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Original Paper

Peanut Allergens Alter Intestinal Barrier Permeability and Tight Junction Localisation in Caco-2 Cell Cultures[1]

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Key Words
Allergen • Peanut • Caco-2 • Tight junction • Epithelia • Permeability • Transport

Abstract
Background/Aims: Allergen absorption by epithelia may play an important role in downstream immune responses. Transport mechanisms that can bypass Peyer's patches include transcellular and paracellular transport. The capacity of an allergen to cross via these means can modulate downstream processing of the allergen by the immune system. The aim of this study was to investigate allergen-epithelial interactions of peanut allergens with the human intestinal epithelium. ***Methods:*** We achieved this using the human Caco-2 cell culture model, exposed to crude peanut extract. Western and immunofluorescence analysis were used to identify the cellular and molecular changes of peanut extract on the intestinal epithelium. ***Results:*** Following exposure of Caco-2 cells to peanut extract, binding of the peanut allergens Ara h 1 and Ara h 2 to the apical cellular membrane and transcytosis across the monolayers were observed. Additionally, the co-localisation of the transmembrane tight junction proteins occludin, JAM-A and claudin-1, with the intracellular adhesion protein ZO-1 was modified. ***Conclusion:*** Disruption of Caco-2 barrier integrity through tight junction disruption may enable movement of peanut proteins across the intestinal epithelium. This accounts for peanut's increased allergenicity, compared to other food allergens, and provides an explanation for the potency of peanut allergens in immune response elicitation.

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Introduction

Peanut proteins have several features that contribute to their allergenicity. These include, thermo-stability [1, 2], protease resistance to digestive enzymes [3] and a stable structure with many IgE binding epitopes [4-7]. These features are not unique to peanuts alone, and many other allergens typically share these traits. In fact, allergens are often defined by these characteristics. So, if most allergens share these characteristics, then why compared to other food allergies, does peanut allergy often result in anaphylaxis and is also infrequently outgrown? To explore this question, we commenced from the very point of allergen contact with the intestinal epithelium. Normally, luminal contents, including macromolecules are sampled by specialised differentiated epithelial cells, termed M cells within Peyer's patches. Here, luminal antigens are presented to the underlying immune cells, specifically antigen presenting cells (APC), which will begin the process of antigen or non-antigen recognition through downstream B and T cell activation.

The notion that allergen sensitisation potentially begins prior to APC recognition, for example at the site of absorption, was attributed to a comparative study, including allergic and non-allergic siblings. Here, the HLA class II complex was observed to be identical in the allergic and non-allergic pairs of siblings [8]. This may indicate that the allergen sensitisation process begins prior to APC presentation. Considering this, the epimmunome [9], a new concept introduced to categorise the immune stimulatory molecules produced by epithelial cells in response to an external stimulus, has been investigated in response to stimulation by the major peanut allergen Ara h 2. Interestingly, Caco-2 cells incurred an increased stress response to 100ug/mL pure native Ara h 2, which included a stressed phenotype when compared to equivalent levels of lipopolysaccharide. However, heated Ara h 2 also induced an immune modulating phenotype capable of influencing the sensitising capability of Ara h 2 [10]. Despite these findings, peanut protein was observed to cross the intestinal epithelium of BALB/c mice only in M cells at intestinal Peyer's patches, and was not observed in the paracellular spaces of normal intestinal epithelium [11]. These findings may simply highlight the different reactions to peanut allergens among different species or it may reflect the form in which the allergens were presented, where Caco-2 cells were treated with purified Ara h 2 and mice were treated with peanut extract. This may indicate that intestinal epithelial cell (IEC) allergen transport is influenced by other mediators present within the natural food matrix of the allergen [12-14]. Therefore, Ara h 2, similarly with the remaining allergens present within the peanut, may interact with the intestinal epithelium differently when combined with their food matrix or in unison, thus allowing M cell-independent IEC transport across the intestinal epithelium. There is insufficient data to establish whether peanut allergens cross the human intestinal epithelium, from where they may influence downstream immune reactions, as through direct access to the immune cell rich lamina propria. Other allergens, including Ber e 1 and Ses i 1, from Brazil nut and sesame, respectively [15], as well as the wheat allergen ω5-gliadin [16], have been observed to cross the intestinal epithelium in the absence of M cells. Thus, it seems likely that peanut allergens have the capacity to cross the intestinal epithelium.

Also of interest is the likelihood that Ara h 2, or the remaining panel of peanut allergens, may act as an adjuvant for the elicitation of peanut allergy. For example, cholera toxin is a common immune adjuvant administered to elicit sensitisation for a substance in experimental procedures [17-19]. Similarly, the house dust mite allergen Der p 1, which causes a reduction in intestinal barrier integrity through tight junction (TJ) disruption [20], is likely to exacerbate sensitisation to house dust mite through enhanced allergen delivery to underlying immune cells. Therefore, peanut allergen absorption and interaction with the intestinal epithelium may play a key role in sensitisation process of peanut allergy. Additionally, this may account for the increased allergenicity of peanuts compared to other allergenic foods. The present study utilised the Caco-2 cell culture model of intestinal epithelial transport, as a means to identify the transport of peanut allergens across the human intestinal epithelium and the effect of peanut extract on epithelial barrier integrity to identify any possible adjuvant properties of peanut.

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Materials and Methods

Antibodies and other reagents

Primary antibodies: Mouse monoclonal anti-Zonula occludin 1(ZO-1) (Cat. No. 339100), rabbit polyclonal anti-occludin (Cat. No. 71-1500), rabbit polyclonal anti- junctional adhesion molecule A (JAM-A) (Cat. No. 36-1700) and rabbit polyclonal anti-claudin 1, (Cat. No. 51-9000) were purchased from Invitrogen. Rabbit polyclonal anti-Ara-h 1 and rabbit polyclonal anti-Ara h 2, were gifts from Burks and colleagues at the University of North Carolina. Mouse monoclonal Anti-β-actin (Cat. No. A5441) was purchased from Sigma Aldrich.

Secondary antibodies: Goat anti-mouse Alexa Fluor 647 (Cat. No. A21237), goat anti-rabbit Alexa Fluor 488 (Cat. No. A11070), goat anti-rabbit IgG (HRP) (Cat. No. 65-6120) and goat anti-mouse IgG (HRP) (Cat. No. M30107) were purchased from Invitrogen.

Cell culture

The human intestinal cell line Caco-2 (ATCC, Cat. No. HTB-37) was maintained 1:8 by serial passage in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4,500 mg/L-1)(HiClone/Thermo Scientific, Cat No. SH30081.01), supplemented with 1X L-glutamine, 10% foetal bovine serum (FBS), 1% non essential amino acids, penicillin (10,000U mL-1) and streptomycin (10,000 U mL-1). Culture conditions were maintained at 37°C, 95% humidity and 5% CO_2. Only Caco-2 cell passages of 20-50 were used for this study, and cell viability was always more than 95%, as assessed with trypan blue exclusion.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was used to assess differentiation, according to a previously described protocol [21]. Briefly, Caco-2 cells were seeded into 6-well plates and grown for 25 days after seeding. On days 7, 14, 21 and 25, cells were lysed in ALP lysis buffer (10mM Tris-HCl, 0.5mM $MgCl_2$, 0.1% Triton-X 100, pH 7.5) on ice. The lysate was microcentrifuged at 13,000 rpm and the pellet discarded. The supernatant was passed through a fine gauge needle and stored at -80°C until required.

Total protein (60ug) was added in triplicate to the wells of a 96-well plate, before being topped up to 100uL with ALP lysis buffer. Shrimp ALP (1U/uL , Roche, Cat. No. 04898133001) was used as a standard. A total of 10uL of each standard was added to the wells before being topped up to 100uL with ALP lysis buffer. 100uL of substrate solution (*p*-Nitrophenyl Phosphate, Sigma, Cat. No. N7653) was added to all sample and standard wells and allowed to incubate for 20 minutes. The absorbance was read at 405 nm using a POLARstar Omega plate reader (BMG LABTECH, Offenburg, Germany), and the amount of ALP in lysates was calculated as ALP activity (U)/ug total cellular protein.

Peanut extract (PE) preparation

Commercially available roasted peanuts (25g) were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was dissolved in 60mL ddH_2O and incubated overnight at 4°C. The peanut suspension was centrifuged at 5000 x g. The pellet was discarded and the supernatant microcentrifuged at 13,000 rpm for 2 X 10 min. The extract was lyophilised and resuspended in 5mL of ddH2O. The solution was dialysed against dH_2O for 24 hours with two changes of dH_2O. The pH was checked and adjusted using pH indicator strips (MERCK, Cat. No. 1.09535.0001) and then filtered through a Millex-GV 0.22um low protein binding syringe filter (Millipore, Cat. No. SLGV033RS). The protein concentration was estimated using a bicinchoninic acid (BCA) protein estimation kit (Pierce/Thermoscientific, Cat. No. 23227). The ionic concentration (ppm) was compared to isotonic Hank's balanced salt solution (HBSS) (6720ppm) and saline (9000ppm) to ensure comparable hydrostatic pressure (ppm), and to prevent cell stress as a result of fluctuations in hydrostatic pressure. The PE was stored at -20°C until required.

Determining treatment concentrations

To determine optimal treatment concentrations of PE for experimental use, confluent Caco-2 cultures maintained for 21 days were treated with 1, 3, 5 and 10mg/mL peanut extract for 3 hours. The cells were trypsinised and cell viability determined with trypan blue. A lower treatment concentration of 1mg/mL and an upper of 3mg/mL were chosen for use in subsequent experiments. Both of these treatment concentrations did not reduce cellular viability below 95%.

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Transport experiments

The capacity of peanut proteins to cross the Caco-2 cell culture model of the intestinal epithelium was investigated. All transport experiments were conducted according to Hubatsch and colleagues [22] with some modifications. Briefly, 80-90% confluent Caco-2 cells were trypsinised in 0.025% Trypsin with 0.2% EDTA in PBS without Ca^{2+}/Mg^{2+}. The cell suspension was centrifuged at 500 x g for 5 min to pellet the cells. The cells were resuspended in 5mL fresh media and counted using the Countess automated cell counter (Invitrogen, Cat. No. C10227). The cells were seeded at 2 X 10^4 cells/cm^2 onto 24mm cell culture inserts with PET membranes (Millicell/Millipore, Cat. No. PIHT30R48). Only cells with viability of more than 95% were used for all experiments. Media was replaced after 5 hours, and then every other day for a total of 21-22 days ensuring that media was always replaced the day prior to permeability experiments. The transepithelial resistance (TEER) was constantly monitored throughout the experiments using an EVOM epithelial resistance meter (World Precision Instruments, Cat. No. EVOM), fitted with an EndOhm tissue resistance chamber (World Precision Instruments, Cat. No. ENDOHM-24SNAP). On day 21-22, media was replaced with pre-warmed HBSS and left to equilibrate for 15min at 37°C. The HBSS was replaced with fresh HBSS and the TEER recorded as previously mentioned and adjusted to obtain $\Omega \bullet cm^2$. TEER minimum values ranged anywhere from 165 to 1000 $\Omega \bullet cm^2$ in the literature [15, 22-24]. Therefore, for this study, a minimum TEER of 500 $\Omega \bullet cm^2$ was chosen, and any monolayers with TEER below this value were discarded.

2mL of either 1mg/mL or 3mg/mL PE was diluted in HBSS and pre-warmed to 37° before being added to the apical chamber, and incubated whilst shaking gently for 3 hours at 37°C. A 1mL aliquot was removed from the basal chamber each hour and replaced with HBSS. Aliquots were stored at -80°C until analysis. After 3 hours, the HBSS and peanut test solutions were stored and the membranes were washed 3 X 5 minutes in HBSS and the TEER was recorded and adjusted as described previously.

Monolayer integrity and viability analyses

Lucifer Yellow exclusion. To determine the integrity of the monolayer after treatment with PE, Lucifer Yellow exclusion was performed accordingly [25], with some modification. 2mL of the impermeable, polar, fluorescent dye Lucifer Yellow (100ug/mL, diluted in HBSS,) was added to the washed monolayers in the apical chamber and incubated at 37° whilst shaking for 1 hour. Fluorescence was recorded using a POLARstar omega (BMG Labtech) fluorescent plate reader with excitation/emission of 430/520, respectively. Percentage exclusion was calculated with the following formula:

$$\% \text{ Lucifer Yellow rejection} = 100\ (1-(RFU_{[basolateral]}/RFU_{[apical]}))$$

Monolayers were deemed intact if leakage was not more than untreated control monolayers.

Lactate dehydrogenase release. To investigate whether TEER decrease was a result of cell death, lactate dehydrogenase (LDH) release was used as a marker for cell toxicity/death. The amount of lactate dehydrogenase released into the apical solution was measured using a Cytotoxitcity Detection Kit (LDH) (Roche, Cat. No. 04744926001) according to manufacturer's instructions with the following modifications: 100uL of apical solution was incubated with 100uL of dye/catalyst for 30min at room temperature in the dark. A stop solution (50uL) was added to stop the reaction. An upper high control was used to assess for total cell toxicity/lysis, which involved the addition of 2mL 20% Triton-X100 in HBSS for 3 hours, to confluent monolayers to induce total cell lysis. All experiments were performed in triplicate. LDH release was measured at absorbance 492nm and a reference wavelength of 650nm. Background absorbance of solvents was subtracted, and LDH release by test monolayers were compared to normal cell death of control monolayers and appropriately proportioned compared to high control.

Trypan blue dye exclusion. To establish that cell death after peanut protein treatment was not the cause of TEER decrease, confluent 21-day-old cultures grown in T25 culture flasks were treated with 1mg/mL and 3mg/mL of PE or media alone for 3 hours. Cells were washed twice with Phosphate buffered saline (PBS) and trypsinised as normal. Cells were counted as previously mentioned and cell viability was recorded.

TEER recovery. To determine the extent of TEER recovery after the removal of PE, confluent 21-day-old Caco-2 monolayers were treated with 1mg/mL or 3mg/mL PE (diluted in standard growth media) in the apical compartment. Initial TEER readings just prior and after the addition of PE were taken, then every 30 min for the next 6 hours, and once at 24 and 48 hours. At 3 hours, the PE was replaced with fresh media. Initial TEER readings were adjusted to 100% and all values expressed as proportions of initial TEER.

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Detection of peanut allergens

Detection of peanut allergens using western blot analysis. To confirm the transport of peanut allergens across the Caco-2 cell cultures, 20uL each of apical chamber (starting solution as positive control), and 40uL of basal chambers (control, 1hr, 2hrs, 3hrs, 24hrs and 48hrs) for both 1mg/mL or 3 mg/mL were separated by electrophoresis on 14% SDS-polyacrylamide (w/v) gels (SDS-PAGE). The proteins were transferred to nitrocellulose using the iBlot dry blotting system for 7.5 mins, using program P3 (Invitrogen). The membrane was blocked to prevent non-specific binding in 5% (w/v) non-fat dry milk in Tris buffered saline (TBS). The membrane was then probed for the major peanut allergens Ara h 1 and Ara h 2, overnight at 4°C, using rabbit polyclonal anti-Ara h 1 and anti-Ara h 2, respectively, both diluted 1:5000 in 1% (w/v) non-fat skim milk powder in TBS. After 3 X 10 minute washes in TBS, the membrane was incubated in anti-rabbit (HRP) at a 1:5000 dilution in TBS. This was then followed with 3 X 10 minute washes in TBS, and the image captured using the Luminescent Image Analyser LAS-3000 (Fujifilm).

Detection of peanut allergens using liquid chromatography mass spectrometry (LC-MS/MS). Peanut allergens and proteins present in the basal transport media were reduced and alkylated with 10mM DTT and 0.5mM iodoacetamide followed by trypsin digestion according to the procedure described by Ang and colleagues [26, 27]. Trypic digestion products were captured, purified and eluted from a C18 Sep Pak cartridge (Waters, Milford, MA, USA) according to the manufacturer's instructions. The trypic digestion products were then analysed by LC-MS/MS. LC-MS/MS was carried out on a LTQ Orbitrap Velos (Thermo Scientific, FL, USA) equipped with a nanospray interface coupled to an Ultimate 3000 RSLC nanosystem (Dionex, CA, USA). Typically 1ug of the peptide mix (~0.5uL) was loaded onto the enrichment (trap) column at an isocratic flow of 3 uL/min of 3% CH_3CN/0.1% formic acid for 4 min before the enrichment column is switched in-line with the analytical column. The eluents used for the liquid chromatography were 0.1% v/v formic acid (solvent A) and 100% CH_3CN/0.1% v/v TFA (solvent B). The flow rate was 0.3 uL/min and the following gradient was used: 3 – 5% B for 1 min, 5 – 25% B in 40 min, 25 – 80% B in 10 min and maintained at 80% B for the final 5 min. The column was then equilibrated with 3% B for 10 min prior to the next analysis. The LTQ-Orbitrap Velos mass spectrometer was operated in the data dependent mode with nano ESI spray voltage of +1.6 kV, capillary temperature of 250°C and S-lens RF value of 60%. All spectra were acquired in positive mode with full scan MS spectra scanning from m/z 150 – 2000 in the FT mode at 60,000 resolution after accumulating to a target value of 1.00×10^6 with maximum accumulation of 500 ms. Lock mass of 445.120024 from ambient air was applied. The 20 most intense peptide ions with charge states ≥ 2 were isolated at a target value of 5000 and fragmented by low energy CID with normalized collision energy of 30, activation Q of 0.25 and activation time of 10 ms. Dynamic exclusion settings of 2 repeat counts over 30 s and exclusion duration of 70 s. At all times, monoisotopic precursor selection was enabled. All LC-MS/MS data was then searched using the Mascot v 2.2.04 (Matrix Science, London, UK) using the NCBInr database with Viridiplantae (i.e. Green plants) as the taxonomy. The allowed variable modifications were oxidation of methionine and static modifications were caramidomethyl; missed cleavages allowed, 2; and peptide and MS/MS tolerances, 10ppm and 0.5Da, respectively, with a peptide charge 2+.

Detection of Ara h 1 and Ara h 2 by confocal immunofluorescence microscopy. Monolayers were stained for the peanut allergens Ara h 1 and Ara h 2. Briefly, 21-day-old monolayers were treated for 3, 24 or 48 hours with 1mg/mL or 3mg/mL PE prior to 2 X 10 min washes in PBS. Monolayers were then fixed for 30 minutes in 3% paraformaldehyde followed by 2 X 5 min washes in PBS. This was followed by an incubation in 3% triton-X100 in PBS for 5 minutes. The membrane was washed in PBS, and the monolayers were blocked for non-specific binding in 3% BSA in PBS for 1 hour at room temperature. The Caco-2 monolayers were subjected to an overnight incubation in either rabbit anti-Ara h 1 (1:5000) or rabbit anti-Ara h 2 (1:5000) and Mouse anti-ZO-1 (1:2000), diluted in 1%BSA in PBS, at 4°C. The monolayers were then washed 3 X 10min in PBS with 0.1% Tween-20 (PBST). The membranes were then subjected to a 1 hour incubation in goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 647, both diluted 1:1000 in 1% BSA in PBS. The membranes were mounted with anti-fade reagent (Invitrogen) after 3 additional washes in PBST, and viewed using a LEICA confocal microscope (LEICADMIRE2) and analysed using the Leica confocal imaging software (version 2).

Tight junction analysis

Western blot analysis. Caco-2 cells, treated with 1mg/mL or 3mg/mL PE for 3 hours or 1mg/mL PE for 24 or 48 hours, or media alone, were scraped from the membrane and placed into a sterile eppendorf tube

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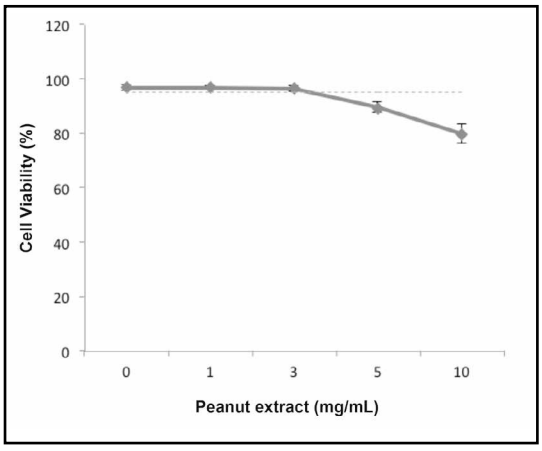

Fig. 1. Effect of peanut protein on Caco-2 cell viability. Caco-2 monolayers, maintained for 21 days were treated with 1, 3, 5 or 10mg/mL of PE for 3 hours. Following the treatment, cell viability was assessed after trypsinisation using trypan blue. After 1mg/mL peanut protein treatment, cell viability was still more than 95% (96.8%). Increasing the treatment to 3mg/mL peanut protein only marginally decreased viability (96.5%), but cell viability was still maintained above 95%. Only after an increase in treatment to 5mg/mL peanut protein, did cell viability fall below 95% (89.3%) and significantly fall after treatment with 10mg/mL peanut protein (79.7%). Dotted line represents 95% cellular viability.

with 500uL cell lysis buffer (50mM Tris-HCl, 10mM EDTA, 1% SDS, 1 X Protease inhibitor cocktail (Roche), pH 7.4) and endured a freeze thaw cycle. The extract was then centrifuged at 13,000 x g. The extract endured 2 X 20 second rounds of sonication on ice using a Microson Ultrasonic cell disrupter (Misonix Inc, NY, USA). The protein concentration was estimated, as previously mentioned, and equal volumes of protein for each treatment were loaded with 5X SDS-PAGE gel loading buffer. Proteins were separated electrophoretically using 8 or 12% w/v polyacrylamide gels, where appropriate. Proteins were then transferred to nitrocellulose as previously described. The membrane was probed with primary antibodies towards occludin, JAM-A or claudin-1 (all 1:5000) and ZO-1 (1:200) followed by secondary (HRP) antibody described previously. Densitometry was performed and adjusted for internal equal protein loading against β-actin (1:5000) and compared to control using a Student's *t*-test.

Immunofluorescence. Caco-2 monolayers maintained for 21 days, and treated with 1mg/mL PE for 3, 24 or 48 hours, were subjected to an overnight incubation in primary antibodies towards occludin (1:3000), claudin-1 (1:5000) or JAM-A (1:4000) and ZO-1 (1:2000), as described previously. Monolayers were then probed using the secondary antibodies goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 647 (both diluted 1:1000) and images captured as described above. Co-localisation analysis was performed using Image J (NIH, public domain) using the "co-localisation threshold" plug-in (Authors: Tony Collins and Wayne Rasband) on 16-bit grey scale scans from each channel. The Mander's co-localisation coefficient after threshold tM1 (RED: ZO-1) and tM2 (GREEN: Occludin, Claudin-1 or JAM-A) was used.

Results

Peanut allergen epithelial transport

Cell viability experiments identified the optimal PE treatment to be in the range of 1-3mg/mL (Fig. 1). An upper range of 3mg/mL was the maximum treatment to maintain cellular viability. The ionic concentration of the lower 1mg/mL peanut solution was 6976ppm while the 3mg/mL peanut solution was 7104ppm, both of these being within the ionic concentrations of HBSS (6720ppm) and saline (9000ppm).

After treatment of Caco-2 cells with 1mg/mL PE, Ara h 1 was faintly detected at 65kDa (Fig. 2A), and levels slightly decreased over the next 2 hours, but steadily increased up until 48 hours. When treatment was increased to 3mg/mL PE, Ara h 1 was strongly detected at 1 hr and continued to cross the Caco-2 monolayer and accumulate up until 24 hours, at which point Ara h 1 levels decreased until 48 hours. Ara h 1 was not detected in control chambers.

Ara h 2 demonstrated slightly different transport capabilities (Fig. 2B). After treatment with 1mg/mL peanut protein, levels of Ara h 2 were not significantly different from control at 1mg/mL. However, at 3mg/mL the 17kDa doublet was consistently detected throughout the 48 hours of treatment, with levels significantly more than control and substantially increasing at 24 hours before returning to standard levels.

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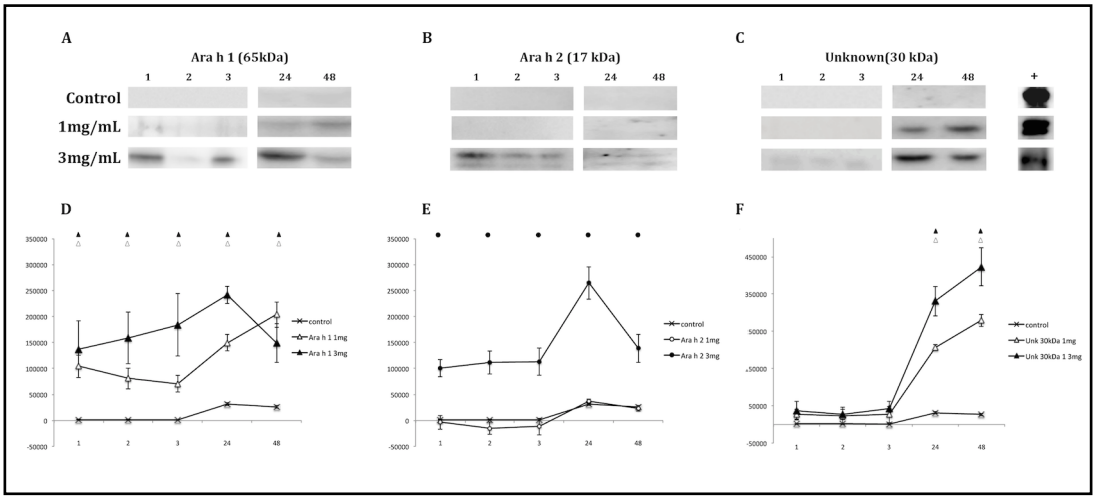

Fig. 2. Detection of peanut allergens after transport across Caco-2 monolayers. Caco-2 monolayers, maintained on permeable filters for 21 days, were treated with either 1 or 3mg/mL peanut protein for 3-48 hours. Aliquot of the apical chamber was taken immediately after addition of peanut treatment to act as a positive control (+) and analysed for Ara h 1, Ara h 2 and unknown 30kDa protein. Aliquots of the basal chamber were taken at 1hr, 2hrs, 3hrs, 24hrs and 48hrs (lanes 1, 2, 3, 24 and 48, respectively), for both peanut treated and control chambers. 40uL of each aliquot was analysed using Western blot analysis (A-C). Transport of the major peanut allergens Ara h 1 (D) and Ara h 2 (E), along with an unknown protein of 30 kDa (F) were quantified with densitometry. Overlaying respective shapes, in the graphs, indicate significance <0.05.

Interestingly, an unknown 30kDa protein was detected with the Ara h 1 antibody (Fig. 2C). This protein was shown to be approximately 30kDa. After the first 3 hours, this protein was only detected for the higher treatment concentration of 3mg/mL but not for the lower 1mg/mL. After 24 and 48 hours of treatment the unknown protein was detected at significantly high levels.

In addition to western blot analysis, we confirmed the presence of Ara h 1, and additionally identified the presence of Ara h 3 and Ara h 6 in the basal transport media by LC-MS/MS analysis. We identified Ara h 1, Ara h 3 and Ara h 6 with the following significant Mascot MOWSE (Molecular Weight Search) scores [27] 815, 722 and 399, respectively in the basal transport media. LC-MS/MS analysis also revealed the presence of other peanut proteins in the basal transport media including: Arachin Ahy-1 (score 1056), Arachin 6 (score 899), Arachin Ahy-4 (score 855), Conarachin (~Ara h 1 (score 764)), Glycinin (score 469) and Conglutin (~Ara h 2 (score 357)).

Peanut allergen epithelial interactions

Both Ara h 1 and Ara h 2 bound to the membrane of Caco-2 monolayers (Fig. 3A and 4A, respectively), as observed by immunofluorescence microscopy. Binding was especially evident at 24 and 48 hours for both Ara h 1 and Ara h 2. However, binding was only slightly detected for Ara h 2 at 3 hours compared to Ara h 1. In addition, apical binding increased at 48 hours for both allergens tested at 3mg/mL, however binding was observed to decrease by 48 hours when treated with 1mg/mL Ara h 1.

Co-localisation of both Ara h 1 and Ara h 2 with the tight junction protein ZO-1 was also evident at both treatment concentrations at both 24 and 48 hours. Ara h 2 exhibited increased colocalisation with ZO-1 compared to Ara h 1.

At 3 hours, both allergens could be detected by immunofluorescence microscopy within the cytoplasm of Caco-2 cells (Fig. 3B and 4B, arrow heads), internalised allergens were not detected at 24 and 48 hours within Caco-2 cell cytoplasm.

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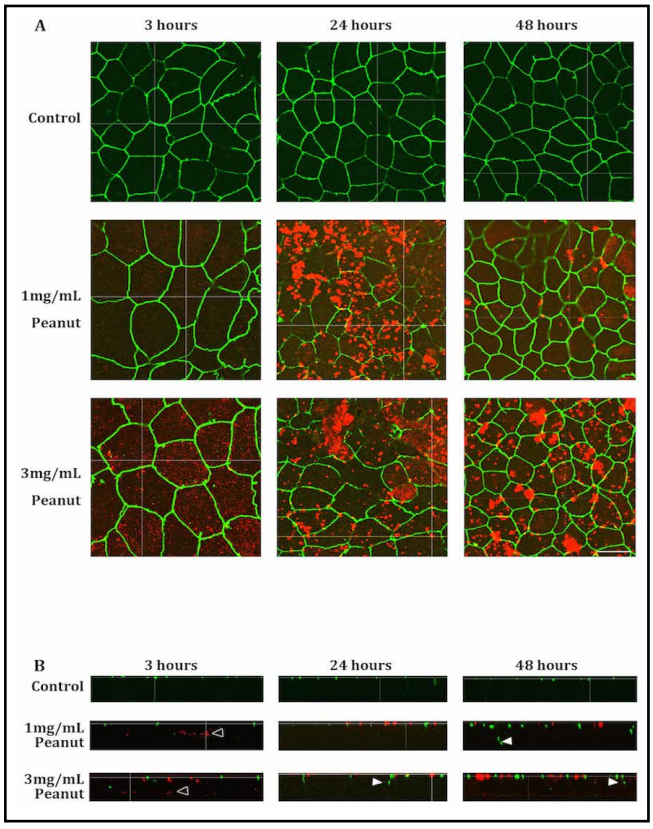

Fig. 3. Analysis of the attachment of Ara h 1 to Caco-2 monolayers by immunofluorescence microscopy. (A) 21-day old Caco-2 monolayers were treated for 3, 24 and 48 hours with either 1 or 3mg/mL peanut extract or media alone (control), and stained for both Zonula occludin-1 (ZO-1) to outline cellular morphology (green), and the major peanut allergen Ara h 1 (red). Empty arrowheads at 3 hours indicate internalised Ara h 1. Solid arrowheads indicate irregular staining of ZO-1. Bar indicates 15um. (B) X-Z sections of representative images shown in A.

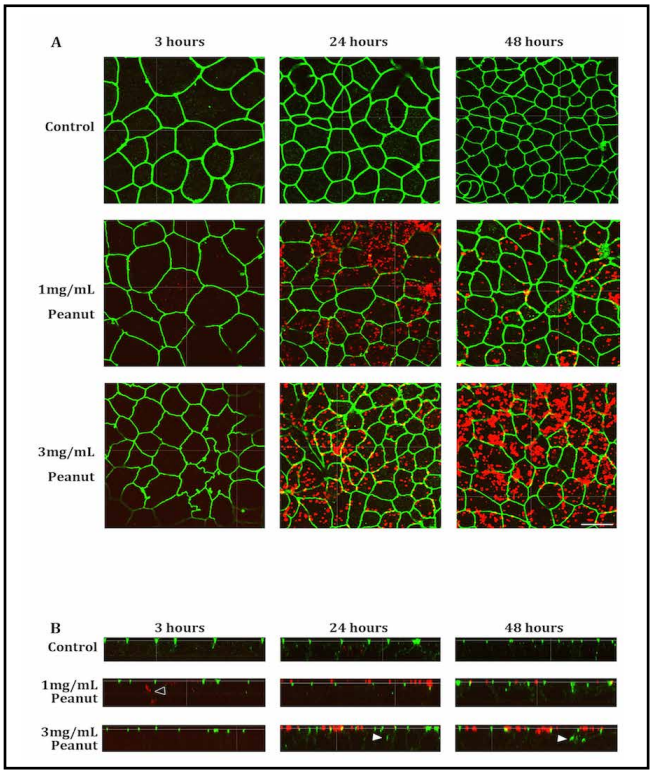

Fig. 4. Analysis of the attachment of Ara h 2 to Caco-2 monolayers by immunofluorescence microscopy. (A) 21-day old Caco-2 monolayers were treated for 3, 24 and 48 hours with either 1 or 3mg/mL peanut extract or media alone (control), and stained for both Zonula occludin-1 (ZO-1) to outline cellular morphology (green), and the major peanut allergen Ara h 2 (red). Empty arrowhead at 3 hours indicates internalised Ara h 2. Solid arrowheads indicate irregular staining of ZO-1. Bar indicates 15um. (B) X-Z sections of representative images shown in A.

Peanut extract effect on intestinal epithelial barrier integrity

Initial transepithelial electrical resistance (TEER) was relatively high. TEER decreased as treatment concentration increased (Fig. 5A). TEER had significantly decreased after

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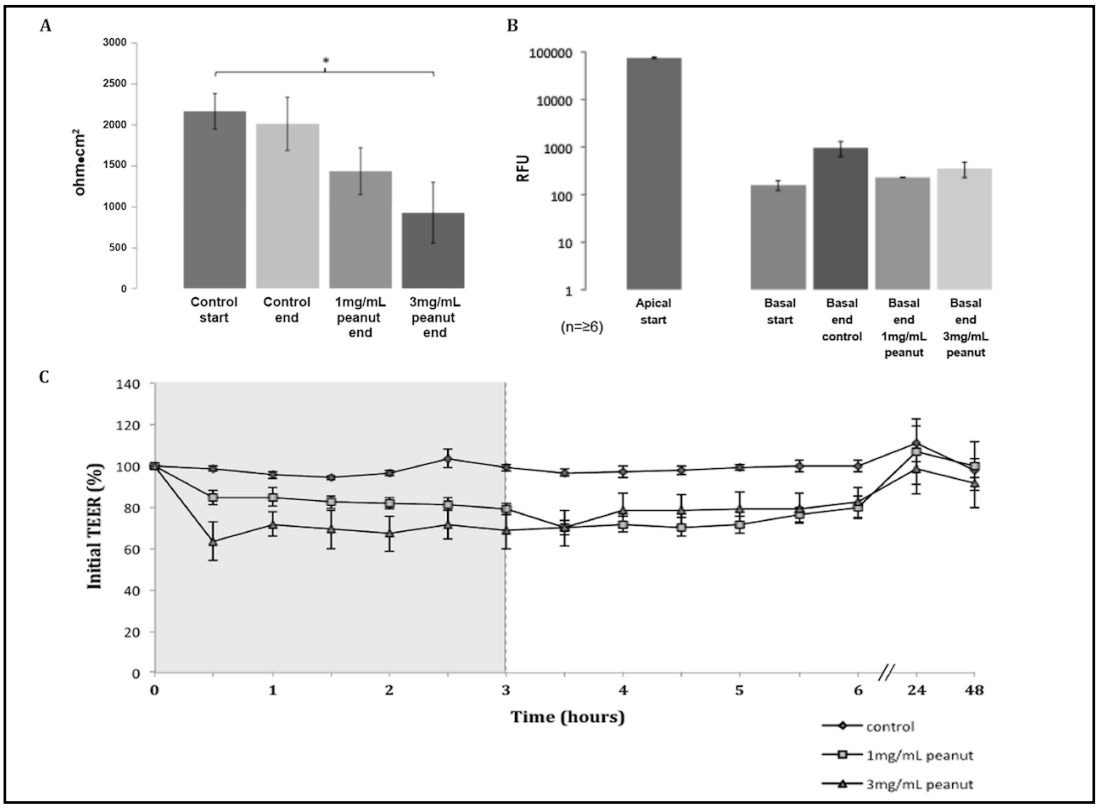

Fig. 5. Effect of peanut protein on Caco-2 barrier integrity. (A) Effect of peanut protein on the transepithelial resistance (TEER) of Caco-2 monolayers. 21-day old Caco-2 monolayers were treated with either 1 or 3mg/mL peanut extract or HBSS for three hours. TEER was recorded immediately before and after treatments. Although not significant, treatment with 1mg/mL peanut extract decreased TEER by ~500 ohm/cm2. A significant decrease in initial TEER was observed with treatment with 3mg/mL peanut extract. There was no significant difference between before and after TEER of control monolayers (n= >6 for each treatment group). (B) Assessment of Caco-2 monolayer integrity using Lucifer Yellow, after treatment with peanut protein for 3 hours. The impermeable fluorescent marker Lucifer Yellow was used to detect for paracellular leakage after treatment of 21-day old Caco-2 monolayers with 1 or 3mg/mL crude peanut extract respectively or HBSS only (control). No significant difference was observed between the paracellular leakage of Lucifer Yellow for all treatment groups and control. The % rejection of Lucifer Yellow was 98.7%, 99.7% and 99.5% for control, 1mg/mL peanut and 3mg/mL peanut, respectively (n= >6 for each treatment group). (C) TEER recovery of Caco-2 monolayers after treatment with peanut protein. 21-day old Caco-2 monolayers were treated for three hours with either 1 or 3mg/mL peanut protein or media only (control). Media was changed after 3 hours treatment and TEER recorded for a further 3 hours. TEER was recorded every 30 minutes and the average of two reading taken (n= 6 for each treatment group). Values shown are proportional to % of TEER at time = 0. Both treatment groups show an initial rapid decrease in TEER, with 3mg/mL peanut demonstrating the biggest drop in TEER. During the 3-hour recovery period, only the 3mg/mL treatment shows a slight TEER recovery. Treatment with 1 and 3mg/mL peanut protein decreases TEER by 20% and 30%, respectively after 3 hours of peanut protein treatment. After a 3-hour recovery period, TEER is still 20% lower than initial starting TEER.

3 hours with 3mg/mL peanut protein, but not with the lower treatment of 1mg/mL, compared to initial TEER. Despite this, the decrease in TEER did not drop below 500 •cm^2, the minimum value chosen to represent compromised epithelial barrier integrity.

Interestingly, TEER did not completely recover after removal of PE and replacement with fresh media until 48 hours after peanut removal (Fig. 5C). TEER after treatment with 3mg/mL PE recovered to a comparable level to TEER after treatment with 1mg/mL PE after

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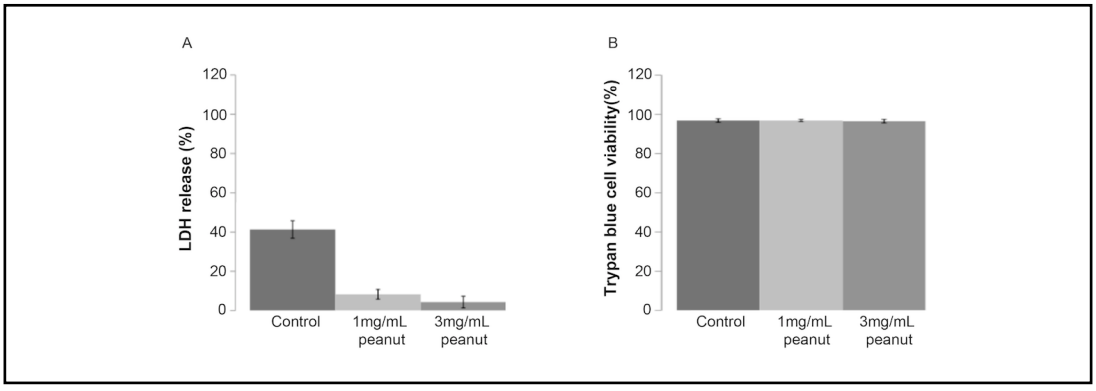

Fig. 6. Effect of peanut protein on Caco-2 cell viability. (A) Lactate dehydrogenase release by Caco-2 monolayers after treatment with peanut protein. 21-day old Caco-2 monolayers were treated for 3 hours with either 1 or 3mg/mL peanut or Hank's balanced salts (control). A high control was performed using 20% Triton-X100 for total cell death/lysis (data not shown). LDH release by peanut-treated monolayers was less than the LDH released by the control monolayers, indicating no increased cell toxicity caused by treatment with peanut protein (n= >6). (B) Trypan blue cell viability analysis of peanut treated Caco-2 monolayers. 21-day old Caco-2 monolayers were treated for 3 hours with either 1 or 3mg/mL peanut protein or media alone (control). Cells were trypsinised and viability analysed using Trypan blue exclusion. There is no significant difference between cell viability of control monolayers and those treated with either 1 or 3mg/mL peanut protein for 3 hours.

3 hours recovery. However, the TEER for both treatments at this stage was still 20% less than control. Despite this, TEER appeared to be fully recovered after 48 hours post PE removal.

Regardless of the decrease in TEER, and slow partial recovery of TEER over 3 hours, epithelial barrier integrity was still maintained throughout the treatment. Exclusion of Lucifer Yellow during treatment was comparable to the Lucifer Yellow excluded by untreated control monolayers (Fig. 5B).

Peanut extract effect on intestinal cell viability

Cell viability was not compromised after treatment with either 1 or 3mg/mL PE. Levels of LDH released as a result of cell lysis/death did not indicate reduced viability compared to levels of LDH released as a result of normal cell death (Fig. 6A). In fact, LDH released by peanut treated cells was less than that released by untreated control monolayers. Uncompromised Caco-2 cell viability was confirmed with trypan blue exclusion (Fig. 6B), as an additional means of testing cellular viability. Here, cellular viability was the same as untreated control Caco-2 monolayers.

Tight junction analysis

Total cellular protein levels of the TJ proteins occludin, ZO-1, JAM-A and Claudin-1 were analysed by western blot analysis (Fig. 7). Over the 3-hour treatment with PE, all cellular TJ levels remained unchanged compared to untreated control Caco-2 monolayers. However, localisation of the same TJ elements by confocal microscopy, illustrates changes to the TJ compared to untreated control. The transmembrane protein occludin (Fig. 8), was observed to strongly co-localise with the intracellular protein ZO-1 for control monolayers, as expected. However, internal staining of occludin at the lateral membrane decreased after 3 hours treatment with PE, compared to control, with a simultaneous decrease in the co-localisation of occludin with ZO-1 at this time. Co-localisation remained decreased at 24 hours, but staining improved at the lateral membrane. Co-localisation returned to normal after 48 hours with lateral membrane staining exceeding that detected for untreated control Caco-2 monolayers.

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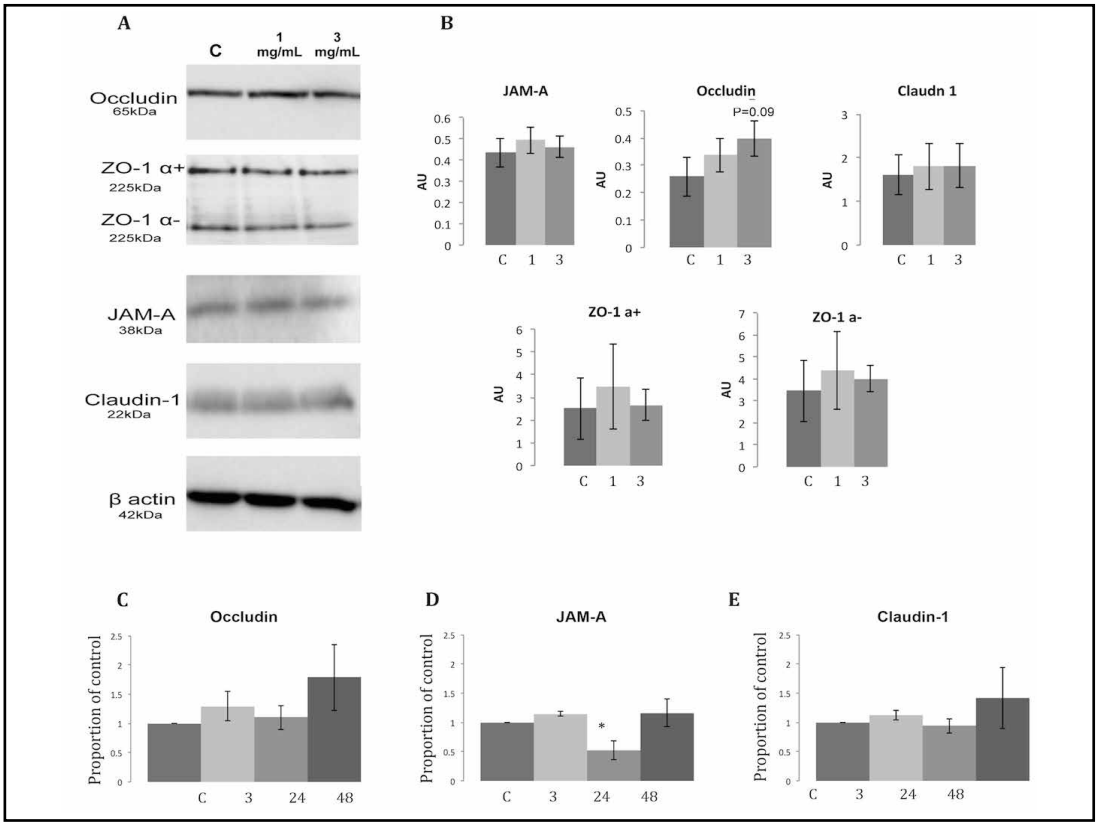

Fig. 7. Effect of peanut protein on tight junction expression of Caco-2 monolayers. The transmembrane tight junction proteins occludin, junctional adhesion molecule A (Jam-A) and claudin-1, as well as both isoforms of the intracellular tight junction protein Zonula occludin 1 (ZO-1), of 21-day old Caco-2 monolayers, treated for 3 hours with 1 or 3 mg/mL peanut protein (A), were analysed using western blot analysis. Densitometry of the western blot analysis of the expression of tight junction proteins, after a 3-hour treatment with peanut protein is shown in (B). There appears to be no significant change to the protein levels of the tight junction proteins occludin, ZO-1, JAM-A and cludin-1 after 3 hours exposure to peanut protein. Despite non-significance, occludin expression after 3 hours does appear to increase. (C-E) represent the protein levels of occludin, JAM-A and claudin-1, respectively at 3, 24 and 48 hours treatment with 1mg/mL peanut extract obtained via western blot and densitometric analyses. Values are adjusted to represent proportion of untreated control.

Another transmembrane TJ protein, JAM-A, demonstrated significant displacement from the TJ compared to control after 3 hours treatment (Fig. 9). By 24 hours, the co-localisation of JAM-A with ZO-1 had improved, but was still significantly different from the co-localisation of control monolayers. Similarly, total cellular protein levels also reflect this finding at 24 hours, with significant decreases observed for the total cellular protein levels of JAM-A. Interestingly, non-co-localised JAM-A present on the entire lateral membrane, as well as on the apical membrane, was completely removed after 3 hours with PE. After 24 hours treatment, JAM-A returns to the lateral membrane, and the same linear conformation is observed. At this stage, an increase in JAM-A is also observed throughout the cytoplasm of the monolayer and in particular at the apical membrane. JAM-A continues to increase in abundance after 48 hours treatment.

Claudin-1, another transmembrane TJ protein and the only type of Claudin analysed in this study, demonstrated co-localisation with ZO-1 at the TJ, as well as strong lateral membrane staining throughout control monolayers (Fig. 10). Co-localisation of Claudin-1 with ZO-1 significantly decreased after 3 hours treatment with peanut protein. Diffusion

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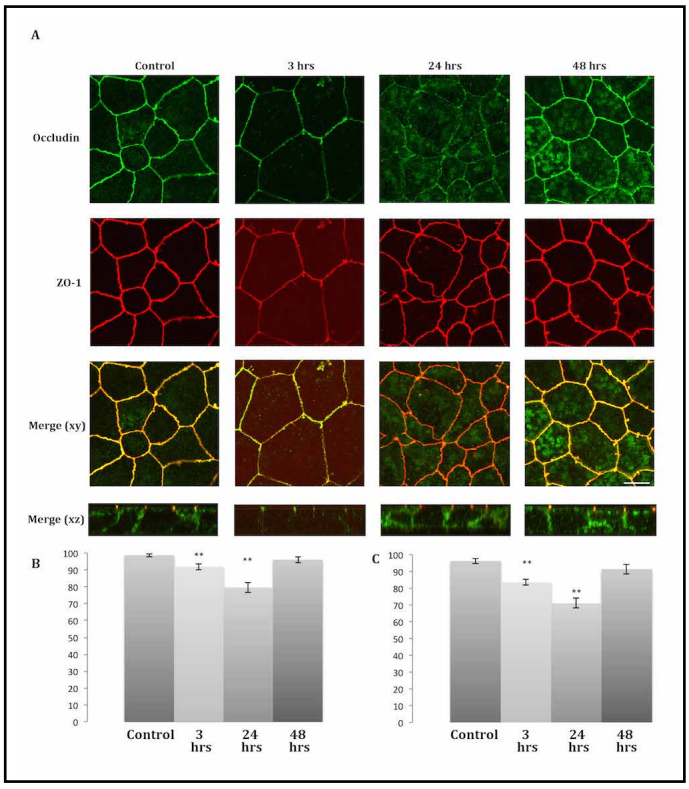

Fig. 8. Identification and localisation of Caco-2 tight junctions by immunofluorescence staining of occludin and ZO-1. (A) 21-day old Caco-2 monolayers were treated for three hours with either 1 or 3mg/mL crude peanut or media (control), and stained with both occludin (green) and Zonula occludin-1 (ZO-1) (red). Co-localisation (overlay) shows both tight junction proteins localised to the cell membrane. Bottom charts indicate the co-localisation analysis of ZO-1 and occludin using Image J. Red channel co-localised with green channel (B), and green channel co-localised with the red channel (C). Bar indicates 7.5um.

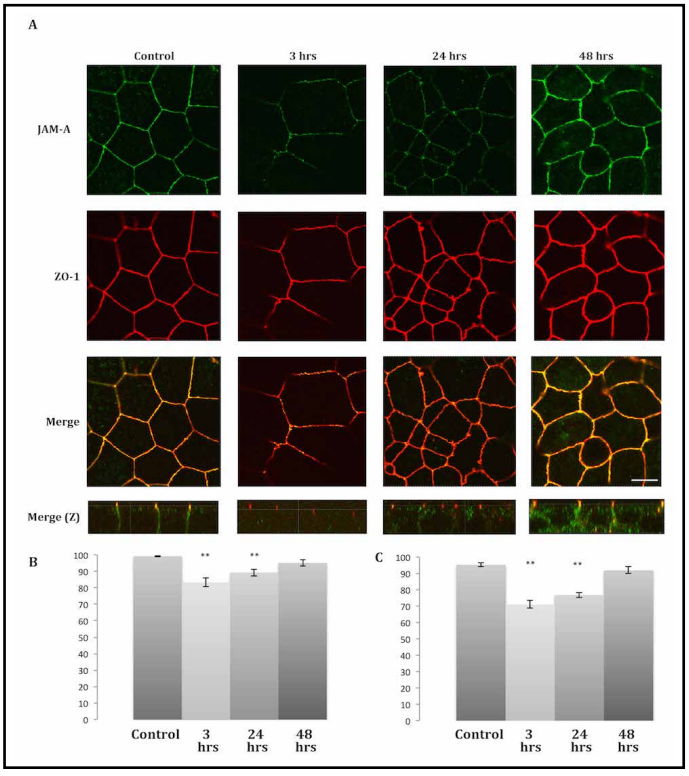

Fig. 9. Identification and localisation of Caco-2 tight junctions by immunofluorescence staining of JAM-A and ZO-1. (A) 21-day old Caco-2 monolayers were treated for three hours with either 1 or 3mg/mL crude peanut or media (control), and stained with both JAM-A (green) and Zonula occludin-1 (ZO-1) (red). Co-localisation (overlay) shows both tight junction proteins localised to the cell membrane in control. Bottom charts indicate the co-localisation analysis of ZO-1 and JAM-A using Image J. Red channel co-localised with green channel (B), and green channel co-localised with the red channel (C). Bar indicates 7.5um.

of Claudin away from the lateral membrane was also observed at 3 hours treatment. Co-localisation improved by 24 hours, and was completely restored to the TJ by 48 hours, with diffuse cytoplasmic deposits within the monolayer, and improved localisation at the lateral membrane.

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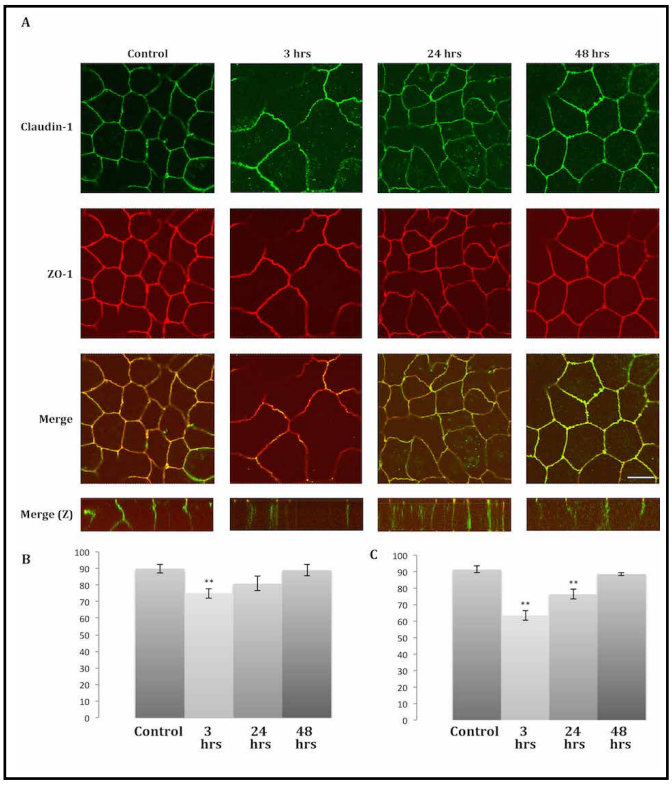

Fig. 10. Identification and localisation of Caco-2 tight junctions by immunofluorescence staining of Claudin-1 and ZO-1. (A) 21-day old Caco-2 monolayers were treated for three hours with either 1 or 3mg/mL crude peanut or media (control), and stained with both claudin-1 (green) and Zonula occludin-1 (ZO-1) (red). Co-localisation (overlay) shows both tight junction proteins localised to the cell membrane. Bottom charts indicate the co-localisation analysis of ZO-1 and claudin-1 using Image J. Red channel co-localised with green channel (B), and green channel co-localised with the red channel (C). Bar indicates 7.5um.

Discussion

Peanut allergy persists as one of the major contributors to anaphylactic deaths caused by food ingestion [28], and with increased incidence within Westernised societies [29-33] and limited treatment options, the development of novel therapeutic intervention is dependant on further improving the understanding of the mechanism of this disease at an intrinsic level.

The absorption of allergens across the intestinal mucosa has not been extensively investigated. The epimmunome, a relatively novel concept applied to all immune stimulating factors produced by epithelium in response to foreign stimuli, has resurfaced the current lack of understanding that the role of the intestinal epithelium may play in the disease process of peanut allergy. Therefore, our present study explored the ability of peanut allergens to transpose the intestinal epithelium and the effect of peanut allergens, within their food matrix, on the barrier integrity and TJ assembly of the intestinal Caco-2 cell culture model.

Determining optimal treatment conditions

A treatment range of 1-3mg/mL of total PE was identified. This range did not affect Caco-2 cellular viability. Exceeding the range to 5mg/mL did however decrease cell viability to approximately 90%. It is likely that, because peanut is a highly proteinaceous material, the protein content exposed to the Caco-2 cells may have altered the osmolarity of the treatment solution. It is unlikely that osmolarity was affected by the upper and lower treatments of PE, as both the treatments were within the isotonic ranges of the neutral HBSS transport medium and isotonic saline.

Intestinal epithelial transport of peanut allergens

This study showed that both the major peanut allergens Ara h 1 (63.5kDa) and Ara h 2 (17kDa) crossed the Caco-2 monolayer, as well as Ara h 3 and Ara h 6. Ara h 1 was observed to have a different capacity to cross the intestinal epithelium compared to Ara

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h 2. This may simply reflect the different transport mechanisms unique to each allergen, which may contribute to the allergenicity of the peanut through multifactorial properties. For example, Ara h 2's transport appears to increase at higher treatment concentrations, which is consistent with simple diffusion. However, Ara h 1 was detected consistently at both the lower and higher treatment concentrations, suggesting that transport may have been facilitated in some manner. Some method of facilitated transport (e.g. endocytosis) would be likely, especially given the larger size of Ara h 1, and its tendency to aggregate into insoluble complexes, especially when heated [1, 10, 34, 35]. It has been previously demonstrated that soluble molecules are more easily absorbed across the intestinal epithelium compared to larger insoluble aggregates, which are limited to M cell passage [36]. Another legume allergen, soybean Gly m 1, has recently been shown to be endocytosed by IPEC-J2 cells *in vitro* [37]. Investigation into a similar means of transport by Ara h 1 requires future investigation. Despite this, it must be taken into consideration that Ara h 1 comprises, on average, 12-16% of the total protein content within peanut [38]. Therefore, the differences observed between the transport capacities of both allergens may simply reflect the total individual allergen content within the peanut. Also, the apparent decrease of Ara h 1 and Ara h 2 present in the basal chamber after 24hrs is most likely the result of continued recycling and/or degradation of the allergens in the basal chamber. Additionally of interest, an unknown protein was detected with the Ara h 1 antibody, this protein was shown to be roughly 30kDa. It is likely that this is a break down product of Ara h 1, although further investigation is required to confirm this. This protein was also observed at higher and longer duration treatments with PE.

Immunofluorescence microscopy revealed internalisation and significant binding of both Ara h 1 and Ara h 2 to the apical Caco-2 membrane that increased with treatment duration. Both Ara h 1 and Ara h 2 were internalised by Caco-2 cells in the first 3 hours of treatment. Previously, peanut allergens were not observed to be internalised by intestinal epithelial cells of mice, unless they were located near Peyer's patches [11]. Also, because Ara h 1 and Ara h 2 can be internalised by IECs, these can then be subjected to IEC processing for potential downstream immune stimulation [39-42]. On the other hand, this may be an important element of increased potency of peanut allergens, whereby binding facilitates allergen transport across the intestinal epithelium, in a manner very similar to an adjuvant. Ara h 1 was observed to bind more strongly in the first 3 hours compared to Ara h 2, which may again be due to the higher levels of Ara h 1 compared to Ara h 2. Binding of food proteins to the intestinal epithelium is not uncommon. Other food proteins such as lectins, including peanut lectin, adhere to the intestinal epithelia [43], and are used to improve drug delivery [44]. Lectins have also been observed to increase the intestinal passage of other food ingredients [45]. The membrane binding of Ara h 1 and Ara h 2 observed in this study may increase allergen transport across the intestinal epithelium. How the attachment of the peanut allergens to the intestinal epithelium may play a role in the sensitisation process of peanut allergy, as well as the mechanism of transport, requires further investigation, which is currently being investigated in our laboratory.

Effect of peanut extract on intestinal epithelial barrier integrity

Epithelial barrier integrity plays a key role in regulating the immune response. Compromised barrier integrity has been linked to several diseases, as reviewed by Liu et al. [46], including allergy, diabetes, asthma, inflammatory bowel disease and celiac disease. To observe for alterations in the barrier integrity of the Caco-2 monolayers treated with PE, we investigated changes in the transepithelial electrical resistance (TEER) and flux of the paracellular marker Lucifer Yellow. Interestingly, TEER decreased as treatment concentration increased. More specifically, TEER had significantly decreased after 3 hours with 3mg/mL PE, but not with the lower treatment of 1mg/mL, compared to initial TEER. However, the drop in TEER did not drop below 500 $\Omega \bullet cm^2$, which was the chosen limit for intact Caco-2 monolayers for this study. Thus, it appears that treatment with peanut at higher concentrations, significantly decreases the TEER of the Caco-2 monolayers, but

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not to the extent that the barrier integrity was completely compromised. Also, TEER was observed to fully recover 24 hours post PE removal. Thus, the extent of disruption to the intestinal epithelial barrier may be limited to changes only in ion flux, as the passage of Lucifer Yellow, a large impermeable tracer molecule with limited capacity to cross the Caco-2 monolayers, was comparable to untreated control monolayers. Also, TEER is an acute measure of epithelial barrier integrity compared to paracellular tracer molecules such as Lucifer Yellow, which have restricted paracellular passage depending on their hydrodynamic radius, polarity and ionic charge. Although epithelial barrier integrity was not completely disrupted by peanut treatment in this study by the lower concentrations of peanut, there was still a significant decrease at higher concentrations to justify further investigation into barrier integrity alteration of intestinal epithelia after exposure to peanut.

TEER decrease is often the result of a compromised monolayer, either as a result of TJ disruption or cell death. Both TJ disruption and cell death were investigated to identify the possible cause of TEER decrease. In this study, cell death was not the cause of TEER decrease, as viability analysis via both LDH release and trypan blue exclusion demonstrated no significant difference between the viability of untreated control monolayers and those treated with PE. In fact, treatment with PE resulted in significantly less LDH release suggesting a suppression of normal cell death. This finding requires further investigation.

In addition to cell death as a result of decreased TEER, Caco-2 TJ were also investigated. TJ are composed of two main units including the transmembrane protein complex that spans the paracellular space. This is comprised of occludin [47], JAM-A[48] and members of the claudin family, which differ with tissue type [49]. All of these interact with the intracellular protein complex that attaches to the structural elements of the cytoskeleton, such as actin. This complex includes ZO-1 [50, 51], ZO-2 [51, 52] and ZO-3 [53], just to name a few. There are numerous other proteins, which have been observed to localise to the TJ and are involved in cytoskeletal adhesion, but were not explored by this study due to time constraints.

To investigate whether altered TJ were the cause of TEER decrease, total cellular protein levels of the TJ proteins were analysed. The TJ proteins investigated in this study included occludin, JAM-A, Claudin-1 and ZO-1. No significant changes to the cellular levels of these proteins was observed after 3 hours of peanut treatment, indicating no protein degradation during this time. However, at 3 hours, the co-localisation of occludin, Claudin-1 and JAM-A with ZO-1 significantly changed after peanut treatment compared to control monolayers. These observed changes are likely responsible for the observed decrease in TEER.

We consider the changes in colocalisation of JAM-A with ZO-1 significant as, to the best of our knowledge, this is the first time that JAM-A has been observed to redistribute after treatment with peanut protein and to increase at the apical membrane in differentiated Caco-2 monolayers after peanut treatment. Relocation of JAM-A to the cell surface has been observed in endothelial cells in response to treatment with inflammatory mediators, where visible redistribution of JAM-A from the TJ was observed, despite no changes to protein levels [54]. Movement of JAM-A to the apical membrane in response to inflammatory mediators has also been observed in brain endothelial cells [55]. It is possible that the inflammatory response produced after exposure of Caco-2 cells to peanut allergens Ara h 1 [56] and Ara h 2 [10] may be the cause of the redistribution of JAM-A within the cell.

Other members of the JAM family have been identified as ligands for the endothelial migration of leukocytes [57]. Specifically, JAM-C and a JAM-like protein (JAML) appear to play a role in polymorphonuclear (PMN) leukocyte-epithelial migration [58, 59]. However, evidence suggests that JAM-A itself may not be involved in PMN transepithelial migration, as observed in the intestinal epithelial cell line T84 with anti-JAM-A monoclonal antibodies [60]. Whether the relocation of JAM-A to the apical membrane observed in this study, has a similar immune-signalling role as with endothelial cells is unknown and requires further investigation. In addition, the homodimerisation of JAM-A has been observed to regulate epithelial proliferation, where a decrease in JAM-A homodimerisation results in an increase in cellular proliferation [61]. It is worth noting here that peanut lectin has the capacity to increase cellular proliferation [62-64]; primarily in individuals who express the Thomsen-

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Friedenreich antigen [63, 64], through a mechanism that involves the activation of MAPK through binding to specific glycosylated variants of CD44v6 [62]. However, it is unlikely that JAM-A displacement is the result of lectin-induced proliferation as Caco-2 has been shown not to demonstrate a proliferative response to peanut lectin, despite surface binding [65], and does not typically express CD44v6 [62]. Therefore, if the redistribution or loss of JAM-A homodimerisation at the TJ results in increased epithelial proliferation, then it is likely that peanut lectin is not the cause but may be the additional constituents contained within the PE treatment. In contrast to this, Ara h 1 has recently been observed to prevent cell proliferation [56], therefore any proliferative response as a result of decreased JAM-A homodimerisation may be ameliorated by the preventative action of Ara h 1. This remains an area for more extensive research to ascertain the role of JAM-A in intestinal epithelial barrier integrity reduction and cellular proliferation after exposure to peanut.

In addition to the alterations observed for JAM-A at the TJ, significant changes in occludin were also observed. Not only was the co-localisation of occludin with ZO-1 diminished at the TJ after 3 hours with peanut protein treatment but there was also a decrease in the lateral membrane staining observed in control monolayers. Interestingly, JAM-A has been observed to regulate the expression of occludin [66]. We cannot therefore exclude the possibility that the observed changes in occludin localisation in the present study may be a direct result of the redistribution of JAM-A.

Although lateral staining was improved after 24 hours treatment, occludin and ZO-1 co-localisation was still significantly less than control. Re-establishment of co-localisation was not observed until 48 hours. Occludin does not appear to be critical in maintaining the barrier integrity of the intestinal epithelium, as observed with occludin knockout mice [67]. Occludin disruption has been observed after the addition of small peptides targeted at the extracellular binding domains of occludin, which results in the decrease of TEER and increase of the paracellular flux of tracer molecules in a *Xenopus* kidney epithelial cell line A6.2 [68]. The peptide sequence used in the same study was matched against all known sequences for *Arachis hypogaea* using BLAST. This resulted in 35 significant alignments, of which, many included non-specific seed storage proteins including Glycinin and Arachin (data not shown). We therefore cannot exclude the possibility that some degree of occludin disruption may be caused by binding affinity of significantly similar protein sequences between *Arachis hypogaea* and the extracellular binding domain of occludin. This area of research requires future investigation.

Also of interest is the size selectivity of occludin to macromolecule paracellular passage, where knockdown of occludin in Caco-2 allowed for increased passage of larger tracer molecules (70kDa dextran) compared to smaller molecules such as mannitol, glucose, urea, 10kDa dextran and inulin, with little effect on TEER [69]. The change in localisation of occludin observed in the present study may help to explain why despite no increased flux of Lucifer Yellow, peanut allergens were able to pass through the Caco-2 monolayer, in spite of the large size of Ara h 1 at 63.5kDa. Thus, it is likely that the delocalisation of occludin from the TJ is not responsible for TEER reduction, but may play a role in the passage of the peanut allergens. Therefore, it is worthwhile exploring the role that occludin may potentially play in the paracellular passage of larger allergens like Ara h 1.

Additionally, claudin-1, another transmembrane TJ protein and the only type of claudin analysed in this study, demonstrated co-localisation with ZO-1 at the TJ, as well as strong lateral membrane staining throughout the monolayer. Peanut protein decreased the co-localisation of claudin-1 with ZO-1 after 3 hours. However, partial and full recovery of the co-localisation was observed by 24 and 48 hours, respectively. Since a similar decrease in localisation was observed for both JAM-A and claudin-1, it is worth investigating common binding motifs involved in their interaction with ZO-1. The PDZ binding motif, present in both JAM-A [70] and claudin-1 [71] interacts with the intracellular TJ plaque protein ZO-1 at its PDZ1 binding domain. It appears that this interaction is not shared by occludin, which also binds ZO-1 [50]. JAM-A and claudin-1 also share another common binding partner MUPP-1 [72], where again the PDZ domain is the likely mechanism of interaction. Investigation

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into the potential interference of the PDZ binding of both claudin-1 and JAM-A may further improve our understanding of the displacement of JAM-A and claudin-1 at the TJ after peanut treatment in Caco-2 cells. Limitations in identifying any potential interference are hindered because of the lack of full genome sequence of *Arachis hypogaea* until just recently.

As presented here, investigation into the mechanism of TJ displacement in intestinal epithelial cells by peanut is of high importance in the understanding of the etiology of peanut allergy. Further investigation into the role peanut plays in modifying epithelial barrier integrity is also beneficial in exploring any unknown adjuvant properties peanut may have. Understanding these mechanisms is of profound importance for not only improving the understanding of both the elicitation and manifestation of the disease, but for the development of new therapeutic targets to combat the condition, both aimed at the prevention and treatment of peanut allergy in the future.

Abbreviations

ALP (alkaline phosphatase); APC (antigen presenting cell); DMEM (Dulbecco's modified Eagle's medium); FBS (foetal bovine serum); IEC (intestinal epithelial cell); JAM (junctional adhesion molecule); JAML (JAM-like protein); LDH (lactate dehydrogenase); TEER (transepithelial electrical resistance); TJ (tight junction); ZO (Zonula-occludin).

Disclosure Statement

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