Final Report

29th November 2010

T07042

STUDY OF T CELLS IN ALLERGY AND RESOLUTION

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ABBREVIATIONS AND DEFINITIONS

Abbreviations:

APC	Allophycocyanin or antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDI	Cell division index
CFSE	Carboxyfluorescein succinimidyl diacetate ester
CFSEnet	Final %CFSE value after subtracting the background %CFSE of untreated cultures from %CFSE of stimulated cultures
CO_2	Carbon dioxide
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetra acetic acid
dH_20	Distilled water
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent activated cell sorting
HBSS	Hank balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
FCS	Fetal calf serum
FSC	Forward scatter
HI-	Heat-inactivated
hr	Hour
Ig	Immunoglobulin

IFN	Interferon
IL-	Interleukin
kDa	Kilodalton
L	Litre
Lfu	Lytic forming units
µg	Microgram
µM	Micromolar
mg	Milligram
Min	Minute
ng	Nanogram
NR	Non responders
OD	Optical density
OFC	Oral food challenge
OVA	Ovalbumin
OVAhi	High dose ovalbumin
OVAlo	Low dose ovalbumin
OM	Ovomucoid
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-Tween	PBS containing 0.1% Tween-20
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	Proliferation Index
PMA	Phorbol myristate acetate
PMT	Photomultiplier tube
R	Responders
RPM	Revolutions per minute

RT	Room temperature
SPT	Skin prick test
SSC	Side scatter
TET	Tetanus toxoid
Th1	T helper type 1
Th2	T helper type 2
Treg	T Regulatory cell
[^{3}H]-TdR	Tritiated thymidine

Definitions:

NEG	Negative reaction to liquid egg challenge
POS	Positive reaction to egg challenge
EA	Egg allergic (Sponge POS and/or Liquid POS)
FA	Fully egg allergic (sponge POS; well cooked egg reactive)
PA	Partially allergic (Liquid POS; uncooked egg reactive)
PR	Partially resolved (UNK; cooked egg tolerant)
ER	Egg resolved (egg tolerant; cooked egg and uncooked egg tolerant)
UNK	Unknown (Sponge NEG, Liquid Egg ND)

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EXECUTIVE SUMMARY

Background

The immunological mechanisms underlying resolution of egg allergy are poorly characterised. A better understanding of the relationship between cellular and humoral responses to egg and clinical sensitivity may lead to a more accurate understanding of the pathogenesis of egg allergy and the mechanisms underlying natural resolution.

Objective

The aim of this project was to follow immune responses to the egg allergen ovalbumin (OVA) as children progress to resolution or persistence of their egg allergy. The longitudinal changes in allergen –specific T cell proliferation and intracellular cytokine expression (IL-4 and IFNγ) were measured as well as specific serum immunoglobulins (IgE, IgG, IgG1 and IgG4) in individuals whose egg allergic status had been confirmed by open oral challenge. In addition, the project aimed to characterise the rate at which the children's allergies resolved to well-cooked and uncooked egg.

Method

Egg-allergic subjects underwent repeated annual open oral egg challenges with well cooked egg (sponge cake) and if negative, uncooked (pasteurized) egg over time to confirm resolution or persistence of allergy. Non-allergic controls were recruited from hospital patients. Skin prick tests were performed and blood samples taken at each challenge occasion for immunological assays. CFSE (carboxyl-fluorescein diacetate succinimidyl ester) labelled peripheral blood mononuclear cells (PBMC) were cultured *in vitro* for 8 days with OVA (200µg/ml or 1mg/ml) or Tetanus toxoid (8.21Lfu/ml) culture medium containing 10% autologous plasma. Using multi-colour flow cytometry, the proportion of allergen-specific T cells (CD3+/CFSElo) expressing intracellular IL-4 and IFNγ cytokines was measured. Total and egg-specific IgG1 and IgG4

antibody subclasses were assayed by ELISA and egg white IgE antibodies were measured by ImmunoCAP.

Results

T cell proliferation**:**

- PBMCs from egg-allergic individuals proliferated more rapidly in the presence of egg allergen (ovalbumin) compared to those from non-allergic controls. There was no significant difference when PBMCs were stimulated by non-allergic antigen (tetanus toxoid). Unstimulated PBMCs from egg-allergic subjects were more reactive overall.

Intracellular Cytokine secretion:

- Stimulation of separated PBMC cells with the allergens tested gave rise to no significant differences in the proportion of interleukin-4 (IL-4) producing allergen-specific T cells (%IL-4+/CD3+/CFSElo cells) between EA and ER subjects.
- Higher proportions of IFNγ+ secreting cells were observed in response to stimulation with OVAhi in previously egg-allergic subjects whose allergy had resolved (p=0.0092**).
- The percentage of IFNγ producing cells in response to stimulation with OVAhi was higher in ER subjects than in partially allergic (p=0.0149*) and partially resolved subjects (p=0.0062**).
- The ratio of IFNγ/IL-4 producing cells and log of the same ratio confirmed a T-helper 1 cell skew (Th1 skew) with IFNγ as the dominant cytokine in ER compared with EA subjects (p=0.0113* and p=0.0172* respectively)
- In EA subjects stimulation with TET (p<0.0001***), OVAhi (p<0.0001***) and OVAlo (p<0.0001***) gave rise to a higher proportion of IL-4+ producing cells than incubation with culture medium alone.
- In the longitudinal analysis, no significant changes in IL-4+ or IFNγ+ producing egg specific T cells were observed for EA or ER over time, by Wilcoxon's matched paired test.

However, Fisher's exact, two-tailed test showed that the persistently EA subjects (p=0.0133*) had higher levels of IL-4 than egg resolved subjects on their final egg challenge, whereas ER subjects (p=0.0214*) had higher levels of IFNγ+ than EA subjects on their final challenge.

Humoral analysis

- Egg-specific immunoglobulin E levels decreased between first and last oral challenge in children who had resolved egg allergy compared with children who had persistent active egg allergy p=0.0003***.
- Immunoglobulin G4 levels in children with resolving egg allergy increase overtime (Wilcoxon Matched Pairs test p=0.0119*; Mann-Whitney U-test: not significant; p=0.057)
- Paired data show a trend towards a decrease in IgE/IgG4 ratio in active allergy, but this ratio significantly decreases in patients whose allergy resolves (p=0.0081**)

Clinical analyses

- 181 hen's egg challenges were performed during the course of the study in children whose median age of onset was 12 months.
- Pre-enrolment reactions occurred to baked egg in 5 (5%), lightly cooked egg in 58 (61%) and uncooked egg in 9 (9%) subjects. The worst symptoms in pre-study reactions were: cutaneous only in 57 (60%), gastrointestinal in 20 (21%), respiratory in 11 (12%) and 7 (7%) had anaphylaxis. Adrenaline was used during five reactions.
- There were 77 well cooked and 104 uncooked egg challenges conducted during the study. Of these 28/77 (37%) were positive challenges to well cooked egg of which , 65% had cutaneous symptoms, 68% gastrointestinal and 39% rhinitis, with no other respiratory reactions. Of the 61/104 (59%) positive uncooked egg challenges, 75% had cutaneous symptoms, 56% gastrointestinal and 15% respiratory. Adrenaline was not required.

- Oral Tolerance was gained twice as rapidly to well-cooked than uncooked egg (median 5.6yr v 10.3yr; p<0.0001) and continued to 13yrs; hazard ratio 2.23 (95%CI 1.6-3.9). Over 1/4 had resolved allergy to well-cooked egg at 3 years and 2/3 at 6 years. Of 28/77 (37%) positive well-cooked egg challenges, 65% had cutaneous symptoms, 68% gastrointestinal and 39% rhinitis, with no other respiratory reactions. Adrenaline was not required.

Conclusions

It is already known that production of the cytokine IFNγ antagonizes the production of IgE antibody and provides a balance to IL-4, a potential promoter of IgE responses. We have provided evidence which indicates that the resolution of egg allergy is associated with a change in the cytokine producing phenotype of OVA-specific T cells from T-helper 2 (Th2) to T-helper 1 (Th1), which is accompanied by an increase in the production of OVA-specific IgG4 and reduction in the production of egg-specific IgE.

The study has also shown that resolution of egg allergy takes place over many years, with children outgrowing allergy to well-cooked egg approximately twice as quickly as they outgrow allergy to uncooked egg. There were no severe reactions to well-cooked egg challenge during the study period, and adrenaline was not required. Our data support initiation of home reintroduction of well-cooked egg from 2-3yr of age in children with previous mild reactions and no asthma. Resolution continues to occur in older children, so that despite an earlier positive challenge, attempts at reintroduction should be continued.

1 INTRODUCTION

Over recent years the prevalence of food allergy in early childhood has steadily increased and it now affects 7-8% of children [1,2]. One of the most common childhood food allergies is to hens egg (referred to as egg hereafter) [3, 4] with a childhood population prevalence estimated at 1-2% [5, 6].

Current treatment of egg allergy involves complete dietary avoidance of egg-containing foods [10, 11] which can be difficult to achieve as egg products are often present in baked goods or processed foods [12].

Egg allergy is considered to resolve relatively quickly during early childhood [5] in comparison to other food allergies such as to peanuts or nuts [6]. One longitudinal study estimated that 50% of children lose reactivity to uncooked egg by 35 months [7]. However, there is a wide variation in the speed of resolution between individuals.

Allergy to cooked egg resolves earlier than allergy to uncooked egg in individuals [8, 9]. Families find it useful to define when well cooked egg allergy resolves as this can result in considerable relaxation of avoidance measures. The recently published British Society of Allergy and Clinical Immunology's guidelines on the management of egg [74] allergy made recommendations about reintroduction of egg: children who have had only mild symptoms (only cutaneous symptoms) on significant exposure (e.g. a mouthful of scrambled eggs) with no ongoing asthma may have well-cooked egg (e.g. sponge cake) introduced from the age of about 2–3 years at home. If this is tolerated then reintroduction of lightly cooked egg (e.g. scrambled) may be attempted from about 3–4 years [10]. These recommendations were based on limited data, clinical experience and expert opinion. Data are lacking to guide clinicians when to advise reintroduction of well cooked egg. The outcome variable in most

studies has been the resolution of allergy to uncooked egg where factors predictive of resolution (such as egg white IgE, age at diagnosis, skin prick test wheal size, including end-point titration and severity of reactions) have been identified [7, 11, 12, 13].

This study was designed to follow egg allergic children prospectively and perform serial challenges to both well-cooked and uncooked egg to define the stages of resolution and aid clinical decision making as well as to inform advice to those affected by egg allergy .

This longitudinal prospective study was also designed to examine changes in cellular and humoral responses to food allergens and clinical patterns of resolution in young children with egg allergy. This might reveal important mechanistic clues which would shed light on this poorly understood disease. Patients with any type of allergy generally have a period of 'pre-sensitisation' (where they make IgE against the allergen), before the development of clinical allergy. This is followed by the emergence of clinical allergy. Finally as the clinical allergy resolves the patients become less clinically reactive but they still make IgE in response to the allergen. Understanding the mechanism by which clinical allergy develops and then subsides, in the presence of continuing IgE production is clearly important and involves investigation of other cellular and humoral facets of the immune system.

Early life events appear to shape the maturation and development of the acquired immune response [13]. Egg allergy usually develops in children with a history of atopic eczema or other food allergy (e.g. to cow's milk), but is also important as a marker of progression of the allergic march with affected children being at greater risk of peanut allergy and/or sensitization to aeroallergens and asthma later in life [14].

Dysfunctional immunity in early development in allergic children appears to result in inappropriate T cell responses to allergens. Functionally distinct T cell subsets (CD4+) can be

characterized by differentiated expression of cytokines [15]. Type 1 (Th1) T cells secrete predominantly interleukin (IL) -2 and interferon gamma (IFNγ) while type 2 (Th2) T cells secrete predominantly IL-4, IL-5, IL-6 and IL-10. Additionally, Th0 cells thought to be progenitors of Th1 and Th2 cells have been described to produce IL-2, IFNγ, IL-4 and IL-5 simultaneously. Th2 cells play an essential role in the promotion of allergen-specific IgE synthesis by B cells [16, 17, 18]. The cytokines produced by activated T lymphocytes can influence the type of immune response to food allergens that results. Type 2 Th cell responses are thought to predominate in subjects with food allergy while Th1 cells can induce tolerance [19]. Increased IFNγ (secreted by Th1 cells) in the circulation during and after oral egg immunotherapy in egg allergic subjects is thought to induce clinical tolerance to egg protein. However, the effect of egg immunotherapy on allergen specific T cell cytokine production is currently unknown.

Oral tolerance implies a specific suppression of cellular and/or humoral immune reactivity to a food antigen taken through the oral route. Failure of oral tolerance is attributed to the development and pathogenesis of several immunologically based diseases including food hypersensitivity. Although in most cases IgE-mediated egg allergy resolves spontaneously with age the contributing factors and underlying immune mechanisms are largely unknown. Studies with animal models have been used to help understand and identify some potential mechanisms. The mechanisms being proposed include the induction of T regulatory (Treg) cells including the CD4+/CD25^{hi} subset [39, 40] associated with low antigen dose [41], and either clonal anergy (unresponsiveness) or deletion, of memory and effector T cells associated with high dose tolerance [42, 43].

Immediate type-1 food hypersensitivity is mediated by allergen-specific IgE antibody directed against allergenic proteins. These antibodies bind to high-affinity IgE antibody receptors on

circulating basophils and tissue mast cells present throughout the body, including the skin, gastrointestinal tract and respiratory tract. Subsequent allergen exposure binds and cross links IgE antibodies on the mast cell surface, resulting in receptor activation and intracellular signalling that initiates the release of inflammatory mediators (e.g. histamine, serine proteases, proteoglycans) and synthesis of additional factors (e.g. cytokines and chemokines) that promote allergic inflammation. The effect of these mediators on surrounding tissue is what gives rise to the range of local (e.g. itching, swelling, nausea) and systemic symptoms (e.g. airway obstruction, hives, low blood pressure) observed during allergic reactions to food [20,21].

Egg white protein contains 23 different glycoproteins. Many of these egg proteins have been well characterized and ovomucoid (Gal d1), ovalbumin (Gal d2), ovotransferrin (Gal d3) and lysozyme (Gal d4) have been identified as the main egg allergens [22, 23, 24]. Of these ovomucoid has been shown to be the dominant allergen [25] despite comprising less than 10% of total egg protein compared to 50% for ovalbumin. However, in our patient cohort, during set up experiments Ovalbumin was found to be recognised much more frequently by immune cells than ovomucoid, and for this reason we studied ovalbumin responses. Egg allergic individuals generate specific IgE antibodies against egg proteins with both conformational (e.g. ovalbumin) and/ or sequential epitopes (e.g. ovomucoid). Recent findings suggest heating generally decreases protein allergenicity by destroying conformational epitopes [26]. This is supported by the suggestion that some children with egg allergy can tolerate cooked egg, but are still sensitive to uncooked egg [27, 28]. Similarly, studies on children who had outgrown cow's milk allergy had milk specific IgE antibodies primarily directed at conformational epitopes (tertiary structure), whereas children with persistent milk allergy had a significant proportion of their IgE antibodies directed at sequential (linear) epitopes [29, 30, 31, 32].

Recently, Wang et al, [33] used peptide microarray studies to evaluate the correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. The group showed that subjects with severe milk allergy had greater IgE epitope diversity and higher affinity compared with those who outgrew their allergy. In sublingual grass pollen immunotherapy low dose allergen exposure is thought to be responsible for IgE mediated symptoms. Whereas, repeated high dose allergen exposure seems to redirect the allergic response to one dominated by T cells that favour the production of IgG and IgA antibodies [34, 35, 36].

Increases in production of non-IgE (IgG) competitive antibodies during resolution of allergy may suppress the allergic response by competitively counter-acting the pathological effects of IgE antibody [37].

This study was designed to follow egg allergic children prospectively for several years and perform serial well cooked and uncooked egg challenges annually to define the stages of acquisition of tolerance, as well as identifying risk factors for persistence or early resolution of egg allergy.

2. MATERIALS AND METHODS

2.1 STUDY POPULATION

2. 1.1 Egg-allergic participants

This was a longitudinal study performed at the Clinical Research Facility at the Cambridge Biomedical Research Campus between 2004 and 2010. Permission was granted by the Local Research Ethics Committee. Children were recruited to the study from the Addenbrooke's Hospital Allergy clinic and by advertisement in a national patient support group newsletter (the Anaphylaxis Campaign).

Children (2-15 years old) with a history of a typical type-1 hypersensitivity reaction to egg and/or skin prick test (SPT) weal diameter >/=3mm to egg extract (ALK-Abello, Horsholm, Denmark), and/or serum egg specific IgE ≥0.35kU/l (ImmunoCap FEIA, Phadia, Uppsala, Sweden) at the time of diagnosis were invited to participate. Subjects who had never eaten egg were enrolled if they had a positive oral challenge to egg and a positive SPT and/or positive serum specific egg IgE.

Subjects were excluded from the study if they had a history of major immunodeficiency or were on medication that could affect their immune system. Children with a history of severe allergy were not excluded. Written informed consent was obtained in accordance with ethics guidelines for research in children.

A detailed allergic history and physical examination was performed on the day of consent and oral challenge. Blood samples were collected on the same day as SPT performed; 12-15ml was collected by venipuncture into heparinised tubes for transport at room temperature.

2.1.2 Clinical History

Data on sex, age of first reaction, symptoms, other food allergies, asthma, eczema and hay fever were recorded for each subject.

2.2 Oral Food Challenge (OFC) Protocol

Supervised one day open oral food challenges were performed in the Wellcome Trust Clinical Research Facility to determine the subject's clinical reactivity to egg. Subjects were asked to stop taking anti-histamines 72h before the challenge. Briefly, the oral egg challenges were conducted by administering in a titrated manner up to a maximum of 12g (equivalent to ¼ of whole egg) of 'well cooked egg' (baked at 180^0C for 20 min) in the form of well cooked cake or 21.5g of 'uncooked egg' (equivalent of ½ whole egg) of pasteurised whole egg pellet (Noble Foods Ltd, UK) in ~50ml of flavoured milk as liquid vehicle. Following an initial lip rub, the first dose was either ~0.4g of well cooked egg or 0.5g of uncooked egg and the dose was increased sequentially at 10 min intervals (0.8g, 1.5g 3g, 6g of well cooked egg or 1g, 2g, 6g, 12g of uncooked egg). Subjects were monitored throughout, and for 2 hours after the completion of the oral challenge. The challenge was stopped when all doses were tolerated or an objective reaction occurred i.e. development of two or more symptoms distant to mouth e.g. skin reddening, hives, swelling, runny nose, wheezing, abdominal pain or vomiting).Treatment was initiated immediately, mild reactions were treated with oral antihistamines, mild airway symptoms were treated with nebulised salbutamol and adrenaline was available in case of severe reactions.

The type of egg challenge (whether cooked or uncooked) was dependent upon whether each subject had previously tolerated well cooked egg. If this was the case, then an uncooked egg challenge (with whole pasteurized uncooked egg) was undertaken. All other subjects underwent a well cooked egg challenge initially, and if they passed went on to an uncooked egg challenge on another day. Subjects were then classified according to table 1 and figure 2.

Table 1: Classification of Outcome of egg challenge

Clinical Status at time point	Outcome of challenge to well cooked or uncooked egg	
	Well cooked egg	Uncooked egg
Fully allergic	Positive	Positive /ND*
Partially allergic	Negative	Positive
Partially resolved**	Negative	**Challenge pending
Resolved	Negative	Negative

*ND= not done, usually unnecessary because of positive challenge to well cooked egg at that time point.

**Challenge pending= in these subjects there was a time gap between the negative well cooked egg challenge and the uncooked egg challenge being undertaken. Therefore they may have been allergic or tolerant to uncooked egg at the time of their well cooked egg challenge, but it was unknown. They are therefore considered separately, as partially resolved.

The subjects were re-challenged annually until they either reached complete resolution or were persistently allergic to egg at the end of the 6 year study (Figure 1 and 2) [44].

Longitudinal follow up

Participants were followed up longitudinally and underwent annual egg challenges, skin prick tests and serum egg specific IgE measurements. Subjects who reacted to the well cooked or uncooked challenges had repeat challenges every 12 months to the same form of egg. Either

full or partial resolution was recorded if subjects had no reaction to uncooked or well cooked egg challenge respectively (Figure 2).

Cooked egg–tolerant subjects were instructed to introduce cooked egg products into their diet until they returned for re-challenge with uncooked egg. Subjects who were tolerant to uncooked egg were advised to introduce any form of egg into their diet.

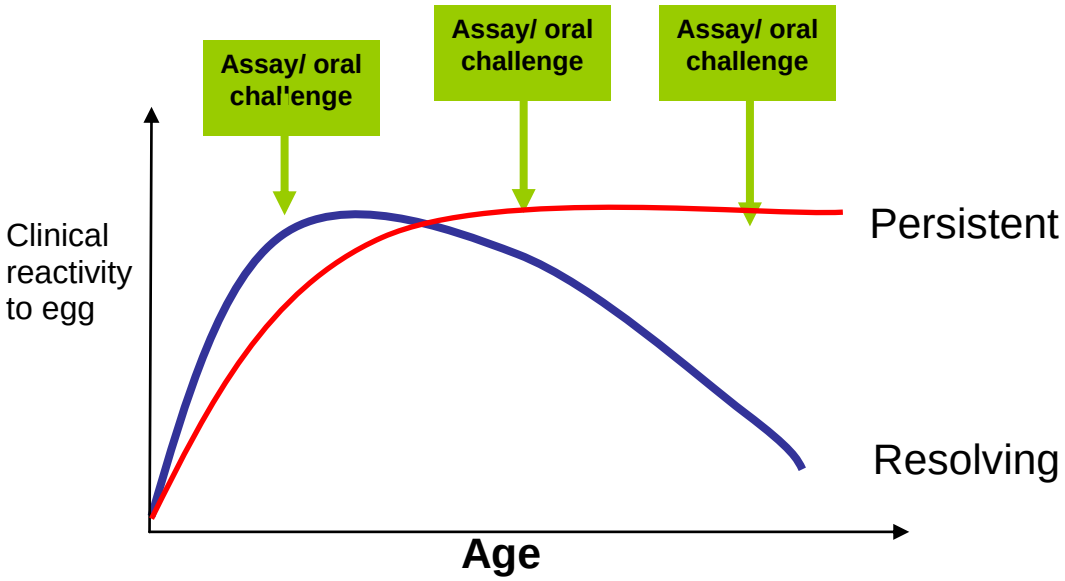
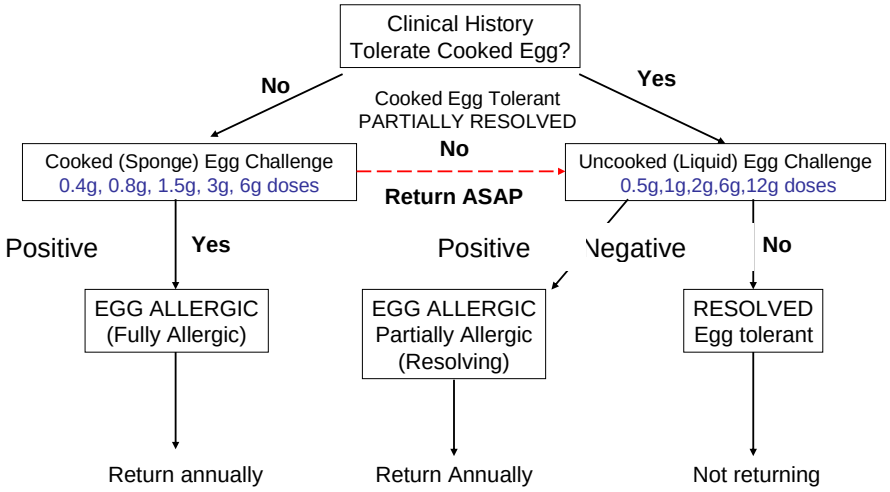


Figure 1: Overview of longitudinal study. Children were invited back for oral challenge and to take blood for completion of assays on an annual basis.

2.2.2 Clinical measurements at each egg challenge

Skin prick tests

Skin prick tests (SPT) with whole egg (ALK-Abello, Horsholm, Denmark) were performed. Histamine and saline served as positive and negative controls, respectively. The largest and perpendicular diameter was measured. A 3mm or larger weal was regarded as positive. All SPTs were performed and interpreted by one of two trained operators.



Flow diagram: Study design and characterisation of clinical egg reactivity

Figure 2: Flow diagram showing study design and characterization of clinical egg groups Clinical history was used to determine which type (well cooked egg in the form of cake or pasteurised raw egg) of oral challenge would be performed. Clinical group was based on the egg challenge outcomes.

2.2.3 In vitro IgE tests

Serum samples were collected at the time of each challenge visit. Total IgE and egg-white specific IgE levels were simultaneously measured by ImmunoCAP system (Phadia, Uppsala, Sweden). Egg white IgE levels of ≥ 0.35kU/l were considered positive. The lower limit of the latter assay is 0.35kU/l, and the upper limit for reporting results was 100kU/l; for calculation purposes, values of 0.34 and 100.1 kU/l were used if concentration results were beyond these limits.

2.2.4 Blood Sample

Blood samples were also obtained for serum and plasma which were stored at -20^{o}C and peripheral blood mononuclear cells isolated and cryopreserved in liquid nitrogen. Serum was isolated from a small volume of non-heparinised blood, allowed to clot in glass vials, then transferred to a centrifuge tube and spun for 10 min at 4000 rpm (3130 x g) at room temperature with the brake on. The upper serum layer was aliquoted into 2ml tubes and stored at -20^{o}C. (Please see section 2.5 for an explanation of PBMC and plasma isolation and sections 2.5.1 to 2.5.3 for an explanation of cryopreservation and thawing).

2.3 Immunologic Evaluation: Laboratory Studies

2.3.1 Assays for total IgG, IgG1 and IgG4 subclasses

Mr John Deighton previously published data on the study of IgG subclass antibodies in patients allergic to wasp or bee venom [75] which was based on an earlier publication by Rieben and Blaser [76] on the quantification of IgG and IgG4 antibodies to bee venom phospholipase A2 by competitive inhibition in ELISA. More recently,,Mr J. Deighton published cross-sectional data on the patterns of immunoglobulin G responses to egg and peanut allergens (45). Longitudinal assays of total IgG, OVA-specific IgG1 and OVA-specific IgG4 (µg/ml) were measured in individual serum samples by in-house ELISA (see section 2.3.2). All ELISA's were developed and performed by Mr J. Deighton. Appropriate controls were included for all assays performed.

2.3.2 Quantification of specific antibody titres in reference anti-sera

Sera from 23 egg-allergic subjects with high specific IgE titre against egg (egg-specific IgE>5.0kU/l) was used to create a reference pool. Total IgG and egg-specific subclasses IgG1 and IgG4 titres in the reference pool were quantified by a modified competitive ELISA method, as follows [45]. Multibind microtitre plates (Greiner Bio-one, Gloucester, UK) were coated for 2hr at room temperature (RT) with Ovalbumin (OVA) (5µg/ml) in 100µl carbonate/bicarbonate buffer (pH 9.6). Plates were washed three times in phosphate buffered saline (PBS)-containing 0.1% (v/v) Tween 20 (PBST) and blocked with 5% (v/v) heat-inactivated fetal calf serum (Sigma) in PBST for 1h at RT. One dilution of reference anti-sera was added to all wells and incubated overnight at 4^{o}C. Serial two-fold dilutions of the monoclonal anti-human IgG, anti-IgG1 or anti-IgG4 (WHO/IUIS clones HP6064/8a4, HP6012/NL16 or HP6011/RJ4, respectively) antibodies were added in triplicate overnight at 4^{o}C. Plates were washed three times in PBST, and the bound monoclonal antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma 1:1000) incubated for 3hr at RT. After two PBST washes and a final wash in distilled water, 100µl of *p*-nitrophenyl phosphate (1mg/ml (Sigma) in diethanolamine buffer pH 9.8) was added. Optical densities at 405nm were read using BioRad Model 550 (BioRad, Hemel Hempstead, UK). An optimal concentration of the monoclonal antibody was selected based on the titration curve, and the assay was repeated in the presence of pre-titrated amounts on inhibiting purified human IgG (Calbiochem, San Diego, CA, USA), $IgG1_K$ (Sigma) or $IgG4_K$ (Sigma). The OVA-specific IgG titres in the reference sera pool was calculated from the derived inhibition curve. Five independent measurements were made. The antibody titres (mean ± standard deviation in µg/ml) of the OVA-specific pool were 92.18 ± 0.69 (IgG), 7.47± 0.33 (IgG1) and 83.6 ± 4.14 (IgG4). The sensitivity limits (ng/ml) were 4.0 (IgG), 6.0 (IgG1) and 4.0 (IgG4). Inter-assay co-efficient of variations (CVs) were 6 % (IgG), 8.8% (IgG1) and 9.1% (IgG4) [45].

2.3.3 Quantification of OVA-specific IgG antibody titres in patient sera

Serial dilutions of reference anti-sera and at least three dilutions of patient sera (1:20 to 1:1600) were incubated with OVA-coated plates in duplicate overnight at 4^{o}C. Each subclass of bound anti-sera was detected with monoclonal anti-human IgG (8a4), anti-IgG1 (NL16) or anti-IgG4 (RJ4) used at previously selected concentrations, overnight at 4^{o}C. Bound monoclonal antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (1:1000; Sigma) for 3hr at RT, followed by *p*-nitrophenyl phosphate substrate addition and reading of optical densities at 405nm. Washes and incubation conditions were performed as for quantification of reference anti-sera pools. Specific antibody titres in µg/ml were extrapolated from the standard curve of reference anti-sera. All samples from an individual subject were run on the same plate on the same day. All dilutions were performed in duplicate.

2.4 Preparation of allergen extracts and endotoxin decontamination

Lyophilised ovalbumin (OVA) and ovomucoid (OM) of grade V purity (Sigma, Dorset, UK) was reconstituted in PBS at 10mg/ml and stored at -80^{o}C. Protein concentrations of OVA and OM were measured by the bicinchoninic acid assay (BCA; Pierce Biotechnology). Bacterial endotoxins are able to stimulate the immune system and frequently contaminate commercially available reagents and may mask the true effect of the protein antigen/allergen. Endotoxin was depleted from OVA and OM using EndoTrap™ Red affinity columns (Profos International, Regtensburg, Germany) following the manufacturer's instructions. Tetanus Toxoid (TET) (821 Lfu/ml; 2105Lf/mg) was purchased from the Statens Serum Institute (Copenhagen, Denmark). All protein preparations, cell culture media and a human plasma pool were tested independently for endotoxin (Limulus amebocyte assay; Cambrex BioScience, Verviers, Belgium). 1 endotoxin unit (EU) is approximately 100pg of endotoxin/lipopolysaccharide. Endotoxin levels were 0.263 EU/mg OVA, 0.364 EU/mg OM and not detected in TET, cell culture media or plasma. Therefore endotoxin levels were always <0.364 EU/ml (<3.6pg/ml

per culture), i.e. at concentrations below those known to stimulate PBMC (<1EU). Polymyxin B is an antibiotic which binds and inactivates endotoxins and their ability to activate T cells. If addition of Polymyxin B reduces T cell proliferation it is an indicator that endotoxin contamination is high enough to affect the assay. We found OVA-stimulated T cell responses were not inhibited by polymyxin B (2ug/ml) in randomly tested culture assays indicating that endotoxin levels are too low to affect the T cell response [44].

2.4.1 Optimization of allergen dose for stimulation of PBMC samples

Optimization of allergen doses for stimulation of allergen/or antigen specific T cells in PBMC samples was originally assessed by Tay et al [44] using small scale fresh peripheral blood mononuclear cell (PBMC) cultures ($1x10^5$/well) in RPMI-1640 culture media with 10% heat-inactivated human AB serum (96U plates, Falcon; BD Biosciences, San Jose, CA, USA). After 6 days, ^{3}H-thymidine (1.0Ci/well, Amersham Biosciences, Amersham, UK) was added for 16hr. Radioactivity retained (cpm/well) was quantified by scintillation counting. Optimal concentrations for PBMC culture were selected based on T cell proliferation index (PI) and number of responders. PI is the ratio of T cell proliferation in response to allergen divided by T cell proliferation in control medium lacking allergen. TET was used as a positive control at 8.21Lfu/ml based on PI of 16.6±3.5 at 12Lfu/ml, 11.9±0.6 at 6Lfu/ml and 11.7±0.6 at 3Lfu/ml TET and 3/3 responders at all three doses. OVA- and OM-stimulated PI and number of responders were OVA: 1mg/ml, 5.5±1.0 (12/12); 0.5 mg/ml, 3.7±0.7 (7/12);0.25mg/ml, 2.5±0.5 (3/12); OM: 1mg/ml, 2.7±1.1 (6/6); 0.5mg/ml, 1.6±0.3 (4/6);0.25mg/ml, 1.2±0.2 (1/6). Thus, 1mg/ml OVA and OM were required to reveal all positive responders [44].

For the longitudinal study OVA, OM and TET concentrations were re-tested on cryopreserved PBMC. This ensured that all PBMC samples from a single individual were simultaneously assayed under the same tissue culture and monoclonal antibody labelling conditions and the

only variable was the length of time each PBMC sample had been cryopreserved. Pilot experiments were performed to compare T cell responses between fresh and frozen PBMCs from the same donor and no differences in proliferation or cytokine production were detected. PBMC from egg allergy subjects were isolated and cryopreserved between 2005 and 2009. T cell assays were performed on the cryopreserved PBMC samples between 2008 and 2010. Potentially PBMC samples may have been cryopreserved for up to 5 years in liquid nitrogen. Thawed CFSE labelled PBMC (1 x10^6/ml) were cultured in 24-well plates (Falcon, UK) for 8 days with varying doses of OVA (0.1 to 1mg/ml) in complete medium (RPMI-1640 medium supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100ug/ml streptomycin (Sigma, UK) and 10% autologous plasma (data not shown). Cells permitting, two doses of OVA were used, OVAhi (1mg/ml) and OVAlo (200ug/ml), OM (1mg/ml) and TET (8.21Lfu/ml: 1:50dil stock).

2.5 Peripheral blood mononuclear cell separation

PBMC were purified from heparinised whole blood after spinning for plasma at 1819 rpm (640 x g) for 15 min at room temperature with the brake on. Blood was diluted with equal volume of Hank's Balanced Salt Solution (HBSS) (Sigma, UK) into 50ml conical tubes (BD Falcon, UK). The blood solution was gently mixed before overlaying onto 15ml of Lymphoprep™ (Axis-Shield, Oslo, Norway). The tubes were centrifuged at 2022 rpm (800 x g) for 22 min at room temperature with no brake on. The buffy coat PBMC layer at the interface was aspirated and transferred into a fresh 50ml conical tube. The PBMC were washed twice with HBSS, pooled, and counted using 0.4% Trypan Blue solution (Sigma, St Louis, MO). Using established techniques [13, 46] which have been shown not to alter cell function, isolated PBMC were cryopreserved in 7.5% dimethyl sulphoxide (DMSO) (Sigma, UK) supplemented with 43.5% human male AB serum (Sigma, UK) and stored in liquid nitrogen.

2.5.1 Media used for cryopreservation

To generate the media containing 43.5% human AB serum, human AB serum (Sigma, UK) was inactivated at 56^{o}C water bath for 30min. DMSO was added to a final concentration of 7.5% (v/v).

2.5.2 Cryopreservation of human peripheral blood mononuclear cells.

Lymphoprep isolated PBMC were resuspended at concentrations of 12-15 million PBMC per ml of 4^{o}C cooled freezing mix media. Once aliquoted, cryovials were placed on ice and then transferred into a freezing container (Nalgene, Rochester, NY), and stored at -80^{o}C for 24h, cryovials were then transferred into liquid nitrogen for long term storage.

2.5.3 Thawing of cryopreserved cells

Nine ml of cell culture media (serum free RPMI-1640 supplemented with HEPES buffer, antibiotics, and glutamine), warmed to 37^{o}C, was aliquoted into 30ml centrifuge tube (Sterilin, UK). No more than 2 cryovials were thawed at the same time. The cryovials were thawed rapidly in a 37^{o}C water bath until the cell suspension was almost completely melted. One ml of cell culture media was slowly added to the thawed cells and then transferred to a fresh centrifuge tube and the remaining eight ml of media was slowly added drop wise. This is a standard method used in tissue culture i.e. to avoid diluting cells when cold. Cells need to be diluted at room temperature or above with serum containing medium however, long periods at or above room temperature should also be avoided to minimise DMSO toxicity, hence 'rapid' thawing and gentle dilution was used. The tubes were centrifuged at 1819 rpm (640 x g) for 10 min at RT and then the cells were resuspended in 3ml of media. Cell viability was assessed using 0.4%Trypan blue dye (Sigma, UK), the viability was over 80% at the time of seeding i.e. when adding cell to TC culture, if the viability was less than 70% the cells were not used.

2.6 Preparation and stimulation of PBMCs

PBMCs were thawed, washed and viable cells counted using 0.4% Trypan Blue exclusion dye. For CFSE labelling, PBMC were resuspended at $1x10^7$cells/ml in serum-free RPMI-1640 (Sigma, UK). Labelled PBMC cultures were incubated with endotoxin free allergens and 10% autologous plasma for 8 days at 37^{o}C in a 5%CO_2 humidified incubator (see section 2.6.1).

2.6.1 Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining and cell culture

Labelling of the proliferating PBMC with the fluorescent proliferation dye, 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden) was performed according to Turcanu et al (2003) [47] with some modifications. Titration of CFSE to determine the optimal concentration for CFSE staining was determined using 0.5µM, 1µM and 2.5µM CFSE cultured PBMC with or without tetanus toxoid (8.21Lfu/ml) for 7 days, collected and stained with anti-CD4-PE. The cell division index (CDI) was used to compare effectiveness of staining [48].Maximum CD4+T cell proliferation among PBMC samples was seen with 2.5µM CFSE. The cell division index was calculated, based on a fixed number (usually 30000 events) of CD3+/CFSEhi T cells with the following formula:

<u>Number of CD3+/CFSElo T cells with antigen (allergen)</u>

A CDI ratio of >2 was considered to represent a doubling or more above background proliferation (in the absence of allergen) and therefore a positive proliferative response..

For staining with CFSE, briefly, thawed PBMC (1×10^7/ml) were washed, labelled with 2.5µM CFSE in the dark for 15min with gentle shaking in a 37^{o}C water bath. Labelling was stopped with 1ml autologous plasma and excess dye was washed away. CFSE labelled cells were recounted using a haemacytometer with 0.4% Trypan blue and added to 24-well flat bottomed plate (BD falcon, UK) at a density of $1x10^6$/ml/well with or without endotoxin free

allergens (OVA (200ug/ml and/or 1mg/ml) and TET (8.21Lfu/ml) in medium (RPMI-1640 with 2mmol/L L-glutamine, 25mMol/L HEPES buffer containing 10% autologous plasma, 100IU/mL penicillin, and 100µg/mL streptomycin). Cells were cultured for 8 days at 37^{o}C in 5%CO_2 humidified atmosphere to expand the allergen specific T cells.

2.7 Flow Cytometry detection of intracellular cytokines

After 8 days of culture in the presence of allergens, PBMC were re-stimulated for 4hr with the phorbol ester, phorbol-12-myristate-13-acetate (PMA; 50ng/ml, Sigma) and calcium ionophore, ionomycin (1ug/ml, Sigma, UK) to induce intracellular cytokine gene expression in the presence of GolgiStop™ (0.7µl/ml; BD Pharmingen, UK) which contains monensin blocking protein transport through the Golgi apparatus. The cells were fixed and permeabilised in 200µl Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4^{O}C. Cells were washed twice and stained with fluorescent monoclonal antibodies: CD3-Peridinin chlorophyll protein (PerCP) (3ul; Clone UCHT); IL-4-Phycoerythrin (PE) (1:4; Clone 8D4-8) and IFNγ-Allophycocyanin (APC) (1:6 or 1:24; Clone B27) (all BD Pharmingen, UK) for 30min at 4^{o}C in 100µl Perm/Wash buffer (BD Pharmingen). The concentration of each fluorescent monoclonal antibody in the cocktail mix was separately titrated to maximize effective surface and intracellular staining. After two washes in Perm/Wash buffer the samples were run for 4-colour detection in a FACSCalibur flow cytometer (BD Biosciences, UK) on the same day or within 24 hr. An autofluorescent control (unstained PBMC) and compensation controls (single antibody stained PBMC) were included in all experiments to set the optimal instrument settings for voltage gain and compensation respectively. A threshold of 110 on FSC (forward scatter) was set to exclude cell debris and FSC PMT Amp gain on 1.04 to visualize all lymphocytes and proliferating lymphoblasts on the FSC vs. SSC (side scatter) dot plot. At least 100,000 events were acquired for each sample and stored as list mode data using CellQuest software (BD Biosciences), and data analysed using WinMDI 2.9 software.

Cytokine production was determined for all the subjects in whom we could collect sufficient cells.

2.7.1 Flow cytometric data analysis

The proliferation dye, CFSE, was used to differentiate between the non-proliferating parental resting T cells (CD3+/CFSEhi) and the proliferating allergen-specific activated daughter T cells (CD3+/CFSElo). The gating strategy we used to assess the intracellular cytokine expression by the allergen specific T cells involves 1) gating on viable PBMC (R1) on forward scatter (FSC) vs. side scatter (SSC) plot (Figure 3); 2) then identify two T cell subsets on the CFSE vs. CD3 plot, the proliferating CD3+/CFSElo cells (R3), represents the allergen specific T cells, and the non-proliferating CD3+/CFSEhi parental cells (R2); 3) each T cell subset are then analysed on a IL-4 vs. IFNγ plot to show the percentage of single positives for Th1 IFNγ+ and Th2 IL-4+ cytokines using quadrant markers. T cell responses were considered to be positive if >400 CD3+/CFSElo cells (R3) were collected.

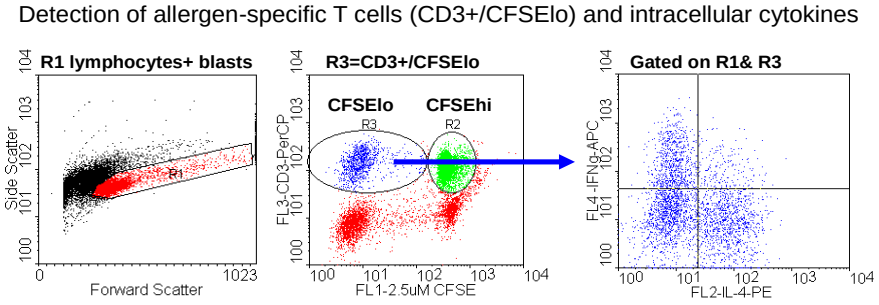


Figure 3: Gating strategy employed to detect allergen-specific T cells (R3) and intracellular cytokine analysis. Allergen/ antigen stimulated PBMC were gated based on their size and granularity on the Forward –Side scatter plot (R1: red dots). These cells were separated based on their expression of the T cell receptor (CD3) and the fluorescent dye, CFSE. Two T cell subsets are identified, the parental non-proliferating CD3+/CFSEhi T cell subset (R2: green dots) and the proliferating daughter CD3+/CFSElo T cell subset (R3: blue dots). The allergen-specific T cells (R3) are further separated based on their expression of the T helper cytokines IL-4 and

IFNγ using quadrant markers to identify the single positive and double positive cells. The upper left (UL) quadrant shows IFNγ single positive cells and the lower right (LR) quadrant shows IL-4 single positive cells only. The upper right (UR) quadrant shows the double positive cells producing both IFNγ and IL-4 cytokine.

The CFSEnet was calculated by subtracting the percentage of CFSElo cells in the CD3+ subset (%CD3+/CFSElo) found in unstimulated cultures from %CD3+/CFSElo found in allergen stimulated cultures, e.g. in OVA-stimulated cultures CFSEnet $_{OVA}$= [(%CD3+/CFSElo)$_{OVA}$ – (%CD3+/CFSElo) $_{UNSTIMULATED}$]. CFSEnet is an absolute % value of the final (total) net % proliferation of allergen specific T cells after subtracting the % of background non-specific proliferation (negative control). Cell division index (CDI) is a ratio of proliferation in response to antigen (allergen) divided by proliferation of the unstimulated medium control (PBMC, RPMI and 10% autologous plasma). The ratio of %CD3+/CFSElo of OVA-stimulated cultures/ %CD3+/CFSElo of unstimulated cultures after normalizing for the same number of cells in the non-proliferating parental CD3+/CFSEhi population (R2) gate for each condition. A CDI > 2 is considered a significant positive response to allergen (see section 2.6.1).

CD3+ T cells were analysed as CD4 expression was down-regulated by PMA described previously [49]. To define T cell subsets in cultures on day 8, some cultures were not re-stimulated with PMA and stained with CD4-PE, CD8-PerCP and CD3-APC (all BD Pharmingen). Analysis of CD3+/CFSElo and CD3+/CFSEhi revealed that CD8+ cells do not make a significant contribution (4.1±0.5%) to the CD3+/CFSElo subset; and a significant number of CD8- cells were CD3-/CD4- (12± 2.6%) or CD3+/CD4- (16.5±3.2%) published in [44].

The binding specificities of IL-4-PE and IFNγ-APC antibodies were tested by pre-incubating antibodies with excess IL-4 or IFNγ (1µg each, R&D Systems, UK) [50]. Cytokines completely inhibited antibody binding to TET specific T cells expressing both cytokines (data not shown).

2.8 Measurement of Interleukin-10

For cytokine production 1 x 10^6 PBMC/ml were cultured with or without endotoxin free allergens in 24 well culture plates and were maintained as indicated above (see section 2.6.1). Culture supernatants were collected on day 8 and kept frozen at -20^{o}C until quantitative cytokine determination. Interleukin-10 levels in culture supernatants were analysed in duplicate by an in-house ELISA in a subgroup (fully EA; partially EA and fully ER) of subjects**.** Briefly, wells of a 96-well microtitre plate were coated 2hr at RT with 2ug/ml IL-10 anti-human cytokine monoclonal antibody (R&D systems, Europe) in 100µl carbonate/bicarbonate buffer, pH 9.6, washed three times with phosphate-buffered saline (PBS)-containing 0.1% Tween 20 (PBST) and blocked for 1 hr at RT with PBST containing 5% heat-inactivated fetal calf serum (FCS). Each well was washed three times with PBST. Culture supernatants were used undiluted in duplicate and incubated overnight at 4^{o}C. After washing each well three times with PBST; biotinylated anti-human IL-10 antibody at 1 in 400 dilution (1.25ug/ml) (Sigma) was added for 2hr 37^{o}C. After another incubation for 2 hr at RT with ExtraAvidin-Alkaline Phosphatase (1/400dil; Sigma), substrate solution (*p*-nitrophenyl phosphate (1mg/ml (Sigma) in diethanolamine buffer pH 9.8) was added and optical density was measured at 405nm in a microtitre plate reader (Biorad). Results were related to a standard curve obtained with recombinant human IL-10 standard (BD Pharmingen, Europe) at different concentrations. The ovalbumin-stimulated d8 PBMC cultures were also analyzed by flow cytometry using IL-10-PE (Clone JES3-9D7; BD Pharmingen, Europe) in place of IL-4–PE to detect intracellular IL-10 in the OVA-specific T cells subset (CD3+/CFSElo). Detection of intracellular IL-10 at present has been unsuccessful using IL-10-PE (Clone JES3-9D7)

2.9 Excel databases

Two databases in Excel were produced recording 1) clinical measurements (e.g. Age, Sex, symptoms, SPT results, IgE results, onset of allergy and duration); 2) *In vitro* longitudinal assay results for each subject, each challenge and each allergen (e.g. T cell proliferation (% CD3+/CFSElo; CFSEnet, CDI); T cell cytokine expression (%IL-4+, %IFNγ+, ratio of IFNγ/IL-4, log of ratio;%IL-10+, ratio o IFNγ /IL-10), Serum antibody titres (Total IgG, specific-IgG1, specific-IgG4, IgG1/IgG4, total IgE, egg-specific IgE,); Extracellular cell supernatant IL-10 levels (pg/ml)

2.10 Statistical analyses

Most data were not normally distributed; therefore, differences in memory T cell proliferation and intracellular cytokine production between unpaired groups were compared by the Mann-Whitney *U* test. Two-tailed unpaired Student's *t* test was used to analyze the mean differences between groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed in the parameter being assessed between data sets before using the two-tailed paired or unpaired Student's *t* test.

Differences within individuals over time in each egg group were compared with Wilcoxon matched pairs test. A probability level of less than 5% ($p < 0.05$) was considered statistically significant. Fisher's exact test (two tailed) was used to assess the significance of cytokine data in terms of predominance of one cytokine (IFNγ or IL-4) over the other in samples from persistently egg allergic subjects compared with fully resolved subjects on their last challenge visit. All tests were performed using GraphPad Prism (Version 5, GraphPad Software Inc., San Diego, CA, UK). Graphs were generated in GraphPad Prism and Excel.

3.0 RESULTS:

3.1 Clinical data

There were 181 hen's egg challenges (77 to well cooked egg in 58 children, and 104 to uncooked egg in 74 children) over a six year period. Ninety five children were studied overall as several had more than one challenge during this time.

3.1.1 Clinical characteristics of participants

The age of onset of egg allergy (defined as first clinical reaction to egg) was median 12m (25^{th} -75^{th}centiles 8-13m). There was no significant difference in the age of onset of allergy between subjects undergoing well cooked or uncooked egg challenge. Median age at enrolment was 55m (25^{th} -75^{th}centiles 32-87m). Male: female ratio was 1.56.

The type of egg causing the original historical reaction was well cooked (typically cake or biscuit) in 5 (5%), lightly cooked (typically scrambled eggs) in 58 (61%) and uncooked (e.g. mayonnaise) in 9 (9%). Five subjects (5%) reacted to only skin contact to raw egg and 15 (16%) had never knowingly eaten or reacted to egg, but the diagnosis had been suggested by allergy testing.

The symptoms reported during each subject's worst ***pre-study*** reaction were cutaneous only in 57 (60%), gastrointestinal in 20 (21%), respiratory in 11 (12%) and 7 (7%) had anaphylaxis. Of subjects who had anaphylaxis before being enrolled in the study, 5 reacted to lightly cooked egg, one to ingestion of uncooked egg and one was unknown. Adrenaline had been used to treat reactions in five subjects, antihistamines alone were used in 16 subjects, and there was no information about treatment for 56 subjects.

Challenges were performed at median age of 55m (16-288m) for well-cooked egg and median 66m (17-184m) for uncooked egg (p=0.009 for difference between medians; Mann-Whitney U-test). Overall, 28/77 (37%) of well cooked egg and 61/104 (59%) of uncooked egg challenges were positive.

3.1.2 Symptoms elicited during positive challenges

The symptoms experienced during challenges are shown in table 2a and b.

For well cooked egg challenges, 3/28 (11%) reacted to the first oral dose (~0.4g, all oral itching). In 23/61 (38%) of uncooked egg challenges the subject reacted to the first oral dose (oral itching in 10, nausea in 1 and cutaneous rash in 12).

Table 2a Symptoms experienced during *positive egg challenges* (numbers of subjects are shown with percentages in parentheses). Symptoms are shown according to type of egg challenge. *=excluding rhinitis. Cutaneous symptoms were recorded if erythema, urticaria or cutaneous angioedema occurred. Gastrointestinal symptoms were recorded if nausea, vomiting or abdominal pain (sufficient to alter behaviour) occurred and respiratory symptoms were recorded if wheezing or cough occurred during challenge.

Type of egg challenge	Total positive challenges	Cutaneous	Gastrointestinal	Respiratory*	Rhinitis
Cooked	28	18 (65)	19 (68)	0	11 (39)
Uncooked	61	46 (75)	34 (56)	9 (15)	20 (36)

Table 2b Medication used during positive egg challenges (numbers of subjects are shown with percentages in parentheses). Treatment is shown according to type of egg challenge.

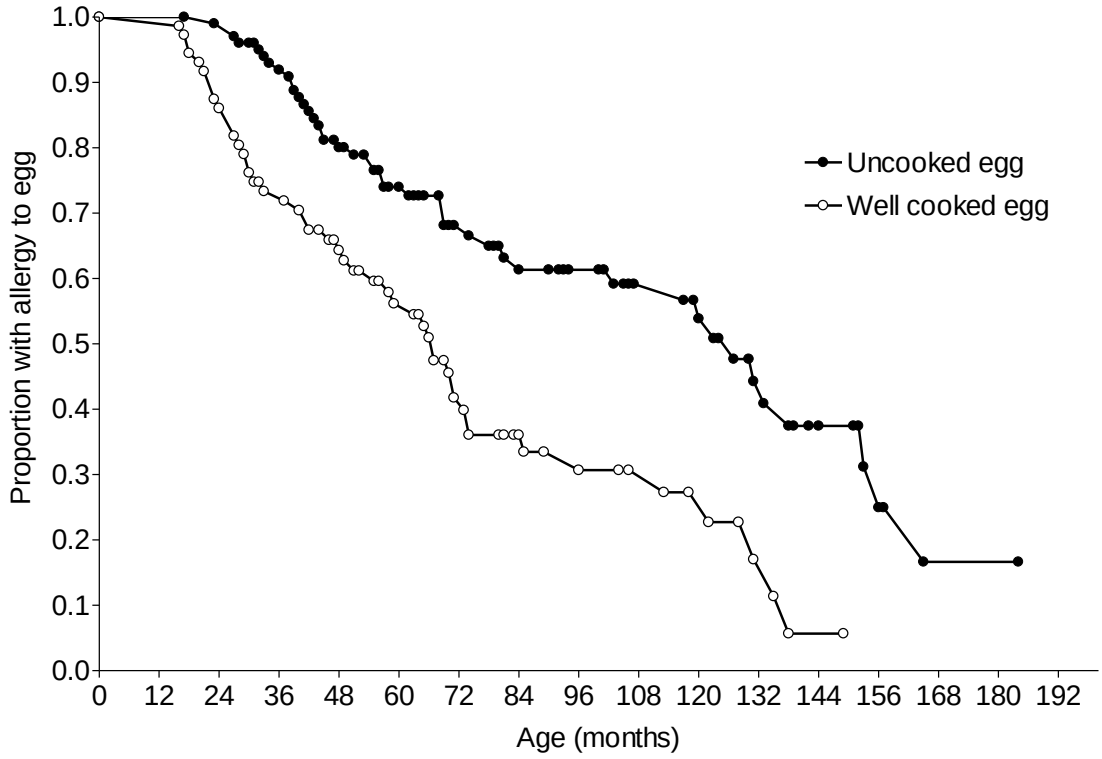
Type of egg challenge	Total positive challenges	Oral antihistamines	Oral corticosteroids	Nebulised bronchodilator	Intramuscular adrenaline	No treatment
Cooked	28	19 (68)	0	0	0	9 (32)
Uncooked	61	48 (79)	1	3 (5)	0	21 (33)

3.1.3 Resolution of egg allergy

The age of acquisition of tolerance to well-cooked and uncooked egg was analysed using Kaplan Meier survival curves (figure 4). Tolerance was gained more rapidly to well-cooked rather than to uncooked egg (logrank test $p<0.0001$). The median age at which tolerance occurred was 67m for well-cooked and 127m for uncooked egg The hazard ratio was 2.23 (95%CI 1.6-3.9), indicating the rate of resolution of allergy to well-cooked egg was approximately twice that of uncooked egg.

The curves show a rapid early acquisition of tolerance to both well-cooked and uncooked egg in the majority followed by a slower rate of resolution in the remaining minority. There was an early separation in the curves. In the older and slower resolvers there was a bigger difference in the slope of the curves such that the rate of resolution to uncooked egg was even slower. Nonetheless, resolution still continued to occur to cooked egg up to the age of 158m (13.2yr) and for uncooked egg up to 182m (15.2y).

Figure 4 Cumulative persistence (survival) of allergy to well cooked and uncooked egg.



3.2 Experimental data

3.2.1 Characteristics

Over the total time period of the study, we investigated the relationship between the frequency of cellular (allergen specific T cell proliferation and intracellular cytokine secretion) and humoral responses (serum IgE, IgG, IgG1 and IgG4) and the phenotype of egg allergy in a group of 109 subjects' characterized with respect to their clinical reactivity to cooked (baked sponge cake) or uncooked (pasteurized raw) egg. Subjects were defined as fully allergic (reactive to well cooked egg; Sponge POS), partially resolved (well cooked egg tolerant), partially allergic (reactive to uncooked egg; Liquid POS) and fully resolved (egg tolerant) (Figure 6)

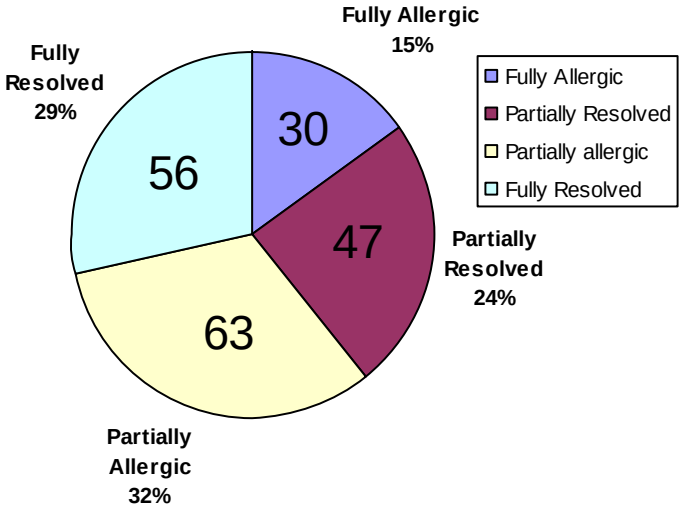


Figure 6: Pie chart showing cross-sectional data for the number and proportion of subjects in each challenge group

15% were fully allergic (Well cooked POS); 24% had partially resolved (cooked egg tolerant); 32% were partially allergic (uncooked POS; uncooked egg reactive) and 29% had fully resolved (egg tolerant).

Immunologic responses to oral egg challenge at baseline initial challenge

Of 55 subjects who underwent an initial egg OFC at baseline (i.e. as part of their initial assessment and diagnosis), and also provided a blood sample for analysis, 9 were fully allergic, 23 had partially resolved egg allergy, 11 were partially allergic to egg and 12 had fully resolved their egg allergy (Table 3).

Table 3: Distribution of male and female subjects on initial oral egg challenge

Clinical Group	Male	Female
Fully Allergic	4	5
Partially Resolved	14	9
Partially Allergic	7	4
Fully Resolved	8	4

Immunologic responses to egg on final oral egg challenge

For egg allergic subjects there was an average of 24.54m (2.12yr) between first and last challenge. For the egg-resolved subjects the average difference was 23.45m (1.95yrs). Of the 39 subjects whose blood was available for processing and analysis (only subjects with two samples included), upon their final oral egg challenge of the study 3 were found to be fully allergic, 3 had partially resolved, 18 were partially allergic and 15 had fully resolved (Table 4).

Table 4: The distribution of boys and girls between the four clinical sub-groups at study completion.

Clinical Group	Male	Female
Fully Allergic	1	2
Partially Resolved	1	2
Partially Allergic	15	3
Fully Resolved	10	5

3.2.2 OFC attendance, recall and withdrawals

Twenty-two subjects did not return for annual oral egg challenges. Twelve subjects stopped returning after their second visit. Attempts to contact parents of 25 subjects resulted in 6 out of 11 ER subjects successfully being recalled to provide a blood sample and 7 out of 14 EA subjects recalled for an egg challenge.

Cross-sectional comparison of fully allergic (well cooked POS) and partially resolved (well cooked NEG) subjects

CFSE staining allowed us to detect allergen specific T cells in blood samples collected from these subjects and compare the frequency of these cells between fully allergic (well cooked POS; cooked egg reactive) and partially resolved (well cooked NEG; cooked egg tolerant) subjects. No difference in age of subjects (data not shown) or proportion of ovalbumin-specific T cell (%CD3+/CFSElo) proliferation (Figure 7) was observed between the fully allergic and partially resolved subjects. However, there was a significant difference in age between partially resolved subjects compared with partially allergic (uncooked POS; uncooked egg reactive) and fully resolved (egg tolerant) subjects ($p=0.0074^{**}$ and $p=0.0226^{*}$) respectively (**Figure 8**). It appears that the natural steps to resolution are cooked egg allergic (FA) → cooked egg tolerant (PR) →uncooked egg allergic (PA) → egg tolerant (ER).

Cryopreservation maintains functional status of PBMC

We performed allergen-specific stimulations on cryopreserved PBMCs in-vitro with autologous plasma. Viability after thawing (measured by Trypan blue dye exclusion) was consistently above 70% at the time of seeding [51]. PBMCs were labelled with 2.5uM CFSE and treated with monensin for 4hrs after 8 day culture period. For lymphocyte cultures 70% viability was considered a sufficient number of memory cells as antigen presenting cells were also present in the culture and would also proliferate in the presence of the allergen over the 8 day in vitro expansion period.

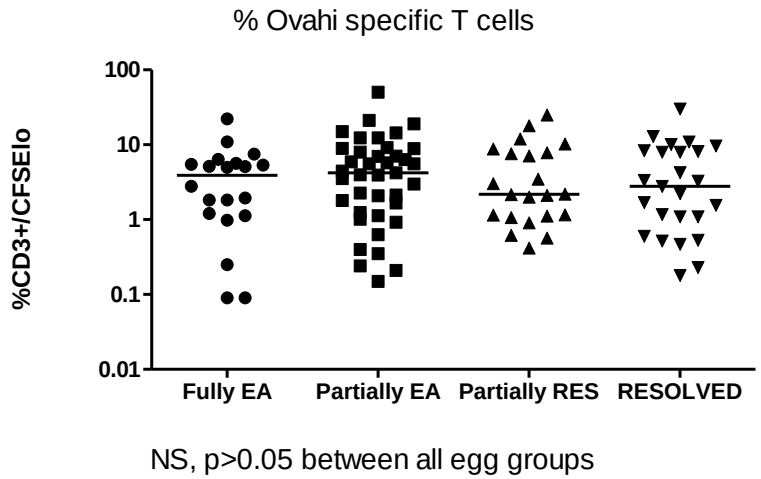


Figure 7: The proportion of proliferating OVAhi-specific T cells (CD3+/CFSElo) in each clinical sub-group. No statistically significant differences in the proportion of proliferating high dose ovalbumin specific T cells are seen between the clinical sub- groups.

3.2.3 T cell proliferation

OVA-specific proliferation does not change significantly as egg allergy resolves

Each time a CFSE-labelled T cell divides (proliferates) the staining intensity is decreased by half. Cells that have proliferated can therefore be detected and counted by flow cytometry. In the CFSE assay, the number of cells that have proliferated per a fixed number (e.g. 30,000 events) of undivided cells (CD3+/CFSEhi), is determined for cultures with or without allergen. The magnitude of the response is expressed as the number of cells that have proliferated in response to allergen divided by the number of cells that have proliferated in the absence of allergen (Figure 8). This ratio is called the cell division index (CDI) and is a measure of the rate of proliferation.

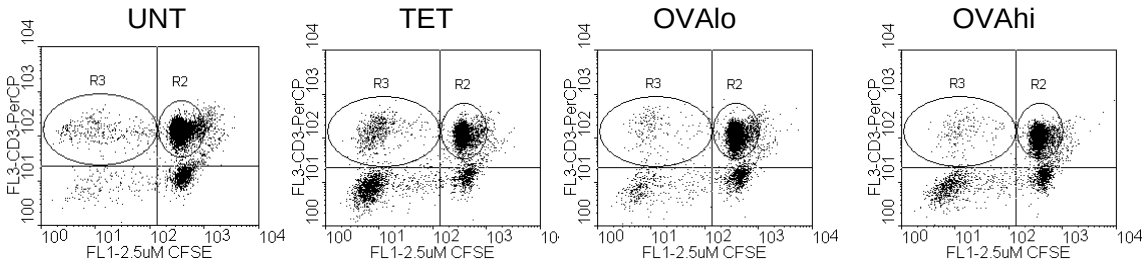
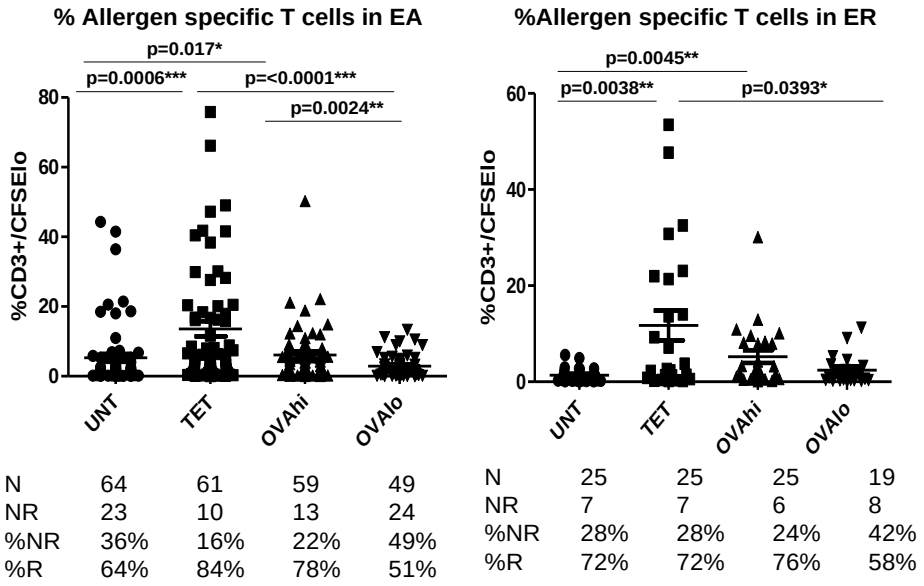


Figure 8: Differences in proportion of CD3+/CFSElo in response to allergen. The percentage of proliferating allergen specific-T cells (R3: CD3+/CFSElo) and non-proliferating parental cells (R2: CD3+/CFSEhi) are identified on a CFSE versus. CD3 dot plot by flow cytometry (by circled areas). The proportions of these cells normalized to a fixed number of CD3+/CFSEhi (R2) for each allergen and untreated control is used to calculate the CDI.

T cell proliferative responses to culture medium alone (negative control), tetanus toxoid (TET; 8.2lLfU/ml (positive control)), low dose ovalbumin (OVAlo; 200ug/ml) and high dose ovalbumin (OVAhi; 1mg/ml) from egg allergic (EA) and egg resolved (ER) subjects show quantitative and qualitative differences between the allergens/ antigen in each egg group (Figure 9).



N	64	61	59	49
NR	23	10	13	24
%NR	36%	16%	22%	49%
%R	64%	84%	78%	51%

N	25	25	25	19
NR	7	7	6	8
%NR	28%	28%	24%	42%
%R	72%	72%	76%	58%

Figure 9: Comparison of the percentage of allergen-specific T cells (CD3+/CFSElo) in EA and ER subjects. N is the total number of samples assayed for each allergen/antigen tested. NR is the number of non-responders who failed to proliferate and expand their allergen T cell population to >400 events in the CD3+/CFSElo T cell subset sufficient enough to detect intracellular cytokines. The percentage of non-responders (%NR) and % of responders (%R) is also shown. Median values=horizontal line (Mann-Whitney U test $p<0.05$ is considered significant).

Differences in T cell proliferation were detected in PBMCs stimulated by different antigen/ allergens. The levels of proliferation by TET (positive control) and OVAhi in both EA and ER subjects were significantly above untreated background negative controls (EA: $median_{TET}$ 6.17 (range 0.03-75.86) p= 0.0006***; ER: $median_{TET}$ 2.69 (range 0.17-53.45) p=0.0038**) and (EA: $median_{OVA}$ 4.22 (range 0.09-50.29) p=0.017*; ER $median_{OVA}$ 2.78 (range 0.18-30.10) p=0.0045**) (Figure 9). However, **OVAlo** did not stimulate T cell proliferation above untreated background negative controls in the EA or ER group (Figure 9).

Comparison of T cell proliferative responses measured as %CD3+/CFSELo to TET, OVAlo and OVAhi showed no significant difference between EA and ER subjects (Figure 10). Surprisingly, we observed a significantly higher level of non-specific proliferation in untreated

negative control cultures from EA (n=64: median 1.54 (0.05-44.30) p=0.0382*) compared with ER (n=25: median 1.14 (0.06-5.59)) subjects (Figure 11A)). Further detailed analysis of the EA group (Figure 11B) identified CD3+/CFSElo cells from partially allergic (PA: n=6) uncooked egg POS) and partially resolved PR: n=14) subjects were responsible for the higher levels of proliferation $median_{PA}$ 5.55 (0.78-44.30) p=0.0152*) and $median_{PR}$ 2.1 (0.68-29.86) p=0.0233*) respectively and not due to the fully allergic (FA: n=8; $median_{FA}$ 3.4 (0.05-20.56) subjects.

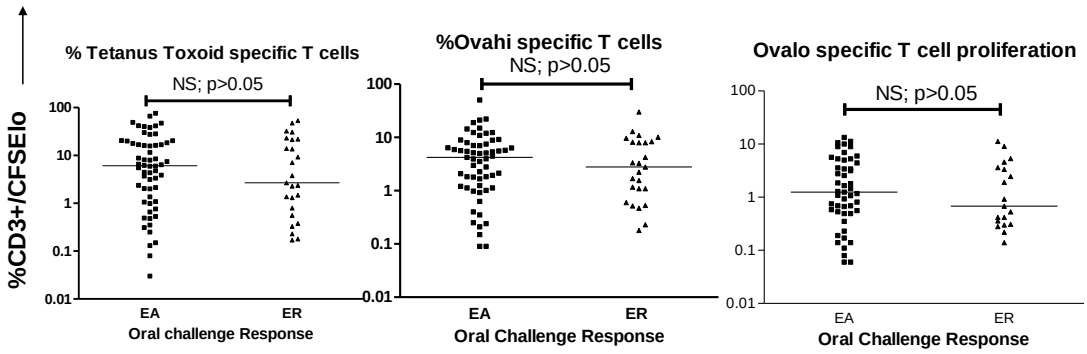


Figure 10: Cross- sectional comparison of antigen/allergen specific T cell proliferation between EA and ER subjects. No differences in the proportion of antigen/ allergen- specific T cell (%CD3/CFSElo) proliferation for TET (positive control), OVAhi or OVAlo was observed between EA and ER groups. Median value =horizontal line.

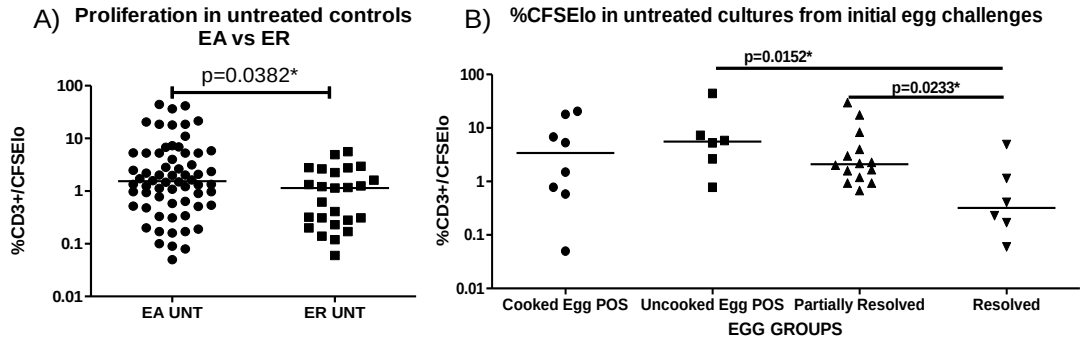


Figure 11: A) Comparison of proliferation of untreated PBMC from EA (n=64) and ER (n=25) subjects and B) clinical sub-groups on initial challenge visit ((FA (n=8); PA (n=6); PR (n=14) and RES (n=6)) Median values=horizontal line; Mann-Whitney U test p<0.05 considered significant.

In addition, analysis of proliferation measured as either CFSEnet (i.e. final net proliferation) or cell divison index (i.e. the rate of proliferation), showed the same significant differences between the antigen/ allergens in both egg allergic (EA) and egg resolved (ER) subjects. The CFSEnet for tetanus toxoid (positive control) and high dose ovalbumin (1mg/ml) were significantly higher than for low dose ovalbumin (200µg/ml). In EA subjects (median $_{TET}$ 3.5 (range -38.70-73.32) p=0.0035**; median $_{OVA}$ 1.19 (range -39.70-45.02) p=0.0035**) and in egg resolved subjects (median$_{TET}$ 2.43 (range -3.43-52.28) p=0.0107*); median$_{OVA}$ 1.02 (range -3.26-25.19) p=0.0236*) (Figure 12). Similarly, CDI for TET and OVAhi was significantly higher than for OVAlo (EA median$_{TET}$ 6.63 (range 0.07-95) p=0.035**; median$_{OVA}$ 3.18 (range 0.03-147) p=0.0060**) and (ER: median$_{TET}$ 4.07 (range 0.29-387) p=0.0060*; median$_{OVA}$ 3.03 (range 0.14-92.34) p=0.0120*) respectively (Figure 13). This indicates that the memory T cell population in PBMC cultures from EA or ER subjects respond more rapidly to tetanus toxoid and high dose ovalbumin than to low dose ovalbumin. No statistical differences in CFSEnet or CDI were observed between TET and OVAhi (Figure 12 and 13).

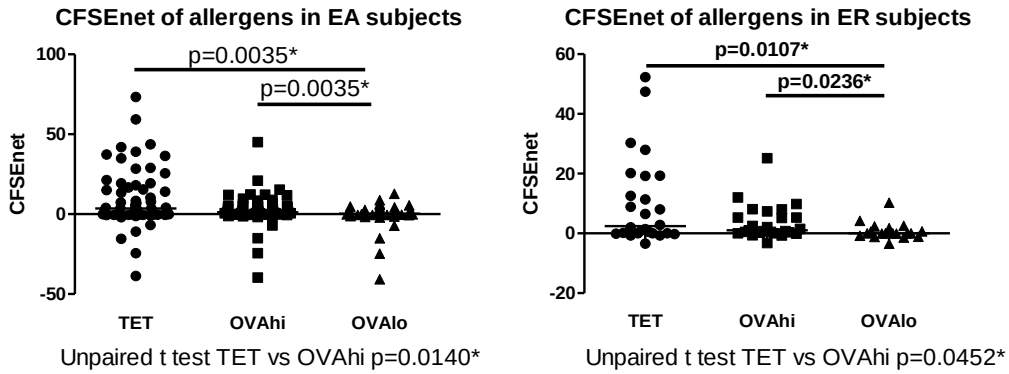


Figure 12: Cross-sectional comparison of specific T cell proliferation of allergens/antigen measured as CFSEnet (final net proliferation) in EA and ER subjects. Median value= horizontal lines. Mann-Whitney U test to compare medians p<0.05 is considered significant. Unpaired t test comparing mean values shows significant difference between tetanus toxoid (positive control) and high dose ovalbumin (1mg/ml). Unpaired t test to compare means p<0.05 is considered significant.

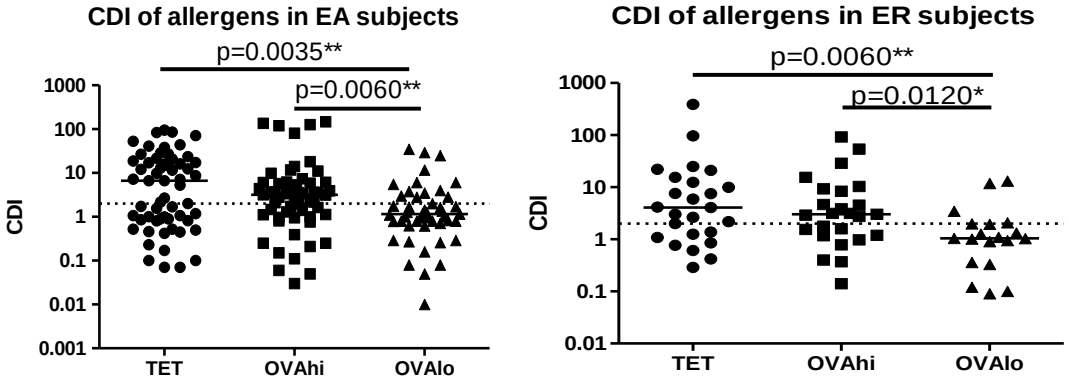


Figure 13: Cross-sectional comparison of the rate of proliferation measured as the cell division index (CDI) between allergens/antigen in EA and ER subjects. Data shows the rate of proliferation in tetanus toxoid and high dose ovalbumin treated PBMC cultures is significantly higher than PBMC treated with low dose ovalbumin in EA and ER subjects This indicates that memory T cells proliferate and expand more rapidly to tetanus toxoid and high dose ovalbumin than to low dose ovalbumin. Median value= horizontal lines. Mann-Whitney U test to compare medians p<0.05 is considered significant.

Longitudinal changes in OVA specific T cell proliferation were not detected during resolution of egg allergy

Longitudinal analysis of T cell proliferation was performed on 33 persistent egg allergic subjects (including 20 paired samples) and 28 egg tolerant subjects (including 11 paired samples) Paired samples were from the first and final challenge from the same individual. We analysed the data using paired (Wilcoxon matched pairs test) and unpaired (Mann-Whitney U test) non-parametric tests to detect any significant changes in OVAhi T cell proliferation measured as %CD3+/CFSElo, CFSEnet or CDI between the same individual's first challenge and their final challenge. No significant longitudinal changes were observed in %CD3+/CFSElo T cells or CFSEnet in response to high dose ovalbumin in both EA and ER groups (Figure 14 and 15). However, a significant increase in CDI_{OVA} was seen in persistent EA subjects in paired (p=0.0283*) and unpaired (p=0.0064*) data, but not in either analysis for ER subjects (Figure 16). The data suggested that the memory T cell response to high dose ovalbumin is more rapid in EA subjects than in ER subjects.

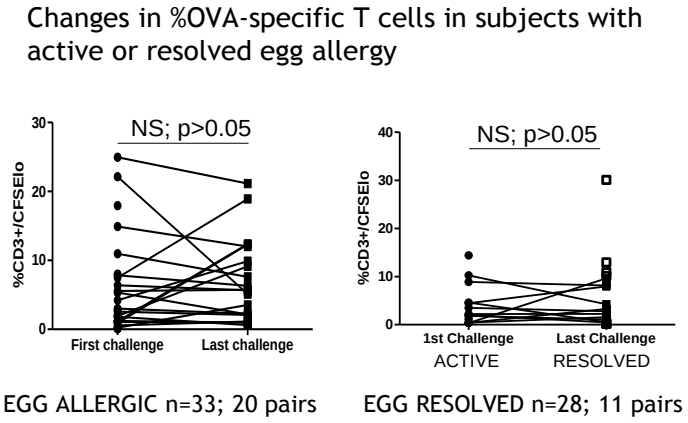


Figure 14: Graphs showing longitudinal changes in the proportion of OVAhi specific T cell proliferation (%CD3+/CFSElo) within individual subjects between their first and last challenge with either persistently active or fully resolved egg allergy. Data is shown from egg allergic (n=33; 20 pairs) and egg resolved (n=28; 11 pairs) subjects. No significant differences in the

proportion of OVAhi specific T cells were observed between a subject's first and last challenge (Wilcoxon matched pairs test; p<0.05 is considered significant).

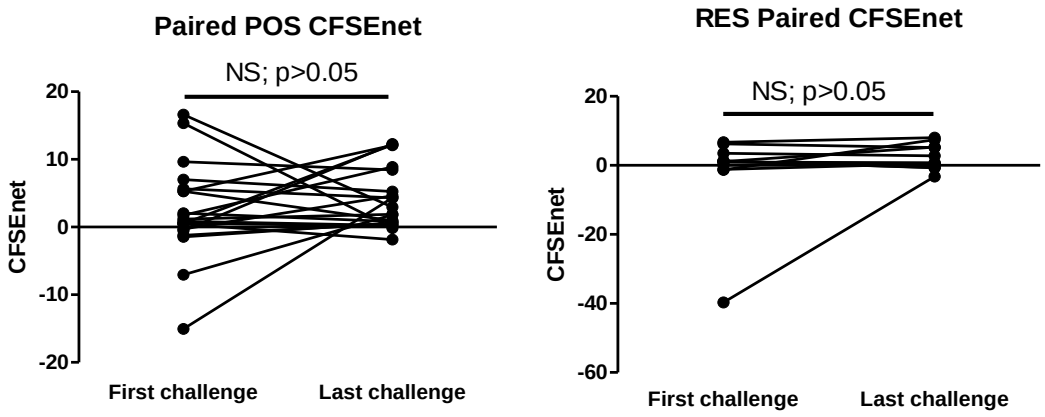


Figure 15: Graphs showing longitudinal changes in CFSEnet for OVAhi treated PBMC from individuals between their first and last challenge. Data is shown from persistently egg allergic (POS) (n=33; 20 pairs) and fully egg resolved (RES) (n=28; 11 pairs) subjects. Paired analysis showed no significant change in CFSEnet within individuals' over time. (Wilcoxon matched pairs test; p<0.05 is considered significant).

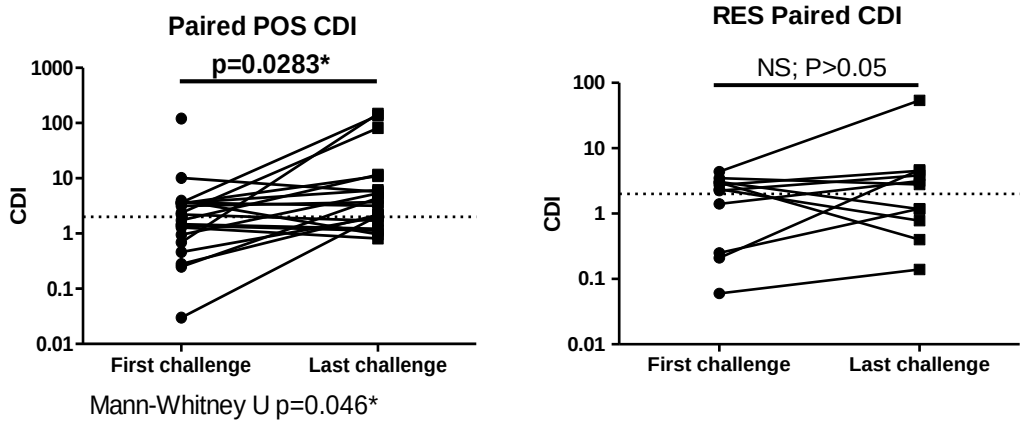


Figure 16: Longitudinal changes in the rate of proliferation measured as the cell division index (CDI) in children with persistently active (n=33; 20 pairs) or fully resolved (n-=28; 11 pairs) egg allergy. The rate of proliferation increases in subjects with persistent active egg allergy over time (Wilcoxon's matched pairs test; p=0.0283*).

3.2.4 Cytokine expression

Differences were observed in the proportion of egg-specific IFNγ producing cells identified in PBMC samples from resolved subjects

In high-dose ovalbumin (OVAhi) treated PBMC cultures, our data showed no statistical difference in the proportion of IL-4+ OVAhi-specific T cells between egg allergic (EA: n=59) and egg resolved (ER: n=41) subjects. The spread in the proportion of IFNγ+ OVAhi specific T cells in EA and ER subjects is similar. However, we did detect a significant increase in the proportion of IFNγ+ OVA-specific T cells in ER subjects compared with EA subjects (median $_{EA\ OVA}$ 6.37 (range 0-56.04) and median $_{ER\ OVA}$ 16.09 (range 0.43-68.23) p=0.0155*) (Figure 17).

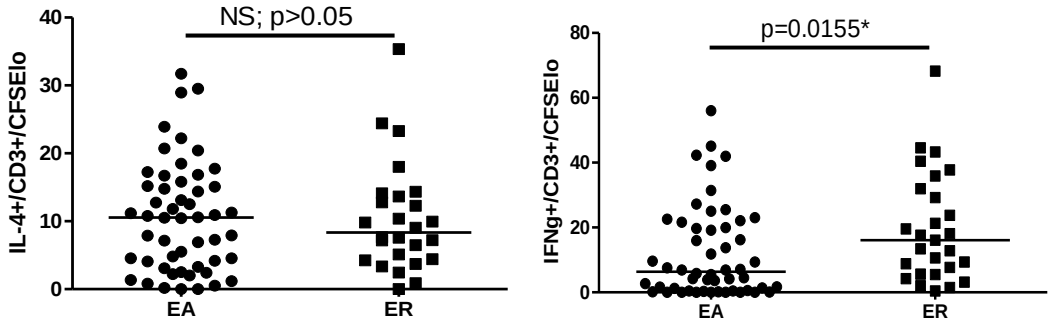


Figure 17: Cross-sectional comparison of the proportion of IL-4+ and IFNγ+ producing OVAhi-specific T cells between egg allergic (EA) and egg resolved (ER) subjects. No significant difference in the proportion of IL-4 positive cells was observed between EA and ER subjects. The proportion of IFNγ positive cells was increased in ER subjects (Mann- Whitney U test p=0.0155*).

Separating the egg allergic subjects into smaller clinical sub-groups based on their reactivity to well cooked egg (n=20) or uncooked egg (n=44) allowed a more detailed analysis of any differences in intracellular cytokine expression. We observed no difference in the proportion of IL-4+/CD3+/CFSElo in response to high dose ovalbumin between the clinical sub-groups. However, we did observe significant differences in the proportion of IFNγ+/CD3+/CFSElo

between subjects reactive to uncooked egg (liquid POS) or tolerant of cooked egg (UNK) compared to subjects who had fully resolved their egg allergy. The proportion of IFNγ producing OVAhi specific T cells were found to be lower in liquid POS subjects (uncooked egg reactive; median$_{OVA}$ 6.815 (range 0-56.04) p=0.0149*) and UNK (partially resolved) subjects (UNK; cooked egg tolerant): n=21 median$_{OVA}$ 2.390 (range 0-43.91) p=0.0062**) compared with ER subjects (NEG; egg tolerant): n=25 median$_{OVA}$ 17.65 (range 0.43-68.23).(Figure 18).

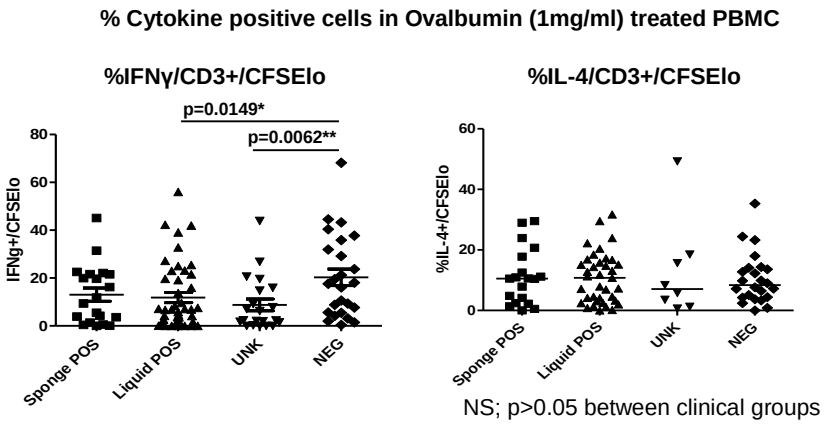


Figure 18: Cross–sectional comparison of the proportion of IFNγ+ or IL-4+ OVAhi –specific T cells in clinical sub-groups defined by their reactivity to well cooked egg (sponge cake) or to uncooked egg (pasteurised raw egg). The proportion of IFNγ or IL-4 producing OVAhi-specific T cells in Sponge POS (n= 20: well cooked egg reactive i.e. fully allergic), Liquid POS (n=44: uncooked egg reactive i.e. partially allergic), UNK (n=21;well-cooked egg tolerant; partially resolved) and NEG (n=25; uncooked egg tolerant i.e. fully resolved) subjects is shown. A significantly higher proportion of IFNγ producing OVAhi specific T cells were found in fully resolved subjects compared with subjects that reacted to uncooked egg (p=0.0149*) or tolerated cooked egg (p=0.0062*). No differences in the proportion of IL-4 producing OVAhi specific T cells were found between the clinical sub-groups (Mann-Whitney U test $p<0.05$ is considered significant)

Quantitative and qualitative differences in cytokine expression were found in allergen-specific T cells

In subjects with active egg allergy our results showed that the proportion of IL-4+/CD3+/CFSElo expressing cells in antigen/allergen (TET (positive control), OVAhi and OVAlo) treated PBMC cultures was greater than untreated PBMC (negative control). (TET (positive control) ($median_{TET}$10.51 (range 0-47.43) p<0.0001***), OVAhi ($median_{OVAhi}$ range 10.44 (0-31.70) p<0.0001***) OVAlo ($median_{OVAlo}$ 4.575 (range 0-28.57) p<0.0001***) untreated PBMC (negative control) ($median_{UNT}$ 1.04 (range 0-30.43)). PBMC cultures treated with TET antigen had similar proportions of IL-4+/CD3+/CFSElo expressing cells as those treated with OVAhi allergen. Tetanus toxoid and OVAhi treated cultures had higher proportions of IL-4+/CD3+/CFSElo expressing cells than OVAlo treated cultures (TET_{MEDIAN} 8.70 (range 0-48.95); p=0.0166*, $OVAhi_{MEDIAN}$ 8.35 (range 0-35.35); p=0.0084** $OVAlo_{MEDIAN}$ 5.465 (range 0-38.17)) (Figure 19). In subjects with resolved egg allergy we also found significantly higher proportions of IL-4+/CD3+/CFSElo expressing cells in antigen/allergen treated PBMC (TET; p=0.0002***, OVAhi; p=0.0001*** and OVAlo; p=0.0102*) compared with untreated PBMC (UNT_{MEDIAN} 1.11(range 0-28.57).. In ER subjects the proportion of IL-4+ /CD3+/CFSElo expressing cells between high and low dose ovalbumin did not differ, however in EA subjects the proportion of IL-4+/CD3+/CFSElo expressing cells is lower in PBMC cultures treated with low dose ovalbumin (Figure 19).

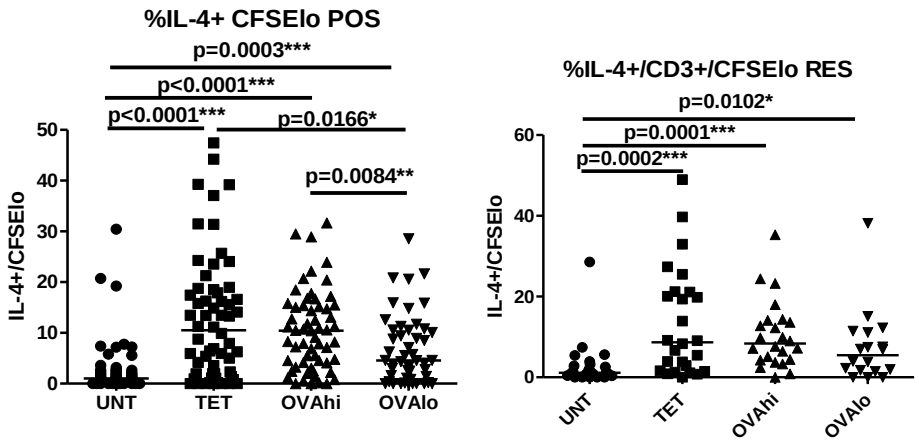


Figure 19: Cross-sectional comparison of the proportion of IL-4+ specific T cells between antigen/ allergens (i.e. Tetanus toxoid, high and low dose ovalbumin) in egg allergic (POS) (n=63) and egg resolved (RES) (n=26) subjects. We observed significant quantitative differences in the proportion of IL-4+ expressing cells in both egg groups. Significant higher proportion of IL-4+/CD3+/CFSElo cells were seen for tetanus toxoid (positive control), high and low dose ovalbumin treated PBMC compared with untreated PBMC (negative control) in both egg groups. In EA subjects we observed a significant decrease in the proportion of IL-4+/CD3+/CFSElo cells in PBMC treated with low dose ovalbumin (200ug/ml) compared with high dose ovalbumin (1mg/ml). Median values =horizontal line; Mann-Whitney U test p<0.05 considered significant.

The proportion of IFNγ+/CD3+/CFSElo expressing cells in PBMC cultures treated with TET(positive control) ($median_{TET}$ 6.53 (range 0-58.91); p<0.0001***), OVAhi ($median_{OVAhi}$ 6.9 (range 0-56.04); p<0.0001***) or OVAlo ($median_{OVAlo}$ 3.485 (range 0-56.90); p<0.0001***) was significantly higher than in untreated PBMC cultures (negative control) ($median_{UNT}$ 0.26 (range 0-28.57) in egg allergic (POS_) subjects (Figure 20). In egg resolved (RES) subjects TET ($median_{TET}$ 15.32 (range 0-55.03); p<0.0001***) and OVAhi ($median_{OVAhi}$ 16.87 (range 0.43-68.23); p<0.0001***) treated PBMC cultures also showed significantly higher proportions of IFNγ+/CD3+/CFSElo expressing cells than untreated PBMC cultures ($median_{UNT}$ 0.71 (range 0-20.38). In ER subjects comparing median values, OVAlo treated cultures (median $_{OVAlo}$ 4.56 (range 0-41.06) showed no difference in proportion of IFNγ+/CD3+/CFSElo expressing cells (Mann-Whitney U p=0.0571) compared with untreated cultures. However, in ER subjects a

significant difference in the proportion of IFNγ/CD3+/CFSElo cells between OVAlo and untreated PBMC (no allergen) cultures was seen when comparing mean values (Unpaired t test; p=0.0173*) suggesting a higher proportion of IFNγ+/CD3+/CFSElo expressing cells in allergen treated cells. No differences were observed between high and low dose ovalbumin in EA subjects. In ER subjects, no differences were observed between TET and OVAhi treated cultures but both these antigen/ allergens gave rise to significantly higher proportion of IFNγ+/CD3+/CFSElo expressing cells than cultures treated with OVAlo (p=0.0195* and p=0.0085**) respectively (Figure 20).

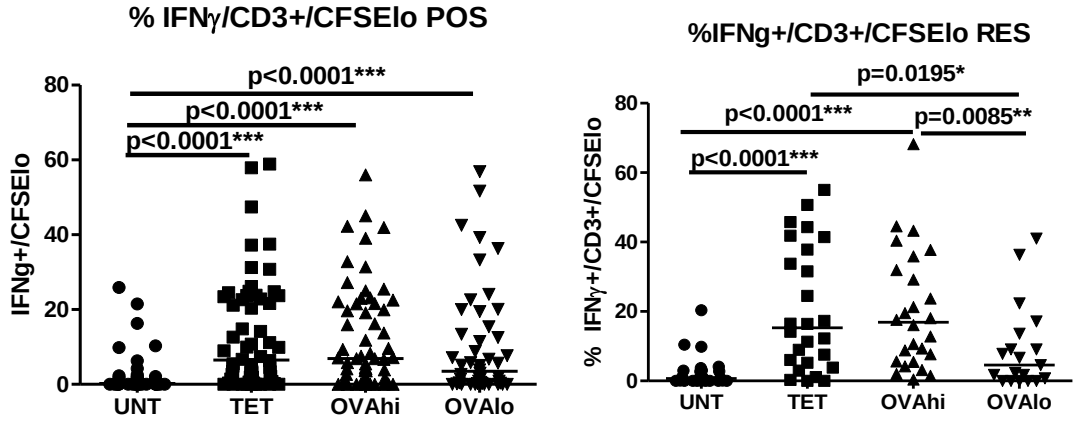


Figure 20: Cross-sectional comparison of the proportion of IFNγ+ specific T cells between antigen/ allergens (TET, OVAhi and OVAlo) in EA (n=63) and ER (n=26) subjects. Significantly higher proportion of IFNγ+/CD3+/CFSElo cells is seen in TET, OVAhi and OVAlo treated PBMC in EA subjects compared with untreated (no allergen) control PBMC. Similarly, in ER subjects TET and OVAhi both had significantly higher proportion of IFNγ expressing cells than untreated controls but OVAlo did not. Comparison of high (1mg/ml) and low (200µg/ml) dose ovalbumin showed significant differences in ER subjects only. In both egg groups the range of %IFN+/CD3+/CFSElo cells for TET and OVAhi was similar but for OVAlo the range was reduced in ER subjects.

The ratio of IFNγ+/IL-4+ expression by T cells is altered by culture with different antigen/ allergens.

Data analysis of the ratio of IFNγ+/IL-4+ expressing T cells in EA compared with ER subjects showed that for cultures treated with TET (EA_{MEDIAN} 0.94 (range 0.01-64.34 vs. ER_{MEDIAN} 1.96 (range 0.15-23.94); p=0.0139*) and OVAhi (EA_{MEDIAN} 0.96 (range 0.004-4.94) vs. ER_{MEDIAN} 1.5 (range 0.15-27.96); p=0.0113*) there was a statistical increase in the ratio but a decrease was seen in cultures treated with OVAlo (EA_{MEDIAN} 1.935 (range 0.02-12) vs. ER_{MEDIAN} 0.67 (0.02-5.5); p=0.0360*)(Figure 21). In OVAhi stimulated PBMC, the difference observed was due to the uncooked egg POS (partially allergic; p=0.0187*) clinical sub-group of EA subjects (Figure 22).

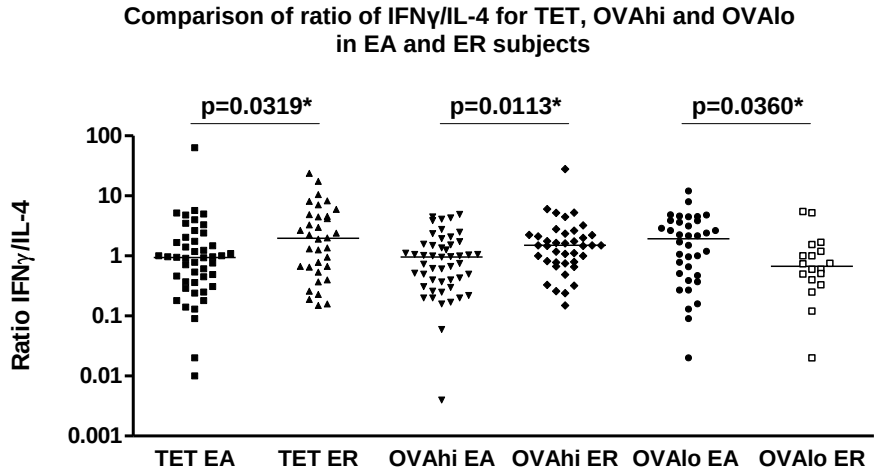


Figure 21: Cross-sectional comparison of the ratio of IFNγ/IL-4 for TET, OVAhi and OVAlo in EA and ER subjects. The IFNγ/IL-4 ratio was found to be higher in ER subjects for TET (p=0.0319*) and OVAhi (p=0.0113*) but lower for OVAlo (p=0.0360*) using Mann Whitney U test $p<0.05$ is considered significance. The change in cytokine skew for the systemic antigen, tetanus toxoid, from a T helper Th2 response to a T helper Th1 response and for low dose ovalbumin from Th1 to Th2 skew were both unexpected and difficult to explain. A possible explanation is that a ratio cannot be calculated if one of the cytokines has a zero value. In the literature tetanus toxoid is often used as a positive control for T helper type 2 responses seen in cell culture supernatants. In our T cell assay both Th1 and Th2

cytokines are assessed simultaneously intracellularly and therefore able to detect the more subtle cytokine skew.

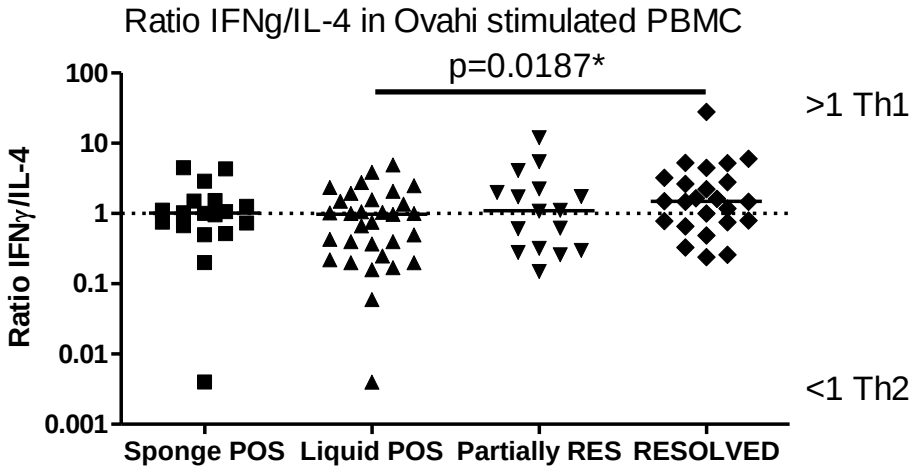


Figure 22: Comparison of IFNγ/IL-4 ratio in OVAhi treated PBMC from subjects in different clinical sub-groups. Median ratio values above >1 indicate that the OVAhi-specific T cells have a T-helper type 1 skew with IFNγ as the dominant cytokine. This data shows in egg resolved subjects the median IFNγ/IL-4 ratio is greater than one (>1) and significantly higher than partially allergic subjects reactive to uncooked egg (p=0.0187*)

Comparison of the log of ratio of IFNγ/IL-4 expressing T cells between EA and ER subjects showed statistical differences for cultures treated with TET (p=0.0373*), OVAhi (p=0.0172*) and OVAlo (p=0.0333*). For TET and OVAhi the log of ratio showed a change from a Th2 cytokine skew in EA subjects to Th1 cytokine skew in ER subjects but unexpectedly the opposite was observed for OVAlo i.e. a switch from Th1 in EA subjects to Th2 cytokine skew in ER subjects.(Figure 23).

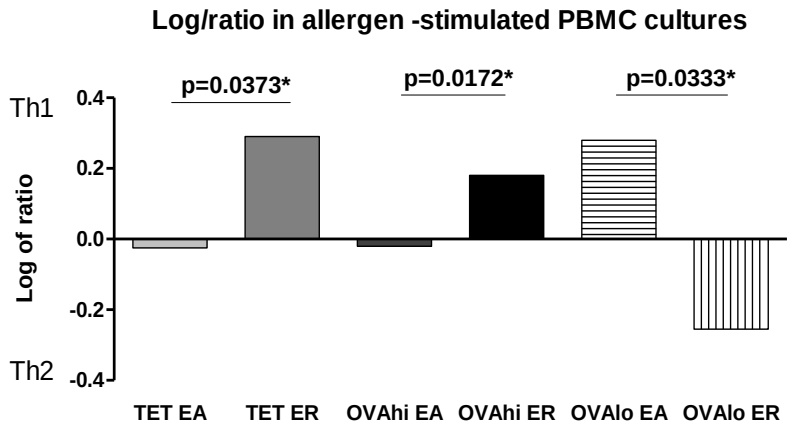


Figure 23: Bar chart shows cross-sectional comparison of the log of ratio (IFNγ/IL-4) of allergens between EA and ER subjects. The results show TET (p=0.0373*) and OVAhi (p=0.0172*) switching from Th2 to Th1 cytokine skew and OVAlo (p=0.0333*) switching from Th1 to Th2 in EA versus ER subjects.

No significant change was found in the proportion of IL-4 producing T cells with resolution of egg allergy but there is up-regulation of IFNγ producing T cells

Paired and unpaired analysis showed no statistically significant changes in the proportion of IL-4+/CD3+/CFSElo expressing cells between first and last challenge within individuals with persistent (EA: n=33; 20 pairs) or resolved (ER: n=28; 11 pairs) egg allergy (Figure 24).

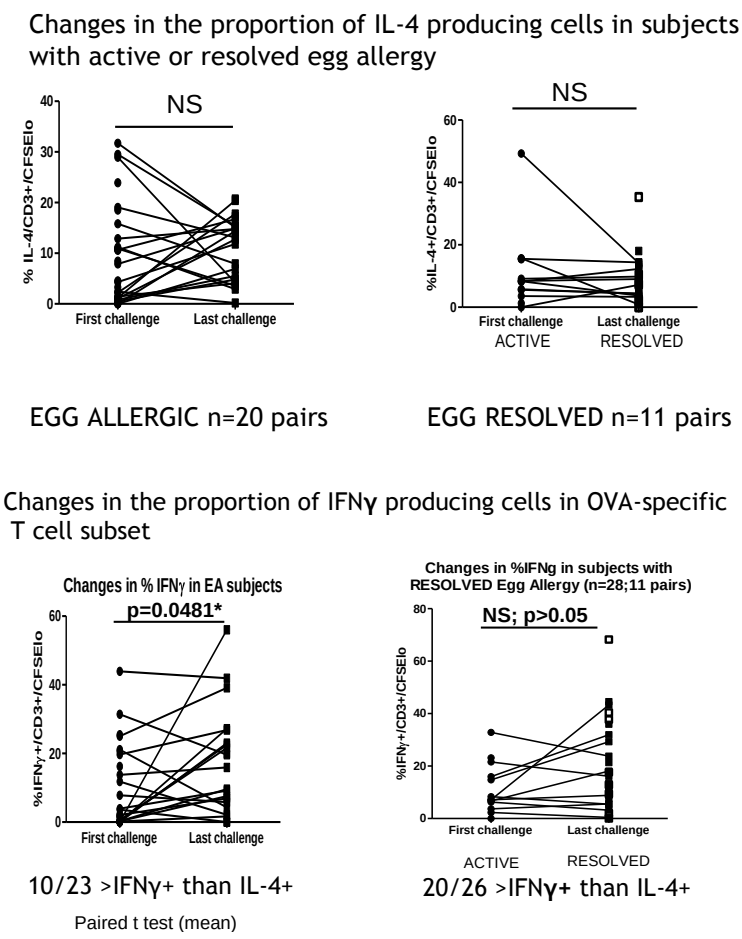


Figure 24: Longitudinal changes in %IL-4+ and %IFNγ+ OVAhi-specific T cells in EA and ER subjects. Paired data showed no change in %IL-4+ OVAhi specific T cells in EA (20 pairs) and ER (11 pairs) subjects but an increase in % IFNγ+ in EA subjects. (Students paired t test; p=0.0481*) NB. Open symbols represents subjects that resolved on their first challenge (equivalent to last challenge).

However, 12/23 (53%) EA subjects on their final challenge expressed more IL-4 than IFNγ producing T cells while only 4/26 (15%) ER subjects did (Fisher's exact test, 2-tailed p=0.0133*) (Figure 27). Surprisingly, no significant changes in the proportion of IFNγ+/CD3+/CFSElo expressing T cells in paired data were observed for EA (p=0.0559) or ER subjects. However, analysis of unpaired data showed a significant increase in the proportion of IFNγ+/CD3+/CFSElo expressing cells in EA subjects (p=0.0316*). Further analysis based on comparison of the mean values demonstrated a significant increase

between first and final challenges in paired (n=20; p=0.0481*) and unpaired (n=33; p=0.0463*) data in EA subjects only (Figure 24). Comparing the number of ER subjects where OVAhi-specific T cells express more IFNγ than IL-4 occurs (20 out of 26)(77%)) is significantly higher than in EA subjects (10 out of 23 (43%) using Fisher's exact test (2-tailed) (p=0.0214*). This suggests that on their final challenge the dominant cytokine in ER subjects is IFNγ. This is not the case for persistently EA subjects (Figure 25).

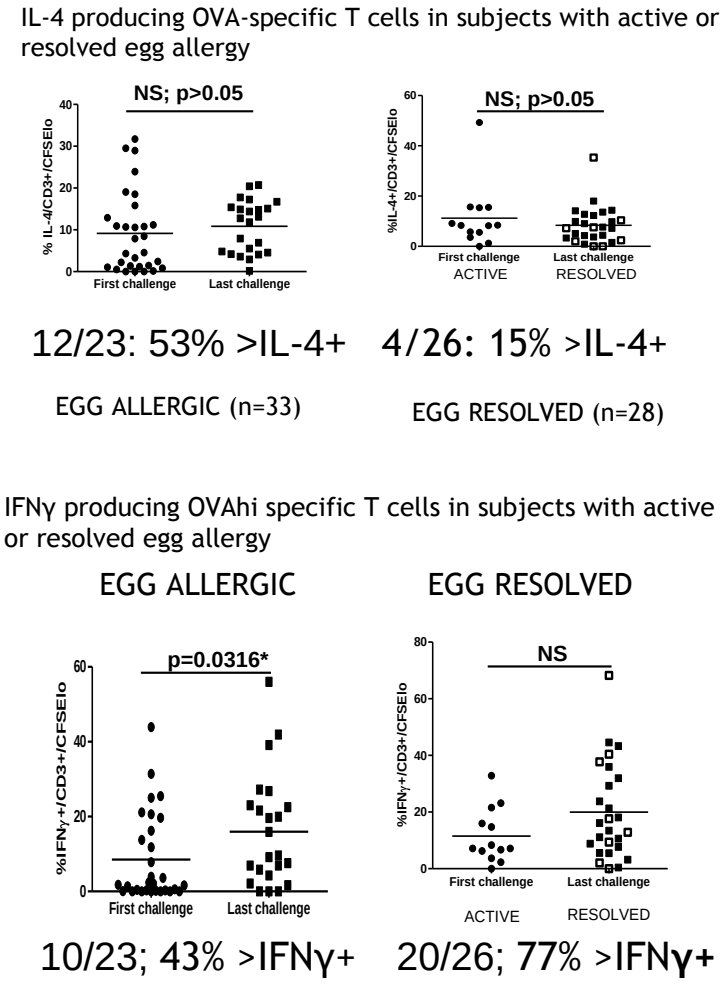


Figure 25: Comparison of the proportion of IL-4+ compared to IFNγ+ in EA and ER subjects. No differences observed by paired analysis (Wilcoxon matched pairs test), but unpaired analysis (Mann-Whitney U) showed an increase in IFNγ+/CD3+/CFSElo cells in EA subjects. Fisher's exact 2- tailed test showed the majority of ER subjects expressed more IFNγ than IL-4 (0.0214*) but EA subjects

expressed more IL-4 than IFNγ (p=0.0133*) NB. Open symbols represents subjects that resolved on their first challenge (equivalent to last challenge.

Changes were observed in the ratio of IFNγ/IL-4 in OVA–specific T cells in subjects with active egg allergy

Unpaired analysis of the ratio of IFNγ/IL-4 in ovalbumin specific T cells was shown to statistically increase between the first and last challenge in EA subjects (n=33; 14 pairs) (Mann Whitney U test: p=0.0332*) and not in ER subjects (n=28; 10 pairs). Paired analysis only suggested an increasing trend in EA subjects (NS; p=0.0515) again no significant changes among ER subjects were observed (Figure 26). The log of this cytokine expressing T cell ratio increased in subjects with persistent egg allergy over time (p=0.0258*) (Figure 27).

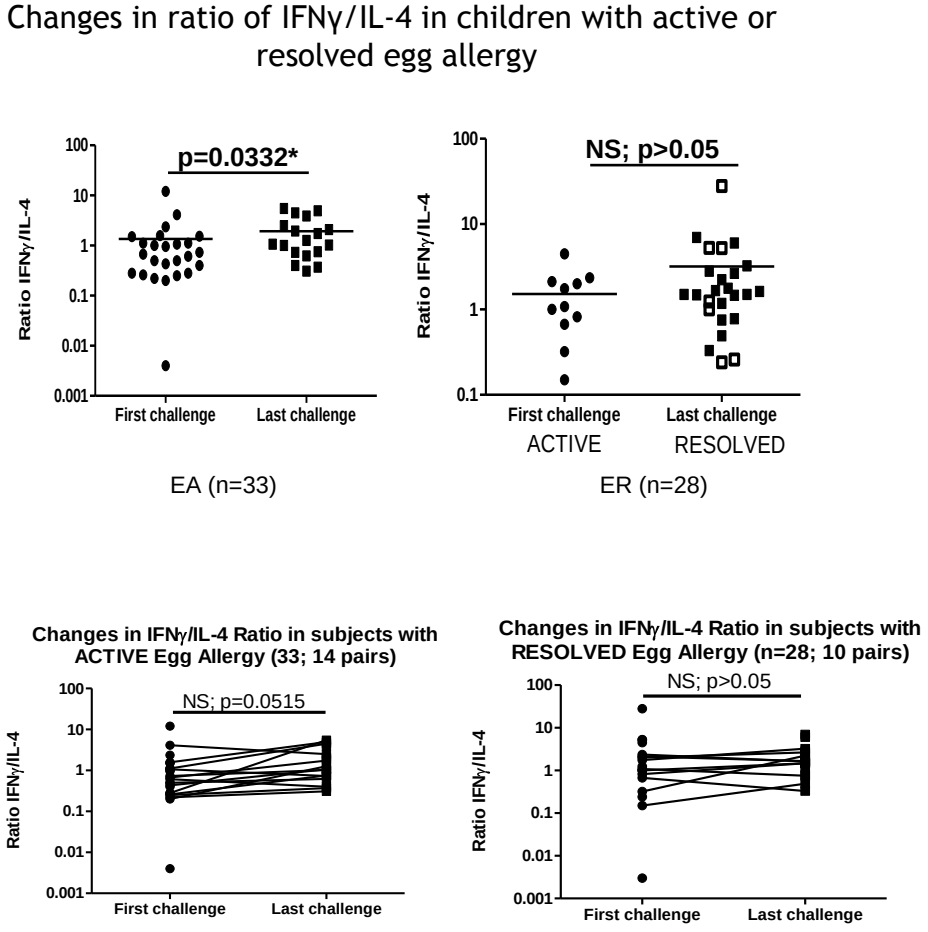


Figure 26: Comparison of IFNγ/IL-4 ratio in unpaired and paired data in EA and ER subjects

Significant increase in IFNγ/IL-4 ratio was seen in subjects with persistent egg allergy by Mann-Whitney U test (unpaired: p=0.0332*) and a trend of an increase by Wilcoxon matched pairs test (paired: NS, p=0.0515). No change was observed in ER subjects, Statistical significance may not have been reached in paired data analysis due to the small paired sample size NB. Open symbols represents subjects that resolved on their first challenge (equivalent to last challenge.

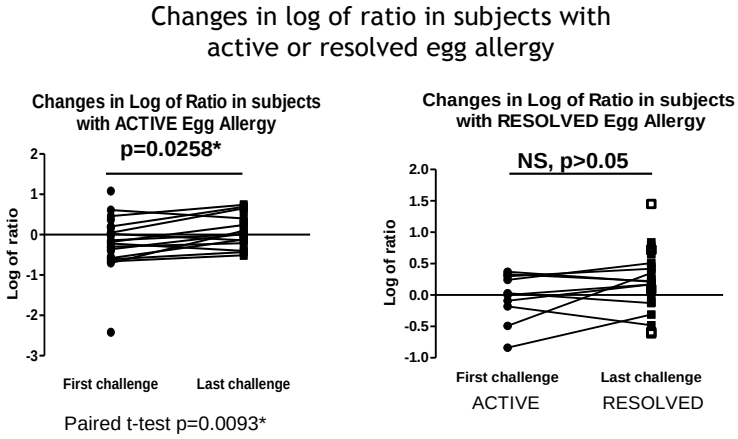


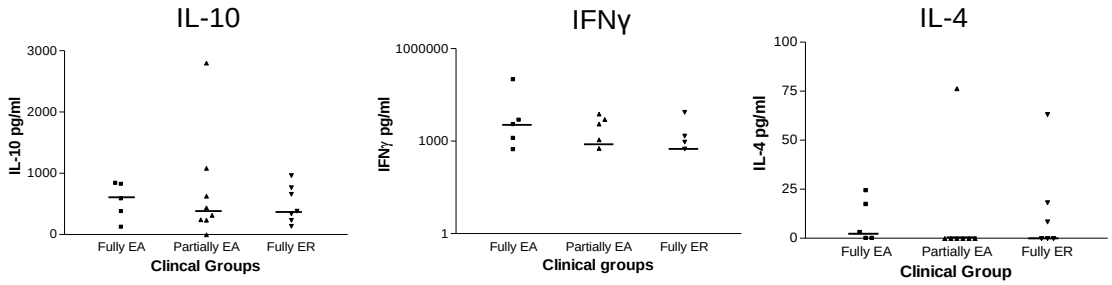
Figure 27: Log of ratio (IFNγ/IL-4) in subjects with persistent egg allergy increases within individuals over time. Comparison of log of ratio (IFNγ/IL-4) of subjects between first and last challenges in EA and ER subjects analysed using Wilcoxon matched pairs test. A significant increase was seen only in EA subjects overtime (p=0.0258*). Use of logarithmic data reduces the scale and allows data with extreme data points to be more easily presented graphically. NB. Open symbols represents subjects that resolved on their first challenge (equivalent to last challenge.

No differences were observed between clinical subject groups in extracellular cytokine levels (of IL-10, IFNγ or IL-4) among 8 day PBMC cell culture supernatants treated with high dose ovalbumin (OVAhi)

We examined the levels of extracellular IL-10, IFNγ and IL-4 cytokines produced in OVAhi-treated PBMC day 8 cell culture supernatants by ELISA from subjects who were fully allergic (n=5), partially allergic (n=8) or had fully resolved (n=7) their egg allergy Comparison of

median values for each cytokine between clinical groups showed no statistical differences (Figure 28).

Simultaneous analysis of extracellular cytokines in OVA-treated day 8 supernatants



NS; p>0.05 between clinical groups for all cytokines evaluated

Figure 28: Analysis of day 8 cell culture supernatants for extracellular cytokines by ELISA method. No differences in cytokine production between clinical sub-groups were detected. The levels are shown following subtraction of background cytokine levels of untreated PBMC cultures. The limit of detection for IL-10 is 20 pg/ml, for IFNγ is 40 pg/ml and IL-4 is 10 pg/ml.

3.2.5 Immunoglobulin expression

Total serum IgG levels increased and egg-specific IgE levels decreased over time in subjects with resolved egg allergy.

Data from EA and ER subjects were analysed using paired and unpaired data analysis. There is an unexpected increase in total IgE levels within individuals over time with resolved egg allergy (13 pairs; p=0.0327*) which is not seen in subjects with persistent egg allergy (21 pairs). However, comparison of unpaired data shows no significant differences in total IgE levels between first and last challenge in subjects with active or resolved egg allergy.

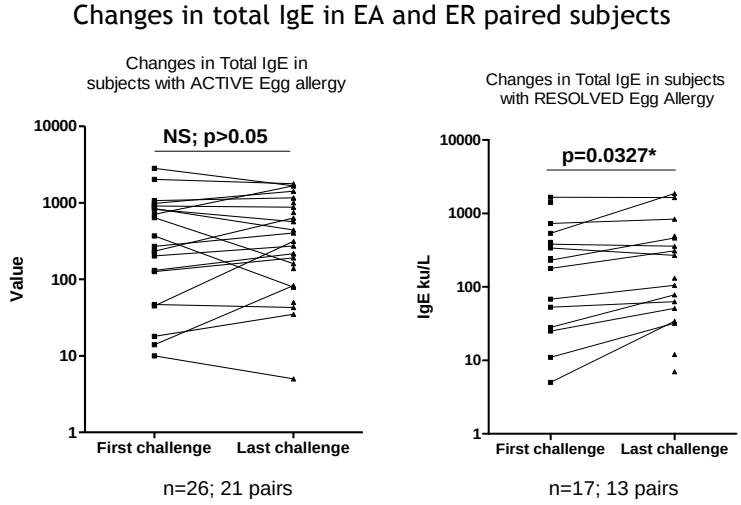


Figure 29: Changes in total IgE in EA and ER subjects. Graphs show paired data (upper panel of graphs) and unpaired data (lower panel of graphs). No significant differences were seen in subjects

with persistent egg allergy but an increase is observed in subjects with resolved egg allergy by paired data analysis (Wilcoxon matched paired test, $p<0.05$ is considered significant)

However, egg-specific IgE was significantly down-regulated in ER subjects compared with those with active (persistent) egg allergy (EA $median_{EA}$ 2.94 (range 0.45-81.10) range; ER $median_{ER}$ 0.58 (0.35-13.50) p=0.0003*** (Figure 30). Paired analysis was performed to detect changes in egg-specific IgE between the first and final egg challenge of EA (n=27) and ER (n=18) subjects Paired analysis of egg-specific IgE in ER subjects confirms a decrease in levels with resolution (p=0.0244*) and a trend towards decreasing in EA subjects (NS; p=0.0665) (Figure 31).

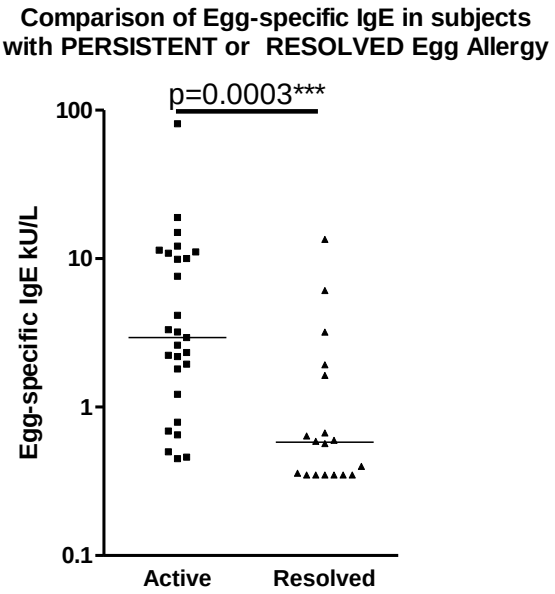


Figure 30: Egg-specific IgE declines in subjects with resolved egg allergy. Egg-specific IgE measured in 27 subjects with persistent active egg allergy and 18 subjects with resolved egg allergy were compared and shown to decrease significantly in resolved subjects (Mann-Whitney U : p=0.0003***).

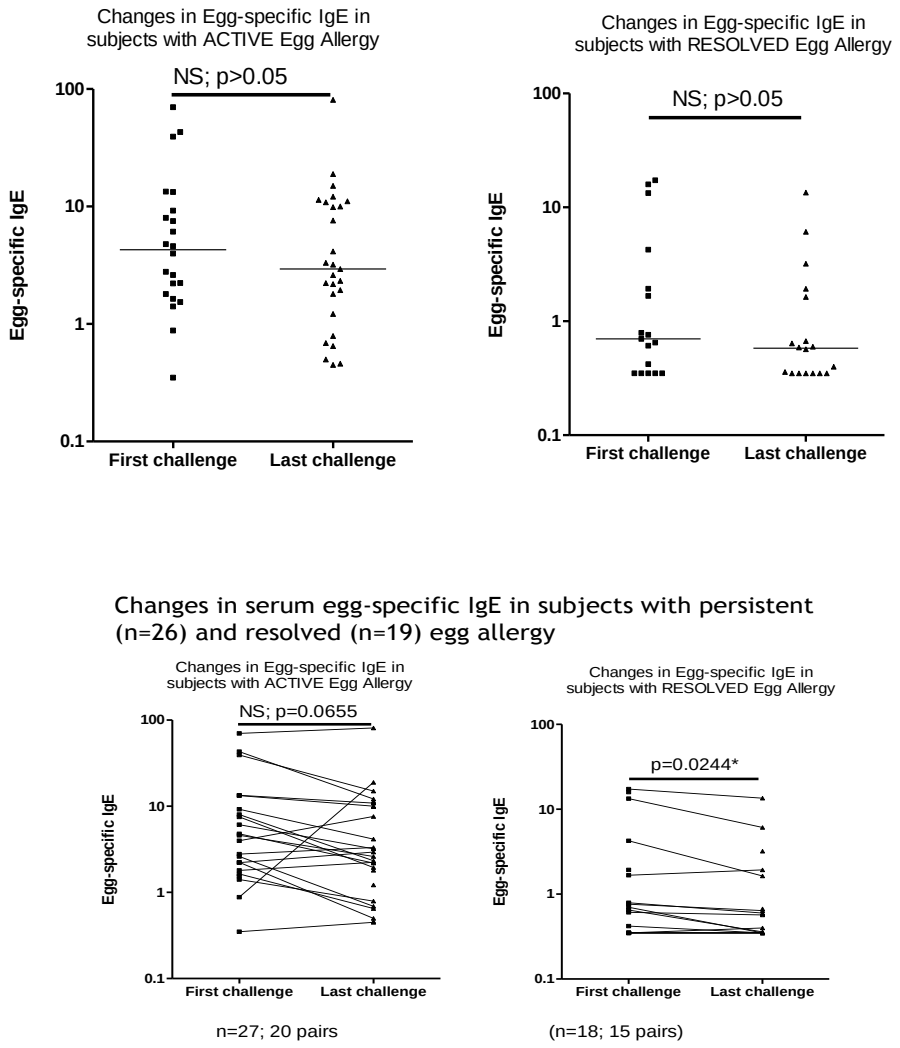


Figure 31: Changes in egg-specific IgE in subjects with active or resolved egg allergy. A significant decrease p=0.0244*) in egg-specific IgE in ER subjects acquiring tolerance to egg was detected by paired analysis (Wilcoxon matched pairs test; p<0.05 is considered significant).

Serum OVA-specific IgG4 levels increased as egg allergic subjects' allergy resolves.

No changes in total IgG between the first and last oral challenge were detected in EA (n=28; 21 pairs) or ER (n=20; 19 pairs) subjects by paired or unpaired analysis (Figure 32). Similarly, no statistically significant changes were observed in OVA specific IgG1 levels in EA (n=28; 24 pairs) or ER (n=20; 19 pairs) subjects over time, although in resolved subjects there may be a trend towards increasing IgG1 (NS; p=0.06) (Figure 33). 'Resolved' subjects were those who

on the final egg challenge showed they were clinically tolerant of uncooked egg whereas the subjects with 'active' egg allergy were clinically still reactive to egg whether cooked or uncooked at the time of their last challenge.

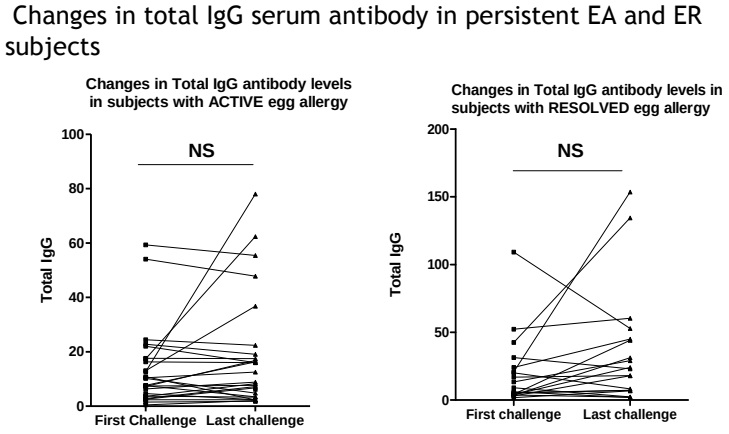


Figure 32: Changes in total serum IgG antibody levels in subjects with active and resolved egg allergy. Paired analysis using Wilcoxon matched paired test (p,0.05 is considered significant) showed no significant change in total IgG levels in EA (21 pairs) or ER 19 pairs) over time..

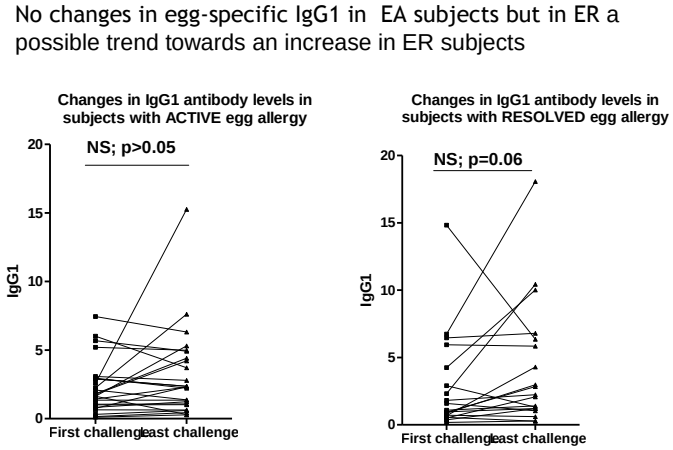


Figure 33: Changes in OVA-specific IgG1 antibody levels in subjects with active or resolved egg allergy. Paired data analysis of serum OVA-specific IgG1 levels showed no change in subjects with active egg allergy (n=21 pairs) over time, however, a possible trend to increase may exist in subjects with resolved egg allergy (n=19 pairs) (NS; p=0.06) by Wilcoxon matched paired test (p<0.05 is considered significant)

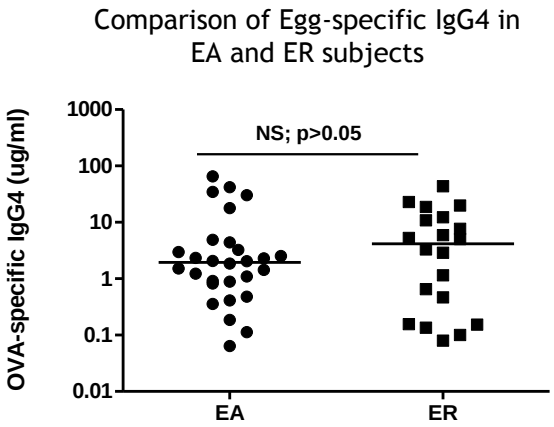
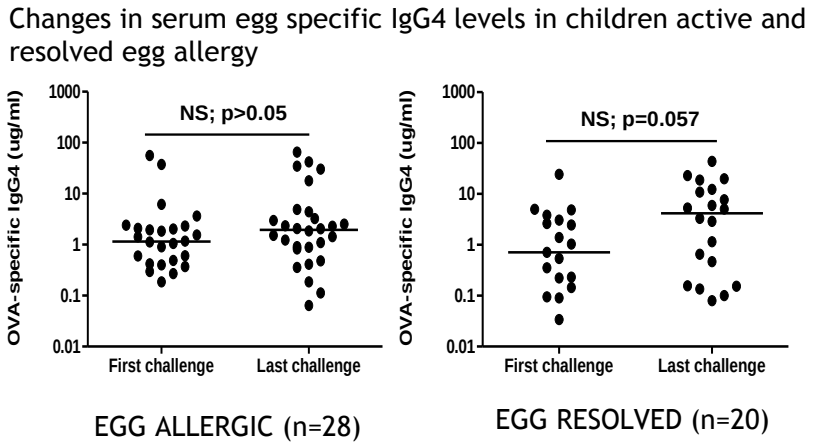


Figure 34: Cross-sectional comparison of serum OVA-specific IgG4 antibody levels in egg allergic (EA) (n=28) and egg resolved (ER) (n=20) subjects. No differences in serum OVA-specific Ig4 levels were detected between the EA and ER subjects .Comparison of median values by Mann-Whitney U test.($p<0.05$ is considered significant). Horizonal line respresents the median value.

Comparison of median serum OVA-specific IgG4 levels cross-sectionally between EA and ER showed no differences (Figure 34). However, paired data analysis showed no changes in egg specific IgG4 levels in EA subjects (n=28; 26 pairs) but in ER subjects (n=20; 19 pairs) the levels increased with resolution (p=0.0119*) (Figure 35).



Changes in egg-specific IgG4 in subjects with persistent and resolved egg allergy

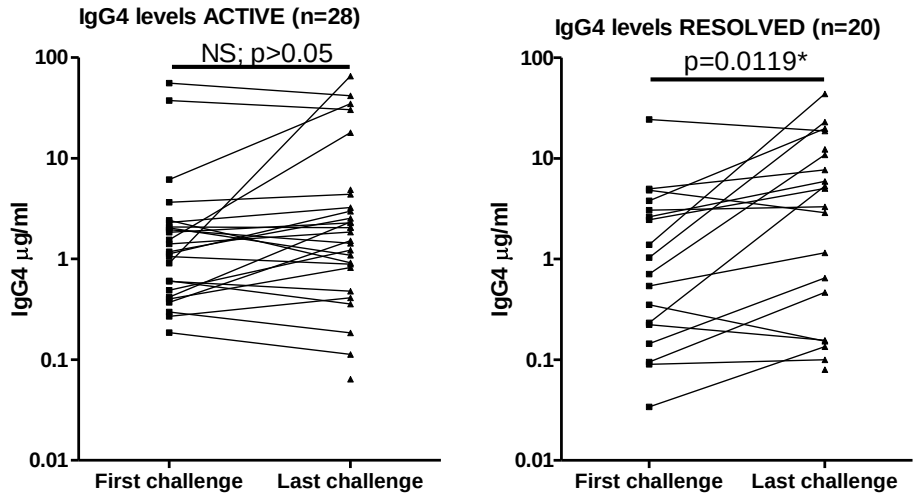


Figure 35: Changes in OVA-specific serum IgG4 levels in subjects with persistent (n=28) and resolved (n=20) egg allergy between first and last challenge. Unpaired data analysis using Mann-Whitney U test showed no significant difference (upper panel, top left) or by paired analysis (bottom left, lower panel) using Wilcoxon matched paired test in EA subjects. **I**ncreasing trend in OVA-specific IgG4 antibody levels were detected in unpaired data (NS, p=0.0579) and a significant increase in paired data (p=0.0119*) from ER subjects. P values <0.05 considerd significant for both types of non-parametric tests.

Paired data analysis of the ratio of IgG1/IgG4 ratio between the first and last challenge showed a trend towards decreasing in persistent egg allergic subjects (NS p=0.05) and a significant decrease in subjects with resolved egg allergy (p=0.0076**) (Figure 36). IgG4 is thought to be an IgE blocking antibody preventing cross-linking with allergen and involved in the development of tolerance. In persistent EA, a trend towards a decrease in IgE/Ig4 ratio between first and last challenge (p=0.0503) suggests a tendency for IgE levels to decrease while simultaneously IgG4 levels increase. This is more pronounced in resolved subjects where the decrease in IgE/IgG4 between first and last challenge is significant p=0.0081** (Figure 37).

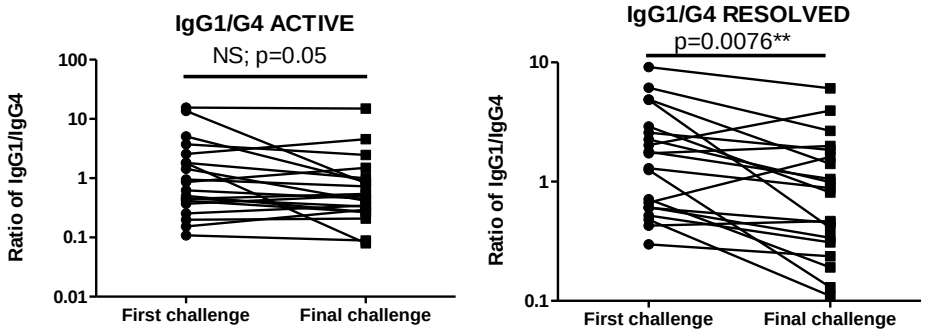


Figure 36: Changes in ratio of IgG1/IgG4 within subjects overtime with either active or resolved egg allergy between their first and final challenge. Significant decrease in IgG1/IgG4 ratio in ER subjects (Wilcoxon matched pairs test; p=0.0076**).

Changes in IgE/IgG4 ratios in subjects with persistent or resolved egg allergy

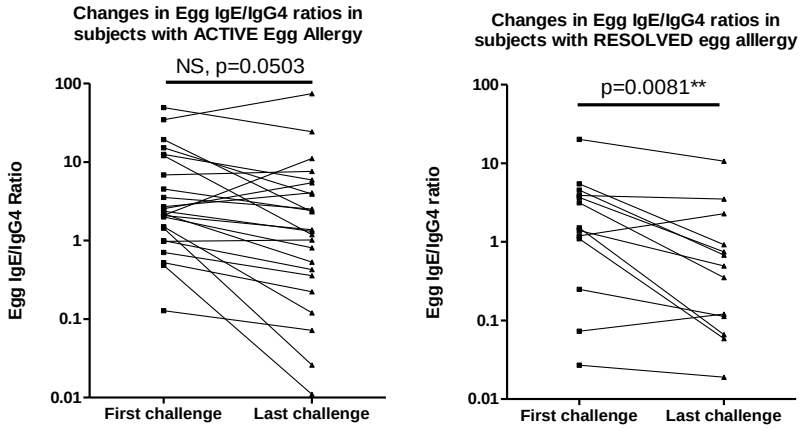


Figure 37: Changes in IgE/Ig4 ratio overtime within individuals with either persistent active egg allergy or resolved egg allergy. Paired data show a trend towards a decrease in IgE/IgG4 ratio in active allergy (NS; p=0.0503) and a significant decrease in resolved egg allergy (p=0.0081**).

3.2.6 Data Summary

T cell proliferation data

- Untreated peripheral blood mononuclear cell cultures from egg allergic subjects had a higher proportion of non-specific background T cell proliferation than untreated peripheral blood mononuclear cells from egg resolved subjects (p=0.0382*). Detailed analysis of the egg allergic clinical sub-groups on initial oral food challenge showed untreated peripheral blood mononuclear cells from partially allergic (uncooked egg reactive; p=0.0152*) and partially resolved (cooked egg tolerant; p=0.0233*) subjects had higher percentages of allergen/antigen specific T cells than egg resolved subjects but not fully allergic (well cooked egg reactive) subjects.

- No differences in the proliferation of allergen/antigen specific T cells were detected between egg allergic or egg resolved subjects for the systemic allergen tetanus toxoid (8.21Lfu/ml) or oral egg allergen ovalbumin (low dose (200 micrograms per millilitre and high dose (1milligram per millilitre). Detailed analysis of egg allergic clinical sub-groups showed no differences in proliferation compared with egg resolved subjects for high dose ovalbumin.

- Carboxyfluorescein succinimydyl ester net value and cell division index also showed no differences in proliferation or rate of proliferation respectively between egg allergic and egg resolved subjects.

- Quantitative differences in T cell responses were observed between the allergens (tetanus toxoid versus egg allergen at high or low dose) in peripheral blood mononuclear cell cultures from egg allergic and egg resolved subjects. Stimulation with tetanus toxoid and high dose ovalbumin showed similar levels of T cell proliferation in egg allergic and egg

resolved subjects. Both these allergens had higher proliferative capacity than low dose ovalbumin in egg allergic subjects (p=0.0001*** and p=0.0024**)..In egg resolved subjects only tetanus toxoid had a higher proliferative capacity than low dose ovalbumin (p=0.0393*). Analysis of carboxyfluorescein succinimydyl ester net value and cell division index confirmed the statistical differences in proliferation between allergens in egg allergic and egg resolved subjects.

Intra-cellular cytokine expression of T cells

- Proportion of interleukin-4 expressing cells was not significantly different among peripheral blood mononuclear cell cultures treated with the different allergens (tetanus toxoid,, high and low dose ovalbumin) between egg allergic and egg resolved subjects
- Proportion of interferon-gamma expressing cells was higher in egg resolved subjects stimulated with high dose ovalbumin (p=0.0092**).
- The ratio of interferon-gamma to interleukin-4 positive cells was higher in egg resolved compared with egg allergic subjects for tetanus toxoid and high dose ovalbumin but lower for low dose ovalbumin..
- The logarithmic value of the ratio of interferon-gamma to interleukin-4 displayed a similar pattern with tetanus toxoid and high dose ovalbumin having a predominantly T helper type 1 skew and low dose ovalbumin having a T helper type 2 skew in egg resolved subjects.
- In egg allergic and egg resolved subjects stimulation with all the allergens produced more intracellular cytokine production than untreated controls. Differences in the proportion of interleukin-4 expresing cells and interferon-gamma expressing cells exist between the allergens in egg allergic and egg resolved subjects.
- Longitudinal analysis of paired egg allergic (n=33; 20 pairs) and egg resolved (n=28; 11 pairs) data showed no differences in the percentage of ovalbumin-specific T cells or carboxyfluorescein succinimydyl ester net value.
- Cell division index was increased in subjects with persistent egg allergy.

- Cytokine analysis broadly correlates with egg allergy status with just over half (12/23) with active egg allergy (p=0.013*) showing a T helper 2 (interleukin-4) cytokine skew compared with the majority with resolved egg allergy (20/26) (p=0.0214*) displaying a T helper 1 (interferon-gamma) cytokine skew.
- Increased proportion of the ovalbumin-specific T cells producing interferon-gamma in children with resolved compared to active egg allergy (p=0.0115*)

Extra-cellular cytokine data

- Simultaneous analysis of day 8 peripheral blood mononuclear cell culture supernatants collected from high dose ovalbumin treated peripheral blood mononuclear cell cultures from fully allergic, partially allergic and fully resolved subjects showed no significant differences in the levels of extracellular cytokines interleukin-10, interleukin-4 or interferon-gamma.

Immunoglobulin data

- Our results showed egg-specific immunoglobulin E levels decreased between first and last oral challenge in children who had resolved their egg allergy compared with children who had persistent active egg allergy p=0.0003***.
- Paired analysis of egg resolved subjects showed an unexpected increase in total immunoglobulin E (p=0.0327*) between the first and final challenge
- No differences in total immunoglobulin G or immunoglobulin G1 was observed between egg allergic and egg resolved subjects.
- Longitudinal analysis of matched pairs of persistently active or resolved subjects showed no changes in total immunoglobulin G or egg-specific immunoglobulin G1, although a trend towards increasing immunoglobulin G1 may occur in resolving subjects (not significant; p=0.06).

- In contrast, immunoglobulin G4 levels in children with resolved egg allergy appear to increase within individuals overtime (Wilcoxon Matched Pairs test p=0.0119*; Mann-Whitney U-test: not significant; p=0.057)
- Ratio of immunoglobulin G1 to immunoglobulin G4 was shown to significantly decline in egg resolved subjects (p=0.0076**) and a trend towards decreasing in egg allergic subjects (not significant; p=0.05)
- Ratio of immunoglobulin E to immunoglobulin G4 significantly decreases in egg resolved (p=0.0081**) and a trend towards decreasing in egg allergic subjects (not significant; p=0.0507)

This is the first study defining both the clinical and immunological longitudinal changes which occur during resolution of clinical egg allergy. Additionally, this study identified and examined the responses of allergen-specific T cells as opposed to the non-specific pool of peripheral blood mononuclear cells, using a novel technique of intracellular staining. This avoids confounding influences from other cell types. Our experimental work has shown that during resolution of egg allergy there is a switching to an interferon gamma dominant response by allergen specific T cells; although interleukin-4 secreting cells are not reduced in number they are less dominant in the resolved state. The ratio of interferon gamma to interleukin-4 therefore tends to increase longitudinally during resolution. These changes are associated with a reduction in levels of serum specific immunoglobulin E and increase in specific immunoglobulin G4 as clinical tolerance is attained.

4 DISCUSSION

4.1 Experimental Data

The immunological changes which take place during resolution of food allergy are poorly characterized. The aim of our prospective study was to define the changes in T cell responses longitudinally (proliferation and cytokine secretion) during either resolution or persistence of one of the commonest food allergies, egg allergy.

The development of methods for fluorescent cell division tracking by Lyons and Parish (1994) [52] allowed researchers to examine for the first time the proliferation of lymphocyte subsets within complex mixtures of cells, e.g. peripheral blood mononuclear cells (PBMC), which could be easily quantified by using appropriate cell surface markers to identify the lymphocyte subpopulation. Significant progress in understanding the important role of T cell derived cytokines in the induction and maintenance of the allergic status has been made [16, 17, 18]. Previous studies have presented evidence that immunotherapy with house dust mite or grass pollen may act by induction of an altered pattern of cytokine synthesis by T cells [53, 54]. Induction of tolerance is considered to involve a complex array of interacting mechanisms. Hence, an alteration of the Th1/Th2 cytokine imbalance in egg allergic subjects is regarded as just one approach. Thus, one aim of our study was to investigate whether resolution of hen egg allergy is partly a consequence of changes in cytokine secretion from a predominantly T helper type 2 (IL-4) cytokine response to a predominantly T helper type 1 (IFNγ) by allergen specific T cells

One hundred and nine egg allergic (EA) subjects were recruited with a known clinical history of type I mediated hypersensitivity responses, positive skin prick test and egg-specific serum IgE antibodies. The subjects were challenged annually over a 6 year period with either well

cooked egg or uncooked egg. Peripheral blood mononuclear cells isolated from subjects at each challenge visit were stimulated *in vitro* with ovalbumin (OVA), one of the major hen egg allergens, or with a control antigen Tetanus toxoid (TET). Previously, our group demonstrated stronger proliferative T cell responses to OVA than to ovomucoid (OM) [44]). This may reflect differences in individual reactivity to these egg allergens. Cytokine secretion was measured 4 hours after stimulation with phorbol myristate acetate (PMA) and ionomycin after 8 days of culture.

An unexpected finding was a higher level of non-specific background proliferation observed in PBMC cultures from EA subjects but not in resolved subjects. The explanation is not clear, but one possibility may be stimulation by very small levels of egg protein in the autologous plasma used in cultures. However, against this hypothesis there was no difference between allergic and resolved subjects in proliferation when OVA (up to 1mg/ml) was to added cultures. Another point is that the observed proliferation was non-specific as these cells were not producing the intracellular cytokines of interest.

The oral allergen ovalbumin (OVA) and positive control antigen tetanus toxoid (TET) used to stimulate PBMCs showed no differences in the T cell proliferative responses they gave rise to i.e. generation of allergen-specific T cells (CD3+/CFSElo) between subject groups. Previous studies with [^{3}H]-thymidine assessed proliferation as DNA synthesis of whole PBMC cultures [77, 44] whereas the CFSE assay is more sensitive and well suited to quantifying proliferation of specific T cells directly in response to allergen. In combination with flow cytometry we were able to analyse the phenotype and function of the allergen responsive T cells.

In OVA-stimulated cultures, egg resolved (ER) subjects showed an increase in the proportion of IFNγ secreting cells compared with egg allergic (EA) subjects. In TET stimulated cultures we observed similar levels of cytokines in EA and ER subjects. We conclude that resolution of

hen egg allergy involve changes in allergen-specific T cell responsiveness resulting in IFNγ up-regulation but no change in lymphoproliferation to OVA.

In fact, in this study, we demonstrated that resolution of hen egg allergy is associated with a significant increase in IFNγ secretion by allergen-specific T cells *in vitro*. However, as seen in previous studies with bee venom [55] we did not observe a simultaneous decrease in allergen-specific IL-4 secretion. Our data suggest that IL-4 production levels may be equally maintained regardless of egg allergic status. A higher frequency of IFNγ positive cells and therefore a switch to IFNγ dominance is associated with development of resolution and tolerance. This finding does suggest a shift towards a more Th1-like cell response resulting in down-regulation of clinical reactivity in subjects with egg allergy. This pattern has also been observed in studies on skin biopsy samples during grass pollen immunotherapy [56, 57, 36] which indicated enhanced mRNA expression of Th1 type cytokines, but found no effect on Th2 type cytokine secretion pattern.[17, 18].

Suppression of the allergic response by several humoral and basic immunological mechanisms has been proposed. One of these mechanisms is an increase in the production of immunoglobulin (Ig) G competitive antibodies, which reduces the pathological effects of IgE via several different mechanisms [34]. The different immunological effects relate to clinical outcome as reduction in symptoms and the need for treatment, and reduced inflammatory response. The size of induction of IgG following egg immunotherapy may be related to the cumulative dose used for challenge treatment and the clinical effects of the oral challenge. Our data suggest that the rise of IgE blocking antibodies, particularly IgG4 during resolution of egg allergy, is the main reason for protection [58, 59]. Evidence from previous studies suggest production of IgE antibodies, which are considered to be critical as an indicator of immediate hypersensitivity, is mainly dependent on the ratio between IL-4 and IFNγ cytokines, which are secreted by activated T cells [60, 61, 62, 63]. Human B cell clones can be induced to

proliferate in vitro and switch to IgE and IgG4 synthesis by IL-4 and a co-stimulatory signal provided by activated CD4+T cell clones. Our data shows that egg-specific IgE production is reduced in ER subjects, perhaps suppressed by increasing IFNγ [64]. Our paired longitudinal data suggest a protective effect of IgG4 antibodies in some egg allergic subjects as they progress towards tolerance induction. The IgE/IgG4 ratio declined significantly in ER subjects and a similar trend is seen in some subjects with persistent egg allergy who may go on to resolve later.

4.2 Clinical data

We have described the clinical features, outcomes and natural history of resolution of egg allergy in a large representative sample of young children in the UK. This will aid clinical decision making. We have demonstrated that during resolution of hen egg allergy, tolerance to well cooked egg is achieved before uncooked egg; and that well cooked egg challenges are well tolerated with no severe reactions. We have identified a serum IgE level that confers a high probability of predicting tolerance to egg and propose (below) an algorithm for reintroduction of well cooked and lightly cooked egg based on serum IgE levels .

The natural history of egg allergy is for the majority to undergo spontaneous resolution over time [7]. A recent prospective challenge study showed the median time to raw egg tolerance was 35 months and 66% had resolved after 5 years of follow-up [9]. In contrast we found that ¾ had not resolved by five years of age and the median age at complete resolution of egg allergy was 125 months. This discrepancy may be partly explained by methodological differences, as the population we studied was older. Nonetheless, both studies underestimate the true rate of resolution because subjects were likely to have achieved tolerance to egg in advance of the egg challenge.

In our study, tolerance to well cooked egg was achieved well before tolerance to uncooked egg, in the same population. There is a considerable difference, with median age at tolerance of 67m and 125m for well cooked and uncooked egg respectively. A recent study of egg-allergic patients aged between 0.5 and 25 years showed that 70% were able to tolerate challenges with well-cooked egg [65]. Therefore during resolution of allergy, gradual and stepwise reintroduction should be attempted with well cooked egg first [74]. In the present study, nearly 1/4 had resolved allergy to well cooked egg at 3 years and 2/3 at 6 years. It is

important to note that resolution can occur in older children, so that despite an earlier positive challenge, attempts at reintroduction should be continued.

Families find it helpful to establish when their children have achieved tolerance to well cooked egg, so that they no longer worry about well cooked egg as a hidden ingredient in foods, and allergen avoidance becomes less onerous. Additionally, after a negative challenge, well cooked egg should be introduced into the diet on a regular basis: this is shown to be well tolerated with no adverse effect on growth, allergy or intestinal permeability [65]. In a previous study the food allergy related quality of life score improved for children who underwent food challenge in a clinic setting [67]. The improvement in quality of life occurred after both positive and negative challenges, implying that all families found the challenge process helpful regardless of the outcome.

Data advising on the timing of reintroduction are scarce, recently published BSACI guidelines [74] recommend that 'children who have had only mild symptoms (cutaneous) on significant exposure (e.g. a mouthful of scrambled eggs) with no ongoing asthma could have well-cooked egg (e.g. sponge cake) reintroduced from the age of about 2-3 years at home' [74]. In our study, tolerance to well-cooked egg was achieved approximately twice as quickly as tolerance to uncooked egg, in the same population. There is a considerable difference, with median age at tolerance of 5 years and 10 years for well-cooked and uncooked egg respectively. Our data therefore support this guideline: at three years 27% had no reaction to a cooked egg challenge and reactions were universally mild. Furthermore, nearly 2/3 had resolved allergy to well-cooked egg at 6 years. It is important to note that resolution can occur in older children, so that despite an earlier positive challenge, attempts at reintroduction should be continued. Our data support undertaking a well-cooked egg challenge as this is usually tolerated even when the skin test is positive (e.g. 3-5mm wheal diameter; table 2).

There are a number of safety factors built into this approach. The proportion of egg in the food is very small for well-cooked egg reintroduction. The age when resolution is demonstrated by challenge is likely to be greater than the age at which resolution actually occurs therefore a higher proportion would have resolved than is apparent. Our data are based on full dose challenges of egg given over 2 hours, whereas the home reintroduction recommendation is over days or weeks. Thus the dose of allergen is significantly less and the rate of reintroduction significantly lower. Lastly, for lightly cooked reintroduction, children would have already introduced well-cooked egg into their diet; therefore the process of resolution has begun.

The safety of performing home reintroduction of well cooked egg can be considered by examining previous challenge studies, although there are important methodological differences between home reintroduction and egg challenge protocols. There have been no studies of egg reactions after home reintroduction. In studies employing hospital based egg challenges the onset of reactions is rapid, with most symptoms occurring in the first 30 minutes of ingestion [68]. Urticaria and/or angio-oedema occur in up to 90% of subjects (within minutes) and gastrointestinal symptoms are described in 10-44% (within 2 hours) [6, 68, 69]. The majority of reactions however are mild. Reporting of more severe reactions with significant respiratory symptoms varies widely from 2-34% in challenge studies [68-73]. One study which reported a 7% rate of severe reactions including 21% with respiratory reactions, but this involved administration of a whole raw egg at once [73]. A retrospective chart review of clinical practice challenges reported a severe reaction rate of 17%, however the type of egg used in challenges was not reported [18]. Most of these studies employed raw or lightly cooked egg challenges and/or large single doses and/or short dose intervals. A cautious regime using sequential well cooked then lightly cooked egg with stepwise reintroduction as we suggest is better tolerated. We emphasize that the BSACI algorithm involves slow stepwise reintroduction over several days instead of challenge over a few hours [74].

In the present study, reactions to well cooked egg were mild to moderate with no serious respiratory features, the majority having only cutaneous or gastrointestinal reactions. This should provide reassurance for clinicians planning to reintroduce well cooked egg back into the diet of children with mild egg allergy, at home.

A recent publication cautioned against any home introduction of well cooked egg, after reporting that injectable adrenaline was administered to a number of children during hospital-based challenges to cooked egg [65]. However the challenge involved a large dose of egg given over a few hours. Whereas our first day's dose is about 120 fold less**.** In addition the cohort challenged contained significant numbers of children with severe egg allergy and asthma, two factors which would lead one to consider a hospital supervised challenge. We recommend that children who have had only mild symptoms (cutaneous) on significant exposure (e.g. a mouthful of scrambled eggs) with no ongoing asthma could have well cooked egg (e.g. sponge cake) introduced from the age of about 2-3 years at home in accordance with the recently published BSACI egg allergy guidelines [74].

Data from our group and others [28] show that children who tolerate well cooked egg may still react to uncooked egg. In case series severe reactions have been reported [27]. The speed with which allergy to uncooked egg resolves varies greatly between individuals; therefore the timing and appropriateness of reintroduction should be individually assessed. Biomarkers could be helpful in this respect. Recent studies have suggested that the absolute level [10] and the rate of fall [38] of egg white IgE may help to identify patients in whom the egg allergy has resolved. Children with a peak level of egg IgE <2kU/l had the fastest rate of resolution [10], and a reduction in serum egg-specific IgE level of 50% over 12 months was associated with a 0.52 probability of tolerance [38].

5 CONCLUSIONS

This longitudinal study of atopic children with egg allergy has shown up-regulation of Th1 responsiveness (shown by increased egg specific interferon gamma) and IgG4 levels and decreased egg-specific IgE with acquisition of clinical tolerance. This is the first longitudinal clinical study which defines the ages at resolution of egg allergy, for both well-cooked and uncooked egg. The findings support the recently published BSACI guidelines on egg reintroduction.

Acknowledgements

We are grateful to the Foods Standards Agency for their funding of the study and salary support for Dr Islam, Dr Tay and Sr King. We would like to thank the staff at the Wellcome Trust Clinical Research Facility where the clinical work conducted. We would also like to thank the Cambridge Biomedical Research Centre for their continuing support in hosting this research. We also thank the Anaphylaxis Campaign for helping to publicize the study. Finally we are indebted to all the participants and their families who volunteered for this research project.

Publications

Tay SS, Clark AT, Deighton J, King Y, Ewan PW. Patterns of immunoglobulin G responses to egg and peanut allergens are distinct: ovalbumin-specific immunoglobulin responses are ubiquitous, but peanut-specific immunoglobulin responses are up-regulated in peanut allergy**.** Clin Exp Allergy 2007;37:1512-8

Tay SS, Clark AT, King Y, Deighton J, Ewan PW. T cell proliferation and cytokine responses to ovalbumin and ovomucoid detected in children with and without egg allergy. Clin Exp Allergy 2007;37:1519-27

Clark A, Islam S, King Y, Deighton J, Szun Sun Tay, Anagnostou K, Ewan P. A longitudinal study of resolution of allergy to well-cooked and uncooked egg. Clin Exp Allergy in press.

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