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Peanut allergen Ara h 2-specific T cells are activated via Ras-Erk MAP kinase pathway signalling and identified by CD154 expression

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Background. Peanut allergy is driven by peanut-specific Th2 cells. Ara h 2, a peanut 2S albumin, is one of the major peanut allergens. **Methods.** An Ara h 2-specific T cell line was generated from peanut sensitised C3H/HeJ mice and used to analyse allergen-induced cytokine secretion and activation of signalling molecules. **Results.** Peanut stimulated splenocytes from peanut sensitised mice had strong proliferation responses and highly elevated IL-4 secretion. The Ara h 2-specific T cells generated from sensitised mice were mainly CD4⁺ T helper cells with a Th2 cytokine profile. The expression of CD154 on the surface of these T cell clones was detected following Ara h 2 stimulation. Ara h 2 stimulation induced phosphorylation of ZAP-70 and Erk1/2 in the T cells. **Conclusions.** CD154 is a useful marker for identifying activated, allergen-specific T cells. The Ras-Erk mitogen-activated protein kinase pathway is involved in the activation of Ara h 2-specific Th2 cells.

Keywords: peanut; allergen; food allergy; Th2 cell; Ras-Erk; CD154

1. Introduction

Peanut allergy affects approximately 1% of the US population (Sicherer, Munoz-Furlong, Godbold, & Sampson, 2010) and is rarely outgrown (Skolnick et al., 2001). Ara h 2 has been classified as a major peanut allergen with greater than 90% of peanut allergic subjects having IgE reactivity to the protein (Burks et al., 1992; McDermott et al., 2007; Stanley et al., 1997). Ara h 2 shares sequence and structural homology with the 2S albumin class of seed storage proteins, which comprises several known allergens (Moreno & Clemente, 2008).

The central role of Th2 cells in the development of food allergy is well documented (Akdis & Akdis, 2007; Akdis et al., 2004; Chehade & Mayer, 2005). The differentiation of Th2 cells from naïve T cells requires both the recognition of antigen by T cell receptors (TCR) and the activation of downstream signal-transduction molecules of the interleukin 4 receptor (IL-4R) pathway. TCR-mediated activation of the Ras/mitogen-activated protein kinase pathway alters IL-4R function and is required for Th2 cell differentiation (Yamashita et al., 1999, 2005). We

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hypothesised that this pathway is involved in the activation of peanut-specific CD4+ T cells and Th2 cytokine expression following peanut allergen stimulation.

A pivotal role in the activation and function of dendritic cells (DCs) and B cells is thought to be played by the CD40–CD154 partnership (MacDonald, Straw, Dalton, & Pearce, 2002). Activated, but not resting, CD4+ T cells express CD154, providing co-stimulatory signals to B cells and DCs. To this end, *de novo* CD154 expression following antigen stimulation has been used to identify antigen-specific CD4+ T cells (Chattopadhyay, Yu, & Roederer, 2005). Birch pollen allergens induced CD154 expression on CD3+ lymphocytes only in atopic individuals (Nakstad, Kahler, & Lyberg, 1999). We hypothesised that CD154 can be used to study peanut allergen-specific CD4+ T cells. Identifying allergen-specific T cells is critical in defining the properties of these cells, which has unfortunately proved challenging because of their low frequency within the T-cell repertoire (Akdis et al., 2004).

In the present work, an Ara h 2-specific T cell line was established from peanut sensitised mouse splenocytes. In the cell line, the activation of Ras-Erk MAPK was investigated following peanut allergen stimulation. The utility of CD154 expression as a marker of Ara h 2-specific T cells was determined. These results will be useful for future studies with peanut immunotherapy to understand alterations in T cell signalling that lead to a decrease in peanut-specific Th2 responses.

2. Methods

2.1. Mouse model of peanut allergy

The mouse model was based on previous work establishing the utility of the model and demonstrating Ara h 2 responses (Li et al., 2000; Pons et al., 2004). Five-week-old female C3H/HeJ mice purchased from The Jackson Laboratory were maintained under specific pathogen-free conditions. Standard guidelines for the care and use of animals were followed (Institute of Laboratory Animal Resources Commission of Life Sciences & Council, 1996). Crude peanut extract (CPE) and peanut allergen Ara h 2 were prepared according to previous reports (Pons et al., 2004; Sen et al., 2002). Peanut sensitisation was performed by intraperitoneal (ip) injection of 0.5 mg of CPE with 2.0 mg of Alum on days 0, 7 and 21. Naïve mice received PBS plus 2.0 mg Alum. Peanut challenges were performed with 1.0 mg CPE given ip on day 35. Hypersensitivity reactions were assessed by scoring anaphylactic symptoms (on a 0–5 scale, with 0 meaning no symptoms and 5 meaning anaphylactic death) and measuring body temperatures at 30 minutes post-challenge, according to previous work (Li et al., 2000; Pons et al., 2004).

2.2. CFSE dilution assay and [³H]-thymidine incorporation to study T cell proliferation

Splenocytes were labelled with CFSE as described previously (Akdis et al., 2004; Turcanu, Maleki, & Lack, 2003). CFSE labelled cells in IMDM media supplemented with 10% FBS (IMDM-10) were seeded at the concentration of 5×10^6 /well in U-bottom 96-well plates and left unstimulated or stimulated with CPE (100 µg/ml) or Ara h 2 (100 µg/ml). After 48 hours incubation at 37°C in a 5% CO₂ humidified incubator, cells were stained for CD4 status and analysed by flow cytometry.

Alternatively, cells were cultured as just described, without adding CFSE; at 48 hours, 1 $\mu\text{Ci}/\text{well}$ [^3H]-thymidine was added and cells were cultured for an additional 18 hours. Incorporation of [^3H]-thymidine was measured on a scintillation counter.

2.3. Ara h 2-specific T cell line establishment

Splenocytes from sensitised mice were cultured in IMDM-10, seeded at a concentration of $4 \times 10^6/\text{well}$ in 24 well, flat bottom culture plates, then stimulated with 100 $\mu\text{g}/\text{ml}$ Ara h 2 for 7 days at 37°C in a 5% CO_2 humidified incubator. Supernatants were removed and replaced with fresh medium; 5 ng/ml mouse IL-2 was added to each well, followed by another 7 days of culturing. Cells were then re-stimulated with Ara h 2 for 7 days in the presence of 5×10^7 cells/ml antigen presenting cells (APC). Mitomycin C (50 $\mu\text{g}/\text{ml}$, Sigma) treated splenocytes were used as APC. T-cell lines were maintained by alternating one week of IL-2 (5 ng/ml) treatment and one week allergen stimulation in the presence of APC.

2.4. Cytokine expression using intracellular staining

For polyclonal stimulation of Ara h 2-specific T cells, triplicate cultures of 4×10^5 cells/well were stimulated in a 96-well round bottom culture plate with anti-CD3 monoclonal antibody (BD Pharmingen, clone 145-2C11) and anti-CD28 monoclonal antibody (BD Pharmingen, clone 37.51) at final concentrations of 5 $\mu\text{g}/\mu\text{l}$ and 1 $\mu\text{g}/\mu\text{l}$, respectively, in IMDM-10. For CPE and Ara h 2 stimulation, 2×10^5 cells/well in triplicate cultures were stimulated in 200 μl of IMDM-10 with CPE or Ara h 2 (final concentration, 100 $\mu\text{g}/\text{ml}$ each) in the presence of 1×10^6 APC. After 3 days of stimulation, the cells were re-stimulated with Leukocyte Activation Cocktail (containing GolgiPlug, BD Pharmingen) for 6 hours. Cells were harvested and centrifuged at 3500 rpm for 5 minutes, then suspended in staining buffer (PBS without Mg^{2+} or Ca^{2+} , 1% FBS and 0.09% sodium azide). Cell suspensions were incubated with Fc antibody for 15 minutes at 4°C . After blocking, cells were washed twice and re-suspended in 100 μl staining buffer, then stained with anti-CD3-PerCP and anti-CD4 (FITC or PE, depending on cytokine of interest) (BD Pharmingen) for 30 minutes at 4°C . The cells were fixed and permeabilised with Fixation/Permeabilisation buffer (BD Cytofix/Cytoperm), then stained with FITC-conjugated anti-mouse IFN- γ or PE-conjugated anti-mouse IL-4 antibody (BD Pharmingen).

2.5. ELISA for the quantification of mouse IL-4

Cytokine production in supernatant was analysed by mouse IL-4 ELISA MAX kit (Biolegend, Cat.431101) according to manufacturer's instructions.

2.6. Co-culture method to detect Ara h 2-specific T cell by CD154

In round bottom 96 well plates, cells were cultured at 37°C , 5% CO_2 for 24 hours with the following conditions: 5×10^4 T cells, 2.5×10^5 APC cells, 100 $\mu\text{g}/\text{ml}$ Ara h 2, 1 $\mu\text{g}/\text{ml}$ anti-CD154-PE (BD Pharmingen, MRI) and 2 μM monensin (BD Bioscience) (Chattopadhyay, Yu, & Roederer, 2006). After stimulation, the contents of each well were transferred to V bottom plates and spun for 3 minutes at 3000 rpm

at 4°C. Supernatant was aspirated and cells re-suspended in 50 µl staining buffer containing anti-CD4-FITC and analysed by flow cytometry.

2.7. Detection of ZAP70 and Erk1/2 phosphorylation by intracellular staining

T cell lines were washed and counted after a 7-day rest. These cells were then equilibrated for 4 hours in IMDM without FBS and incubated with antigen-loaded APC in the ratio of 1:5 and plated in a 96 well plate (5×10^4 Ara h 2-specific T cells in each well) for 5–30 minutes. Cells were fixed, permeabilised and stained with Alexa Fluor 488 conjugated anti-Phospho-ERK1/2 (T202/Y204) (BD PhosFlow) or PE conjugated anti-Phospho-Zap70 (Y319)/Syk (Y352) antibody (BD PhosFlow) with PE or FITC conjugated anti-CD4 antibody (BD Pharmingen) at 4°C to detect the activation of ERK1/2 or ZAP70.

2.8. Flow cytometric analysis

Flow cytometric analysis of surface and intracellular markers were performed using a three colour FAC Scan flow cytometer (Becton Dickson) and FlowJo 6.4.1 software (TreeStar). Isotype matched mAbs, mouse FITC, PE or PerCP conjugated IgG1/IgG2a were used as control. At least 10^4 cells per sample were analysed.

3. Results

3.1. Establishment of an Ara h 2-specific T cell line from peanut sensitised mice

Mice were sensitised to peanut allergens and then given a provocation challenge with peanut extract to verify that they were hypersensitised. Peanut sensitised mice had a median symptom score of four compared to naïve mice with a median score of zero (data not shown). Body temperature decrease is also a hallmark response of acute allergic reaction in mice, and as expected, peanut sensitised mice had a large decrease (mean of 7.6°C) in body temperature while naïve mice did not (data not shown).

The proliferation of CD4+ T cells from sensitised mice was strongly up-regulated relative to naïve CD4+ T cells (Figure 1A). Following CPE stimulation, sensitised mouse CD4+ T cell proliferation is approximately nine times greater than naïve mouse splenocyte proliferation. With Ara h 2 stimulation, this difference is greater than 5-fold. The content of IL-4 in the supernatants of cultured splenocytes from sensitised mice following CPE stimulation was greater than 10 times higher than naïve mice (data not shown). Ara h 2 also induced IL-4 secretion, although the effect was not as dramatic as measured for CPE, likely due to the low number of Ara h 2-specific T cells.

In order to characterise peanut allergen activated T cells, we established an Ara h 2 specific T cell line. The cell line was derived from peanut sensitised mouse splenocytes through six stimulation cycles *in vitro*. By flow cytometry, we determined that 83% of the Ara h 2-specific cells were CD4+ T cells (Figure 1B). The Ara h 2-T cell line proliferated in response to both CPE and Ara h 2 (Figure 1C).

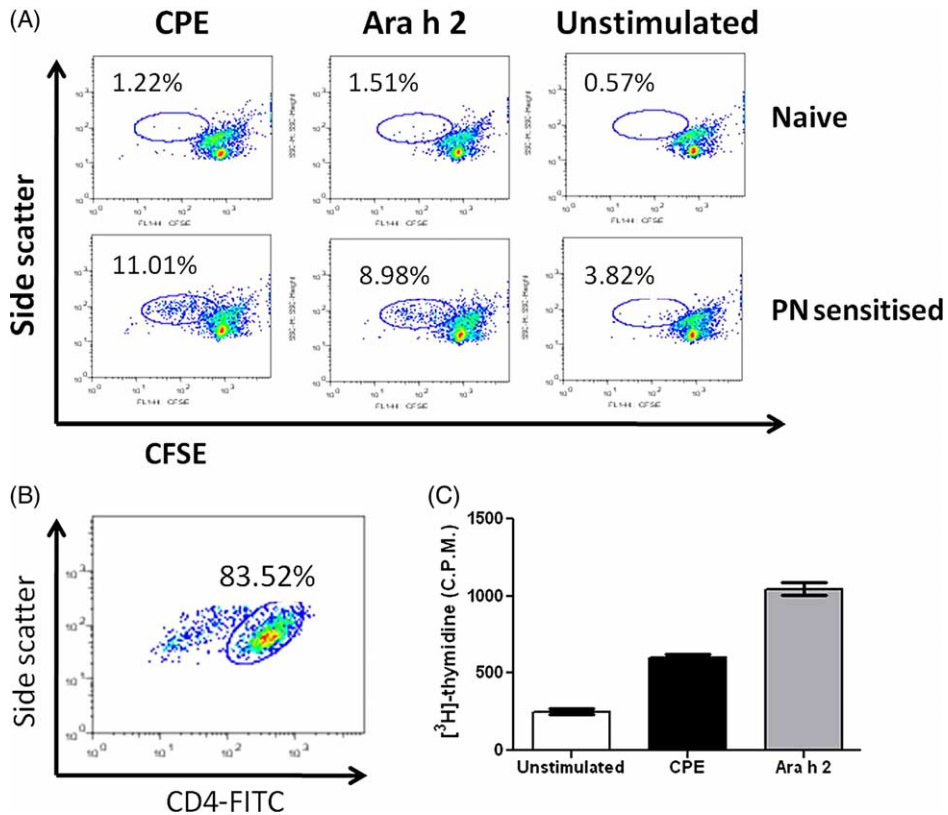


Figure 1. Establishment of an Ara h 2-specific T cell line. (A) Splenocytes from peanut (PN) sensitised and naïve mice were assessed for proliferation response to crude peanut extract (CPE) and Ara h 2 by CFSE dilution assay. An Ara h 2-T cell line was produced *in vitro* from splenocytes and assayed for CD4 as a measure of purity (B) and proliferation response to CPE and Ara h 2 (C).

3.2. Ara h 2-specific T cell lines are Th2-skewed

On stimulation with CPE or Ara h 2, Ara h 2-specific T cells showed a strong Th2 skewing. Intracellular staining for IL-4 and IFN- γ demonstrated that greater than 11% of cells were IL-4+, whereas less than 1% were IFN- γ + (Figure 2A). IL-4 was also detected in large amounts from cultured supernatants, indicating that the Ara h 2-specific T cells can secrete this important Th2-type cytokine (Figure 2B).

3.3. CD154 is expressed on activated Ara h 2-specific Th2-skewed cells

Activated CD4+ T cells express CD154 (CD40L), which can be used to identify antigen-specific CD4+ T cells in a stimulation assay (Chattopadhyay et al., 2005). For Ara h 2-specific T cells, peak expression of CD154 was detected after 12 hours of stimulation with CPE or Ara h 2. The percentage of CD154+ cells was 66% following CPE stimulation and 68% following Ara h 2 stimulation (Figure 3A).

To verify that the CD154+ activated Ara h 2-T cells were Th2-polarised, intracellular cytokine measurements were made. Approximately 50% of the CD154+

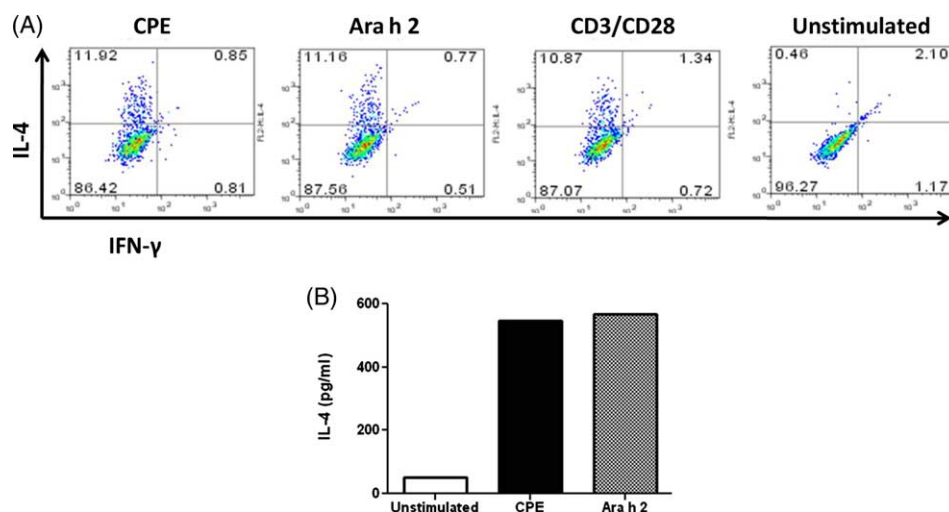


Figure 2. Ara h 2-specific T cells are Th2-polarised. Intracellular IL-4 and IFN- γ were measured (A) and secreted. IL-4 was quantified in culture supernatants via ELISA (B) following stimulation with crude peanut extract (CPE), Ara h 2, CD3/CD28 or unstimulated controls.

cells were positive for IL-4, while only $\sim 8\%$ were positive for IFN- γ (Figure 3B). This result demonstrates that activated Ara h 2-specific T cells can be identified by CD154 expression on the cellular surface, and importantly, cytokine production can be assessed by a simple flow cytometry assay.

3.4. Activation of Ara h 2-specific T cells induces phosphorylation of the Ras-Erk MAP kinase signalling pathway

Th2 cell differentiation is highly dependent on the extent of TCR mediated activation of the Ras-Erk MAP kinase (MAPK) signalling cascade. The change in MAPK phosphorylation was measured by Alexa Fluor 488 conjugated Phospho-ERK1/2 antibody (Figure 4A). After 5 minutes of incubation with CPE or Ara h 2, Erk1/2 phosphorylation was noticeably increased in Ara h 2-T cells. Stimulation with a non-related egg allergen, ovomucoid (OVM) induced no changes in phosphorylation.

Since tyrosine phosphorylation of ZAP-70 occurs upstream of the Ras-Erk pathway, we examined whether the Ras-Erk cascade was activated because of ZAP-70 phosphorylation following peanut allergen stimulation. The results demonstrated that tyrosine phosphorylation of Zap-70 was induced by CPE or Ara h 2 at 5 minutes, which then gradually declined with time (Figure 4B). The T cells stimulated with OVM were refractory to phosphorylation of ZAP-70.

4. Discussion

Peanut allergy is a growing public health concern because of the increase in prevalence, the unlikely chances of spontaneous resolution and the life-threatening allergic reactions provoked by accidental ingestion (Burks, 2008). Currently there is

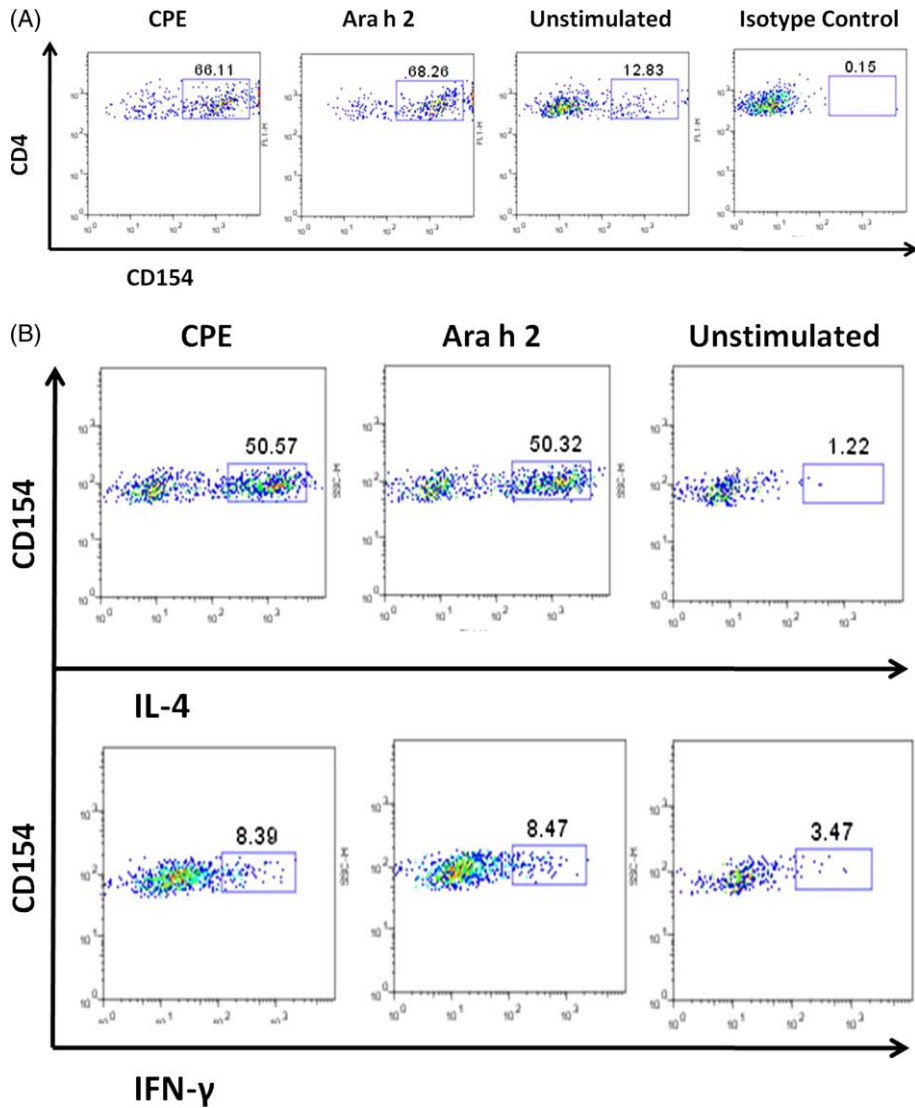


Figure 3. CD154 expression is a useful marker of Ara h 2-specific T cell activation. (A) The Ara h 2-specific T cell line was stimulated with crude peanut extract (CPE) and Ara h 2 to determine percentage of cells expressing the activation marker, CD154. (B) CD154⁺ T cells were assessed for intracellular IL-4 and IFN- γ production following stimulation with CPE or Ara h 2.

no proactive treatment for peanut allergy, although investigations into oral immunotherapy have shown promising results in clinical trials (Jones et al., 2009). To better define and understand peanut allergy, and food allergy in general, tools need to be developed to assess allergen-specific T cell responses. Once developed, these tools will direct experiments to better define the abnormal T cell responses seen in peanut allergic subjects, while allowing investigators to follow alterations in T cell responses following immunotherapy.

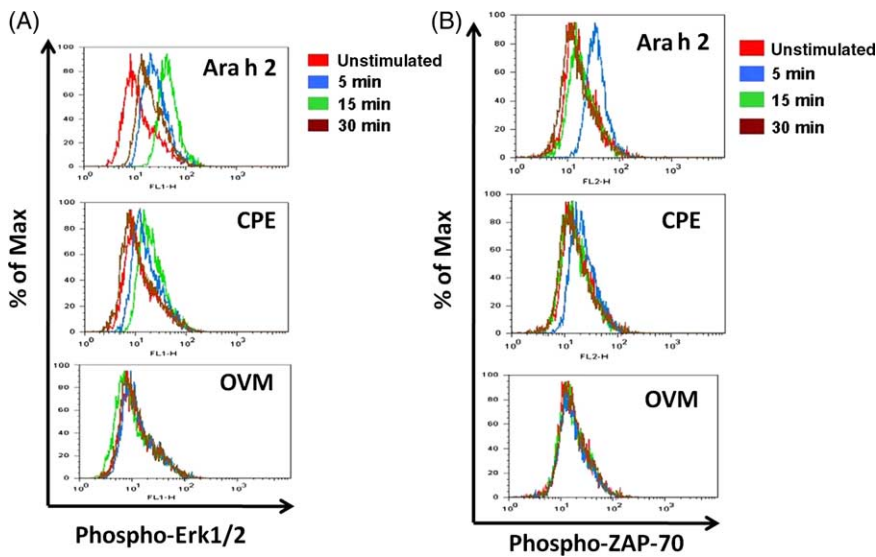


Figure 4. Cellular signalling studies in Ara h 2-specific T cells following activation. (A) Phosphorylation of Erk1/2 was determined in response to crude peanut extract (CPE), Ara h 2 and an unrelated egg allergen ovomucoid (OVM) at several time points. (B) ZAP-70 activation was determined for CPE, Ara h 2 and OVM stimulation following the same time course.

In this report, we have developed a method to identify Ara h 2-specific T cells. Through the use of an Ara h 2-T cell line, we demonstrated that activated, but not resting, CD4+ T cells express CD154 on their cell surface. This marker has been used previously to identify birch pollen allergen-specific T cells in humans (Nakstad et al., 1999). We believe that this marker will be useful for future investigations of murine T cells in our animal model of peanut allergy, and has obvious potential as a way to develop assays to study Ara h 2-specific T cell responses in our clinical trials related to peanut allergy (Jones et al., 2009). Importantly, this method is a simple flow cytometry-based assay, which should be applicable to unfractionated cells (i.e. whole mouse splenocytes or human PBMCs) and can be used in any lab studying food allergy. Also of note, the staining for CD154 is compatible with intracellular cytokine staining (see Figure 3B), which can be used to characterise these allergen-specific T cells.

Our initial attempts to quantify cell signalling events in Ara h 2-specific T cells by Western blotting failed. No differences in the phosphorylated Erk1/2 signals were detected when T cells were simulated with CPE or Ara h 2. This is likely due to the presence of APC, since APC outnumbered T cells 4:1, causing a very high background signal, giving poor signal to noise readings. To overcome this difficulty, we developed a flow cytometry-based approach to study the signalling events in the T cell line (Chow, Patel, & Hedley, 2001). Intracellular staining with CD4 and phosphorylation-specific antibodies was used to detect single cell phosphorylation patterns.

The findings from the cell signalling study were encouraging, as the results demonstrated signalling cascade events expected for Th2-type cells. ZAP-70 became activated after a short, 5-minute, stimulation with Ara h 2. A profound increase in

Erk1/2 phosphorylation was demonstrated after 15 minutes of stimulation. Activation of Erk1/2 is known to result in transcription of *c-Fos* and *JunB*, whose protein products heterodimerise to form the AP-1 complex that leads to transcription of Th2 cytokines (Zheng & Flavell, 1997). Additionally, T cell antigen receptor-mediated activation of the Ras/MAPK pathway controls IL-4 receptor function and Th2 cell differentiation (Yamashita et al., 1999). Our results indicate that Erk1/2 signalling is up-regulated in Ara h 2-specific T cells upon activation, which helps explain the Th2-polarisation in the Ara h 2-specific T cell line.

Since peanut allergen Ara h 2 can activate the Erk1/2 MAPK pathway, which is responsible for Th2 cytokines induction, it is conceivable that the down-regulation of activation in the Erk1/2 MAPK pathway could result in decreased allergy responses to peanut. Future studies in this area will be directed at following Ara h 2-specific T cell signalling events, via CD154 expression, following immunotherapy in mouse models and ultimately on clinical samples with human subjects undergoing peanut immunotherapy.

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