The role of peanut-specific T cell responses in children with peanut allergy and in children who are tolerant to peanuts

Final Technical Report

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Abbreviations:

- BSA – Bovine serum albumin;
- CFSE – carboxyfluorescein succinimidyl ester;
- CLA – Cutaneous Lymphocyte-associated Antigen;
- FAP – facilitated antigen presentation;
- FCS – Foetal calf serum
- HBSS – Hank's balanced salt solution
- IFNγ – interferon gamma
- IgE – Immunoglobulin E
- IgG - Immunoglobulin G
- NA – non-allergic (tolerant to peanut consumption);
- OG – outgrown (of peanut allergy);
- PA – peanut allergic;
- PBMC – Peripheral blood mononuclear cells;
- PBS – Phosphate-buffered solution
- PPD – Purified protein derivative (of *Mycobacterium tuberculosis*)
- PS – peanut sensitised (tolerant to peanut consumption but immunologically reactive to peanuts, as evidenced by skin reactivity and/or the presence of peanut-specific IgE antibodies);
- Th – T helper cell;
- TNFα – Tumor necrosis factor alpha
- TT – Tetanus toxoid

EXECUTIVE SUMMARY:

Background and aims of the study

Peanut allergy is a severe, potentially life-threatening condition, characterised by anaphylactic reactions triggered by even minute quantities of peanut. Furthermore, peanut allergy prevalence increased significantly in the UK over the last decade, so safety advice regarding peanut-containing foods as well as the use of dietary interventions for preventing peanut allergy (by avoidance or early consumption) is now sought by the public and especially by parents from government bodies and from medical professionals.

However it is still unclear:

● why only a small minority of the children who are exposed to peanuts end up developing peanut allergy while the others develop tolerance to peanuts.

● whether consumption of peanuts early in life or conversely rigorous peanut avoidance and elimination of peanut traces in foods and in the environment during the first three years are better for preventing peanut allergy.

● finally, as most children react to peanuts the first time they eat them, it is still unknown how did they became allergic and what was the route of their initial allergenic exposure (the skin or the gut).

By investigating these problems, the present study aims to provide scientific evidence regarding the immunological mechanisms involved in the induction, development and persistence of peanut allergy.

Main findings of the study and their significance

Our findings are related to the four objectives that we achieved in this study:

Objective 01: Investigation of four immunological mechanisms that could explain the differences in the kinetics of proliferative responses to peanut of peanut allergic (PA) and non-allergic (NA) donors.

In a previous project we found significantly higher and earlier proliferative responses to peanut in PA compared with NA donors. These differences could be explained by four non-exclusive immunological mechanisms:

i. PA have higher peanut-specific IgE levels that increase proliferation;
ii. PA have higher numbers of peanut-specific T cells than NA individuals;
iii. PA have memory responses to peanut while NA have naïve responses;
iv. NA have suppressor cells that inhibit peanut-specific responses.

In the present study we investigated these mechanisms and we found that the differences between PA and NA donors peanut-specific responses can indeed be explained by the presence of peanut-specific IgE that facilitates antigen presentation (FAP). Higher peanut-specific IgE levels may also be the cause of the higher frequencies of peanut-specific circulating Th cells that we found in PA when compared with PS and NA individuals, so both these mechanisms contribute to the higher proliferative responses we saw in PA individuals.

Conversely, we found that in both PA and NA donors peanut-specific responses were driven by memory Th cells and not by naïve T cells. We could not find any difference regarding the levels of suppressor cytokines produced in peanut-specific responses by PA and NA donors either. Whilst the

experimental model that we used to measure these responses (in vitro peanut-stimulated PBMC cultures) poses significant difficulties for measuring cytokine production because of the high background cytokine levels, it still appears that T cells from PA and NA individuals do not differ significantly with respect to their production of suppressor cytokines. However, in our model we have nevertheless observed significant differences between PA and NA donors with respect to the peanut-specific proliferation of their T cells. Therefore it seems very unlikely that suppressor cytokines are the cause of the differences between PA and NA, at least with respect to the immunological parameters that we measured in our in vitro experimental system.

Our results are significant in revealing the role of peanut-specific IgE for maintaining the allergic response by FAP. Thus anti-IgE antibodies that can block IgE-mediated FAP could break the positive feedback mechanism that supports peanut allergy and could lead to the resolution of this allergy.

From the standpoint of food safety and dietary interventions, IgE-mediated FAP could explain why extremely low levels of peanut proteins that may not trigger allergic reactions could nevertheless be sufficient to maintain peanut allergic responses. The differences between PA and NA individuals that we revealed regarding circulating T cell frequency represent another important finding and we plan to use this novel technique to monitor the immunological effects of dietary interventions (early introduction of peanuts into the children diet or avoidance of peanuts in the diet).

Objective 02: We stored plasma from all donors at minus 70 degrees so that we shall be able to measure cytokines or antibody levels that may reflect peanut allergy or tolerance. We plan to establish further collaborative projects looking at antibody affinities, epitope spread and biomarkers of tolerance.

Objective 03: Correlate peanut-specific IgE production and T cell function in PA and NA children.
We found that B cell responses (reflected by peanut-specific IgE) and T cell responses to peanut antigens are correlated whereas B and T cell responses (to a control antigen) are uncoupled in PA individuals. Conversely, in NA individuals peanut-specific responses are uncoupled.

Our results confirm the hypothesis that B cell responses to allergens (but not those to non-allergenic proteins) are on-going responses that are closely linked with allergen-specific T cell responses, possibly through the positive feedback circuit triggered by IgE-mediated FAP.

Objective 04: Characterisation of proliferative T cell responses to peanut amongst the Cutaneous Lymphocyte-associated Antigen (CLA) expressing (skin homing) and beta 7 integrin expressing (gut homing) memory T cells subpopulations in peanut allergic, non-allergic and sensitised children.
We found that in PA donors the peanut-specific response is predominantly generated by skin-homing CLA+ memory T cells that have initially seen peanut antigens in the skin. Conversely, peanut-specific responses in NA

individuals are mixed, being generated by both skin-homing and gut-homing memory Th cells. These differences are specific for peanut responses since we could not see any difference between PA and NA responses to a control food antigen (ovalbumin): in these donors control responses show no clear subset predominance of skin-homing or gut-homing memory Th cells.

This result is very significant for designing interventions aimed at preventing peanut allergy since these argue in favour of skin sensitisation. Thus skin exposure to peanuts, presumably through inflamed, eczematous skin, may lead to peanut allergy development.

Future work and further developments of the results of the study

The present study provides an explanation on how low dose exposure to peanut allergens leads to on-going peanut-specific IgE production through the positive feedback mechanism driven by IgE-mediated FAP. This could underlie the persistence of peanut allergy despite stringent peanut avoidance.

Our findings also suggest that the primary route of peanut antigen exposure may be crucial in determining the allergy versus tolerance outcome. Thus, our results support the notion that allergic sensitisation may occur through the skin and oral tolerance results from gut exposure.

The investigation of the immunological mechanisms underlying peanut allergy and tolerance allowed for the establishment of novel approaches that will be used for the monitorisation of participants in the LEAP (Learning Early About Peanut allergy) study – a randomized interventional trial aimed at finding whether avoidance or early exposure to peanuts are better for preventing peanut allergy in order to provide solid scientific information for advising parents about the safety of peanut-containing food consumption by children.

In the LEAP study, that is also supported by the FSA, we shall be able to monitor, using antibody and T cell assays, the emergence of tolerance or allergy to peanuts and the underlying immune responses including FAP.

INTRODUCTION

Peanut allergy is a severe, potentially life-threatening condition in children and is characterised by severe anaphylactic reactions triggered by even minute quantities of peanut allergen.

From an epidemiological point of view it has been found that peanut allergy is increasing in many developed countries. In the UK, the prevalence of peanut sensitisation rose from 1.3% in 1989 to 3.2% in 1995 (32). This finding led to significant concerns since food allergies are overall a leading cause of anaphylaxis – in the USA for example, food allergies account annually for about 30,000 anaphylactic episodes, 2,000 hospital admissions and around 200 deaths (33).

Unfortunately, the reasons for the increased prevalence of peanut allergy (and of food allergies in general) are not clear. Several public health strategies aimed at decreasing the prevalence of peanut allergy have been considered in different countries and the usual advice was peanut avoidance for infants and small children (for example the UK Government advisory Committee on Toxicity of Chemicals in food, Consumer Products and the Environment (COT) report on peanut allergy from June 1998). Nevertheless, this advice does not seem to have caused as yet a significant impact upon the prevalence of peanut allergy in British children (34).

Therefore it is very important to explore in greater depth the immunological mechanisms that underlie peanut allergy and conversely peanut tolerance (ability to consume peanuts without ill effect), in order to gather scientific evidence that might subsequently be used to formulate further advice.

In this respect, the T lymphocyte cells represent the immunological component whose activity determines the outcome of an immune response to a food (45). There may be several factors that modulate the initial T cell response to a food allergen (such as the antigen presenting cells) as well as processes that take place later in the immune response and involve B cells and other effectors, but it is the multiple T cell subsets which orchestrate the overall immune response to antigens, including food allergens, as described in detail in figure 1.

Indeed, T cells can be differentiated, depending upon the cytokines that they secrete into several subsets (46): Th1 (that secrete interferon gamma), Th2 (that secrete IL4, IL5, IL13 etc), Th17 (that secrete IL17), Th3/Treg (less well characterised, thought to act through IL10, TGF beta and identified by the expression of FoxP3).

A Th2-dominated immune response to a food leads to food allergy because Th2 cells induce the production of food-specific IgE antibody by B cells. Conversely, Th1 and Th3/Treg cells inhibit Th2 cells and therefore may prevent / inhibit food allergies.

In the current project, we aimed to find out more about the types of T and B lymphocyte responses that underlie peanut-allergic and and peanut-tolerance phenotypes, respectively. We will then be applying this information, in a subsequent intervention study that we are now conducting, in order to monitor the immunological development of a large group of 640 high-risk children who will either eat peanuts or avoid them until 3 years of age (research project T07049). Naturally, the results of these immunological studies on their own are not likely to be sufficient to allow the formulation of new dietary recommendations for the prevention of peanut allergy. Nevertheless, by identifying the effects of different diets upon the peanut-specific T cells that underlie the states of allergy or tolerance to foods and by correlating the immunological data with the clinical outcome of the respective diets (prevention of peanut allergy or other effects), we could provide additional scientific arguments that could be useful for guiding food consumption policies in children towards the prevention of food allergy.

Data from previous FSA funded work (FSA project code - T07001) allowed us to make clinical and immunological observations relating to the different states of peanut allergy and tolerance, which formed the basis for the further research questions which this current project was aimed at addressing.

Thus, PBMC isolated from blood from a PA donor typically shows a high and early proliferative response similar to that encountered in the case of the recall antigens tetanus toxoid and PPD. The proliferative response that we measure represents immune cell division triggered by culture of the PBMC in the presence of the respective antigen. We have demonstrated in the past, using CFSE / anti-CD4 staining of peanut-stimulated PBMC cultures, that T helper cells represent the proliferating cell subset amongst PBMC in such cultures (5). In the present work, in order to characterise the kinetics of the proliferative response, we added the peanut antigens when setting the PBMC in culture and then we collected aliquots at different time points (days 3, 5, 7 and 9 after setting the culture). We further transferred these aliquots to a separate plate, added tritiated thymidine and incubated the plates for an additional 6h period in order to allow the labelled thymidine to be incorporated into the DNA of the proliferating cells (as described in more detail in the methods chapter - page).
We present our findings in this respect in figures 2 and 3.

Thus, we found that the peanut-specific proliferative response peaks in peanut allergic donors on day 5 of culture with peanut antigen and declines before day 7. The T cell immune response against other food antigens (beta-lactoglobulin, ovalbumin) was shown to be similar to the response against peanut antigens in NA donors in that peanut specific T cell proliferation is much lower and peaks on day 7 or later (Fig. 2A).

Conversely, in NA children the T cell proliferative response to peanut reaches its maximal value later, usually on day 7 of culture (Fig 2B).

In previous studies, we have simultaneously assessed PBMC proliferation induced by the control antigens and found no significant difference between

the PA donors and the NA controls with respect to the kinetics and intensity of the respective responses. As control antigens we chose two food antigens – (lactoglobulin and ovalbumin) that were tolerated by the children whom we investigated, as they were not allergic to milk or egg and two recall positive control antigens: PPD and tetanus toxoid (TT). The level of PBMC proliferation to lactoglobulin and ovalbumin would reveal the immune responses to milk and eggs – foods that were tolerated by the donors in our study. The responses to PPD (that contains antigens from Mycobacterium tuberculosis) and tetanus toxoid would reveal the immune responses to recall antigens. (All children would have come in contact with commensal Mycobacteria and were vaccinated to tetanus in the UK, according to normal childhood vaccination policies.)
We used these control antigens in order to confirm that the differences between PA and NA donors that we observed were indeed peanut-specific and did not represent, for instance, a non-specific response of PA individuals that would manifest itself regardless of the antigen their cells were exposed to. We purchased lactoglobulin and ovalbumin from Sigma-Aldrich (Poole, UK) and the PPD and TT from the State Serum Institute (Copenhagen, Denmark).

In the previous T07001 study, when comparing peanut-specific proliferation of PBMC isolated from n=17 PA and n=10 NA donors respectively (Fig. 3), we found that on day 5 the median stimulation indices were 88.35 (range: 13.8-523.26) for PA and 6.07 (range: 1.18-38.8) for NA (i.e. 93.12% lower than in PA) whereas on day 7 the corresponding median values were 35.26 (range: 3.46-428.5) for PA and 34.71 (range: 2.6-239.6) for NA donors:

Thus, the differences in peanut-specific proliferation reflect the allergic status of the donors when measured on day 5 but not on day 7 of the PBMC culture in the presence of the peanut antigen (Figure 3).

Thus, due to the different T cell proliferation kinetics existing in PA *versus* NA donors, only the early (day 5 in culture) but not the late (day 7 in culture) assessment allows for the different clinical phenotypes to be revealed by these proliferation assays; according to the Mann-Whitney U-test the differences between PA and NA were statistically significant on day 5 ($p=0.0002$) but not significant on day 7 ($p=0.58$).Thus, in a typical PA donor peanut-specific responses are similar to those elicited by recall antigens such as PPD and tetanus toxoid (TT) and different from those elicited by non-allergenic control food antigens such as beta-lactoglobulin or ovalbumin.

There are several points of scientific interest that warranted an in depth investigation in the case of these findings:

(1) We identified a clear difference between PA and NA individuals with respect to one of their immunological response parameters (i.e. T cell proliferation) to peanut antigens. Indeed, peanut-specific T cell responses appear to be higher in PA than in NA individuals and similar to responses elicited by memory antigens such as PPD and TT. We then used the T cell differences that we found as a starting point to investigate the mechanisms that underlie peanut allergy and tolerance

respectively. We focused our investigation upon peanut-specific T cell responses because it is the balance between different T cell subsets that ultimately determines whether the individual will be peanut allergic or tolerant to peanuts.

(2) Regarding peanut-specific proliferative responses in peanut allergy, conflicting results were reported in the past by different groups. Thus, some authors found that PBMC isolated from both PA and NA children show high but statistically similar levels of proliferation when cultured in the presence of a crude peanut antigen extract or purified Ara h2 (39, 40). Conversely, de Jong et al. (41) and Hourihane et al (42) described significantly higher levels of peanut-specific proliferation in PBMC isolated from PA children, while the latter group found no peanut-induced proliferation of cells from NA donors. Our finding could explain why these results may have been different – distinct kinetics of peanut-specific responses in PA and in NA individuals lead to significant differences between PA and NA to be perceptible on day 5 but not if measured on day 7 of the PBMC cultures.

In the present project we aimed to build on these findings in order to:

(1) Further our understanding of the T cell responses (that represent, as previously described in the introduction and in Figure 1, the key controlling element of the allergic / non-allergic immune response, that may tip the balance towards food allergy and tolerance) in the pathogenesis of food allergy;
(2) Understand the mechanisms that underlie T cell responses in peanut tolerant individuals and
(3) To devise new immunomodulatory strategies that could allow us to normalise T cell responses in future therapies leading to prevention and/or treatment of peanut allergy. Indeed if early consumption of high amounts of peanut in childhood could lead to oral tolerance to this food as a result of the development of a large and stable population of peanut-specific regulatory T cells, this could prevent the development of allergenic Th2 peanut-specific cells. Conversely, if peanut-specific Th2 cells have already emerged and induce peanut allergy, an optimal therapy would be to induce an additional, dominant, Th1 / Treg response that could override the Th2 subset and lead to peanut-specific tolerance. Such an approach, evidenced by the appearance of responses to novel epitopes, has already been shown to be successful in treating grass pollen allergy (43).

In this project, the distinct clinical phenotypes of donors' reactivity to peanuts were determined in order to underpin the immunological characterisation of T cell responses.

• **Peanut allergy (PA)** was thus clinically defined by type I hypersensivity symptoms induced by peanut consumption. It was diagnosed by skin reactivity to peanuts (at least 6mm diameter wheal) or the presence of peanut-specific IgE (above 15kU/l) or a positive oral challenge. Indeed, the > 6mm SPT wheal was what gives a 95% positive predictive value with a

positive challenge to peanut as described by Eigenmann and Sampson (Interpreting skin prick tests in the evaluation of food allergy in children. Paediatric Allergy and Immunology 1999, 9:186-191). In other words a wheal greater than 6mm is virtually certain of predicting peanut allergy. We have validated this in the ALSPAC population as well as in our tertiary clinic population in a previous FSA project (FSA project code - T07001).

• **peanut tolerance** was defined as the ability to eat peanuts without developing any clinical symptoms. Whilst peanut tolerance is as such a clearly straightforward phenotype from a clinical point of view, it can be further subdivided in three subtypes when considering the immunological responses to peanuts that tolerant individuals may express. Thus some individuals, that we define as being peanut sensitised (PS), may be eating peanuts without any clinical symptoms despite having measurable peanut-specific IgE antibodies and/or positive skin prick tests (SPT) to peanuts. Another subtype of tolerant individuals have outgrown peanut allergy (OG), i.e. they have reacted in the past but are able to eat peanuts at present without any clinical symptoms. Finally, we define NA individuals as tolerant individuals who do not have peanut-specific IgE nor positive SPT to peanuts.

In this study, we focused on peanut-specific T helper cells because the activity of this T cell subset is the most likely to provide us with insight regarding:

a) the site of peanut allergic sensitisation,
b) the characteristic of the on-going immune responses that underlie the states of peanut allergy and tolerance respectively and
c) immune mechanisms that maintain peanut allergy and prevent its resolution.

Peanut-specific memory T cells are the most likely subset that could give us information regarding the initial site of peanut sensitisation because at the time of this initial sensitisation they acquire the expression of a homing receptor (such as CLA for T cells sensitised in skin-draining lymph nodes and alpha 4 beta 7 for T cells sensitised in the gut associated lymphoid tissue – GALT). The acquisition of these homing receptors allows these memory T cells to recirculate predominantly in the tissues where they are the most likely to re-encounter their specific antigen. Homing markers such as CLA have been used in the past to characterise responses to allergens encountered in the skin, for example nickel (44).
Conversely, other immune cell subsets (such as the dendritic cells) are not sufficiently long lived to provide information regarding a sensitisation event that may have occurred many years earlier.

Thus, in this project we investigated the role of circulating peanut-specific T helper (Th) cell responses in peanut allergic (PA) and non-allergic (NA) children because Th cells are the key immunological component that underlies the state of allergy or tolerance to peanuts (and to foods in general)[1-2].

Indeed, whilst the defining pathogenic factor in peanut allergy is peanut-specific IgE, the production of such IgE is entirely dependent upon T helper

type 2 cells production of cytokines and stimuli that induce B cells to secrete IgE. Indeed, Th2 cells (which are defined by their production of cytokines such as IL-4, IL-5 and IL-13 and lack of production of interferon gamma) induce naïve B cells to proliferate and switch their antibody class to IgE as described in Figure 1 (45, 46).

The key role of Th2 cells in allergy is clearly demonstrated for example by 'natural experiments' in which the transfer of peanut specific T cells from a peanut allergic individual to a non allergic one (in the case of organ transplantation) also transfers peanut allergy[3]. Conversely, in the case of successful immunotherapy that leads to allergy resolution the T cell function is modulated first while specific IgE levels may remain high or even increase temporarily[4].

Main objectives, rationale and expected results of the study

The four objectives of our study were:

Objective 01: Investigation of four immunological mechanisms that could explain the differences in the kinetics of proliferative responses to peanut of PA and NA/OG donors.

As part of a previous FSA-funded project[5], (FSA project code T07001) we have established novel methods to investigate T helper cell activity in peanut allergy using flow cytometry and we found differences in the kinetics of proliferative responses to peanut of T cells isolated from peanut allergic (PA), non-allergic (NA) and from children who outgrew their allergy (OG). Flow cytometry (FACS) was used to identify the peanut-specific cells amongst the peanut -stimulated PBMC by using CFSE labelling as previously described (5). FACS methodology also allowed us to measure the degree of allergenic polarization (Th2 skewing of the cytokine production phenotype) of these peanut-specific T cells using intracellular cytokine staining.

We hypothesised that these differences could be explained by four possible immunological mechanisms:

- IgE-mediated modulation of allergen-specific T helper responses;
- Increased frequency of peanut-specific precursor T helper in PA relative to peanut tolerant individuals;
- Predominance of memory Th cell responses in PA children compared with naïve responses in NA/OG;
- maintenance of tolerance in peanut tolerant subjects by regulatory T cells.

We aimed to investigate these explanations and to determine the characteristics of Th cells involved in peanut allergy as opposed to peanut sensitisation and tolerance.

We expected to gain insight into the immunological mechanisms that maintain peanut allergy and to use this information to further develop diagnostic and therapeutic strategies.

Objective 02: Separate and store plasma from all donors at -70 degrees.

Having plasma from physically well-characterised donors should allow us to determine whether cytokines or antibody levels may reflect the states of allergy or tolerance to peanuts and establish further collaborative projects looking at antibody affinities, epitope spread and biomarkers of tolerance.

Indeed, the characterisation of distinct antibody affinities and epitope spread has been used previously to characterise the immune responses of peanut-allergic children. It was found that more severe peanut allergy was correlated with a higher level of epitope spread (45). This information could theoretically provide insight as to whether food allergic children are progressing towards the resolution of their allergy or not (i.e. whether the allergen-recognition epitope spread is decreasing, staying unchanged or increasing). Based upon such information, clinical interventions aimed at speeding allergy outgrowing could be decided. Further studies such as identifying triggers of basophil degranulation could identify other parameters that correlate with allergy or tolerance to foods (biomarkers of tolerance). Some of these studies will be attempted as part of a larger intervention trial that our group is conducting (the LEAP study) in which the development of peanut-specific allergy or tolerance and the effect of peanut consumption will be investigated longitudinally.

Objective 03: Correlate peanut-specific IgE production and T cell function in PA and NA children.

We wanted to find out whether peanut-specific B cell activity (as measured by IgE levels) and T cell function is linked in PA and NA individuals.

We expect to use this information in the future, in order to design a therapeutic intervention aimed at inducing peanut allergy resolution since such intervention is likely to target peanut-specific T helper cells in order to act indirectly upon the pathogenic peanut-specific IgE.

Objective 04: Characterisation of proliferative T cell responses to peanut amongst the Cutaneous Lymphocyte-associated Antigen (CLA) expressing (skin homing) and beta 7 integrin expressing (gut homing) memory T cells subpopulations in peanut allergic, non-allergic and sensitised children.

In order to provide advice aimed at preventing peanut allergy it is essential to know what is the route of exposure leading to peanut sensitisation in children.

Previous FSA-funded work has revealed that peanut allergy is increased in children with eczema who have used arachis oil-containing emollient creams (FSA project code - T07001)[6]. Another FSA-funded project further demonstrated that, in a mouse model, cutaneous exposure to peanut induces a Th2-type allergic immune response (FSA project code - T07022)[7].

We took the next logical step in this investigation by determining if the peanut-specific T cells from PA and those from NA first encountered peanut antigens in the skin or in the gut. In this respect, we took advantage of the fact that memory T cells that were first activated in skin-draining lymph nodes express the cutaneous lymphocyte-associated antigen (CLA) marker whilst those homing to the gut express the adhesion molecule alpha 4 beta 7.

By isolating these subsets form PBMC of PA and NA donors we aimed to identify the initial allergic sensitisation site as a step towards establishing a novel approach to prevent potential peanut allergic sensitisation through the eczematous skin.

The scientific and practical relevance of this part of our investigation is the following: after the initial exposure to any food, the immune system responds in a number of ways. In the majority of cases tolerance results, but in others this exposure leads to the development of allergy. Interestingly, despite the requirement for prior contact with an allergen for sensitization to occur, over 80% of individuals allergic to peanut react on their first known peanut ingestion (35), suggesting prior occult exposure. Apart from oral exposure to peanut, sensitisation may potentially occur in utero, via lactation or cutaneous exposure to environmental peanut antigen. Whilst it remains unclear how the route by which antigen exposure occurs influences the underlying immune response, this understanding is crucial for the development of effective prevention strategies.

Both eczema and topical exposure to preparations containing arachis (peanut) oil have been independently identified as risk factors for the development of peanut allergy (36), suggesting the possibility that exposure to low doses of peanut antigen through inflamed skin can lead to allergic sensitization. This is further supported by scientific evidence that sensitization may occur cutaneously (37), whilst in contrast animal models have shown that oral tolerance induction using high doses of food antigens can prevent subsequent allergic sensitization to these foods (38).

To examine the hypothesis of cutaneous allergic sensitisation and oral tolerance induction in vitro, we exploit the fact that memory T lymphocytes express homing receptors on their surface that reflect the site where they were initially sensitized. The Cutaneous Lymphocyte-associated antigen (CLA) is a skin homing receptor on memory T cells, the expression of which implies sensitization in the skin, and α4β7 integrin is a gut homing receptor, the expression of which implies sensitization in the gastrointestinal tract.

Therefore if PA individuals were initially sensitised to peanut through the skin, we would expect their peanut-specific response to be derived mainly from the CLA+ T cell memory population. Conversely, if T cells were initially sensitised in the gastrointestinal tract (as we would expect to be the case in NA individuals), then we would expect that their peanut-specific response to be derived mainly from the alpha 4 beta 7+ T cell memory population.

MATERIALS AND METHODS:

Peanut allergy diagnostic. The diagnosis of peanut allergy was based on a characteristic history of immediate hypersensitivity reactions occurring soon after peanut ingestion (as assessed by a paediatric allergy clinician) and at least one of the following diagnostic criteria: peanut skin prick test wheal ≥6mm or peanut-specific IgE ≥15 kU/L or a positive peanut challenge. The non-allergic NA children had never reacted to peanut ingestion and were currently consuming peanuts as part of their diets, tolerating the equivalent of at least one peanut butter sandwich. Comparatively, peanut sensitised (PS) individuals and tolerant individuals who had outgrown peanut allergy (OG) could also eat similar amounts of peanuts without any reaction, but PS had

measurable peanut-specific IgE antibodies and/or positive skin prick tests (SPT) to peanuts while OG had reacted in the past.

Reagents. Peanut defatted extract was kindly provided by Dr. Henning Løwenstein (ALK Abelló, Denmark). The lyophilised protein was dissolved in phosphate buffered saline (PBS), sterilised by filtration and added to the cell culture at a final concentration of 100 micrograms /ml. This antigen concentration ensured consistent cellular proliferation *in vitro*, as determined by titration in a preliminary experiment. Tetanus toxoid (TT, Staatens Serum Institute, Copenhagen, Denmark) was used at 10micrograms/ml (27). Fluorochrome-labelled antibodies specific for human cytokines (IFN gamma, IL-4, IL-13 and TNFalpha) were from BD Pharmingen (Cowley, UK); the corresponding clones used were B27 (IFN gamma), MP4-25D2 (IL-4), JES10-5A2 (IL-13) and MAb11 (TNFalpha). CFSE was from Molecular Probes (Eugene, Oregon).

Antibody levels. Peanut-specific IgG was measured using a direct ELISA system adapted according to the method described by RG Hamilton and NF Adkinson (31). Briefly, NUNC MaxiSorp ELISA plates were coated overnight with 50 microliters/well of 0.1mg/ml defatted whole peanut extract dissolved in bicarbonate buffer (pH=9.6). The plates were than washed twice with PBS-Tween (0.1%) and blocked with 1% BSA for 1h at 37°C. Multiple dilutions of plasma were then added to the wells, incubated 2h at 37°C, washed and then bound IgG was measured using a biotinylated anti-human IgG antibody and streptavidin-horseradish peroxidise with tetramethylbenzidine as substrate for detection. Peanut-specific IgG is expressed as relative units / ml using a standard curve obtained using human purified IgG as reference. Antibody levels were determined for all the patients in whom we could collect sufficient plasma. Peanut-specific and Tetanus Toxoid (TT)-specific IgE antibody levels in plasma were measured using the Phadia UniCAP and TT-specific IgG antibody levels were assessed by a commercial ELISA (Binding Site Birmingham UK).

Measurement of IgE antibodies. The measurement of IgE antibodies by Phadia UniCAP is done as follows, according to the manufacturer's recommended procedure: the specific allergen of interest, covalently coupled to ImmunoCAP reacts with the specific IgE in the patient sample. After washing away non-specific IgE, enzyme-labeled antibodies against IgE are added to form a complex. After incubation, unbound enzyme-anti-IgE is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured. The fluorescence is directly proportional to the concentration of specific IgE present in the sample. To evaluate the test results, the response units for patient samples are compared directly to the response for the calibrators. The entire process is automatic, so the serum sample is loaded into the UniCAP machine together with the controls and the results are obtained at the end.

PBMC isolation. Venous blood was collected using citrate dextrose (Sigma, Poole, UK) as anticoagulant and centrifuged (600g, 10 min, room temperature) within 2h from collection in order to separate plasma. PBMC were further isolated by density gradient separation using Histopaque-1077 and washed in Hank's buffered salt solution (HBSS) containing 20mM HEPES (HBSS+HEPES). PBMC were always set in culture immediately after isolation and CFSE labelling, without an intermediate cryopreservation step.

PBMC labelling with CFSE (carboxyfluorescein succinimidyl ester) was done immediately after separation. CFSE dissolved in dimethyl sulfoxide was used at a final concentration of 5micromoles/l and the labelling was done for 10 minutes at 37ºC (water bath) with continuous shaking in order to ensure homogeneous cell staining.
The steps followed for CFSE staining were the following:
CFSE labeling:

- PBMC concentration is adjusted at 10 million / milliliter.
- A CFSE aliquot of 10 microliters is diluted 1/100 with PBS.
- 10 microliters of the diluted CFSE are added to the PBMC.
- PBMC are incubated for 10 minutes at 37C
- 2ml FCS are added to quench the unbound CFSE
- 40ml PBS are added to dilute the CFSE
- PBMC are washed by centrifugation (10 minutes at 620g)
- The unbound CFSE-containing supernatant is discarded
- The CFSE-labelled PBMC are resuspended in RPMI 1640 containing 5% autologous plasma (prefiltered through a 0.2 nm filter to remove platelets) and antibiotics (penicillin / streptomycin / gentamycin).
- The PBMC are then distributed at 3 million live cells per 2ml per well on a 24-well plate. Peanut defatted extract dissolved in RPMI 1640 is added to the wells (final concentration 100 micrograms peanut /ml).

A diagram showing the proliferation of antigen-stimulated CFSE-labelled PBMC is shown on page 18.

Peanut-specific T cell responses were investigated *in vitro* using PBMC culture in the presence of peanut antigens (sub-optimal stimulation using defatted peanut antigens at a final concentration of 100 micrograms / ml. Cultures were set in RPMI medium supplemented with 5% autologous plasma, either depleted or not of IgE antibodies. By comparing the peanut-specific T cell responses that occur in the presence of the IgE antibodies that are contained in the plasma with the same responses that occur in the absence of these IgE antibodies (using IgE-depleted plasma) we can find out what, if any, is the contribution of IgE antibodies to allergic responses. We are interested to obtain this information because a new drug (omalizumab, a monoclonal antibody that binds to IgE and blocks its activity) has been shown to be efficient for treating asthma and other allergies including peanut allergy (48).
We now wanted to dissect the possible mechanisms by which interfering with the IgE may have a therapeutic effect in allergy and, having a well defined in vitro experimental system, we chose to remove IgE instead of adding additional molecules that could increase its complexity.
Our hypothesis, that we aimed to investigate, is that in peanut allergy there is a positive feedback circuit that maintains this allergy so that it is not

spontaneously outgrown (as is the case with milk and egg allergies). The feedback circuit that we postulate would be formed by peanut-specific Th cells that induce and amplify the production of peanut-specific IgE. In their turn IgE facilitate peanut antigen presentation to Th cells and therefore amplify Th2 responses. By removing the IgE arm of this potential feedback circuit we aim to find out whether such circuit is indeed active in our experimental system and how could it be modulated.

Plasma-depletion of IgE antibodies was done by mixing plasma with anti-human IgE coated magnetic beads.

Peanut-specific PBMC T cell proliferation was determined by measuring methyl thymidine incorporation into DNA during cell division. Indeed, when cells divide they synthetise new DNA and use for this the [^{3}H]-radioactive labelled thymidine added to the culture medium. At the end of the culture, cells are harvested on glass filters, so that unbound [^{3}H]-thymidine (which is a small molecule) is washed away but the [^{3}H]-thymidine that is incorporated into DNA is trapped on the filter. By measuring the amount of radioactive [^{3}H] on the filter we can thus determine the level of DNA synthesis that occurred in the cells which is proportional with their proliferative response (cell division). In practice, at different time points after setting the PBMC cultures in the presence of peanut antigens, [^{3}H]-methyl thymidine (0.5 microCi/well) was added to the cells and [^{3}H]-methyl thymidine incorporation into DNA was measured after a 6h-incubation period The step-by-step procedure used was the following: at different time points (days 3, 5 and 7) after setting up the cell cultures, supernatants were carefully removed from the culture plates and stored for cytokine assessment. The cells in cultures were then mixed and a 100 microliter aliquot was transferred to another plate. Then [3H]-methyl thymidine (tritiated thymidine, 10µl, equivalent to 0.5 microCi/well) was added to each of the occupied wells on the cell culture plate. Alternately, if the cultures were initially established in 96-well plates, the thymidine was added directly to the cultures. After incubation,tritiated thymidine uptake was assessed using the Packard (now Perkin Elmer) FilterMate™ Harvester, and counted on the Packard TopCount® microplate scintillation and luminescence counter.

The cells were harvested from the 96 well culture plates to 96-well Packard UniFilter® GF/C® plates using the Packard FilterMate™ Harvester. 20µL of MicroScint 20 (Perkin Elmer Life and Analytical Sciences, Boston , MA, USA) was added to each well. The plates were sealed with TopSeal-A (Perkin Elmer), and read on the Packard TopCount NXT Microplate scintillation and luminescence counter Model 9904V (Perkin Elmer, USA). The Packard TopCount simultaneously counts up to 12 wells of the standard 96-well microplate. The results were expressed as a Stimulation index (SI) of stimulated against unstimulated no antigen controls.

Cytokine-producing phenotype of peanut-specific cells was determined as previously described (Turcanu et al., J Clin Invest. 2003; 111: 1065-72). Briefly, CFSE-labelled PBMC were cultured in the presence of peanut

antigens (100 micrograms/ml) for 7 days. Over this period, peanut-specific T cells have divided several times and as a consequence they exhibit lower fluorescence than non-specific T cells. Cytokine production was then stimulated with phorbol-12-myristate-13 acetate (50nM) and ionomycin (1 micromolar) in the presence of brefeldin A (2 micrograms/ml) as secretion inhibitor. The cells were then washed, fixed (20 minutes, 4% paraformaldehyde), permeabilised (10 minutes, 0.2% saponin) and stained with anti-cytokine antibodies. The percentage of peanut-specific T cells that produce cytokines was determined by flow cytometry, gating on the low-fluorescence, peanut-specific T lymphocytes.

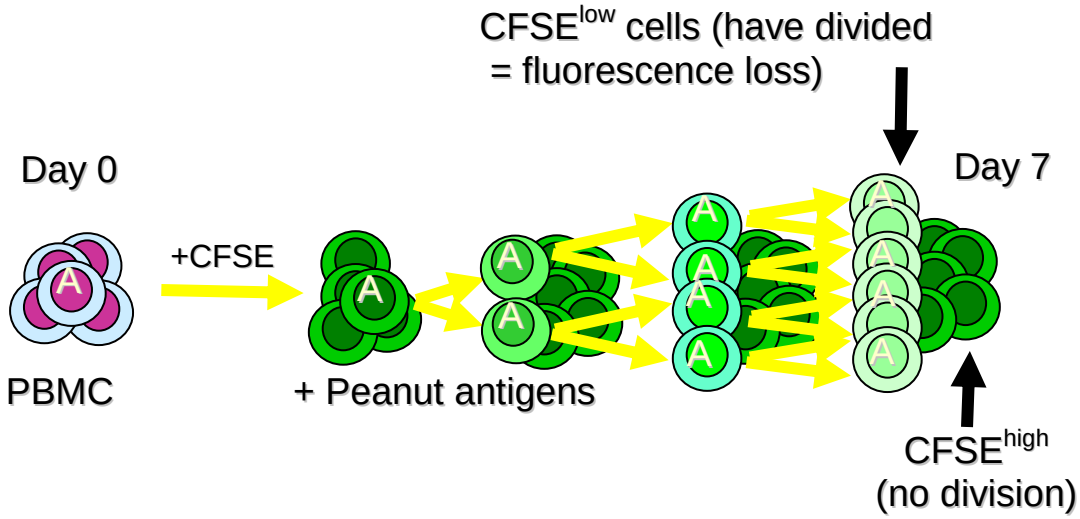


Figure 20: T cell subset purification and staining for detection of subset purity by flow cytometry.

We separated initially the CD4+ T helper subset from PBMC using the negative CD4+ kit separation from Miltenyi Biotech. The memory Th cells subset (CD4+CD45RO+) from this was further negatively selected using anti-CD45RA magnetic beads. We then further purified the CLA+ and the beta7+ memory (CD45RO) T cell subsets using the CLA isolation kit (Miltenyi Biotech) and the PE-labelled anti-beta 7 antibody followed by anti-PE microbeads. The purity of each subset was assessed by flow cytometry. At least 10,000 events were collected for each experimental condition and data were analysed using the WinMDI 2.8 software (Scripps Research Institute: http://facs.scripps.edu/software.html).

Antigen-specific T cell precursor frequency assessment. Immediately after setting the CFSE-labelled cells in culture, an aliquot of 250 microliters was collected and stained with CD4 PE antibodies. 25 microliters of count standard beads (Serotec, UK) were added, then the amount of T helper cells in the culture was determined by flow cytometry. The percentage of $CFSE^{low}$ peanut-specific T helper lymphocytes was again determined by flow cytometry on day 7 of the cell culture using the same amount of count standard beads. The method used to calculate the precursor frequency was that described by Beeler, Engler et al. (reference 19). The significance of calculating the frequency of allergen-specific cells is that it allows for the monitorization of the

allergy state of the individual. Indeed, allergic individuals have significantly higher frequencies of allergen-specific T cells circulating in their blood than non-allergic individuals (16, 17). Furthermore, when allergy resolves, the precursor frequency decreases, so we aimed to develop this novel diagnostic tool for the monitorization of peanut-specific responses in peanut allergic children and also in the participants in the LEAP interventional trial.

T cell subset purification and staining for detection of subset purity by flow cytometry.
We separated initially, in our first experiments, the CD4+ T helper subset from PBMC using the negative CD4+ kit separation from Miltenyi Biotech. The memory Th cells subset (CD4+CD45RO+) from the was further negatively selected using anti-CD45RA magnetic beads. We then further purified the CLA+ and the beta7+ memory (CD45RO) T cell subsets using the CLA isolation kit (Miltenyi Biotech) and the PE-labelled anti-beta 7 antibody followed by anti-PE microbeads.

IgE depletion from plasma using magnetic beads. Autologous plasma was separated from citrate anticoagulated blood by centrifugation, then was filtered through a 0.8/0.2 micron Acrodisk filter in order to remove any remaining particles. Heat-inactivation of plasma was done for 2h in a water bath heated at 56ºC, then the plasma was centrifuged (5 minutes at 1000g) and filtered through a 0.8/0.2 micron Acrodisk. For specific IgE-depletion, plasma was incubated for 1h at room temperature under gentle rotation together with M-450 Tosylactivated Dynabeads (from Dynal, Paisley, UK), coated according to the manufacturers' protocol with goat polyclonal anti-human IgE antibodies from Becton Dickinson (Cowley, UK). 8-16 x 10^8 coated Dynabeads were used per mililiter of plasma for each cycle of IgE depletion. IgE-depleted plasma was then collected, sterilised by filtration and added to the cell culture at 5% final concentration. Captured IgE was eluted from the Dynabeads using a 0.02M acetate buffer pH 2.5. The buffer was then exchanged into RPMI 1640 cell culture medium using a 10kDa cutoff concentrator (from Vivascience AG, Hannover, Germany). The concentration and peanut specificity of the isolated IgE was subsequently determined using the Pharmacia UniCAP assay and the IgE was added back to control cultures in which IgE-depleted plasma was used as supplement.
In order to decrease the number of experimental steps and thus improve cell viability at the end of the purification procedure, we optimised the MACS (Magnetic Activated Cell Sorting) protocol – that we describe in more detail below - as follows:
- we replaced the CD4+ kit and anti-CD45RA magnetic beads with the Memory T cell isolation kit (also from Miltenyi Biotech), thus shortening the procedure and avoiding one incubation step;
- we added the anti-CLA PE antibody during the incubation with the Memory T cell isolation kit;
- we replaced the anti- beta 7 PE antibody with and anti-beta 7 APC antibody;
- we used anti-APC magnetic beads instead of the anti-PE magnetic beads.

Isolation of dendritic cells from PBMC - Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec, Bisley, UK)

The Blood Dendritic Cell Isolation Kit II concurrently isolates plasmacytoid and myeloid dendritic cells from human PBMCs. This process is performed in 2 stages. Initially, B cells and monocytes are magnetically labeled so that they are retained within the magnetic column using a cocktail of CD19 and CD14 MicroBeads. Then the pre-enriched dendritic cells in the effluent fraction are magnetically labeled and enriched with a cocktail of antibodies against the dendritic cell markers CD304 (BDCA-4/Neuropilin-1), CD141 (BDCA-3), and CD1c (BDCA-1). The highly pure enriched cell fraction comprises of plasmacytoid dendritic cells, CD1c (BDCA-1)$^{+}$ type-1 myeloid dendritic cells (MDC1s), and CD1c (BDCA-1)$^{-}$ CD141 (BDCA-3)^{bright} type-2 myeloid dendritic cells (MDC2s). The B cells and monocytes are depleted in advance because a subpopulation of B cells expresses CD1c (BDCA-1), and monocytes express CD141 (BDCA-3) at low levels.

The procedures were performed as follows. 300µl of MACS buffer was added per 100million PBMCs, followed by 100µl each of FcR Blocking Reagent and Non DC Depletion Cocktail per 100 million cells. This was incubated at 4-8°C for 15 minutes in the refrigerator. This magnetically labels the B cells and monocytes. The cells were washed and up to 125 million cells resuspended in 500µl of buffer. This was then passed through a MACS LS column pre-rinsed with 3mls of buffer and placed in a magnetic field of a MACS separator on a MACS stand. The magnetically labeled B cells and monocytes are retained within the column. 3 washes were performed with 3 mls of MACS buffer per wash, with each wash occurring when the column was empty. The effluent (negative fraction), free from B cells and monocytes, was collected.

The effluent was centrifuged at 1800rpm for 5 minutes at room temperature and the supernatant carefully pipetted off before the cells were resuspended in 400µl of MACS buffer and 100 µl DC enrichment cocktail per 100 million original cells. This magnetically labels the plasmacytoid and myeloid dendritic cells, enabling them to be positively selected later on. The cells were incubated at 4-8°C for 15 minutes, then washed and passed through a pre-rinsed MACS MS column. 3 washes with 500 µl MACS buffer were performed and the negative fraction collected in the effluent for subsequent T cell separation. The magnetically-labeled dendritic cells were retained in the column whilst in the magnetic field of the MACS separator. The column was removed from the magnetic field so that the positively isolated dendritic cells could be recovered by adding 1ml of MACS buffer and collecting the cells displaced from the column outside the magnetic field. This was performed by inserting the plunger into the MACS column and exerting pressure until the contents were expelled. The dendritic cells were counted and an aliquot removed for FACS analysis. The rest of the cells were prepared for culture.

Isolation of memory T cells from PBMC - Memory CD4+ T cell isolation kit (Miltenyi Biotec, Bisley, UK)

The Memory CD4^{+} T Cell Isolation Kit is an indirect magnetic labeling system for the isolation of untouched memory T helper cells. Naive CD4+ T cells and

non-CD4+ T cells (ie CD45RA+CD4+ T cells, CD8+ T cells, B cells, NK cells, γ/δ T cells, monocytes, DCs, granulocytes, platelets, and erythroid cells) are incubated with a cocktail of biotinylated CD45RA, CD8, CD14, CD16, CD19, CD56, CD36, CD123, anti-TCRγ/δ, and CD235a (glycophorin A) antibodies and then magnetically labeled with Anti-Biotin MicroBeads, enabling them to be depleted from the cell fraction and leaving the target memory CD4+ T cells unlabelled in the effluent.

The dentritic cell-free PBMC effluent from the above step is used for subsequent T cell isolation. The cells were counted, centrifuged and the supernatant carefully removed. They were resuspended in 40 µl of MACS buffer per 10 million cells and 10 µl of Biotin Antibody cocktail and 20 µl of PE-labelled anti-CLA antibody (Miltenyi Biotec, Bisley, UK) was added per 10 million cells. Incubation took place at 4-8°C for 10 minutes. A further 30 µl of MACS buffer and 20 µl of Anti-Biotin Microbeads were added per 10 million cells and incubated for 15 minutes at 4-8°C.

The cells are washed with MACS buffer, centrifuged, resuspended in 1mls of buffer and passed through a pre-rinsed MACS LS column held on a MACS separator. The column is washed 3 times with 3mls of MACS buffer and the effluent containing the negative fraction, comprising the memory CD4+ T cells, was counted and used for the next step. The magnetically labeled cells are retained within the column and are discarded.

Isolation of CLA+ memory T cell subset - Anti CLA Microbead kit (Miltenyi Biotec, Bisley, UK)

The memory CD4 + T cells were centrifuged and resuspended in 60 µl MACS buffer before 20 µl of Anti-PE microbeads were added per 10 million cells. The CLA+ cells labeled with Anti-CLA PE in the previous step therefore become magnetically labeled with Anti-PE Microbeads. 20-30 µl of APC-labelled anti-β7 antibody (BD Biosciences Pharmingen) was also added and cells were incubated for 15 minutes at 4-8°C. Cells were then washed, centrifuged and resuspended in 500 µl MACS buffer before they were passed through a pre-rinsed MACS MS column. 500 µl washes were performed 3 times and the effluent, comprising unpurified β7 + cells, was collected for the next step of the isolation proceure. The magnetically labeled CLA+ memory T cells were recovered from the column once it was removed from the magnetic field as before. An aliquot of CLA+ memory T cells is removed for FACS analysis whilst the remainder is used for culture.

Isolation of β7+ memory T cell subset - Anti APC MicroBeads (Miltenyi Biotec, Bisley, UK)

The unpurified β7+ fraction is centrifuged and resuspended in 80 µl of MACS buffer with 20 µl of Anti-APC microbeads 20µl per 10 million cells and incubated at 4-8°C for 15 minutes. The APC-labelled β7 cells from the previous step become magnetically labeled with Anti-APC MicroBeads. The

cells were then washed, centrifuged and resuspended before passing through a pre-rinsed MACS MS column. The β7+ cells retained within the positive fraction are held in the column as 3 washes are performed and are recovered once the magnetic field is removed. These cells are used for culture once an aliquot is removed for FACS analysis.

These modifications improved the efficiency of our cell purification protocol; we thus reached over 90% purity in the CLA positive population and over 70% purity in the beta 7 positive population. These values represent typical levels of purity that were obtained in our study, as shown in the table below:

Table 1. Purity levels of CLA positive and respectively alpha 4 beta 7 positive T helper cells used for cell cultures. Each set of values represents an independent experiment using cells isolated from a different donor. Cell purity was assessed by flow cytometry at the end of the MACS separation procedure.

	CLA purity %	B7 purity %
	95.14	72.83
	98.59	71.7
	95.14	72.83
	84.73	78.81
	83.15	83.02
	94.19	88.47
	96.4	69.89
	96.4	80.14
	94.83	84.41
	86.28	77.7
	95.69	77.66
	93.37	70.1
	97.16	74.78
	92.16	81.91
	85.5	73.7
	87.43	73.53
Average	92.26	76.9675

These levels of T cell subset purity are comparable with those reported by other investigators who compared CLA+ versus β7+ responses.

Assessment of cytokine production in cell cultures

The cell culture supernatants were collected on day 5, stored frozen at 80ºC and batched for cytokine analysis on the Luminex 100 IS system (Luminex Corporation, Austin, Texas, USA) using STarStation V2.0 software (Applied Cytometry, UK). Analyses were performed to IL1a, IL1b, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL10, IL12p40, IL12p70, IL13, IL15, IP10, Eotaxin, Interferon γ, GM-CSF, MCP-1, MIP1a, RANTES, TNFα and TGFβ1, 2 and 3 using the Beadlyte Human 22-plex Multi-Cytokine Detection System and Beadlyte TGFβ1, β2, β3 Detection System (Upstate, CA, USA). Analyses were performed in duplicate to supernatants collected on Day 0 and Day 5 (unstimulated and stimulated with 400µg/ml of peanut)

Procedure for Beadlyte Human 22-plex Multi-Cytokine Detection System

The Beadlyte Human 22-plex Multi-Cytokine Detection System was used to simultaneously detect to IL1a, IL1b, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL10, IL12p40, IL12p70, IL13, IL15, IP10, Eotaxin, Interferon γ, GM-CSF, MCP-1, MIP1a, RANTES and TNFα secretion in the cell culture supernatants. Analyses were performed in duplicate to supernatants collected on Day 0 and Day 5 (unstimulated and stimulated with 400µg/ml of peanut. Each Beadlyte 96-well Filter Plate had the capacity to run 40 samples (8 patients) in duplicate.

The standards were prepared and serially diluted as a basis for the standard curve. 50µl of either standard or sample was placed into each well of the Beadlyte 96-well Filter Plate. This was performed in duplicate for each standard or sample. 25µl of Beadlyte Human 22plex multi cytokine beads was added to each well, mixed and incubated for 2 hours in the dark on a plate shaker at room temperature. The plate was then washed twice. 75µl of Beadlyte Cytokine Assay buffer and 25µl of Beadlyte Human 22 plex Multicytokine biotin was added to each well, then mixed and incubated for 1.5 hours in the dark on a plate shaker at room temperature. 25µl of Beadlyte Streptavidin-Phycoerythrin was added per well, mixed and incubated for 30 minutes in the dark on a plate shaker at room temperature. 25µl Beadlyte Stop solution was added to each well and 125µl sheath fluid was used to resuspend the contents of each well before proceeding to read the results on the Luminex 100 IS system.

Luminex 100 IS system multiplex assay detection system

The Beadlyte 96-well Filter Plates were read using the Luminex's 100™ multiplex assay detection system and STarStation V2.0 software.

The equipment was cleaned and calibrated prior to each use. Separate templates were created for the Beadlyte Human 22-plex Multi-Cytokine and the TGFβ1, β2, β3 detection plates. The data is acquired and. analysed. The output, comprising the average concentration of each cytokine from each duplicate pair of wells (in pg/ml), is displayed in a Microsoft Excel (Microsoft Corporation, USA) spreadsheet. This data is analysed as described in the following section. The shutdown procedure is run at the end of each session.

RESULTS:

The results will be presented in relation to each of the project objectives.
We shall list the objectives, briefly state the main outcomes and then discuss in detail the approach, the techniques that we used and our findings:

Objective 01: Investigation of four immunological mechanisms that could explain the differences in the kinetics of proliferative responses to peanut of PA and NA/OG donors. (The clinical phenotypes of these donors were characterised by an experienced paediatric allergologist according to the criteria described in the methods section).

As described in the introduction, four immunological mechanisms that may act simultaneously or separately may indeed explain the differences in the kinetics of proliferative responses to peanut of PA and NA/OG donors that we reported previously. Indeed, we found that overall peanut-specific proliferative responses were higher and occurred earlier in PA than in NA/OG donors.

These differences could be explained by four non-mutually exclusive immunological mechanisms that may act simultaneously or in isolation.

The first hypothesis that we investigated is that the differences we found are caused by IgE-mediated modulation of allergen-specific T helper responses – specifically because peanut-specific IgE induces higher proliferation in PA individuals because it facilitates peanut antigen presentation - facilitated antigen presentation (FAP). FAP is an immune process characterised by the 'concentration' of antigens into the antigen presenting cells, such as the dendritic cells, that leads to increased presentation of these antigens to T cells and stronger T cell responses. IgE has been shown to exert FAP effects because it binds to Fc receptors on the surface of antigen presenting cells . When subsequently binding to allergens, IgE is internalized and directs the bound allergen into and antigen processing pathway (22-24). The consequences of IgE-mediated FAP in allergy is the amplification of the allergen-specific response and the maintenance or even the increased severity of allergic reactions. At least part of the success of allergen-specific immunotherapy for treating allergy may indeed be attributed to the inhibition of IgE-mediated FAP (27, 28).

In order to investigate FAP we depleted total IgE from PA donors plasma and used the depleted plasma for the PBMC cultures to determine peanut-specific proliferation. We chose to deplete total IgE rather than peanut-specific IgE only because we did not want to interfere with the peanut-binding activity of these antibodies, in order to be able to reintroduce these IgE into our experimental system at a later stage. Indeed, in order to demonstrate that the FAP effects were definitely caused by IgE we then added the IgE back and determined whether peanut-specific proliferation was restored as an indication of the role of IgE in FAP.
Finally, we performed the same assay in NA control donors to confirm that the FAP effect that we found was peanut-specific.

In order to fulfil this objective, we had to carry out a preliminary task - the establishment of a method allowing depletion of IgE antibodies from PA donors' plasma:

We established a method, based IgE binding on anti-IgE antibody-coated magnetic beads, allowing us to calculate the frequency of circulating peanut-specific T cells in peanut allergic and non-allergic donors. We measured (using the UniCAP Elisa method) the level of peanut-specific IgE antibodies in the untreated plasma and after IgE depletion to confirm the efficiency of our approach.

Autologous plasma was filtered through a 0.8/0.2 micron Acrodisk filter in order to remove any particles. Heat-inactivation of plasma was done for 2h in a water bath heated at 56ºC. For IgE-depletion, plasma was incubated for 1h at room temperature under gentle rotation together with M-450 Tosylactivated Dynabeads (from Dynal, Paisley, UK), coated according to the manufacturers' protocol with goat polyclonal anti-human IgE antibodies from Becton Dickinson (Cowley, UK). 8-16 x 10^8 coated Dynabeads were used per mililiter of plasma for each cycle of IgE depletion. IgE-depleted plasma was then collected, sterilised by filtration and added to the cell culture at 5% final concentration. Captured IgE was eluted from the Dynabeads using a 0.02M acetate buffer pH 2.5. The buffer was then exchanged into RPMI 1640 cell culture medium using a 10kDa cutoff concentrator (from Vivascience AG, Hannover, Germany). The concentration and peanut specificity of the isolated IgE was determined using the Pharmacia UniCAP assay and the IgE was added back to control cultures in which IgE-depleted plasma was used as supplement.

We show in figure 4 the efficiency of our method for peanut-specific IgE depletion: if multiple rounds of successive IgE depletion were used, peanut-specific IgE was decreased from a median value IgE= 9.35 kIU/l (range: 0.57 – 434 kIU/l) to a median value IgE= 3.5 kIU/l (range: 0.39 – 100 kIU/l).

Using this depletion method, we investigated the effect of IgE depletion upon peanut-specific Th proliferation and we found that IgE-depletion from plasma using magnetic beads leads to a decrease in peanut antigen-driven PBMC in PA but not in NA donors (Figure 5). We also show that proliferation to control antigen remains unaffected.

PBMC culture in the presence of peanut antigens and IgE-depleted plasma led to a significant decrease of peanut-specific PBMC proliferation in PA but not in NA individuals, while tetanus toxoid-specific proliferation remained unaffected, further demonstrating that the decrease in proliferation is allergen-specific and IgE-dependent. The effect of IgE-depletion and subsequent IgE add-back to the culture upon peanut-specific proliferation in PA (n=5) and NA donors (n=4) respectively is described (Fig 5A and 5B).
On the vertical axis peanut-specific proliferation (expressed as stimulation indices) is shown. Each point represents one donor and bars represent median values. In a number of cases insufficient amounts of IgE could be

eluted, so in fig 5A there are fewer data points in which peanut-specific proliferation in the presence of reconstituted plasma could be measured.
We further found that the add-back of IgE to IgE-depleted plasma leads to the restoration of the peanut antigen-driven PBMC proliferation in PA but not in NA donors, while proliferation to control antigen remains unaffected (data not shown).

Thus, after the IgE-capture magnetic beads were used to deplete IgE from plasma, they were washed and the attached IgE was eluted from the beads using 0.02M acetate buffer pH 2.5, neutralised, then added to PBMC cultured in the presence of peanut antigens and of intact or (alternately) of IgE-depleted plasma. In peanut allergic donors, the average peanut-specific IgE concentration per well in the case of intact plasma was 15.11 IU/well. For IgE-depleted plasma the IgE decreased with 93.2 – 45.5% of the intact plasma values (average decrease 66.57%) and for the plasma with added-back IgE, the average IgE level represented 101.9% of IgE levels in intact plasma.
In a typical PA individual, we observed that IgE-depletion (labelled as IgE^-) led to a decrease in peanut-specific proliferation while adding-back purified IgE (labelled as IgE^+) restored proliferation. Overall, we found that adding back purified IgE that was eluted from the magnetic beads to the IgE-depleted plasma leads to an increase of PBMC proliferation in PA donors to levels similar to those observed in non-IgE-depleted plasma.
In the figures below, 5% plasma is added to the cell culture medium as a supplement, in order to ensure antibody levels reflect those in the organism.

Added plasma described in Figure 6 belongs to one of three conditions i.e. is either intact **(condition 1)** or IgE-depleted **(condition 2)** or IgE-depleted and then IgE is eluted and added back to it **(condition 3)**: and proliferation was determined to two distinct antigens: either peanut antigens or tetanus toxoid (TT) as a positive control antigen. These experiments were done using autologous plasma from the patient whose PBMC were used; we preferred to avoid the use of pooled plasma in order to avoid adding additional unknown factors (such as blood-group specific antibodies, whose effects could potentially be very complex) into the experimental system.
This finding further confirms that the variations in peanut-specific proliferation found are caused by IgE-dependent FAP.

A second hypothesis that could explain the differences of proliferative responses that we observed between PA and NA individuals is an increased frequency of peanut-specific precursor T helper cells in PA.

In order to investigate this hypothesis we had to establish a method allowing the calculation of the frequency of circulating (precursor) peanut-specific T cells in peanut allergic and non-allergic donors.

Outcome: we established a method, based upon CFSE staining and PBMC culture in the presence of peanut antigens, allowing us to calculate the frequency of circulating peanut-specific T cells in peanut allergic and non-allergic donors, as summarised below.

The frequency of peanut-specific Th cells (precursor frequency) was determined by adapting to our *in vitro* experimental conditions the method established by Givan and her collaborators and further developed by Rimaniol et al. and by Tsuge et al. (references 15-17) for the study of allergen-specific cells. Briefly, after the PBMC were isolated from PA and NA donors respectively, (using the method described in the methods section) they were labelled with carboxyfluorescein succinimidyl ester (at a final concentration 5micromolar, for 10 minutes, in a shaking water bath at 37°C). We have previously shown that the dividing lymphocytes are peanut-specific and they halve their fluorescence after each division. After a 7-day culture, we identified the emergent $CFSE^{low}$ population and calculated the number of precursor cells that lead to the respective number according to the number of cell divisions underwent by the $CFSE^{low}$ cells. In order to correct those values for cell death during culture, we determined the number of PBMC existing at the beginning of the culture using standard count beads from Serotec (Kidlington, UK). The method used to calculate the precursor frequency was that described by Beeler and his collaborators (reference19). At least 10,000 events were collected for each experimental condition and data were analysed using the WinMDI 2.8 software (from the Scripps Research Institute website: http://facs.scripps.edu/software.html).

In figure 7 we show the process of identification of live cells and correction for cell death occurring *in vitro* by comparing the cell numbers on day 0 and on day 7 of the culture. We then describe the method of calculation for the precursor frequency, done by analysing the number of divisions undergone by $CFSE^{low}$ cells.
We applied this method to investigate the precursor Th Cell frequencies in PA and in NA individuals. We found that *in vitro* IgE-dependent FAP is reflected by a higher precursor frequency of peanut specific T cells amongst PBMC in PA individuals (V. Turcanu, A. C. Stephens, S.M.H. Chan, F. Rancé and G. Lack IgE-mediated facilitated antigen presentation underlies higher immune responses to peanut in allergic individuals compared with tolerant individuals. *Manuscript in preparation – data shown as Fig.8*). We investigated the peanut-specific responses in NA as controls for the PA individuals, as published by other authors who used this method to investigate allergic responses to other allergens (16, 17), rather than use additional control antigens in PA individuals.

We thus used the method that we developed, based upon CFSE staining and culture in the presence of peanut antigens that we established to calculate the frequency of circulating peanut-specific T cells in peanut allergic and non-allergic donors.

The frequency of peanut-specific Th cells (precursor frequency) was thus determined by adapting to our *in vitro* experimental conditions the method established by Givan and her collaborators and further developed by Rimaniol et al. and by Tsuge et al. for the study of allergen-specific cells.

In comparison with these authors who studied respiratory allergies to grass pollens, we found much lower numbers of peanut-specific precursor Th cells,

suggesting that food allergies (or at least peanut allergy) may be reflected by lower levels of allergen-specific cells or alternately that such cells may be homing in the associated lymphoid level.

Another difference, notable especially when comparing our results with those published by Rimaniol et al. (17) who found ~10-20% grass pollen-specific T cells in the circulation in allergic individuals, may be caused by the fact that we corrected for cell death during *in vitro* cultures, so our results may reflect the biological reality more accurately. Our data are much more similar to those published by Beeler, Engler et al. regarding drug-specific circulating cells (2006), when they also used CFSE labelling followed by allergen stimulation.

We found that PA individuals have indeed around 10 times more peanut-specific T helper cells amongst circulating PBMC than the NA donors: the median precursor frequency values were 0.608 for PA (n=14) and 0.076 for NA (n=9) individuals. We expressed the precursor frequency values that we found as percentages of circulating CD4+ Th cells because the normal values of circulating CD4+ Th cells vary within well-defined limits in otherwise healthy children. This is a very robust measure for precursor frequencies, which is presumably why the other authors used the same approach (reference 19).

The difference between the two groups (of n=14 PA and n=9 NA individuals) was highly statistically significant ($p<0.001$), as shown in Figure 8 . Our findings are similar to those recently published by other groups who used the same comparison approach (between allergic and non allergic individuals) when studying other allergic diseases such as grass pollen rhinitis, cow's milk allergy, drug allergy etc. These authors used this experimental approach to define and further characterise the allergic status of their patients and to monitor the success of immunotherapeutic interventions (15-17, 19, 49).

A third hypothesis that could explain the differences of proliferative responses that we observed between PA and NA individuals is that we observe memory Th responses in PA children compared with naïve responses in NA. It had already been shown by others, when investigating other allergic responses, that allergen-specific PBMC proliferation in vitro is almost entirely due to the memory T cells amongst PBMC (44). Nevertheless, we could not exclude without any experimental evidence the possibility that peanut-specific responses might be different. However we did not expect having to investigate large numbers of PA and NA individuals because we would anticipate that in most of them the memory response would account for more than 80% of peanut-specific proliferation regardless of their clinical phenotype.

Therefore, in order to test this hypothesis we characterised the memory *versus* naïve responses of peanut-specific T cells from peanut allergic and non-allergic donors.
We separated therefore memory and naïve T cell subsets from N=5 PA and n=5 NA donors and compared the peanut-specific proliferation using tritiated thymidine incorporation. We found that the memory T cell subsets accounted

for more than 80% of peanut-specific responses in both PA and NA donors (Figure 9) We expressed these results as proportional of total peanut-specific proliferation in each donor because the levels of peanut specific responses differed greatly between the group of PA individuals (high proliferation) and the NA individuals (low proliferation). Peanut-specific proliferation was measured on day 5 of the cultures in the presence of peanut antigens..
Each column represents an individual. Total proliferation (100% represents the sum of memory + naïve Th responses).

We could not find any statistically significant difference between PA and NA individuals with respect to their peanut specific memory responses (Figure 10).
Therefore is seems unlikely that the differences between PA and NA could be explained by memory responses in PA and naïve responses in NA; in fact peanut-specific responses seem to be largely driven by memory T cell responses in both groups.

A fourth hypothesis that could explain the differences in proliferative T cell responses that we observed between PA and NA individuals is that NA individuals have circulating regulatory / suppressor T cells that produce suppressive cytokines that inhibit peanut-specific T cell proliferation.

In order to test this hypothesis, we collected cell culture supernatants from n=11 patients with peanut allergic, tolerant and sensitised phenotypes. The supernatants represented cell-free culture medium collected from the culture wells without mixing the cell cultures. We measured the concentration of cytokines (IL-10 as well as IL-7, IL-17 and the chemokine IP-10) produced due to peanut stimulation using Cytobeads.

We could not find any significant difference or trend in cytokine production between peanut allergic and tolerant individuals, at the different time points we investigated, for any of the cytokines we studied, suggesting that the clinical phenotype of the patients is not reflected by their *in vitro* peanut-specific cytokine secretion in the experimental model tested. However, in our experimental model where we investigated peanut-stimulated *in vitro* PBMC cultures we were able to distinguish between peanut allergic and non-allergic individuals on the basis of Th1/Th2 polarisation of their cytokine responses. This has been demonstrated using intracellular staining of cytokines in CFSE-labelled PBMC (5). Conversely, we did not observe any significant difference between PA and NA with respect to the suppressor cytokines measured. Therefore we deduce that the activity of the suppressor cytokines investigated is not different between PA and NA individuals and therefore is not responsible for the peanut allergic *versus* peanut tolerant phenotype. Another explanation for our failure to detect significant differences between PA and NA individuals with respect to the suppressor cytokines that their PBMC produce may be that cytokines participate in the allergic response in vivo but are not produced in our experimental system.

Objective 02: Separate and store plasma from all donors at -70 degrees.

Having plasma from well-characterised donors shall allow us to conduct further work to determine whether cytokines or antibody levels may reflect the states of allergy or tolerance to peanuts and establish further collaborative projects looking at antibody affinities, epitope spread and biomarkers of tolerance, outside the current project.

Objective 03: Correlate peanut-specific IgE production and T cell function in n= 19 PA and n=12 NA children.

The aim of this part of the project is to determine the correlations (if any) between peanut-specific B cell responses (IgE) and T cell function. Indeed, whilst it is known that the maturation of B cell responses directly depends upon T cell help (45, 46) there is limited information on the relationship between antibody levels to a recall antigen, such as peanut, and specific T cell response in humans. Since the only successful immune intervention to date, that is used on a large scale and is able to induce allergy resolution (allergen-specific immunotherapy) targets T cells in order to modify B cell function (i.e. IgE antibody production) it is important that we obtain more information on the correlations between T and be cell responses for designing therapeutic interventions in peanut allergy.

For this reason, we collected blood samples from n=19 PA and n=12 NA children. We measured their B cell responses (expressed as serum antibodies) as well as their T cell responses (proliferation and cytokine production phenotypes) to peanut and to tetanus toxoid (as positive control antigen) according to the procedures described in the methods section.

We found that in peanut allergic donors, peanut-specific IgE (average 21kU/l, median 2.27kU/l, range 0.34-100kU/l) but not peanut-specific IgG was positively correlated with T cell proliferation ($r' = 0.751$, $p = 0.003$). In these donors, specific IgE was positively correlated with peanut-specific Th2 cytokines production: $r' = 0.635$, $p = 0.02$ for IL-4 and $r' = 0.641$, $p = 0.025$ for IL-13 and negatively correlated with Th1 cytokines ($r' = -0.71$, $p = 0.007$ for IFN gamma and $r' = -0.746$, $p = 0.005$ for TNF alpha respectively). However, peanut-specific IgE was not correlated with T cell proliferation or cytokine production in non-allergic individuals.

Conversely, when measuring the response to tetanus toxoid as a control antigen, we found that tetanus-specific IgG did not correlate with lymphocyte proliferation (Spearman rank correlation coefficient $r' = 0.08$, $p = 0.74$) nor with tetanus-specific cytokine production (IFN gamma: $r' = 0.198$, $p = 0.285$; TNFalpha: $r' = 0.274$, $p = 0.146$; IL-4: $r' = -0.007$, $p = 0.96$; $p = 0.221$; IL-13: $r' = 0.363$, $p = 0.056$).

We shall describe below, in more detail, the correlations between peanut-specific IgE production and T cell function in PA and NA children that we found:

IgE (Humoral) allergic responses to peanut antigens (B-cell responses) are correlated with the cytokine profile of peanut-specific T cell responses in PA donors.

There was a strongly positive statistically significant correlation between the Th2 response (IL4, IL13) measured in the peanut-specific $CFSE^{low}$ population with the specific IgE responses to peanut measured in the plasma. Conversely, there was a strongly negative statistically significant correlation between the Th1 cytokine response (IFN gamma, TNF alpha) and the levels of peanut-specific IgE. We show the analysis of the correlations between B and T cell peanut-specific and tetanus toxoid-specific responses for all patients investigated regardless of their allergic phenotypes (Figures 12, 13). We also compare the PA and NA groups separately with respect to their peanut-specific and TT-specific antibody responses (Figures 14, 15). Finally, we also show these correlations separately for PA and NA individuals (Table 1, below) and the values from this table are the values given throughout the text because we feel that these are more relevant for defining the distinct allergic phenotypes.

Peanut-specific B and T cell responses are correlated in PA but not in NA individuals.

In table 2 (page 33) we compare the level of peanut-specific B cell responses (expressed as specific IgE level) with several peanut-specific T cell response parameters (T cell proliferation and specific intracellular cytokine production respectively.) We found that indeed there is a strong correlation between B and T cell responses in PA individuals suggesting a permanent on-going B stimulation by T cells while this correlation has been lost in the case of NA individuals. Thus, in the case of PA individuals, there is a highly significant correlation between peanut-specific T cell proliferation and IgE (Spearman r'=0.751, p=0.003). The correlations between peanut-specific T cell cytokine production and IgE levels are negative in the case of Th1 cytokines (r'=-0.71, p=0.007 for IFN gamma and r'=-0.746, p=0.005 for TNF alpha respectively). Conversely, peanut-specific IgE levels are positively correlated with Th2 cytokines: r'=0.635, p=0.02 for IL-4 and r'=0.641, p=0.025 for IL-13. Conversely there is no correlation between peanut-specific B cell responses (reflected by the IgE values) and T cell responses in NA individuals.

In the case of the control antigen TT, TT-specific T and B cell responses (lymphocyte proliferation and respectively IgG and IgE levels) are normal and equivalent in PA and NA donors

We compared the T cell and the B cell responses to TT in PA and NA children (Fig.14). T cell responses were determined by assessing TT-specific lymphocyte proliferation, expressed as stimulation index (SI) measured using tritiated thymidine incorporation after 5 days of PBMC culture in the presence of 10micrograms/ml TT. B cell responses to tetanus were determined as tetanus-specific IgG levels, measured by ELISA.

Tetanus-specific lymphocyte proliferation as well as tetanus-specific plasma IgG levels were equivalent in both PA and NA donors, meaning that we did not observe any statistically significant difference between the two groups. It does not appear therefore that PA are somehow intrinsically biased towards

generating more IgE and/or IgG to unrelated control antigens than the NA individuals. Tetanus specific IgE was not detected in either the PA or NA donors, with the exception of a few donors who had relatively low IgE levels. Thus the peanut allergic status to peanut does not affect the normal immune response to tetanus antigens in children who have received a routine immunisation schedule.

Peanut-specific T and B cell responses (lymphocyte proliferation and respective IgE levels) are higher in PA than in NA donors

T cell responses to lyophilised peanut extract and peanut-specific IgE levels as measured by Pharmacia CAP were different in PA and NA children. We found that T cell proliferative responses to peanut were significantly greater in children with PA as compared with their NA controls (Fig. 15). Cell proliferation was again expressed as stimulation indices (SI). SI represent the ratio between the averages of triplicate measurements of antigen-stimulated wells and control wells where no antigen was added in order to take into account background proliferation. SI are widely used for expressing antigen-specific proliferation because they allow for the normalization between different experiments in which background proliferation may differ. Indeed, SI has been used to present proliferation data in many publications that describe peanut-specific responses in cultured PBMC in vitro (references 9-11) and no other way of expressing proliferation data has been proposed, to our best knowledge, that could be more robust than SI.

Regarding peanut-specific counts-per-minute absolute values, proliferation reached median values of 1523 (range 228-4203) in NA and 16359 (range 411-39817) in PA donors. For comparison, in the case of tetanus toxoid-specific proliferation, the respective cpm values were 13711 (range 150-36676) in NA and 12788 (range 1337-59136) in PA donors. Not surprisingly, specific IgE levels to peanut were significantly elevated in the PA group and absent in the NA group (Fig.15). IgG response to peanut was equivalent in both groups.

There is no correlation between humoral responses to TT and TT-specific cytokine responses.

We identified TT-specific Th lymphocytes using CFSE-labelling as previously described. Indeed, in a CFSE–labelled, ($CFSE^{high}$) PBMC population cultured in the presence of antigens we observed the emergence of a less fluorescent, $CFSE^{low}$ subset which represents the dividing antigen-specific cells. In a previous paper (reference 5), we have shown by cloning that this emerging $CFSE^{low}$ population is indeed antigen-specific. Identification of the (rare) antigen-specific Th cells amongst the PBMC in culture also allows us to determine their cytokine-production phenotype while using a non-specific boost with phorbol-12-myristate-13-acetate and ionomycin in the presence of brefeldin A as secretion inhibitor. We thus characterise the antigen-cytokine response as the percentage of cytokine-producing cells amongst the antigen-specific T cell population. We examined the relationships between tetanus antigen responses and IL-4, IL-13, IFN gamma and TNFalpha in the tetanus-specific $CFSE^{low}$ population by intracellular cytokine staining and flow cytometry. We did not observe any correlation between the humoral response

to tetanus (TT-specific IgG level) and TT-specific cytokine responses (figure 13).

In order to avoid repeating the negative results regarding the lack of correlation between certain components of the immune response, the positive information obtained in this part of the study can be summarised in the table below:

Table 2 …Correlation between peanut-specific IgE and peanut-specific T cell responses in non-allergic (NA) and in peanut allergic (PA) donors.

PA (n=19) sp IgE values versus	**Spearman correlation**	**P-value**
T cell proliferation (expressed as SI)	0.751	0.003
IFN gamma (% expression in CFSElow)	-0.71	0.007
TNF alpha (% expression in CFSElow)	-0.746	0.005
IL4 (% expression in CFSElow)	0.635	0.02
IL13 (% expression in CFSElow)	0.641	0.025
NA (n=12) sp IgE values versus	**Spearman correlation**	**P-value**
T cell proliferation (expressed as SI)	0.445	0.317
IFN gamma (% expression in CFSElow)	0.259	0.574
TNF alpha (% expression in CFSElow)	0.319	0.538
IL4 (% expression in CFSElow)	0.556	0.195
IL13 (% expression in CFSElow)	0.406	0.425

In conclusion, in peanut allergic individuals B and T cell responses to peanut antigens are correlated whereas in non-allergenic subjects, responses B and T cell responses are uncoupled. We aim to use this information in the future, in order to design a therapeutic intervention aimed at inducing peanut allergy resolution since our findings support the hypothesis that such intervention is likely to be successful by targeting peanut-specific T helper cells in order to act indirectly upon the pathogenic peanut-specific IgE.

Objective 04: Characterisation of proliferative T cell responses to peanut amongst the Cutaneous Lymphocyte-associated Antigen (CLA) expressing (skin homing) and beta 7 integrin expressing (gut homing) memory T cells subpopulations in peanut allergic, non-allergic and sensitised children.

The approach that we chose to address this objective was to use immunomagnetic bead separation followed by peanut-stimulation of T call subsets. Briefly, immunomagnetic beads were used to isolate CLA+ and α4β7+ T cells from PBMCs. The cells were then stimulated with peanut extract in the presence of dendritic cells as antigen presenting cells. In the experiments described under the previous objectives, unseparated PBMC contain of course the dendritic cells that act as APC. However, since in this

objective we had to isolate the CLA and α4β7+ T cells and then assess their proliferation. Since for their proliferation T cells need APC, we separated the dendritic cells first and then proceeded with the isolation of the T cell subsets. Thymidine incorporation was assayed on days 5 and 7 to measure lymphocyte proliferation. Peanut-specific proliferation was measured as the stimulation indices to peanut that were calculated as the ratio between counts per minute measured in peanut-stimulated cultures and in unstimulated control cultures. Stimulation indices in the CLA+ cells were then compared to those in the α4β7+ cells in both peanut allergic and peanut tolerant children.

In order to achieve this objective we had initially to establish a reliable method that could allow us to reliably separate PBMC into CLA+ & alpha 4 beta 7+ memory T cell subsets achieving highly pure subsets.

We had to overcome multiple difficulties related to the low numbers of CLA+ & alpha 4 beta 7+ memory T cells in circulation and to optimise the reagents and the protocol.

In the end, we consistently achieved cell viability of over 90% after cell separation. Various parameters were explored to optimize cell purity. A number of experiments were carried out to reassess the methods and optimise conditions for cell purity.

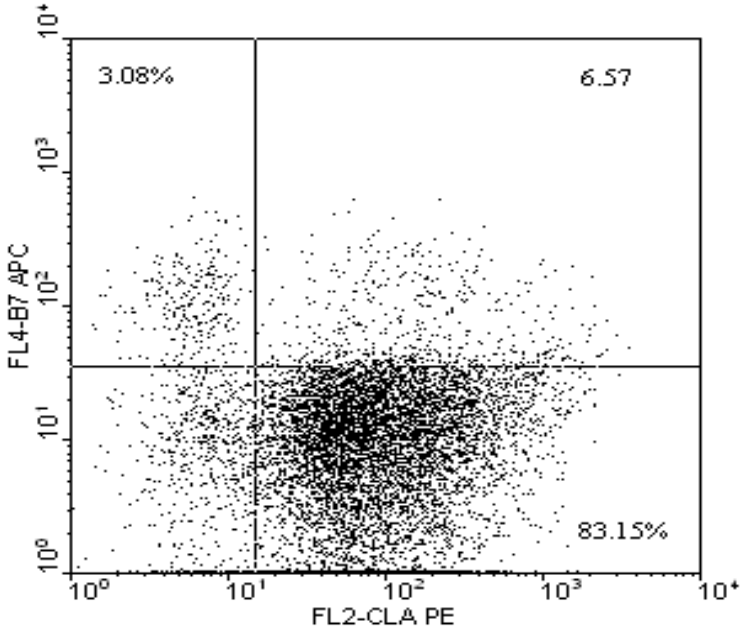
Thus, for example, the FL-3 channel fluorochrome-conjugated antibody was changed from CD45RoCychrome to CD45RoPECy5 to see if better results could be achieved. Changing the label in this way made interpretation easier and led to a more defined result. The β7 fluorochrome label and associated MicroBeads were also changed from PE to Allophycocyanin (APC) and the CD4 fluorochrome and MicroBeads from APC to FITC.

As another example of purification optimization, Miltenyi Biotec introduced a new Memory CD4+ T cell isolation, an indirect magnetic labelling system for the isolation of untouched memory T helper cells from human peripheral blood. For magnetic depletion of naive T cells, $CD8^+$ T cells, B cells, NK cells, γ/δ T cells, monocytes, DCs, granulocytes, platelets, and erythroid cells, PBMCs are incubated with a cocktail of biotinylated CD45RA, CD8, CD14, CD16, CD19, CD56, CD36, CD123, anti-TCRγ/δ, and CD235a (glycophorin A) antibodies. These cells are subsequently magnetically labeled with Anti-Biotin MicroBeads for depletion. This kit which was adopted as a replacement for the CD4+ T Cell Negative Isolation Kit II and CD45RA MicroBeads, to isolate memory CD4+ T cells. This yielded better purity of the intermediate memory T cells (94.74% vs the 58.6% previously achieved)

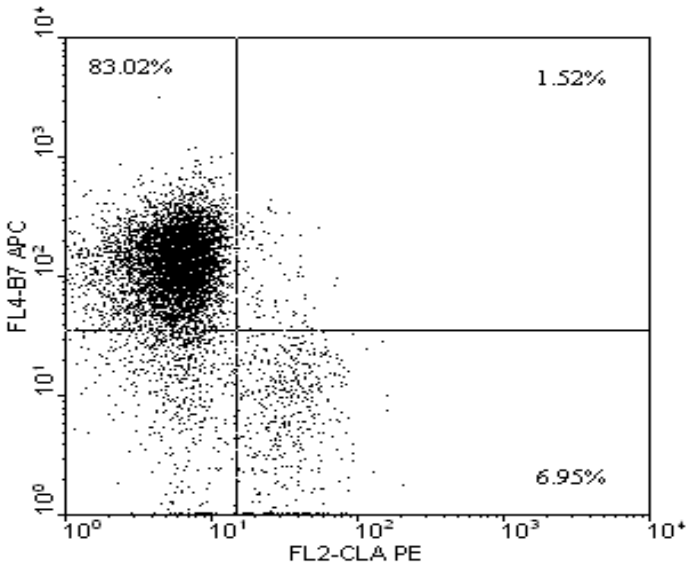
Whilst CLA purity improved with these measures to 87.7%, issues with β7 yield arose at this stage and it was considered that, taking into account the emission intensities of the different available fluorochromes, labelling with allophycocyanin (APC) may be less favourable than phycoerythrin (PE) labelling of this T cell subset. Experiments to assess the integrity of the antibody labelling were performed. B7 was labelled with 5µl, 10µl and 20µl APC and then subsequently with PE such that any β7 unbound by the APC

label would be bound by the PE label. Flow cytometry was performed and demonstrated that β7 APC labelling predominated, indicating that the β7 APC antibody rapidly and adequately saturated β7. The gating strategy used on flow cytometry assessment was also improved and new antibody was purchased to rule out the risk of chemical deterioration.
We achieved over 80% purity in our target CLA+ and α4β7+ T cell populations. The variations regarding the final T cell population purity that we achieved result from the fact that a small percentage of the memory T lymphocytes express neither the CLA nor the beta7 marker (are double negatives). Depending upon the order of the purification (we could either purify the CLA+ cells first and the beta 7 + subsequently or vice-versa) these double-negative cells were found in one or the other population.

Typical example of CLA subset purification (achieving 89.72% purity – reflecting the sum of the CLA+ subsets i.e. 83.15 + 6.57%):



Typical example of alpha 4 beta 7 subset purification (achieving 84.54% purity – reflecting the sum of the two beta 7+ subsets i.e. 83.02 + 1.52%):



Having established a reliable method to isolate Th memory subsets, we then characterised the proliferative T cell responses to peanut amongst the CLA+ (skin homing) and beta 7 integrin expressing (gut homing) subpopulations in peanut allergic and in peanut non-allergic children.

We found that the *in vitro* peanut-specific response of n=10 PA individuals occurs predominantly in the CLA+ skin homing subset whilst the response in n=10 NA individuals occurs predominantly in the α4β7+ subset.

We show in Figure 16 the peanut-specific proliferation of T cell subsets isolated from a typical PA and from a typical NA individual. We added increasing concentrations of peanut proteins to the cultures in order to observe the dose-response curve. Peanut-specific proliferation is expressed as stimulation indices.

We analysed the responses to peanuts in n=10 PA donors and found that the CLA+ subset was responsible for more than 50% of the peanut-specific proliferation in 9 out of 10 of the donors (Fig 17). Indeed, in 8/10 donors CLA responses account for at least 80% of the response. (In this figure each column represents a different patient. Total peanut-specific proliferation is shown as 100% proliferation.)

We also analysed the responses to peanuts in n=10 NA donors and found that the α4β7+ subset was responsible for more than 50% of the peanut-specific proliferation in 4 out of 10 donors. Interestingly, some NA individuals show a rather mixed peanut-specific response, with some proliferation being observed in the CLA+ subset too.

One could speculate that the peanut-specific response in NA individuals may be more mixed because, once they have established oral tolerance to peanuts as a consequence of developing a subset of gut-homing alpha 4 beta 7 regulatory T cells, they can eat peanuts and consequently they came in contact with peanut antigens through the skin too. Conversely, peanut allergic

individuals became peanut allergic because their initial exposure to peanuts occurred through (possibly eczematous) skin. They then developed CLA positive allergenic T cells that dominate their peanut-specific responses. Since they are allergic, these individuals avoid eating peanuts and therefore do not have the possibility of developing an alpha 4 beta 7 peanut-specific response.

In order to compare the peanut-specific proliferative responses of the PA and NA individuals, we calculated the ratios of CLA/ α4β7+ proliferation and we found a statistically significant difference between the two groups ($p=0.009$). In order to confirm the specificity of this response we compared the responses of PA and NA individuals to a control antigen (ovalbumin – Ova). We did not find a statistically significant difference between ova-specific responses of PA and NA individuals ($p=0.162$). This finding demonstrates that the CLA response seen in PA individuals is not a general characteristic of their responses but is specific to peanuts. There is no difference between PA and NA with regard to their response to an unrelated antigen.

This result supports our hypothesis that the cutaneous sensitization route may be involved in the induction of peanut allergy in children whilst the oral route is presumably involved in the establishment of tolerance to peanut. We attempted to investigate further the peanut-specific responses of the CLA and alpha 4 beta 7 subsets pf PA and NA individuals. Therefore we measured cytokine production in the supernatants from peanut-stimulated cultures. We could not however find any statistically significant difference between these groups with respect to their cytokine production (figures shown in the annexe).

DISCUSSION

In the past several authors have compared the *in vitro* peanut-specific proliferative responses of PBMC isolated from PA and NA donors, reaching different conclusions - some found equal levels of peanut-specific proliferation in both PA and NA (PA=NA), others described higher proliferation in PA donors (PA>NA). Thus Dorion et al. 1994[10] and Higgins et al. 1995[8] found that proliferation in PA was equivalent to that in NA while, conversely, de Jong et al. 1996[11], Laan et al. 1998[9] and Hourihane et al.1998[12] all found that PA>NA i.e. PA donors have higher proliferative responses than NA controls. Our results are similar to those published by de Jong et al. 1996[11], Laan et al. 1998[9] and Hourihane et al.1998[12]; in fact we used similar reagents, stimulation protocols and we expressed our data in the same way. Furthermore, our work provides additional insights that could explain the differences between the different authors quoted above. Indeed, we investigated peanut-specific proliferation in more depth – for example we measured the kinetics of the proliferation on days 3, 5, 7 and 9 after adding the peanut antigens at the beginning of the culture. We could thus observe that PA and NA individuals have different kinetics of their peanut-specific responses, as we discuss below. Since the authors quoted did not measure the kinetics of the response, it is conceivable that they may have measured the peanut-specific response at a time point when the differences between PA and NA individuals were not significant (as we show to be the case on day 7 of the cultures – Figure 3).

In order to accurately determine the characteristics of *in vitro* peanut-specific proliferative T cell responses of PA and NA donors, we used the system described by Plebanski et al. for the investigation of human T cell responses to soluble antigens[14] because its use allows for a relatively low level of background proliferation.

We took advantage of the features of this assay system (flat bottom culture plates that prevent high background proliferation and the use of autologous plasma that makes the system more biologically relevant) and we determined proliferation at several time points (culture days 3, 5, 7 and 9) in order to obtain peanut-specific proliferation kinetics in PA and NA donors. We found that there is a clear difference between the *in vitro* responses of PBMC from PA and NA donors: PBMC from PA donors show greater proliferation and their response peaks earlier (on day 5 after the beginning of the culture) than PBMC from NA donors. Moreover, PBMC from NA donors proliferate when cultured in with peanut antigens but they display lower levels and reach peak proliferation at a later time point (day 7) than PBMC from PA.

For many antigen-stimulated PBMC proliferation experiments reported in the past it is notable the use of foetal calf serum (FCS) or AB (IV) human serum as cell culture supplements rather than autologous serum or plasma.

We aimed to maintain our *in vitro* assay system as close to the biological microenvironment as possible by using autologous plasma for cell culture. Autologous plasma (plasma obtained from the same donor as the cells) also contains the antibodies that may influence T cell proliferation so that

differences between PA and NA donors can be better identified. Switch experiments , carried out outside this project, in which serum from a PA donor was added to PBMC from an NA donor led us to the finding that a serum factor existing in PA but not in NA plasma was responsible for the higher levels of proliferation seen in PA. We further identified this serum factor as being IgE by depletion-repletion and blocking experiments: when IgE-containing plasma from a PA monozygotic twin was added to the PBMC isolated from his NA brother, there was an increase of peanut-specific proliferation. This increase was abrogated if IgE was destroyed by heating 2h at 56˚C before the plasma was added to the culture. The role of IgE in amplifying peanut-specific proliferation was further demonstrated in IgE-depletion experiments using magnetic beads coated with anti-IgE antibodies.

It is known that persistent food allergy is characterised by high specific IgE levels and high T cell proliferative responses whereas resolution of food allergies is characterised by declining IgE levels and decreasing T cell proliferative responses. One possible explanation for this is that T cells (through the elaboration of pro-allergic cytokines and cognate interaction with B cells) drive the production of IgE. An additional, non-exclusive explanation for this association is that IgE production amplifies T cell proliferative responses through FAP, leading to a positive feedback loop that maintains the allergy.

In the present work we found clear evidence of IgE-mediated FAP leading to an increase in allergen-specific T cell proliferative responses: thus heat-inactivating plasma (which destroys IgE) leads to a decrease in peanut-specific proliferative responses while adding plasma from a PA individual to the cells of a NA person leads to an increase in peanut-specific proliferation. The role of IgE in stimulating peanut-specific proliferation is further confirmed by our depletion / add-back experiments.

The current literature on IgE-mediated FAP suggests that allergen presentation to the specific T cells amongst the PBMC is indeed increased when higher amounts of allergen-derived peptides are present on the surface of antigen presenting cells[21]. In this respect, the presence of allergen-specific IgE antibodies leads to FAP due to a higher rate of allergen capture and internalisation due to the endocytosis of IgE-allergen complexes. Mechanistically, it has been shown[22-25] that the high-affinity IgE receptors on dendritic cells deliver IgE-bound antigens into organelles containing MHC class II, HLA-DM and lysosomal proteins (a cathepsin S-dependent pathway of MHC class II presentation).

The relationship between the presence of allergen-specific IgE in the serum of allergic individuals and facilitated antigen presentation that leads to increased allergen-driven T lymphocyte proliferation has been clearly established *in vitro*[26-28]. The *in vivo* functional role of this phenomenon is yet unclear, nevertheless it has been shown that antigen endocytosis mediated through the high affinity IgE receptor turns interferon gamma-treated mouse mast cells into potent antigen presenting cells[29]. These results suggest that IgE-mediated allergen endocytosis leads to an increased and possibly

qualitatively different presentation due to the activation of signalling pathways in the antigen presenting cells[30], eventually resulting in stronger, Th2-skewed T cell responses.

The increased peanut-specific T cell precursor frequency we have demonstrated in PA individuals compared with NA individuals arguably results from increased peanut-specific T cell proliferation which we have shown, in our *in vitro* experimental system, to occur through IgE-mediated FAP. In this respect, our results are in accordance with observations of higher levels of circulating allergen-specific precursor T cells in allergic individuals when compared with non-allergic controls[16]. Similar correlations have been observed in the case of IgE decreases and allergy resolution as a consequence of immunotherapy[31].

The importance of IgE-dependent FAP for the activation of allergen-specific T cells has also been demonstrated in the past using blocking antibodies. Indeed, the addition of such anti-IgE antibodies prevents the activation of allergen-specific T cells in an *in vitro* cell culture experimental model[28].
Therefore anti-IgE blocking antibodies may interfere with the allergy immune mechanisms not only by preventing the IgE-triggered mast cell degranulation that underlies allergic reactions but also facilitated antigen presentation that maintains the on-going allergic immune response. This possibility opens the perspective of using long-term treatments with anti-IgE blocking antibodies to modulate the allergic immune response by interrupting the pro-allergic positive feedback dependent upon FAP.

We also found (albeit in a small number of individuals) that the peanut-specific response in PA individuals tends to be generated to a significant extent by CLA+ cells that were sensitised in the skin. Conversely, responses in NA individuals tend to be generated by gut-sensitised cells. This finding supports our initial hypothesis that allergic sensitization to peanut might occur through the skin, leading to Th2 responses and peanut allergy. On the other hand, it is likely that early exposure to peanut protein through the gastrointestinal tract might lead to regulatory and Th1 responses to peanut and consequently to oral tolerance. Whilst these preliminary data are indeed very encouraging, further studies are nevertheless needed before a definitive conclusion could be put forward.

In any case, the specificity of our results relating to peanut responses is proven by the fact that responses to a control antigen (ovalbumin) are similar in PA and NA children and result from both the CLA+ and alpha 4 beta 7 + T cell subsets.

Therefore our results showing that *in vitro* peanut-specific response of peanut allergic individuals occurs predominantly in the CLA+ skin homing subset whilst the response in non-allergic individuals is more variable but tends to occur in the α4β7+ subset suggest that the cutaneous route of sensitization may be indeed involved in the pathogenesis of peanut allergy whilst the oral route is involved in tolerance induction. These data represent the first immunological characterisation of food-specific immune responses in

allergy and tolerance that reflect the potential existence of different pathways of exposure that lead to allergy induction.

This finding may be significant for the development of future strategies for preventing peanut allergic sensitisation by avoiding exposure to peanut antigens through eczematous skin. Further insights into the role of eczema for the development of food allergies could also be obtained through trials that involve large numberes of children with eczema, such as the LEAP study.

We attempted to investigate further the peanut-specific responses of the CLA and alpha 4 beta 7 subsets pf PA and NA individuals. Therefore we measured cytokine production in the supernatants from peanut-stimulated cultures. We could not however find any statistical significance between these groups with respect to their cytokine production (figures shown in the annexe).

CONCLUSIONS

In the present study we have investigated four immunological mechanisms that could explain the differences between PA and NA responses to peanuts. We found that these differences can indeed be explained by the presence of peanut-specific IgE that facilitates antigen presentation (FAP). Higher peanut-specific IgE levels may also be the cause of the higher frequencies of peanut-specific circulating Th cells that we found in PA when compared with PS and NA individuals, so both these mechanisms contribute to the higher proliferative responses we saw in PA individuals.

Conversely, we found that in both PA and NA donors peanut-specific responses were driven by memory Th cells and not by naïve T cells. We could not find any difference regarding the levels of suppressor cytokines produced in peanut-specific responses by PA and NA donors either, therefore these two mechanisms do not seem to cause the differences between PA and NA.

We also stored plasma from all donors at minus 70 degrees so that we shall be able to measure cytokines or antibody levels that may reflect peanut allergy or tolerance. We plan to establish further collaborative projects using these stored samples looking at antibody affinities, epitope spread and biomarkers of tolerance.

As another objective of the study, we investigated the correlation between peanut-specific IgE production and T cell function in PA and NA children. We found that B cell responses (reflected by peanut-specific IgE) and T cell responses to peanut antigens are correlated whereas B and T cell responses (to a control antigen) are uncoupled in PA individuals. Conversely, in NA individuals peanut-specific responses are uncoupled.

Our results confirm the hypothesis that B cell responses to allergens (but not those to non-allergenic proteins) are on-going responses that are closely linked with allergen-specific T cell responses, possibly through the positive feedback circuit triggered by IgE-mediated FAP.

Finally, we analysed the contribution of skin homing versus gut-homing T cell subsets in peanut-specific responses on PA and NA children.
We found that in PA donors the peanut-specific response is predominantly generated by skin-homing CLA+ memory T cells that have initially seen peanut antigens in the skin. Conversely, peanut-specific responses in NA individuals are mixed, being generated by both skin-homing and gut-homing memory Th cells. These differences are specific for peanut responses since we could not see any difference between PA and NA responses to a control food antigen (ovalbumin): in these donors control responses show no clear subset predominance of skin-homing or gut-homing memory Th cells.

This result is potentially very significant for designing interventions aimed at preventing peanut allergy since these argue in favour of skin sensitisation. Thus skin exposure to peanuts, presumably through inflamed, eczematous skin, may lead to peanut allergy development.

FURTHER WORK

Our investigation of the immunological mechanisms underlying peanut allergy and tolerance have led to the establishment of novel approaches that will be used for the monitorisation of participants in the LEAP (Learning Early About Peanut allergy) study – a randomized interventional trial aimed at finding whether avoidance or early exposure to peanuts are better for preventing peanut allergy.

FSA funded work under project T07049 is using blood samples from the LEAP study to characterise the immunological mechanisms of prophylactic oral tolerance induction. This may provide the FSA with the scientific information necessary for designing new approaches to prevent food allergies and would provide a scientific basis for policies and advice on peanut consumption by children.

Additionally, our finding that in PA individuals most of peanut-specific memory Th cells express the CLA skin-homing receptor (indicating sensitisation occurred via the skin),opens the way for investigating the correlation between environmental levels of peanut antigens and the development of peanut allergy with the aim to prevent peanut allergy. Thus, one approach to prevent peanut sensitization might be to avoid children's environmental 9dermal) contact with peanuts.

Planned future work will address this strategy, initially by assessing the level of level of peanut proteins (a measure of 'environmental' exposure to peanut allergens), in households that consume peanut-containing foods and households that avoid peanuts. Depending upon these future findings, we hope to find out whether peanut sensitisation is related to environmental exposure and we shall investigate the eventual role of bacterial superantigens that may act as adjuvants for allergic responses.

PUBLICATIONS:

The following publications arose from the present grant:

1. V. Turcanu, M. Winterbotham, P. Kelleher and G. Lack, Peanut-specific B and T cell Responses Are Correlated in Peanut Allergic but not in Non-allergic Individuals. *Clin Exp Allergy,* **38**:1132-1139, 2008.

2. V. Turcanu, A. C. Stephens, S.M.H. Chan, F. Rancé and G. Lack IgE-mediated facilitated antigen presentation underlies higher immune responses to peanut in allergic individuals compared with tolerant individuals. *Manuscript in preparation*

3. S.M.H. Chan, V. Turcanu, A.C. Stephens, G. Lack, In Vitro Evidence For Different Routes Of Sensitization To Peanut In Children. *Manuscript in preparation*

The following abstracts also arose from the present grant:

1. S.M.H. Chan, V. Turcanu, A.C. Stephens, G. Lack, In Vitro Evidence For Different Routes Of Sensitization To Peanut In Children *J Allergy Clin Immunol* **121**: S214, 2008.

2. S. Hingley, G. Du Toit, G. Roberts, V. Turcanu, G. Lack, H. Fisher, and P. Lau, Peanut-specific IgG4 and its association with peanut allergy. *J Allergy Clin Immunol* **117**: S33, 2006.

3. V. Turcanu, A.C. Stephens and G. Lack IgE-mediated facilitated antigen presentation augments immune responses to peanut antigens in allergic individuals compared with tolerant individuals - The annual meeting of the European Academy of Allergy, Asthma and Clinical Immunology, June 2006

4. V. Turcanu and G. Lack, Levels of peanut-specific precursor T cells reflect peanut allergy status. *J Allergy Clin Immunol* **115**: S62, 2005.

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