity.

Asthma is described as the presence of characteristic symptoms, including airway hyperresponsivness (AHR) to a variety of inhaled bronchoconstrictor stimuli. There are two different ways to measure altered airway functions in mice. The first method is *in vitro* measurement of tracheal smooth muscle contractility after electrical stimulation. Its drawback is that it cannot take into account the influence of mucus production and other *in vivo* changes in the lungs of allergic mice. The second method is to measure AHR in mice placed in a barometric plethysmograph. This method presents the advantage of being performed on live animals, allowing multiple measurements on the same mouse.

<u>Figure 10</u> : Barometric plethysmograph : changes in box pressure waveform after metacholine challenge

(Hamelmann E et al. Am J Respir Crit Care Med. 1997)



The barometric plethysmograph is composed of 2 chambers (Figure 9). Pressure is constant in chamber B, the reference chamber. Pressure differences between chamber B and chamber A, where the mouse is placed, are measured by the pressure transducer called C on the scheme.

Figure 9 shows that the box pressure decreases as the animal inspires and it increases as the animal expires.

Penh (enhanced pause) = Pause* PEP/PIP

Pause = (Te - Tr) / Tr

Te is the time of expiration, meaning: the time of increased pressure in the box. PIP or peak inspiratory pressure is the maximum box pressure signal occuring during inspiration, thus in the negative direction. PEP or peak expiratory pressure is the maximum box pressure signal occuring during expiration, thus in the positive direction. Tr is the relaxation time, time of pressure decay to 36% of the total expiratory signal, as indicated on the graph.

Figure 10 represents changes in box pressure with a control mouse. Box pressure changes when methacholine is administered. Metacholine is a bronchoconstrictive agent. Changes in the waveform of the pressure signal can be quantified by the parameters PEP and PIP. In the presence of methacholine, PEP is higher and PIP is smaller than in the control mouse. Thus, allergic mice have increased enhanced pauses than control mice.

In our experiments, doses of metacholine (0, 10 and 20 mg/ml) were administered by nebulization. For each dose, Penh were measured every minute over seven minutes. Controls included naive mice challenged with PBS.

7. Histology and determination of lung inflammation scores.

Lungs were fixed in 10 % buffered formaldehyde, paraffin-embedded and cut into sections of 5 mm thickness. The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin for the evaluation of inflammation. The presence of eosinophils in tissue sections was determined by the cyanide-resistant peroxidase activity as previously described (76). Briefly, lung sections were incubated for 1 min at room temperature in 10 mM KCN pH 6.5. Slides were then rinsed in PBS and incubated for 15 min with the peroxidase substrate 3,3'-diaminobenzine (Vector Laboratories, Burlingame, CA). After washes in PBS, tissue sections were counterstained with hematoxylin. The eosinophils, which express a cyanide-resistant peroxidase activity, are stained dark brown.

For quantification of lung inflammation, the slides were coded, and peribronchial and perivascular inflammation was scored in a blinded fashion. A value of 1 was given when slides showed no sign of inflammation. Slides were graded from 2 to 4 when bronchi were surrounded by a thin layer of inflammatory cells (2: few bronchi; 3: more bronchi: 4: most bronchi). They were graded from 5 to 7 according to the number of bronchi that were surrounded by a thick layer of inflammatory cells (5: few bronchi; 6: more bronchi: 7: most bronchi). Finally, slides were graded 8 or 9 when inflammation spread into the interstitial area (8: severe; 9: extreme).

8. Flow cytometry.

Whole lung tissue was dissociated by digestion with 1 mg/ml of collagenase type V (Sigma) in RPMI-1640 (Cellgro Mediatech, Washington, DC), supplemented with 10 mM HEPES, 2 mM L-glutamine, 5x10-5 M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml

streptomycin (RPMI supplemented) to obtain single cell preparations. Mononuclear cells were collected at the 20-75% interface of discontinuous Percoll gradient and stained with anti-CD3, anti-CD4, anti-B220, anti-CD11c, anti-MAC-1 or anti-MHC class II Abs (BD PharMingen). After washes and fixation, samples were analyzed by flow cytometry.

9. Purification of $CD4^+$ T cells.

Whole lung tissue was dissociated by digestion with collagenase as described above. Mononuclear cells were collected and washed in RPMI-1640 supplemented. The $CD4^+$ T cells were separated using the automated magnetic cell sorting (autoMACS) according to the protocol provided by the manufacturer (Miltenyi Biotech, Auburn, CA). Briefly, single cell suspensions were incubated with a biotinylated anti-CD4 mAb (BD-PharMingen) for 30 min at 4 °C and washed in PBS containing 2 mM EDTA and 0.5% bovine serum albumin. Streptavidin-conjugated MicroBeads (Miltenyi Biotech) were then added to cells. After a 30 min incubation at 4 °C, cells were washed and $CD4^+$ T cells were purified by positive selection using autoMACS.

10. Quantification of cytokine and chemokine mRNA by real-time PCR.

Lung tissue was dissociated as described above, and mononuclear cells were collected, washed in RPMI supplemented and RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX). The reverse transcription was performed with superscript II reverse transcriptase, dNTPs and poly(dT) oligos. The real-time PCR (Lightcycler, Roche, IN) was

performed with primers generated with Oligo software (Plymouth, MN) and the SYBR green detection system according to the manufacturer. Results are expressed as crossing point (CP), defined as the cycle at which the fluorescence rises appreciably above the background fluorescence as determined by the Second Derivative Maximum Method (Roche Molecular Biochemicals LightCycler® Software). The formula: 20 - (CPcytokine - CPb-actin) was used to represent the logarithm of the relative mRNA levels of a given cytokine. This formula allows the normalization of all results against b-actin levels to correct for differences in cDNA concentration between the starting templates. Differences of crossing points above two cycles were considered significant.

11. Bronchoalveolar lavage and cytospin.

Bronchoalveolar lavage fluids (BALF) were obtained via cannulation of the exposed trachea, by infusion of 0.6 ml of RPMI supplemented through a 22-gauge catheter into the lungs, followed by aspiration of this fluid into a syringe. A volume of 0.4 ml of fluid was consistently recovered. Aliquots were centrifuged and supernatants collected and stored at -70ºC until analyzed. Cell pellets were subjected to cytospin and the slides stained with Giemsa (Sigma).

12. Cytokine ELISA.

Cytokines were measured in the supernatants of BALF by ELISA. Nunc MaxiSorp Immunoplates (Nunc, Napersville, IL) were coated with anti-mouse TNF (MP6-XT22), IFN-γ (clone R4-6A2), IL-4 (clone BVD4-1D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3),

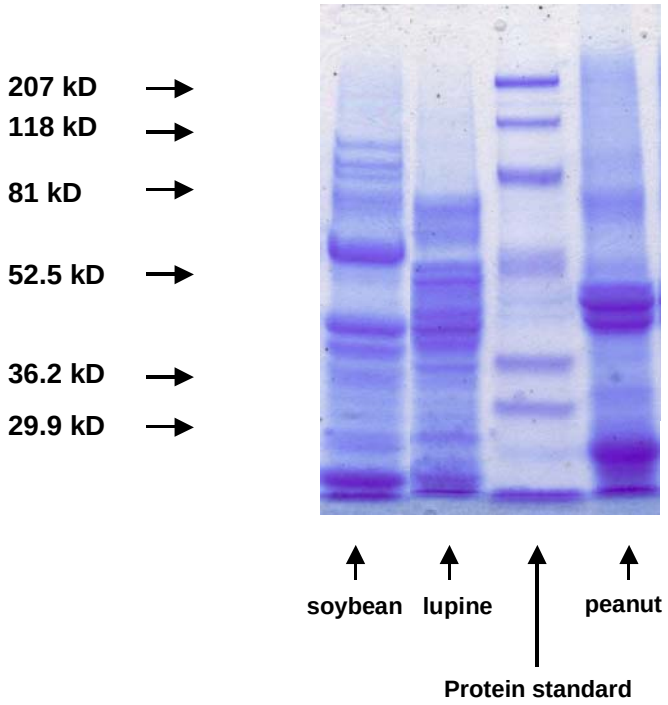
IL-10 (clone JES5-2A5) mAbs (BD PharMingen) or IL-13 (R&D systems, Minneaplois, MN) in 0.1 M sodium bicarbonate buffer (pH 9.5), and incubated overnight at 4ºC. After blocking with PBS-3% BSA, cytokine standards and serial dilutions of supernatants of BALF were added in duplicates. The plates were incubated with biotinylated anti-mouse TNF (MP6-XT3), IFN-γ (clone XMG-1.2), IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), IL-10 (clone JES5-16E3) (BD PharMingen) or IL-13 mAbs (R&D systems), followed by HRP-labeled goat anti-biotin Ab (Vector Laboratories, Burlingame, CA). The colorimetric reaction was developed with the addition of 2,2'-azino-bis (3)-ethylbenzylthiazoline-6-sulfonic acid substrate and H2O2. Standard curves were generated using murine rIFN-γ, rIL-5, rIL-6, rIL-10 (Genzyme, Cambridge, MA), rIL-4 (Endogen Corp., Boston, MA), rTNF-a and rIL-13 (R&D systems). The ELISAs were capable of detecting 10 pg/ml of IL-5, 3 pg/ml of IL-4; 5 pg/ml of IL-6 and 20 pg/ml of IFN-γ and IL-10. A quantikine ELISA kit (R&D systems) was used for detection of IL-1β.

13.Statistics.

The results are reported as the mean ± SD. Statistical significance ($p < 0.05$) was determined by Student's t test and by the Mann-Whitney U test of unpaired samples. The results were analyzed using the Instat statistical program (San Diego, CA).

RESULTS

Figure 11 : SDS-PAGE of legume extracts



1. Proteins in peanut and legume extracts

The mechanisms that control cross-reactive allergic reactions between legumes are only partially understood. Most likely, cross-reacting food and environmental products have similar allergenic molecules or determinants. Thus, we first analyzed electrophoretic profiles of the PPE and several legume extracts were analyzed by SDS-PAGE (Figure 11).

The SDS-PAGE of the PPE confirmed the presence of proteins migrating at the same molecular masss as the major allergens Ara h1 (~ 63 kD) and Ara h2/Ara h3 (17-14 kD). The presence of additional proteins of molecular mass ranging from 7 to 100 kD showed that the PPE is a complex mixture of proteins, most of them having no known role in allergy.

Each of the legume extracts exhibited a complex electrophoretic pattern with bands of molecular mass ranging from about 7 to 100 kD.

2. Reactivity of anti-peanut Abs against legumes

Since protein fractions of similar molecular mass were found in peanut and other legumes, we asked to what extent peanut-specific IgE but also IgG were able to react with legume proteins. In these studies, mice were sensitized to peanut by intraperitoneal injection.

The intraperitoneal route of sensitization has been extensively studied in animal models of food allergy and was shown to efficiently induce systemic food antigen-specific Abs. Thus, C57BL/6 mice were sensitized by ip injection of PPE and CT in order to develop IgG and IgE Abs specific to peanut proteins and study their potential cross-reactivity with other legumes. The injection of 100 μg of PPE and 1 μg of CT on days 0, 5 and 10 induced

<u>Figure 12</u> : Peanut-specific IgG (panel A), IgE (panel B) and IgG-subclass (panel C) titers after IP sensitization

Mice were IP sensitized with 100 μg of PPE and 1 μg of CT on day 0, 5 and 10. OD_{415} of 1/32 diluted sera was read for analysis of peanut-specific IgE. Results are expressed as Mean +/- SD from 2 groups of 5 mice

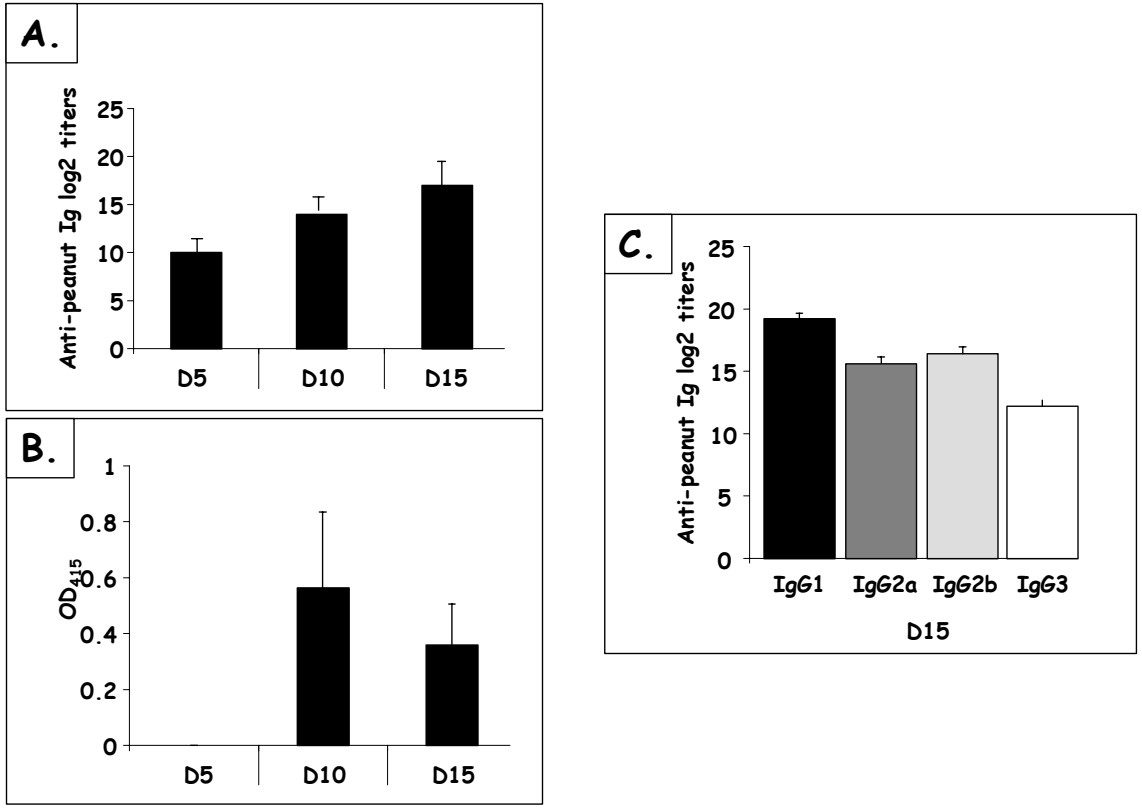


Figure 13 : Peanut-specific IgG (panel A), IgE (panel B) and IgG-subclass (panel C) titers after IP sensitization with a high dose of PPE

Mice were IP sensitized with 200 μg of PPE and 1 μg of CT on day 0, 5 and 10. OD_{415} of 1/32 diluted sera was read for analysis of peanut-specific IgE. Results are expressed as Mean +/- SD from 2 groups of 5 mice

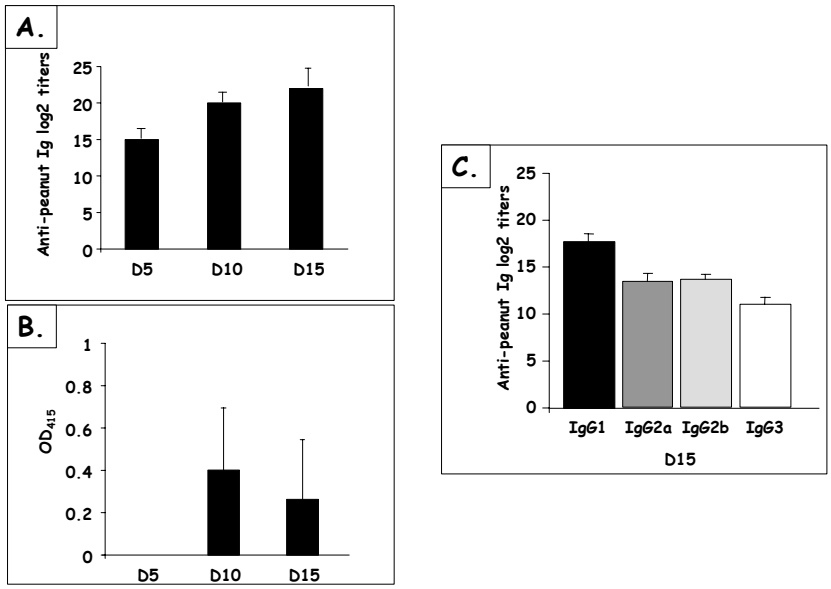
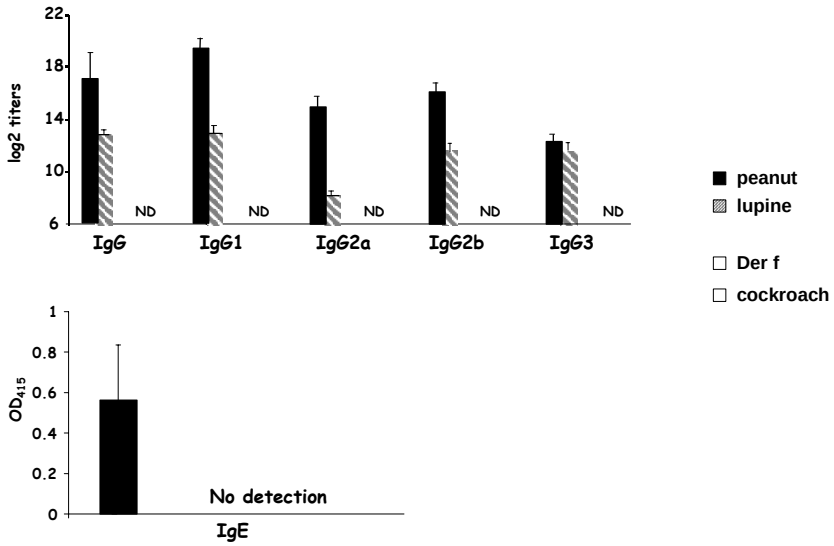


Figure 14 : IgG, IgG subclass and IgE responses to PPE in IP sensitized mice and reactivity with other legumes

Mice were IP sensitized with 100 μg of PPE and 1 μg of CT on day 0, 5 and 10. The cross-reactive potential of peanut-specific IgE and IgG Abs was analyzed 10 and 15 days, respectively. Results are expressed as Mean +/- SD from 2 groups of 5 mice



significant levels of serum peanut-specific IgG and IgE (Figure 12). Peanut-specific IgG responses reached their maximum levels on day 15 after the first sensitization. In contrast, IgE responses peaked at day 10. The IgG-subclass profile was characterized by high IgG1 levels followed by similar titers of IgG2a and IgG2b and lower levels of IgG3 Abs. Injection of higher doses of antigen (Figure 13) neither improved the magnitude of Ab response nor changed its characteristics since neither the kinetic nor the titers and profile of anti-peanut Ab responses were affected.

Based on these results, the cross-reactive potential of peanut-specific IgE and IgG Abs was analyzed on samples collected on day 10 and day 15, respectively, after ip injection of the optimal dose of 100 μg of PPE and 1 μg of CT (Figure 14). Serum IgG Abs of mice ip sensitized with peanut showed significant reaction with proteins of lupine and soybean, although at lower levels than titers of Abs that reacted with peanut. The same IgG-subclass profile was observed when sera were tested against peanut or other legumes. However, no cross-reactive IgE could be detected. Further studies including western blotting and competitive ELISA will be needed to identify legume proteins that react with serum from mice sensitized to peanut.

3. Role of mucosal routes of sensitization in peanut-specific Ab responses

Intraperitoneal injection does not represent a natural mode of sensitization to food allergens. Since this process generally occurs through contact with mucosal surfaces, we examined the development of peanut-specific Ab responses in mice sensitized by nasal or oral administration of PPE together with CT.

Figure 15 : Kinetics and levels of peanut-specific IgG (panel A), IgE (panel B) and IgG subclass (panel C) responses after IP and nasal sensitization

Mice noted IP100-CT1 (3x) were IP sensitized with 100 μg of PPE and 1 μg of CT on day 0, 5 and 10. Mice noted N100-CT1 (2x) were nasally sensitized with 100 μg of PPE and 1 μg of CT on day 0 and 7. OD_{415} of 1/32 diluted sera was read for analysis of peanut-specific IgE. Results of the IP group are expressed as Mean +/- SD from 2 groups of 5 mice (B and C) or as the mean titers of pooled sera (A). Results of the nasal group are expressed as Mean +/- SD from 9 groups of 5 mice.

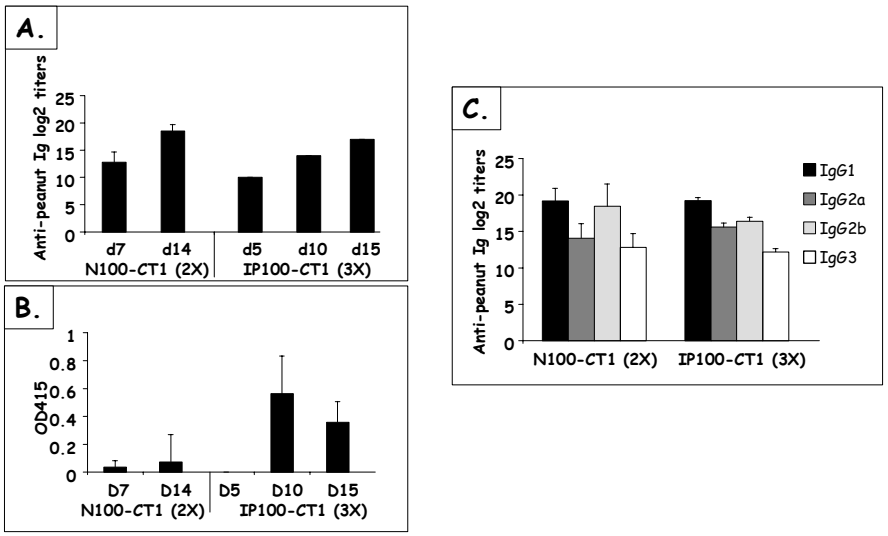
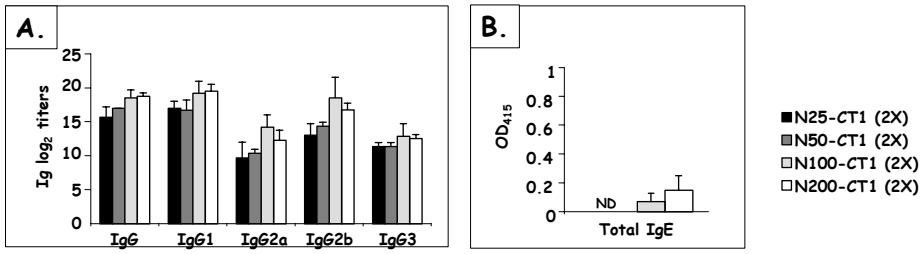


Figure 16 : Influence of nasal doses of PPE on specific IgG (panel A) and total IgE (panel B) responses

Mice were nasally sensitized with 25, 50, 100 or 200 μg of PPE and 1 μg of CT on day 0 and 7. Sera were analyzed on day 14 and OD_{415} of 1/256 diluted sera was read for analysis of total IgE. Results are expressed as Mean +/- SD from 1 group of 5 mice for each dose.



3.1. The nasal route of sensitization

Nasal administration of 100 μg of PPE and 1 μg of CT induced similar IgG Ab levels than in our ip sensitization studies described above (Figure 15A). However, the kinetics of response was different and faster after nasal sensitization. Significant IgG levels were already seen at day 7. In addition, two sensitizations seven days apart were sufficient to induce the same IgG levels than those observed after 3 sensitizations by ip injection. On the other hand, nasal delivery of 100 μg of PPE and 1 μg of CT only induced minimal peanut-specific IgE responses (Figure 15B). High levels of IgG1 and lower but significant levels of IgG2a and IgG3 were induced after nasal sensitization, as seen after ip sensitization (Figure 15C). However, IgG2b levels were significantly higher after nasal sensitization than after ip injection ($p < 0.01$). IgG levels were influenced by antigen doses, the optimal dose being 100 μg of PPE (Figure 16A). The magnitude of the IgE response was not improved when lower Ag doses were used (Figure 16B), suggesting that if the PPE contained a substance that induces a swift towards a Th1 response, it was still in sufficient amount to have the same effect when lower PPE doses were used. In addition, the magnitude of the IgE response was not improved either when higher doses of Ag or CT were used (Figure 17) .

3.2. The oral route of sensitization

The gastrointestinal track is a much harsher environment for allergens and requires higher doses of antigen or adjuvant molecules in order to optimally reach the target organs. Therefore, mice were orally sensitized to 1 mg of PPE and 15 μg of CT. The IgG levels measured at day 7 were slightly higher in nasally sensitized mice than in orally sensitized mice (Figure 18A) and were further enhanced after the second nasal sensitization whereas the

<u>Figure 17</u> : Influence of nasal doses of CT on total IgE responses

Mice were nasally sensitized with 100 μg of PPE and 1 or 5 μg of CT on day 0 and 7. Sera were analyzed on day 14 and OD_{415} of 1/256 diluted sera was read for analysis of total IgE. Results are expressed as Mean +/- SD from 1 group of 5 mice for each dose.

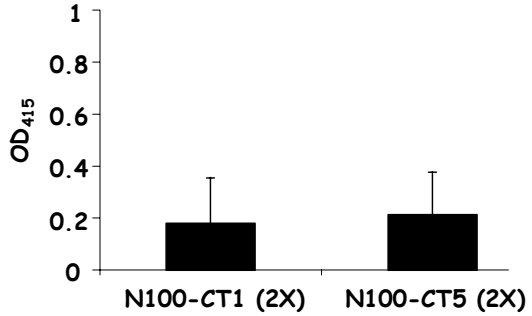


Figure 18 : Kinetics and levels of peanut-specific IgG (panel A) and IgG-subclass (panel B) responses after nasal and oral sensitization

Mice noted O1000-CT15 (2x) were orally sensitized with 1000 μg of PPE and 15 μg of CT on day 0 and 7. Mice noted N100-CT1 (2x) were nasally sensitized with 100 μg of PPE and 1 μg of CT on day 0 and 7. Results of the oral group are expressed as Mean +/- SD from 15 groups of 5 mice. Results of the nasal group are expressed as Mean +/- SD from 9 groups of 5 mice.

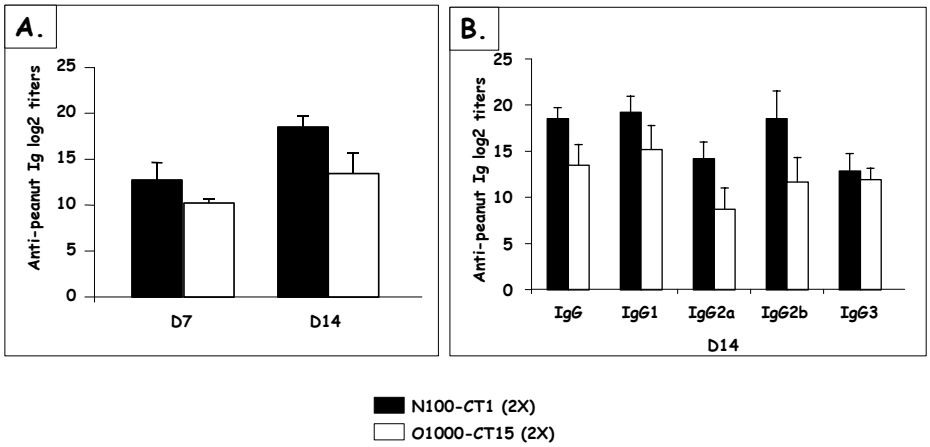
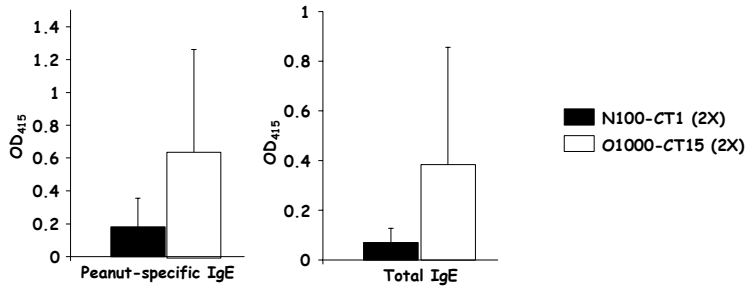


Figure 19 : Levels of total and peanut-specific IgE after nasal and oral sensitization

Mice noted O1000-CT15 (2x) were orally sensitized with 1000 μg of PPE and 15 μg of CT on day 0 and 7. Mice noted N100-CT1 (2x) were nasally sensitized with 100 μg of PPE and 1 μg of CT on day 0 and 7. Sera were analyzed on day 14 and diluted 1/62 for detection of peanut-specific IgE and 1/256 for detection of total IgE. Results of the oral group are expressed as Mean +/- SD from 15 groups of 5 mice. Results of the nasal group are expressed as Mean +/- SD from 9 groups of 5 mice.



increase was modest after the second oral sensitization. Thus, at day 14, IgG levels were significantly lower after oral sensitization than nasal sensitization (Figure 18B) with lower levels of IgG1, IgG2a and IgG2b. The levels of IgG3 remained unchanged. Nasal and oral sensitization also induced different IgG-subclass patterns with a significantly higher IgG1/IgG2a ratio in orally sensitized mice (1.8 ± 0.3 vs 1.3 ± 0.1). Oral sensitization induced a lower IgG2b response. Consistent with the higher IgG1/IgG2a ratio in orally sensitized mice, both total and peanut-specific IgE levels were significantly higher in this group (Figure 19, $P < 0.01$). Increasing the antigen dose enhanced the IgE but not the IgG response (Figure 20). Higher doses of CT lowered both the IgE and IgG3 responses (Figure 21).

Taken together, these results show that oral and nasal sensitization with PPE and CT induced distinct Ab responses at both the qualitative and quantitative levels. Nasal sensitization effectively primed for IgG Abs and induced a mixed profile of IgG-subclass Ab response. In contrast, oral sensitization effectively primed for IgE Abs and induced an overall lower IgG response characterized by a high IgG1/IgG2a ratio. It is interesting to note that unlike other IgG subclasses, the IgG3 response was not dependant on the sensitization route.

The modest IgE Ab levels and the mixed profile of IgG subclasses after nasal sensitization were unexpected since CT is well described as a mucosal adjuvant, promoting Th2 responses to co-administered protein antigens. These surprising responses seemed to be unique to peanut proteins, as sensitization with 100 μg of OVA and 1 μg of CT induced clear OVA-specific Th2-type responses with low levels of IgG2a and high levels of IgG1 and IgE regardless of the route of sensitization. Antigen-specific IgG2b and IgG3 were also significantly lower in OVA-sensitized mice than in peanut-sensitized mice (Figure 22).

Figure 20 : Influence of oral doses of PPE on specific IgG (panel A) and IgE (panel B) and total IgE (panel B) responses

Mice were orally sensitized with 1000 or 2000 μg of PPE and 15 μg of CT on day 0 and 7. Sera were analyzed on day 14 and diluted 1/62 for detection of peanut-specific IgE and 1/256 for detection of total IgE. Results are expressed as Mean +/- SD from 5 group of 5 mice for each dose.

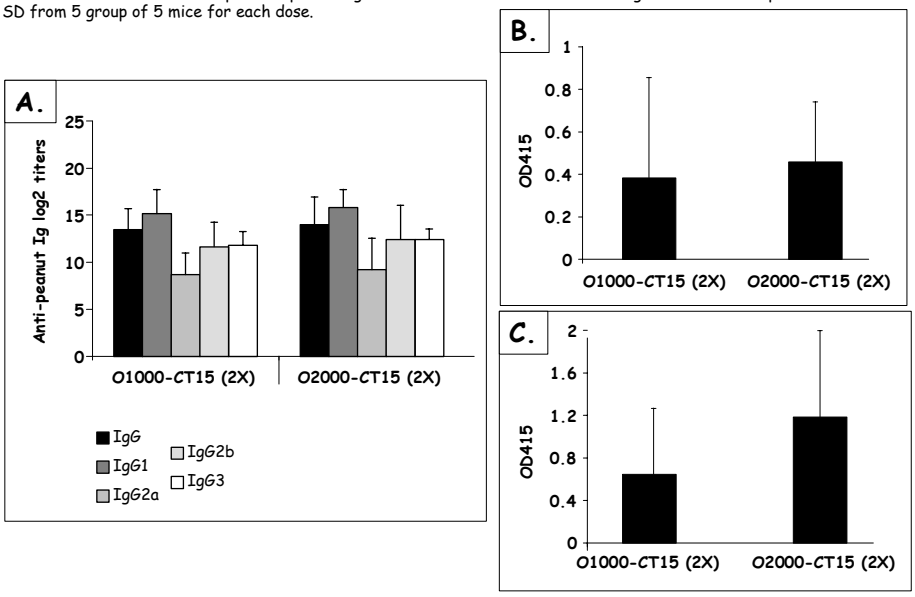
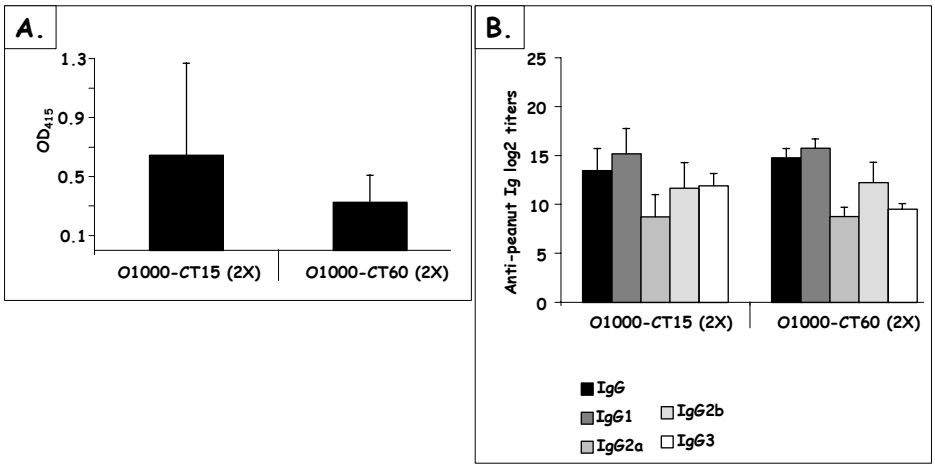


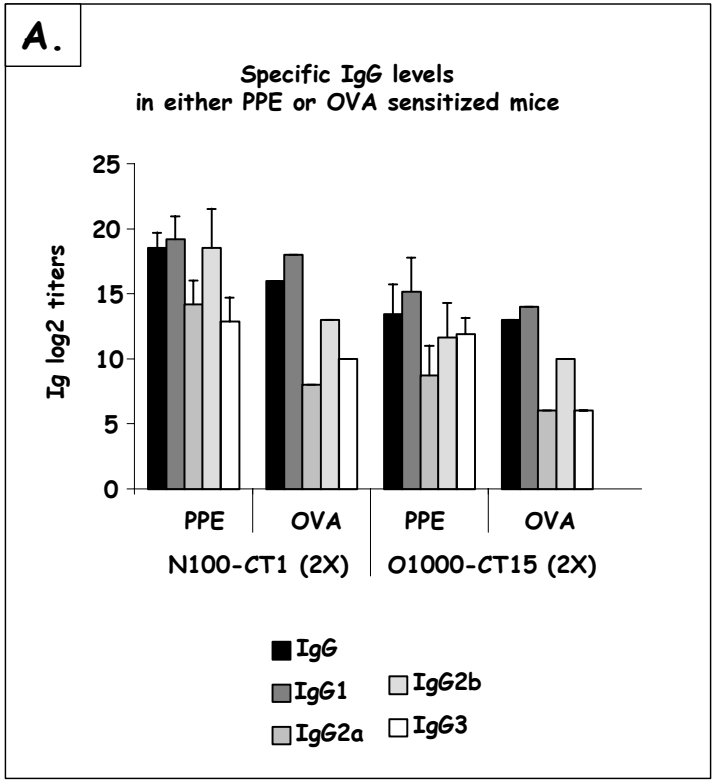
Figure 21 : Influence of oral doses of CT on total IgE (panel A) and specific IgG (panel B) responses

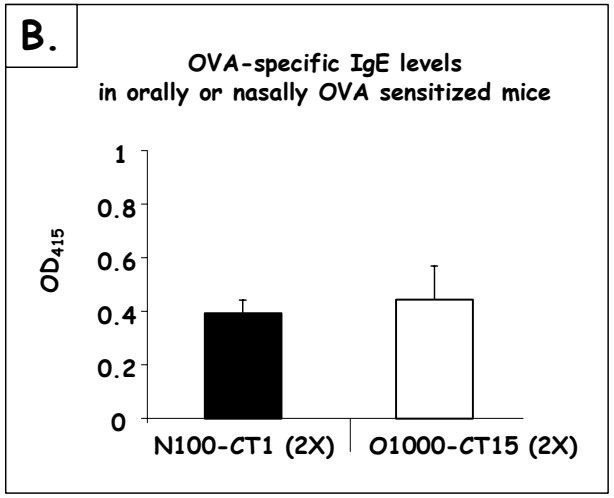
Mice were orally sensitized with 1000 μg of PPE and 15 or 60 μg of CT on day 0 and 7. Sera were analyzed on day 14 and OD_{415} of 1/256 diluted sera was read for analysis of total IgE. Results are expressed as Mean +/- SD from 1 group of 5 mice for each dose.



<u>Figure 22</u> : Influence of the nature of the antigen on the specific IgG (panel A) and IgE (panel B) response in nasally or orally sensitized mice

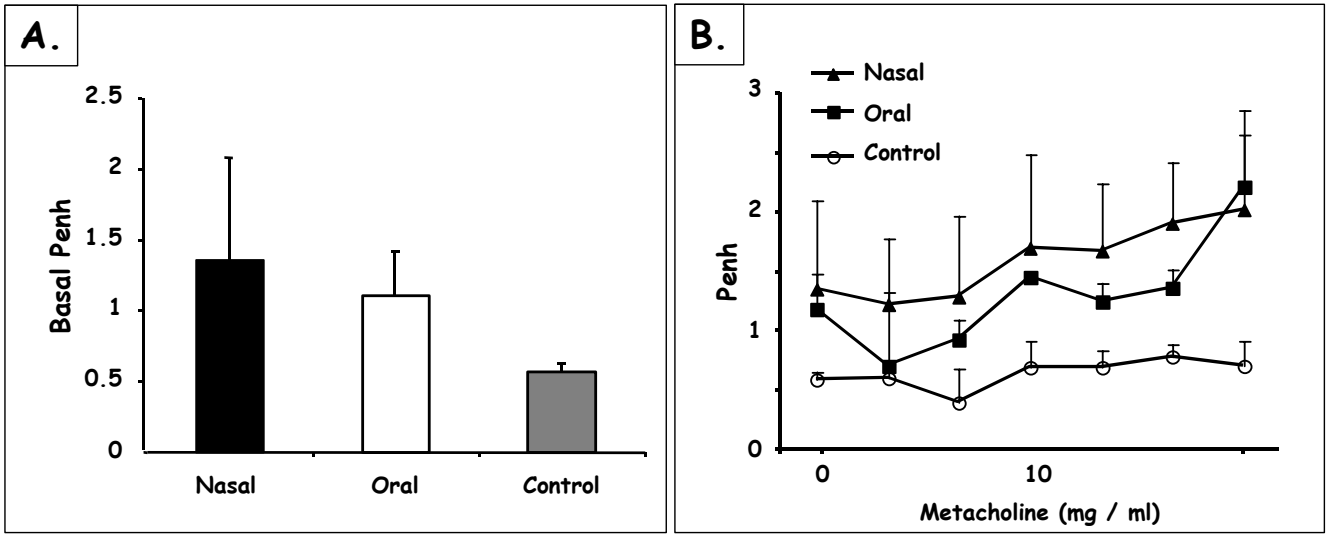
Mice noted O1000-CT15 (2x) were orally sensitized with 1000 μg of either PPE or OVA and 15 μg of CT on day 0 and 7. Mice noted N100-CT1 (2x) were nasally sensitized with 100 μg of either PPE or OVA and 1 μg of CT on day 0 and 7. Sera were analyzed on day 14 and OD_{415} of 1/32 diluted sera was read for analysis of specific IgE . Results are expressed as Mean +/- SD from 1 groups of 5 mice for each condition.





<u>Figure 23</u> : Airway hyper-reactivity after nasal challenge of orally or nasally sensitized mice

Mice were orally or nasally sensitized with PPE and CT on day 0 and 7 and nasally challenged with PPE on day 15 and 16. (Panel A) Baseline Penh was measured six hours after the last challenge. (PanelB) AHR to metacholine: Penh values were measured every minute over 7 minutes. Controls included sham-sensitized and sham-sensitized mice. Values are expressed as mean ± SD of four mice.



4. Influence of the mucosal route of sensitization on airway responses to secondary exposure to peanut

We next asked whether the distinct immune status observed after oral or nasal sensitization could induce distinct responses during the reactivity phase following secondary exposure to the antigen. For this purpose, we characterized the lung cell composition in both groups of mice before and after nasal peanut challenge and we evaluated their airway hyperreactivity, as a clinical symptom of asthma induced by impaired lung functions.

4.1. Both nasally and orally sensitized mice experienced AHR after nasal peanut challenge

The Penh values of mice orally or nasally sensitized were measured 6 h after the last peanut nasal challenge (Figure 23). Mice orally or nasally sensitized exhibited AHR with baseline Penh values of 1.1 ± 0.31 and 1.36 ± 0.72, respectively, which were significantly higher than Penh values of control mice (0.57 ± 0.06). In addition, both orally and nasally sensitized mice showed increased Penh responses to metacholine challenge. Mice sensitized nasally exhibited higher Penh than their counterparts sensitized orally although the difference failed to reach statistical difference (Figure 23).

<u>Figure 24</u>: Cell recruitment in lung and BALF of mice orally or nasally sensitized with peanut

Mice were sensitized by the nasal (5 µl per nostril) or oral route with PPE and CT as adjuvant on days 0 and 7. (Panel A) Lung tissue (top) and BALF (bottom) were collected 10 days after the last sensitization and before challenge. Histology sections were stained with hematoxylin and eosin (40x magnification) and cytospin preparations of BALF were stained with Giemsa (200x magnification). (Panel B) Lung inflammation scores on histology sections. The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate)

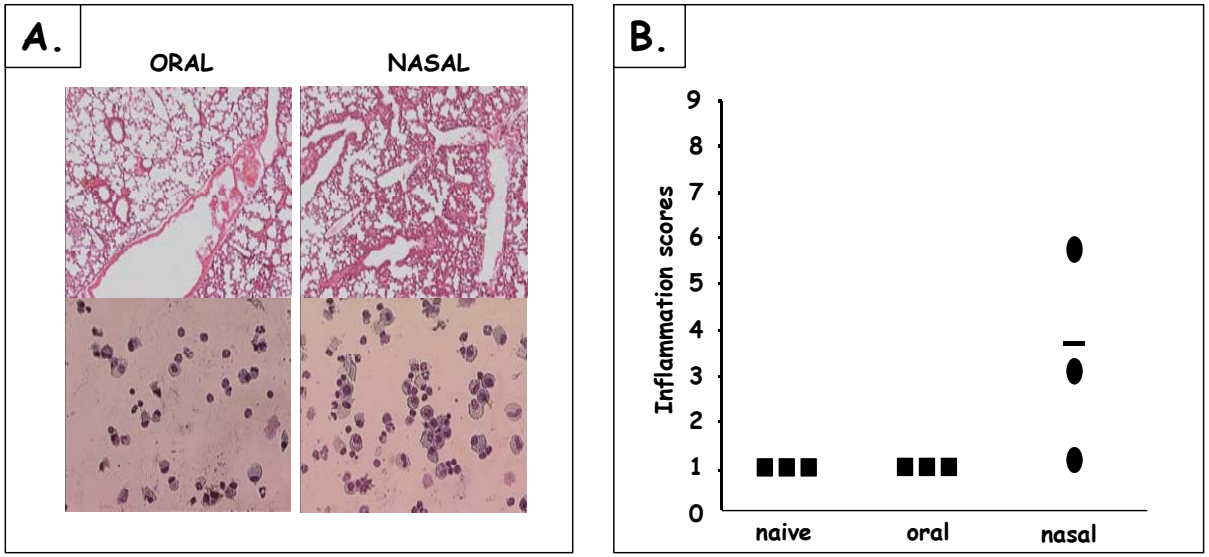
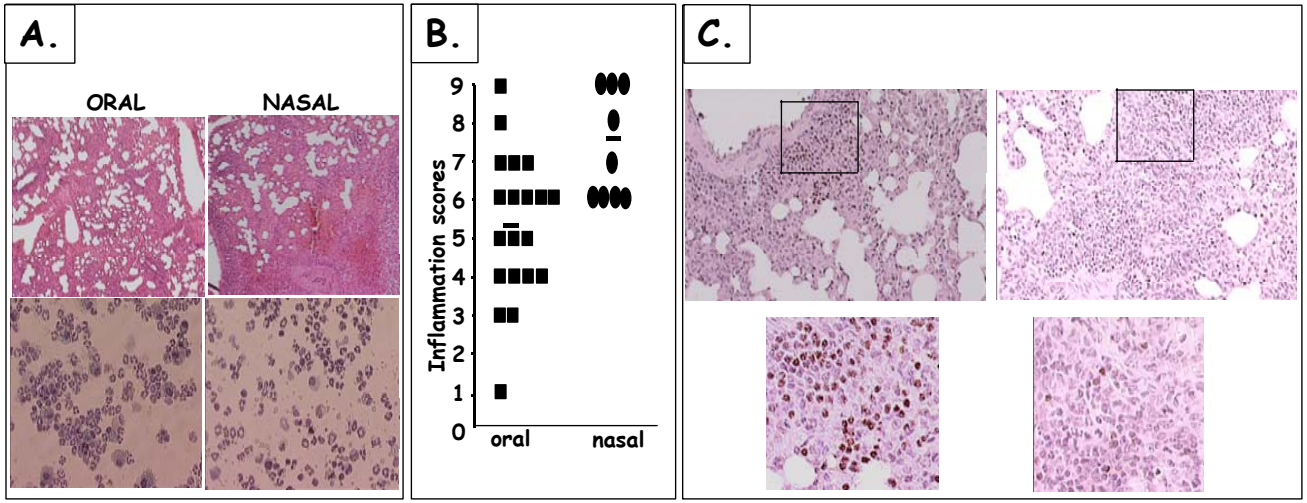


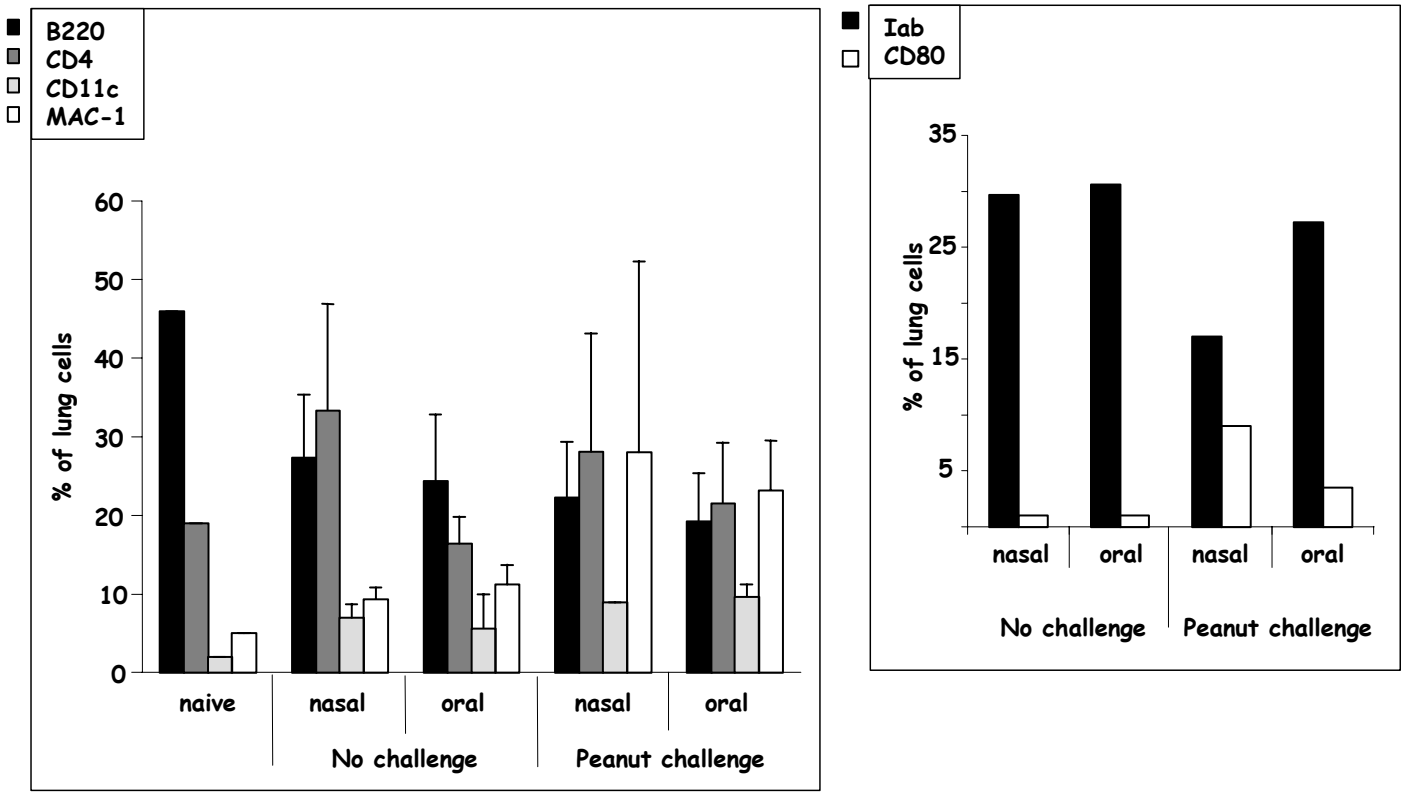
Figure 25: Cell recruitment in lung and BALF after nasal peanut challenge

Mice were sensitized by the nasal (5 μl per nostril) or oral route with PPE and CT as adjuvant on days 0 and 7. (Panel A) Lung tissue (top) and BALF (bottom) were collected on day 17 after nasal peanut challenges on days 15 and 16. Histology sections were stained with hematoxylin and eosin (40x magnification) and cytospin preparations of BALF were stained with Giemsa (200x magnification). The BALF from nasally sensitized mice depicted in this panel were diluted 10-fold before the cytospin. (Panel B) Lung inflammation scores on histology sections. The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate). (Panel C) Lung eosinophilia after nasal challenge of mice orally or nasally sensitized with peanut. The eosinophils were visualized by peroxidase staining of histological sections pretreated with KCN. Magnification: top (100x) and bottom (200x).



<u>Figure 26</u>: Effect of the nasal peanut challenge on the phenotype of lung mononuclear cells

Mice were sensitized by the nasal (5 μl per nostril) or oral route with PPE and CT as adjuvant on days 0 and 7. Lung tissues were collected on day 17 from mice orally or nasally sensitized with PPE and either not challenged or nasally challenged with PPE on days 15 and 16. After collagenase digestion of the tissues and purification on discontinuous Percoll gradient, the cells were collected at the 20-75% interface and analyzed by flow cytometry.



4.2. Nasal and oral sensitization promoted distinct lung inflammatory responses to secondary nasal antigen exposure.

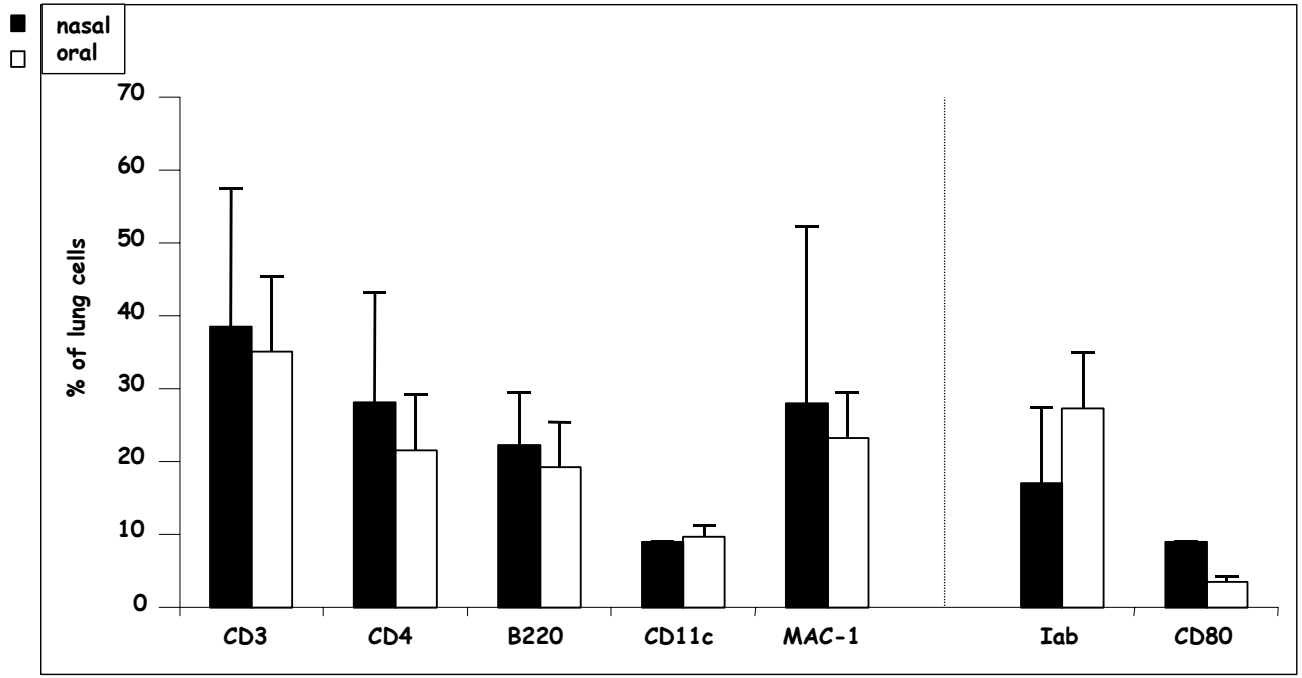
When compared to naïve mice, lungs of mice sensitized orally showed no sign of inflammation ten days after the last immunization (mean score 1). In contrast, lungs of mice nasally sensitized to peanut and CT exhibited a moderate inflammation (mean score 3.3), although nasal sensitization consisted in the administration of 5 μL per nostril, a volume that does not allow for antigen diffusing into the lung. Furthermore, the cell density was higher in BALF of nasally sensitized mice (≈ 20 x 10^4 cells/ml) when compared with orally sensitized mice (≈ 6 x 10^4 cells/ml). BALF of both groups of mice contained mostly mononuclear cells and consisted of small lymphocytes and few macrophages in orally sensitized mice whereas BALF of those nasally sensitized contained bigger size cells that could be either activated lymphocytes or macrophages (Figure 24).

After nasal peanut challenge, mice sensitized by either the oral or the nasal route showed significant lung inflammations when compared to unchallenged controls (Figure 25). Lung inflammation was significantly higher in nasally sensitized mice than in orally sensitized mice (mean inflammation scores of 7.3 and 5.1, respectively). Furthermore, BALF of both groups showed a drastic change of cell composition after the nasal peanut challenge with an important recruitment of polymorphonuclear cells. The cell density in BALF of nasally sensitized mice were more than 10 fold higher (≈ 400 x 10^4 cells/ml) than that seen in BALF of in orally sensitized mice (≈ 30 x 10^4 cells/ml).

The peroxidase staining of tissue sections treated with potassium cyanide showed the recruitment of eosinophils in the lung of mice that received peanut plus CT, by the oral route.

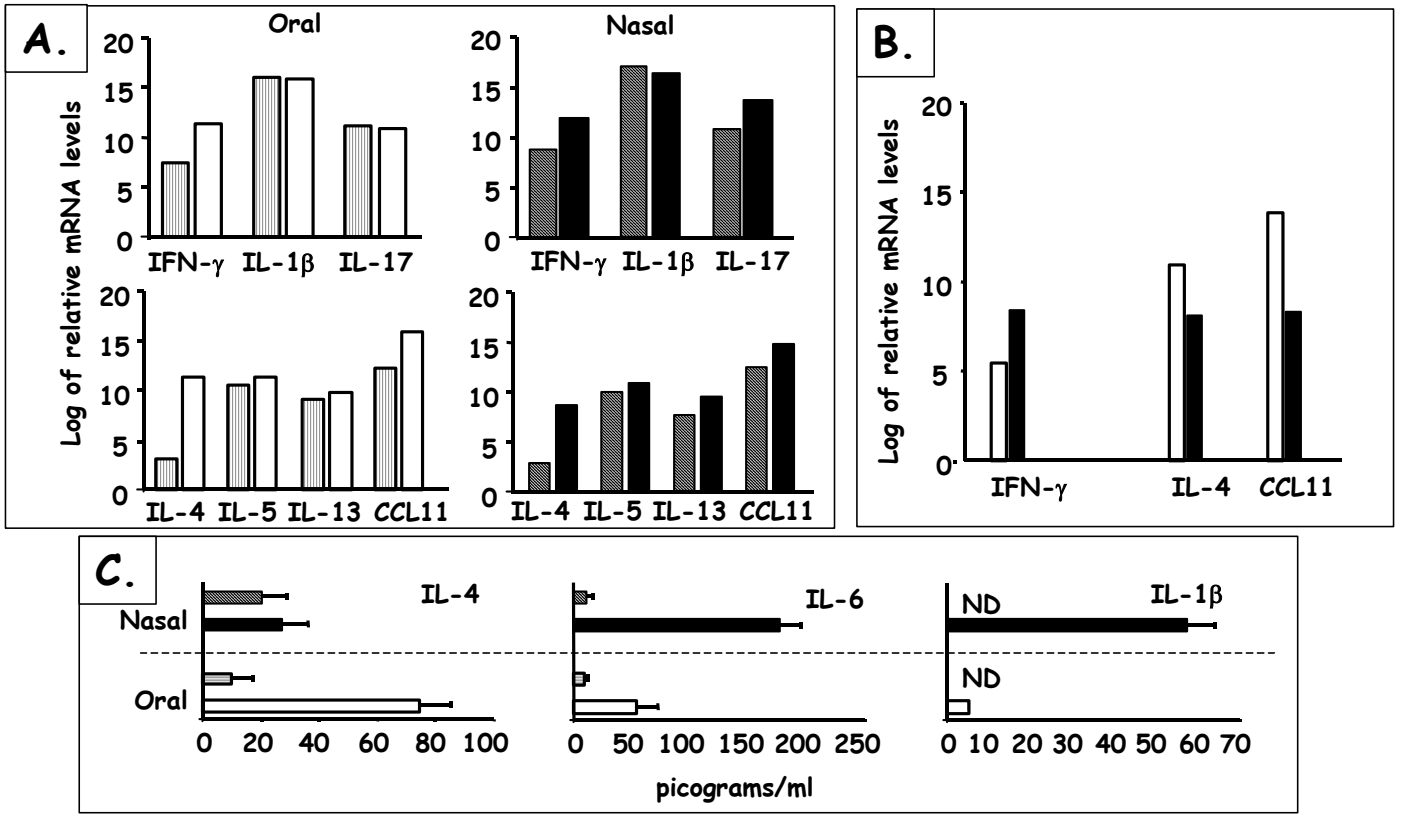
Figure 27: Influence of the route of sensitization on the phenotype of lung mononuclear cells

Mice were sensitized by the nasal (5 µl per nostril) or oral route with PPE and CT as adjuvant on days 0 and 7. Lung tissues were collected on day 17 from mice orally or nasally sensitized with PPE and nasally challenged with PPE on days 15 and 16. After collagenase digestion of the tissues and purification on discontinuous Percoll gradient, the cells were collected at the 20-75% interface and analyzed by flow cytometry.



<u>Figure 28</u>: Cytokine responses in lungs and BALF of mice sensitized orally or nasally with peanut

(Panel A) Relative cytokine mRNA expression in whole lung tissue before (dotted pattern) or after (open or solid pattern) nasal peanut challenge. (Panel B) Relative cytokine mRNA expression by $CD4^+$ lung T cells collected after nasal peanut challenge of mice sensitized by the oral (open bars) or nasal (solid bars) routes. Results are expressed as $20 - (CP_{cytokine} - CP_{\beta\text{-actin}})$ with CP = crossing points. Differences of crossing points above two cycles are considered significant. The results are representative of three separate experiments. (Panel C) Cytokine secretion in BALF collected before (dotted pattern) or after (open or solid pattern) nasal peanut challenge. Cytokine levels were determined by ELISA. The levels of IL-5, IL-10, IL-13, TNF-α and IFN-γ were below detection (ND).



In contrast, mice sensitized nasally showed no or low eosinophilia but exhibited important lung neutrophilia.

4.3. Phenotype of lung mononuclear cells after nasal peanut challenge

One week after the last sensitization and before any challenge was performed, mononuclear cells of the lungs were mainly B and T cells in both nasally or orally sensitized mice (Figure 26). Macrophages (i.e., $CD11b^+$ cells) and $CD11c^+$ cells represented less than 20% of total lung mononuclear cells in both groups of mice. Interestingly, the frequency of $CD11c^+$ and $MAC1^+$ cells was still significantly higher than that seen in the lungs of naïve mice. The frequency of B220 positive cells (B cells) was also significantly different in naive mice compared to that of orally or nasally sensitized mice, before challenge was performed. Nasal challenge of either orally and nasally sensitized mice induced the recruitment of high numbers of macrophages in the lungs. Consequently, the nasal peanut challenge decreased the percentage of B and T cells compared to their frequency in sensitized but not challenged mice.

The frequency of lung CD3-, CD4- and B220-positive cells was higher in mice nasally sensitized compare to groups sensitized orally (Figure 27). This result suggests that the mechanisms of recruitment of B and T cells in the lung differ according to the route of sensitization. Innate factors, including neutrophils are known to influence B and T cell recruitment. Consistent with the massive recruitment of neutrophils in the lung of nasally sensitized mice, the low IgG1/IgG2a ration and the lack of IgE induced by nasal sensitization, we hypothesized that B and T cells were recruited via signals provided by neutrophils. In contrast, one could hypothesize that other recruitment signals for B and T cells occur in orally

sensitized mice, which developed high IgE responses and lung eosinophilia. Of interest, 27 ± 7 % of cells recruited in the lung of orally sensitized mice expressed high levels of MHC class II molecules (I-A^b). On the other hand, only 17 ± 9 % of cells recruited after nasal challenge expressed high levels of MHC class II molecules in the lung of nasally sensitized mice. This further suggests that nasal sensitization induced the recruitment of non-activated cells via an innate mechanism. The expression of CD80 in nasally sensitized mice may reflect the recruitment of macrophages more than their activation status.

5. Distinct Th cell and inflammatory cytokine responses in mice sensitized by the oral or nasal routes

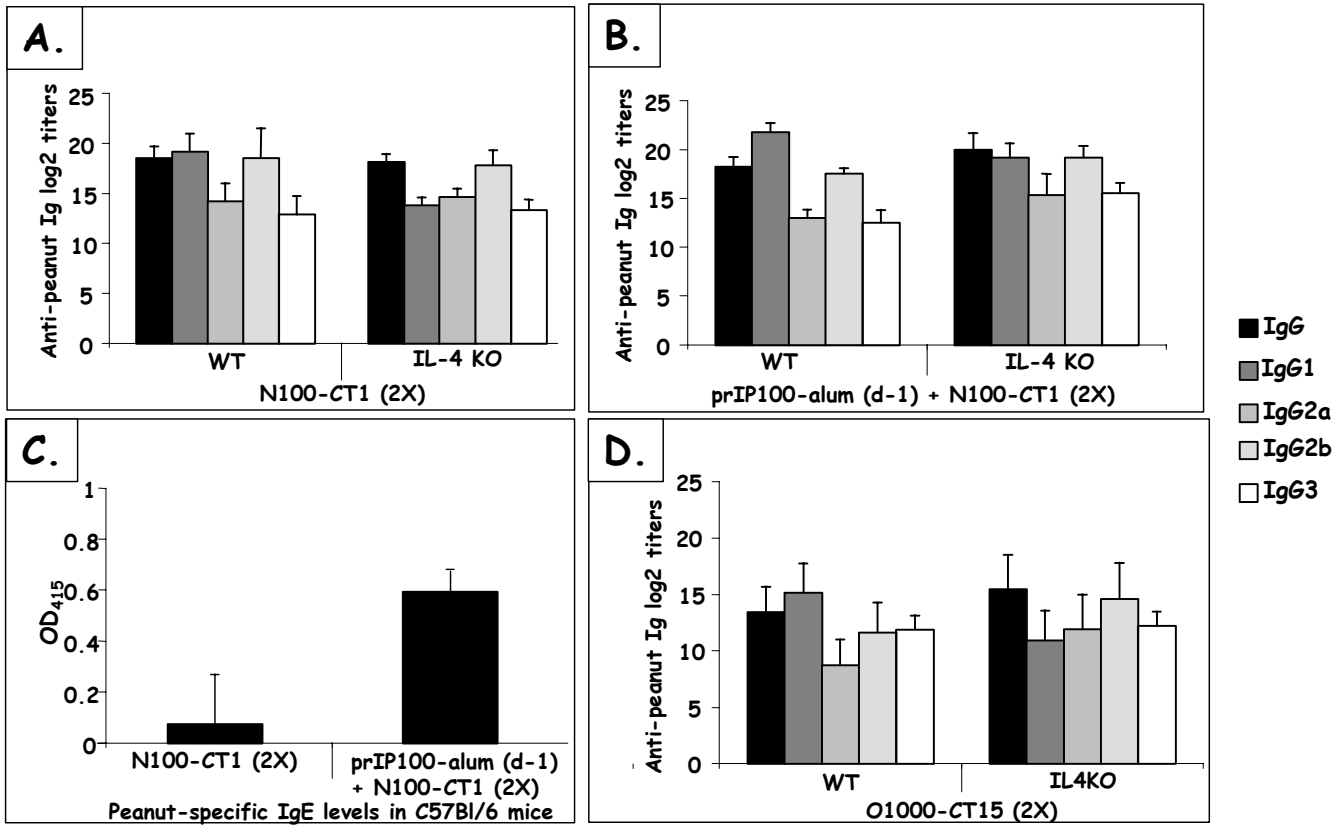
5.1. Nasal peanut challenge induces higher Th2-type cytokine responses in the lungs of orally sensitized mice

Before nasal challenge, nasally and orally sensitized mice exhibited similar levels of both Th1 and Th2 cytokines. High levels of IL-1β mRNA followed by CCL-11 and IL-17 and lower levels of IL-5, IL-13, IFN-γ and IL-4 were measured in the lungs of both groups (Figure 28). Nasal challenges with peanut only slightly increased IL-5, IL-13 and IL-1β mRNA. In contrast, a significant increase of IL-4, IFN-γ, and CCL-11 mRNA was detected after nasal challenge of mice sensitized by either the oral or nasal routes. Interestingly, the increase of the Th2-associated cytokines IL-4 and CCL-11 was higher in orally sensitized mice (ΔCP oral/nasal of 2.33 and 1.3, respectively), whereas the increase of Th1-associated cytokine IL-17 was higher in nasally sensitized mice (ΔCP nasal/oral = 2.27).

Since mRNA for the $CD4^+$ T-associated cytokines IL-4, IFN-γ and CCL-11 were the most up-regulated after nasal peanut challenge, we next analyzed the contribution of $CD4^+$ T

Figure 29: Plasma Ab responses to oral or nasal peanut sensitization in WT and IL-4 KO mice

Mice noted O1000-CT15 (2x) were orally sensitized with 1000 μg of PPE and 15 μg of CT on day 0 and 7. Mice noted N100-CT1 (2x) were nasally sensitized with 100 μg of PPE and 1 μg of CT on day 0 and 7. Mice noted prIP100-alum (d-1) + N100-CT1 (2X) had an ip priming (100 μg of PPE in the presence of alum) one day before the beginning on the nasal sensitization protocol. Sera were analyzed on day 14. Results of are expressed as Mean +/- SD from 1 group of 5 mice for each condition.



cells to lung cytokine and chemokine responses. Purified lung $CD4^+$ T cells from nasally sensitized mice expressed significantly higher mRNA levels of the Th1-associated cytokine IFN-γ. On the other hand, higher mRNA levels of the Th2-associated cytokines IL-4 and CCL-11 were seen in lung $CD4^+$ T cells from orally sensitized mice.

Before challenge, the BALF of mice orally or nasally sensitized to peanut showed low and similar levels of IL-4 and IL-6 while IL-1β levels were below detection (Figure 28). Nasal peanut challenge significantly increased IL-4 secretion in BALF of orally but not nasally sensitized mice. In contrast, only nasally sensitized mice showed significantly increased IL-1β and IL-6 secretion in BALF upon nasal peanut challenge (Figure 28).

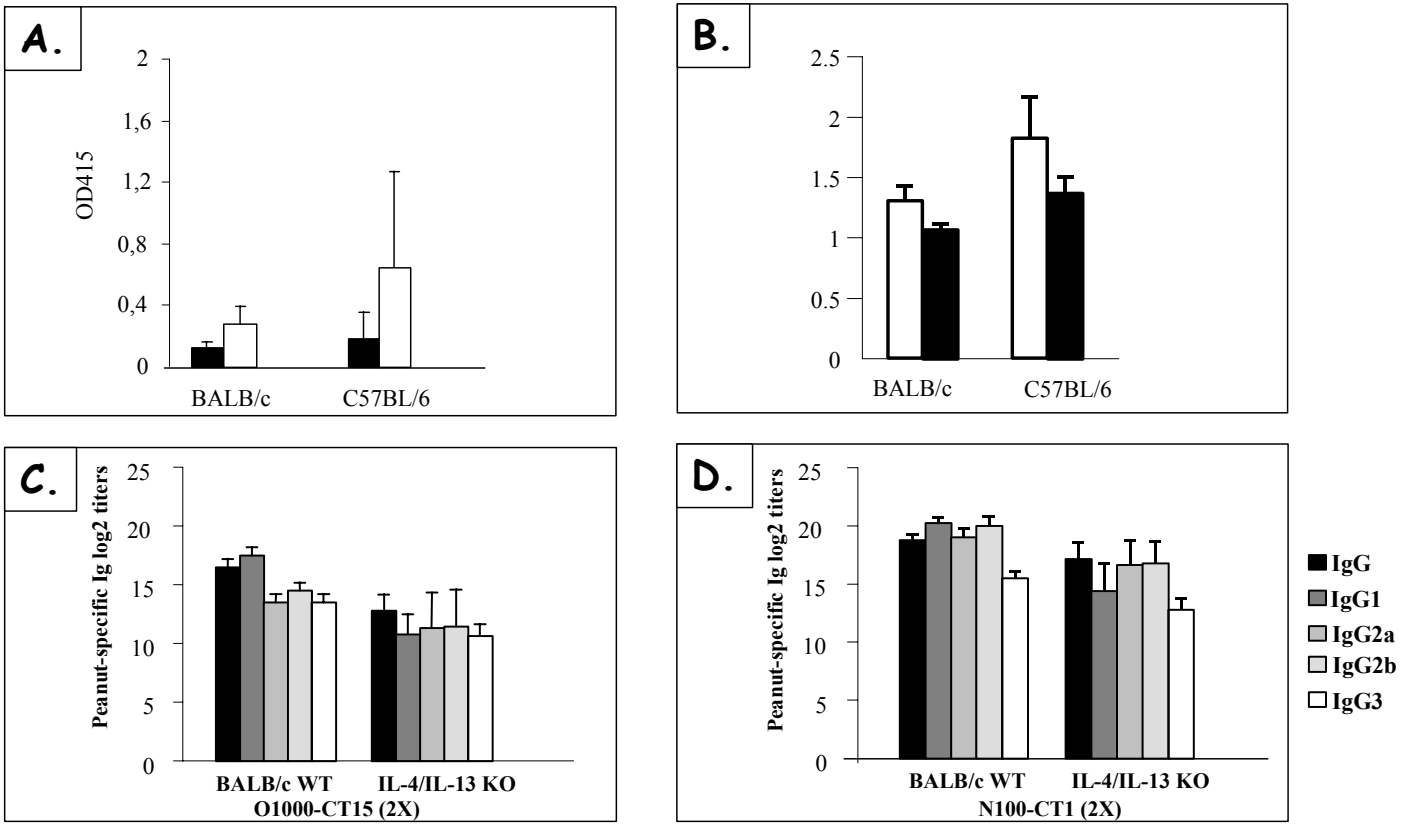
5.2. Role of Th1 and Th2 cytokines in the Ab response to peanut sensitization

5.2.1. Antibody responses in C57BL/6 WT and IL-4 KO mice

Consistent with the role of IL-4 in IgE class switching, no peanut-specific or total IgE Ab could be detected in IL-4 KO mice after either oral or nasal sensitization. Our results show that IL-4 played a role in IgG1 responses of both nasally and orally sensitized mice, since IgG1 levels were significantly lower in IL-4 KO mice regardless of the route of sensitization (Figure 29 A and D). The production of IgG2a, IgG2b and IgG3 was not altered in IL-4 KO mice nasally sensitized (Figure 29 A). In contrast, IgG1 decrease in C57BL/6 IL-4 KO mice sensitized by the oral route was compensated by an increased IgG2a and IgG2b response (Figure 29 D). This result suggests that IL-4 plays a more important role in IgG Ab responses in the model of oral sensitization than in the model of nasal sensitization.

<u>Figure 30</u>: Plasma Ab responses to oral or nasal peanut sensitization in WT and IL-4/IL-13 double KO mice.

Total IgE levels (A) and IgG1/IgG2a ratio (B) in orally (white) or nasally (black) peanut sensitized BALB/c and C57BL/6 mice. IgG-subclass responses to oral (C) or nasal (D) peanut sensitization in BALB/c WT and IL-4/IL-13 double KO mice. Results are expressed as Mean +/- SD from 1 group of 5 mice for each condition



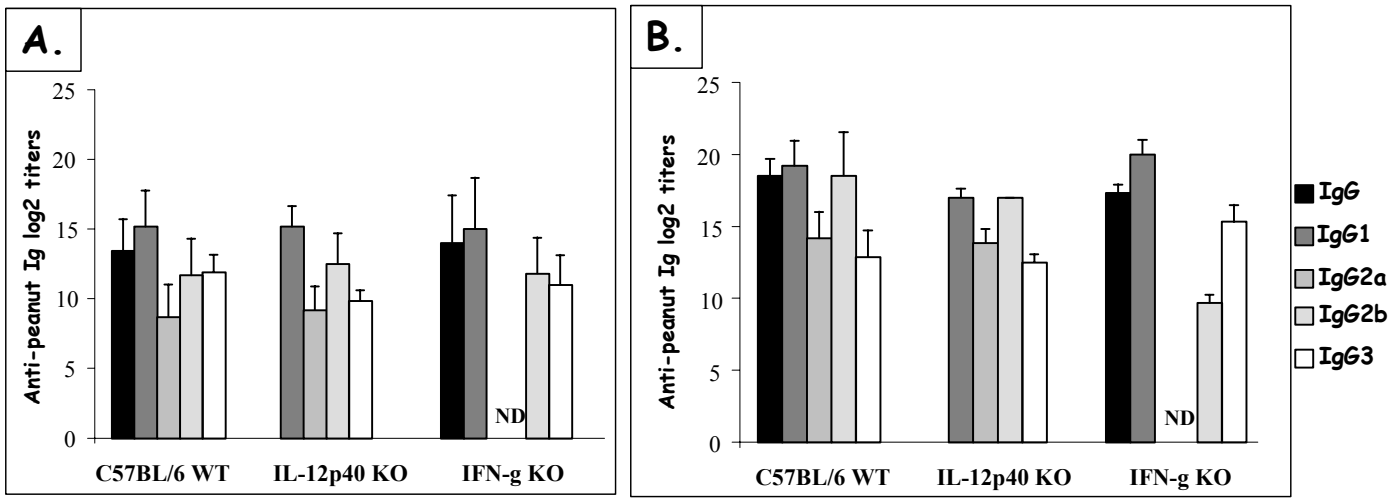
Additional groups of mice were intraperitoneally administered 100 μg of PPE in the presence of the Th2 adjuvant, alum, one day before the beginning of the usual nasal sensitization protocol, which consisted of two administrations of 100 μg of PPE plus 1 μg of CT (Figure 29 B and C). Mice intraperitoneally primed with PPE in alum exhibited a Th2 antibody profile with a high IgG1/IgG2a ratio, which contrasted with the lower IgG1/IgG2a ratio of unprimed mice. It was interesting to note that Ab compensation did occur in nasally sensitized IL-4 KO mice that were previously ip primed in the presence of the Th2 adjuvant alum.

5.2.2. Antibody responses to peanut sensitization in BALB/c WT and IL-4/IL-13 double KO mice

As IL-4/IL-13 KO mice from the C57BL/6 background were not available, we used BALB/c mice in order to investigate the effect of IL-4 and IL-13 deficiency in our models. We first asked whether distinct immune responses to oral or nasal peanut were induced in BALB/c mice, as they were previously described in C57BL/6 mice. Figure 30A shows that our protocols of oral and nasal sensitization induced lower IgE levels in BALB/c mice than in C57BL/6 mice. This was unlikely the result of an unsufficient immune response in BALB/c mice as the extent of the total peanut-specific IgG response was similar in both strains (Figures 29 and 30). Similar to what was observed in C57BL/6 mice, peanut-specific IgE levels were significantly higher after oral sensitization of BALB/c mice compare to nasally sensitized mice. In addition, the difference observed between orally and nasally sensitized C57BL/6 mice in their IgG1 / IgG2a ratio was maintained in BALB/c mice (Figure 30B). Thus, IL4/IL-13 KO mice from the BALB/c background were suitable in our study.

<u>Figure 31</u>: Plasma IgG-subclass responses to oral (A) or nasal (B) peanut sensitization in C57BL/6 WT, IL-12p40 KO and IFN-γ KO mice

Orally sensitized mice were given 1000 mg PPE and 15 mg CT on day 0 and 7. Nasally sensitized mice were given 100 mg PPE and 1 mg CT on day 0 and 7. Sera were analyzed on day 14. Results of are expressed as Mean +/- SD from 1 group of 5 mice for each condition (WT: wild-type)



As for IL-4 KO mice, no total or peanut-specific IgE were detected in double KO mice. Figures 30 C and 30 D shows that unlike in IL-4 KO mice, there was a decrease in the overall IgG response after both oral and nasal sensitization of double KO mice compare to their WT counterparts. Consequently, there was a reduction in all subclass levels. However, IgG1 levels diminished most dramatically, making the IgG1/IgG2a ratio significantly lower in double KO mice compare to WT mice (0,99 +/- 0,17 and 1,3 +/- 0,12 respectively, in orally sensitized mice; 0,86 +/- 0,06 and 1,07 +/- 0,05 respectively, in nasally sensitized mice). Thus, compensatory mechanisms made it possible for the Th1 component to develop in IL-4/IL-13 KO mice.

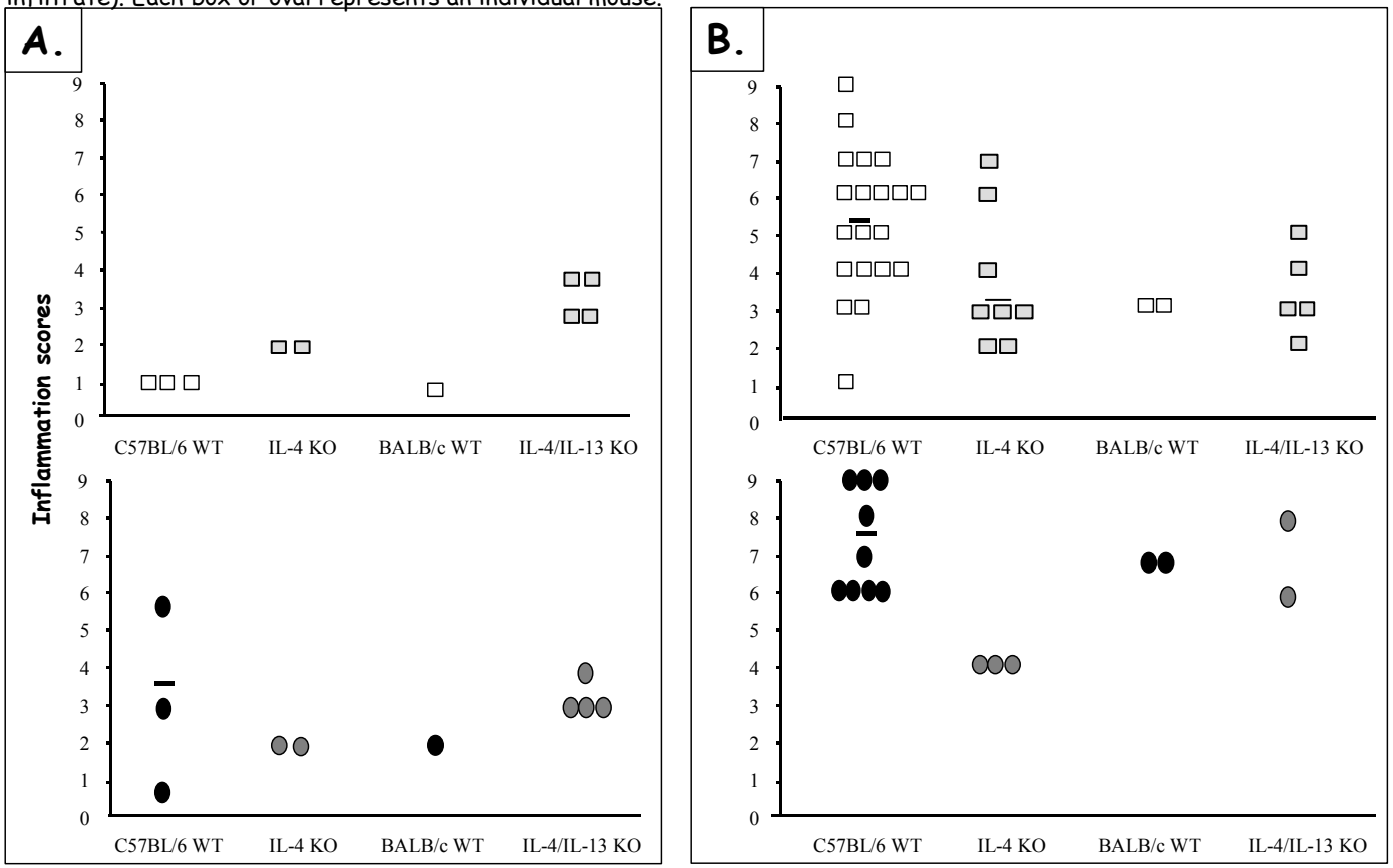
5.2.3. Antibody responses in C57BL/6 WT, IL-12p40 KO and IFN-γ KO mice

Unexpectedly, there was no increase in IgE levels of either IL-12p40 KO and IFN-γ KO mice compare to their WT counterparts.

Figure 31A shows that orally sensitized IL-12p40 KO mice displayed lower levels of peanut-specific IgG3 than WT mice. It is interesting to note that peanut-specific IgG3 levels also decreased in WT mice orally sensitized with a high dose of CT, whereas levels of other IgG-subclasses remained unchanged (Figure 21). As CT has been shown to reduce the expression of IL-12 receptor [187], both results in WT and orally sensitized IL-12p40 KO mice suggested that peanut-specific IgG3 levels were dependent of IL-12 in orally sensitized mice. Figure 31B shows that nasally sensitized IL-12p40 KO mice had lower levels of peanut-specific IgG1 and IgG2b. As peanut-specific IgG3 levels were unaffected, it is possible that nasal sensitization induced a Th1 environment able to compensate for the absence or the reduced levels of IL-12.

Figure 32: Lung inflammatory responses to peanut in WT, IL-4 KO and IL-4/IL-13 double KO mice.

Mice were orally (squares) or nasally (circles) sensitized by administration of PPE and CT on days 0 and 7. Lung tissue was collected on day 17 before (A) or after peanut nasal challenges on days 15 and 16 (B). The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate). Each box or oval represents an individual mouse.



Unlike in IL-12p40 KO mice, IgG2a levels were below detection in both orally sensitized and nasally sensitized IFN-γ KO mice (Figure 31), confirming that IFN-γ KO mice display a complete loss of the Th1 component. Levels of other subclasses remained unchanged in orally sensitized IFN-γ KO mice, while peanut-specific IgG3 increased and peanut-specific IgG2b decreased in nasally sensitized IFN-γ KO mice. Thus, IFN-γ differentially regulates the levels of IgG sublasses in orally and nasally sensitized mice. Involved only in IgG2a production in orally sensitized mice, it modulated the levels of all Abs measured except IgG1 in nasally sensitized mice. In particular, the high levels of IgG2b observed in WT mice after nasal but not oral sensitization may be dependant on IFN-γ.

5.3. Role of Th1 and Th2 cytokines in lung inflammatory responses to peanut challenge

5.3.1. Lung inflammatory responses in IL-4 KO and IL-4/IL-13 double KO mice

As described previously, lungs of OS WT mice showed no sign of inflammation before challenge, while NS WT mice exhibited a moderate inflammation. Inflammation scores were not significantly different in IL-4 KO mice compare to WT mice (Figure 32A). In contrast, lung cell recruitment in unchallenged IL-4 / IL-13 KO mice was higher than that of unchallenged WT mice, and differences between NS and OS double KO mice were attenuated (Figure 32A).

After challenge (Figure 32B), there was a reduction of inflammation in both orally and nasally sensitized IL-4 KO mice compare to their WT counterparts. But such attenuation was not observed in double KO mice. Thus, while partial abolition of the Th2 pathway

Figure 33 : Lung mononuclear cell phenotype after nasal challenge of BALB/c WT (A) and IL-4/IL-13 KO (B) mice

BALB/c mice were immunized by the nasal (5 ml per nostril) or oral route with PPE and CT as adjuvant on days 0 and 7. Lung tissues were collected on day 17 from mice orally or nasally immunized with PPE and either not challenged or nasally challenged with PPE on days 15 and 16. After collagenase digestion of the tissues and purification on discontinuous Percoll gradient, the cells were collected at the 20-75% interface and analyzed by flow cytometry.

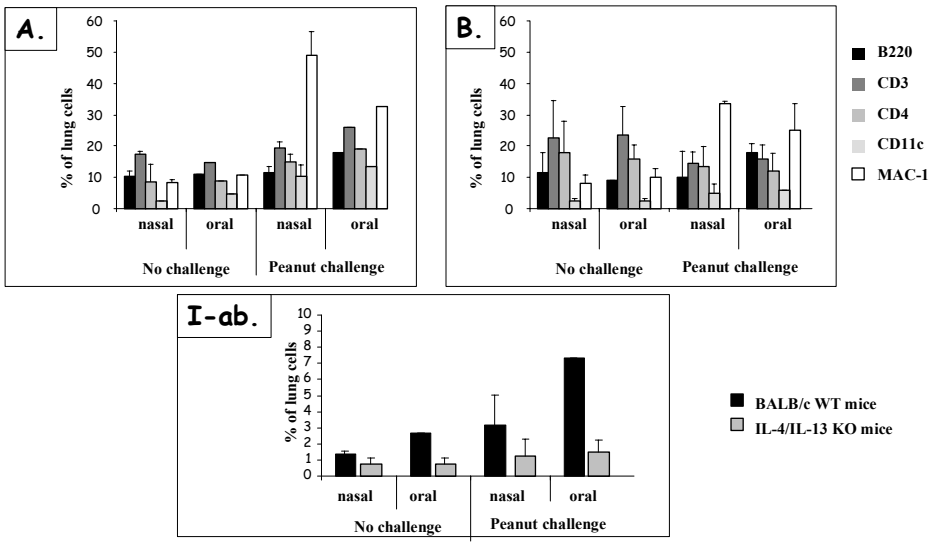
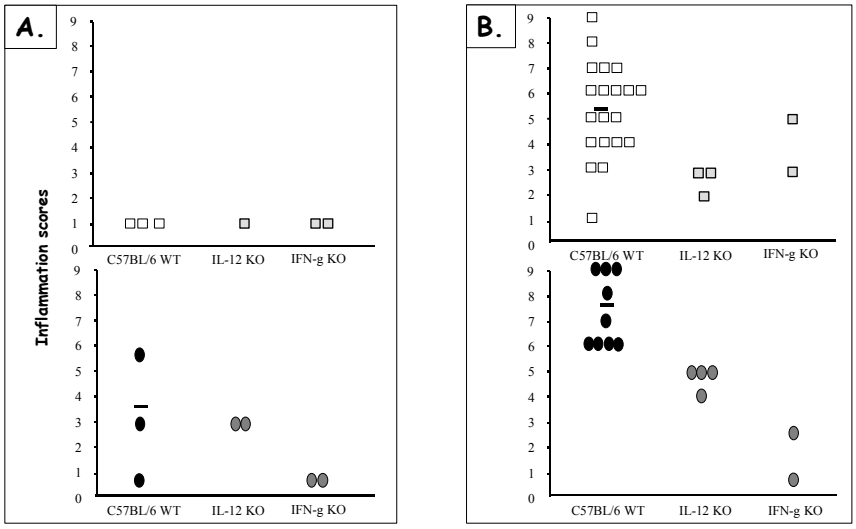


Figure 34: Lung inflammatory responses to peanut in WT, IL-12 KO and IFN-γ KO mice.

Mice were orally (squares) or nasally (circles) sensitized by administration of PPE and CT on days 0 and 7. Lung tissue was collected on day 17 before (A) or after peanut nasal challenges on days 15 and 16 (B). The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate). Each box or oval represents an individual mouse.



induced a weakening of the inflammatory reaction, its stronger impairment in double KO mice may have resulted in the development of the Th1 component, as suggested by the IgG-subclass profile, and induced a pro-inflammatory state in both orally and nasally sensitized mice, which was noticeable before challenge and made it possible to maintain a substantial lung inflammation after challenge.

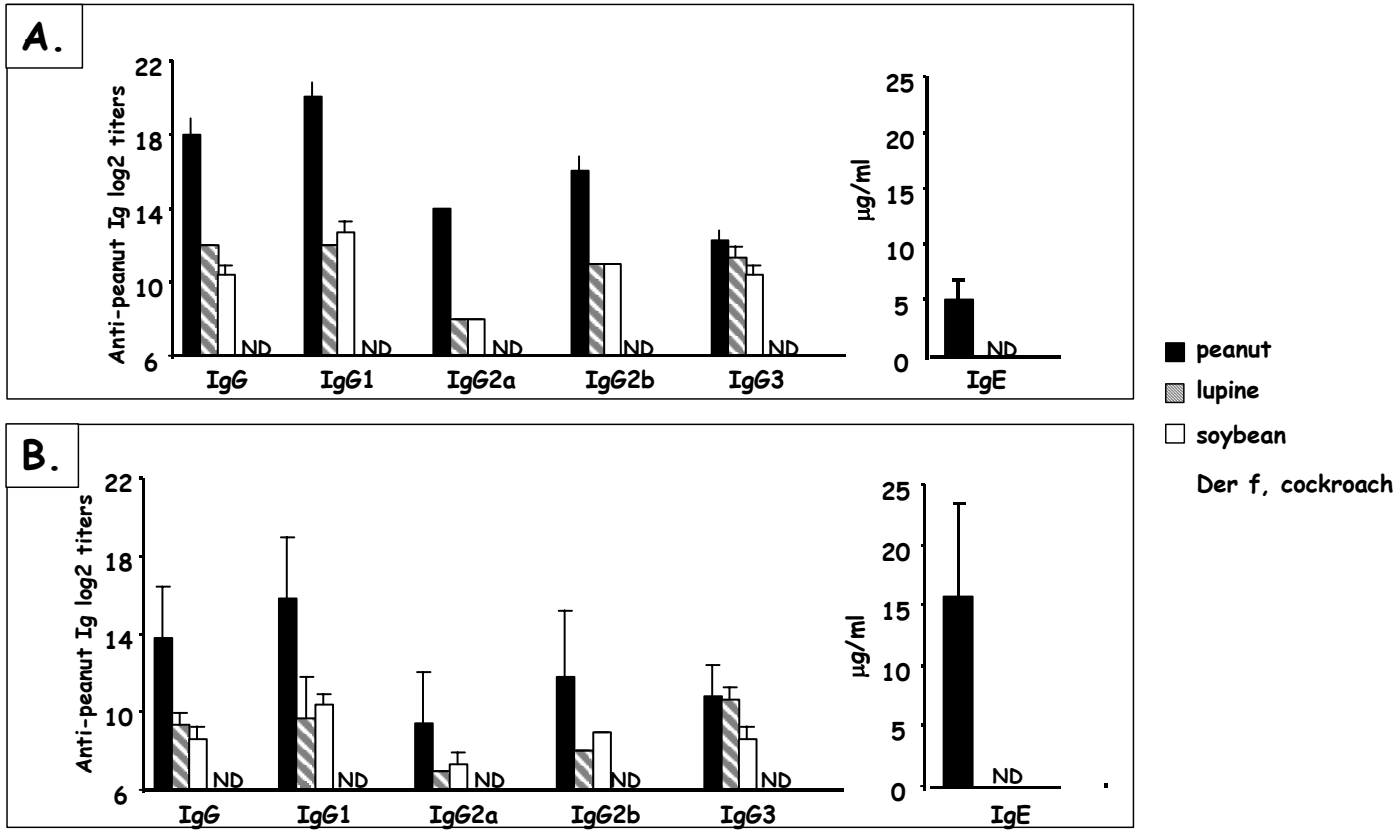
The lung mononuclear cell composition after nasal challenge of BALB/c WT and IL-4/IL-13 KO mice is shown in Figure 33. As in C57BL/6 WT mice, the major event induced by the challenge of BALB/c WT mice was the recruitment of macrophages, representing 50 and 30% of mononuclear cells of nasally and orally sensitized mice, respectively. Consistent again with results in C57BL/6 mice, a higher percentage of lung cells were Iab-positive in orally sensitized than in nasally sensitized mice. The most striking effects of the lack of IL-4 and IL-13 was the weakening of the macrophage recruitment in nasally sensitized mice after peanut challenge and the considerably lower expression of costimulatory molecules in both groups of mice.

5.3.2. Lung inflammatory responses in IL-12 KO and IFN-γ KO mice

Before challenge (Figure 34A), there was no sign of inflammation in orally sensitized WT, IL-12 KO or IFN-γ KO mice. The moderate lung inflammation observed in both WT and IL-12 KO nasally sensitized unchallenged mice, was abrogated in IFN-γ KO nasally sensitized unchallenged mice. After challenge (Figure 34B), the absence of IL-12 resulted in a substantial reduction of airway inflammation in orally sensitized mice, whereas it was reduced but still significant in nasally sensitized mice. Unlike IL-12, IFN-γ absence resulted in a complete abrogation of lung inflammation in nasally sensitized mice, while airway inflammation was only partially reduced in orally sensitized mice.

<u>Figure 35</u> : IgG, IgG subclass and IgE responses to PPE in orally or nasally sensitized mice and reactivity with other legumes

Mice were orally (A) or nasally (B) sensitized with peanut protein extract and CT as adjuvant on days 0 and 7. The cross-reactive potential of peanut-specific IgE and IgG Abs was analyzed 10 and 15 days, respectively. Results are expressed as Mean +/- SD from 2 groups of 5 mice



6. Biological significance of immune responses induced by oral vs nasal sensitization

6.1. *In vitro* reactivity of Abs from mice nasally or orally sensitized to peanut with legumes or unrelated antigens

Plasma from mice orally or nasally sensitized to peanut were also tested for their ability to react with unrelated antigens or other legumes (Figure 35). Plasma IgG of mice nasally or orally sensitized to peanut reacted with garden pea, lupine or soybean *in vitro*. However, the titles of Abs were that reacted with the other legumes were consistently lower than those of peanut-specific IgG isotypes and subclasses. No difference was seen between the levels of IgG and IgG subclasses that reacted with garden pea, lupine and soybean. In addition, the same pattern of IgG subclasses reacting with garden pea, lupine or soybean was seen after nasal and oral sensitization with peanut.

Despite significant levels of IgG that reacted with unrelated legumes, no detectable IgE reactivity to garden pea, lupine or soybean was seen in mice either nasally or orally sensitized to peanut.

No cross-reacting IgG or IgE Abs could be detected against unrelated antigens, including OVA, Der f and cockroach protein extracts.

Figure 36: Lung inflammatory responses to nasal challenge with legume proteins

Mice were sensitized orally (A) or nasally (B) with peanut protein extract and CT as adjuvant on days 0 and 7 and nasally challenged with legume proteins, on days 15 and 16. Lung tissues were collected at day 17 and inflammation was examined on sections stained with hematoxylin and eosin (40x magnification).

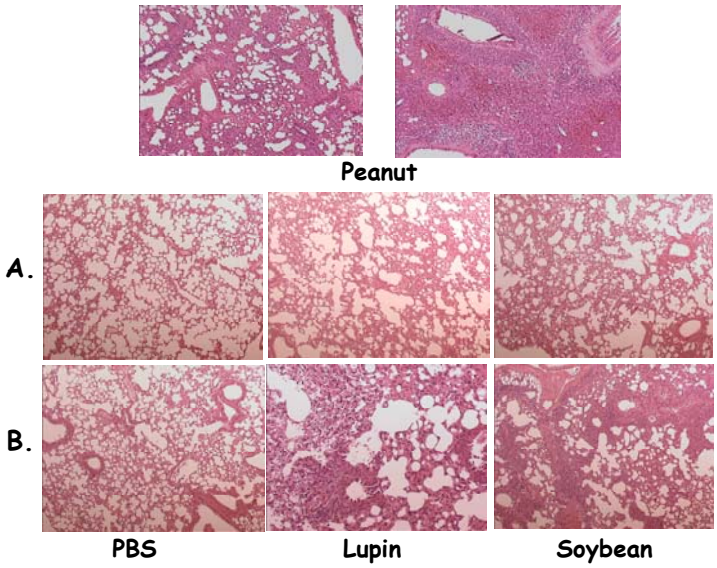
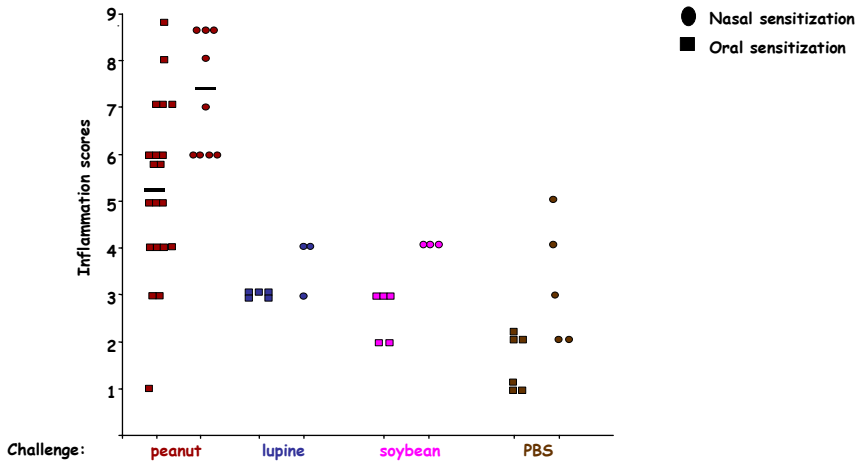


Figure 37: Lung inflammatory scores after nasal challenge with legume proteins

Mice were sensitized orally or nasally with peanut protein extract and CT as adjuvant on days 0 and 7 and nasally challenged with legume proteins, on days 15 and 16. Lung tissues were collected at day 17 and inflammation was examined on sections stained with hematoxylin and eosin (40x magnification). The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate)



6.2. Differential lung inflammatory responses to nasal challenge with legumes or unrelated antigens in mice nasally and orally sensitized to peanut

We next investigated whether the environment induced by oral versus nasal sensitization with peanut would influence inflammatory responses to legumes or unrelated food and environmental proteins. For this purpose, mice were challenged two consecutive days with 200 μg of legume or unrelated proteins. In contrast with their reactivity to legume and non-reactivity to unrelated proteins *in vitro*, mice nasally and orally sensitized to peanut exhibited lung inflammations for all related (Figure 36 and 37) and unrelated (Figure 38) antigens tested. These inflammations were significantly higher than those seen with PBS and significantly lower than those induced by peanut. Interestingly, mice nasally and orally sensitized to peanut differentially reacted to nasal challenge with legumes or unrelated proteins, *in vivo*. In fact, low levels of lung inflammation were seen when orally sensitized mice were challenged with lupine, soybean, OVA or Der f proteins. On the other hand, nasal challenge with these food or environmental antigens induced high lung inflammatory responses in mice nasally sensitized to peanut.

6.3. Nasal challenge with legume or unrelated antigens induced the recruitment of MAC-1+ cells in the lungs of nasally and orally sensitized mice.

After nasal sensitization, challenge with legumes or unrelated antigens tend to induce a higher recruitment of T and B cells than challenge with peanut (Figure 39). This result is consistent with the hypothesis of antigen non-specific inflammation after nasal sensitization. In contrast, challenge with legumes or unrelated antigens tend to induce a lower recruitment

Figure 38: Lung inflammatory responses to nasal challenge with unrelated proteins

Mice were sensitized orally (A) or nasally (B) with peanut protein extract and CT as adjuvant on days 0 and 7 and nasally challenged with OVA or Der f proteins, on days 15 and 16. Lung tissues were collected at day 17 and inflammation was examined on sections stained with hematoxylin and eosin (40x magnification).

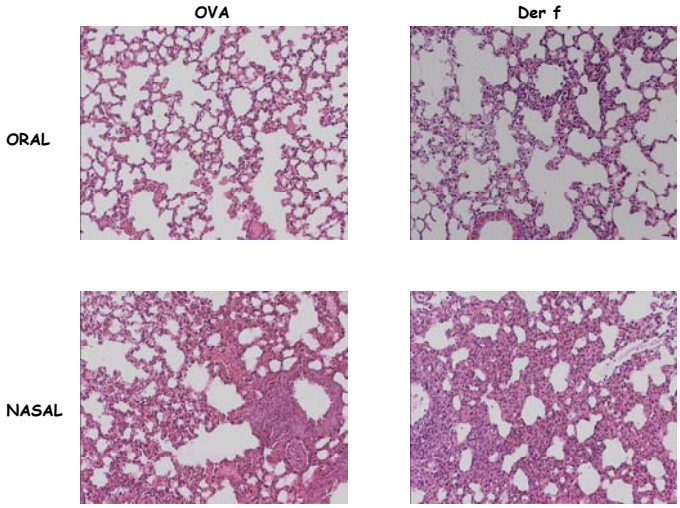
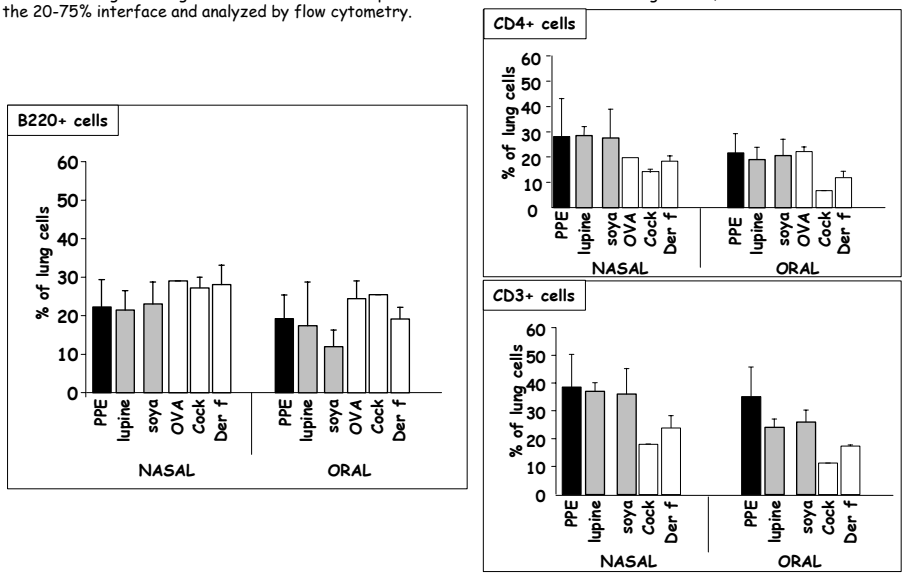


Figure 39: Recruitment of B220 -, CD3 - or CD4 – positive cells in the lung after nasal peanut challenge with legume proteins or unrelated antigens

Mice were sensitized by the nasal or oral route with PPE and CT as adjuvant on days 0 and 7. Lung tissues were collected on day 17 from mice orally or nasally sensitized with PPE and nasally challenged with legume or unrelated proteins on days 15 and 16. After collagenase digestion of the tissues and purification on discontinuous Percoll gradient, the cells were collected at the 20-75% interface and analyzed by flow cytometry.

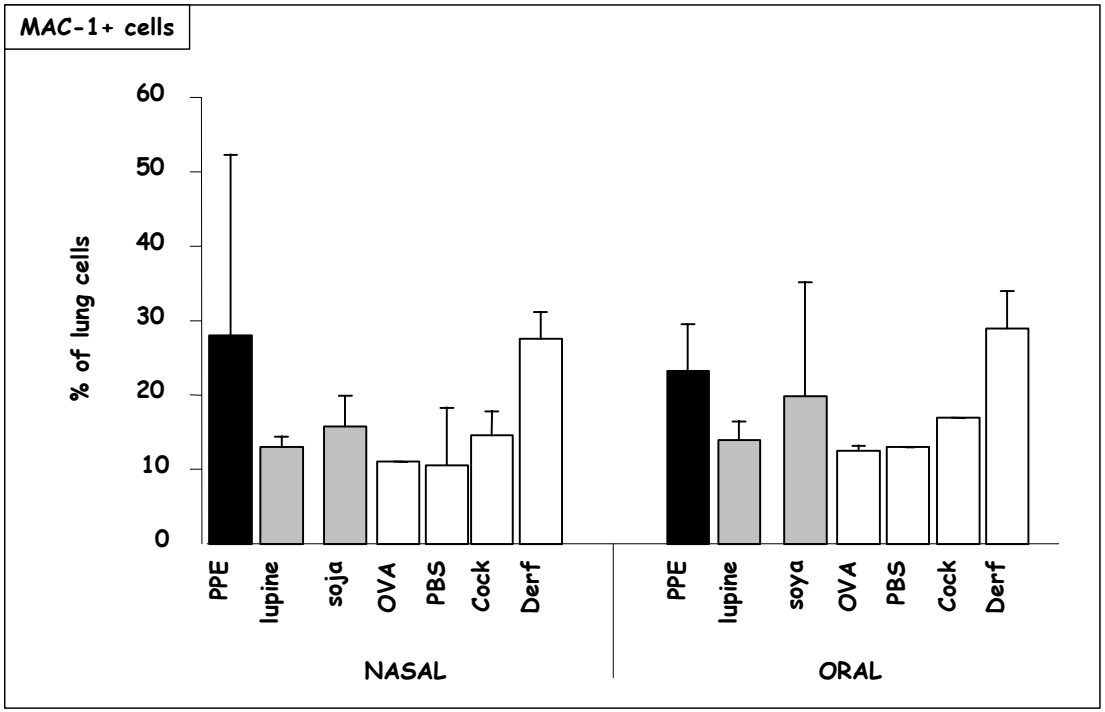


of T and B cells than challenge with peanut in orally sensitized mice. This result suggests an antigen-specific response in the lung of orally sensitized mcie.

The recruitment of MAC-1 $^{+}$ cells is higher after challenge with peanut than after challenge with legumes or unrelated antigens (Figure 40). It may reflect an antigen-specific recruitment mediated by RANTES secreting antigen-specific T cells. High numbers of macrophages were seen in the lungs of peanut-sensitized mice that were challenges with Der f. This result may not indicate a higher macrophage recruitment but the activation and proliferation of lung resident macrophages by pro-inflammatory molecules included in the protein extract. This hypothesis could be tested by analyzing cytokines and chemokines in the BAL fluids, which reflect the cell population recently recruited in the lung.

<u>Figure 40</u>: Recruitment of MAC-1 - positive cells in the lung after nasal peanut challenge with legume proteins or unrelated antigens

Mice were sensitized by the nasal or oral route with PPE and CT as adjuvant on days 0 and 7. Lung tissues were collected on day 17 from mice orally or nasally sensitized with PPE and nasally challenged with legume or unrelated proteins on days 15 and 16. After collagenase digestion of the tissues and purification on discontinuous Percoll gradient, the cells were collected at the 20-75% interface and analyzed by flow cytometry.



DISCUSSION

Although the route of sensitization could have important implications for allergic and inflammatory responses to food antigens, no animal model has addressed how oral and nasal sensitizations affect allergic manifestations to peanut. In particular, the cellular and molecular events following nasal peanut challenge have not been extensively investigated. We developed a mouse model of peanut allergy in order to investigate mechanisms underlying allergic reactions to ingested or inhaled allergens and to assess the potential of legume or unrelated environmental proteins to trigger allergic cross-reactions.

1. Proteins in PPE and other legume protein extracts

Major peanut allergens (Ara h1, Ara h2, Ara h3) have been identified, cloned and mapped for B and T cell specificity [188-190], as well as major allergens of legume extracts. However, they are not the only proteins present in PPE and legume extracts. Thus one cannot exclude the possibility of cross-reactive immunological reactions involving a number of proteins that are not recognized as major allergens. In addition, direct effects of most food proteins on innate immunity remain to be elucidated.

Electrophoretic comparison of PPE and legume extracts clearly showed molecular basis for potential cross-reactive immunologic reaction. While most previous studies focused on the presence of common epitopes between major peanut and legume allergens, we show that peanut and legumes contain a number of proteins of similar molecular mass. Based on their relative concentration in PPE and legume extracts these proteins could be major targets of Abs in sera of mice sensitized to peanut by ip injection in the presence of CT. Competitive ELISA and western blot analysis have been performed, although technical problems prevented us from reliably identifying legume proteins that react with serum from mice

sensitized to peanut by ip or mucosal sensitization in the presence of CT. Our initial mass spectrometry analysis suggests that several of these proteins are vicilins, like Ara h1. However, this and other families of molecules found in PPE and legume extracts have not been thoroughly investigated for potential regulatory effects on innate immune responses.

Taken together, we confirmed the existence of molecular grounds for cross-reactive Ab responses between peanut and other members of the legume family. Our findings also suggest the role of proteins other than major allergens for the development of allergic symptoms and will benefit from the availability of relevant animal models.

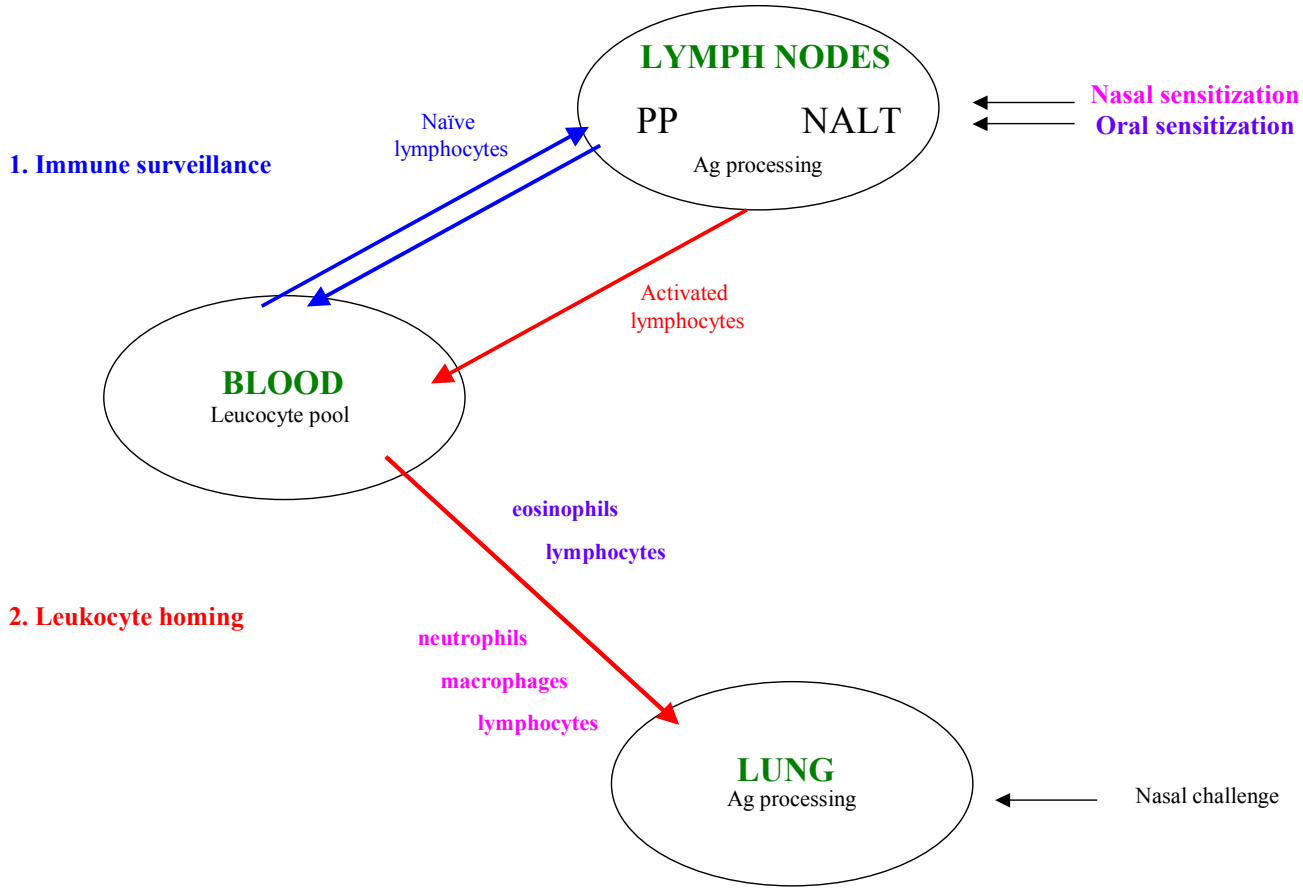
2. Mucosal routes of sensitization

2.1. Rational: natural routes of allergic sensitization

A number of studies on food allergy involved systemic sensitization in the presence of an adjuvant such as alum or CT. This approach favors the initiation of vigorous serum IgG1 and IgE responses and has proven useful to dissect the mechanisms required for the induction of allergic symptoms following secondary exposure to the allergen. However, this system may not be appropriate to study the sensitization phase, since IgE levels are artificially mounted. Recent models of peanut allergy involve oral sensitization, which is a natural route of sensitization to a food allergen. As mentioned earlier, the nasal route could represent another natural mode of sensitization to a food allergen.

Mucosal tissues of the gut or the lung display unique features. In addition, ingested food antigens are significantly modified after digestion, whereas inhaled food antigens reach the priming sites directly. Thus, it is likely that ingestion and inhalation of food allergens have different effects on the immune response induced by sensitization. Furthermore, the route of

Figure 41: Homing of effector cells induced in the NALT or GALT



sensitization could influence the reactivity phase as well. In fact, distinct mechanisms govern the homing of effector cells induced by nasal and oral sensitizations [191] (Figure 41).

2.2. Influence of the route of sensitization on Ab responses

2.2.1. Differences in the quantitative response

Here we show that nasal sensitization with PPE and CT induced higher antigen-specific IgG levels than oral sensitization. This result was maintained when different doses of antigen or adjuvant were used and when OVA was used instead of PPE. This result is consistent with other studies that aimed at characterizing the antibody response after nasal vaccination and comparing it with that after vaccination by the oral route. Moldoveanu et al. [192] showed that the specific serum antibody response to Influenza virus appeared later but persisted much longer after nasal vaccination. Hirabayashi et al. [193] found that nasal vaccination induced remarkably higher levels of antiviral IgG and IgA antibodies in the serum than did the oral route of vaccination. Similarly, nasal sensitization with a plant virus expressing a peptide from HIV [194] or with cowpea mosaic virus [194] generated higher titers of specific IgG in serum than oral sensitization, in the presence of CT.

Although knowledge is scarce about the immune responses after oral versus nasal sensitization, one possible explanation is that the antibody response in the intestine is more tightly regulated than that in the upper respiratory tract, in order to minimize the risk of reacting against the heavy load of food antigens and commensal bacteria. Another possibility could be that inductive sites are more accessible in the NALT than in the GALT or that the antigen is trapped more efficiently and is presented for a longer time in the upper respiratory tract than in the intestinal tract.

2.2.2. Differences in the qualitative response

The quantitatively distinct Ab responses induced by oral or nasal sensitization were further characterized by analyzing the profiles of the IgG-subclass responses. In mice, the Th2 response results in IgG1 production, while the Th1 response leads to IgG2a production [33]. Therefore, quantification of IgG1 and IgG2a allows following the Th1 and Th2 responses, respectively. Nasal sensitization induced a Th1/Th2 mixed IgG-subclass response and a minimal IgE response. In contrast, oral sensitization effectively primed for IgE Abs and the IgG response was characterized by a higher IgG1/IgG2a ratio. This result is not likely to be due to intrinsic features of the GI and the respiratory tracts because sensitization with OVA induced a Th2-type IgG-subclass response and high IgE levels regardless of the route of sensitization, consistent with the results of previous studies [195-197]. It may be due to unique features of the PPE that contains a large number of proteins, which biological activity has not been carefully investigated. The use of purified peanut allergens instead of the whole PPE will allow us investigate this point.

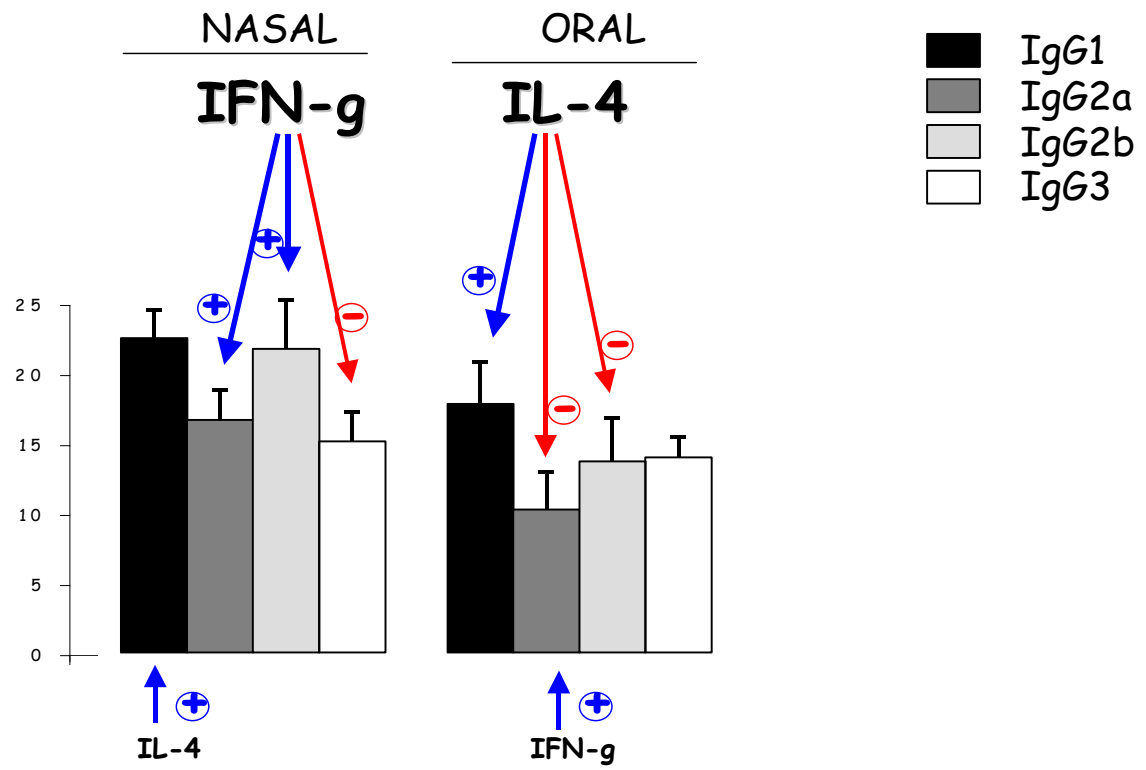
The qualitatively distinct responses to PPE and OVA after oral sensitization in the presence of CT are consistent with previous studies indicating that the immune response is dependent on the ability of the mouse strain to process and recognize a particular antigen in the presence of CT [198].

2.2.3. Lessons from cytokine KO mice

Our findings show that Th2 cytokines play a more important role in orally sensitized than in nasally sensitized mice Ab responses

It has long been established that IL-4 is a major inducer of IgE [39, 199]. As expected, and in line with many previous data [200-205], no IgE Abs were detected in orally or nasally sensitized IL-4 KO mice. The role of IL-4 in the magnitude and profile of the IgG response is less clear-cut. Serum IgG responses of to oral Ag administered in the presence of CT in IL-4 KO mice were analyzed in two previous studies. Vajdy et al. [205] found no or poor anti-KLH or anti-OVA serum B cell responses and attributed the lack of responsiveness to the failure of IL-4 KO mice to develop germinal centers in PPs. In contrast, the overall extent of the response to tetanus toxoid, a stronger immunogen than KLH or OVA, was similar in WT and IL-4 KO mice [204]. The profile of IgG subclasses observed in the absence of IL-4 was characterized by increased IgG2a and IgG2b Ab responses but no detectable IgG1 levels, suggesting a major role of IL-4 in IgG1 class switching, as shown in other gene-targeting experiments, reviewed by Stavnezer J. [206]. We also found that the total Ag-specific IgG response was of similar magnitude in WT and IL-4 KO mice, which may be due to the fact that PPE is a potent allergen. Peanut-specific IgG1 levels , although significantly lower than in WT mice, were still substantial in both groups. It indicates that peanut sensitization in the presence of CT induced IL-4 independent IgG1, which existence has also been demonstrated by Kuhn R, 1991. We originally assumed that the residual IgG1 production may be caused by IL-13, as shown in a previous study where Ag-specific IgG1 levels were below detection in IL-4 and IL-13 double KO mice, whereas they were only partially reduced in IL-4-deficient mice [207]. However, our study in IL-4 and IL-13 double KO mice clearly shows that, if these two

Figure 42: Th1 and Th2 cytokines differentially affect Ab responses in orally and nasally sensitized mice



cytokines are responsible for the dominance of IgG1 Abs, they are not essential for its production.

Interestingly, our data demonstrate that IL-4 differentially regulates the levels of peanut-specific IgG sublasses in orally and nasally sensitized mice (Figure 42). In fact, in addition to induce Th2-associated Abs, IL-4 has an inhibitory effect on the Th1-associated IgG2a and IgG2b Abs in orally sensitized mice. In contrast, it up-regulates the levels of IgG1 Abs, while having no effect on IgG2a and IgG2b in nasally sensitized mice. An underlying explanation of such results may be that IFN-γ in nasally sensitized mice is strong enough to counteract IL-4 inhibitory effects.

Conversely, our findings show that Th1 cytokines play a more important role in nasally sensitized than in orally sensitized mice Ab responses

Unlike IL-12 deficiency, IFN-γ gene targeting made it possible to completely block the Th1 pathway, as shown by the lack of detectable IgG2a Abs. Interestingly, our data demonstrate that IFN-γ differentially regulates the levels of peanut-specific IgG sublasses in orally and nasally sensitized mice (Figure 42). In fact, IFN-γ up-regulates the levels of IgG2a Abs, while having no effect on other subclasses in orally sensitized mice. In contrast, it up-regulates IgG2a levels while having a stimulatory effect on the production of IgG2b and an inhibitory effect on IgG3 levels in nasally sensitized mice. Given our previous results, we assume that IL-4 in orally sensitized mice is strong enough to counteract IFN-γ effects during the primary exposure to the Ag.

3. Influence of the sensitization route on the response to antigen secondary exposure

3.1. Relevance of the nasal route for allergen re-exposure

The nasal route was chosen over the oral route for allergen re-exposure because inflammatory reactions are more readily detectable in the respiratory tract than in the gastrointestinal. As an example, eosinophil recruitment in the gut can only be detected when very high doses of allergen are administered, inducing diarrhea [167]. The nasal route provides a suitable model to dissect mechanisms of the induction of allergic symptoms after administration of much lower doses of allergen. Nasal challenges were also performed for their biological relevance. As mentioned earlier, airborne peanut has been described to induce asthma in peanut-allergic patients.

Last, the nasal route of exposure made it possible to study mechanisms involved in asthma. Asthma, a pulmonary disease characterized by airway inflammation and increased airway responsiveness to a variety of stimuli [92], is a major health problem that affects about 12 million people in the United States. Th2 cells have been long recognized as having a key function in the pathogenesis of asthma by producing IL-4, IL-5, IL-9 and IL-13, and leading to IgE-mediated eosinophil inflammation of the lungs [208]. But there is now increasing evidence that asthma can also take place in the absence of IgE and eosinophilia. This other form of asthma is characterized by a crucial role of Th1 cells, which regulate the production of a number of cytokines including IL-1, IL-8 and IL-17, and lead to neutrophil infiltration in

the lungs [114, 115, 209]. In the majority of current murine models of asthma, animals are sensitized by intraperitoneal injection of ovalbumin together with a Th2-skewing adjuvant, before being intranasally challenged [210, 211]. This protocol results in IgE production, eosinophil recruitment in the airways and hyperresponsiveness, thus making it possible to thoroughly investigate the role of the Th2 pathway in asthma. However, only few studies [53, 212, 213] have developed animal models of Th1-induced airway inflammation and the mechanisms involved in this process remain to be elucidated.

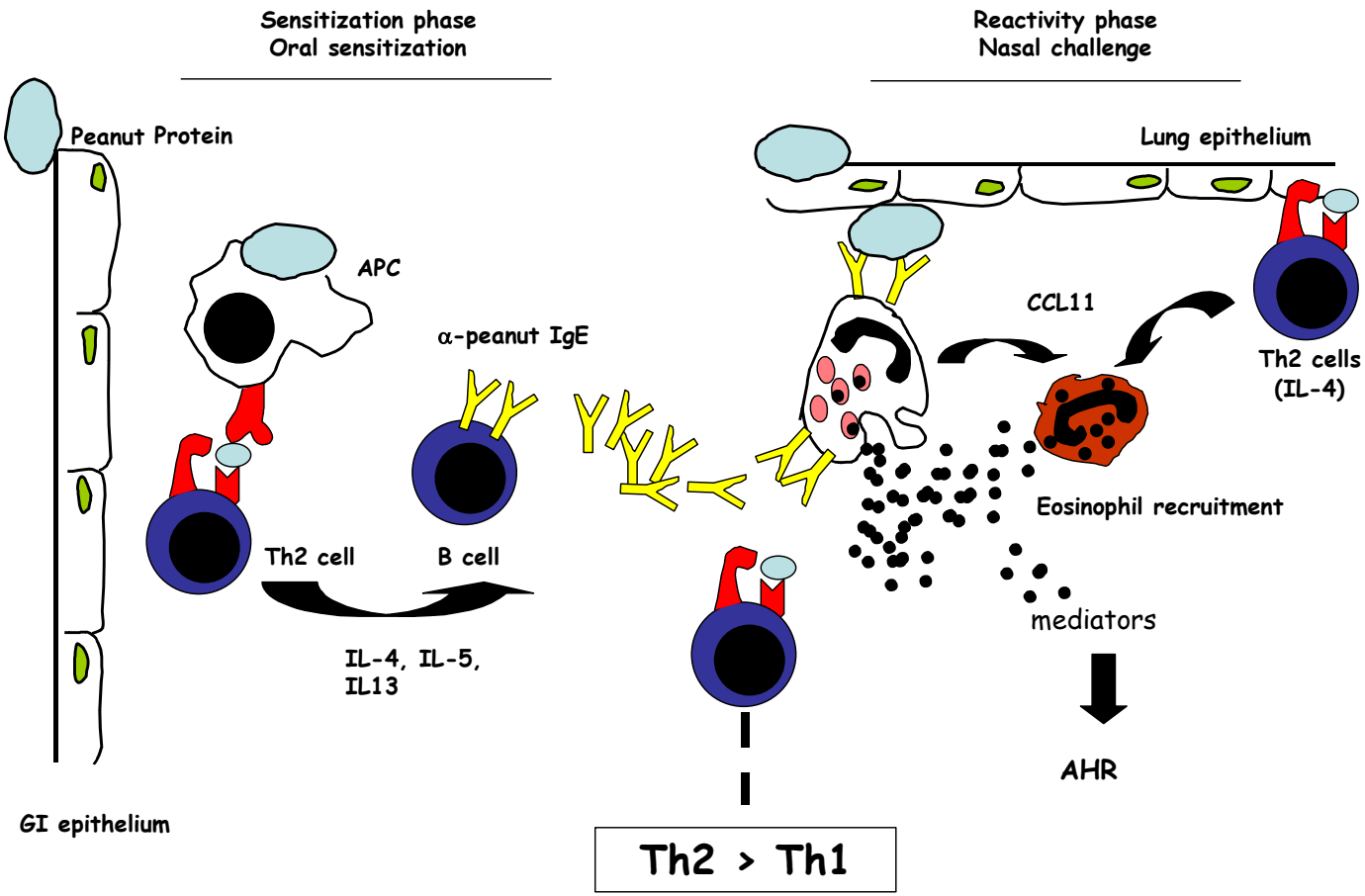
3.2. Nasal and oral sensitization promoted differential lung inflammatory responses

3.2.1. Eosinophil- and neutrophil-associated asthma models

Ten days after the last sensitization and before any challenge was performed, the composition of lung immune cells was significantly different in orally and nasally sensitized mice compared to that of naïve mice. This is in accordance with the concept of a common mucosa-associated immune system [214] with the hypothesis that cells primed in one mucosal inductive site may home to different mucosal effector sites. This proposal is supported by vaccination studies showing that intestinal priming can lead to protection of the respiratory tract [215-218].

Lung inflammatory responses have been shown to be under control of cytokines and chemokines in mice [219] as well as in human [208, 220]. For example, the Th2-type cytokines IL-4 [39, 40, 202] and IL-13 enhances IgE Abs responses [41], an effect antagonized by IFN-γ [221]. A

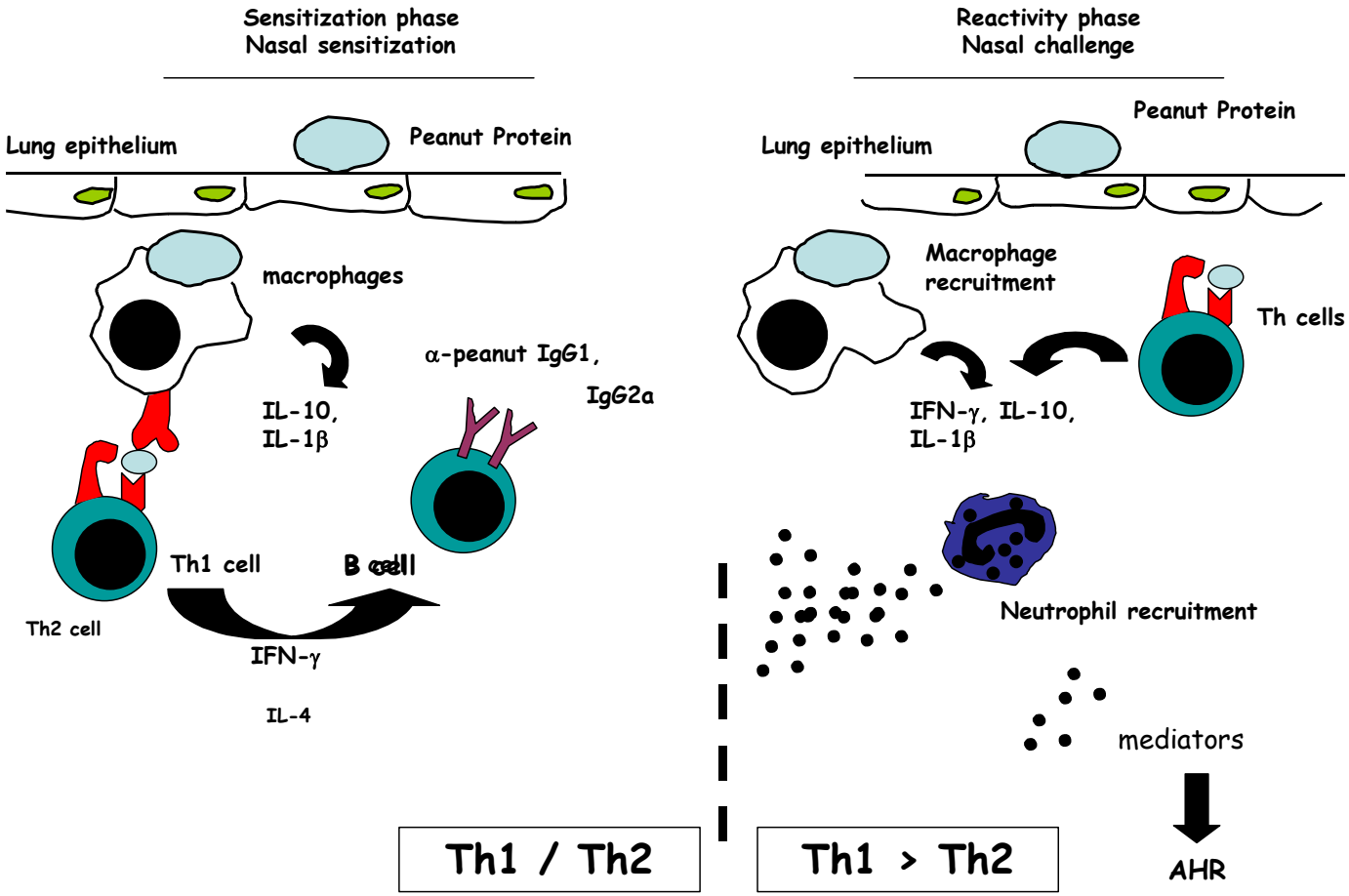
Figure 43: Summary of cellular and molecular events underlying the oral sensitization / nasal challenge model



number of studies have demonstrated that IL-5 and CCL11 regulate mucosal (i.e., respiratory and GI) eosinophilic inflammations [45, 46, 111]. IL-1, IL-6 and IFN-γ are known for their role in inflammatory responses [222]. IL-17 is a Th1 cytokine involved in the recruitment of neutrophils into the airways [114, 223].

Both orally and nasally sensitized mice experienced AHR, a clinical symptom of asthma caused by impaired lung functions. However, significant differences were seen in terms of cells infiltrating airway tissues and cytokine responses in the groups sensitized orally and nasally. Thus, nasal challenge significantly increased lung IL-4 and CCL-11 mRNA levels and IL-4 secretion in the BALF of orally sensitized mice. These findings were consistent with the higher lung eosinophilia seen in these mice and with the previously reported role of IgE for eosinophil-mediated airway hyperactivity [224, 225]. Allergic asthma is described as an atopic disease involving IgE, with Th2 responses and eosinophilic airway inflammation, resulting in enhanced bronchial reactivity [112]. Our results suggest that nasal peanut challenge of mice sensitized by the oral route can lead to lung inflammatory responses that resemble allergic asthma (Figure 43). In contrast with orally sensitized mice, those sensitized by the nasal route showed increased IL-17 and IFN-γ mRNA levels in the whole lung samples and infiltrating $CD4^+$ T cells, respectively. These mice also showed increased IL-6 secretion in the BALF after nasal peanut challenge. Unlike orally sensitized mice, no significant lung eosinophilia was seen after nasal challenge of nasally sensitized mice. Our results are consistent with studies suggesting that IgG antibodies counter allergen-triggered eosinophil recruitment [226]. There is now evidence that asthma can also take place in the absence of IgE and eosinophilia. As seen after nasal peanut challenge of mice sensitized by the nasal route, this other form of asthma is characterized by a massive infiltration of neutrophils in the lung [113, 114]. It has also been shown that IL-1, IL-6 and IL-17 secreted by epithelial cells and macrophages play a major role in neutrophil-associated asthma [115, 116]. In this regard, the leading cellular event

Figure 44: Summary of cellular and molecular events underlying the nasal sensitization / nasal challenge model



after nasal challenge of mice sensitized by the nasal route is a massive recruitment of neutrophils and macrophages in the lung. Taken together, these results suggest that IgE-mediated asthma is induced after nasal challenge of mice orally sensitized to peanut while non-IgE-mediated asthma occurred in mice sensitized by the nasal route (Figure 44).

3.2.2. Lessons from cytokine KO mice

Th2 cytokines are required for full lung reactivity of both models of asthma

IL-4-deficient mice in the model of allergen-induced eosinophilic airway inflammation have given contrasting results. They developed substantially less peribronchial inflammation and eosinophilia than their WT counterparts in some investigations [227-229] while still displaying severe eosinophilic inflammation in other studies [230, 231]. In a similar manner, conflicting results have been reported for the development of allergic asthma in IL-13 KO mice [232, 233]. These discrepancies may be explained by overlapping functions of IL-4 and IL-13, which use a common signaling pathway via the IL-4 receptor a subunit [234] and the subsequent activation of STAT-6 [235] and reveal the need to use IL-4 and IL-13 double knock-out mice in order to clarify the role of the Th2 pathway in this pathology. However, few studies have used these mice in the model of allergen-induced eosinophilic airway inflammation. To our knowledge, those mice were not used either in models of neutrophil-induced airway inflammation. The attenuation of lung cell recruitment observed in IL-4 KO mice in the two models of our study suggests a key role for IL-4 in both inflammation processes, which action may be mediated by IgE Abs, as indicated by others [224, 225], in orally sensitized mice. However, nasally sensitized mice, which do not display detectable IgE levels, suggest that other mechanisms are involved in IL-4-mediated action on pulmonary

inflammation. Unexpectedly, lung inflammation was enhanced in double KO mice. This observation led us to believe that compensatory mechanisms resulting into the development of the Th1 component, as demonstrated by the low IgG1/IgG2a ratio in IL-4/IL-13 KO mice, were stronger in double KO mice, whose Th2 pathway was more impaired than in IL-4 KO mice and lead to a pro-inflammatory state in both orally and nasally sensitized mice.

Th1 cytokines are required for full lung reactivity of both models of asthma

IL-12 is a heterodimeric cytokine made of two subunits, p35 and p40, both of which are required for functional activity [236]. Gene targeting studies that aimed at assessing the role of IL-12 in mouse models of eosinophilic asthma have given contrasting results. In some investigations [237], IL-12 KO mice gave support to the well-known role of IL-12 in promoting Th1 responses and inhibiting Th2 responses [238] by showing enhanced eosinophil recruitment to the airways. However, a recent study demonstrated that IL-12-deficient mice have substantially reduced airway recruitment of eosinophils, compare to their WT counterparts. Furthermore, levels of Ag-specific IgE, IgG1 and IgG2a were the same in both groups of mice [239]. In line with this last study, IL-12p40 KO mice in our system of eosinophilic lung inflammation showed significantly reduced cell infiltration, and levels of peanut-specific IgE were not increased. Knowing that IL-12 favors the Th1 pathway mainly via the induction of IFN-g production in WT mice [238, 240] and assuming that this pathway was impaired in IL-12 KO mice, two interpretations may be proposed in order to explain our results. IL-12 KO mice may have overcome the defect of Th1 responses, possibly through the action of IL-18, a cytokine that promotes IFN-g responses [241]. Alternatively, Th1 cells that are generally believed to only protect against allergic reactions by attenuating the activity of Th2 cells [212] may also support Th2 cell-induced allergic asthma [52, 53, 242]. However, it is also possible that

IL-12p40 KO mice were characterized by a weaker Th2 environment compare to WT mice, as suggested by their lower peanut-specific IgE and IgG1 levels. In fact, studies indicate that IL-12p40 homodimers are produced in WT mice and act as antagonists of IL-12 mediated signaling pathway [243]. Consistent with this hypothesis, IL-12p40 has recently been shown to contribute to the generation of a Th2-type environment in a mouse model of allergic diarrhea [244]. Our data also show that IL-12 plays a role in the model of neutrophilic asthma, although not essential since it is only partially reduced in IL-12p40 KO mice.

4. Non specific reactions to food and environmental antigens

As indicated earlier, peanut allergy is frequently associated with pollen allergy [28, 148-150] and peanut allergens could share sequence homology with environmental antigens [151]. Therefore, it was important to determine whether the immune status resulting from oral or nasal sensitization with peanut affected airway responses to unrelated antigens. Interestingly, only mice nasally sensitized to peanut exhibited lung inflammatory responses to nasal challenges with unrelated antigens, including OVA, which do not have known similarities with peanut proteins. This finding strongly suggests that the cytokine environment and subsequent innate responses dictate the potential of non-specific airway inflammation of mice sensitized by the nasal route. Of interest, these results are consistent with the greater level of inflammatory cytokine responses in the lungs (i.e., IL-17 and IFN-γ mRNA) and BALF (i.e., IL-6) in these mice. Our separate studies suggest that the dose of peanut used for the nasal sensitization does not explain the tendency of nasally sensitized mice to develop non-specific inflammatory responses. It has been reported that Der f protein extracts stimulate macrophages and inflammatory responses [245]. Our results suggest that PPE can also stimulate cells in the upper respiratory tract and trigger a state of pro-inflammation. These data indicate

that nasal sensitization with peanut leads to a lasting proinflammatory status, which could modify bronchial reactivity and favor non-specific lung inflammation to unrelated environmental antigens.

The Th2 environment induced by oral sensitization suggests that B and T cells were recruited via Th2 cells in orally sensitized mice. This recruitment may be antigen-specific. As antigen-specific B and T cells are primed in the lymph nodes, there may be a delay before they reach the lung.

To note, although oiur protocol of oral sensitization induced significant levels of peanut-specific IgE, no cross-reactive IgE could be detected. Several reasons may explain this result. Our flours are complex mixtures of antigenic, nonantigenic and allergenic substances. We don't know if all components, in particular allergens, were absorbed on the ELISA plate. Second, our failure to detect cross-reactive IgE could be due to their weak concentration in the serum. Third, it may be that only highly peanut-specific IgE are cross-reactive. Repeated contact with the primary antigen, resulting in an increase in antibody affinity, may also result in an increase in cross-reactivity.

In conclusion, our research has shown that sensitization with peanut proteins in the presence of cholera toxin by the oral or the nasal route has distinct effects on the immune response. The plasma antibody response induced by oral sensitization was clearly a Th2-type response with high levels of peanut-specific IgE and a high IgG1/IgG2a ratio. In contrast, the plasma antibody response induced by nasal sensitization was a mixed response with high levels of both IgG1 and IgG2a and low levels of IgE. Furthermore, distinct inflammatory responses to subsequent exposure to peanut in the respiratory tract were observed, with mice

sensitized by the oral route being more prone to allergic-type responses, characterized by a high eosinophilia. Nasal sensitization, on the other hand, favored non-allergic inflammation and innate responses to unrelated environmental antigens. Our two mouse models of adverse reactions to food proteins might thus be usefull tools to study the IgE-mediated and the non-IgE asthma, two forms of the same disease, observed in humans.

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PUBLICATIONS ET COMMUNICATIONS

- **Publications**

Romy Fischer, Jerry R. McGhee, Huong Lan Vu, T. Prescott Atkinson, Raymond J. Jackson, Daniel Tomé, and Prosper N. Boyaka. *Oral and nasal sensitization promote distinct immune responses and lung reactivity in a mouse model of peanut allergy.* American Journal of Pathology. 2005 Dec, Vol. 167, No. 6

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Boyaka PN, Tafaro A, **Fischer R**, Fujihashi K, Jirillo E, McGhee JR. *Therapeutic manipulation of the immune system: enhancement of innate and adaptive mucosal immunity*. Curr Pharm Des. 2003;9(24):1965-72. Review

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American Association of Immunologists (AAI) meeting, Denver, Colorado, mai 2003. **Romy Fischer**, Jerry R. McGhee, Huong Lan Vu, T. Prescott Atkinson, Raymond J. Jackson, Daniel Tomé, and Prosper N. Boyaka. *Mucosal routes of sensitization influence the immune response in a mouse model of peanut-induced asthma.*

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ARTICLE 1

Oral and nasal sensitization promote distinct immune responses and lung reactivity in a mouse model of peanut allergy

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Immunopathology and Infectious Diseases

Oral and Nasal Sensitization Promote Distinct Immune Responses and Lung Reactivity in a Mouse Model of Peanut Allergy

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Despite structural and functional differences between the initial sites of contact with allergens in the gastrointestinal and nasal tracts, few animal models have examined the influence of the mucosal routes of sensitization on host reactivity to food or environmental antigens. We compared the oral and nasal routes of peanut sensitization for the development of a mouse model of allergy. Mice were sensitized by administration of peanut proteins in the presence of cholera toxin as adjuvant. Antibody and cytokine responses were characterized, as well as airway reactivity to nasal challenge with peanut or unrelated antigens. Oral sensitization promoted higher levels of IgE, but lower IgG responses, than nasal sensitization. Both orally and nasally sensitized mice experienced airway hyperreactivity on nasal peanut challenge. The peanut challenge also induced lung eosinophilia and type 2 helper T-cell-type cytokines in orally sensitized mice. In contrast, peanut challenge in nasally sensitized mice promoted neutrophilia and higher levels of lung MAC-1$^+$ I-A$^{b\ low}$ cells and inflammatory cytokines. In addition, nasal but not oral, sensitization promoted lung inflammatory responses to unrelated antigens. In summary, both oral and nasal peanut sensitization prime mice for airway hyperreactivity, but the initial mucosal route of sensitization influences the nature of lung inflammatory responses to peanut and unrelated allergens. ***(Am J Pathol 2005, 167:1621–1630)***

The prevalence of peanut allergy has doubled in the last decade, and it now affects more than 3 million individuals in the United States.[1] This health care problem is further enhanced by potential cross-reactive allergens. Thus, clinical symptoms were reported in peanut allergic patients who had ingested food of the same botanical family[2–4] or even taxonomically unrelated products.[5] Allergic respiratory symptoms have also been described in peanut-allergic patients after inhalation of airborne peanut particles in school[5] or on airline flights.[6,7] In this regard, food allergens are now well recognized to play a significant role as aeroallergens in the etiology of asthmatic symptoms in individuals with food allergies.[8]

Sensitization to food allergens such as peanut generally occurs in the gastrointestinal (GI) tract. However, it could also occur as a consequence of direct or cross-sensitization by inhalational exposure to peanut or cross-reactive environmental antigens. For example, peanut allergy is frequently associated with pollen allergy,[9–12] and peanut allergens share sequence homologies with environmental antigens.[13] A study on children with a history of at least one acute allergic reaction showed that initial reactions to peanut occurred at 24 months of age, with the large majority resulting from a first oral exposure.[5] Because IgE-mediated allergic reactions require prior exposure to the allergen, one cannot rule out earlier sensitization through inhalation of airborne peanut particles. In addition, the presence of cross-reactive IgE to pollen and peanut antigens in pollen-allergic patients[14] and the reports that these individuals can develop positive skin tests to peanut[15,16] suggest that allergic symptoms to peanut may also be caused by respiratory sensitization with cross-reactive allergens. Structural and functional differences have been described between the gut-associated lymphoid tissues and the nasopharyngeal-associated lymphoid tissues[17] that are the first sites

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of contact with ingested and inhaled antigens, respectively. But it remains unclear how priming through each site could influence subsequent allergic or inflammatory reactions.

It is widely accepted that IgE and cytokines produced by Type 2 helper T (Th2) cells play a pivotal role in allergic manifestations.[18,19] However, recent studies suggest that a larger number of parameters contribute to allergic responses. For example, in addition to IgE, antibodies (Abs) of the IgG isotype could exert a regulatory effect on allergic reactions;[20] however, underlying mechanisms are still poorly understood.[21] Th1 cells that were believed to only protect against allergic reactions by attenuating the activity of Th2 cells[22] now appear to also support Th2 cell-induced allergic asthma.[23–25] In addition, Th1 cells have been shown to recruit and activate neutrophils for subsequent airway hyperreactivity (AHR).[26] The route of allergen sensitization may influence the pattern of Ab and T-cell responses and, therefore, the nature of potential adverse reactions. This increasing complexity of mechanisms underlying allergic and nonallergic inflammatory responses further limits our understanding of adverse effects that occur in individuals with allergies.

Peanut allergy has been mostly investigated in animal models sensitized by the subcutaneous,[27] the intraperitoneal,[28,29] or the oral route[30–32] and challenged by the oral route.[27,28,30,31] The nasal route has been less extensively investigated. Furthermore, to our knowledge no study has compared inflammatory lung reactions to unrelated food or respiratory antigens in animal models sensitized by the oral and nasal routes. We compared Ab and T-cell responses induced by oral or nasal sensitization with whole-peanut protein extract (PPE) and cholera toxin (CT) as adjuvant. We then examined the influence of these responses on airway reactivity to nasal challenge with PPE or unrelated antigens. Our data show that the initial mucosal route of peanut sensitization affects the nature of the immune response and the lung reactivity to peanut but also to unrelated antigens.

Materials and Methods

Mice

Female C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water *ad libitum*. Studies were performed in accordance with institutional guidelines to avoid pain and distress.

Mucosal Sensitization

Whole-peanut protein extracts (PPE) were obtained as previously described by ammonium bicarbonate treatment of defatted peanut extracts.[2] Mice 8 to 12 weeks of age were sensitized on days 0 and 7 with whole PPE and CT as adjuvant. Anesthetized mice were nasally administered 100 μg of PPE and 1 μg of CT in a total volume of 10 μl with 5 μl placed into each nare. This volume of the nasal vaccine is retained in the nasal cavity after nasal administration to anesthetized mice.[33] For sensitization by the oral route, mice were deprived of food for 2 hours and then orally treated with 250 μl of sodium bicarbonate as previously described.[34] Oral sensitization consisted of intragrastric administration of 1 mg of PPE plus 15 μg of CT in 250 μl of phosphate-buffered saline (PBS). Other doses of CT (5 μg nasal or 60 μg oral) and PPE (25, 50, or 200 μg nasal or 2 mg oral) were tested in separate experiments. Some experiments included mice given ovalbumin (OVA) (Sigma Chemical, Saint Louis, MO) as antigen instead of PPE. In these experiments, mice were then either nasally administered 100 μg of OVA plus 1 μg of CT or given 1 mg of OVA plus 15 μg of CT by the oral route. Plasma samples were collected 1 week after each sensitization, on days 7 and 14, for analysis of peanut-specific Ab responses.

Nasal Challenge with Peanut and Unrelated Proteins

Mice nasally or orally sensitized to peanut were nasally challenged on days 15 and 16 with 200 μg of PPE in a total volume of 100 μl. More specifically, anesthetized mice were given 25 μl of PPE per nare, four times at 2- to 3-minute intervals. For analysis of lung responses to unrelated proteins, mice were challenged with 200 μg of OVA or 40 μg of *Dermatophagoides farinae* (Der f) protein extract (Greer Laboratories, Lenoir, NC) instead of PPE.

Plasma Antibody Responses

Plasma levels of peanut-specific Abs were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates (Falcon) were coated with 50 μg/ml PPE in PBS and incubated overnight at 4°C. After blocking with PBS-1% bovine serum albumin, serial dilutions of plasma samples were added and incubated overnight at 4°C. Peanut-specific IgG Abs were detected using 0.3 μg/ml of horseradish peroxidase (HRP)-labeled goat anti-mouse γ-heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL). Biotin-conjugated rat anti-mouse γl (clone A85-1), γ2a (clone R19-15), γ2b (clone R12-3), or γ3 (clone R40-82) heavy chain mAbs (BD PharMingen, San Diego, CA) were used at 0.5 μg/ml; and streptavidin-HRP (BD PharMingen) was diluted at 1:2000 for the detection of peanut-specific IgG subclasses. The colorimetric reaction was developed by the addition of 2,2′-azino-bis(3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma) and H_2O_2. Endpoint titers were expressed as the $\log_2$ of plasma dilution giving an optical density at 415 nm of $\geq$0.1 above those obtained with control plasma. To determine the potential of plasma of peanut-sensitized mice to react with irrelevant pro-

tein antigens, plasma samples were added to ELISA plates coated with OVA (1 mg/ml) or Der f protein extract (10 μg/ml).

The removal of IgG has been shown to improve the detection of IgE Abs.[35] Thus, dilutions of plasma samples were first depleted of IgG by overnight incubation at 4°C in protein G-coated 96-well plates (Reacti-Bind plates; Pierce, Rockford, IL). Total and antigen-specific IgE levels were then analyzed by ELISA. For detection of antigen-specific IgE Abs, IgG-depleted samples were added to ELISA plates coated with PPE (50 μg/ml, 100 μl/well). The IgE were detected with 0.5 μg/ml biotin-conjugated rat anti-mouse IgE (clone R35-118; BD PharMingen) followed by streptavidin-HRP (1:2000). The levels of total IgE Abs were determined using capture and detection antibodies, as well as IgE standard, from the BD OptEIA Set mouse IgE kit (BD Biosciences, San Diego, CA).

Airway Hyperreactivity

Enhanced pause (Penh), an index that reflects changes in amplitude of pressure wave form and expiratory time, was measured 6 hours after the last nasal peanut challenge in mice placed in a barometric plethysmograph according to a previously described method.[36] Doses of metacholine (0, 10, and 20 mg/ml) were administered by nebulization. For each dose, Penh were measured every minute over 7 minutes. Controls included sham-sensitized and sham-challenged mice.

Histology and Determination of Lung Inflammation Scores

Lungs were fixed in 10% buffered formaldehyde, paraffin-embedded, and cut into sections of 5 μm thickness. The sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin for the evaluation of inflammation. The presence of eosinophils in tissue sections was determined by the cyanide-resistant peroxidase activity as previously described.[37] Briefly, lung sections were incubated for 1 minute at room temperature in 10 mmol/L KCN, pH 6.5. Slides were then rinsed in PBS and incubated for 15 minutes with the peroxidase substrate 3,3′-diaminobenzine (Vector Laboratories, Burlingame, CA). After washes in PBS, tissue sections were counterstained with hematoxylin. The eosinophils, which express a cyanide-resistant peroxidase activity, appeared as containing dark brown granules, and their frequency was estimated by microscopic observation at $\times$200 magnification. The neutrophils, which do not express a cyanide-resistant peroxidase, were segregated based on their characteristic morphology.

For quantification of lung inflammation, the slides were coded, and peribronchial and perivascular inflammation was scored in a blinded fashion by two independent investigators. A value of 1 was given when slides showed no sign of inflammation. Slides were graded from 2 to 4 when bronchi were surrounded by a thin layer of inflammatory cells (2, few bronchi; 3, more bronchi; and 4, most bronchi). They were graded from 5 to 7 according to the number of bronchi that were surrounded by a thick layer of inflammatory cells (5, few bronchi; 6, more bronchi; and 7, most bronchi). Finally, slides were graded 8 or 9 when inflammation spread into the interstitial area (8, severe; and 9, extreme).

Flow Cytometry

Whole-lung tissue was dissociated by digestion with 1 mg/ml collagenase type V (Sigma) in RPMI-1640 (Cellgro Mediatech, Washington, DC), supplemented with 10 mmol/L HEPES, 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (supplemented RPMI) to obtain single cell preparations. Mononuclear cells were collected at the 20 to 75% interface of discontinuous Percoll gradient and stained with anti-CD3 (clone 145-2C11), anti-CD4 (clone GK1.5), anti-B220 (clone RA3-6B2), anti-CD11c (clone HL3), anti-MAC-1 (clone M1/70), or anti-MHC class II Abs (I-A^b, clone AF6-120.1) (BD PharMingen). After washes and fixation, samples were analyzed by flow cytometry.

Purification of $CD4^+$ T Cells

Whole-lung tissue was dissociated by digestion with collagenase as described above. Mononuclear cells were collected and washed in supplemented RPMI. The $CD4^+$ T cells were purified using the automated magnetic cell sorting (autoMACS) according to the protocol provided by the manufacturer (Miltenyi Biotech, Auburn, CA). Briefly, single cell suspensions were incubated with a biotinylated anti-CD4 mAb (BD PharMingen) for 30 minutes at 4°C and washed in PBS containing 2 mmol/L EDTA and 0.5% bovine serum albumin. Streptavidin-conjugated MicroBeads (Miltenyi Biotech) were then added to cells. After a 30-minute incubation at 4°C, cells were washed, and $CD4^+$ T cells were purified by positive selection using autoMACS.

Quantification of Cytokine and Chemokine mRNA by Real-Time PCR

Lung tissue was dissociated as described above; mononuclear cells were collected and washed in supplemented RPMI; and RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX). The reverse transcription was performed with superscript II reverse transcriptase, dNTPs, and poly(dT) oligos. The real-time PCR (Lightcycler; Roche, Indianapolis, IN) was performed with primers generated with Oligo software (Plymouth, MN) and the SYBR green detection system according to the manufacturer. Results are expressed as crossing point (CP), defined as the cycle at which the fluorescence rises appreciably above the background fluorescence as determined by the Second Derivative Maximum Method (Roche Molecular Biochemicals LightCycler Software). The formula $20 - (CP_{cytokine} - CP_{\beta\text{-actin}})$ was used to

represent the logarithm of the relative mRNA levels of a given cytokine. This formula allows the normalization of all results against β-actin levels to correct for differences in cDNA concentration between the starting templates. Differences of crossing points above two cycles were considered significant.

Bronchoalveolar Lavage and Cytospin

Bronchoalveolar lavage fluids (BALF) were obtained via cannulation of the exposed trachea, by infusion of 0.6 ml of supplemented RPMI through a 22-gauge catheter into the lungs, followed by aspiration of this fluid into a syringe. A volume of 0.4 ml of fluid was consistently recovered. Aliquots were centrifuged, and supernatants were collected and stored at −70°C until analyzed. Cell pellets were subjected to cytospin, and the slides were stained with Giemsa (Sigma).

Cytokine ELISA

Cytokines were measured in the supernatants of BALFs by ELISA. Nunc MaxiSorp Immunoplates (Nunc, Napersville, IL) were coated with anti-mouse tumor necrosis factor-α (TNF-α) (clone MP6-XT22), interferon (IFN)-γ (clone R4-6A2), interleukin-4 (IL-4) (clone BVD4-1D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3), or IL-10 (clone JES5-2A5) mAbs (BD PharMingen) or IL-13 (R&D Systems, Minneapolis, MN) in 0.1 mol/L sodium bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. After blocking with PBS-3% bovine serum albumin, cytokine standards and serial dilutions of supernatants of BALFs were added in duplicates. The plates were incubated with biotinylated anti-mouse TNF-α (clone MP6-XT3), IFN-γ (clone XMG-1.2), IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), IL-10 (clone JES5-16E3) (BD PharMingen), or IL-13 mAbs (R&D Systems), followed by HRP-labeled goat anti-biotin Ab (Vector Laboratories). The colorimetric reaction was developed with the addition of 2,2′-azino-bis(3)-ethylbenzylthiazoline-6-sulfonic acid substrate and H_2O_2. Standard curves were generated using murine rIFN-γ, rIL-5, rIL-6, rIL-10 (Genzyme, Cambridge, MA), rIL-4 (Endogen Corp., Boston, MA), rTNF-α, and rIL-13 (R&D Systems). The ELISAs were capable of detecting 3 pg/ml IL-4; 5 pg/ml IL-6; 10 pg/ml IL-5; 20 pg/ml IFN-γ, TNF-α, and IL-10; and 30 pg/ml IL-13. A quantikine ELISA kit (R&D Systems) was used for detection of IL-1β.

Statistics

The results are reported as the mean ± 1 SD. Statistical significance ($P < 0.05$) was determined by Student's *t*-test and by the Mann-Whitney *U*-test of unpaired samples. The results were analyzed using the InStat statistical software (San Diego, CA) for Apple computers.

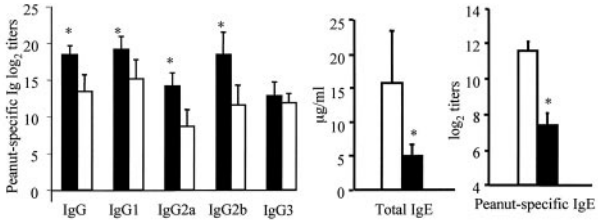

Figure 1. Plasma antibody responses in mice orally or nasal sensitized to peanut. Mice were orally (□) or nasally (■) sensitized by administration of PPE and CT on days 0 and 7. Plasma samples were collected on day 14, and Ab responses were analyzed by ELISA. Results are expressed as mean $\log_2$ titers or mean mg/ml ± 1 SD and are from nine separate experiments with five mice per group. *$P < 0.01$.

Results

Oral Sensitization with PPE and CT Induces Higher IgE but Lower IgG Antibody Responses Than Nasal Sensitization

Although mucosal surfaces of the GI and respiratory tracts are considered the primary sites of sensitization to food antigens, it remains unclear how peanut priming through each site could influence subsequent allergic or inflammatory reactions. We first compared the plasma levels of peanut-specific Ab responses in mice that received whole PPE and CT as adjuvant by the oral and the nasal route. Both mucosal routes of sensitization promoted peanut-specific plasma IgG Abs, but higher levels of IgG responses were measured in mice sensitized by the nasal route (Figure 1). In addition, nasal and oral sensitization also induced different patterns of peanut-specific IgG subclass responses with a lower IgG1-to-IgG2a ratio in nasally sensitized mice (1.3 ± 0.1 vs. 1.8 ± 0.3, $P < 0.01$) (Figure 1).

In contrast with plasma IgG responses, higher levels of peanut-specific IgE Ab responses were measured in orally sensitized mice (Figure 1). The difference in the levels of IgE responses between mice sensitized by the oral and nasal routes was maintained when lower or higher doses of antigen or adjuvant were used (data not shown). In addition, no difference was seen in antigen-specific IgE Ab responses between mice that were given OVA (1 mg orally or 100 mg nasally) instead of PPE (data not shown). Taken together, these results suggested that oral sensitization with PPE favors IgE Ab responses, whereas nasal sensitization more effectively primes for IgG Abs.

Both Orally and Nasally Sensitized Mice Experience AHR after Nasal Peanut Challenge

The Penh values of mice orally or nasally sensitized with PPE were measured 6 hours after nasal challenge to determine whether these routes of mucosal sensitization primed for different AHR responses. Despite the difference in IgG and IgE Ab responses, both orally and nasally sensitized mice exhibited similar baseline AHR 6 hours after the last nasal peanut challenge with Penh

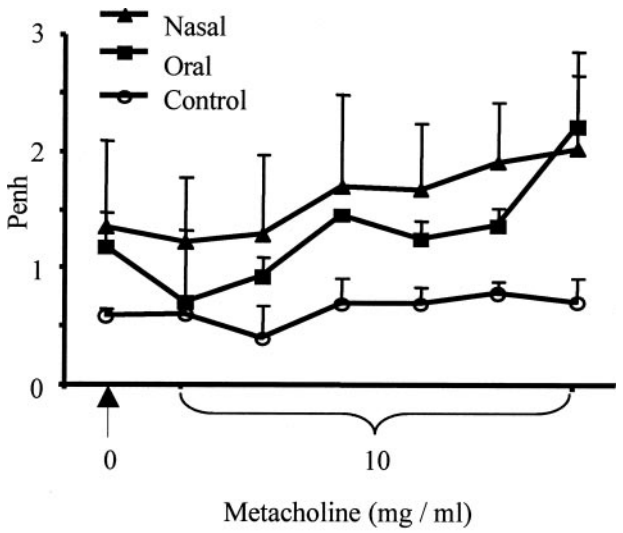

Figure 2. Airway hyperreactivity after nasal challenge of orally or nasally sensitized mice. Baseline Penh was measured 6 hours after the last peanut challenge in mice sensitized to peanut by the oral or nasal route. Penh values of AHR to metacholine were measured every minute over 7 minutes. Controls included sham-sensitized and sham-challenged mice. Values are expressed as means ± SD of four mice.

values of 1.1 ± 0.31 and 1.36 ± 0.72, respectively (Figure 2). These Penh values were significantly higher than those of control sham-sensitized mice (0.57 ± 0.06). In addition, both orally and nasally sensitized mice showed a similar increase of Penh responses to metacholine challenge (Figure 2). Although the difference of Penh values failed to reach statistical difference, nasally sensitized mice consistently exhibited higher Penh than their counterparts sensitized by the oral route.

Nasal Sensitization Promotes Higher Lung Inflammatory Responses

Lungs of orally sensitized mice showed no sign of inflammation when analyzed 10 days after the last administration of PPE and CT (mean score 1; Figure 3, A and C). In contrast, nasally sensitized mice exhibited a moderate inflammation (mean score 3.3; Figure 3, A and C). Furthermore, the cell density was higher in BALF of nasally sensitized mice ($\approx 20 \times 10^4$ cells/ml) when compared with orally sensitized mice ($\approx 6 \times 10^4$) (Figure 3A). Nasal peanut challenge induced a massive recruitment of polymorphonuclear cells in the BALF of both groups of mice (Figure 3, B and C). The cell density in BALFs of nasally sensitized mice were more than 10-fold higher (400×10^4 cells/ml) than that seen in BALFs of orally sensitized mice (30×10^4 cells/ml) (Figure 3B). In addition, lung inflammation was significantly higher in nasally sensitized mice than in those orally sensitized (mean inflammation scores of 7.3 and 5.1, respectively) (Figure 3, B and C). Lung inflammation was not seen after nasal peanut challenge of control naïve mice or mice given CT only by either the oral or nasal route (not shown).

Tissue sections were also subjected to the cyanide-resistant peroxidase staining to further characterize the recruitment of eosinophils in the lungs after the nasal peanut challenge. Eosinophils were not detected in the lungs of control PBS-challenged or unchallenged mice (results not shown). Significant lung eosinophilia was observed in the lung of mice sensitized by the oral route (Figure 3D). In contrast, nasal peanut challenge promoted only rare eosinophils but significant neutrophilia in the lung of nasally sensitized mice (Figure 3D).

Nasal Peanut Challenge Induces Higher Th2-Type Cytokine Responses in the Lungs of Orally Sensitized Mice

Cytokine-specific mRNA responses were analyzed on whole-lung tissues before and after nasal challenge with PPE. Before the nasal challenge, lung tissues of nasally and orally sensitized mice exhibited similar levels of both Th1 and Th2 cytokine mRNA, including IL-1β mRNA followed by CCL-11 and IL-17 and lower levels of IL-5, IL-13, IFN-γ, and IL-4 (Figure 4A). Nasal peanut challenge significantly increased IL-4, IFN-γ, and CCL-11 mRNA in the lungs of mice sensitized by either the oral or nasal routes (Figure 4A). Interestingly, the increase of mRNA for Th2-associated cytokines IL-4 and CCL-11 was higher in orally sensitized mice (oral-to-nasal CP ratio of 2.33 and 1.3, respectively). On the other hand, mRNA levels of the Th1-associated cytokine IL-17 were higher in nasally sensitized mice (nasal-to-oral CP ratio of 2.27).

We next analyzed the contribution of $CD4^+$ T cells to lung cytokine and chemokine responses after nasal peanut challenge. Purified lung $CD4^+$ T cells from nasally sensitized mice expressed significantly higher mRNA levels of the Th1-associated cytokine IFN-γ (Figure 4B). On the other hand, higher mRNA levels of the Th2-associated cytokines IL-4 and CCL-11 were seen in lung $CD4^+$ T cells from orally sensitized mice (Figure 4B).

Cytokine responses to nasal peanut challenge were also analyzed at the protein levels in BALFs. Before challenge, the BALFs of mice orally or nasally sensitized with PPE showed low and similar levels of IL-4 and IL-6 (Figure 4C). Nasal peanut challenge significantly increased IL-4 secretion in BALFs of orally sensitized mice but not in samples from those nasally sensitized. In contrast, only nasally sensitized mice showed significantly increased IL-1β and IL-6 secretion in BALFs on nasal peanut challenge (Figure 4C).

Nasal Peanut Challenge Induces a Massive Recruitment of MAC-1^+ I-$A^{b\ low}$ Cells in the Lungs of Nasally Sensitized Mice

Because IL-1 and IL-6 responses after nasal peanut challenge were much higher in BALFs of nasally sensitized mice, we more carefully examined the phenotype of cells that contributed to these responses. Before the nasal peanut challenge, the lungs of orally and

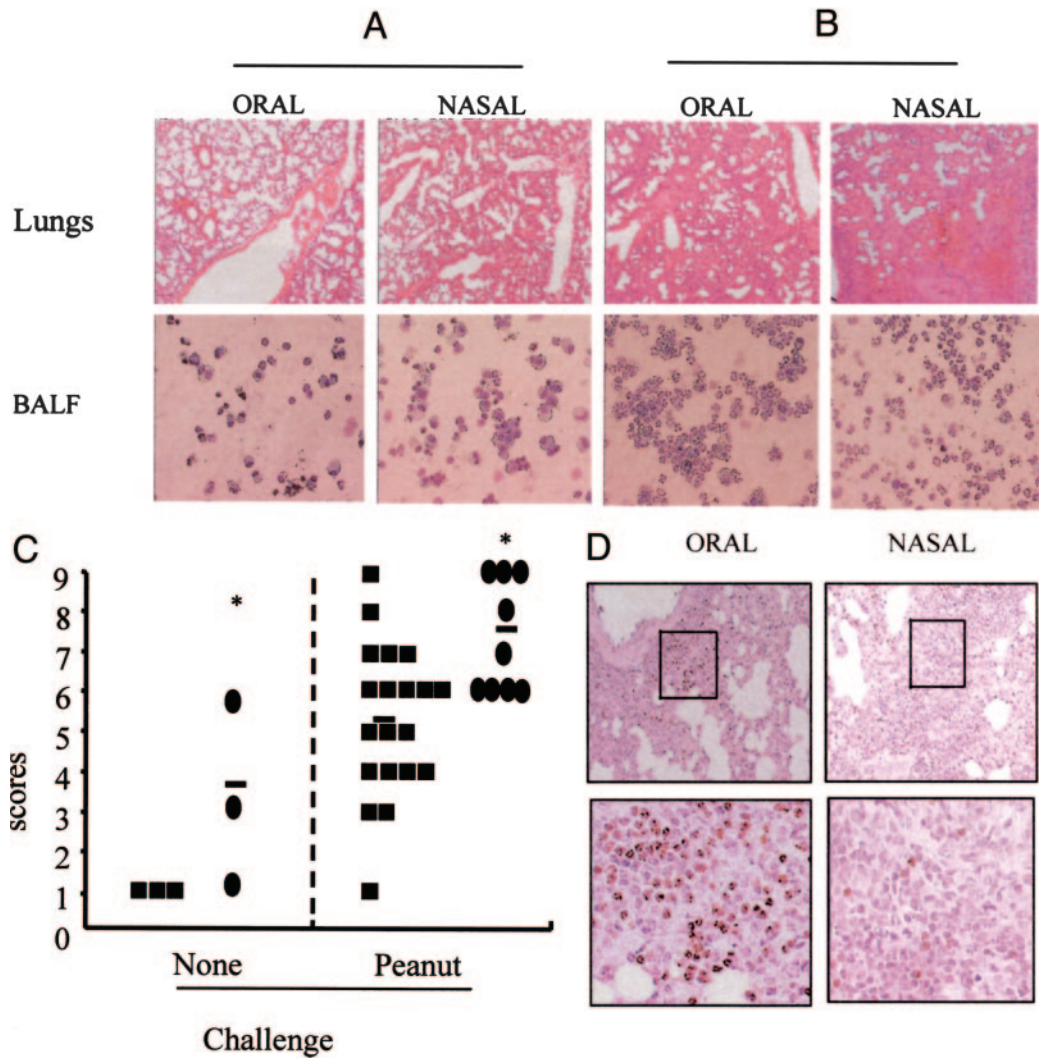

Figure 3. Cell recruitment in lung and BALFs of mice orally or nasally sensitized to peanut. Mice were given PPE and CT as adjuvant on days 0 and 7 by the nasal (5 μl per nostril) or oral (250 μl) route. **A:** Lung tissue (**top**) and BALFs (**bottom**) were collected 10 days after the last sensitization and before challenge. **B:** Lungs and BALFs were collected on day 17 after nasal peanut challenges on days 15 and 16. Histology sections were stained with hematoxylin and eosin (magnification ×40), and cytospin preparations of BALFs were stained with Giemsa (magnification ×200). The BALFs from nasally sensitized mice depicted in this panel were diluted 10-fold before the cytospin. **C:** Lung inflammation scores on histology sections. The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1, minimal infiltrate; 9, massive infiltrate). Boxes represent mice given peanut proteins orally, whereas ovals represent nasal administration. **D:** Lung eosinophilia after nasal challenge of mice orally or nasally sensitized with PPE. The eosinophils were visualized by peroxidase staining of histological sections pretreated with KCN (magnification: top, ×100 and bottom, ×200).

nasally sensitized mice exhibited the same frequency (ie, 9%) of $CD11b^+$ (MAC-1) in the lungs (Figure 5A). Nasal challenge induced macrophage recruitment in the lungs, and their percentage rose to ~30% and ~60% in orally and nasally sensitized mice, respectively (Figure 5B). We also observed a difference in the phenotype of $CD11b^+$ cells recruited in the lung after the nasal challenge. Thus, whereas one-third of $CD11b^+$ cells recruited in the lung of orally sensitized mice expressed high levels of MHC class II molecules ($I\text{-}A^b$), only a small fraction (one-tenth) of those recruited in the lung of nasally sensitized mice expressed this phenotype associated with activated macrophages (Figure 5B).

Lung Inflammatory Responses of Mice Mucosally Sensitized with Peanut after Nasal Challenge with Unrelated Antigens

Peanut allergy is frequently associated with pollen allergy,[9–12] and peanut allergens could share sequence homology with environmental antigens.[13] Therefore, we next investigated whether the change of airway environment induced by oral versus nasal sensitization to peanut would influence inflammatory responses to unrelated food or environmental proteins. Interestingly, mice sensitized to peanut by the oral and nasal route differentially reacted to nasal challenge with unrelated proteins. Thus, low levels of lung inflammation (ie, mean inflammation

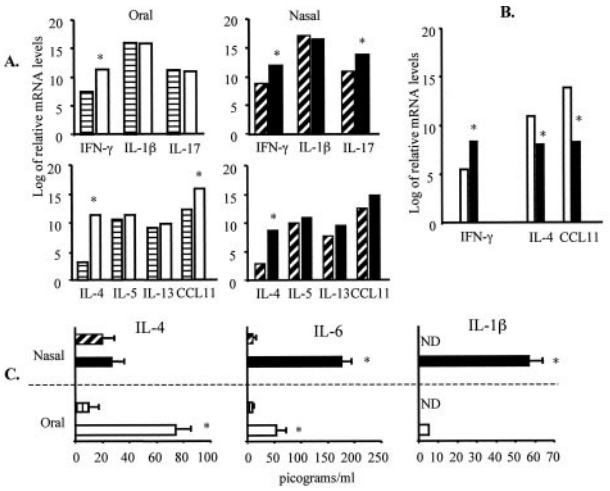

Figure 4. Cytokine responses in lungs and BALFs of orally or nasally sensitized mice. **A:** Relative cytokine mRNA expression in whole-lung tissue before (**dotted pattern**) or after (**open** or **solid pattern**) nasal peanut challenge. **B:** Relative cytokine mRNA expression by $CD4^+$ lung T cells collected after nasal peanut challenge of mice sensitized by the oral (□) or nasal (■) routes. All of the results were normalized against β-actin expression to correct for differences in cDNA concentration of the starting template and are expressed as $20 - (CP_{cytokine} - CP_{\beta\text{-actin}})$. Differences of crossing points above two cycles are considered significant. The results are representative of three separate experiments. **C:** Cytokine secretion in BALFs collected before (**dotted pattern**) or after (**open** or **solid pattern**) nasal peanut challenge. Cytokine levels were determined by ELISA. The levels of IL-5, IL-10, IL-13, TNF-α, and IFN-γ were below detection limit (ND).

score = 2) were seen when orally sensitized mice were challenged with OVA or Der f proteins. On the other hand, nasal challenge with the unrelated food or environmental antigens induced high lung inflammatory responses (ie, mean inflammation score = 4) in mice previously sensitized to peanut by the nasal route (Figure 6).

Discussion

Food allergies have been mostly investigated by sensitization and challenge of animal models via the subcutaneous, parenteral, or oral routes.[28,38–41] Although peanut allergy has been thus far studied on animal models after oral sensitization or challenge,[31,42] the possibility of allergic reaction after airborne sensitization and/or challenge is now being considered.[43] The mucosal tissues of the GI tract are not readily accessible, making difficult the investigation of mechanisms governing adverse mucosal reactions to food and environmental antigens/allergens. Animal models could be developed to address mucosal mechanisms of allergy by taking advantage of the greater accessibility of the mucosal tissues of the respiratory tract. However, it is now well established that distinct mechanisms govern the homing of effector cells induced in the nasopharyngeal and GI tissues.[44] To date, no animal model has addressed how oral and nasal sensitization to peanut affects allergic manifestations and, more specifically, the cellular and molecular events after nasal challenge with peanut or unrelated antigens. We hypothesized that mucosal sensitization in the presence of CT, a mucosal adjuvant that promotes IgE Ab and Th2-type responses,[34,45,46] could aid the development of mouse models of peanut allergy by either oral or nasal sensitization. We found that oral sensitization of mice induced higher levels of peanut-specific plasma IgE Ab responses and higher lung eosinophilia after nasal challenge with peanut. In contrast, nasal sensitization led to higher levels of peanut-specific plasma IgG Ab responses and increased lung inflammation with a massive recruitment of macrophages after nasal challenge with peanut. More importantly, nasal but not oral peanut sensitization favored inflammatory responses to nasal challenge with unrelated antigens.

Vaccine studies in humans and animal models have shown that the oral and nasal routes of priming promote distinct profiles of IgG and IgA Ab responses in the respiratory and genito-urinary tract.[41,47–49] Few studies have directly compared the profile of serum Ab responses after oral and nasal sensitization to the same

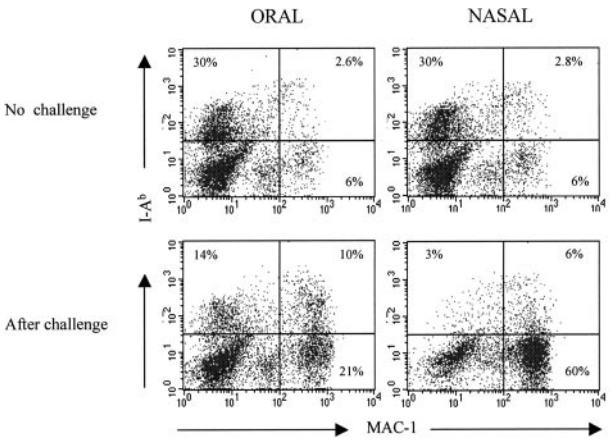

Figure 5. The frequency of MAC-1^+ cells in the lung of mice mucosally sensitized to peanut. Orally or nasally sensitized mice were either challenged or not challenged with PPE on days 15 and 16. Lung tissues were collected on day 17 and digested with collagenase. After purification on a discontinuous Percoll gradient, the cells were collected at the 20 to 75% interface and analyzed by flow cytometry.

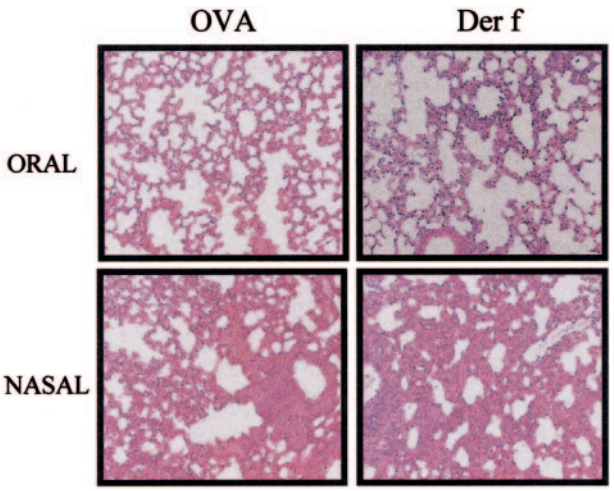

Figure 6. Lung inflammatory responses to nasal challenge with unrelated antigens. Mice were nasally or orally sensitized by administration of PPE and CT as adjuvant on days 0 and 7 and nasally challenged with OVA or the respiratory antigen Der f on days 15 and 16. Lung tissues were collected at day 17, and inflammation was examined on sections stained with hematoxylin and eosin (magnification ×100).

antigen/allergen. Our finding that oral delivery of PPE and CT promotes higher levels of peanut-specific IgE responses than nasal delivery is consistent with earlier reports suggesting that Peyer's patches of the GI tract are preferred sites of IgE Ab responses.[50,51] High IgE responses have been reported after nasal administration of mice with several protein antigens in the presence of CT.[34,52–54] High levels of antigen-specific IgE Abs were also measured when OVA was used instead of PPE in our nasal sensitization studies (data not shown). Peanut contains a large number of proteins, and the biological activity of most of them has not been carefully investigated. Thus, unique biological activity of some peanut proteins may counteract the Th2-inducing effect of the CT adjuvant, as suggested by others.[32] In addition, different subsets of antigen-presenting cells are present in the inductive site of the GI- and nasopharyngeal-associated lymphoid tissues[17] and their respective contribution to IgG and IgE responses remain to be elucidated. Together, these results suggest that oral sensitization with whole PPE and CT is more effective at promoting systemic IgE Abs and Th2-associated responses than when this regimen is delivered by the nasal route.

Antibody and lung inflammatory responses are under the control of cytokines and chemokines in mice[55] as well as in humans.[21,56] For example, the Th2-type cytokines IL-4[57–59] and IL-13 enhance IgE responses,[60] an effect antagonized by IFN-γ.[61] A number of studies have demonstrated that IL-5 and CCL-11 favor mucosal (ie, respiratory and GI) eosinophilic inflammations.[62–64] IL-1, IL-6, and IFN-γ are known for their role in inflammation,[65] and IL-17 is a Th1 cytokine involved in the recruitment of neutrophils into the airways.[66,67] The signs of mild lung inflammation, which were noticeable in nasally sensitized mice before any challenge, were unlikely due to the diffusion of PPE and CT into the lungs. In fact, these observations were made 10 days after sensitization with a small volume of solution that most likely did not diffuse outside the nasal cavity.[33] Furthermore, lung and BALF proinflammatory cytokine responses of mice sensitized orally and nasally were not different before challenge. The evaluation of other parameters such as chemokines may be needed to explain the higher number of cells in the lungs of mice sensitized by the nasal route.

Mice sensitized both orally and nasally to peanut experienced AHR on nasal peanut challenge. Despite recent arguments about the limitation of the whole-body plethysmography as a reliable method for evaluation of lung functions,[68–70] our data show similar alteration of Penh in orally and nasally sensitized mice. However, significant differences were seen in terms of cells infiltrating airway tissues and cytokine responses in the two groups. Thus, nasal challenge significantly increased lung IL-4 and CCL-11 mRNA levels and IL-4 secretion in the BALFs of orally sensitized mice. These findings were consistent with the higher lung eosinophilia seen in these mice and with the previously reported role of IgE for eosinophil-mediated airway hyperactivity.[71,72] Allergic asthma is described as an atopic disease that involves IgE, Th2 responses, and eosinophilic airway inflammation, resulting in enhanced bronchial reactivity.[19] Our results suggest that nasal peanut challenge of mice sensitized by the oral route can lead to lung inflammatory responses that resemble allergic asthma. In contrast with mice sensitized orally, those sensitized by the nasal route showed increased IL-17 and IFN-γ mRNA levels in the whole-lung samples and in infiltrating $CD4^+$ T cells, respectively. These mice also showed increased IL-1 and IL-6 secretion in the BALFs after nasal peanut challenge. Unlike orally sensitized mice, no significant lung eosinophilia was seen after nasal challenge of those nasally sensitized. Our results are consistent with studies suggesting that IgG antibodies counter allergen-triggered eosinophil recruitment.[73] There is now evidence that asthma can also take place in the absence of IgE and eosinophilia. As seen after nasal peanut challenge of mice sensitized by the nasal route, this other form of asthma is characterized by a massive infiltration of neutrophils in the lung.[66,74] It has also been shown that IL-1, IL-6, and IL-17 secreted by epithelial cells and macrophages play a major role in neutrophil-associated asthma.[75,76] In this regard, the leading cellular event after nasal challenge of mice sensitized by the nasal route is a massive recruitment of macrophages in the lung. Taken together, these results suggest that IgE-mediated asthma is induced after nasal challenge of mice orally sensitized with PPE, whereas non-IgE-mediated asthma occurred in those sensitized by the nasal route.

As indicated earlier, peanut allergy is frequently associated with pollen allergy,[9–12] and peanut allergens could share sequence homology with environmental antigens.[13] A recent report showed that Th2-type responses induced in the GI tract can influence immunophysiological responses in distant noninflamed mucosal tissue and regulate airway responsiveness.[77] Therefore, it was important to determine whether the immune status resulting from oral or nasal sensitization with peanut affected airway responses to unrelated antigens. Interestingly, only mice sensitized to peanut by the nasal route exhibited lung inflammatory responses to nasal challenges with unrelated antigens, including OVA, without known similarities with peanut proteins. This finding strongly suggests that the cytokine environment and subsequent innate responses dictate the potential of nonspecific airway inflammation of mice sensitized by the nasal route. Of interest, these results are consistent with the greater level of inflammatory cytokine responses in the lungs (ie, IL-17 and IFN-γ mRNA) and BALFs (ie, IL-6) in these mice. Our separate studies suggest that the dose of peanut used for the nasal sensitization does not explain the tendency of nasally sensitized mice to develop nonspecific inflammatory responses. It has been reported that Der f protein extracts stimulate macrophages and inflammatory responses.[78] Our results suggest that peanut protein extracts can also stimulate cells in the upper respiratory tract and trigger a state of pro-inflammation. These data indicate that nasal sensitization with peanut leads to a lasting proinflammatory status, which could modify bronchial reactivity and favor nonspecific lung inflammation to unrelated environmental antigens.

In summary, we have shown that peanut sensitization via the oral and nasal routes leads to distinct inflamma-

tory responses to subsequent exposure to peanut in the respiratory tract, with mice sensitized by the oral route more prone to allergic-type responses. Nasal sensitization on the other hand, favors nonallergic inflammation and innate responses to unrelated environmental antigens. These findings have important implication for the development of animal models that more accurately reflect allergic pathologies in humans.

Acknowledgments

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ARTICLE 2

Th1 and Th2 cells are both required for eosinophil-associated and neutrophil-associated asthma in the mouse

En préparation

Th1 and Th2 cells are both required for eosinophil-associated and neutrophil-associated asthma in the mouse

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Abbreviations: AHR, airway hyper-reactivity; CT, cholera toxin; KO, knock-out; OS, orally sensitized; NS, nasally sensitized; PPE, peanut protein extracts; WT, wild-type.

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Abstract

Objective: Although two distinct inflammatory subtypes of asthma have been described in patients, the vast majority of current animal models mimic the features of eosinophil-mediated asthma. In a previous study, we succeeded in developing mice models of both types of asthma, by nasal challenge of mice sensitized either orally or nasally with whole peanut protein extract in the presence of cholera toxin. We now aimed at characterizing the role of Th1 and Th2 cytokines in the two models. **Methods**: Our protocols of nasal or oral sensitization were tested in mice deficient in Th1 (IL-12 and IFN-g) or Th2 (IL-4 and IL-13) cytokines. Serum antibody responses to peanut sensitization and lung inflammatory responses to peanut challenge were compared in wild-type and cytokine knock-out mice. **Results**: In the model of eosinophil-associated asthma, antibody responses were primarily controlled by Th2 cytokines, whereas they were primarily controlled by Th1 cytokines in the model of neutrophil-associated asthma. Furthermore, both Th1 and Th2 environments were required during priming for full lung reactivity in each model. **Conclusion**: By reproducing the features of the human diseases, our mouse models should prove useful to investigate the mechanisms involved in eosinophil- and neutrophil -associated asthma.

Introduction

Asthma, a pulmonary disease characterized by airway inflammation and increased airway responsiveness to a variety of stimuli [1], is a major health problem that affects about 12 million people in the United States. Th2 cells have been long recognized as having a key function in the pathogenesis of asthma by producing IL-4, IL-5, IL-9 and IL-13, and leading to IgE-mediated eosinophil inflammation of the lungs [2]. But there is now increasing evidence that asthma can also take place in the absence of IgE and eosinophilia. This other form of asthma is characterized by a crucial role of Th1 cells, which regulate the production of a number of cytokines including IL-1, IL-8 and IL-17, and lead to neutrophil infiltration in the lungs [3-5]. In the majority of current murine models of asthma, animals are sensitized by intraperitoneal injection of ovalbumin together with a Th2-skewing adjuvant, before being intranasally challenged [6, 7]. This protocol results in IgE production, eosinophil recruitment in the airways and hyperresponsiveness, thus making it possible to thoroughly investigate the role of the Th2 pathway in asthma. However, only few studies [8-10] have developed animal models of Th1-induced airway inflammation and the mechanisms involved in this process remain to be elucidated.

We have recently developed two mice models of asthma, by nasal challenge of either orally or nasally sensitized mice. Whole peanut protein extract and the adjuvant cholera toxin were used in both protocols. Orally sensitized (OS) mice displayed an asthma phenotype featuring Th2-induced AHR, associated with high levels of serum peanut-specific IgE, and a lung environment characterized by high eosinophilia and the predominance of Th2-type cytokines. On the other hand, nasally sensitized (NS) mice displayed an asthma phenotype featuring Th1-induced AHR, associated with a mixed serum Ab profile, and a lung environment characterized by high neutrophil and macrophage infiltration and the predominance of inflammatory cytokines. Of note, although cytokines from one or the other

Th subset were predominantly secreted in each of our mouse model, both types were expressed in the lungs, in the same manner that both Th1 and Th2 T cells can be found in the lungs of asthmatic patients [11, 12]. Further, OS and NS mice showed no difference in lung cytokine mRNA levels before nasal challenge. Thus, we hypothesized that both Th1 and Th2 environments are required during the oral or nasal priming to peanut for lung reactivity to nasal challenge. In the present study, we aimed at verifying this hypothesis and tested our two sensitization protocols in mice deficient in IL-4 or IFN-γ, which are cytokines playing critical roles in the induction and regulation of the Th2 and Th1 pathways, respectively. We also used IL-4/IL-13 double KO and IL-12 KO mice in order to evaluate the roles of these cytokines in our models of Th1- and Th2-type induced asthma.

Materials and Methods

Mice

Female IL-4 KO, IL-12 KO and IFN-γ KO mice, generated on a C57BL/6 background, and female IL-4/IL-13 double KO mice, generated on a BALB/c background, were purchased from the Jackson Laboratories and provided to the immunocompromised-mouse facility in the University of Alabama at Birmingham Immunobiology Vaccine Center. Control wild-type C57BL/6 and BALB/C mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water *ad libitum*. Studies were performed in accordance with Institutional guidelines to avoid pain and distress.

Mucosal sensitization

Whole peanut protein extracts (PPE) were obtained as previously described by ammonium bicarbonate treatment of defatted peanut extracts [13]. Mice 8 to 12 weeks of age were sensitized two times a week apart with whole PPE and CT as adjuvant. Anesthetized mice were nasally administered 100 μg of PPE and 1 μg of CT in a total volume of 10 μl with 5 μl placed into each nare. This volume of the nasal vaccine is retained in the nasal cavity after nasal administration to anaesthetized mice [14]. For sensitization by the oral route, mice were deprived of food for two hrs, and then orally treated with 250 μl of sodium bicarbonate as previously described [15]. Oral sensitization consisted in intragrastric administration of 1 mg of PPE plus 15 μg of CT in 250 μl of PBS. Plasma samples were collected one week after each sensitization on day 7 and 14 for analysis of peanut-specific Ab responses.

Nasal challenge with peanut

Mice nasally or orally sensitized to peanut were nasally challenged on days 15 and 16 with 200 μg of PPE in a total volume of 100 μl. More specifically, anesthetized mice were given 25 μl of PPE per nare, four times at 2-3 min intervals.

Plasma antibody responses

Plasma levels of peanut-specific Abs were measured by ELISA. Briefly, 96-well microplates (Falcon) were coated with 50 μg/ml of PPE in PBS and incubated overnight at 4ºC. After blocking with PBS-1% BSA, serial dilutions of plasma samples were added and incubated overnight at 4ºC. Peanut-specific IgG Abs were detected using 0.3 μg/ml of HRP-labeled goat anti-mouse γ-heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL). Biotin-conjugated rat anti-mouse γ 1 (clone A85-1), γ2a (clone R19-15), γ2b (clone R12-3) or γ3 (clone R40-82) heavy chain mAbs (BD PharMingen, San Diego, CA) were employed at 0.5 μg/ml and streptavidin-HRP (BD PharMingen) was diluted at 1:2000 for the detection of peanut-specific IgG subclasses. The colorimetric reaction was developed by the addition of 2,2'-azino-bis (3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma) and H_2O_2. Endpoint titers were expressed as the $\log_2$ of plasma dilution giving an optical density at 415 nm of ≥ 0.1 above those obtained with control plasma.

The removal of IgG has been shown to improve the detection of IgE Abs [16]. Thus, dilutions of plasma samples were first depleted in IgG by overnight incubation at 4ºC in protein G coated 96-well plates (Reacti-Bind plates, Pierce, Rockford, IL). Total and antigen-specific IgE levels were then analyzed by ELISA. For detection of antigen-specific IgE Abs, IgG-depleted samples were added to ELISA plates coated with PPE (50 μg/ml, 100 μl per

well). The IgE were detected with 0.5 μg/ml biotin-conjugated rat anti-mouse IgE (clone R35-118; BD PharMingen) followed by streptavidin-HRP (1:2000). The levels of total IgE Abs were determined using capture and detection antibodies, as well as IgE standard, from the BD OptEIA Set mouse IgE kit (BD Biosciences, San Diego, CA).

Histology and determination of lung inflammation scores

Lungs were fixed in 10 % buffered formaldehyde, paraffin-embedded and cut into sections of 5 μm thickness. The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin for the evaluation of inflammation. The presence of eosinophils in tissue sections was determined by the cyanide-resistant peroxidase activity as previously described [17]. Briefly, lung sections were incubated for 1 min at room temperature in 10 mM KCN pH 6.5. Slides were then rinsed in PBS and incubated for 15 min with the peroxidase substrate 3,3'-diaminobenzine (Vector Laboratories, Burlingame, CA). After washes in PBS, tissue sections were counterstained with hematoxylin.

For quantification of lung inflammation, the slides were coded, and peribronchial and perivascular inflammation was scored in a blinded fashion by two independent investigators. A value of 1 was given when slides showed no sign of inflammation. Slides were graded from 2 to 4 when bronchi were surrounded by a thin layer of inflammatory cells (2: few bronchi; 3: more bronchi: 4: most bronchi). They were graded from 5 to 7 according to the number of bronchi that were surrounded by a thick layer of inflammatory cells (5: few bronchi; 6: more bronchi: 7: most bronchi). Finally, slides were graded 8 or 9 when inflammation spread into the interstitial area (8: severe; 9: extreme).

Flow cytometry

Whole lung tissue was dissociated by digestion with 1 mg/ml of collagenase type V (Sigma) in RPMI-1640 (Cellgro Mediatech, Washington, DC), supplemented with 10 mM HEPES, 2 mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (RPMI supplemented) to obtain single cell preparations. Mononuclear cells were collected at the 20-75% interface of discontinuous Percoll gradient and stained with anti-CD3 (clone 145-2C11), anti-CD4 (clone GK1.5), anti-B220 (clone RA3-6B2), anti-CD11c (clone HL3), anti-MAC-1 (clone M1/70) or anti-MHC class II Abs ($I\text{-}A^b$, clone AF6-120.1) (BD PharMingen). After washes and fixation, samples were analyzed by flow cytometry.

Statistics

The results are reported as the mean + one standard deviation (SD). Statistical significance ($p < 0.05$) was determined by Student's t test and by the Mann-Whitney U test of unpaired samples. The results were analyzed using the Instat statistical program (San Diego, CA) for Apple computers.

Results

1) Th1 and Th2 cytokines influencing the Ab response to peanut sensitization

Serum antibody responses to peanut sensitization in C57BL/6 WT and IL-4 KO mice

Consistent with the role of IL-4 in IgE class switching, peanut-specific IgE Abs were not detected in IL-4 KO mice after either oral or nasal immunization (Figure 1A). The peanut-specific IgG response was found to be of similar magnitude in WT and IL-4 KO mice, but to differ in isotype distribution (Figure 1B). In fact, both OS and NS mice displayed significantly lower peanut-specific IgG1 compare to WT mice. While NS mice had the same levels of peanut-specific IgG2a, IgG2b and IgG3 in the presence or absence of IL-4, there was an increase of peanut-specific IgG2a and IgG2b in OS IL-4 KO mice. Thus, IL-4 differentially regulated the levels of peanut-specific IgG sublasses in OS and NS mice. Involved only in IgE and IgG1 production in NS mice, it modulated the levels of all Abs except IgG3 in OS mice.

Serum antibody responses to peanut sensitization in BALB/c WT and IL-4/IL-13 double KO mice

As IL-4/IL-13 KO mice from the C57BL/6 background were not available, we used BALB/c mice in order to investigate the effect of IL-4 and IL-13 deficiency in our models. We first asked whether distinct immune responses to oral or nasal peanut were induced in BALB/c mice, as they were previously described in C57BL/6 mice. Figure 2A shows that our protocols of oral and nasal sensitization induced lower IgE levels in BALB/c mice than in C57BL/6 mice. This was unlikely the result of an unsufficient immune response in BALB/c mice as the extent of the total peanut-specific IgG response was similar in both strains

(results not shown). Similar to what was observed in C57BL/6 mice, peanut-specific IgE levels were significantly higher after oral sensitization of BALB/c mice compare to nasally sensitized mice. In addition, the difference observed between OS and NS C57BL/6 mice in their IgG1 / IgG2a ratio was maintained in BALB/c mice. Thus, IL4/IL-13 KO mice from the BALB/c background were suitable in our study.

As for IL-4 KO mice, no total or peanut-specific IgE were detected in double KO mice (results not shown). Figure 2B shows that unlike in IL-4 KO mice, there was a decrease in the overall IgG response after both oral and nasal sensitization of double KO mice compare to their WT counterparts. Consequently, there was a reduction in all subclass levels. However, IgG1 levels diminished most dramatically, making the IgG1/IgG2a ratio significantly lower in double KO mice compare to WT mice (0,99 +/- 0,17 and 1,3 +/- 0,12 respectively, in OS mice; 0,86 +/- 0,06 and 1,07 +/- 0,05 respectively, in NS mice). Thus, compensatory mechanisms made it possible for the Th1 component to develop in IL-4/IL-13 KO mice.

Serum antibody responses to peanut sensitization in C57BL/6 WT, IL-12p40 KO and IFN-γ KO mice

Unexpectedly, there was no increase in IgE levels of either IL-12p40 KO and IFN-γ KO mice compare to their WT counterparts (results not shown).

Figure 3A shows that OS IL-12p40 KO mice displayed lower levels of peanut-specific IgG3 than WT mice. It was interesting to note that peanut-specific IgG3 levels decreased some more in WT mice orally sensitized with a high dose of CT, whereas levels of other IgG-subclasses remained unchanged (results not shown). As CT has been shown to reduce the expression of IL-12 receptor (Braun MC, 1999 J Exp Med), both results in WT

and OS IL-12p40 KO mice suggested that peanut-specific IgG3 levels were dependent of IL-12 in OS mice.

Figure 3B shows that NS IL-12p40 KO mice had lower levels of peanut-specific IgG1 and IgG2b. As peanut-specific IgG3 levels were unaffected, it is possible that nasal sensitization induced a Th1 environment able to compensate for the absence or the reduced levels of IL-12.

Unlike in IL-12p40 KO mice, IgG2a levels were below detection in both OS and NS IFN-γ KO mice (Figure 3), confirming that IFN-γ KO mice display a complete loss of the Th1 component. Levels of other subclasses remained unchanged in OS IFN-γ KO mice, while peanut-specific IgG3 increased and peanut-specific IgG2b decreased in NS IFN-γ KO mice. Thus, IFN-γ differentially regulates the levels of IgG sublasses in OS and NS mice. Involved only in IgG2a production in OS mice, it modulated the levels of all Abs measured except IgG1 in NS mice. In particular, the high levels of IgG2b observed in WT mice after nasal but not oral sensitization may be dependant on IFN-γ.

2) Th1 and Th2 cytokines influencing lung inflammatory responses to peanut secondary exposure

Lung inflammatory responses to peanut secondary exposure in IL-4 KO and IL-4/IL-13 double KO mice

As described previously, lungs of OS WT mice showed no sign of inflammation before challenge, while NS mice exhibited a moderate inflammation (Figure 4A). Inflammation scores were not significantly different in IL-4 KO mice. In contrast, lung cell recruitment was higher than that of unchallenged WT mice in IL-4 / IL-13 KO mice, and

differences between NS and OS mice were attenuated. After challenge (Figure 4B), there was a reduction of inflammation in both OS and NS IL-4 KO mice compare to their WT counterparts. But such attenuation was not observed in double KO mice. Thus, while partial abolition of the Th2 pathway induced a weakening of the inflammatory reaction, its stronger impairment in double KO mice may have resulted in the development of the Th1 component, as suggested by the IgG-subclass profile, and induced a pro-inflammatory state in both OS and NS mice, which was noticeable before challenge and made it possible to maintain a substantial lung inflammation after challenge.

The lung mononuclear cell composition after nasal challenge of BALB/c WT and IL-4/IL-13 KO mice is shown in Figure 5. As in C57BL/6 WT mice, the major event induced by the challenge of BALB/c WT mice was the recruitment of macrophages, representing 50 and 30% of mononuclear cells of NS and OS mice, respectively. Consistent again with results in C57BL/6 mice, a higher percentage of lung cells were Iab-positive in OS than in NS mice. The most striking effects of the lack of IL-4 and IL-13 was the weakening of the macrophage recruitment in NS mice after peanut challenge and the considerably lower expression of costimulatory molecules in both groups of mice.

Lung inflammatory responses to peanut secondary exposure in IL-12 KO and IFN-γ KO mice

Before challenge (Figure 6A), there was no sign of inflammation in OS WT, IL-12 KO or IFN-γ KO mice. The moderate lung inflammation observed in both WT and IL-12 KO NS unchallenged mice, was abrogated in IFN-γ KO NS unchallenged mice. After challenge (Figure 6B), the absence of IL-12 resulted in a substantial reduction of airway inflammation in OS mice, whereas it was reduced but still significant in NS mice. Unlike IL-12, IFN-γ absence resulted in a complete abrogation of lung inflammation in NS mice, while airway inflammation was only partially reduced in OS mice.

Discussion

Although two distinct inflammatory subtypes of asthma have been described in patients, the vast majority of current animal models mimic the features of eosinophil-mediated asthma. The few studies that aimed at describing a Th1-induced airway inflammation used adoptive transfer strategies that were quite artificial and lead to conflicting data [8-10]. As a result, while the role of Th2 cells in asthma is quite well documented, the influence of Th1 cells and interactions between Th1 and Th2 cells in asthma remain unclear. The mechanism of action of neutrophils inducing airway hyperresponsiveness is also unknown [18]. In a previous study, we succeeded in promoting a Th1-polarised and a Th2-polarised lung inflammation in mouse models of asthma by using a physiological approach. In the present study, we show that both Th1 and Th2 pathways are required in our models for full lung reactivity, while confirming a more important role of IL-4 and IFN-γ in the eosinophil-associated asthma model and in the neutrophil-associated asthma model, respectively.

Before characterizing the role of Th1 and Th2 cytokines in our models of asthma, we aimed at verifying that it was possible to develop them in another mouse strain. Several investigators used the same protocol of sensitization with OVA and alum in BALB/c and C57BL/6 mice in order to determine the influence of the genetic background in allergen-induced pulmonary responses. Levels of specific IgE and IgG1, Th2 type cytokine mRNA responses as well as lung eosinophilia and hyperresponsiveness were higher in BALB/c mice than in C57BL/6 mice [19-21]. On the contrary, our protocols of oral and nasal sensitization induced higher IgE levels and a higher IgG1/IgG2a ratio in C57BL/6 than in BALB/c mice. In addition, inflammation scores were also higher in C57BL/6 than in BALB/c mice. These

results may be explained by the higher sensitivity of C57BL/6 mice to the mucosal adjuvant activity of CT [22, 23]. Interestingly, distinct immune responses were induced in OS and NS BALB/c mice, as previously described in C57BL/6 mice. In more detail, high levels of serum peanut-specific IgE and a high IgG1/IGg2a ratio was induced in OS mice, while no IgE Abs and a mixed serum Ab profile were measured in NS mice. Consistent again with the results in C57BL/6 mice, NS mice post-challenge lung phenotype was characterized by significantly higher levels of MAC-1+ I-Ab low cells, compare to OS mice. This is in agreement with the fact that alveolar macrophages have been shown to suppress the induction of of IgE responses [24]. Thus, it seemed feasible to develop mice models of Th1-induced and Th2-induced lung inflammation in BALB/c mice, as we did in C57BL/6 mice. This result also made it possible to investigate more closely the role of cytokines in our mouse models in both strains.

Our first important result is that Th2 cytokines play a more important role in OS than in NS mice Ab responses, while being required for full lung reactivity of both models of asthma. It has long been established that IL-4 is a major inducer of IgE [25, 26]. As expected, and in line with many previous data [27-32], no IgE Abs were detected in OS or NS IL-4 KO mice. The role of IL-4 in the magnitude and profile of the IgG response is less clear-cut. Serum IgG responses of IL-4 KO mice to oral Ag administered in the presence of CT were analyzed in two previous studies. Vajdy et al. [32] found no or poor anti-KLH or anti-OVA serum B cell responses and attributed the lack of responsiveness to the failure of IL-4 KO mice to develop germinal centers in PPs. In contrast, the overall extent of the response to tetanus toxoid, a stronger immunogen than KLH or OVA, was similar in WT and IL-4 KO mice [28]. The profile of IgG subclasses observed in the absence of IL-4 was characterized by increased IgG2a and IgG2b Ab responses but no detectable IgG1 levels, suggesting a major role of IL-4 in IgG1 class switching, as shown in other gene-targeting experiments, reviewed

by [33]. We also found that the total Ag-specific IgG response was of similar magnitude in WT and IL-4 KO mice, which may be due to the fact that PPE is a potent allergen. Peanut-specific IgG1 levels, although significantly lower than in WT mice, were still substantial in both groups. It indicates that peanut sensitization in the presence of CT induced IL-4 independent IgG1, which existence has also been demonstrated by Kuhn R, 1991. We originally assumed that the residual IgG1 production may be caused by IL-13, as shown in a previous study where Ag-specific IgG1 levels were below detection in IL-4 and IL-13 double KO mice, whereas they were only partially reduced in IL-4-deficient mice [34]. However, our study in IL-4 and IL-13 double KO mice clearly shows that, if these two cytokines are responsible for the dominance of IgG1 Abs, they are not essential for its production.

Interestingly, our data demonstrate that IL-4 differentially regulates the levels of peanut-specific IgG sublasses in OS and NS mice. In fact, in addition to induce Th2-associated Abs, IL-4 has an inhibitory effect on the Th1-associated IgG2a and IgG2b Abs in OS mice. In contrast, it up-regulates the levels of IgG1 Abs, while having no effect on IgG2a and IgG2b in NS mice. An underlying explanation of such results may be that IFN-γ in NS mice is strong enough to counteract IL-4 inhibitory effects.

IL-4-deficient mice in the model of allergen-induced eosinophilic airway inflammation have given contrasting results. They developed substantially less peribronchial inflammation and eosinophilia than their WT counterparts in some investigations [35-37] while still displaying severe eosinophilic inflammation in other studies [38, 39]. In a similar manner, conflicting results have been reported for the development of allergic asthma in IL-13 KO mice [40, 41]. These discrepancies may be explained by overlapping functions of IL-4 and IL-13, which use a common signaling pathway via the IL-4 receptor a subunit [42] and the subsequent activation of STAT-6 [43] and reveal the need to use IL-4 and IL-13 double knock-out mice in order to clarify the role of the Th2 pathway in this pathology. However, few studies have

used these mice in the model of allergen-induced eosinophilic airway inflammation. To our knowledge, those mice were not used either in models of neutrophil-induced airway inflammation. The attenuation of lung cell recruitment observed in IL-4 KO mice in the two models of our study suggests a key role for IL-4 in both inflammation processes, which action may be mediated by IgE Abs, as indicated by others [44, 45], in OS mice. However, NS mice, which do not display detectable IgE levels, suggest that other mechanisms are involved in IL-4-mediated action on pulmonary inflammation. Unexpectedly, lung inflammation was enhanced in double KO mice. This observation led us to believe that compensatory mechanisms resulting into the development of the Th1 component, as demonstrated by the low IgG1/IgG2a ratio in IL-4/IL-13 KO mice, were stronger in double KO mice, whose Th2 pathway was more impaired than in IL-4 KO mice and lead to a pro-inflammatory state in both OS and NS mice.

Conversely, our second important result is that Th1 cytokines play a more important role in NS than in OS mice Ab responses, while being required for full lung reactivity of both models of asthma. IL-12 is a heterodimeric cytokine made of two subunits, p35 and p40, both of which are required for functional activity [46]. Gene targeting studies that aimed at assessing the role of IL-12 in mouse models of eosinophilic asthma have given contrasting results. In some investigations [47], IL-12 KO mice gave support to the well-known role of IL-12 in promoting Th1 responses and inhibiting Th2 responses [48] by showing enhanced eosinophil recruitment to the airways. However, a recent study demonstrated that IL-12-deficient mice have substantially reduced airway recruitment of eosinophils, compare to their WT counterparts. Furthermore, levels of Ag-specific IgE, IgG1 and IgG2a were the same in both groups of mice [49]. In line with this last study, IL-12p40 KO mice in our system of eosinophilic lung inflammation showed significantly reduced cell infiltration, and levels of

peanut-specific IgE were not increased. Knowing that IL-12 favors the Th1 pathway mainly via the induction of IFN-γ production in WT mice [48, 50] and assuming that this pathway was impaired in IL-12 KO mice, two interpretations may be proposed in order to explain our results. IL-12 KO mice may have overcome the defect of Th1 responses, possibly through the action of IL-18, a cytokine that promotes IFN-γ responses [51]. Alternatively, Th1 cells that are generally believed to only protect against allergic reactions by attenuating the activity of Th2 cells [10] may also support Th2 cell-induced allergic asthma [8, 52, 53]. However, it is also possible that IL-12p40 KO mice were characterized by a weaker Th2 environment compare to WT mice, as suggested by their lower peanut-specific IgE and IgG1 levels. In fact, studies indicate that IL-12p40 homodimers are produced in WT mice and act as antagonists of IL-12 mediated signaling pathway [54]. Consistent with this hypothesis, IL-12p40 has recently been shown to contribute to the generation of a Th2-type environment in a mouse model of allergic diarrhea [55]. Our data also show that IL-12 plays a role in the model of neutrophilic asthma, although not essential since it is only partially reduced in IL-12p40 KO mice.

Unlike IL-12 deficiency, IFN-γ gene targeting made it possible to completely block the Th1 pathway, as shown by the lack of detectable IgG2a Abs. Interestingly, our data demonstrate that IFN-γ differentially regulates the levels of peanut-specific IgG sublasses in OS and NS mice. In fact, IFN-γ up-regulates the levels of IgG2a Abs, while having no effect on other subclasses in OS mice. In contrast, it up-regulates IgG2a levels while having a stimulatory effect on the production of IgG2b and an inhibitory effect on IgG3 levels in NS mice. Given our previous results, we assume that IL-4 in OS mice is strong enough to counteract IFN-γ effects during the primary exposure to the Ag. However, the abrogation of lung cell recruitment in the neutrophil-induced asthma model and its attenuation in the eosinophilic model demonstrates the importance of IFN-γ in both processes during the secondary Ag exposure.

Taken together with the Ab responses of IL-4 KO mice, data from IFN-γ KO mice are consistent with the model that nasal sensitization to peanut favors higher Th1 responses while oral sensitization promotes Th2 responses. They also show that both IL-4 and IFN-γ are important in the regulation of lung eosinophilic or neutrophilic responses during secondary exposure to peanut. However, a limitation to the use of KO mice in our study is that they lack a Th1 or a Th2 cytokine during both the sensitization and challenge phases, making it difficulty to discriminate their role during the primary or secondary exposure to the Ag. The use of anti-cytokine treatment can bypass this problem. But a major disadvantage to injecting neutralizing Abs in vivo is that inactivation of the cytokine may be incomplete or time-restricted. Indeed, a study by Finkelman et al. [56] showed that anticytokine antibodies can act as carrier proteins and prolong the half-life of the cytokine. Thus, cytokine knock-out mice still represented better systems to assess the role of Th1 or Th2 cytokines in our models of asthma.

In conclusion, by reproducing the features of the human diseases, our mouse models should prove useful to investigate the mechanisms involved in both eosinophil-associated and neutrophil-associated asthma.

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Figure legends

Figure 1. Plasma IgE (A) and IgG-subclass (B) responses to oral or nasal peanut sensitization in WT and IL-4 KO mice. C57BL/6 mice were orally or nasally sensitized by administration of PPE and CT on days 0 and 7. Plasma samples were collected on day 14 and Ab responses were analyzed by ELISA. Results of are expressed as Mean +/- SD from 1 group of 5 mice for each condition.

Figure 2. Plasma Ab responses to oral or nasal peanut sensitization in WT and IL-4/IL-13 double KO mice. (A) Total IgE levels and IgG1/IgG2a ratio in orally (white) or nasally (black) peanut sensitized BALB/c and C57BL/6 mice. (B) IgG-subclass responses to oral or nasal peanut sensitization in BALB/c WT and IL-4/IL-13 double KO mice Results of are expressed as Mean +/- SD from 1 group of 5 mice for each condition.

Figure 3. Plasma IgG-subclass responses to oral (A) or nasal (B) peanut sensitization in C57BL/6 WT, IL-12p40 KO and IFN-γ KO mice. C57BL/6 mice were orally or nasally sensitized by administration of PPE and CT on days 0 and 7. Plasma samples were collected on day 14 and Ab responses were analyzed by ELISA. Results of are expressed as Mean +/- SD from 1 group of 5 mice for each condition.

Figure 4. Lung inflammatory responses to oral or nasal peanut in WT, IL-4 KO and IL-4/IL-13 double KO mice. Mice were orally or nasally sensitized by administration of PPE and CT on days 0 and 7. Lung tissue was collected on day 17 before (A) or after peanut nasal challenges on days 15 and 16 (B). The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate). Each box or oval represents an individual mouse.

Figures 5. Lung mononuclear cell phenotype after nasal challenge of BALB/c WT and IL-4/IL-13 KO mice. BALB/c mice were immunized by the nasal (5 □l per nostril) or oral route with PPE and CT as adjuvant on days 0 and 7. Lung tissues were collected on day 17 from mice orally or nasally immunized with PPE and either not challenged or nasally challenged with PPE on days 15 and 16. After collagenase digestion of the tissues and purification on discontinuous Percoll gradient, the cells were collected at the 20-75% interface and analyzed by flow cytometry.

Figure 6. Lung inflammatory responses to oral or nasal peanut in WT, IL-12 KO and IFN-γ KO mice. Mice were orally or nasally sensitized by administration of PPE and CT on days 0 and 7. Lung tissue was collected on day 17 before (A) or after peanut nasal challenges on days 15 and 16 (B). The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1 = minimal infiltrate; 9 = massive infiltrate). Each box or oval represents an individual mouse.

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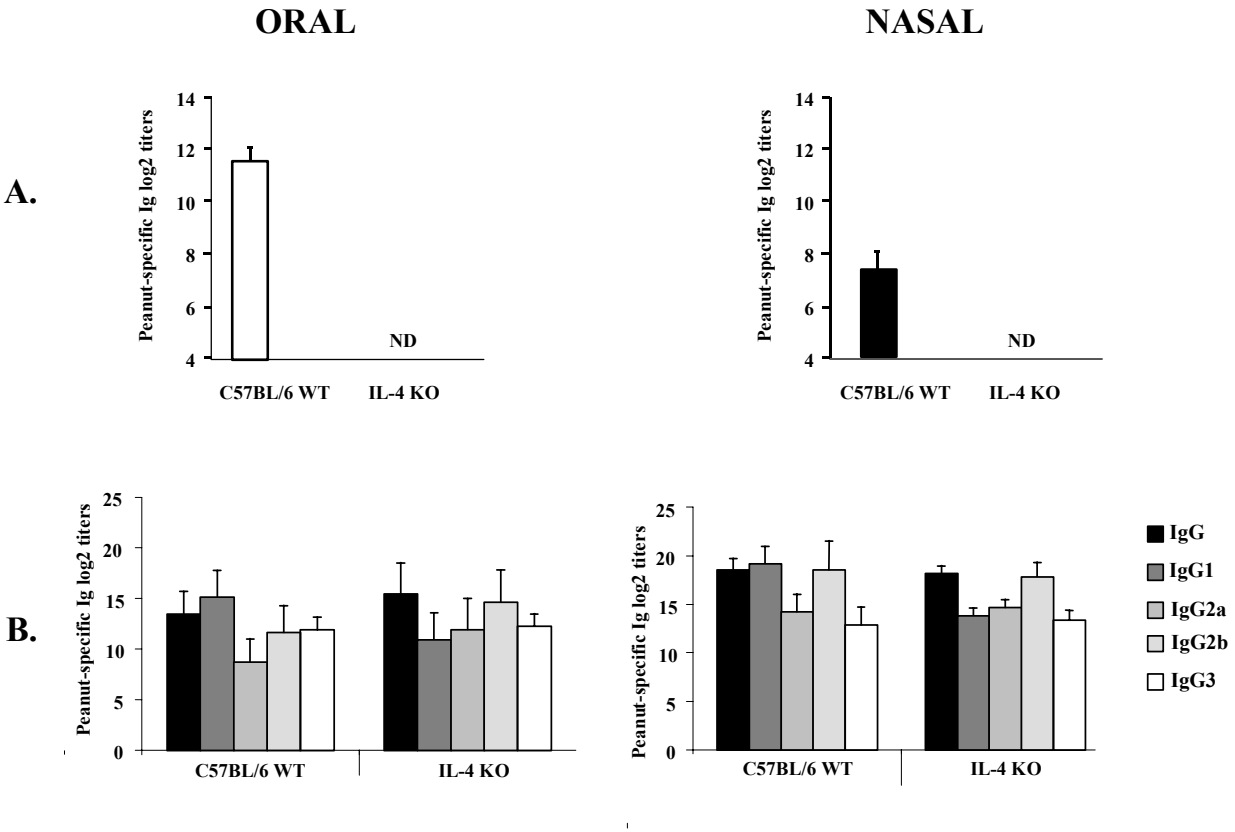
Figure 1


ORAL
NASAL
A.
Peanut-specific Ig log2 titers
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12
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8
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ND
C57BL/6 WT
IL-4 KO
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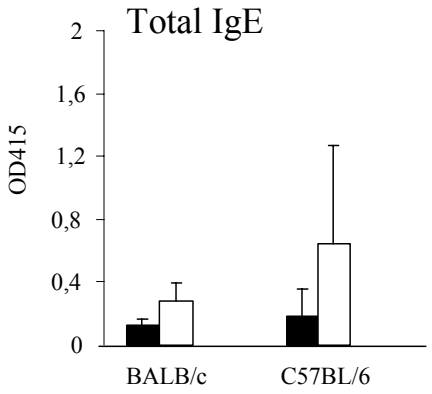
Figure 2

A.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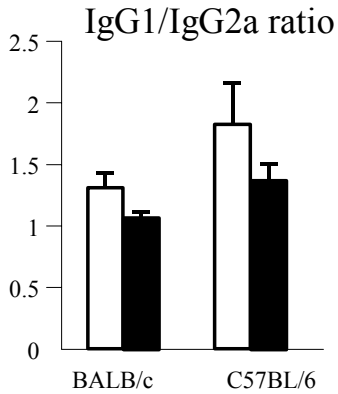
BALB/c
C57BL/6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1.5
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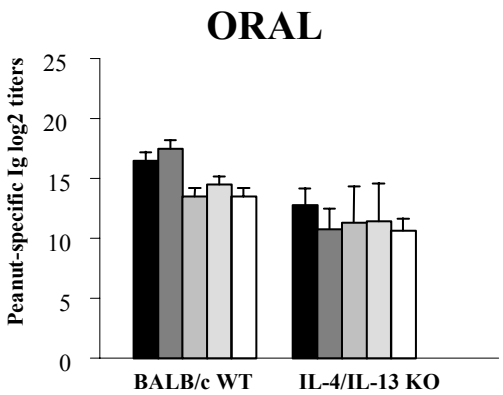
BALB/c
C57BL/6

B.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Peanut-specific Ig log2 titers
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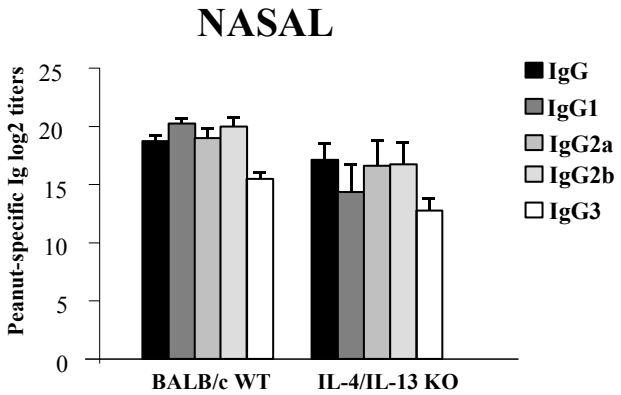
BALB/c WT
IL-4/IL-13 KO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Peanut-specific Ig log2 titers
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BALB/c WT
IL-4/IL-13 KO
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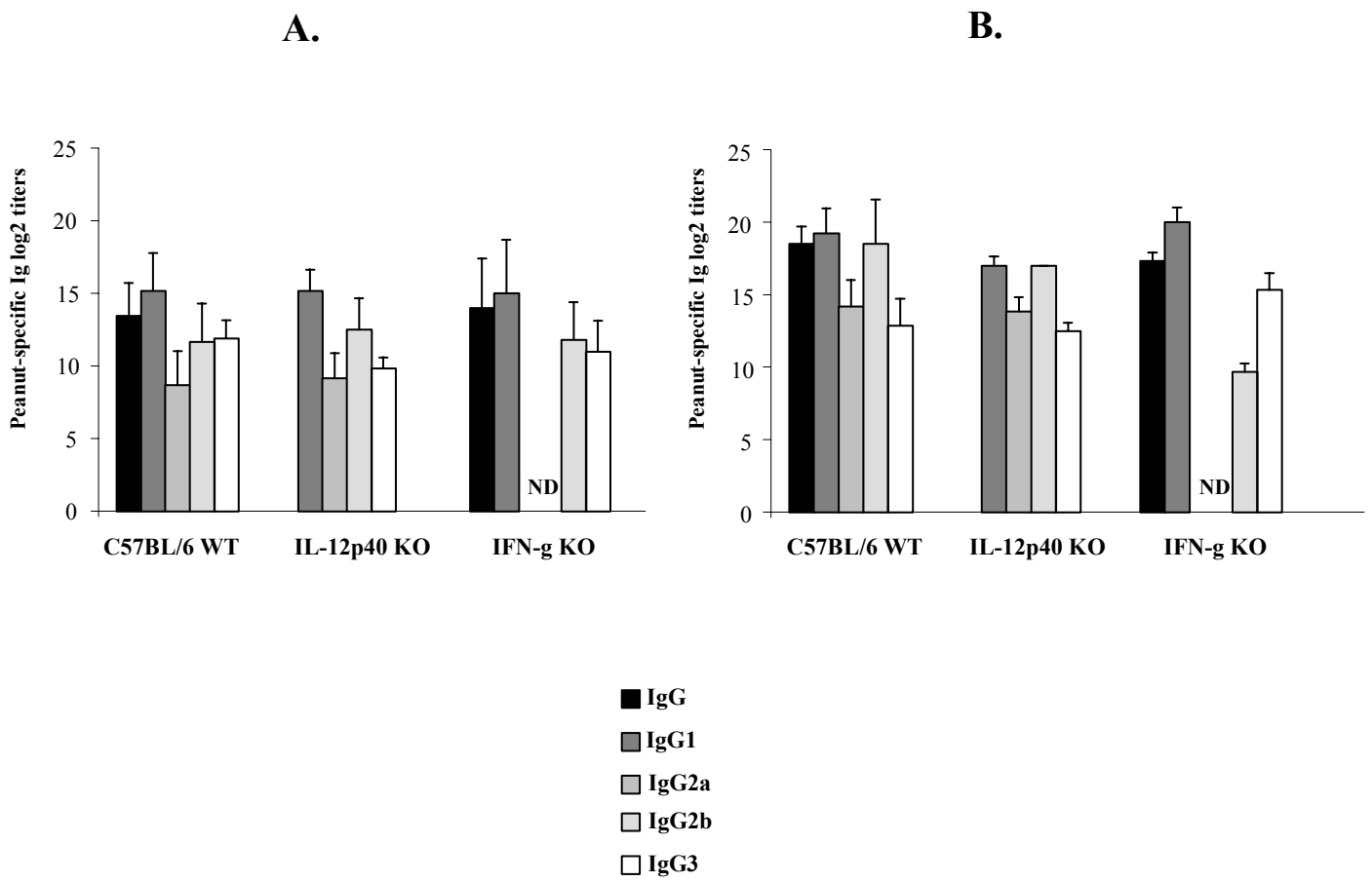
IgG2b
IgG3

Figure 3


A.
B.
Peanut-specific Ig log2 titers
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C57BL/6 WT
IL-12p40 KO
IFN-g KO
IgG
IgG1
IgG2a
IgG2b
IgG3

Figure 4

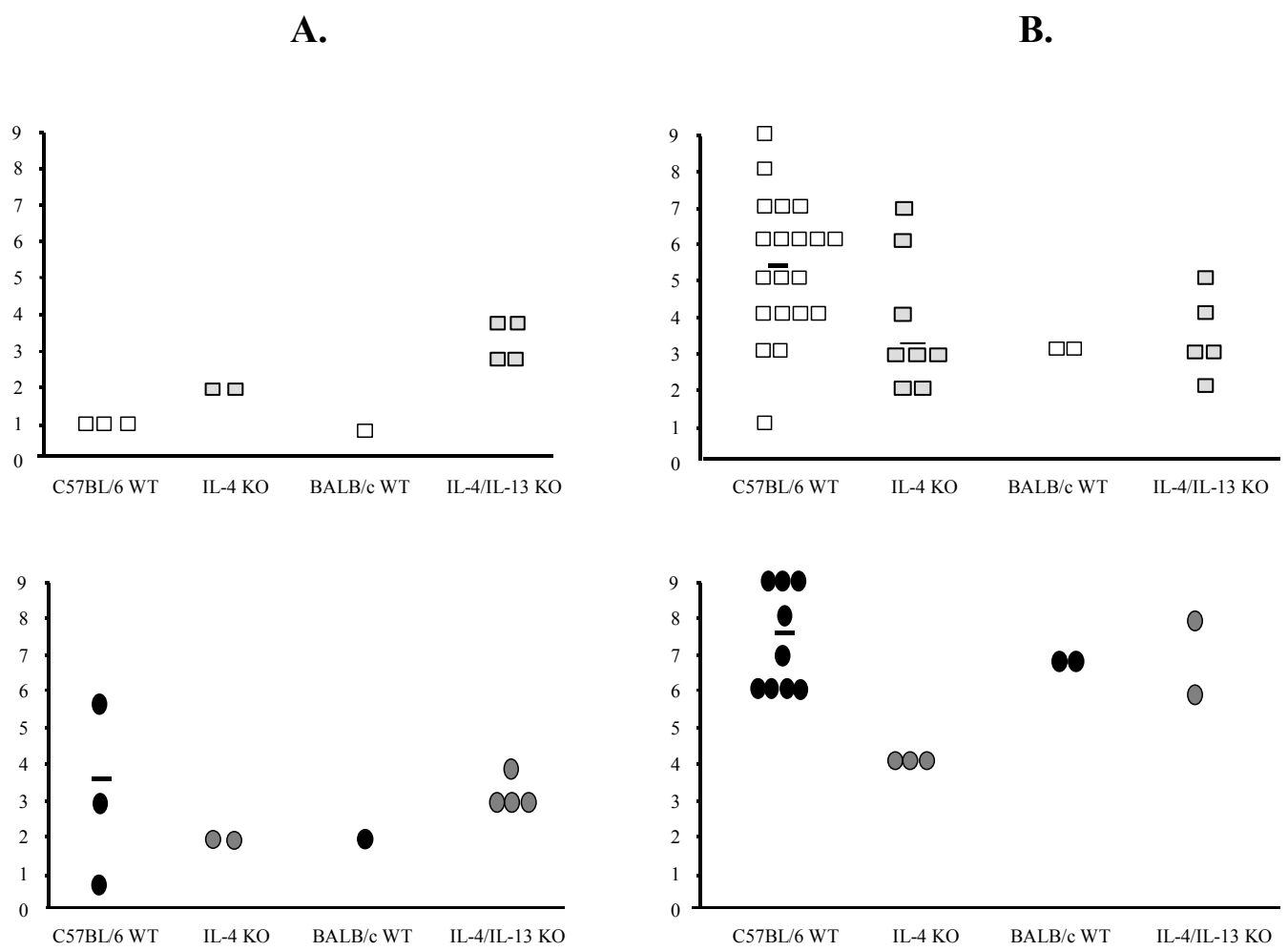


Figure 5

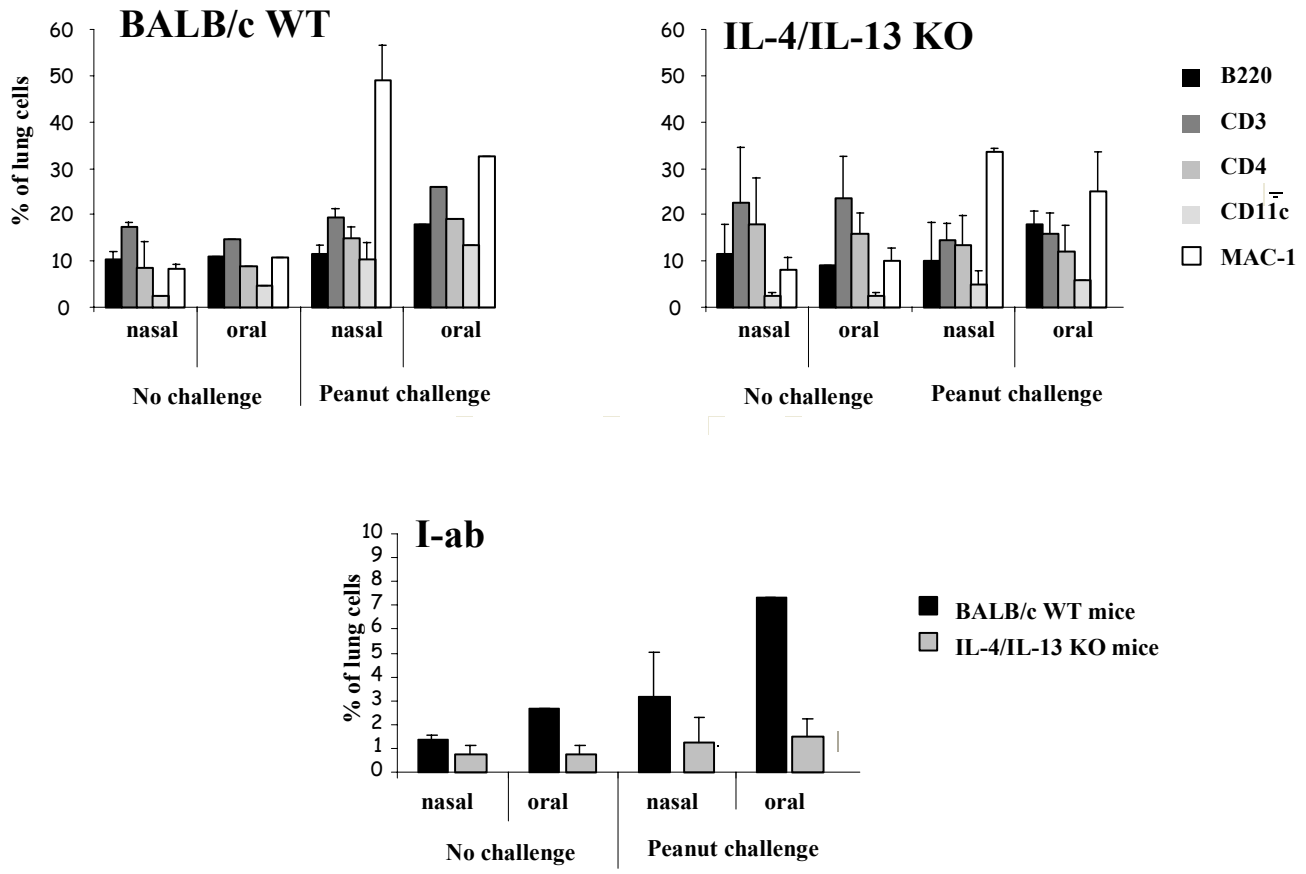
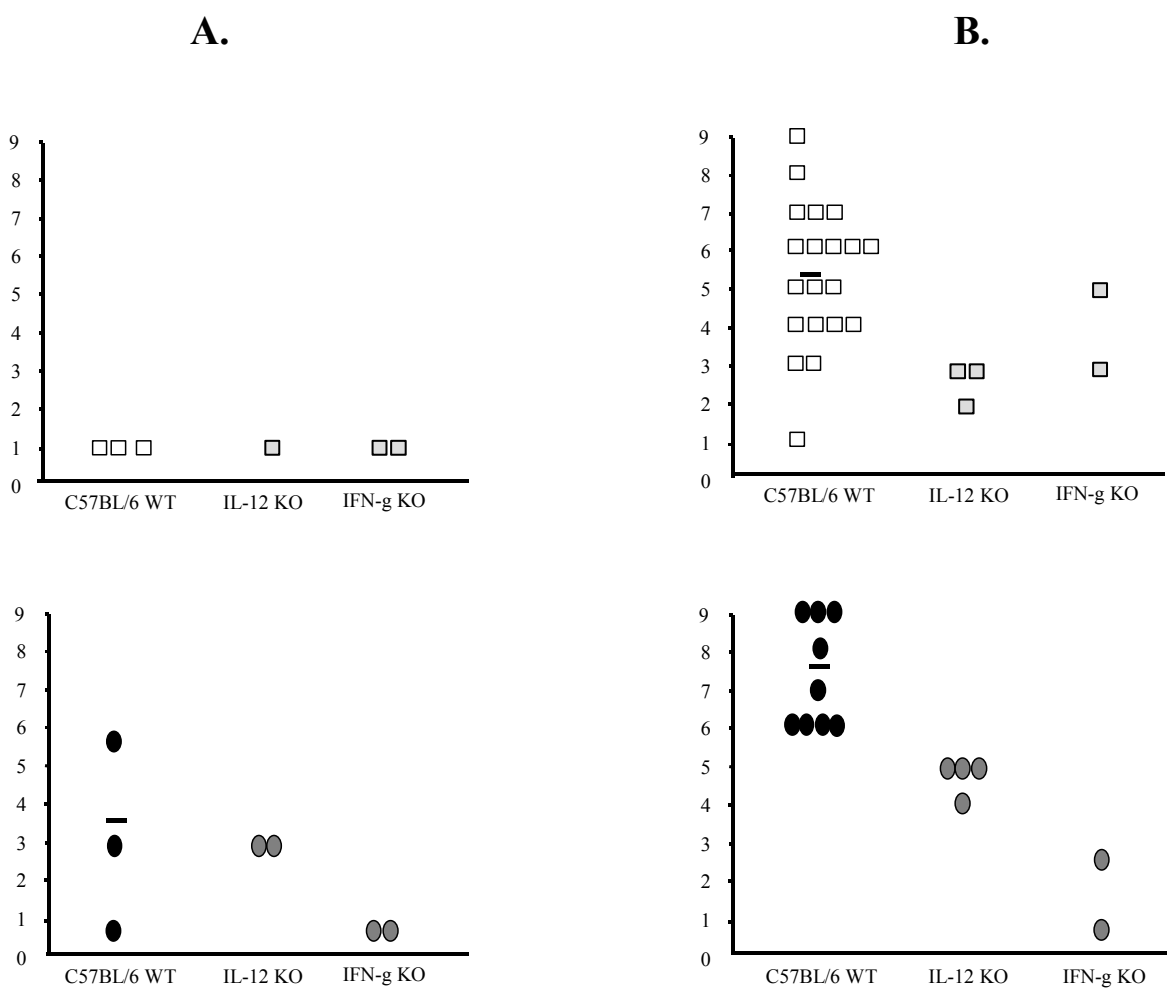

BALB/c WT
% of lung cells
60
50
40
30
20
10
0
nasal
oral
nasal
oral
No challenge
Peanut challenge
IL-4/IL-13 KO
B220
CD3
CD4
CD11c
MAC-1
I-ab
10
9
8
7
6
5
4
3
2
1
0
BALB/c WT mice
IL-4/IL-13 KO mice

Figure 6



ARTICLE 3

Allergic and inflammatory reactions to legumes in mouse models of peanut allergy

En préparation