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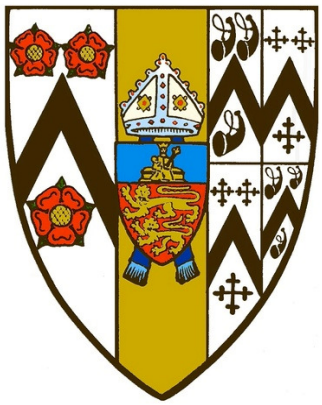

William Rawstron Hillson

The Sir William Dunn School of Pathology

and

Brasenose College

A thesis submitted for the degree of Doctor of Philosophy at the University of Oxford, Michaelmas Term 2013

Abstract

The role of thermal processing and protein oxidation in peanut allergy

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Food allergies are an increasing health problem throughout the developed world. Among these, peanut allergy is particularly significant, due to its exceptional severity and frequent lifelong duration. Much of its aetiology remains unclear. In particular, it remains unknown why, unlike other food allergies, peanut allergy incidence correlates poorly with average dietary peanut consumption. A popular explanation for this discrepancy is that peanut allergy is more common in regions where predominantly dry-roasted (DR) peanuts are consumed, leading to speculation that DR-induced chemical modifications may contribute to pathological T_h2 responses in humans. Yet to date, no research group has demonstrated an enhanced immunogenicity of DR peanuts relative to raw in a murine model of sensitisation.

This thesis begins with the hypothesis that dry-roasting does indeed alter the chemical composition of peanut proteins in such a way as to increase immunogenicity and allergenicity. To test this hypothesis robustly, I have first addressed flaws in previous studies by developing a methodology to thoroughly characterise samples of raw and DR peanut protein, as well as purifying samples of individual peanut allergens. Using these samples, I have demonstrated an enhanced immunogenicity of DR peanut protein relative to raw, in intragastric, subcutaneous and epicutaneous models of mouse sensitisation, and furthermore, that such enhanced responses feature a pronounced T_h2 bias and functional IgE production. I will present evidence that this difference is not caused by either protein aggregation or the presence of other non-protein substances, but is due to an intrinsic property of the DR peanut proteins. I will go on to clarify candidate molecular mechanisms of this effect, examining several putative receptors and probing the effects of roasting on dendritic cell binding and interactions of peanut proteins. I conclude in light of these investigations that the dry-roasting hypothesis remains the most plausible explanation for the epidemiological distribution of peanut allergy, although many additional questions remain regarding the nature of the chemical modifications produced by roasting and the molecular basis of their recognition by the immune system.

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Table of Contents

Abbreviations

Ac-LDL – Acetylated Low Density Lipoprotein

Ag – Antigen

APC (1) – Allophycocyanin

APC (2) – Antigen Presenting Cell

Ara h – *Arachis hypogaea*, binomial designation for common peanut

Balb/c – Bagg Albino strain c

BCA – Biconchonic Acid

BSA – Bovine Serum Albumin

CPH – Crude Peanut Homogenate

C57Bl/6 – C.C. Little 57 Black 6 strain

DAMP – Damage Associated Molecular Pattern

DCs – Dendritic Cells

DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

Dil - 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

dH_2O – De-ionised distilled water

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulfoxide

DNPH – 2.4-dinitrophenyl hydrazine

DR – Dry-roast(ed)

EC - Epicutaneous

ECL – Enhanced Chemiluminescence

EDTA - Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked Immunosorbent Assay

FACS – Fluorescence activated cell sorting

FCS – Foetal Calf Serum

FITC – Fluorescein Isothiocyanate

GA – Glycoaldehyde

GI – Gastrointestinal

GM-CSF – Granulocyte/macrophage colony stimulating factor

HCl – Hydrochloric acid

HEL – Hen Egg Lysozyme

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP – Horseradish Peroxidase

H_2SO_4 – Sulfuric acid

Ig – Immunoglobulin

IG – Intragastric

IL – Interleukin

kDa – kiloDalton

LDL – Low Density Lipoprotein

LOX-1 – Lectin-like Oxidised LDL Receptor

LPS - Lipopolysaccharide

LTA – Lipoteichoic Acid

MALDI – Matrix-assisted laser desorption ionisation

MHC – Major Histocompatibility Complex

MLN – Mesenteric Lymph Nodes

MR – Mannose Receptor

MS – Mass Spectrometry

MWM – Molecular Weight Markers

$NaBH_4$ – Sodium Borohydride

$NaCNBH_4$ – Sodium Cyanoborohydride

$NaCO_3$ – Sodium Carbonate

$NaHCO_3$ – Sodium Bicarbonate

OD – Optical Density

OVA – Ovalbumin

PAGE – Polyacrylamide Gel Electrophoresis

PAMP – Pathogen-Associated Molecular Pattern

PBS – Phosphate-Buffered Saline

PE – Phycoerythrin

PerCP - Peridinin chlorophyll protein

PNAG - p-nitrophenyl N-acetyl-β-D-glucosamide

PPE – Peanut Protein Extract

RAGE – Receptor for Advanced Glycation End Products

RBL – Rat Basophilic Leukaemia

RC – Reactive Carbonyl species

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute (medium)

RT – Room Temperature

SC – Subcutaneous

SD – Standard Deviation

SDS – Sodium Dodecyl Sulfate

SRA – Scavenger Receptor A

TBS – Tween-Buffered Saline

TCA – Trichloroacteic Acid

T_h – T helper cell

T_{reg} – T regulatory cell

TLR – Toll-like Receptor

Tris - Tris(hydroxymethyl)aminomethane

Chapter 1
Introduction

1.1 Scope of Introduction

The purpose of this chapter is to give a general overview of the current state of peanut allergy research, and to place it in its wider context of the global incidence of food allergy. Accordingly, detailed discussions of specific aspects will be reserved for the introductory sections of the appropriate results chapters, where they can be more easily related directly to the relevant experimental data.

1.2 Peanut allergy – a global "epidemic"

1.2.1 Food allergy - a disease of development

It is perhaps ironic that just as the Western world has successfully eliminated many diseases which in the past were leading causes of morbidity and mortality, some diseases seem only to have become more widespread with increasing industrialisation and rising standards of living. One of the most notorious of these exceptions is that of food allergy, a disease whose incidence in the developed world has increased dramatically ever since records began (Sicherer and Sampson 2007). Even taking into account improved diagnosis and the registration of long-term conditions which went previously undetected, such a rapid increase is almost without precedent (Asero, Ballmer-Weber et al. 2007). This increase in the rate of food allergy has had non-trivial social and economic costs – taking into account not only the direct effects on sufferers, but also lost productivity and the necessity of adapting workplaces to avoid exposing allergic employees to substances they cannot tolerate (Miles, Fordham et al. 2005).

Food allergy is a type 1 (IgE-mediated) allergic reaction (Kumar, Verma et al. 2012). Like all allergies, it does not derive from some external assault on the body – rather, it results from the sufferer's own defences against such attacks running out of control. Food allergy consists of the production of deleterious immune responses, in which the patient's body treats a harmless food biomolecule as though it were a dangerous organism, releasing defensive mediators in such quantities that they become toxic (Sicherer and Sampson 2006). Sufferers can experience symptoms ranging from irritation and pain to life-threatening anaphylaxis (Hourihane, Kilburn et al. 1997), sometimes by encountering only

minute quantities of the foodstuff to which they are allergic. Although the existence of food allergy has been documented for nearly 100 years (Saavedra-Delgado 1989), until recently research into possible treatments has remained largely focused on treating the symptoms, primarily with the intent of demonstrating that certain lifestyle modifications are effective in preventing or reducing anaphylactic episodes (de Leon, Rolland et al. 2007). Improvements to this situation have been hampered by an inadequate basic knowledge of the mechanisms of allergic sensitisation, which would otherwise open up potential avenues for therapeutic intervention. Genetic, developmental and environmental factors have all been invoked as explanations for the worldwide rise of food allergy, each with only partial success (Roberts, Patel et al. 2003). And yet whatever else may be said about these hypotheses, even in principle they can only explain changes in the *total* rate of food allergy. They are of little help in explaining the reasons why a few specific foodstuffs seem to be more likely to cause allergy than others.

1.2.2 The food allergy "epidemic"

It has often be remarked that the current incidence of food allergy in the Western world resembles an "epidemic", not because it is contagious, but by the analogy of its dramatic, rapid and seemingly irresistible spread (de Leon, Rolland et al. 2007) (Sicherer and Sampson 2007). Food allergy was first described in a series of anecdotal reports from the 1920s onwards (Saavedra-Delgado 1989; Maleki 2004), but since these initial reports, diagnosis has increased dramatically in the decades following, as testified by longitudinal studies of populations of children in many countries around the world. Today, between 3-4% of Americans are believed to have at least one food allergy (Boye 2012), with figures estimated to be similar or even higher in European nations (Mills, Mackie et al. 2007). Over the past

few decades, evidence of an increase in food allergy has emerged in America (Sicherer, Munoz-Furlong et al. 2010), Europe (Du Toit, Katz et al. 2008) and Australia (Mullins 2007), and there is also evidence of increases in developing nations such as China (Liao, Liao et al. 2009) and South Africa (Zar, Ehrlich et al. 2007), although the accuracy of much information on food allergy prevalence in developing nations remains questionable (Boye 2012). Whilst it is true that at present, food allergy appears to be much more common in the Western world, this does not appear to indicate a genetic linkage. Immigrant communities originating from regions in which food allergy is rare rapidly develop similar food allergy rates to the indigenous population after they begin residing in the West, which would strongly implicate environmental factors in the development of food allergy (Leung, Carlin et al. 1994).

The concept of an ongoing food allergy epidemic is not without its scientific critics. For instance, one recent study found large discrepancies between reported and diagnosed cases of food allergy, and proposed that once these were removed, the difference in allergy rates between their study population and a similar study 20 years previously disappeared (Venter, Pereira et al. 2008). Other scientists have been even more sceptical, questioning whether there is truly a difference in food allergy incidence between the West and the developing world, alleging under-reporting and biased sampling (van de Poel 2009). Whilst such critiques deserve to be answered, they remain at present a minority viewpoint. The majority of researchers maintain that even when such factors are taken into account, a substantial residue of the apparent rise in food allergy remains to be explained (Prescott and Allen 2011).

Accordingly, many researchers have attempted to devise an explanation for the relatively sudden and dramatic rise of food allergy. It has occurred over such a comparatively short period of time that it is implausible to attribute it to the emergence of any novel genetic traits among the affected populations. Rather, it appears to be a pre-existing tendency which has only recently been triggered. Classically, in evolutionary theory such apparently maladaptive traits of organisms are often explained either as imperfect adaptation to a changing environment or alternatively as an unavoidable side-effect of a beneficial trait to which it is tightly linked in some way (Midgley 2002).

Falling into the former category is perhaps the most famous of the suggested explanations for food allergy – the *hygiene hypothesis*. In its original form, it stated that the absence of endemic childhood infections which have been removed from the environment due to improved hygiene has caused the failure of immature human immune systems to prime correctly, causing a hyperactive immune response when harmless food antigens are encountered (Okada, Kuhn et al. 2010). A more recent variant of this hypothesis falls more into the second category of evolutionary explanation, stating that a predisposition to food allergy evolved by natural selection as an unavoidable side-effect of improved immune responses to chronic infection by helminth parasites (Fitzsimmons and Dunne 2009).

Yet although these hypotheses go some way towards explaining the apparent epidemic of food allergy affecting the Western world, they do not explain why allergies to certain foods are much more common than others. Nor do they explain why some food allergens, most notably peanut, produce symptoms of exceptional severity and duration (Scurlock and Burks 2004).

1.2.2 Peanut allergy in context

Peanuts are one of the most important agricultural crops in the world, both for human and animal consumption, as well as the many industrial applications of peanut oil (Woodroof 1996). Their ubiquity in the human diet may well be one contributing factor to the high incidence of peanut allergy (Fox, Sasieni et al. 2009), but even when this is taken into account, peanut allergy still has some puzzling and unique features which set it apart from other food allergies. For one thing, peanut allergy is dramatically more severe than many other allergies to comparable foods – the incidence of life-threatening anaphylaxis is much greater in peanut-allergic patients than among those allergic to soya (Foucard and Malmheden Yman 1999) and red kidney bean (Verma, Kumar et al. 2013), two phylogenetically related plants. Another peculiar feature of peanut allergy is its duration. Many food allergies develop during childhood and are typically resolved by adulthood (Sicherer 2011). Peanut allergy, however, is exceptional in that a substantial number of patients retain the condition lifelong (Bock and Atkins 1989). It has even been claimed that outgrowing peanut allergy is rare, although this has been challenged by more recent epidemiological studies and thus remains at present a subject of some debate (Fleischer, Conover-Walker et al. 2003). Regardless of the outcome of this dispute, peanut allergy is unquestionably an outlier in this regard.

The production of pathological immune responses is a fascinating topic which has exercised some of the best minds in immunology. The fact that a system which evolved specifically to destroy invading pathogens could misfire in such a devastating manner is important not only for the affected patients, but for what it reveals about how the human immune system functions, its structural flaws, and how these could potentially be exploited in therapeutic

interventions. Peanut allergy is thus an ideal model system, not only because it showcases hypersensitivity responses at their most dramatic, but also because of the wealth of research materials which have been generated over the past century by clinicians investigating allergy. A complete explanation of peanut allergy would not only need to explain the generic mechanisms behind sensitisation which the peanut allergens share with countless other foodstuffs, but also the unique features of peanut allergy which so distinguish it from similar conditions.

1.3 Pathogenesis of food allergy

1.2.1 Feeding and the immune system

The many forms of life on earth possess a fabulous variety of immune systems, all of which are united by a common function – to protect the bodies of large, multicellular organisms against attack by smaller and more agile parasitic organisms. These pathogenic species enjoy such a dramatic advantage in both adaptability and rate of reproduction that without such immune systems, no multicellular organism could survive. And yet immune defence is fraught with challenges: owing to their common ancestry, all organisms operate using a very similar biochemistry, frequently sharing proteins and other molecular structures in common. In consequence, immune systems must function based on the recognition of minute and subtle differences between the molecular markers (known as antigens) of their own bodies and those of invaders. This property of any successful immune system is classically described as the "self/non-self" distinction, and is theorised to develop during the early stages of an organism's life (Silverstein and Rose 1997). During this initial phase of acclimation, responses to "self" antigens are systematically downregulated, conferring a protection against the immune system's effects which normally persists lifelong and is referred to as "tolerance", with the various diseases of autoimmunity representing pathological malfunctions of this self-censorship by the immune system (Eberl 2010). The targeting of immune responses against new, specific antigens which the organism later encounters, is referred to as *adaptive immunity*, because it requires the initiation of a novel immune response to a specific antigen distinct from the self-tolerance which has been in place since infancy.

But the “self/non-self” distinction, whilst explanatorily useful in the field of autoimmunity for example, is clearly insufficient by itself as a definition of all immunity, for it immediately raises an enormous practical problem. Organisms also use and lose their own components steadily as they go about their lives, and consequently they must also secure a supply of biomolecules to replenish their own stocks, much of which will be derived from other organisms. And yet the immune system must distinguish those “non-self” proteins which are harmful from those which are essential for their survival. The situation becomes even more vexed if the remains of other organisms which are consumed are themselves contaminated with traces of pathogens.

Therefore, the criterion for initiating an immune response against a foreign biomolecule cannot simply be that it originates from a different organism. It must originate from a *harmful* foreign organism specifically, lest immune responses are triggered indiscriminately against both dangerous interlopers and vital foodstuffs. The solution which mammalian immune systems have hit upon is to interlink adaptive immunity with a much older, non-adaptive parallel system of pathogen recognition (Michallet, Rota et al. 2013). The components of this system are genetically hardwired, and hence are referred to as *innate immunity*. Innate immunity operates primarily by responding to generic molecular markers which are common to wide varieties of pathogens (Pathogen-associated Molecular Patterns or PAMPs), such as chemically distinct viral RNA (Gurtler and Bowie 2013), and lipopolysaccharide (LPS), which is a key component of gram-negative bacterial membranes (Meng and Strober 2010). In essence, using the PAMPs as targeting markers allows the immune system to bypass the constant antigenic shifting of pathogens and respond to invariant cellular components which they are powerless to change, allowing specific

immune responses to “lock on” to broad ranges of related parasites (Blander and Sander 2012).

This model has been heavily criticised by some immunologists, (notably by Polly Matzinger), who instead propose that foreign proteins only invoke immune responses when they are accompanied by signals indicating tissue *damage* (so-called Damage-Associated Molecular Patterns or DAMPs) (Matzinger 2002). The presence of dsDNA, for example, in the extracellular matrix is a clear sign of cell death. Other contributors have taken a more moderate position between the two, granting an important role to both PAMPs and DAMPs in immune activation (Pradeu and Cooper 2012). Still others have attacked the distinction between the two, arguing that such “paradigms” impose artificial boundaries on the highly interrelated immune system components (Vance 2000; Eberl 2010). Whilst the debate over their relative importance is ongoing, for now it should be noted that a large and increasing array of both PAMP and DAMP receptors have been identified in humans (Tang, Kang et al. 2012). Because innate immunity is directly genetically encoded, and does not require experience of antigens to activate, it is invariably the first response which an organism will mount against a foreign invader. Consequently it is of immense importance to the filtering process which distinguishes between harmless food proteins and evidence of pathogens. With this in mind, let us now turn to the filtering process itself.

1.3.2 Tolerisation and the “default” setting for immunity

Broadly speaking, when an organism encounters a novel antigen for the first time, one of two consequences can result. If the components of the immune system come to a consensus that it is harmful, a destructive response will be initiated, aimed at eliminating

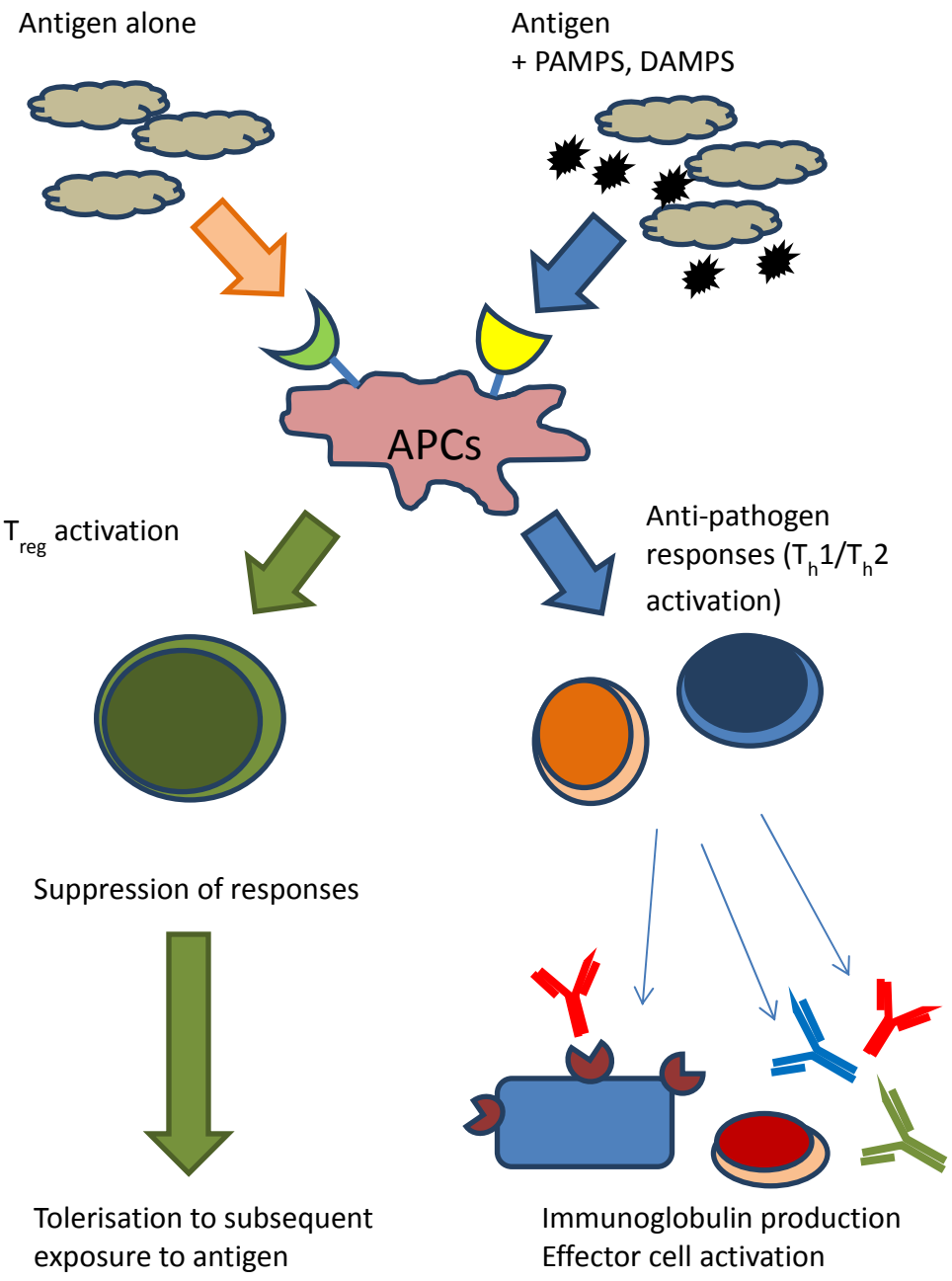


Figure 1.1 Discrimination between harmless and pathogen-related allergens by the innate immune system, leading to tolerance and immune responses respectively.

the antigen in question from the system. Upon success, this response will mature into a form of immunological memory, which will ensure the rapid deployment of an identical response whenever the antigen is encountered again (Tokoyoda, Hauser et al. 2010). The other possibility, in the event that the immune system deems the antigen harmless, is that destructive responses will be actively suppressed, a process known as tolerisation, which will ensure not only that the protein will not be purged, but that it will remain on the organism's list of harmless substances if it is subsequently encountered (Corthay 2009).

The processes by which this discrimination takes place are immensely complex, and large parts remain poorly understood, but several general features can readily be observed. Antigens will generally first be exposed to the immune system via either digestion, or by entering through wounds. Their initial encounter is likely to be with Antigen-Presenting Cells (APC) such as Dendritic Cells (DC) or macrophages (Soyer, Akdis et al. 2013), which prodigiously sample their surroundings via phagocytosis and endocytosis and take up large quantities of extracellular medium by macropinocytosis. The antigen will be chopped up into peptides by the cell's processing facilities, and then transported back to the surface, where it is presented in the cleft of the protein MHC class II, accompanied by the expression of a panel of costimulatory molecules (Lombardi, Singh et al. 2010). Together, these are responsible for priming a class of effector cells of the immune system, known as T-lymphocytes, which express a specific T-cell receptor able to bind to the presented antigen (a vast diversity of such receptor variants is produced naturally during T-cell development via recombination) (Soyer, Akdis et al. 2013). It is the specific T-cells which are activated in this way which go on to co-ordinate the immune response in question – whether this is an aggressive response to the antigen managed by T-helper (T_h) cells, or a suppressive

response by T-regulatory (T_{reg}) cells to block unnecessary attacks on a harmless antigen (Corthay 2009). In turn, these T-cells will go on to either stimulate or suppress B-cells responding to the same antigen and producing soluble antibodies of various types, depending on the type of pathogen which is to be combated. It is the initial "decision" by the APCs about which co-stimulatory molecules and cytokines to express, and hence which antigen-specific T-cell types to activate, which makes the crucial difference between tolerance of an antigen and launching an immune response (Soyer, Akdis et al. 2013).

This APC-mediated selectivity appears to be determined by the activation of a combination of the DAMP and PAMP receptors, together with the quantity of the antigen which is processed by the cell. By default, if a novel protein is unaccompanied by any signs of cellular damage, or any molecules characteristic of pathogens, its presentation by APCs results in a suppressive immune response, co-ordinated by so-called T_{reg} cells. These cells play a key role in preventing autoimmunity, as well as unwanted immune responses more generally, and exert a suppressive effect upon their specific B-cells and other classes of T-cell. This inhibitory effect ensures that an immune response against the cognate antigen will not be initiated in future, unless the regulatory signal is subsequently countermanded by a stronger, opposing activatory signal. This, however, can be difficult to achieve, and the phenomenon known as "breaking tolerance" has been a persistent thorn in the side of vaccine producers in particular, requiring the development of numerous ingenious strategies (embodied in adjuvants) to enhance the immunogenicity of vaccine targets, in order to prevent the onset of tolerisation when they are administered (Coffman, Sher et al. 2010). By contrast, allergy represents the converse – a failure to induce tolerance when this should normally take place.

1.3.3 The T_h1/T_h2 paradigm and allergy

In order to understand how allergic and other immune dysregulation occurs, it is necessary to realise that the mammalian immune system has developed multiple distinct subsystems which specialise in the removal of particular types of pathogens. The classical paradigm for interpreting these systems is that of T_h1/T_h2 polarisation, based on the prototypical subtypes of T-cell which were originally identified as co-ordinating them (Soyer, Akdis et al. 2013). The coadminstration of novel antigens, together with the presence of either DAMPs or PAMPs suggestive of a certain type of infection, acts synergistically to induce the initial phases of the immune response among T-cells and B-cells recognising the antigen in question. The exact type of antibody response is determined by the production of cytokines by the T cells, which induce class switching amongst the antibody coding regions of their cognate B-cells. This alters the invariant regions of the antibody being produced without affecting its antigen-binding variable region, allowing it to retain the same specificity whilst exerting different effects through its invariant region (Zhang, Franklin et al. 2010). IgG1, IgG2 and IgE all share a nearly identical basic structure, but differ sharply in their effector functions because their invariant regions can bind to different receptors.

The T_h1 response is primarily a retaliation to intracellular assault. It plays critical roles in the destruction of viruses and certain bacteria which are able to make their homes in the cytoplasm of eukaryotic cells. The induction of this subtype of T helper cells leads to the synthesis of T_h1-specific cytokines including interferon gamma (IFNγ), which drives the production of IgG1 (in humans) and IgG2a (in mice), with the ultimate goal of containing the parasites before they are able to escape and reproduce. Owing to the mutual antagonism between T_h1 and T_h2 responses, T_h1 antibodies also appear to play a role in the induction of

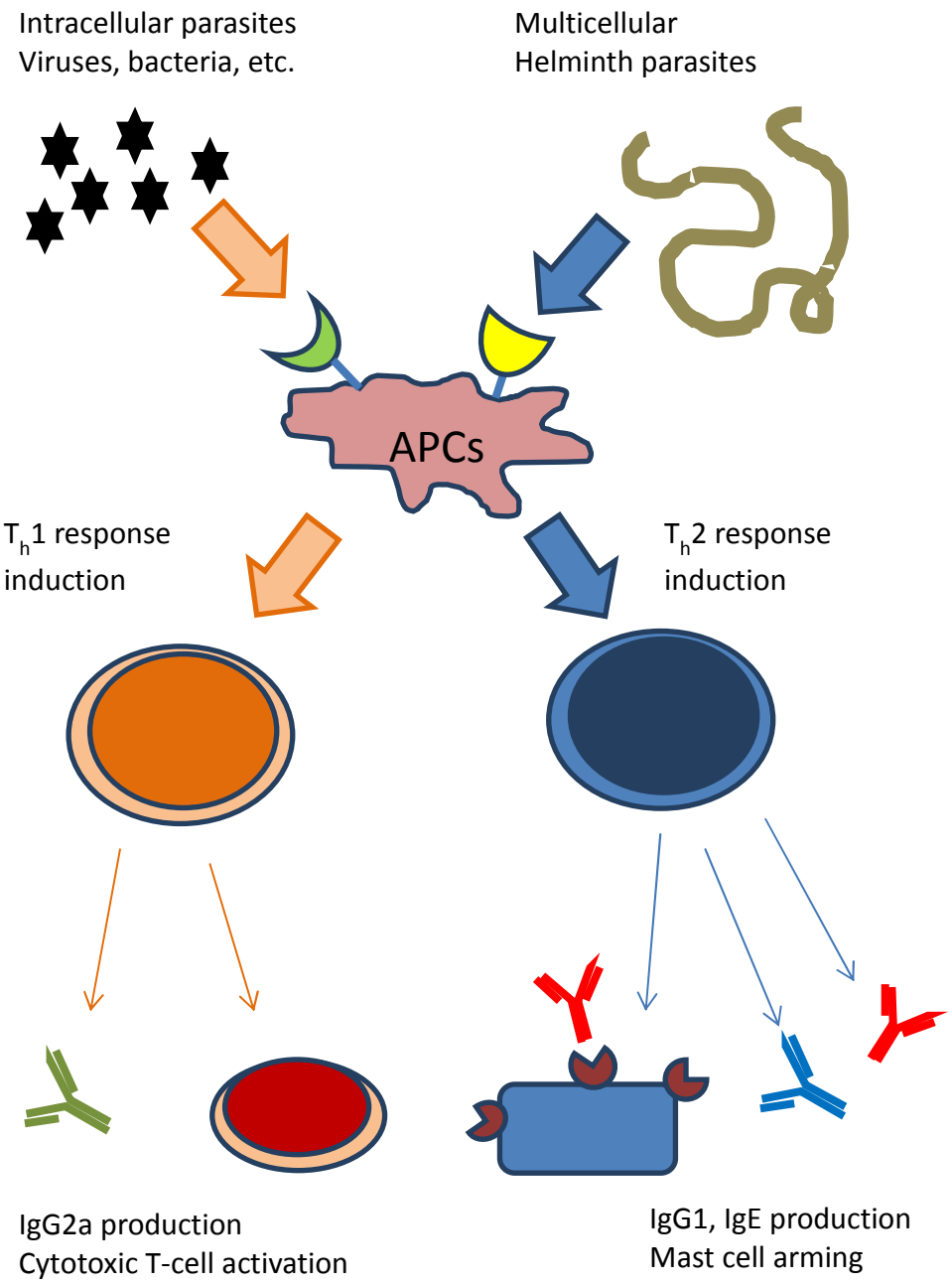


Figure 1.2 The T_h1/T_h2 paradigm, showing the induction of distinct immune responses as a result of the detection of different types of pathogen.

oral tolerance – the creation of such antibodies may have a blocking effect which prevents the generation of potentially dangerous allergic responses (Marth, Breyer et al. 2013).

The T_h2 response on the other hand is primarily an anti-helminth weapon (Fitzsimmons and Dunne 2009). The removal of these large parasites from tissues is beyond the capabilities of targeted cytotoxic mechanisms, and is often achieved by causing such extensive inflammation and damage around the infested area that the parasite is dislodged, whilst secretory cells known as eosinophils bombard it continuously with toxic granules (Harris and Gause 2011). This two-pronged attack is not always completely effective, but it is usually sufficient to keep helminth infestation at acceptably low level (Helmby 2009). A secondary feature of T_h2 responses is the generation of large amounts of IgE, which sticks to the Fcεr1 receptor on mast cells. Once they are "armed" by IgE, mast cells can act rapidly to generate an inflammatory environment, releasing enzymes and cytokines to both generate the initial signalling and recruit other cell types (Galli and Tsai 2010). It is this effect which is responsible for the symptoms of anaphylaxis in food allergy. Once a patient has been "sensitised" by the production of IgE and arming of mast cells, any subsequent exposure to the antigen will produce symptoms. Importantly, the high-affinity IgE necessary for allergy seems not to be directly produced by B-cell class switching, but can only be produced by cells which have first begun producing IgG1 (in mice) or IgG2 (in humans) and then later undergone a second class switching to produce IgE (Xiong, Dolpady et al. 2012).

In essence, food allergy is a misdirected T_h2 response, effectively mistaking a harmless food antigen for a sign of a dangerous helminth parasite. As previously noted, a second important factor in the success of tolerisation is the successful introduction of a sufficiently strong T_{reg} response. If this is obtained, even strongly immunogenic antigens may produce

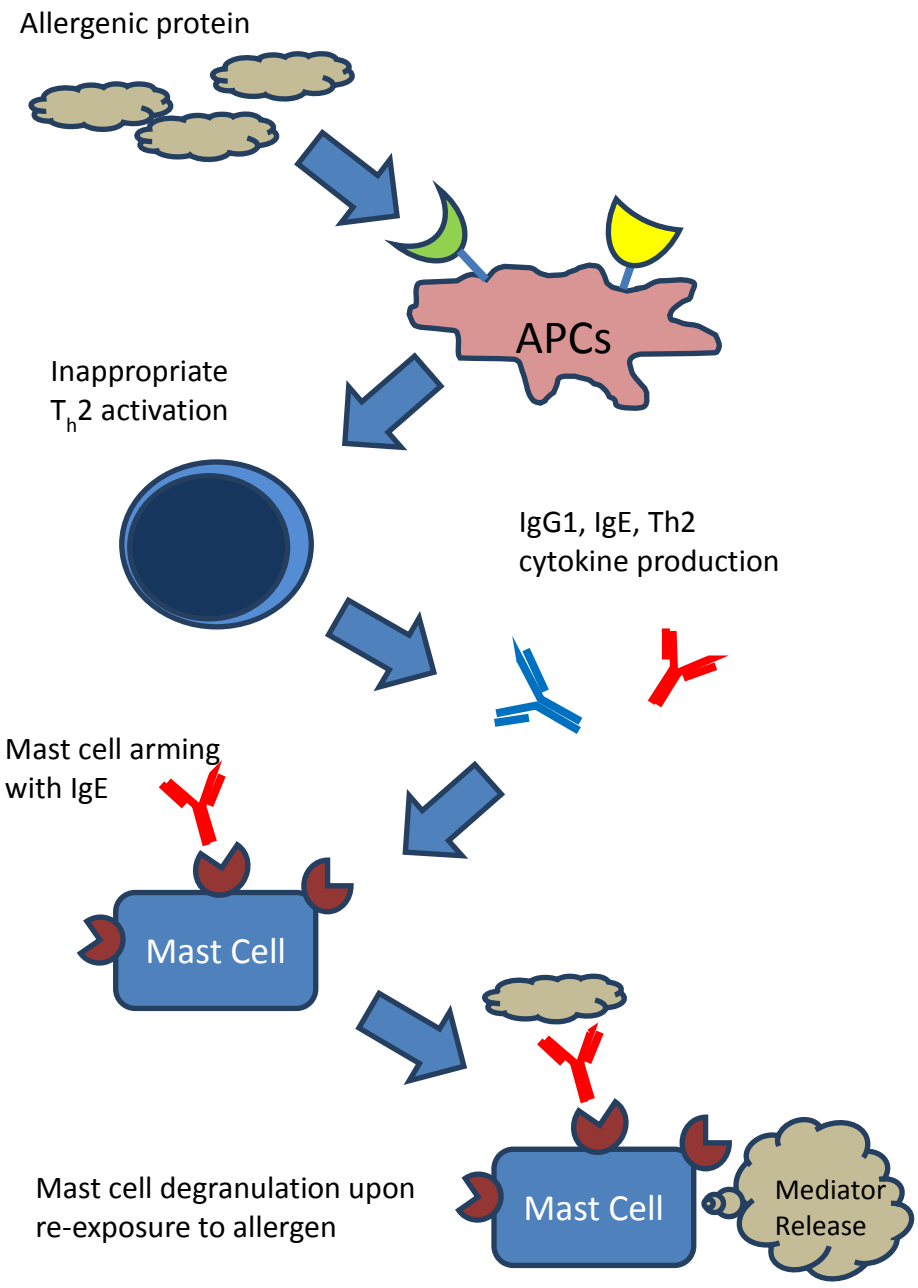


Figure 1.3 The process of allergic sensitisation brought on by the failure of tolerance to a food antigen.

no co-ordinated response – any T_h1 or T_h2 responses generated are countermanded by the T_{reg} (Corthay 2009). Equally, it is possible for a T_h2 response to be initiated, but due to countervailing regulatory signals, be unable to proceed from IgG1 to IgE production. Food allergy is the result when this control system fails, and a misdirected T_h2 response is not correctly shut down, raising two immediate questions. Firstly, why does this regulation malfunction in some individuals, but not in others? Secondly, why does it fail relatively frequently for some antigens, but almost never for others? In other words, what turns an antigen into an allergen?

1.4 Factors affecting allergic sensitisation

By general consensus, the T_h2 dysregulation leading to food allergy is thought to be a stochastic process in which various genetic and environmental factors may increase the chances of the random event of an antigen escaping the normal control systems, thus leading to food allergy.

1.4.1 Genetic factors

Research from twin studies (Sicherer, Furlong et al. 2000; Dreskin 2006) and animal models of allergy (Knippels, van Wijk et al. 2004) have led many researchers to believe that predisposition to allergy is at least partly genetic. In practice however, pinning down specific genetic determinants has proven challenging, and the correlations thus far identified have generally been weak (Tamari, Tanaka et al. 2013), although there are hopes that this situation may improve with the increasing sophistication of genome-wide association studies (Yoon, Ban et al. 2012). It also remains to be seen how and to what extent epigenetic changes as a result of the environment or maternal immunity may interact

with genetic predispositions to allergy, as some have suggested (Lovinsky-Desir and Miller 2012).

1.4.2 Environmental factors

The rapid onset of the food allergy epidemic, and the modest successes of searches for genetic determinants, have led many scientists to conclude that environmental factors are predominant in the causation of food allergy, regardless of any innate predispositions that are eventually uncovered. The most famous of these, the "hygiene hypothesis", has already been mentioned. Evidence for this suggested connection between lack of childhood infection and increased food allergy include the observation that children raised on farms in a more microbe-rich environment appear to be less likely to develop allergies, as are those born into larger family sizes, which are associated with increased childhood infection (Genuneit, Strachan et al. 2013). Nevertheless, today the hygiene hypothesis is rarely advanced in its original form, with proponents typically supplementing it with the more specific hypotheses that either the absence of chronic infestation by helminths or non-pathogenic bacteria in the human microbiota results in a disruption of normal homeostatic mechanisms associated with immune tolerance, resulting in increases in the dysregulation which enables food allergy, among other disorders of immunity (Guarner, Bourdet-Sicard et al. 2006; Jouvin and Kinet 2012).

1.4.3 Consumption of allergenic organisms

A specific category of environmental factors which are thought to be of particular importance are the consumption of allergenic material, either as food or as part of other products. As an initial assumption, it might be expected that allergy to a specific food would

be proportional to its consumption in the relevant population, as is seen in case of wheat allergy (Keet, Matsui et al. 2009), but this is not always the case. Staple foods such as rice and potato are among the most widely consumed human foods on the planet, and yet allergies to these substances are relatively rare (Keet, Matsui et al. 2009; Villalta, Longo et al. 2012). In contrast, disproportionately high contributions to the total allergy rate can be found from relatively obscure plants such as kiwi fruit (Lucas, Grimshaw et al. 2004). One possible explanation for this discrepancy is that the introduction of previously unknown foodstuffs into the human diet may provoke disproportionate immune responses. This would not, however, provide an explanation for why such foodstuffs are allergenic in the first place, given that many other non-native foodstuffs do not generate comparable levels of food allergy.

1.5 What makes an allergen?

Allergens are named according to the standard convention established by the Allergen Nomenclature Sub-Committee of the World Health Organisation, using a four-letter prefix derived from the Linnaean classification of the organism of origin, followed by a number denoting the order in which they were discovered (Chapman, Pomes et al. 2007). For example, the major peanut allergen is named Ara h 1, from *Arachis hypogaea*, and the fact that it was the first peanut allergen to be described in 1981 (Saavedra-Delgado 1989). Allergens may be further classified as "major" or "minor" according to the percentage of allergy patients in which they can elicit allergic symptoms (Chapman, Pomes et al. 2007).

Perhaps surprisingly, despite decades of research into the causes of food allergy, there is still no definitive set of criteria for determining the risk of a biological molecule giving rise to allergy (Breiteneder and Mills 2005). Although the majority of known allergens are members of a relatively small number of protein superfamilies (Radauer, Bublin et al. 2008), these groupings have few structural or functional similarities. Additionally, many homologous proteins from closely related organisms exhibit extremely similar structure and function to known allergens, and yet are not themselves allergenic (Barre, Borges et al. 2005). Accordingly, predicting whether or not a protein is likely to be a potent allergen remains a science in its infancy. Nevertheless, certain characteristics have been repeatedly associated with a diverse range of allergens originating from a broad sample of different organisms, namely:

1.5.1 Abundance

The majority of known allergens are among the most abundant proteins in their respective tissues, often comprising a significant percentage of the total protein content (Bannon 2004). Although some known allergens comprise a relatively small percentage of their tissues of origin, these are in the minority, and it is possible that other factors may explain their potent allergenicity (Herman, Helm et al. 2003). On balance, it would appear that the sheer quantity of an allergen consumed may be an important factor in whether or not enough of it encounters the innate immune system to initiate allergic priming.

1.5.2 Digestion Resistance

Another important factor which many allergens have in common is resistance to digestion. This may be because their structural features make it difficult for the digestive enzymes to break them down, or alternatively because they actively inhibit certain digestive enzymes (Bowman and Selgrade 2008). The overall effect of such traits is an enhancement of the ability of allergens to survive intact through digestion and encounter the immune cells of the gut whilst still intact (Astwood, Leach et al. 1996). In some cases, this may be critical in determining whether and how they are presented, which in turn will determine the resulting immune responses.

1.5.3 Thermal stability

The tertiary structure of many proteins is destroyed by the thermal processing used to prepare many foodstuffs, potentially removing allergenic epitopes (Wal 2003). It is therefore not surprising that the ability to resist this thermal denaturation has been

associated with allergenicity by some researchers, although others vigorously dispute this (Privalle, Bannon et al. 2011). In some cases this property may combine with digestion resistance, as in the case of the peanut allergen Ara h 2, which was found to increase in its resistance to digestion after roasting (Maleki, Viquez et al. 2003).

1.5.4 Tendency to aggregate

The increased immunogenicity of aggregated antigens relative to their native forms is a long-established phenomenon in immunology. However, there is also strong evidence that aggregation of allergens may enhance allergenicity specifically (Sathe, Teuber et al. 2005). The formation of protein aggregates may well increase the avidity with which allergens can bind to the scavenger receptors of the innate immune system, allowing more efficient uptake and presentation of a greater quantity of allergen peptides than would otherwise have been the case, as well as producing stronger responses after sensitisation due to a greater efficiency of binding to B-cell and IgE receptors (Breiteneder and Clare Mills 2005). It therefore remains a plausible contention that the low solubility of some proteins may directly contribute to their allergenicity.

1.5.5 Glycosylation

The role of glycan residues on the surface of glycoproteins in determining their allergenic potential appears somewhat mixed, and appears to vary between different allergens, with studies reporting either increased or decreased immunogenicity upon the removal of glycan residues (van der Veen, van Ree et al. 1997; Altmann 2007). In some cases, such as helminth parasite infections, glycan-specific IgE can be produced in large quantities, although whether this is to the benefit or detriment of the host remains far from clear (van

Die and Cummings 2010). Nevertheless, the existence of whole families of glycan-binding scavenger receptors involved in innate immunity are clear evidence of their immunological significance (Shreffler, Castro et al. 2006), and a specific role for certain glycan recognition receptors in allergy is highly plausible (Al-Ghouleh, Johal et al. 2012).

1.5.6 Specific effects

A few allergens are known to exert their effects through very specific mechanisms. For example, the allergen Der p 1 from house dust mites is a peptidase enzyme, which cleaves the bonds of skin connective proteins and thus efficiently diffuses into the peripheral tissues (Shakib, Schulz et al. 1998), ensuring its exceptionally efficient recovery and presentation by the immune system (Gough, Campbell et al. 2003). This class of effect is, however, relatively rare, suggesting that most allergens are allergenic by virtue of nonspecific effects.

1.6 Peanuts evaluated as a source of allergens

With these criteria in place, let us now return to peanuts specifically, and determine how well the peanut allergens fare when assessed against these predictive criteria for allergenicity. The peanut plant is a leguminous shrub, which grows natively in South America and was first exported to other continents by Europeans under the Spanish and Portuguese empires, becoming truly international when plantations began in the Southern United States as a cash crop to replace the losses to cotton production following the conclusion of the American Civil War (Saavedra-Delgado 1989). From its increasing dominance of the American South, peanuts became a major worldwide export, and it was not long before they were being grown in environments as diverse as China and India (Saavedra-Delgado 1989). Peanuts became valuable not only for human consumption but

also for their ability to fix nitrogen during crop rotation and for the industrial uses of peanut oil (Putnam, Oplinger et al. 1991).

It is no doubt this nearly ubiquitous presence in the human environment (it is notoriously difficult even for those peanut allergic patients who purposely avoid dietary peanut to avoid indirect exposure (Leftwich, Barnett et al. 2011)) which is responsible for a great deal of its representation in the toll of human allergies. But there may well be other factors at work – to take one example; the phylogenetically closely related soya plant is not only genetically very similar, it also contains close homologues of almost all the peanut allergens (Sicherer, Sampson et al. 2000) and is widely grown and consumed as a food crop (Barrett 2006). And yet despite the existence of a significant cohort of soya-allergic patients, these allergies are neither as long-lasting nor as common as those reported to peanut (Barre, Borges et al. 2005).

To date, thirteen proteins have been designated as peanut allergens, though this discussion will concentrate only on those which have been indicated as being particularly clinically significant. The most abundant peanut allergen, Ara h 1, was originally identified in the 1920s under the name of conarachin (Saavedra-Delgado 1989; Pomes, Helm et al. 2003). A 63kDa seed storage vicilin which forms a 190kDa homotrimer, Ara h 1 is one of the principal nitrogen sources for the developing plant embryo, and is accordingly synthesised in large amounts during seed formation (Shin, Compadre et al. 1998) and is deposited within the growing seed (Viquez, Konan et al. 2003). This function may go some way towards explaining its low level of solubility (Lehmann, Schweimer et al. 2006), as it has a strong tendency in solution to convert into very high molecular weight oligomers (Gruppen, Van Boxtel et al. 2006), which may well be related to its native storage in specialised cellular

A

Ara h 1

B

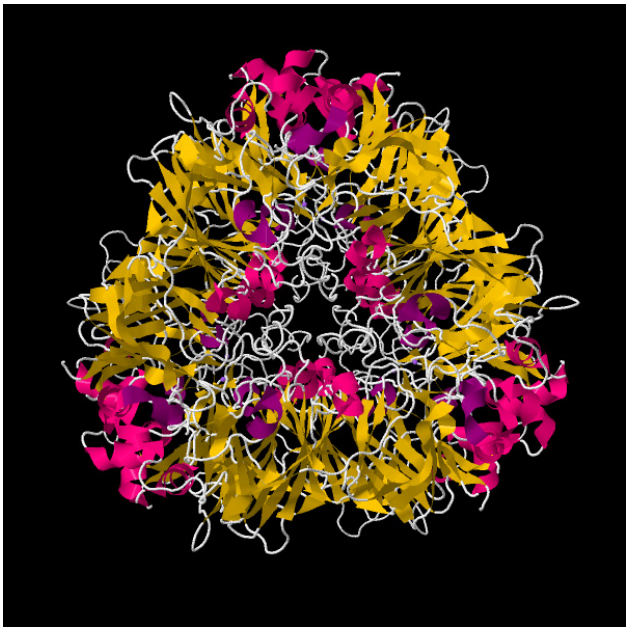
Ara h 2

Figure 1.4 Crystal structures of the major peanut allergens **(A)** Ara h 1 (Cabanos, Urabe et al. 2010), and **(B)** Ara h 2 expressed as a fusion protein with N-terminal Maltose Binding Protein expression tag (Jin, Guo et al. 2009). Visualised using Jmol, an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/

compartments. Ara h 1 has homologues in many other plants in the legume family, although curiously not all of these are allergens, and even those which are allergenic are not so to the same extent (Barre, Sordet et al. 2008). Structural analysis of the protein has revealed nothing particularly unusual to distinguish it from these relatives, suggesting that the differences between closely related proteins which are weaker allergens may be very subtle (Cabanos, Urabe et al. 2010).

Ara h 2 is, like Ara h 1, a seed storage protein, although it belongs to a very different family. It is referred to as a 2s albumin, on the basis of the sedimentation coefficient of the prototypical example of the family (Moreno and Clemente 2008). Generally speaking it is an unremarkable protein, with a highly acidic pI, that exists as a monomer (Moreno and Clemente 2008). One of its most interesting features is that it can act as an inhibitor for the digestive enzyme trypsin, although this does not appear to be related to its native biological function (Maleki, Viquez et al. 2003). Given that one of the known factors used to predict allergenicity is resistance to digestion (Lehmann, Schweimer et al. 2006), and that this property is resistant to thermal processing (another known allergen predictor), it is perhaps not surprising that multiple studies have affirmed Ara h 2 as the most important allergen in triggering responses among peanut-allergic human patients (Porterfield, Murray et al. 2009; Kulis, Chen et al. 2012).

The curious story of the discovery of Ara h 3 and 4 is a cautionary tale for any would-be allergen classifiers (Jin, Guo et al. 2009). Originally identified as separate allergens, when sequences of Ara h 3 and 4 were compared, they were in fact products of the same gene, and appear to represent different cleavage products of the very same protein (Piersma, Gaspari et al. 2005). The protein of which Ara h 3 and 4 are derivatives is a glycinin, another



Ara h 3/4

Ara h 6

Figure 1.5 (A) Crystal structure of the minor peanut allergen Ara h 3/4 (Mueller 2011). **(B)** Nuclear Magnetic Resonance structure of the minor peanut allergen Ara h 6 (Lehmann, Schweimer et al. 2006). Visualised using Jmol, an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/

seed storage protein (Koppelman, Knol et al. 2003). Its role in allergy has been less explored than Ara h 1 and 2, possibly due to the difficulties in purifying and characterising a protein which is processed into such a wide variety of end products. Nevertheless, whilst it has traditionally been classified as a minor peanut allergen, more recent studies have suggested it may be a more important contributor to dangerous allergic reactions than previously thought (Restani, Ballabio et al. 2005).

Ara h 6 and the other 2s albumin, Ara h 7, are highly homologous to Ara h 2 (Suhr, Wicklein et al. 2004; Schmidt, Krause et al.) – indeed, part of the reason for their late discovery is that their biochemical properties are so similar that under many biochemical test conditions, they are indistinguishable. The separation of individual homologues (especially when multiple isoforms exist) is notoriously difficult, and the relative prevalence and allergenic importance of these different 2s albumins remains a subject of ongoing research (Porterfield, Murray et al. 2009). Nevertheless, although these variants are expressed at a much lower frequency than Ara h 2, there is still some evidence which suggests that they share at least some of its allergenic properties. Several studies have shown that peanut-allergic patient sera react to Ara h 6 in its own right, as well as extensively cross-reacting with Ara h 2 (Chen, Wang et al. 2013) (Kulis, Chen et al. 2012).

The remaining peanut allergens are present in relatively small amounts, and are observed to bind to serum IgE from only a minority of patients. Consequently, these are all designated as minor allergens. In summary, we have seen how the two most important peanut allergens possess some of the known factors for allergenicity, including abundance and poor solubility (Ara h 1) as well as digestion resistance (Ara h 2 and the other 2s albumins).

However, the relatively low allergenicity of homologous proteins from closely-related plant species suggest that other factors are also critical.

1.7 The Epidemiology of Peanut Allergy

One of the most fascinating aspects of food allergy is that even when different populations possess a similar overall incidence of the disease, allergies to specific foods can be remarkably restricted to particular geographic regions. Several factors may contribute to this effect. In particular, the introduction of novel foodstuffs to populations with no previous experience may be an important contributor to the development of widespread allergy (Lucas, Grimshaw et al. 2004). However, in contrast, the widespread consumption of a foodstuff may also contribute to the percentage of total allergy it comprises (Rance, Kanny et al. 1999). More interesting however are the numerous allergens which appear to defy these trends, and which appear to vary widely from country to country despite similar rates of consumption. Perhaps unsurprisingly, one of these is the peanut, as it is an exception among allergens in so many ways.

The unusual distribution of peanut allergy first came to light when it emerged that citizens of China and the continental United States of America consume similarly large amounts of peanut per capita (Beyer, Morrow et al. 2001), but nevertheless have almost diametrically opposed rates of peanut allergy. The USA has one of the very highest peanut allergy rates in the world, possibly as high as 1.3% of the population (Sicherer, Munoz-Furlong et al. 2010), whereas in China the incidence appears dramatically lower (Hill, Hosking et al. 1997) (Zeng, Dong et al. 2013). Quite why such an enormous difference exists, seeming to flout the usual models of allergen sensitisation rates, has aroused considerable debate. The observation

that the children of immigrants to Western nations have similar rates of peanut allergens to indigenous children strongly suggests that the cause is environmental, not genetic (Cataldo, Accomando et al. 2006).

A possible solution to this puzzle was uncovered with the realisation that, although the two countries consumed similar amounts of peanuts, they did so in a very different way (Beyer, Morrow et al. 2001) (Maleki, Chung et al. 2000). It remains the case that in China, the vast majority of peanuts are sold raw and are cooked by families themselves, primarily through boiling as part of stews or via frying (Beyer, Morrow et al. 2001). The situation could not be more different in the United States – there, peanuts are primarily cured at the farming site and then dry-roasted (as distinguished from oil-roasting, in which the peanuts are immersed in boiling oil) at very high temperatures before being sold in this form for consumption (Beyer, Morrow et al. 2001).

The correlation is tantalising – and recent studies have shown that it is reproducible across a broad range of different population samples and different panels of countries from the Western and developing world in which peanuts are consumed in large quantities (Lee, Thalayasingam et al. 2013). But, as statisticians never tire of repeating, correlation alone does not necessarily imply causation. For dry-roasting to enhance immunogenicity, it would have to do so in a highly unusual manner. Thermally processed foods which are more immunogenic than their raw counterparts typically exert this effect through the formation of novel epitopes during the cooking process, although it is important to note that this may also work in reverse, with some epitopes being destroyed by the denaturing effects of high temperatures (Davis, Smales et al. 2001). But because peanut allergy does not correlate

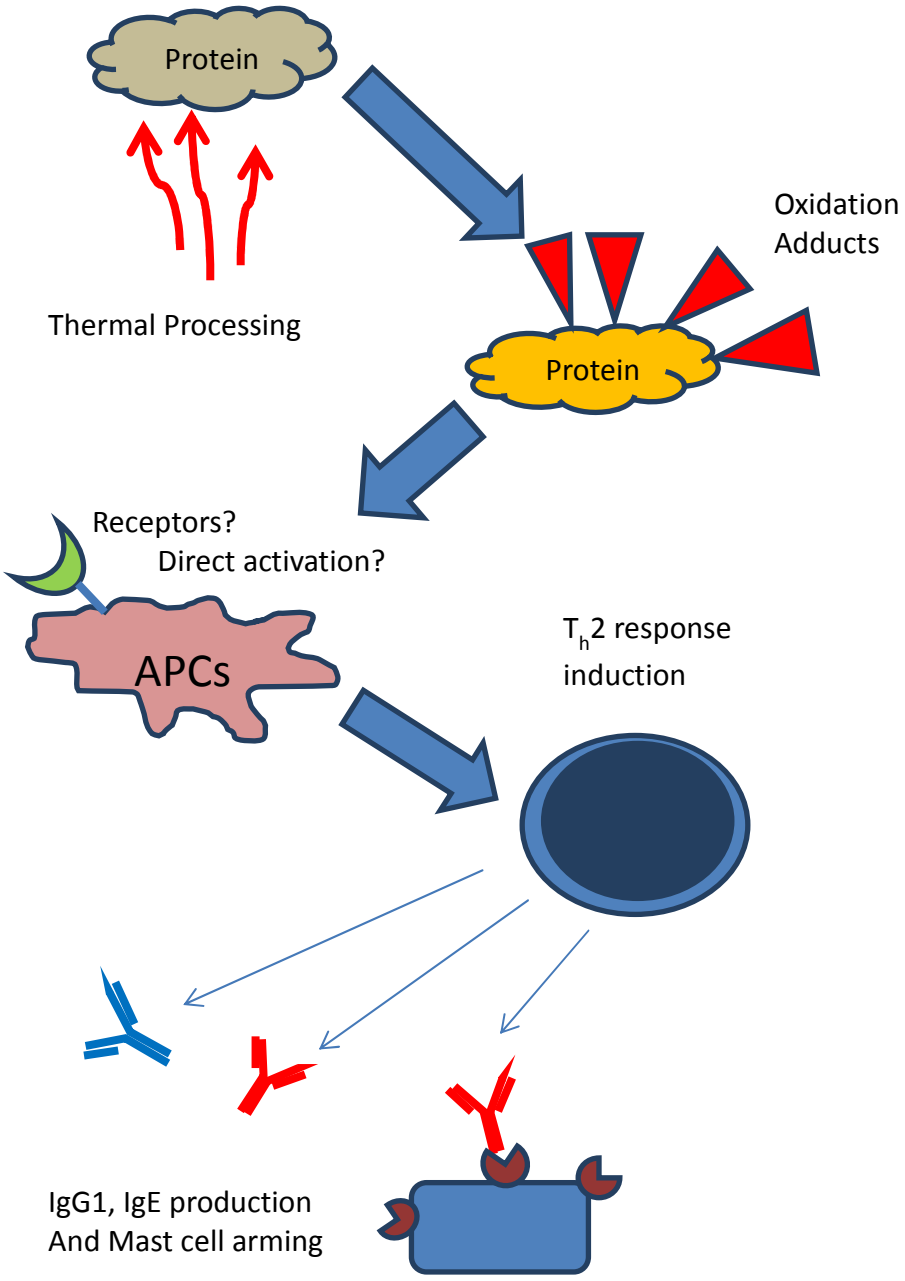


Figure 1.6 Illustration of hypothetical effect of thermal processing on peanut immunogenicity and allergenicity.

with thermal processing *per se*, but only with a particular cooking technique, dry-roasting, it is much less likely to be due to a generic effect of heating (Schmitt, Nesbit et al.).

This question has been examined in considerable detail, and researchers have identified several possible ways in which dry-roasting differs from other cooking methods which could plausibly have immunological significance. For one, dry-roasting is typically of a much longer duration than frying or boiling, and hence might allow the formation of chemical changes requiring prolonged heating that are not formed in sufficient quantities by boiling and frying (Pomes, Butts et al. 2006). Second, the process of dry-roasting, unlike boiling, occurs in an oxygen-rich environment, which may be much more conducive to processes such as protein oxidation. Finally, the presence of an external heating medium (whether oil in the case of frying, or water in the case of boiling) may actually lead to the partial loss of some allergens, as indicated by their recovery from water used to cook peanuts by boiling (Maleki, Casillas et al.). Hence, we might have reason to suspect that the chemical alterations produced by dry-roasting, boiling and frying could be dramatically different.

Even more intriguingly, there is evidence to support the suggestion that the cooking process may alter immunogenicity in more subtle ways. It is plausible that the formation of new molecules through oxidation during cooking, either on the surface of the protein or exposed to the immune system in conjunction with it due to their presence in soluble digested food, may have a co-stimulatory effect which alters the recognition of food proteins by the immune system. Though many thousands of proteins are altered and new reactions formed during cooking, there is one class of modifications in particular which are known to have some immunomodulatory effects, and which are produced in large quantities by the use of dry roasting in particular – the products of the Maillard Reaction.

1.8 Oxidative modifications in health and disease

The non-enzymatic browning reaction, or Maillard Reaction, was first described by the French biochemist Louis-Camille Maillard in 1912 (Maillard 1912). Since its initial discovery it has been discovered to be nearly ubiquitous in cooked food, producing much of the coloration and odour changes associated with cooking (Ashoor 1984), as well as occurring as a side-effect of metabolism in organisms (Dalle-Donne, Rossi et al. 2003). The Maillard Reaction appears to be one of the most important contributors to the chemical changes which occur during cooking, and Maillard Reaction products are often selected in the design of the cooking process due to the improved taste that they often impart (Sanders, Vercellotti et al. 1989).

The Maillard Reaction is in essence a condensation reaction between reducing sugars and amino acids. The reaction itself takes place between the reactive carbonyl groups on the sugar molecules, and the amine groups, which may either be on a free amino acid or as part of a protein. This results in the elimination of water and the formation of a Schiff Base intermediate, which, being highly unstable, immediately undergoes enolisation and is transformed into an Amadori product, which in turn can decompose, forming not only adducts on the surface of the protein, but also soluble products such as dicarbonyls (Thornalley 2005). Crucially, these are then fully capable of reacting with more protein, or even with each other, resulting in a chain reaction which can produce highly complex and varied reaction end products, which are collectively known as Advanced Glycation End-products or AGEs. This classification encompasses a full range from stable cross-links between proteins, to reactive aldehydic carbonyls and aromatic cyclic adducts (Nguyen 2006). The number of reaction combinations and different AGE adducts which can be

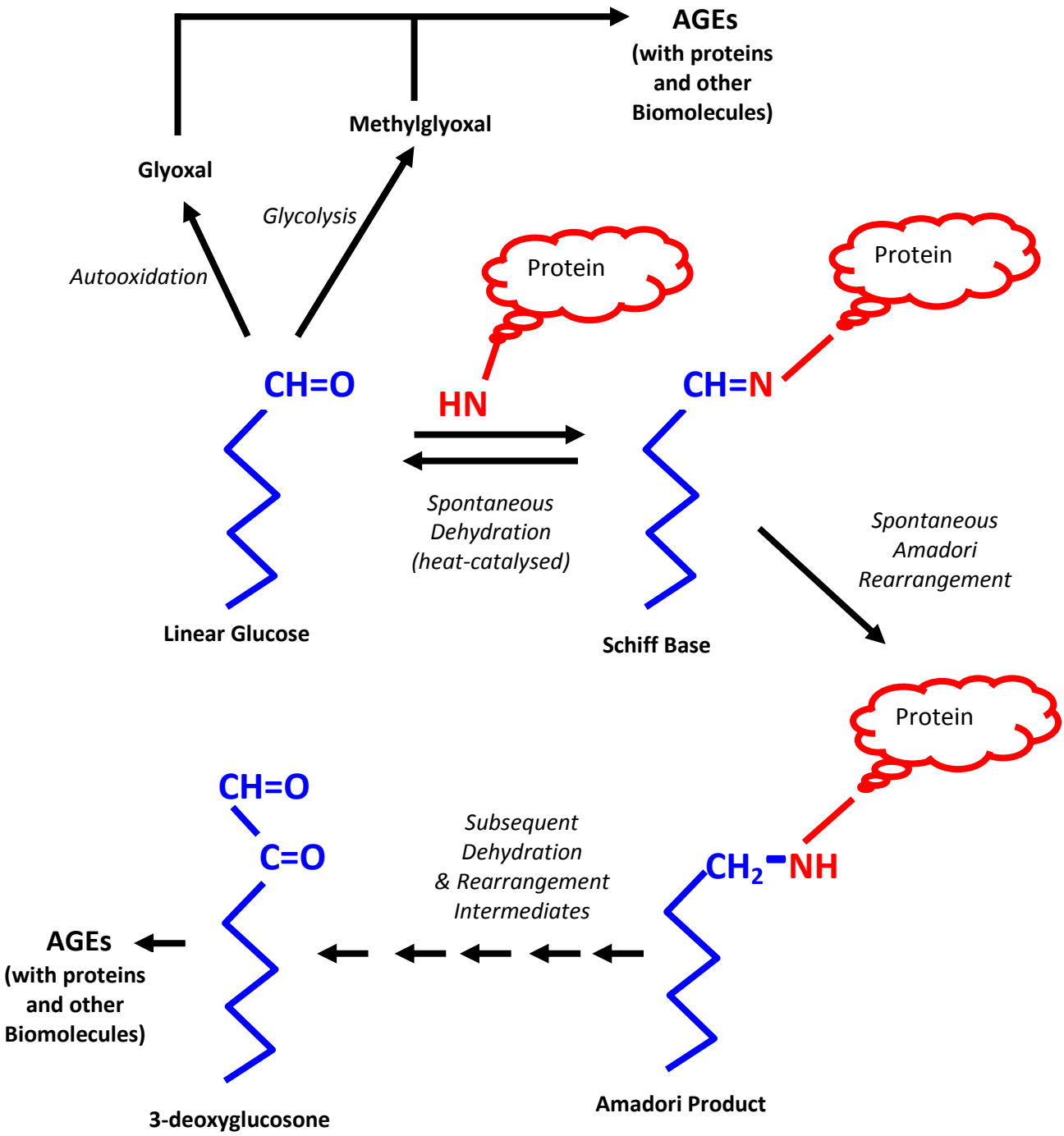


Figure 1.7 Schematic of the key steps of the Maillard Reaction, showing the initial condensation reaction and subsequent elaborations. Based on the simplified reaction overview described by O'Brien, Siraki *et al.* (2005)

produced even from a single protein and a simple aldehyde such as linear glucose is enormous, as can be demonstrated by mass spectrometry analysis of glycated model proteins (unpublished personal communication from the Sattentau Group, University of Oxford; see also Appendix B, Supplementary Figure B4).

Despite being well known for over a century, the health implications of the Maillard Reaction have not received as much attention as its nutritional and olfactory aspects. Nevertheless there has of late accumulated a significant amount of evidence which suggests that its biological activity may not be as benign as has traditionally been assumed (Ilchmann 2010). In particular it has been discovered that an increase in protein oxidation (including the generation of AGEs) has been associated with the pathogenesis of numerous diseases as diverse as atherosclerosis (Ramasamy, Yan et al. 2008), neurodegeneration (Yan, Chen et al. 1994) and diabetes (Negre-Salvayre, Salvayre et al. 2009), which suggests that their endogenous production within the body may be associated with abnormal conditions, and may potentially play a role in the signalling of stress responses (Taki, Takayama et al. 2006). Oxidation often induces dysfunction of proteins or the formation of useless aggregates (Stadtman and Levine 2000), as a result of which proteins which have been oxidised in this way tend to be tagged for destruction and removed from circulation by macrophages leading to destructive processing, or uptake by non-phagocytic cells and elimination by the proteasome (Wells-Knecht, Brinkmann et al. 1996). A situation known as oxidative stress occurs when the rate of production (or intake) of oxidised protein exceeds the organism's natural capacity to neutralise or remove them (Jin and Flavell 2010), resulting in toxicity, tissue damage and other specific effects, depending on the exact nature of the protein which has been damaged (Kar, Ashraf et al. 2008).

Protein oxidation is an inevitable consequence of the many free radicals produced by the respiration which multicellular organisms use to derive energy (Stadtman and Levine 2000), and takes place continuously even in healthy organisms, which have developed numerous mechanisms to efficiently identify and destroy proteins which have been damaged by oxidation (Negre-Salvayre, Auge et al. 2010). The continuous clearance of this oxidised protein is an unavoidable feature of everyday life, and may contribute towards the effects of ageing. However, the situation can become more serious, either when these systems begin to break down, or when they are overwhelmed by huge quantities of oxidised protein (Imai, Kuba et al. 2008). In the case of atherosclerosis, for example, the accumulation of high concentrations of oxidised and modified LDLs in the bloodstream leads to their accelerated precipitation onto atherosclerotic plaques, as well as hindering their clearance by detoxification systems (Hill, Miller et al. 1998).

Several of the receptors which are involved in mediating the uptake of these damaged proteins are also known to be involved in innate immunity. It is therefore tempting to hypothesise that they may also be involved in the uptake and processing of oxidised exogenous proteins, including allergens. These proteins include RAGE, the first AGE receptor to be identified as such, a 5-domain transmembrane signal transduction protein which is known to be involved in pro-inflammatory processes (Ramasamy, Yan et al. 2008). However, other putative binding sites, including DC-SIGN (Shreffler, Castro et al. 2006) and SRA (Ilchmann, Burgdorf et al. 2010) have been proposed, and many of these possibilities are not mutually exclusive. Different receptors acting in a synergistic manner is a common phenomenon, which is consistent with the highly heterogeneous nature of modifications incurred during oxidation (O'Brien, Siraki et al. 2005) (Supplementary Figure B4).

1.10 Model systems for oxidative immunomodulation

Evidence in support of the contention that the modifications induced by dry-roasting may be immunogenic has arisen independently from research conducted outside the peanut allergy field. In particular, the formation of reactive carbonyls (RC), which form one of the principal families of products of the Maillard Reaction, has been studied in relation to their immunogenic properties. These have included an analysis of the role of RC in a failed vaccine for Respiratory Syncitial Virus (RSV) which was trialled in the 1960s but was abandoned when it was revealed to increase symptoms upon subsequent infection, rather than preventing them (Openshaw, Culley et al. 2001). Subsequent research has uncovered evidence that the usage of formaldehyde during the vaccine production process to inactivate the virus resulted in the formation of large amounts of RC, through a chemical process analogous to the early stages of the Maillard Reaction (Moghaddam, Olszewska et al. 2006). These RCs are theorised to act synergistically with an innate trait of RSV capsid proteins, which are thought to have developed T_h2-biasing properties as a means of evading the immune system's T_h1 antiviral responses (Becker 2006). Furthermore, replicating the RC adduction procedure using carbonyl generators such as glycoaldehyde has been shown to increase the immunogenicity of model allergens such as ovalbumin and hen egg lysozyme, as well as enhancing their uptake and presentation by APCs (Moghaddam, Gartlan et al. 2011) (Allison and Fearon 2000).

Consistent with these results have been studies into the immunogenicity of chlorinated proteins, which are analogous to carbonylated ones in several immunologically important respects – they are both formed during periods of oxidative stress, and furthermore have been implicated in the pathogenesis of disease (Pattison and Davies 2006). The formation

of these products in a model system of chlorination by hypochlorous acid resulted in enhanced T-cell responses and improved uptake of the antigens by APCs (Prokopowicz, Arce et al. 2010).

However, studies of the effect of AGE formation as a result of glycation have produced more mixed results, with one experimental model of glycation by glucose suggesting an increased degree of immunogenicity (Hilmenyuk, Bellinghausen et al. 2010; Ilchmann 2010), and another using a different model allergen suggesting a complex effect of decreased IgE binding but increased degranulation capacity (Cucu, Platteau et al. 2011). These conflicting results may be due to a combination of the different allergens used, and the heterogeneity of AGE preparation methods used in both experiments, which may have the potential to induce immunologically significant differences in the populations of AGEs which result.

The chief relevance of these studies to peanut allergy, however, is that they demonstrate how the formation of even small molecular adducts onto the surface of antigens can dramatically alter the manner in which they are recognised by the immune system. Taken together, they strongly suggest that the hypothesis that dry-roasting could produce similar changes in protein immunogenicity is a highly plausible one. It is intriguing to speculate that a similar process to these model systems could be occurring with roasting and peanut allergy – the formation of the oxidation products of dry-roasting acting synergistically with the innate traits of peanut allergens, inducing a more powerful T_h2 response than either alone. If true, this would explain why peanut allergy is typically more severe than that caused by highly related species containing highly homologous allergens, such as soy – because these are not typically consumed after dry roasting.

1.11 Thermal processing and peanut allergy – what is the evidence?

We have seen that chemical modifications similar to those produced during dry-roasting have been shown to exert immunologically significant effects in various models of disease. But this falls short of an indication that they are also relevant in the case of peanut allergy. Is there, then, any direct evidence that protein oxidation can directly contribute to food allergy?

The interactions of food processing with the perception of food proteins by the immune system have long been of interest to allergy researchers. The very first case of identified food allergy was reported as being against raw, but not cooked, fish, and many similar cases have since been reported (Maleki 2004). Since the epidemiological connection between dry-roasting and peanut allergy was first discovered, this interest has also extended to peanut processing. Various studies have attempted to identify the relevant chemical changes taking place in roasted nuts, although thus far they have only attempted to do so in the most general terms (Chung, Butts et al. 2003). More solid evidence, though still conflicting, has emerged from studies of the effects of peanut processing methods on the binding capacity of peanut protein to the sera of peanut-allergic patients. In general, these have shown binding of allergic patient serum IgE to roasted peanut proteins to be significantly higher than binding to raw (Chung and Champagne 2001). Nevertheless, there is large variation in the magnitude of this effect as reported by different research groups, with estimates of the difference in binding between raw and DR peanut proteins ranging from between 2-fold to 90-fold (Maleki, Chung et al. 2000). Still other groups reported no difference in patient serum IgE binding, or increased binding only for purified peanut allergens, not total peanut protein (Mondoulet, Paty et al. 2005). Proponents of the dry-

roasting hypothesis have ascribed these differing results to variation between preparations of DR peanut (Maleki, Casillas et al. 2004).

Furthermore, this effect appears to be specific to dry-roasted peanuts, and is not observed for peanuts which are thermally processed in other methods such as frying or boiling (Beyer, Morrow et al. 2001) (Pomes, Butts et al. 2006). Oil-roasting, a relatively rarely-used cooking technique, appears to have a binding intermediate between that of frying and dry-roasting. Numerous AGEs have been confirmed to exist at higher levels in dry-roasted peanuts, relative to boiled and fried. The production of these during roasting is likely to result from a combination of the long periods at high temperature, together with the oxygen-rich environment of the roasting chambers (Chung, Maleki et al. 2004).

More direct evidence of the immunogenic potential of roasted peanuts has been derived from numerous *in vivo* models of peanut allergy, which have used extracts of roasted peanuts in combination with adjuvants to induce anaphylactic symptoms in mice (Helm and Burks 2002; Song, Qu et al. 2010). However, to the best of my knowledge, to date no research group has used such a murine model to directly compare the immunogenicity of raw versus DR peanut allergens. If such a study was to be conducted, and DR peanut was found to be significantly more immunogenic, it would provide powerful evidence to support the dry-roasting hypothesis. Addressing this hypothesis is the primary purpose of this thesis.

1.12 Hypotheses concerning dry-roasting and peanut allergy

Having established the outlines of contemporary research into the causes of peanut allergy in the preceding section, it is possible to formulate several hypotheses which may guide my own inquiries building on this prior research. If my interpretation of the congruent information from epidemiology, patient serum binding studies and analogous model systems of chemical modification is correct, then it is possible to derive several consequent predictions:

1. **Dry-roasted peanut allergens should be more immunogenic than raw peanut allergens.** That is to say, allergens extracted from dry-roasted peanut should produce stronger immune responses when mice are exposed to them than to their equivalents extracted from raw peanuts.
2. A corollary of the first hypothesis is that **dry-roasted peanut allergens should also be more allergenic than raw peanut allergens**. That is to say, they should not only produce stronger immune responses, but these should exhibit a T_h2 cytokine and antibody bias which is characteristic of allergy.
3. If the previous two hypotheses are correct, it is possible to advance a third prediction: **that the increased immunogenicity of DR peanut allergens should be independent of the route of delivery**. If the changes are indeed due to protein oxidation, the immunogenic modifications would be intrinsic to the proteins and therefore should not be affected by factors such as digestibility or state of aggregation.
4. If RCs are of special importance in allergenicity determination, then **chemical reduction of reactive carbonyl groups should reduce immunogenicity**. The removal

of these groups should return the level of immunogenicity towards that of raw. In contrast, if other changes accompanying roasting are more important, then they should be unaffected by reduction.

5. **Finally, increased immunogenicity of DR proteins should be translated into modified interactions with APCs.** Dry-roasting should result in either increased uptake or altered processing and enhanced presentation of peanut allergens by DCs and other APCs of the immune system.

1.13 Principal aims of the current project

The principal aim of this project is to investigate the hypothesis that the chemical modifications induced by dry-roasting of peanuts can enhance the immunogenicity and allergenicity of peanut proteins, in the hope of either confirming the importance, or dismissing the significance of this effect. In order to test this hypothesis, flaws in previously published studies will be addressed by robustly characterising preparations of peanut protein from both raw and DR peanuts, ensuring reproducibility of results and accurate correlation of any observed effects with protein modification incurred during roasting. Once this process is completed, peanut allergens will be purified to permit study of the effects of these modifications on individual proteins. In order to isolate the contribution of chemical modifications, investigations will be undertaken to quantify the effects of protein aggregation and formation of AGEs, RCs, and modified glycosylation state.

With these extracts prepared, their immunogenicity will be compared for the first time in murine models of sensitisation. The effects of priming with raw or DR total peanut extract and individual allergens will be compared between gastrointestinal, subcutaneous and epicutaneous sensitisation routes. Antigen-specific serum antibody and T cell responses will be analysed both after priming and post-sensitisation challenge with peanut allergens. In particular, signs of the induction of a T_h2 bias in the serum antibody and T-cell cytokine responses will be sought. The role of RCs as a subset of roasting modifications will be explored by chemical reduction of these groups, and the determination of whether this affects sensitisation by reduced peanut allergens.

Finally, *in vitro* investigations will be undertaken in the attempt to specify a putative mechanism or mechanisms accounting for any observed changes in immunogenicity. The binding of raw and DR allergens to various innate immune receptors will be compared, as will their interactions with DCs, in order to determine whether DR peanuts directly activate these APCs or bind preferentially to them and, if so, what mechanism or mechanisms are responsible.

Chapter 2
Materials and Methods

2.1 General Notes

Unless otherwise indicated in the text, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, USA). 96-well plates, either flat or v-bottomed, were obtained from Greiner Bio-one (Wemmel, Belgium) unless otherwise noted. All percentages of liquid mixture constituents are derived from the volume:volume ratio, unless stated otherwise. Primary cells and cell lines were incubated at 37°C in an atmosphere of 5% CO_2, using a Galaxy 170R incubator (Eppendorf, Hamburg, Germany). All other procedures were carried out at room temperature unless otherwise indicated. Media for cell culture were supplemented with 100 units/ml Penicillin and 100µg/ml Streptomycin. Additional antibiotics used are noted in the text. Cell counting was carried out using a Kova Glasstic Slide 10 Haemocytomer (Hycor, Indianapolis, USA). Where centrifuging is referred to, samples were centrifuged using a Heraeus Multifuge 1S-R centrifuge (Thermo Scientific, Waltham, USA) for 5 minutes at 1500rpm unless stated otherwise. Where plate washing is referred to, either an UltraWash Plus (Dynex Technologies, Chantilly, USA) or Columbus (Tecan, Maenndorf, Switzerland) fully automated plate washer was used.

An index of all methods used in any given Chapter of Results is given at the start of that Chapter, to aid the reference of protocols.

2.2 Reagents

The following reagents, sera and cell lines were kindly given or obtained as part of collaborations with the named individuals. Carbonylated and reduced HEL and OVA, and maleylated BSA, were given by Dr Amin Moghaddam, of the Sir William Dunn School of Pathology, Oxford, UK. RBL cell line was donated by Dr Jesse Goyette, of the Sir William Dunn School of Pathology, Oxford, UK. L929 cell line was given by Mr Simon Hackett, of the Sir William Dunn School of Pathology. Epicutaneously-sensitised mouse sera were obtained from Dr Mario Noti, then of the Perelman School of Medicine, University of Pennsylvania, as part of a collaboration.

2.3 Protein Extraction and Characterisation

2.3.1 Sourcing of peanuts

Fresh raw peanuts (*Arachis hypogaea*, Jumbo cultivar) were obtained from an American farm courtesy of a gift by KP Foods Ltd. (Hayes, UK) and were stored at -20°C until they were used. Commercially produced DR peanuts were obtained from Sainsburys Plc (Holborn, UK), Holland and Barrett Plc (Nuneaton, UK) and Farmer Brand (Singapore) and stored at room temperature. Where necessary for subsequent experiments, peanuts were sterilised using a prepaid gamma irradiation sterilisation service by Lillico Plc (Horley, UK). To prepare in-house DR peanuts, fresh raw peanuts were roasted in a laboratory oven (Memmert, Munich, Germany) at either 160 or 178°C, for between 5 and 20 minutes, depending on the experiment.

2.3.2 Determination of protein concentration

Protein concentration of samples was determined using a BCA Assay Kit supplied by Pierce (Rockford, Illinois, USA) according to the protocol supplied by the manufacturer. Briefly, protein samples were diluted to an estimated concentration ranging between 100 and 50µg/ml (in cases where the concentration was completely unknown, a range of serial dilutions was used). A standard curve of diluted BSA (Pierce) was constructed, ranging from 250-550µg/ml. Unknown samples and standards were both mixed 1:8 with Bicinchonic Acid Reagent solution, and incubated at 37°C for 30 minutes. The absorbance at 562nm of each sample was determined using an automated cuvette reader (Molecular Devices, Sunnyvale, USA). The concentration of protein in each unknown sample was determined by interpolation from the absorbance values of the standard curve.

2.3.3 Preparation of Crude Peanut Homogenate (CPH)

To prepare CPH, whole raw or DR peanuts were ground in an airflow cabinet using a pestle and mortar. The resulting paste was solubilized in pyrogen-free pH7.4 PBS (Gibco, Carlsbad, California, USA) and sonicated for two 20-minute periods, with mixing in between. The solution was then filtered through a 75µm tissue filter (BD Biosciences, Franklin Lakes, New Jersey, USA) to remove large particles of debris, and the protein content of the final solution was determined by BCA assay (Pierce, section 2.3.2) according to the manufacturer's instructions. Endotoxin content was determined by commercial testing services (Lonza, Verviers, Belgium), or by TLR4 reporter assay (see Appendix A, Supplementary Protocol A3). Samples were either used immediately or frozen at -80°C for future use.

2.3.4 Preparation of Peanut Protein Extract (PPE)

To prepare PPE, raw and roasted peanuts were ground with a pestle and mortar and the resultant paste was defatted in cold acetone, before drying overnight at 4°C. The resulting powder was solubilized overnight at a concentration of 0.1g/ml in a solution of 50mM $NaCO_3$, pH11, at 4°C under constant agitation. Insoluble debris was removed by centrifugation at 2000g, and fine debris was removed by a second centrifugation of the resulting supernatant at 12000g. The solution was sterilized through a 0.22µm filter (Millipore, Billerica, Massachusetts, USA) and buffer-exchanged into pH7.4 PBS (Gibco) using a 3kDa molecular weight cutoff filter (Millipore). The protein content of the final solution was determined by BCA assay (Pierce), according to the manufacturer's instructions. At a later stage of the project, a protein extract was prepared from commercially-produced soy protein powder (Pulsin' Ltd., Gloucester, UK) using the same protocol.

2.3.5 Endotoxin Depletion

Protein samples were depleted of Endotoxin content by passing through a Detoxi-Gel Endotoxin Removal Column (Pierce) according to the protocol supplied with the product. Briefly, the gel columns were cleaned of any residual endotoxin with five column volumes (CV) of 1% sodium deoxycholate, allowed to flow through by the action of gravity. Afterwards, columns were rinsed with five CV of distilled, pyrogen-free water (Gibco), then equilibrated with five CV pyrogen-free PBS, pH 7.4 (Gibco). Samples were then allowed to flow into the columns, which were then capped and incubated for one hour at room temperature. Samples were then eluted by the addition of further CV of pyrogen-free PBS

(Gibco), after which fractions were collected and their protein content determined by BCA Assay (Pierce, section 2.3.2).

The successful depletion of bacterial Endotoxin was then confirmed by commercial testing services (Lonza), or by TLR4 reporter assay (see Appendix A, Supplementary Protocol A3). Samples were either used immediately or frozen at -80°C for future use.

2.3.6 Thp-1-Blue assay for PRR activation

TLR-expressing THP-1 Blue cell line (THP, Invivogen, Carlsbad, USA), were cultured in RPMI supplemented with 10% FCS. Stimulation of PRRS in this cell line triggers the activation of a secreted alkaline phosphatise via the NFκB transcription factor, which can be quantified through the blue colour produced by the breakdown of its Quanti-Blue substrate (Invivogen), which was used as a proxy readout for bacterial contamination activity in samples. After harvesting, THP cells were transferred onto a 96-well plate at a density of 1million cells/ml, then stimulated with protein samples at a concentration of 20µg protein/ml. Purified LPS (Sigma) was employed as a positive control at concentrations of 100ng/ml, 10ng/ml and 1ng/ml. The resulting mixtures were incubated overnight, and supernatants were removed and either used immediately or frozen at -80°C for future use. Samples of supernatants were mixed 1:4 with Quanti-Blue alkaline phosphatise substrate, and incubated at 37°C for up to five hours to observe the development of blue coloration. When the desired level of intensity was reached, the absorbance of each well was determined using an automated cuvette reader (Molecular Devices, Sunnyvale, USA).

2.3.7 Quantification of Protein Carbonyl Content by Colorimetry

This assay functions based on the change in absorbance of a protein solution when the protein has been conjugated to 2,4 dinitrophenylhydrazine (DNPH). 500µg protein samples were mixed with 500µl of 10mM DNPH in 2M HCl to form a reaction mixture. The resulting mixture was left to stand for 1 hour at room temperature, with vortexing every 10-15 min. Afterwards, proteins were precipitated by adding 500µl of 20% Trichloroacetic acid. The mixture was then centrifuged in a 5417R tabletop microcentrifuge (Eppendorf) for 3 minutes at 11,000g. The resulting pellet was washed three times with 1ml ethanol-ethyl acetate (mixed in 1:1 proportions) to remove residual free DNPH. Following each wash, samples were allowed to stand for 10 minutes before centrifugation. The precipitated proteins were redissolved in 1 ml guanidine solution, using incubation for 15-30 minutes at 37°C if necessary. Any remaining insoluble material was removed by centrifugation. The absorbance of each solubilised sample was determined using an automated cuvette reader (Molecular Devices, Sunnyvale, USA). The protein content of these solubilised samples was simultaneously determined using BCA assay (Pierce, section 2.3.2). Carbonyl content was calculated from the maximum absorbance (*A*, 370 nm), using the DNPH molar absorption coefficient (ε)of 22,000 $M^{-1}cm^{-1}$, following the Beer-Lambert Law – $A = \varepsilon \times c \times l$, where c = solute concentration, and l = path length in millimetres.

2.3.8 Estimation of Protein Carbonyl Content by ELISA

Approximate protein carbonyl content was determined by an ELISA protocol adapted from Buss *et al. (Buss, Chan et al. 1997)* and later refined by Alamdari *et al. (Alamdari, Kostidou et al. 2005)*. 3µg protein was added to 10µl of 10mM DNPH in 2M hydrochloric acid, or 2M HCl

alone, and incubated at room temperature under constant agitation for 45 minutes. Mixtures were neutralised with 0.1M sodium bicarbonate coating buffer (pH 8.3), and pipetted into a 96-well ELISA plate. Plates were incubated overnight at 4°C or for one hour at 37°C. Afterwards plates were washed with PBS (PAA, Pasching, Austria), then blocked at room temperature for 30 minutes with 200µl 1% BSA in PBS. Blocked plates were probed with 1:1000 anti-DNPH IgG detection antibody (Molecular Probes, Invitrogen) for one hour, washed, and then 1:1000 streptavidin-conjugated-horseradish peroxidise (HRP) (GE Healthcare, UK) was added for 30 minutes at room temperature. After a final washing, the wells were developed using ultra-TMB substrate (Thermo Scientific, Rockford, USA). Plates were read using an automatic plate reader (Molecular Devices, Sunnyvale, USA) at 450nm.

2.3.9 Biotinylation of Proteins

Proteins to be labelled with biotin were diluted to 2 mg/ml in PBS, pH 7.4 (Gibco). Immediately prior to use, a 1mg/ml solution of NHS-LC-LC-Biotin (Pierce) linking reagent was reconstituted in DMSO. This reagent was diluted 1:12 in the protein solution to produce a final concentration of 75µg/ml. The reaction vessel was covered with foil and incubated for 2 hours at room temperature with constant agitation. Following this period, the mixture was desalted to remove excess biotinylation reagents using Microcon 10 centrifugal filter units (Millipore). The resulting concentrate was reconstituted to 1 ml in PBS, then the protein concentration was determined using a BCA Assay kit (Pierce, section 2.3.2). Proteins were stored at 4°C with 0.1% sodium azide as a preservative.

To confirm the successful biotinylation, ELISA wells were coated with 5µg of biotinylated protein (together with unmodified controls) in 0.1M sodium bicarbonate coating buffer,

pH8.3 at 37°C for 1 h. The plates were then blocked for 1 hour with 1% BSA, after which the presence of biotin was then detected using biotin-binding streptavidin-HRP (GE Healthcare) and Ultra-TMB substrate (Thermo Scientific). Plates were read using an automatic plate reader (Molecular Devices) at 450nm.

Alternatively, 1µg protein samples were run on a 4-12% Tris-glycine gel (Invitrogen) then transferred to nitrocellulose membrane (GE Healthcare, UK) suing a Transblot semi-dry transfer system (Bio-Rad, Hercules, USA) according to the manufacturer's instructions (please refer to section 3.4). The membrane was blocked overnight at 4°C in 3% BSA then probed for 1 hour with 1:10,000 Streptavidin-HRP (GE Healthcare). Bands were visualised using Lightning-plus ECL substrate (PerkinElmer, Waltham, USA) and R10 X-ray development plates (Fujifilm). A duplicate gel was visualised according to the protocol in section 2.4.3 to control for total protein content.

2.3.10 Reduction of Proteins

Reducing agents were prepared to a stock concentration of 1M in sterile pyrogen-free water (Gibco). Proteins were prepared in sterile, pyrogen-free PBS, pH7.4 (Gibco) to a concentration of 250µg/ml in a total volume of 400µl. Reducing agents were added to produce the final concentrations listed below (table 3.1), and the mixtures were vortexed briefly before incubation for 3 hours at 37°C. In the case of Lysine and N-Acetyl Cysteine, mixtures were instead incubated for 24 or 72 hours. After the reaction time, proteins were buffer exchanged into fresh sterile pH 7.4 PBS (Gibco). Final protein concentration was verified using a BCA Assay Kit (Pierce, section 2.3.2) and an automated plate reader

(Molecular Devices). Residual carbonyl content (as a measure of reduction efficiency) was determined using the colorimetric assay or ELISA outlined in sections 2.3.7-8.

Reducing Agent	*Final Concentration*
NaCNBH4	20mM
NaBH4	10mM

Table 2.1 Reducing agents and their concentrations

2.3.11 Fluoresceination of Proteins

Proteins were fluoresceinated with Alexa-Fluor 488 using a maleimide-based coupling agent, which operates by linking to free cysteine residues. In order to ensure efficient reaction, disulphide bridges in protein samples were broken by pre-incubation with a 10-fold molar excess (0.5mM) of TCEP Bond-breaker reducing agent (Pierce). Alexa Fluor 488 C_5-maleimide (Invitrogen) was reconstituted according to the manufacturer's instructions in DMSO, to a concentration of 5mM, then diluted 1:10 in a reaction mixture of 50µM protein (producing a final 10-fold molar excess). The mixture was incubated overnight at 4°C with constant shaking. The following morning, samples were thoroughly desalted using Microcon 10 centrifugal filter units, then reconstituted to a volume of 120µl. Protein concentrations were determined using a BCA assay kit (Pierce, section 2.3.2).

To confirm the successful fluoresceination, protein samples were diluted to a final concentration of 5µg/ml in sterile pyrogen-free PBS, pH7.4 (Gibco). 50µl volumes were placed in a 96-well flat-bottomed plate, then fluorescence emission at 519nm was determined using an M5 automated fluorescence plate reader (Molecular Devices), with an excitation frequency of 499nm, and an emission cutoff point of 510nm. With reference to

the protein content of the samples, as determined by BCA assay (Pierce, section 2.3.2), the molar ratio of moles fluor/moles carrier protein was calculated by interpolation from a standard curve of samples of free Alexa Fluor 488 C_5-maleimide reagent.

2.3.12 Light scattering measurement

Raw and DR Ara h 1 were diluted to 1mg/ml in PBS pH 7.4 (PAA) and run at 0.4ml/min across a Superose 6 10/30 column (GE Healthcare). The elution was measured with a Dawn Helios II light scattering detection system (Wyatt Technology) and an Optilab rEX refractive index detector (Wyatt Technology). The data were analysed using ASTRA 5.3.4.14 macromolecular characterisation software (Wyatt Technology) assuming a differential refractive index (the amount a solution's refractive index varies per increment of solute concentration) of 0.186 grammes/millilitre according to the manufacturer's recommendations.

2.4 Protein Gel Electrophoresis and Western Blotting

2.4.1 Reducing and non-reducing Denaturing Polyacrylamide Gel Electrophoresis

The composition of protein extracts and purified protein samples were assessed by SDS-PAGE according to a method derived from that originated by Laemmli *(Laemmli 1970)* as recommended by the electrophoresis gel manufacturer Invitrogen. In brief, protein samples were adjusted to equimolar concentrations, mixed with Laemmli buffer (4X solution: 2.5M Tris-HCl, 8% SDS, 40% glycerol, pH 6.8) and then boiled for 2 minutes at 95°C, either in the presence (for reducing gels) or absence (for non-reducing gels) of 2.5% 2-mercaptoethanol.

Samples were then loaded onto either 4-12% gradient gels, or 12% non-gradient gels (Invitrogen) pre-equilibrated with Running Buffer (50mM Tris-HCl, 192mM glycine, 1% SDS, no pH adjustment), using Rainbow pre-stained full-length molecular weight markers (GE Healthcare) for band weight reference. Gels were run using an EV231Power Pack (Consort, Belgium), at 100 volts for 1.5-2 hours at room temperature, depending on the experiment.

2.4.2 Native Polyacrylamide Gel Electrophoresis

For experiments in which denaturing conditions were not desirable, proteins were subjected to electrophoresis under native conditions, according to the Blue-Native-PAGE protocol originally developed by Schägger and von Jagow (Wittig 2006). Samples were dissolved in commercially prepared Native Sample Buffer (Invitrogen), and transferred without heating to wells in a 4-12% Novex Native PAGE gel (Invitrogen) equilibrated into 50mM Bis-Tris, 50mM Tricine (pH 6.8). Nativemark Unstained Protein Standard (Invitrogen) was used as a molecular weight marker. Just prior to running, the central reservoir was supplemented by 1% G-250 dye to act as a charge-shift molecule. After this preparation, gels were run in the usual way, at 100 volts for 1.5-2 hours at room temperature, depending on the experiment.

2.4.3 Visualisation of Protein Bands

Protein bands on both native and denaturing gels were visualised either by Coomassie or Silver staining. For Coomassie staining, gels were briefly rinsed with dH_2O then incubated for one hour in Coomassie staining solution (10% Acetic Acid, 40% Methanol, 0.1% w/v Coomassie Blue), followed by serial washes with Destaining Solution (10% Acetic Acid, 20% Ethanol) until the desired ratio of background staining to band intensity ratio was achieved.

For Silver staining, a SilverQuest staining kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, gels were first fixed overnight at 4°C in Fixing Solution (10% acetic acid, 20% ethanol), before washing in 30% ethanol and incubation with a Sensitising Agent supplied by the manufacturer. After washing with 30% ethanol, the gels were equilibrated into de-ionised water, before staining with a solution of silver ions. Excess silver ions in solution were briefly washed off with de-ionised water, and gels were developed with a reducing agent, which produced staining by depositing metallic silver on the surface of protein bands. Once the desired band intensity was reached, the reaction was halted by the addition of a chelating agent which blocked the reaction of any remaining silver ions.

2.4.4 Western Blot Transfer

For reducing and non-reducing denaturing gels, Western blot transfer was carried out using a modified version of the protocol suggested by Invitrogen for use with their gel products, with additional input from Dr Erdem Akkaya, of the Sir William Dunn School of Pathology. Gels and samples were prepared and run according to the protocol outlined in section 2.4.1, using either reducing or non-reducing conditions, depending on the experiment. After the end point was reached, gels were removed, briefly washed with dH_2O and then equilibrated in Transfer Buffer (5.8g Tris-Base, 2.9g glycine, 0.037% SDS, 20% Methanol, no pH adjustment) for 10 minutes.

Meanwhile, Nitrocellulose Membrane (GE Healthcare) was cut to the appropriate size and also equilibrated in Transfer Buffer, as were blotting paper pads (Whatman, Maidstone, UK). A sandwich was assembled consisting of the gel and membrane, with four pads of Whatman

paper on each side. The assembled sandwich was placed into a Trans-Blot semi-dry transfer system (Bio-Rad), and which was operated at 18 volts for 30 minutes according to the manufacturer's instructions. The appearance of prestained molecular weight markers of various masses onto the membrane was used to estimate the success and rate of protein transfer.

For native gels, different transfer conditions were sometimes used, depending on experimental requirements. If native conditions were only necessary for the running of the proteins but not for subsequent steps, the same protocol was used as for denaturing gels. However, if the proteins were required to be preserved in a native conformation throughout the entire process, the protocol was modified to replace the Transfer Buffer with Native Running Buffer (Invitrogen). Transfer voltage was reduced to 10 volts and transfer time increased to 1 hour.

2.4.5 Anti-carbonyl Western Blot

Anti-carbonyl western blots were carried out according to the general method of Shacter *et al*. (Shacter, Williams et al. 1994), with some modifications to the protocol inspired by the work of Robinson *et al.* (Robinson, Keshavarzian et al. 1999). Protein samples were dissolved in 6% SDS to a final concentration of 2.5-10µg in 10µl, then derivatised using a 10mM solution of DNPH in 10% Trifluoroacetic Acid for 30 minutes with constant agitation. Afterwards, samples were neutralised using a buffer composed of 1M Tris-Base and 30% Glycerol. After thorough mixing, the samples were then run on a 4-12% Tris-Glycine gel (Invitrogen) until the dye front of free DNPH reached the base of the gel.

The gels were equilibrated in Transfer Buffer (5.8g Tris-Base, 2.9g Glycine, 0.037% SDS, 20% Methanol, no pH adjustment), then transferred to nitrocellulose membrane (GE Healthcare) for 20 minutes at 18 volts using a Transblot Semi-dry transfer system (Bio-Rad), according to the manufacturer's instructions. Membranes were blocked for 1 hour in 3% BSA in PBS, and then incubated overnight using a 1:20,000 dilution of biotinylated anti-DNPH detection antibody (Molecular Probes, Invitrogen) in 3% BAS/PBS. The following morning, membranes were washed three times with PBST, then probed for 1 hour with 1:10,000 Streptavidin-HRP complex (GE Healthcare), followed by a final three washes with PBST. Bands were visualised using Lightning Plus ECL (PerkinElmer) and R10 X-ray development plates (Fujifilm). A duplicate gel was stained using a SilverQuest silver staining kit (Invitrogen, section 2.4.3) to control for relative protein concentration.

2.5 Allergen Isolation and Purification

2.5.1 Protein band assignment by mass spectrometry

Proteins were identified using SDS-PAGE and Coomassie staining as described in sections 2.4.1-2, and bands of interest were cut out and finely diced. Samples were decolourised with 25mM ammonium bicarbonate solution containing 50% acetonitrile (solution B), then dehydrated in 100% acetonitrile (ACN). Samples were then evaporated to dryness at 37°C and subsequently reduced using 10mM dithiothreitol for 30 minutes, then alkylated with 55mM Iodoacetamide for one hour and washed twice with solution B.

Samples were dehydrated again with ACN, then evaporated to dryness and digested overnight with 20µl trypsin or chymotrypsin. Digestion was stopped with 1µl formic acid, and supernatant containing peptides removed. Remaining gel pieces were incubated in

Extraction Buffer (50% ACN in water, 0.1% formic acid) for 30 minutes, and both supernatants were then combined and evaporated to dryness. Processed samples were delivered to and data were generated by the Sir William Dunn School Proteomics Facility, Oxford, UK.

2.5.2 Chromatographic Purification of Peanut Allergens

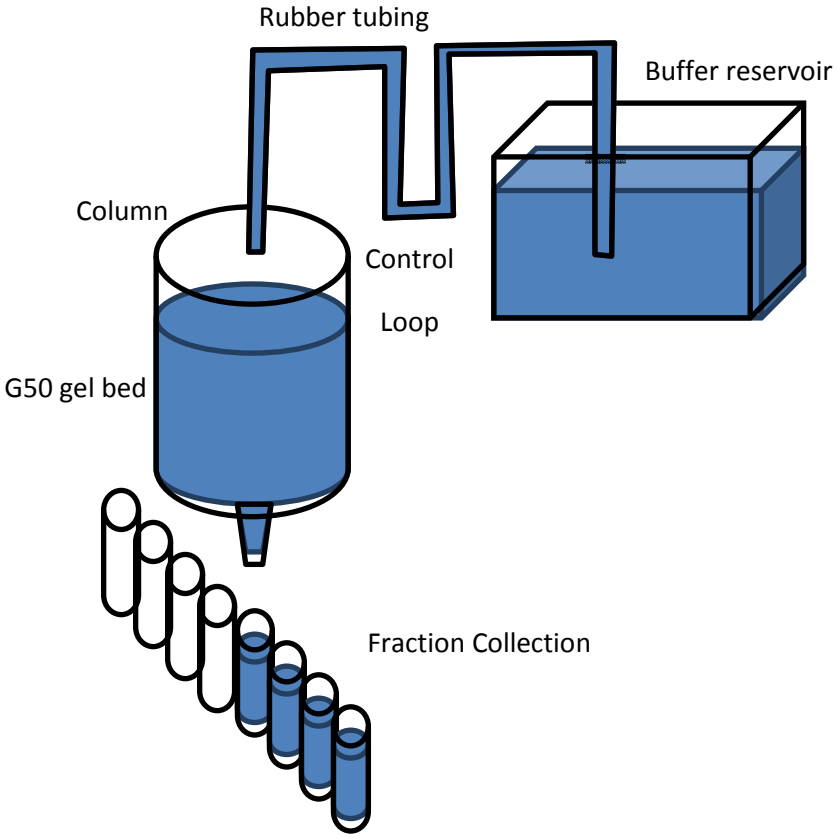
Purification of individual peanut allergens was achieved using a three-step method, derived from an original protocol developed by Mills *et al*. (Mills, Potts et al. 1997), and subsequently modified in collaboration with Dr Steven Johnson and Dr Marcus Bridge, both of the Sir William Dunn School of Pathology, Oxford, UK.

2.5.2.1 Crude Fractionation of PPE

Raw (United Biscuits, Hayes, UK) or DR (roasted in-house, using protocol 2.3.1) peanuts were ground using a pestle and mortar at room temperature. Peanut pastes were defatted with 10 volumes cold acetone before drying to obtain a powder. Proteins were solubilized overnight in 50mM Tris-HCl, 0.2M NaCl, pH8.2, at 4°C at a concentration of 100µg protein /ml extraction buffer. The following morning extracts were filtered (0.22µM filter, Millipore, Billerica, USA) and transferred to a gravity-driven size exclusion column, constructed in-house using a 650ml Chromatography Column (Generon, UK) packed with Sepahadex G-50 Coarse gel filtration medium (Sigma-Aldrich). Samples were loaded onto the column using a tubing feed and flow was established by connecting this feed to a reservoir located above the column (see figure 2.1). Protein samples were then chased through the column by the negative pressure generated by height difference between the column top and surface of the buffer reservoir, and eluting fractions were collected at the base of the column and

analysed by SDS-PAGE (section 2.4). Fractions containing high concentrations of proteins of interest were pooled and, if necessary, concentrated using a 3kDa molecular weight cutoff filter (Millipore).

Figure 2.1 The configuration of gravity-powered size exclusion column system



2.5.2.2 Size-exclusion chromatography resolution of peanut allergens

Further stage chromatographic purification of allergens was carried out using an AKTA-2 FPLC system (GE Healthcare, Waukesha, USA). Pooled protein-containing fractions were transferred to a MonoQ 5/5 column (GE Healthcare), and ion-exchange chromatography was carried out using a rising gradient concentration of NaCl to elute proteins bound to the column at a low-salt starting condition. Proteins were loaded onto the column using 50mM Tris-HCl containing an initial concentration of 0.1M NaCl (pH 8.2, Sigma-Aldrich), which was

increased over a 40 column volume gradient to a final elution concentration of 1M NaCl. Fractions were collected and analysed by SDS-PAGE and MALDI-MS (see section 2.4). In the case of the 2s albumins, the isoelectric point of the proteins was too acidic to allow effective retention on the column under these conditions, so prior to loading these pooled fractions were buffer-exchanged into 50mM Tris-HCl, 0.2M NaCl (pH 8.2, Sigma-Aldrich), using a 3kDa molecular weight cutoff filter (Millipore). Following elution of the samples, fractions were analysed using SDS-PAGE (see section 2.4) and the fractions were sorted by purity, with the fractions containing the least impurities pooled and proceeding to the final step of the purification process.

2.5.2.3 Size-exclusion chromatography purification of peanut allergens

Pooled fractions from the Ion-Exchange step were loaded onto a Superdex 200 10/30 Column (GE Healthcare), which was run under isocratic elution conditions for 1.2 column volumes, using a chromatography buffer containing 50mM Tris-HCl, 0.2M NaCl, pH8.2. Fractions were collected and assayed for the purity of their protein contents. Following confirmation of purity (see section 2.5.3), samples were buffer-exchanged into pH7.4 PBS (Gibco) using a 3kDa molecular weight cutoff filter (Millipore). The protein content of the final solution was determined by BCA assay (Pierce), according to the manufacturer's instructions. Samples were either used immediately or frozen at -80°C for future use.

2.5.3 Confirmation of allergen purity

The purity of allergen preparations isolated using the above method was assessed using multiple criteria. Protein samples were analysed using SDS-PAGE and silver and Coomassie staining as described in section 2.4. Bands were excised in order to further confirm the

purity of the protein content, and were analysed by MALDI-MS as detailed in section 2.5.1. Furthermore, proteins were assayed for endotoxin content, either by commercial testing service (Lonza), or reporter cell assay (see section 2.3.6). Where necessary, samples were endotoxin-depleted, as outlined in section 2.3.5.

2.5.4 Purified Antigen Modification

Purified peanut allergens were chemically modified in matching ways to the CPH and PPE preparations from which they were isolated. In particular, purified antigens from roasted peanuts were reduced according to the protocol given in section 2.3.10, and fluoresceinated according to the protocol given in section 2.3.11. The success of these procedures was evaluated using the methods described in these sections.

2.6 Animal Procedures

2.6.1 Animals

4-5 week old female Balb/c and C57Bl/6 mice were obtained from the breeding colonies in the Pathology Support Building, Oxford, UK, and maintained in that facility on a diet free from peanut proteins. Additional Balb/c mice and C57Bl/6 mice were obtained from Harlan Laboratories, UK.

2.6.2 Termination of Experiments

All *in vivo* murine experiments were performed under the appropriate licenses and in full accordance with the UK Animals (Scientific Procedures) Act 1986, as well as current best practice guidelines issued by the Home Office Inspectorate. Throughout the *in vivo*

experiments, the principle of humane end points was observed (Wolfensohn 2003), and mice were continuously monitored for adverse effects of the procedures conducted. In particular, during sensitisation experiments, mice were regularly monitored using the modified Hypersensitivity Clinical Disease Scoring system devised by Li *et al. (Li 1999)*Table 2.2) to determine any adverse effects (although none were observed throughout the project). Additionally, experiments were terminated as soon as the collection of data allowed. When experiments were terminated, mice were culled by rising concentration of carbon dioxide, and death was confirmed by vertebral dislocation.

Scale	Symptom
1	*Mild scratching, rubbing or both of head, nose or feet*
2	*Eye/snout puffiness/diarrhoea/pilar erecti/reduced activity*
3	*Wheezing/laboured respiration/cyanosis of mouth and tail*
4	*No activity after prodding/convulsions/tremor*
5	*Death*

Table 2.2 Hypersensitivity Clinical Disease Scoring System, adapted to table form from prose in Li et al(Li 1999).

2.6.3 Tissue Sampling

During the experiments, whole blood samples were taken from the main tail vein at the time points indicated on the respective experimental protocols. Animals were restrained using a conical mouse restraint (built in-house by Sir William Dunn School of Pathology Workshop), and samples were taken according to the method described in Wolfensohn *et al*. (Wolfensohn 2003) in which blood was obtained by pricking the primary tail vein with a

scalpel blade and gently stroking the tail to encourage blood flow. Following culling, a final blood sample was collected by cardiac puncture. Whole blood samples were collected in eppendorf tubes (Eppendorf), and were allowed to clot for 30 minutes at 4°C before centrifugation at 13000rpm for 3 minutes to remove cells and coagulation debris. The resulting serum supernatant was stored at -80°C for future use. In some cases, intact whole spleens and mesenteric lymph nodes (MLN) were removed under aseptic conditions and stored in RPMI (PAA) supplemented with 10% FCS (PAA) at 4°C, to be used immediately in the protocols outlined in Section 2.8.

2.6.4 Intragastric immunisation

Groups of 10-12 week old female Balb/c mice (n = 5 per group) were sensitized orally with a single dose of raw or DR CPH as follows: either (1) without adjuvant at a dose of 15mg protein/mouse; or (2) at a dose of 5mg protein/mouse with 10µg Cholera Holotoxin (List Biological, Campbell, California, USA) co-administered using a curved 22 gauge feeding needle (Kent Scientific, Torrington, Connecticut, USA). The mice were challenged orally at week 4 with 5mg of either raw or DR CPH. Subsequent challenges were performed at weeks 8 and 20 with 15mg of either Raw or DR CPH. Whole blood samples were obtained from mice both immediately before and 16 hours after each challenge, as described in section 2.6.3. Additionally, samples were taken at weeks 2, 6, 12, 20 and 22.

2.6.5 Subcutaneous Immunisation

Groups of 10-12 week old female Balb/c mice (n = various, refer to individual experiments) were immunised subcutaneously (SC) into the flank using 10µl samples of Raw or DR PPE or single allergens (depending on the experiment) dissolved in 100µl sterile pH 7.4 PBS (Gibco,

Carlsbad, USA). As positive controls, groups of mice were immunised in the same way using Alhydrogel (Brenntag, Germany) as an adjuvant suspended in a mixture of protein and PBS (Gibco). Each positive control dose contained 2.5mg Aluminium hydroxide, adjusted to a final volume of 100µl. Mice were subsequently challenged orally at 8, 20 and 30 weeks post-immunisation. Whole blood samples were taken as described in section 2.6.3 at 2, 4, 6, 8, 12, and (for some experiments) 30 and 32 weeks. Where additional boosting of the immunisation was conducted, mice were again injected subcutaneously with the appropriate concentrations of protein (refer to individual experiments) in 100µl pH 7.4 sterile PBS (Gibco).

2.6.6 Epicutaneous Sensitisation

This section of the project was carried out in collaboration with Dr Mario Noti, of the Artis Group, Perelman School of Medicine, University of Pennsylvania. All peanut preparations used were produced and supplied by myself, whereas the sensitisation protocol and initial analysis was carried out by Dr Noti at the University of Pennsylvania. Blood samples were then sent to the Sir William Dunn School of Pathology for further analysis, which was conducted by me.

For epicutaneous (EC) sensitization, groups of 10-12 week old female BALB/c mice (n=3) were treated daily with 2nM of the vitamin D_3 analogue calcipotriol (MC903, Tocris Bioscience, Bristol, UK) in 20µL of 100% ethanol (EtOH). The mixture was placed on the ears in the presence of either 10µg raw or DR PPE, daily for 14 days. As a negative control for the effect of the adjuvant, the same volume of EtOH and PPE were applied without the addition of MC903. All mice were challenged intragastrically with 10mg of either raw or DR CPH on

days 14 and 17 of the experiment. Following the first intragastric challenge with CPH, mice were fed *ad libitum* on either raw or roasted peanut kernels for the remainder of the experimental period.

2.7 Serological Studies

2.7.1 Peanut-specific Immunoglobulin G serum ELISA

2.7.1.1 Basic ELISA

96-well flat-bottomed ELISA plates were coated overnight at 4°C with 10µg/ml raw or DR PPE or individual allergens in 0.1M sodium bicarbonate coating buffer (pH 8.3). All serum samples were assayed in triplicate. After coating the plates were washed three times (PBS, 0.05% Tween), then blocked for one hour at room temperature in 1% BSA in PBS. All subsequent incubation steps were carried out in 1% BSA/PBS, and all subsequent washing steps proceeded as described above. After blocking, serum was added to the wells, either at a single fixed dilution (1:100 unless otherwise indicated in figures for individual experiments) or serially diluted according to experimental requirements. Typically, a range of five-fold dilutions ranging from 1:100 to 1:7.8 x 10^6 was used. Sera from non-immunised mice were used as a negative control. Plates were incubated with serum for one hour, washed, then probed for 1 hour with either a 1:1000 solution of HRP-conjugated anti-mouse IgG1 or IgG2a detection antibodies (BD, Oxford, UK), or a 1:10,000 solution of anti-mouse Total IgG detection antibody (Jackson Immunoresearch, West Grove, USA) for one hour, followed by washing. Wells were developed using Ultra-TMB substrate (Thermo Scientific) and read using an automatic plate reader (Molecular Devices) at 450nm). End point titres

were calculated using a sigmoidal dose-response curve upon a logarithmic transform of the data, using a double-background cut-off point.

2.7.1.2 Competition ELISA

Competition ELISA for immunoglobulin G binding followed the general protocol laid out in section 2.7.1.1, with the following modifications. After coating and blocking as described above, serial dilutions of raw or DR PPE in 1% BSA/PBS were produced, ranging from 0.1µg/ml to 1mg/ml. Wells were pre-incubated with these solutions for 5 minutes before the addition of murine serum. Following this step, the protocol was identical to that described in section 2.7.1.1.

2.7.1.3 Allergen binding comparison

96-well flat-bottomed ELISA plates were coated with individual purified raw or DR peanut allergens, prepared as described in Section 2.3, at a concentration of 10µg/ml. Plates were coated overnight and blocked as described in section 2.7.1.1, and probed in the morning with either murine serum, or anti-Ara h 1 and anti-Ara h 2 monoclonal antibodies (Indoor Biotech, Charlottesville, USA) diluted 1:20,000 as a binding control. After 1 hour of incubation at room temperature, secondary detection antibodies were used as described in section 2.7.1.1.

2.7.2 Peanut-specific Immunoglobulin E Sandwich ELISA

ELISA plates were coated for two hours at 37°C with 10µg/ml anti-mouse IgE capture antibody (BD Bioscience) in PBS coating buffer (pH 7.4). All serum samples were assayed in triplicate. After coating, the plates were washed three times (PBS, 0.05% Tween), then

blocked for one hour at room temperature in 1% BSA in PBS. All subsequent incubation steps were carried out in 1% BSA/PBS, and all subsequent washing steps proceeded as described above. After blocking, serum was added to the wells, either at a single fixed dilution (1:20 unless otherwise indicated) or serially diluted according to experimental requirements. Sera from non-immunised mice were used as a negative control. Plates were incubated overnight at 4°C, washed, then probed for 2 hours with a 10µg/ml solution of biotinylated raw or DR PPE/individual allergens (prepared according to the protocols detailed in section 2.3.7) for one hour. After washing, wells were probed with a 1:1000 solution of Streptavidin-conjugated HRP (GE Healthcare) for one hour, then washed. Wells were developed using Ultra-TMB substrate (Thermo Scientific) and read using an automatic plate reader (Molecular Devices) at 450nm). Results were expressed as response units (RU), calculated by dividing sample OD by non-immune sera mean OD + 3 SD.

2.7.3 Peanut-specific Immunoglobulin G Serum Western Blot

After thorough mixing, the samples were then run on a 4-12% Tris-Glycine gel (Invitrogen). Keyhole Limpet Hemocyanin (KLH) (VWR Biotechnology, Radnor, USA) was used as a negative control for serum reactivity. The gels were equilibrated in Transfer Buffer (50mM Tris-Base, 40mM Glycine, 0.37% SDS, 20% Methanol), then transferred to nitrocellulose membrane (GE Healthcare) for 20miutes at 18 volts using a Transblot Semi-dry transfer system (Bio-Rad), according to the manufacturer's instructions. Membranes were blocked for 1 hour in 3% BSA in PBS, then incubated overnight with serum diluted appropriately for each experiment (ranging between 1:10 to 1:10,000) in 3% BAS/PBS. The following morning, membranes were washed three times with PBS (0.05% Tween-20), then probed for 1 hour with either 1:1000 biotinylated anti-murine IgE or 1:10,000 HRP-conjugated anti-

murine IgG detection antibodies (GE Healthcare), followed by three washes with PBST. For IgE detection Western blots, membranes were subsequently incubated with Bands were visualised using Lightning Plus ECL substrate (PerkinElmer) and R10 X-ray development plates (Fujifilm). A duplicate gel was stained using Coomassie staining (section 2.4.3) to control for relative protein concentration.

2.7.4 Rat Basophilic Leukaemia Degranulation Assay

Degranulation assays were carried out in accordance with the method used by the Van der Merwe Group, of the Sir William Dunn School of Pathology, University of Oxford (unpublished personal communication). RBL cells were donated by the Van der Merwe Group, and maintained in DMEM supplemented with 10% FCS. All assays were conducted in triplicate. For degranulation assays, RBL cells were first armed by the addition of immune or naive (negative control) sera diluted 1:333 overnight, then washed and incubated with 100ng/mL raw or DR PPE in phenol-free RPMI (PAA) for 30 minutes. Hexosaminidase (β-HXA) release was determined by incubating a 1:1 mixture of supernatant and 5mM p-nitrophenyl-N-acetyl-β-glucosaminide (p-NAG) in 0.1M pH 4.5 citrate buffer. Reactions were stopped with an equal volume of 0.1M Na_2CO_3/$NaHCO_3$ solution after 1 hour and optical density was determined at 400nm using an automated plate reader (Molecular Devices). Results were expressed as response units (RU), calculated by dividing sample OD by non-immune sera mean OD + 3 SD.

2.8 Measurement of T-Cell Responses

In order to determine the relative proliferation of T-cells from spleen and lymph nodes upon restimulation by peanut antigens, 1-2 weeks after the final IG challenge cells were isolated

from mesenteric lymph nodes (MLNs) and spleens, and cultured in the presence of either 50 µg/mL raw or DR endotoxin-depleted PPE, or without antigen, for 3-5 days. All assays were conducted in triplicate. As positive controls, pooled cells from each group of mice were stimulated with 5µM Concavalin A (Invitrogen). To determine antigen-specific proliferation, cultures were pulsed after 72h over-night with 1 µCi of [3H] thymidine (Perkin-Elmer) and incorporated tritiated thymidine was assessed as average counts per minute (cpm) using a scintillation counter (Skatron, Molecular Devices, Sunnyvale, USA).

For cytokine measurements, supernatants were collected at day 3-5 and assayed using Bio-Plex mouse cytokine kits and Luminex 100 reader (Bio-Rad). T_h1/T_h2 Cytokine Assay cytokine protein standards (Bio-Rad) were reconstituted and diluted according to the instructions supplied with the individual batch, to produce a distinct standard curve for each individual cytokine. Briefly, T_h1/T_h2 Cytokine Assay Kit beads (Bio-Rad) were vortexed thoroughly then added to a 96-well filter plate (Bio-Rad) pre-wet with Bio-Plex Assay Buffer (Bio-Rad), and vacuumed to dryness with a 96-well plate vacuum manifold (Bio-Rad). Wells were washed, and then portions of cytokine standards as well as unknown samples were added, then incubated for 1 hour with constant agitation. Plates were washed three times with Bio-Plex washing buffer (Bio-Rad), and then incubated for 30 minutes with Bio-Rad detection antibody. After three more washes, the plates were incubated for 10 minutes with streptavidin-PE (Bio-Rad). After three final washes, the beads were resuspended in Bio-Plex Assay Buffer and analysed using a Luminex 100 reader (Bio-Rad) and its associated integral software (Bio-Rad).

2.9 Antigen uptake and binding studies

2.9.1 Receptor Binding ELISA

Proteins to be assayed were coated onto 96-well flat-bottomed ELISA plates at a concentration of 5µg/ml in PBS pH7.4 (PAA) for 1 hour at 37°C. Afterwards, plates were washed three times with PBST, then blocked for 1 hour with TBST-Ultrablock (Thermo Scientific) supplemented with 2mM calcium chloride. Receptors to bind the proteins were diluted to 0.5µg/ml in PBS (PAA), and added to the washed wells, then incubated for 1 hour at 37°C. After washing, plates were incubated for one hour at room temperature with an appropriate HRP-conjugated secondary detection antibody. For murine LOX-1 and murine TLR3 His-tag receptor chimeras (R&D Systems), an anti-penta-His-tag HRP-conjugate detection antibody (Immunokontact, Abdingdon, UK) was used. For murine CD36 and RAGE Fc receptor chimeras (R&D systems), an anti-murine IgG Fc HRP conjugate antibody (Jackson Immunoresearch) was used. In either case, wells were developed using Ultra-TMB substrate (Thermo Scientific) and read using an automatic plate reader (Molecular Devices) at 450nm.

2.9.2 Production of GM-CSF Medium

A Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) secreting L929 cell line was given by Mr Simon Hackett, of the Sir William Dunn School of Pathology. Cells were maintained in DMEM (PAA) supplemented with 10% FCS, and expanded to produce large populations for harvesting. When the desired population size for the batch was attained, cells were incubated at full confluence for three days, allowing GM-CSF to accumulate in the cell medium. After this period had elapsed, the supernatant was removed with a pipette (Gilson) and centrifuged to remove any remaining cell debris. The appropriate dilution of GM-CSF medium to use for deriving BM-DC was determined at 3% v/v by Mr Simon Hackett

using a thymidine incorporation cell proliferation assay (for the general principles of this assay, please refer to section 3.8). Batches of GM-CSF medium were stored at -80°C for future use.

2.9.3 Derivation of Dendritic Cells

Mice (Balb/c or C57Bl/6) were euthanized using Schedule 1 procedures, and femora and tibiae were removed and stored at 4°C in RPMI Handling Medium (PAA) supplemented with 10% FCS. The ends of the bones were punctured using hypodermic needles and the bone marrow was flushed out with handling medium. The resulting suspension was filtered through a 70µm filter unit (BD Bioscience), centrifuged and re-suspended into Handling Medium. Cells were counted and plated at a density of 1 million cells/ml in BMDC Derivation Medium (RMPI, 10% FCS, 0.00035% 2-mercaptoethanol, 3% GM-CSF-Medium [see section 2.9.2]), then incubated. After three days, the supernatant was removed, and replaced with fresh Derivation Medium, supplemented by 10ng/ml IL-4 (Invitrogen). After three further days of incubation, supernatants were again removed and the cells loosened using a warm solution of 10mM Lidocaine and 140mM EDTA, pH 7.4. After 10 minutes of incubation, cells were brought into suspension by gentle pipetting, centrifuged and resuspended in Optimem low-protein medium (PAA). Cells were counted and then used immediately in one of the following experimental protocols.

2.9.4 Dendritic Cell Stimulation

BMDCs were harvested as described in section 2.9.2, then re-suspended at a concentration of 2 million cells/ml, then plated onto a 24-well plate in 100µl portions, to give a final cell count of 0.2 x 10^6 cells per well. Cells were then stimulated with preparations of antigens at a concentration of 100µg/ml. LPS (Sigma) was used as a positive control in a titration of doses from 0.001-1ng/ml. As a negative control, cells were pre-incubated with Polymixin B (Invitrogen) at a concentration of 20µg/ml for 5 minutes before the addition of antigens or LPS. When all stimuli had been added, cells were incubated overnight, after which the supernatants were removed by pipette, centrifuged, transferred to a fresh 96-well plate, then frozen at -80°C for future use. Meanwhile, the cells were lifted using warmed 10mM Lidocaine and 140mM EDTA, pH 7.4, followed by gentle pipetting. The resulting suspensions were centrifuged then used for flow cytometry analysis as described in section 2.9.7.

2.9.5 Cell surface binding assay

BMDCs were harvested as described in section 3.9.2, then re-suspended at a concentration of 2 million cells/ml, then plated onto a 24-well plate in 100µl portions, to give a final cell count of 0.2 x 10^6 cells per well. The plates were incubated overnight at 37°C, after which the medium was removed and the cells washed once with warm PBS. DC macropinocytosis was inhibited by the addition of either 10µM Jasplakinolide or 5µM Cytochalasin B. The wells were incubated for 1 hour to ensure that inhibition was complete. Cells were then pulsed with 100µl Optimem medium (PAA), containing 2µg/ml fluoresceinated antigen (prepared as described in section 2.3.11). The plates were incubated for between 30 minutes and 1 hour, depending on the experiment, and then the cells were lifted by pipetting and then washed twice with chilled FACS buffer (see page 89) on ice. Cells were

centrifuged in a 96-well round-bottomed plate, resuspended in 200µl FACS buffer and used immediately for flow cytometry analysis.

2.9.6 Inhibition of Acetylated Low Density Lipoprotein uptake

BMDCs were prepared and re-plated as described in section 2.9.4. After overnight incubation, cells were then pre-incubated with 50µl of antigen in Optimem medium (PAA) for 5 minutes, before being pulsed for 30 minutes with a further 50µl of Optimem containing either 1 or 0.1µg/ml dil-labelled Acetylated LDL (Biomedical Technologies, Inc., Stoughton, USA). Maleylated BSA (Mal-BSA), donated by Dr Amin Moghaddam, was used as a positive inhibition control. Cells were washed twice with warmed Optimem before the cells were lifted using 10mM Lidocaine and 140mM EDTA, followed by gentle pipetting. Cells were centrifuged in a 96-well round-bottomed plate and either used immediately for flow cytometry (section 2.9.7) or fixed in 4% formaldehyde in PBS for 30 minutes at room temperature. Fixed cells were centrifuged again to remove the fixative then resuspended in PBS. They were stored at 4°C for later use.

2.9.7 Flow cytometric analysis of stained cell populations

Upon completion of the relevant experimental protocol, cells were harvested, either through pipetting alone, or after 10 minutes of incubation with warmed Lifting Solution (10mM Lidocaine and 19mM EDTA, pH 7.4), and transferred to a 96-well round-bottomed plate. Cells were then centrifuged and resuspended into FACS Buffer (PBS, pH 7.4, 2% FCS), containing a 1:50 dilution of Fc-Receptor Blocker (BD Bioscience), and incubated on ice for 5 minutes. During this period, solutions of detection antibodies were prepared, at 2X final concentration. Depending on the experiment, different combinations of antibody targets

and dyes were utilised, which are summarised in Table 2.3. Note that in each experiment, only one example of each dye conjugate could be measured at a time, requiring more than one combination to be used in some experiments.

Antibody target	*Labelling dye*	*Dilution (from stock)*	*Manufacturer*
MHC Class II	Peridinin chlorophyll protein (PerCP)	1:300	R&D Systems
CD11/c	Allophycocyanin (APC)	1:100	BD Bioscience
CD40	Fluorescein (FITC)	1:100	BD Bioscience
CD80	Fluorescein (FITC)	1:10	R&D Systems
CD86	Phycoerythrin (PE)	1:100	BD Bioscience
OX40-Ligand	Phycoerythrin (PE)	1:100	BD Bioscience

Table 2.3 Antibodies used for flow cytometry in this project

When 5 minutes had elapsed, the 2X antibody mixtures were added to the wells and mixed thoroughly, then incubated at 4°C with gentle shaking for a further 30 minutes. Afterwards, the plates were centrifuged, and the supernatant was removed. Wells were washed three times with 200µl FACS Buffer, with resuspending each time, before being transferred to 4mm FACS tubes and stored on ice for analysis. Cells were analysed using a FACSCalibur (BD Bioscience) flow cytometry machine, according to the manufacturer's recommendations. Specific gating strategies used are explained and indicated where appropriate in the respective results chapters.

Briefly, using an unstained, untreated cell sample, voltage amplification for Forward and Side-scattering lasers (FSC and SSC), was fine-tuned for the individual cell type, such that the population of live cells formed a distinct group in approximately the centre of the visual

field. The area containing this population was then gated, and subsequent analysis was performed on this gate. Untreated cell samples singly stained in the appropriate colours for a highly-positive cell surface marker were used to compensate for bleeding between the emission spectra of the system's four coloured lasers. With compensation completed, settings were saved and then applied to all of the samples measured in the experiment.

2.10 Statistical Analyses

Data analysis and presentation was performed using a combination of Microsoft Excel 2007 (Microsoft, Redmond, USA), GraphPad Prism version 6.01 (Graphpad Software, La Jolla, California), and Flowjo version 10 (Tree Star Inc., Ashland, USA).

Statistical analyses were performed using GraphPad Prism version 6.01 (Graphpad Software). For normally distributed data groups, statistical significance was assessed using parametric unpaired Student's t-tests. If data groups were not normally distributed, an unpaired Mann-Whitney U-test was used.

Unless otherwise stated, all bar charts throughout this thesis represent the mean of data +/- one standard deviation.

Chapter 3
Characterisation and purification of peanut allergens

3.1 Abstract

Commercial dry-roasted peanuts are widely available but are subject to variability in terms of conditions of roasting and storage. To generate a reproducible source of DR peanut allergens, DR peanuts from commercial sources were homogenised and compared to samples roasted under laboratory conditions from a starting point of raw, fresh peanuts. The two sets were compared in terms of protein oxidation and allergen solubility, and found to be broadly comparable under a set of laboratory roasting and extraction conditions optimised for allergen recovery. Next, samples of raw and DR peanut protein extracts were subjected to serial chromatography, and purified peanut allergen Ara h 1, and a mixture of the peanut 2s albumin allergens (Ara h 2, 6 and 7) were isolated. These were analysed according to the same methodology as their source solutions, and found to be representative of the total population in terms of protein oxidation. Furthermore, the purified Ara h 1 preparations were subjected to additional tests which could not be conducted on a protein mixture, including light scattering and glycosylation state analysis, allowing further clarification of the relevant differences between raw and DR peanut allergens.

3.2 Introduction

Dry-roasting (DR) of peanuts at high temperatures leads to intense protein oxidation via the Maillard Reaction. This leads to the formation of advanced glycation end products (AGEs) (Nguyen 2006), which have been implicated in varied human disease states (Harja, Bu et al. 2008; Negre-Salvayre, Salvayre et al. 2009); and reactive aldehydic carbonyls (RC), which have been directly implicated in the immunomodulation of antigens (O'Brien, Siraki et al. 2005; Moghaddam, Olszewska et al. 2006). One of the principal aims of this project is to develop models of the effects of DR peanuts on human allergy. This will be achieved by a comparison of the relative immunogenicity of raw and DR peanut preparations through intragastric, subcutaneous, and epicutaneous routes. Crude peanut homogenates (CPH) are most desirable for intragastric administration, as it resembles the immediate product of mastication but can also be delivered in accurate doses. However, CPH is disadvantageous for other purposes because it contains many other oxidation products besides modified protein (Moreno, Mackie et al. 2005). To investigate specifically the effects of protein oxidation, peanut protein extracts (PPE) and purified individual peanut allergens will also be prepared for use in subcutaneous immunisation.

It is of vital importance to ensure that any laboratory-prepared DR peanuts are comparable in levels of oxidation to the commercially available DR peanuts which are consumed (Chung, Maleki et al. 2004). Despite being cheap and ubiquitously available (Finkelman 2010), commercially produced DR preparations are potentially problematic when applied to experimental allergy models. This introduction will explain these difficulties and outline a strategy for characterising oxidised peanut preparations for experimental analysis.

3.2.1 Comprehensive characterisation of DR peanut samples

As was previously alluded to in the general introduction, prior studies of DR peanut allergenicity have been frequently marked by highly variable reproducibility of key results between different research groups (Maleki, Chung et al. 2000) (Maleki 2004). I would submit that the most plausible explanation for this effect is inconsistency between the peanut preparations used by different groups. This is because the Maillard Reaction is both critically time and temperature dependent (Thornalley 2005). It could therefore be predicted that DR peanuts, prepared, extracted, and purified under different conditions in different laboratories, should display considerable variability in their biological properties.

Because the extent of protein oxidation is rarely quantified in roasted peanut preparations used in immunogenicity studies (for examples, see (Adel-Patient, Bernard et al. 2005; van Wijk, Nierkens et al. 2005; Sun, Arias et al. 2007; Arias, Chu et al. 2011; Yu 2011)), it is very difficult to compare directly the roasted peanut samples used in different papers, as they have used different peanut cultivars, suppliers, and roasting conditions. Given that other researchers have argued that subtle differences between roasted, boiled and fried peanut proteins maybe be immunologically important (Beyer, Morrow et al. 2001), it would seem that differences between preparations of roasted peanuts are likely to contribute to the inconsistencies between DR peanut allergenicity publications, which have reported divergent patient serum IgE binding (Maleki 2004) and Western blotting (Maleki, Chung et al. 2000; Mondoulet, Paty et al. 2005) reactivity of DR peanut preparations.

Although commercial batches of peanuts are regularly monitored for consistency in such matters as palatability (Oupadissakoon and Young 1984), and the absence of aflatoxin (Lee,

Wang et al. 2004), other factors which may well be physiologically relevant are typically not monitored. The probable heterogeneity between different batches and cultivars of commercially roasted peanuts (Ozias-Akins, Ramos et al. 2009), as well as the widely variable conditions and duration of storage (Lee 2006), render these supplies subject to numerous variables, which makes them far from desirable as a source of allergens for reproducible experiments, especially in cases where the relevant differences between batches may be subtle. On balance, therefore, DR peanut samples prepared under controlled laboratory conditions are preferable to those available commercially.

3.2.2 Recombinant protein is unsuitable for the task at hand

Despite the apparent disadvantages of harvesting protein directly from peanut tissue, relative to recombinant expression (primarily lower yield and greater presence of impurities (Palomares, Estrada-Mondaca et al. 2004)), recombinant peanut allergens are unsuitable in this case. In addition to the usual problem of different glycosylation patterns between the organism of origin and that of expression (Demain and Vaishnav 2009), recombinant allergens can only be produced in their native state without oxidation and AGE modifications (Burks, Cockrell et al. 1995). Because there is no feasible method of dry-roasting a purified protein solution, targeted chemical modifications to generate AGE and reactive aldehydic carbonyl RCs have been developed (Starkl, Krishnamurthy et al. 2011; Vissers, Iwan et al. 2011). However, there are reasons to doubt that any of these methods can fully emulate the range and complexity of modifications produced by dry-roasting. When whole tissues are roasted, a wide variety of biological sugars and metabolic intermediates can react with protein via the Maillard Reaction, producing AGEs of great complexity and variety (Iwan, Vissers et al. 2011). By contrast, models of AGE production by

incubation of proteins with glucose (Chung and Champagne 2001) or modification with glycoaldehyde (Glomb and Monnier 1995), make use of only a single reagent and therefore produce a more limited range of AGEs and RCs. Accordingly, the preparation of protein from whole DR peanut tissues is preferable for modelling of immunogenicity.

3.2.3 Roasting effects can be monitored by allergen solubility and reactive carbonyl formation

To ensure the consistency of oxidation state between CPH and other, more refined peanut preparations such as PPE and single allergens, it is important to find ways to determine the progress of AGE formation through the Maillard Reaction (Verzijl, DeGroot et al. 2000; Uribarri, Woodruff et al. 2010). Whilst many of the products of the Maillard Reaction cannot be detected without specialised antibodies (Singh, Barden et al. 2001) or mass spectrometry (Zhang, Ames et al. 2009), a wide range of reaction products known as RC will readily interact with DNPH (Grimsrud, Xie et al. 2008), which can then be detected by a simple ELISA (Buss, Chan et al. 1997). Accordingly, these RCs can be used as a measure of AGE formation due to protein oxidation (Mondoulet, Paty et al. 2005; Pomes, Butts et al. 2006).

A second change associated with DR which is of particular concern, is the decrease in solubility of known peanut allergens. In particular, the major peanut allergen Ara h 1 has a tendency to aggregate (Blanc, Vissers et al. 2011), which is of dual importance because this propensity has been associated with enhanced immunogenicity (Breiteneder and Mills 2005) and allergenicity (Rosenberg 2006). This effect could be particularly significant for PPE, which contains only soluble protein, and for the purification of individual soluble allergens from PPE (Beyer, Morrow et al. 2001). Accordingly, it is of vital importance to

characterise the relative abundance of Ara h 1 and other peanut allergens by polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970).

3.3.3 Peanut allergens can be separated by a combination of chromatographic methods

As a final step in the separation of peanut proteins from crude homogenates, the two major peanut allergens Ara h 1 and Ara h 2 will be purified from raw and DR PPE to allow the investigation of their individual properties and immunogenicity. The task of purifying the major peanut allergens Ara h 1 and Ara h 2 is made easier by the fact that they are dramatically different proteins. Ara h 1 is a homotrimeric 63kDa protein (Cabanos, Urabe et al. 2010), whereas Ara h 2 is monomeric and its alternate gene products range in weight from 17-24kDa in weight (Moreno and Clemente 2008), meaning that in their respective physiological states, Ara h 1 is almost an order of magnitude larger than Ara h 2. This opens the possibility of separating them based on their size, a long-practiced technique in protein chromatographic purification known as size exclusion (Hagel 2001; Janson 2011). Additionally, both proteins have pronouncedly acidic pI values (the pH at which all of the charges of a protein's amino acid residues are exactly balanced (Berg 2006)), which may be exploited in anion-exchange chromatography (Janson 2011).

Because it is rare for non-homologous proteins to coincide both in molecular weight and pI, it would seem likely that a combination of size exclusion and anion exchange chromatography should produce the best results for purifying raw and DR Ara h 1 and Ara h 2. A similar strategy has been successfully used in the past to purify raw Ara h 1, (Mills, Potts et al. 1997) although whether such techniques can be extended to the highly oxidised forms of DR Ara h 1 remains to be seen, as these will possess different physicochemical

properties in light of their modifications (Chung, Maleki et al. 2004), which may affect the purification process. It also remains to be seen whether the differences between Ara h 2 and the other highly homologous 2s albumin peanut allergens (Ara h 6 and 7) (Schmidt, Krause et al.) are sufficient to allow them to be separated by this method.

3.3 Chapter goals

The following experimental strategy was used to characterise, extract and purify peanut allergen preparations:

1. Preparations of commercially roasted peanuts will be analysed for of their carbonyl content and allergen solubility;
2. Raw, fresh peanuts will be roasted under laboratory conditions to emulate these properties of commercial preparations;
3. An optimal balance of roasting and extraction conditions will be determined for allergen purification;
4. Protein extracts from both raw and DR peanut will be separated by chromatography to isolate individual peanut allergens;
5. The purity of these allergens will be assessed, and their individual properties characterised to ensure consistency with the mixtures from which they were derived.

3.4 Methods used in this Chapter

The follow methods are described in full in the indicated sections of Chapter 3, which contains full protocols and descriptions for all materials and methods used in this project.

- Roasting of peanuts **(2.3.1)**
- Determination of protein concentration **(2.3.2)**
- Preparation of CPH **(2.3.3)**
- Preparation of PPE **(2.3.4)**

- Quantification of Protein Carbonyl Content by Colorimetry **(2.3.7)**
- Estimation of Protein Carbonyl Content by ELISA **(2.3.8)**
- Light scattering measurement **(2.3.12)**

- Reducing Polyacrylamide gel electrophoresis **(2.4.1)**
- Native gel electrophoresis **(2.4.2)**
- Coomassie staining **(2.4.3)**
- Anti-carbonyl Western blot **(2.4.5)**

- Protein band assignment by mass spectrometry **(2.5.1)**
- Chromatographic purification of peanut allergens **(2.5.2)**
- Confirmation of allergen purity **(2.5.3)**

3.5 Results

3.5.1 Dry-roasting induces reactive carbonyl formation and loss of allergen solubility comparable to commercial peanut preparations

A range of typical samples of commercially prepared DR peanut from diverse sources were obtained and analysed according the criteria of known markers of protein oxidation. In order to provide a fair comparison, fresh raw peanuts of the same cultivar (*Jumbo*) were obtained from an American farm. The raw and DR peanuts were crushed and the ground paste solubilised to produce Crude Peanut Homogenate (CPH), and the resulting samples were compared using reducing SDS-PAGE conditions and colorimetric carbonyl assay. The major peanut allergen bands (Ara h 1 and 2) on SDS-PAGE gels were assigned by mass spectrometry (Ara h 1 band total *MASCOT* ion score = 490; Ara h 2 bands total *MASCOT* ion score = 136, 129) and agreement with previous reports (Pomes, Butts et al. 2006) (Kim, Lee et al. 2013). The minor peanut allergen Ara h 3/4 band was also identified from previous publications (Koppelman, Hefle et al. 2010).

As expected, the commercial DR peanut preparations exhibited dramatically increased levels of RC relative to raw (Figure 3.1A); as well as a pronounced relative decrease of the Ara h 1 band, with a corresponding increase of other allergens as a percentage of total protein content (Figure 3.1B). Next, samples of fresh, raw peanuts were submitted to dry roasting in a laboratory oven, at a temperature (178°C) commonly used by peanut industry professionals (Woodroof 1996). Owing to the very small batches of peanuts which were being prepared, the total roasting time was drastically scaled down in order to compensate for the faster heating in the model system. Accordingly, the samples were roasted ranging from five minutes to half an hour. Reactive carbonyl formation began to become noticeable

even after relatively brief periods of roasting (Figure 3.1C), and rapidly increased thereafter, eventually matching and exceeding the carbonyl content of the commercially roasted sample (Brand B).

Because the colorimetric DNPH carbonyl assay requires milligram quantities of protein to be effective (Levine 1990), it was compared to a more economical ELISA-based carbonyl assay based on the same chemical reaction (Alamdari, Kostidou et al. 2005). Carbonyl detection using this method was found to produce highly similar results to the colorimetric assay (Figure 3.1D), and was therefore adopted for all carbonyl assays of valuable purified peanut protein extracts for which milligram quantities could not be spared.

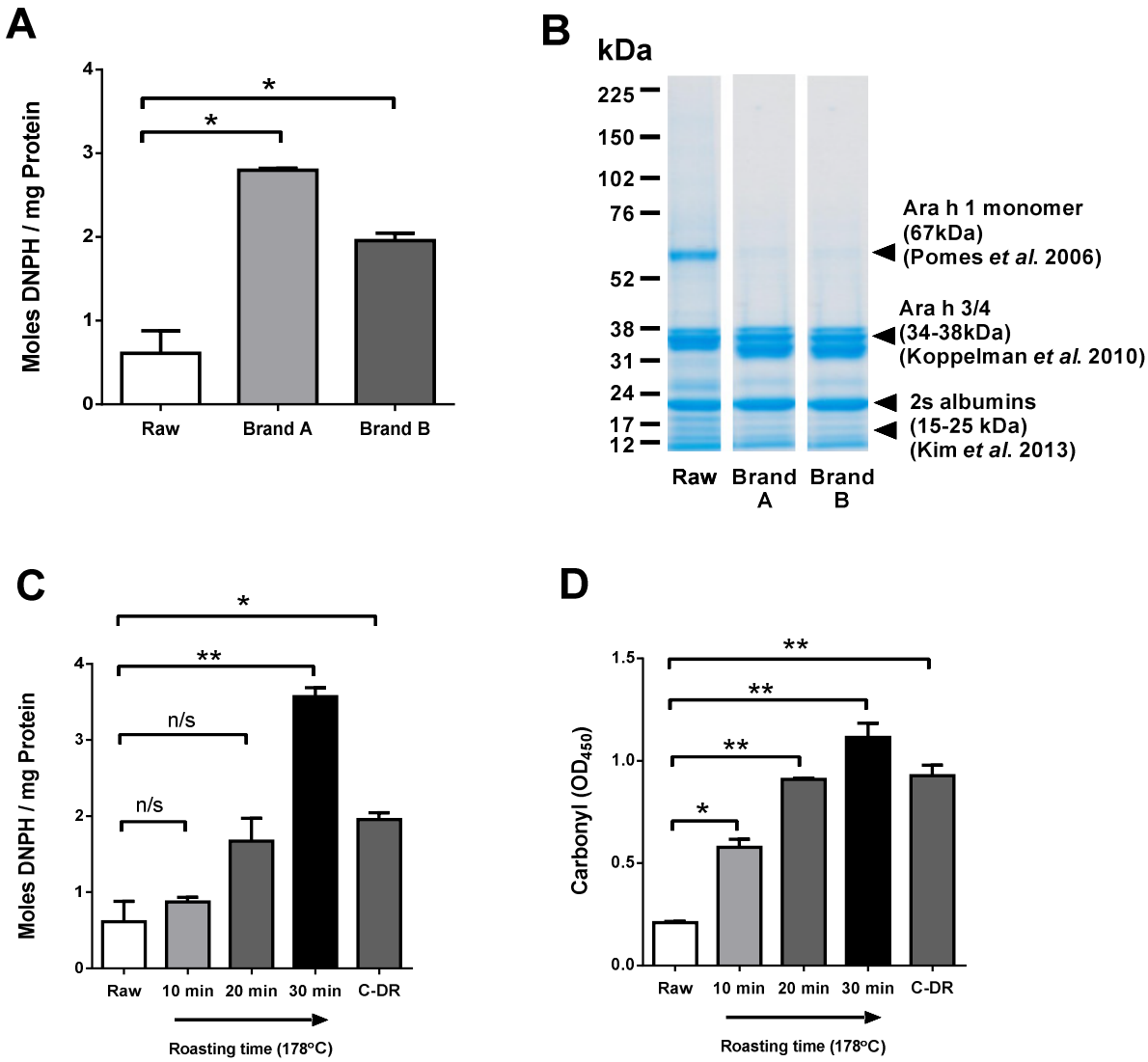


Figure 3.1 Roasting-associated properties of commercial and laboratory DR crude peanut homogenate (CPH). **(A)** Higher carbonyl adduct content in commercial DR CPH relative to raw, as measured by colorimetric carbonyl assay. **(B)** Lower solubility of Ara h 1 in commercial DR CPH, as revealed by reducing 4-12% SDS-PAGE gel. **(C-D)** Formation of reactive carbonyls with increased laboratory roasting time, as measured by **(C)** colorimetric assay, and **(D)** anti-carbonyl ELISA (background OD ≤ 0.12). Commercially roasted Brand B included for comparison. *N* = 3, representative of at least 3 independent experiments. * $p = \leq 0.05$; ** $p = \leq 0.01$; n/s = non-significant; Student's *T*-test

3.5.2 Intermediate roasting conditions are optimal for recovery of soluble roasted allergens

Peanut protein extract (PPE) samples were extracted from the raw and DR peanut samples and analysed according to the same criteria as their corresponding CPH preparations. When using PBS as a solvent, protein yield per gram of peanut flour was drastically lower for DR peanut relative to raw, and the recovery of Ara h 1 was prohibitively low even under moderate roasting conditions (data not shown). As a result, a more potent sodium carbonate extraction buffer was utilised, following the example set in Poms *et al* (Poms, Capelletti et al. 2004). Protein recovery was markedly improved, both in terms of overall quantity and specific recovery of Ara h 1, which came to more closely resemble the band proportions of CPH under intermediate roasting conditions of 20 minutes (Figure 3.2A). At longer roasting times (30 minutes onward) the carbonyl content of the solubilised fraction of peanut proteins began to decrease relative to the earlier time points (Figure 3.2B).

Because the high pH of this extraction buffer was unsuitable for physiological experiments, and also raised concerns regarding possible protein denaturation, following extraction the PPE samples were buffer-exchanged into PBS, and then analysed using NATIVE-PAGE gels to determine the homotrimer formation and overall oligomerisation state of the recovered proteins. This comparison demonstrated that the Ara h 1 trimer and oligomers in both raw and DR PPE ran at identical weights to those found in their CPH equivalents (Figure 3.3), although high molecular weight oligomers and aggregates were seen to be much more common in DR CPH than in DR PPE.

Thus, the following conditions for PPE preparation were adopted – the roasting of raw peanut under moderate conditions (20 minutes at 178°C), followed by defatting and

carbonate buffer extraction, and in turn followed by buffer exchange into PBS. The addition of this intermediate step permitted a far higher efficiency of allergen extraction than would have been possible using a PBS solvent alone. Accordingly, CPH prepared from peanuts roasted under these same conditions was used for all subsequent experiments, in order to ensure that the level of oxidation used in parallel experiments was comparable.

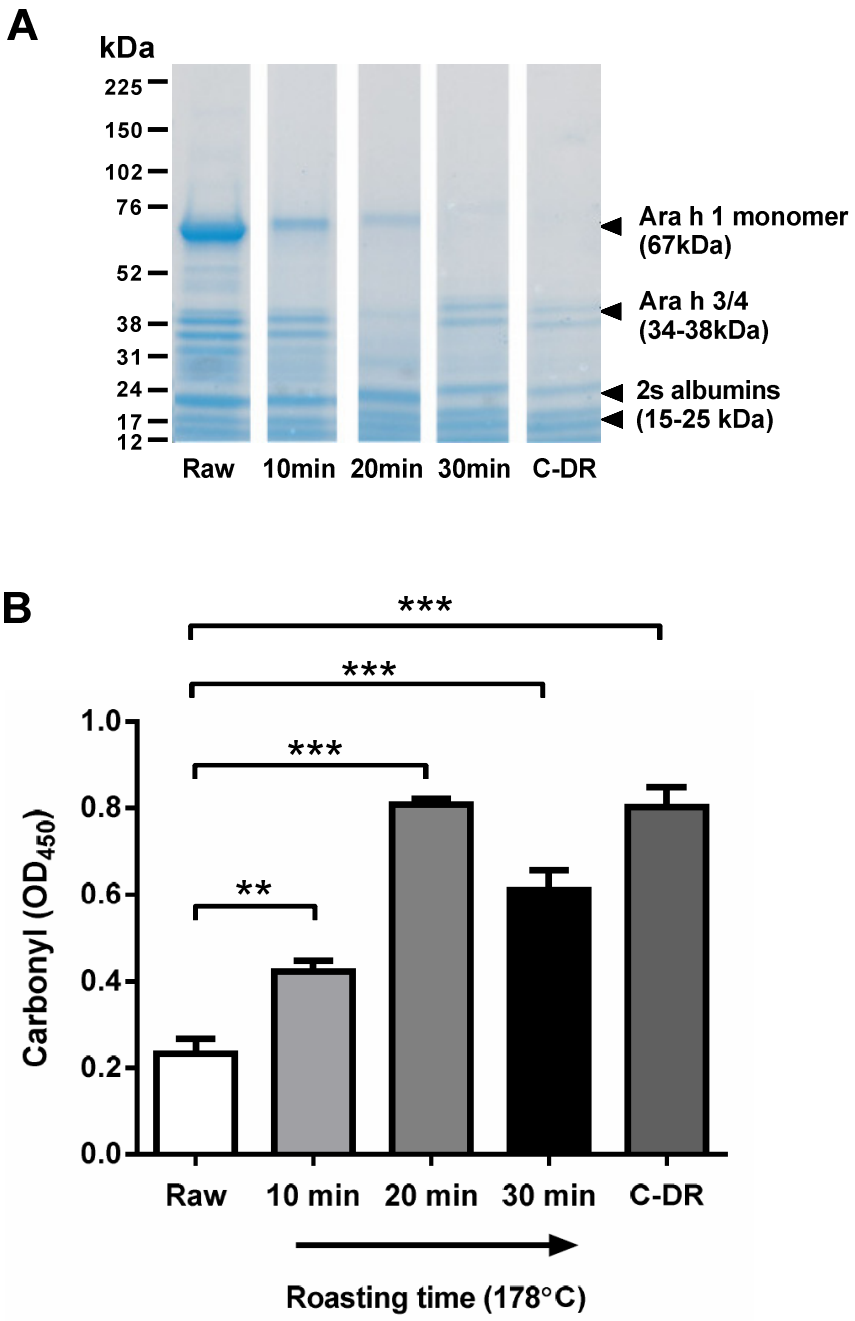


Figure 3.2 Loss of Ara h 1 solubility visualised by reducing 4-12% SDS-PAGE gel **(A),** and formation of reactive carbonyl groups measured by anti-carbonyl ELISA **(B**, background OD ≤ 0.12**),** in peanut protein extract (PPE) with increasing laboratory roasting time. *N* = 3, representative of at least 3 independent experiments. ** $p = \leq 0.01$; *** $p = \leq 0.001$; Student's *T*-test

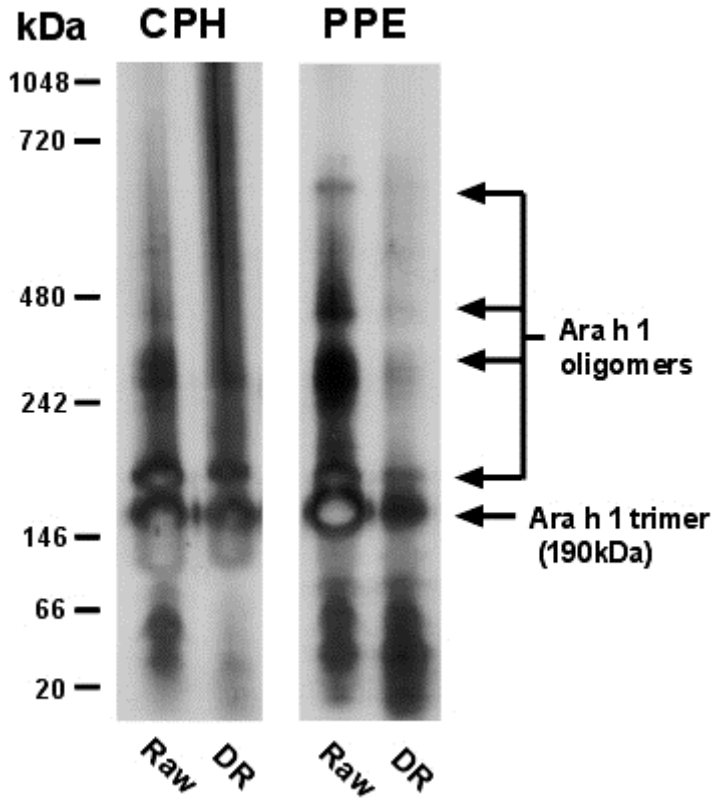


Figure 3.3 Native PAGE gel showing the oligomerisation state and native running masses of peanut allergens in CPH and buffer-exchanged PPE.

3.5.3 Reactive carbonyl formation is distributed evenly across the major peanut allergens

One remaining concern over the PPE extraction process results from a shortcoming of the method of RC detection in whole peanut extracts. Because the colorimetric carbonyl assay can only measure the *total* carbonyl content of a protein mixture as a whole, it cannot be used to determine whether or not the individual peanut allergens are equally oxidised, even in the case of CPH.

This question becomes even more acute in the case of PPE, for which we already have good evidence to suggest that the major Ara h 1 allergen is the least likely to solubilise successfully during the extraction process (Figure 3.2A and B). Before allergen purification could commence, therefore, a method of discerning the carbonylation state of individual protein bands was critical. Such a procedure is available in the form of an anti-RC Western blot. Using this technique, it was found that, taking the relative quantities of protein into account, carbonylation was increased across all three of the most important peanut allergens (Figure 3.4A). This discrepancy was not due to differences in the protein loading concentration (Figure 3.4B).

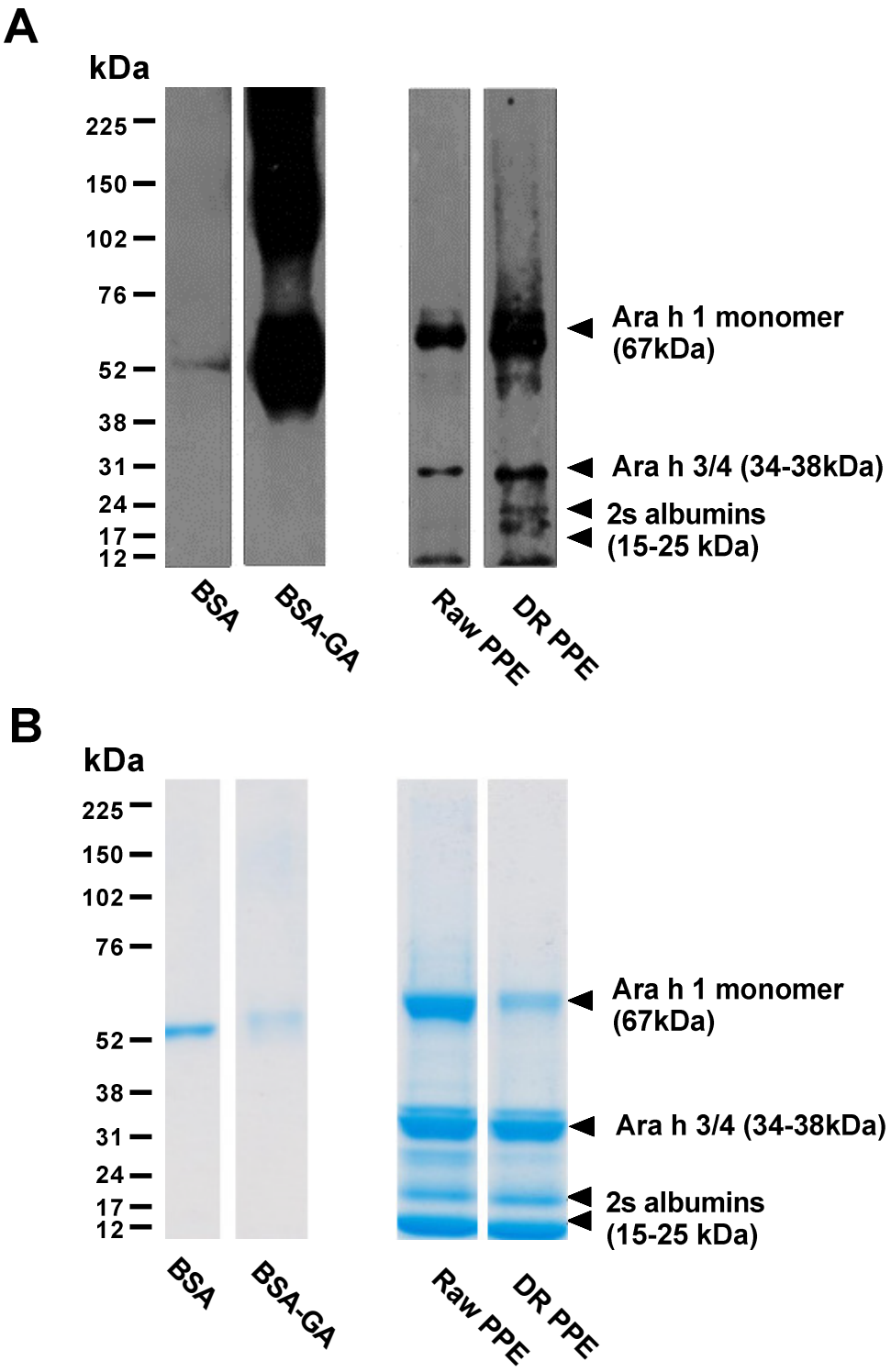


Figure 3.4 (A) Anti-carbonyl western blot of raw and DR PPE, performed on reducing 4-12% gradient SDS-PAGE gel. Controls: unmodified Bovine Serum Albumin (BSA), glycoaldehyde-oxidised BSA (BSA-GA), and reduced BSA-GA (BSA-GA-R), **(B)** Protein loading controls, consisting of a Coomassie stain of a duplicate gel.

3.5.4 The physicochemical differences between raw and DR Ara h 1 dramatically affect their purification

The sequential purification of the two major peanut allergens from the selected raw and DR preparations was undertaken based on a protocol originally devised by Mills *et al*. (Mills, Potts et al. 1997) Peanut allergens were first separated by their mass/charge ratio using anion-exchange chromatography, and secondly by removing the residual impurities in the resultant fractions by size exclusion. Attempts at purification were restricted to Ara h 1 and 2 by virtue of their overwhelming predominance in the literature as the two most common allergens to which peanut-allergic patients respond (Spanhaak, De Jong et al. 1998; Koppelman, Wensing et al. 2004; Flinterman, van Hoffen et al. 2007).

In order to enrich fractions containing these two allergens prior to attempting anion exchange, a preliminary step was introduced in which the aim was to crudely separate broad classes of peanut proteins by molecular weight in a gravity-feed, low-resolution G-50 size exclusion column. Despite the large difference in weight and oligomerisation between the two allergens, when analysed by SDS-PAGE, fractions containing the Ara h 1 band (Shreffler, Castro et al. 2006) and other vicilins could not be resolved from those containing the low molecular weight bands corresponding to Ara h 2 and the other 2s albumins (Vissers, Blanc et al. 2011; Zhuang, Durrani et al. 2012) for either raw or DR PPE (Figure 3.5A and B). Nevertheless, this preliminary step successfully removed other, non-proteinaceous impurities such as carbohydrates and cell debris from these fractions. The fractions containing the greatest amounts of Ara h 1 were transferred to an ion-exchange column, where the differences between raw and DR Ara h 1 became apparent. Whereas raw Ara h 1 could be readily resolved under the conditions used, forming a distinct peak at

approximately 35-40 minutes (Figure 3.6A and B), in the case of DR Ara h 1 this peak was merged with the adjacent peak (Figure 3.7A), which SDS-PAGE revealed to contain Ara h 3 and 4 (Figure 3.7B). Due to near complete overlap of these peaks, there was a substantial residual DR Ara h 3/4 contamination in the majority of these fractions (Figure 3.7B).

In consequence, a third and final purification step was required in which the fractions containing a DR Ara h 1/3/4 mixture were subjected to another run of analytical grade size exclusion. This approach can often be sufficient to separate proteins with a comparable difference in molecular weight to that between the two large peanut allergens (Janson 2011). In this case, the resolution was only partially successful, with some overlap of the two peaks remaining (Figure 3.8A). Accordingly, it was decided to prioritise purity over quantity, and to only retain the fractions at the front of the Ara h 1 peak which contained the purest DR Ara h 1 (Figure 3.8B).

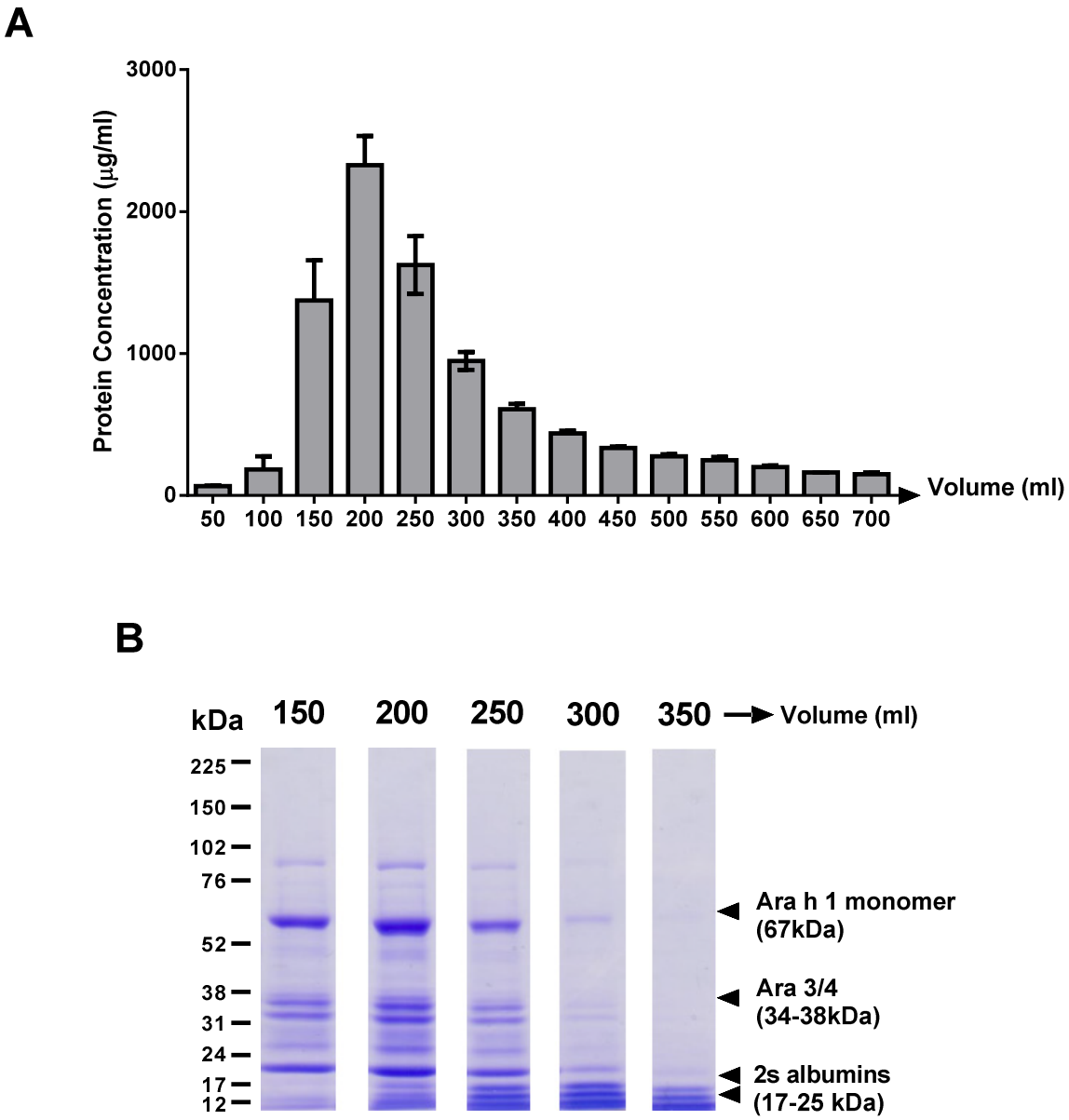


Figure 3.5 Elution profile of raw PPE in the preliminary size exclusion purification step. **(A)** Chromatogram of peanut protein elution from G-50 size exclusion, measured by BCA assay and plotted against time. *N* = 3. **(B)** Reducing 4-12% SDS-PAGE gel of selected fractions from raw PPE elution, indicating the individual peanut allergens. Representative of three independent experiments.

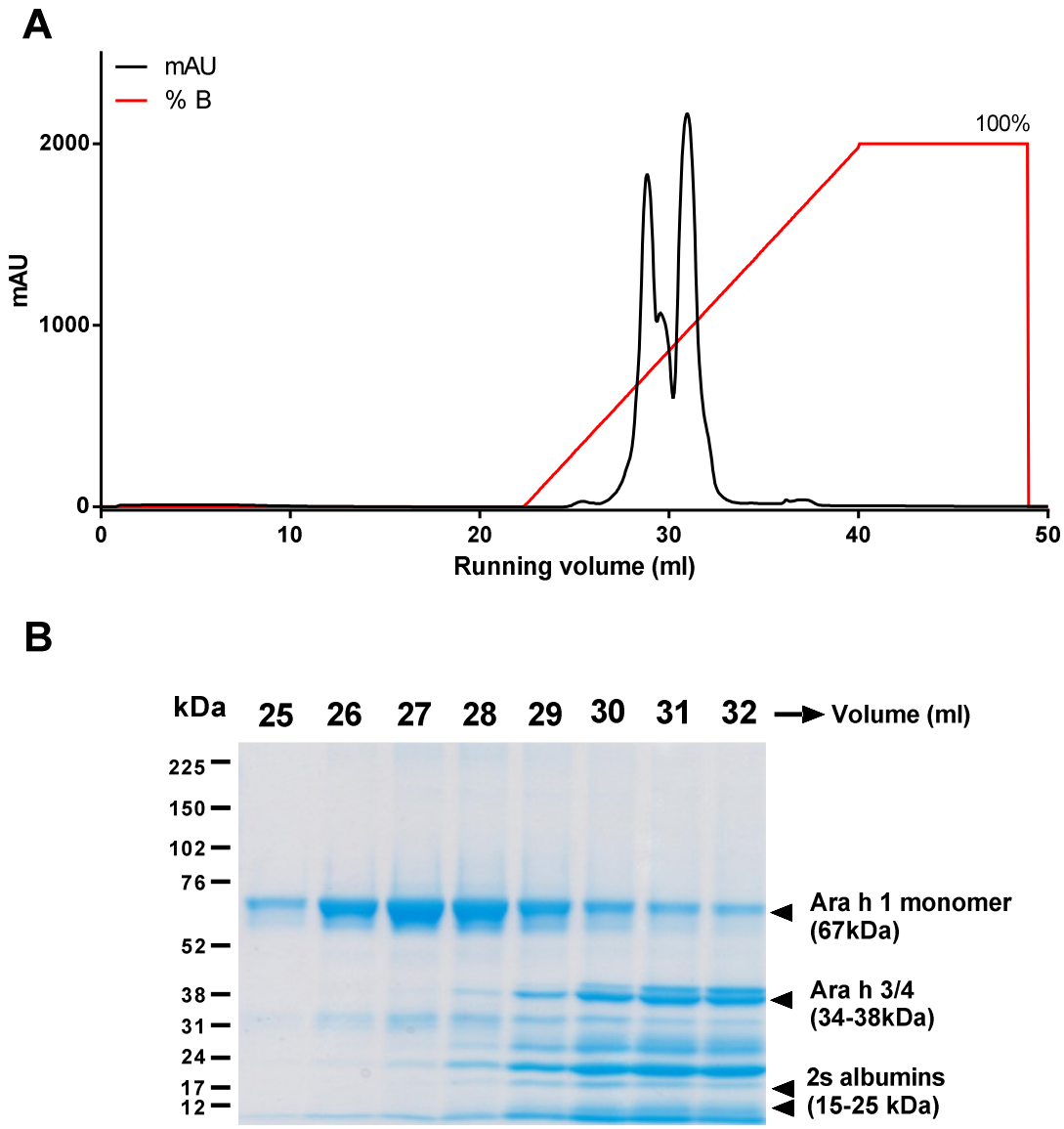


Figure 3.6 Elution profile of Ara h 1-enriched raw PPE fractions in anion exchange chromatography. **(A)** Chromatogram of raw Ara h 1 elution, showing protein concentration (black) measured by UV absorbance, together with percentage rising elution buffer (red). *N* = 3. **(B)** Reducing 4-12% SDS-PAGE gel of selected fractions from raw Ara h 1 elution, indicating the individual peanut allergens. Representative of four independent experiments.

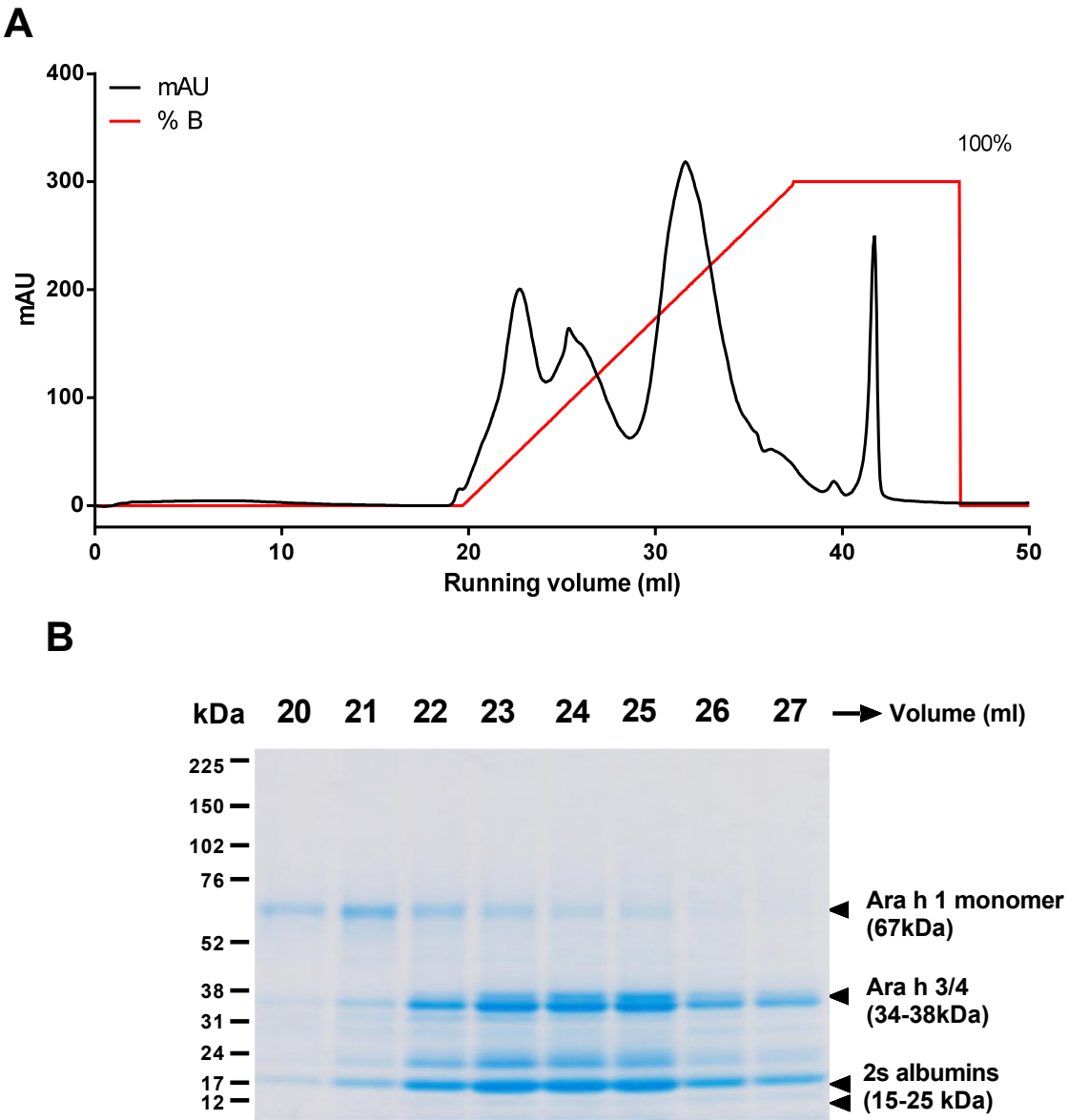


Figure 3.7 Elution profile of Ara h 1-enriched DR PPE fractions in anion exchange chromatography. **(A)** Chromatogram of DR Ara h 1 elution, showing protein concentration (black) measured by UV absorbance, together with percentage rising elution buffer (red). *N* = 3. **(B)** Reducing 4-12% SDS-PAGE gel of selected fractions from DR Ara h 1 elution, indicating the individual peanut allergens. Representative of four independent experiments.

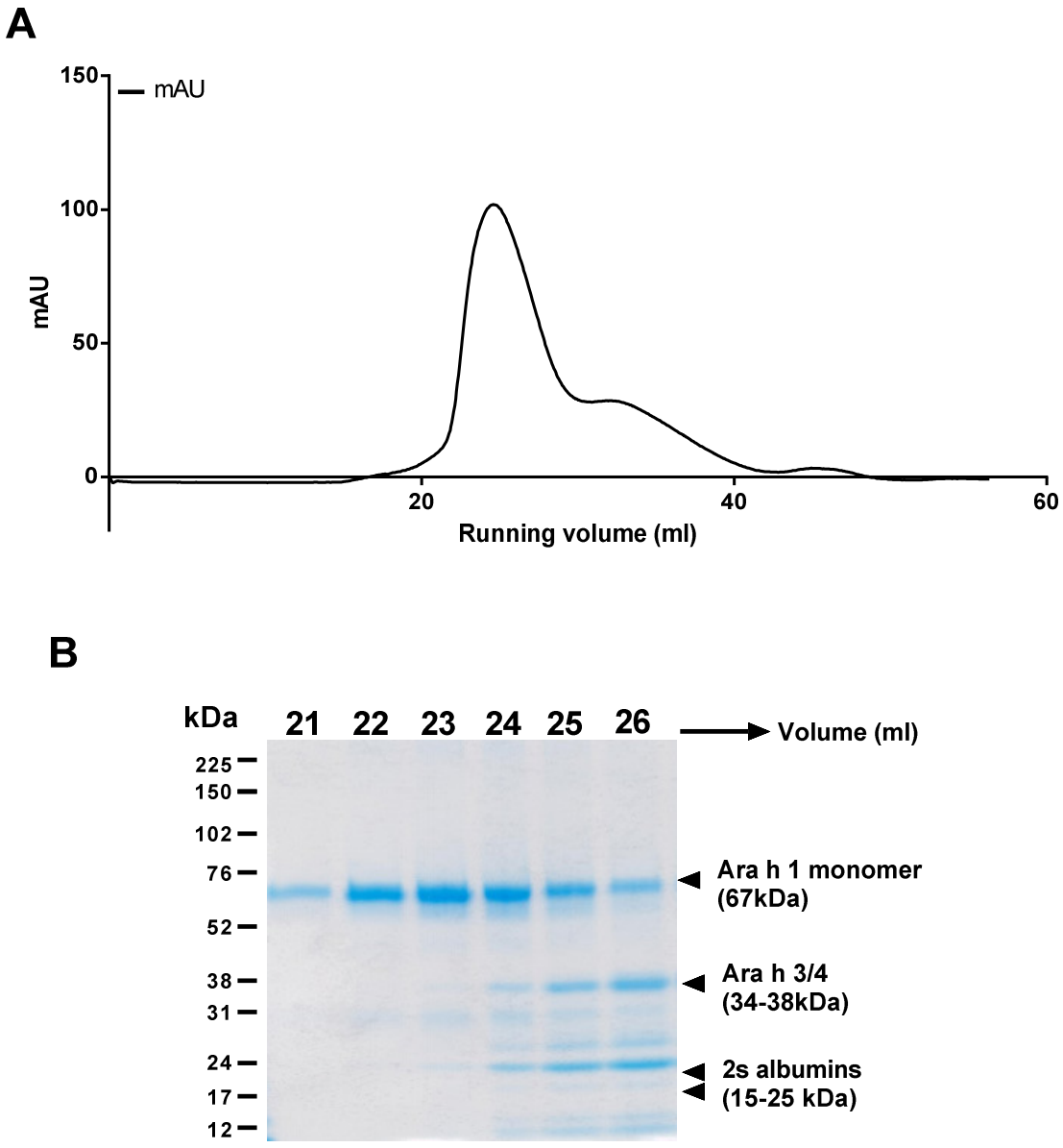


Figure 3.8 Elution profile of pooled DR-Ara h 1-rich fractions in analytical size exclusion chromatography. **(A)** Chromatogram of DR Ara h 1 elution, showing protein concentration (solid black) measured by UV absorbance. *N* = 3. **(B)** Reducing 4-12% SDS-PAGE gel of selected fractions from DR Ara h 1 elution, indicating the individual peanut allergens. Representative of four independent experiments.

3.5.5 The peanut 2s albumins are sufficiently similar that they cannot be resolved by available techniques

Although the three 2s peanut albumin allergens, Ara h 2, 6, and 7, could readily be separated from other high molecular weight peanut proteins such as Ara h 1 (Figure 3.5), neither ion-exchange nor analytical size exclusion could resolve them from each other under the available experimental conditions. Instead, they remained as part of a single peak under ion-exchange conditions (Figure 3.9A). Although some variation was observed in the relative distributions of Ara h 2, 6 and 7 was observed across the peak (Figure 3.9B), a large degree of mutual contamination was present at all points. Due to the difference in molecular weight between these species falling below the resolution threshold of the available size-exclusion columns, this technique was not used to attempt to further separate the 2s albumins.

This observation was further corroborated by MALDI mass spectrometry of peptide digests obtained from excised gel bands of the Ara h 2 doublet, and Ara h 6 band (data not shown), which indicated that all three contained a significant portion of mutual contamination, indicating that even on an SDS-PAGE gel the 2s albumins could not be fully separated by the available techniques. Therefore their fraction was assigned the name “2S albumins”, as they are frequently referred to in previously published literature (Lehmann, Schweimer et al. 2006; Vissers, Blanc et al. 2011).

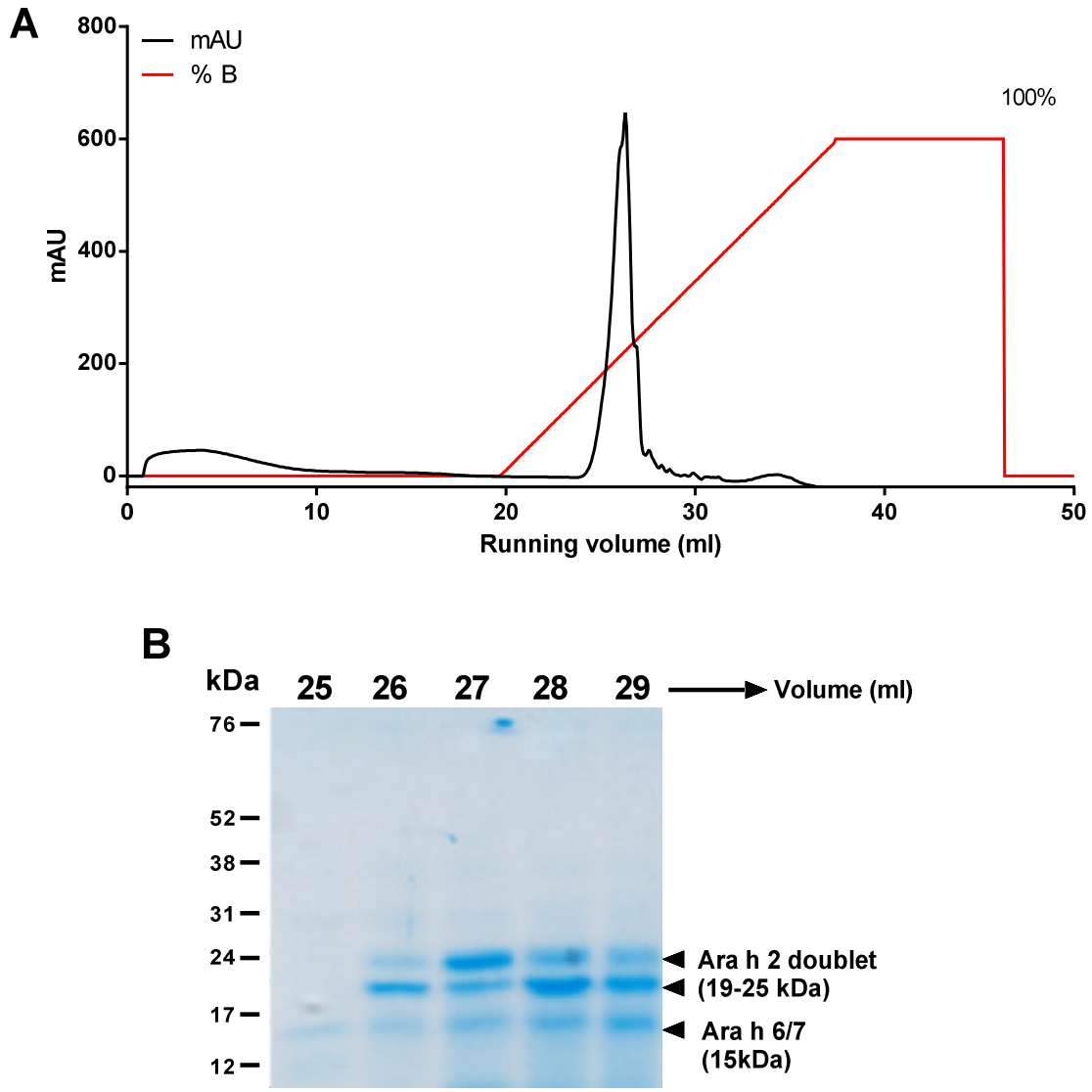


Figure 3.9 Elution profiles of 2s albumin-enriched raw PPE fractions in anion exchange chromatography. **(A)** Chromatograms of raw 2s albumin elution, showing protein concentration (black) measured by UV absorbance, together with percentage rising elution buffer (red), for anion-exchange chromatography. **(B)** Reducing SDS-PAGE gel of selected fractions from raw 2s albumin elution from analytical size exclusion, indicating the individual peanut allergens. Representative of four independent experiments.

3.5.6 Purified Raw and DR allergens are comparable in purity and carbonyl level

Pooled fractions of purified raw or DR Ara h 1, and raw or DR 2s albumins, were analysed by SDS-PAGE to determine their purity, and DNPH-carbonyl ELISA to determine their oxidation state.

DR allergens were found to be of comparable purity to their corresponding raw allergens. Purified raw and DR Ara h 1 and 2s albumins were observed to be highly free of contaminating higher or lower molecular weight allergen species (Figure 3.10A).

Both DR Ara h 1 and 2s albumins were found to be oxidised to a comparable level (as measured by reactive carbonyl ELISA) to the total DR PPE from which they were purified, confirming that their oxidation state was representative of that of the mixture from which they were derived (Figure 3.10B).

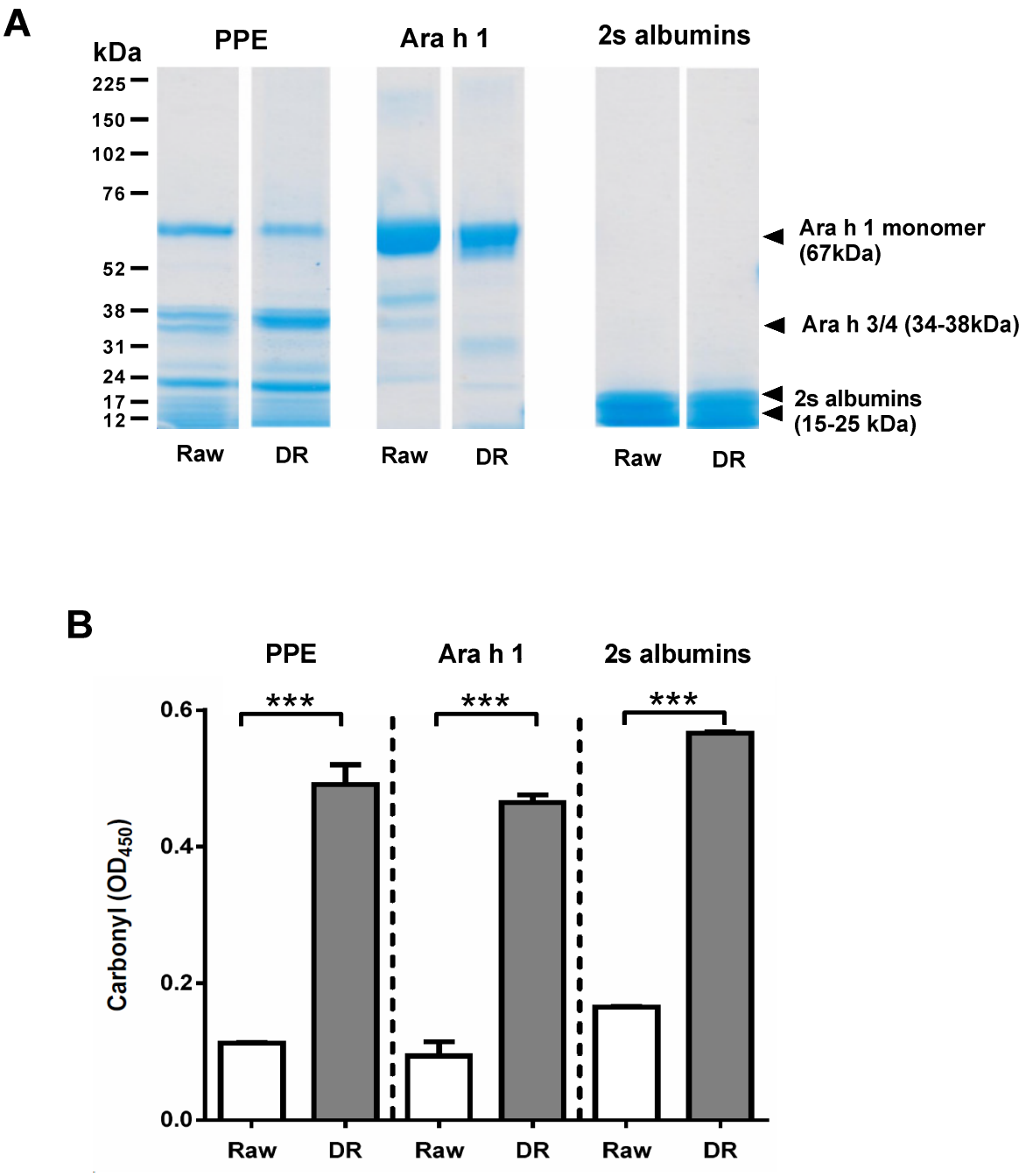


Figure 3.10 Purity comparisons and protein oxidation state of purified Ara h 1 and 2s albumins. **(A)** Reducing 4-12% SDS-PAGE of total PPE and purified allergens from raw and DR peanuts. Representative of three independent batches of purified allergens. **(B)** Anti-carbonyl ELISA comparison of raw and DR Ara h 1 and 2s albumins against raw and DR total PPE (background OD ≤ 0.05). *n* = 3, representative of three independent batches of purified allergens. *** p = ≤ 0.001; Student's *T*-test.

3.5.7 Purified DR Ara h 1 differs in oligomerisation, but not glycosylation, from raw Ara h 1

The successful purification of Ara h 1 from both raw and roasted PPE permitted additional experimental comparisons which would not have been feasible on a heterogeneous mixture of proteins. The first of these was analysis of the protein by light scattering in order to determine its oligomerisation state, which cannot be performed in protein mixtures due to the interfering light scattering effects of other protein species. Consistent with the results of native PAGE (Figure 4.3), purified DR Ara h 1 was found to contain fewer higher molecular weight oligomers and aggregates than raw Ara h 1 (Figure 3.11). The majority of the protein was observed to be in its native trimeric state.

By contrast, when the surface glycan structures of raw and DR Ara h 1 were liberated from the proteins using PNGase F, and identified using mass spectrometry, they were not found to be significantly different between raw and DR Ara h 1 (these experiments were carried out by a collaborator, and can be found in Appendix B). All of the core glycan structures and structural variants listed in previous reports were identified (van Ree, Cabanes-Macheteau et al. 2000; Altmann 2007), and remained at remarkably similar relative proportions in both raw and DR Ara h 1 (Supplementary Figure B1A and B).

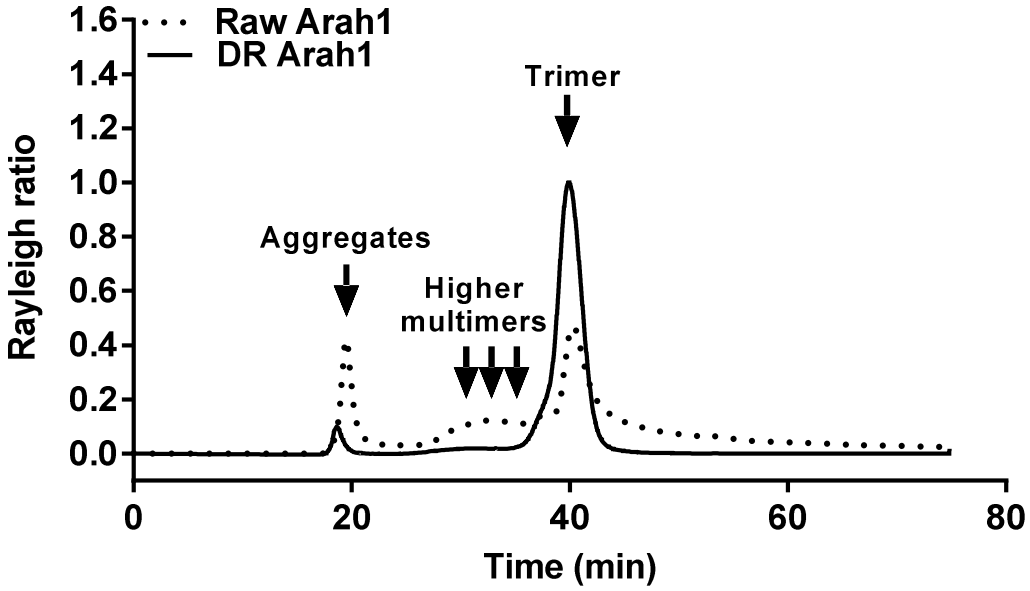


Figure 3.11 Protein oligomer elution profile of purified raw and DR Ara h 1. Protein concentration is represented by the degree of light scattering, expressed as the Rayleigh ratio of incident to scattered light, and is plotted against the time at which the peak containing each oligomer eluted from the attached column. For further details please see Methods and Materials section 2.3.12.

3.6 Discussion

The core goals of this Chapter were firstly; to characterise the dry-roasting of peanut samples by measuring protein oxidation and the loss of solubility of the major peanut allergen Ara h 1, and secondly; to produce a series of progressively refined raw and DR peanut preparations for immunological analysis, from crude peanut homogenate to purified individual allergens. These goals were largely achieved.

The two key properties of commercially-roasted DR peanuts (RC formation (Chung 1999) and protein solubility loss (Kopper, Odum et al. 2005)) were successfully replicated by roasting under laboratory conditions, and crude peanut homogenates, as well as total protein extracts (PPE) were successfully prepared from peanuts roasted in this manner. CPH and PPE prepared from the same raw and DR peanut were found to be highly comparable in both RC content and Ara h 1 solubility. This equivalence is crucial, as it ensures the accuracy of comparisons between different immunogenicity models using complete peanut homogenate (intragastric administration of CPH) and peanut protein (PPE administered subcutaneously). .

The purification of individual allergens from these extracts proved to be more challenging. Plant tissues are often referred to as “recalcitrant” in proteomics literature due to the difficulty of extracting adequate yields of protein from them, most often due to a combination of relatively low protein content per tissue weight and interfering effects of cellulose from cell walls (Carpentier, Witters et al. 2005). Although raw Ara h 1 could be separated from total raw PPE with relative ease due to its abundance (Shin, Compadre et al. 1998), DR Ara h 1 proved much more challenging to adequately purify from other peanut

allergens and contaminating proteins, especially the glycinin Ara h 3/4 (Piersma, Gaspari et al. 2005). The most plausible explanation for this is that the extensive modification of surface residues in DR peanut protein is responsible. It would appear that the changes in physicochemical properties that accompany roasting (for example the adduction of new charged or aromatic moieties such as AGEs (O'Brien, Siraki et al. 2005)) may have altered the mass/charge ratio of portions of the population of these proteins such that they overlap to a greater extent than is present in raw PPE. Despite this difficulty, however, Ara h 1 was successfully isolated from both raw and DR PPE to a comparable degree of purity. Purified DR Ara h 1 also contained comparable levels of RC to its PPE progenitor.

The three 2s albumin allergens, Ara h 2, 6 and 7, were also collectively separated from all other peanut proteins to a comparably high degree of purity from both raw and DR peanut proteins. However, without the availability of affinity chromatography (Porterfield, Murray et al. 2009), they could not be further resolved into individual allergen fractions. A high degree of homology of the 2s albumins of peanut has previously been described by a number of reports (Koppelman, de Jong et al. 2005; Koppelman, Hefle et al. 2010; Kulis, Chen et al. 2012), and it is clearly this high degree of similarity which prevented their separation by either size exclusion or ion exchange techniques. Nevertheless, for the purposes of this project and its investigations of immunogenicity, separation of the 2s albumins, though desirable, is not essential. The 2s albumin allergens may be profitably analysed as a group, due to the extensive cross-reactivity of these proteins described by numerous reports (Koppelman, de Jong et al. 2005; Porterfield, Murray et al. 2009; Chen, Zhuang et al. 2011), which led to Ara h 2 and 6 being described in one recent study as "substantially redundant" (Chen, Wang et al. 2013), due to their equivalent ability to cross-

reactively trigger degranulation of mast cells armed with peanut-allergic patient sera. In addition, some readouts of immunogenicity such as Western blotting of serum immunoglobulins allow the relative immunogenicity of even coadministered allergens to be examined simultaneously (Mondoulet, Paty et al. 2005; Maleki, Casillas et al. 2010). Similar to Ara h 1, the isolated 2s albumins maintained the increased RC level relative to raw seen in PPE.

Both DR total PPE (as measured by native PAGE) and purified DR Ara h 1 (as measured by light scattering) were found to contain fewer high-molecular weight oligomers than their raw counterparts. This is not surprising, as Ara h 1 has a documented tendency to lose solubility during roasting (Chruszcz, Maleki et al. 2011), and other models of protein aggregation suggest that the processes of protein oxidation and aggregation may be linked (Korolainen, Goldsteins et al. 2002). Therefore, the relative lack of high molecular weight oligomers and aggregates in DR PPE and purified Ara h 1 may well be explained by the loss of protein solubility during roasting, which would also explain the lower yields of DR PPE per gram of peanut flour relative to raw. This would, however, imply that the oligomer content of DR PPE is significantly lower than that found in DR CPH, a contention supported by native PAGE comparison via of DR CPH and PPE. Nevertheless, as testified by colorimetry and ELISA of RC content, the heightened oxidation state of the DR peanut protein population relative to raw seen in CPH was maintained in PPE despite the loss of these oligomers.

The glycosylation state of purified Ara h 1 was also investigated to determine any differences between raw and DR Ara h 1. Glycosylation has been observed to be important in determining the allergenicity of some proteins, and is of particular relevance in the case of Ara h 1 specifically, because previous studies have suggested that one of its binding

partners in innate immunity may be the glycan-binding receptor DC-SIGN (Shreffler, Castro et al. 2006). However, when the glycosylation state of raw and roasted Ara h 1 was compared, no changes were detected. This result was surprising, given that cell surface glycans are sugar-based and hence susceptible to attack from the Maillard Reaction during high-temperature thermal processing (Ashoor 1984). However, this finding must be interpreted with an appropriate degree of caution, as without a means to quantify the total glycan moieties on the protein surface, it is difficult to be sure whether the liberated population of glycan species is representative of the whole. Nevertheless, unmodified residues were clearly present on DR Ara h 1 sufficient quantities to be readily detectible by the spectrometer, indicating that even in dry-roasted peanuts, substantial quantities of unmodified glycans remain.

In conclusion, as a result of these studies a full range of raw and DR peanut preparations with comparable levels of oxidation and purity was made available for immunological analysis. These proceeded from CPH through progressive refinement to PPE and isolated peanut allergens, permitting the separate study of the immunogenicity and allergenicity of these components.

Chapter 4
In vivo immunological studies of peanut allergens

4.1 Abstract

To the best of my knowledge, to date no *in vivo* experimental studies have been undertaken to test directly the purported link between roasting and enhanced peanut allergenicity. In this chapter, I will present evidence that dry-roast (DR) but not raw crude peanut homogenate (CPH), without adjuvant, can induce T_h2-biased immune responses after intragastric (IG) administration to Balb/c mice, and produces enhanced immune responses relative to raw even when adjuvant was coadministered. This differential was maintained in the case of Peanut Protein Extract (PPE) injected subcutaneously (SC). In contrast to the IG model, the SC model showed that DR PPE not only induced a T_h2 biased response, but strong functional IgE production as well. The purified peanut allergen Ara h 1 was found to have similar properties when injected SC as the total PPE extract. Similar markers of T_h2 skewing of DR peanut responses were also observed in an epicutaneous (EC) model of mouse sensitisation. Furthermore, DR PPE and CPH were more immunogenic and induced T_h2 bias even in a T_h1-biased strain of mice (C57Bl/6). Subsequent experiments suggest that the increased immunogenicity and T_h2 skewing effects of DR peanut can be lessened by chemical reduction of reactive carbonyl (RC) AGE adducts.

4.2 Introduction

Reports from both epidemiology (Sicherer and Sampson 2007) and laboratory work (Beyer, Morrow et al. 2001; Maleki, Viquez et al. 2003) have converged on the possibility that dry-roasting can increase the allergenicity of peanut proteins. This has been supported by various observations, including apparent increased binding of roasted peanut to the sera of peanut-allergic patients (Maleki, Chung et al. 2000; Chung, Maleki et al. 2004). However, as far as I am aware no research group has yet demonstrated that DR peanut proteins are more allergenic in murine models of peanut allergy.

4.2.1 Immunogenicity and allergenicity

Evidence for the enhanced allergenic potential of DR peanut in immunised mice can be detected through the presence of markers of the T_h2-skewed anti-peanut responses which are associated with allergy (Helm and Burks 2000), including IgG1 and IgE-dominated serum immunoglobulin production, and preferential T_h2-associated cytokine secretion upon restimulation of T-cells from mice immunised with peanut allergen (Song, Qu et al. 2010).

When investigating the *in vivo* immunologic properties of DR peanut, it is important to distinguish between immunogenicity, that is, the tendency to provoke immune responses (De Groot and Martin 2009), and allergenicity, which the tendency to provoke allergy specifically (Larche, Akdis et al. 2006; Tay, Clark et al. 2007). Proteins may be highly immunogenic but simultaneously lack allergenicity (Asselin 1988), which may be affected by factors other than the intrinsic immunogenicity of the allergen in question. Route and frequency of exposure (Dearman, Caddick et al. 2001), the quantities in contact with the immune system (Diesner, Knittelfelder et al. 2008), coadministration with other

immunomodulatory factors (Bashir, Louie et al. 2004), and the general environment and genetic background of the organism (Sicherer and Sampson 2007; Sicherer and Sampson 2009), have also been reported to be significant.

Accordingly, when conducting experimental design for the assessment of DR peanut allergenicity, it is important to separate where possible the many factors which can contribute to allergenicity. For DR, these include both physicochemical effects, such as protein aggregation (Sathe, Teuber et al. 2005) and altered digestibility (Bowman and Selgrade 2008) and potentially allergenic effects which may be receptor-initiated (Allison and Fearon 2000) and alter its detection by the innate immune system (Gerrard 2002; Ilchmann 2010). Therefore it is particularly important to design experiments to distinguish between these two classes of effects.

Genetic factors must also be accounted for. Although specific genetic polymorphisms associated with peanut allergy have not yet been identified in humans (Sicherer, Furlong et al. 2000; Bowman and Selgrade 2009; Sicherer and Sampson 2009), it is well known that certain strains of mice possess an innate bias towards either T_h1 or T_h2 immunity. In particular, the ubiquitous Balb/c strain of white mice is known to be biased towards the T_h2 activity associated with allergy, and as such, it may provide a model for populations of genetically susceptible humans (Knippels, van Wijk et al. 2004). By contrast, other mouse strains such as C57 Black/6 (C57Bl/6) have a more T_h1-oriented bias, making them potentially resistant to allergy and therefore a useful control model for any allergenic effects of DR peanut observed in Balb/c.

4.2.2 Intragastric murine models of peanut allergy

In the first instance, determining the oral immunogenicity of crude peanut homogenate (CPH) administered intragastrically (IG) is desirable, as it most closely resembles the manner in which most peanuts are encountered by humans. Although oral sensitisation of mice to peanut has been extensively practiced (Adel-Patient, Bernard et al. 2005; Song, Qu et al. 2010), prior studies were primarily interested in simulating the effects of anaphylaxis, and accordingly used non-physiologically large doses of Cholera Holotoxin as a mucosal adjuvant (Aldemir, Bars et al. 2009). Given that the priority of this project is to study immunogenicity and allergenicity of peanut proteins, rather than anaphylaxis itself, such rigorous sensitisation protocols would be counterproductive in this context, and might mask subtle differences between antigens in allergenicity. By contrast, the use of much milder oral immunisation protocols is more likely to be of physiological relevance to the immunogenic potential of peanut proteins as they are encountered by humans. Although some researchers hold that sensitisation occurs through the oral consumption of peanuts (Burks, Laubach et al. 2008), others demur (Lack 2008) believing it instead to be tolerogenic, and suggesting instead that a more plausible route is via topical sensitisation (Hsieh, Tsai et al. 2003; Brough, Makinson et al. 2013). Demonstrating enhanced immune responses to IG-administered DR peanut without strong adjuvantation would be of direct relevance to this on-going debate.

However, such a demonstration would not permit a distinction between effects due to intrinsic properties of DR allergens and other effects such as bioavailability in the gut (Bowman and Selgrade 2008), or the synergistic effects of coadministration with other components of the peanut homogenate (Moreno, Mackie et al. 2005). Both oxidised lipids

and carbohydrates have known biological activity, and oxidised phospholipids in particular have been implicated in immune signalling (Gao, Ashraf et al. ; Negre-Salvayre, Auge et al. 2010).

4.2.3 Cutaneous murine models of peanut allergy

In order to disentangle the contribution of other components of CPH from the immunogenic properties of the peanut proteins themselves, additional experimental models are necessary. Subcutaneous (SC) immunisation with peanut protein extract (PPE) is particularly attractive, as it allows allergens to be directly administered whilst bypassing entirely the questions of digestibility and relative bioavailability of proteins in the gut (Helm, Ermel et al. 2003). SC injections therefore offer the cleanest test of the proposition that dry-roasting introduces immunogenic changes to peanut proteins specifically.

However, any changes of intrinsic protein immunogenicity would not exclude the action of other effects such as digestibility, when peanuts are consumed. Indeed it is possible that these factors may interact in a synergistic manner to contribute to overall DR peanut allergenicity. This hypothesis can be readily tested by boosting SC-immunised mice with IG doses of CPH, permitting the study of interactions between initial peanut immunisation and subsequent encounters with peanut protein in the diet (Teuber, Del Val et al. 2002). Because small doses of antigen are often sufficient to elicit responses to SC immunisation, this model also provides the best opportunity to examine the immunogenicity of purified peanut allergens.

Because the primary route of peanut sensitisation in humans is still a matter of considerable research interest and debate, it would be equally desirable to compare the oral and

subcutaneous sensitisation routes with epicutaneous (EC) sensitisation, the chief rival to oral sensitisation as the model for the initial acquisition of peanut allergy in human patients (Lack 2008). To this end, I collaborated with Dr Mario Noti of the Artis Group at the University of Pennsylvania, who used a model of EC sensitisation, which his group had previously developed for other allergens, to test the allergenicity of peanut antigens. The use of this method was designed to permit a direct comparison of the two principle candidate sensitisation routes for peanut allergy, subject of course to the usual caveats of murine research being relevant to the human immune system (Dearman and Kimber 2009).

4.2.4 The contribution of RC can be investigated by chemical reduction

The formation of RC adducts is an important marker of the protein oxidation which accompanies dry-roasting, and is intimately associated through the oxidative Maillard Reaction with the formation of potentially pathogenic AGEs (Chung 1999; Ilchmann, Burgdorf et al. 2010). Additionally, there is evidence to suggest that RC adducts themselves may directly contribute to T_h2 bias of immune responses (Moghaddam, Olszewska et al. 2006).

The development of *in vivo* models of peanut sensitisation would also provide an opportunity to investigate the contribution of RC to DR peanut immunogenicity. This is technically feasible because RCs form a chemically distinct sub-population of AGEs which can be selectively depleted by reduction of RC groups to carboxylic acids, using a suitably powerful reducing agent (Galembeck, Ryan et al. 1977; Glomb and Monnier 1995). The success of RC removal can easily be followed by the same methods of carbonyl detection by colorimetry (Levine 1990) and ELISA (Buss, Chan et al. 1997) used in the previous Chapter.

Once DR peanut preparations have been successfully depleted of RC to a level comparable to raw, they can be used to immunise mice in parallel with untreated samples, allowing the observation of any differences caused by the presence or absence of RC on the surface of DR peanut proteins.

4.3 Chapter Goals

The following experimental strategy was pursued to investigate *in vivo* sensitisation using raw and DR peanut allergens:

1. Compare the immunogenicity of raw and DR CPH administered IG to Balb/c mice, with and without the presence of tolerance-breaking adjuvants;
2. Obtain immune readouts in the form of serum immunoglobulin levels and T-cell restimulation responses, to look for evidence of T_h2 skewing;
3. Compare these responses with those elicited by raw and DR PPE administered SC or EC and subsequently boosted through the IG route with CPH;
4. Compare these findings in Balb/c with C57Bl/6, a mouse strain which does not share their T_h2 bias;
5. Investigate whether any enhancement due to roasting can be prevented by chemical reduction of RC groups.

4.4 Methods used in this Chapter

The following methods are described in full in the indicated sections of Chapter 2, which contains full protocols and descriptions for all materials and methods used in this project.

- Endotoxin depletion **(2.3.5)**
- Quantification of protein carbonyl content by colorimetry **(2.3.7)**
- Estimation of protein Carbonyl Content by ELISA **(2.3.8)**
- Biotinylation of proteins **(2.3.9)**
- Reduction of proteins **(2.3.10)**

- Animal maintenance, termination and tissue sampling **(2.6.1-3)**
- Intragastric immunisation **(2.6.4)**
- Subcutaneous immunisation **(2.6.5)**
- Epicutaneous sensitisation **(2.6.6)**

- Peanut-specific Immunoglobulin G serum ELISA **(2.7.1)**
- Peanut-specific Immunoglobulin E sandwich ELISA **(2.7.2)**
- Rat Basophilic Leukaemia degranulation assay **(2.7.4)**
- Measurement of T-cell responses **(2.8)**

4.5 Results

4.5.1 DR but not raw, CPH evokes strong immune responses in Balb/c mice, with or without adjuvant

To compare the immunogenicity of raw and DR CPH, female Balb/c mice were fed IG with CPH by gavage, and subsequently boosted at regular intervals with further gavage doses (Figure 4.1A). Because it was unknown whether these doses would be sufficient to overcome oral tolerance mechanisms, raw or DR CPH was also administered with Cholera Holotoxin (CT), which has been used successfully to break oral tolerance in similar murine models of food allergy.

DR CPH elicited higher peanut-specific IgG responses than raw, both with and without the presence of CT adjuvant. However, the pattern of this differential was markedly different depending on whether or not CT was coadministered initially. With raw or DR CPH alone, a sub-population of DR-sensitised mice responded strongly, producing stronger responses than any observed in raw, yet the majority only responded weakly, or not at all (Figure 4.1B). By contrast, when CT was added to the initial bolus, robust responses with high IgG production were observed in both raw and DR groups, although these remained more frequent in DR groups compared to raw (Figure 4.1C).

Although initial responses were weak, repeated feedings with DR CPH elicited increased antibody production in responding mice with each successive feeding, indicating the presence of a sizeable population of peanut-responsive B-cells which further increased upon repeated stimulus (Figure 4.1B and C). When multiple repeats of this experiment were considered together, the trend of higher responses to DR CPH among a subset of DR-

immunised mice was sustained across multiple independent experiments for both unadjuvanted CPH (Figure 4.1D), and when Cholera Holotoxin was used as an adjuvant (Figure 4.1E). Serum antibody responses bound equally well to both raw and DR peanut allergens, regardless of the addition of adjuvant (Figure 4.2 A-B). This finding was corroborated by a competition ELISA between raw and DR PPE, which indicated that the overwhelming majority of binding to DR antigens by the sera could be wholly competed with raw antigen (Figure 4.2 C).

Despite this, T-cell responses upon antigen restimulation were weak. T-cells isolated from the mesenteric lymph nodes adjacent to the small intestine did not respond to restimulation by either raw or DR PPE (Figure 4.2D). In contrast, modest proliferation was seen in splenocytes extracted from the same mice (Figure 4.2E). T-cell responses to both raw and DR PPE restimulation were similar, with those to DR PPE being slightly lower on average. Taken together with the serum immunoglobulin binding data (Figure 4.2A-B) this would strongly suggest that the responses raised were primarily against unmodified epitopes.

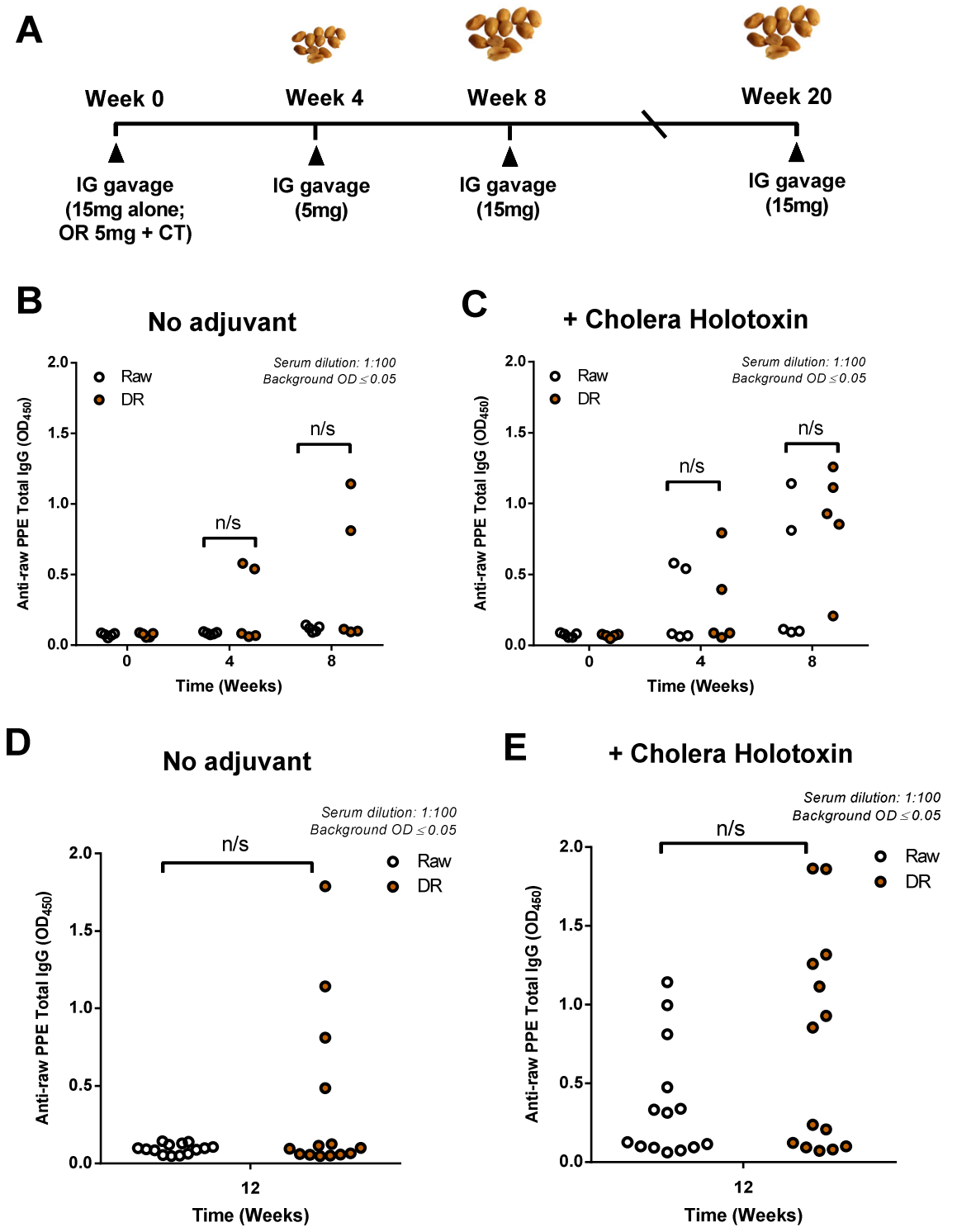


Figure 4.1 Intragastric immunisation of female Balb/c mice. **(A)** Schema of immunisation protocol. **(B-C)** Time course of peanut-specific total serum IgG responses after priming and 1st challenge, **(B)** without and **(C)** with Cholera Holotoxin as a adjuvant. N = 5, representative of 3 independent experiments. **(D-E)** Anti-peanut total serum IgG responses four weeks after the 2nd challenge, **(D)** without and **(E)** with Cholera Holotoxin as an adjuvant. N = 14, three pooled independent experiments. * $p = \leq 0.05$; n/s = non-significant; Mann-Whitney *U*-test

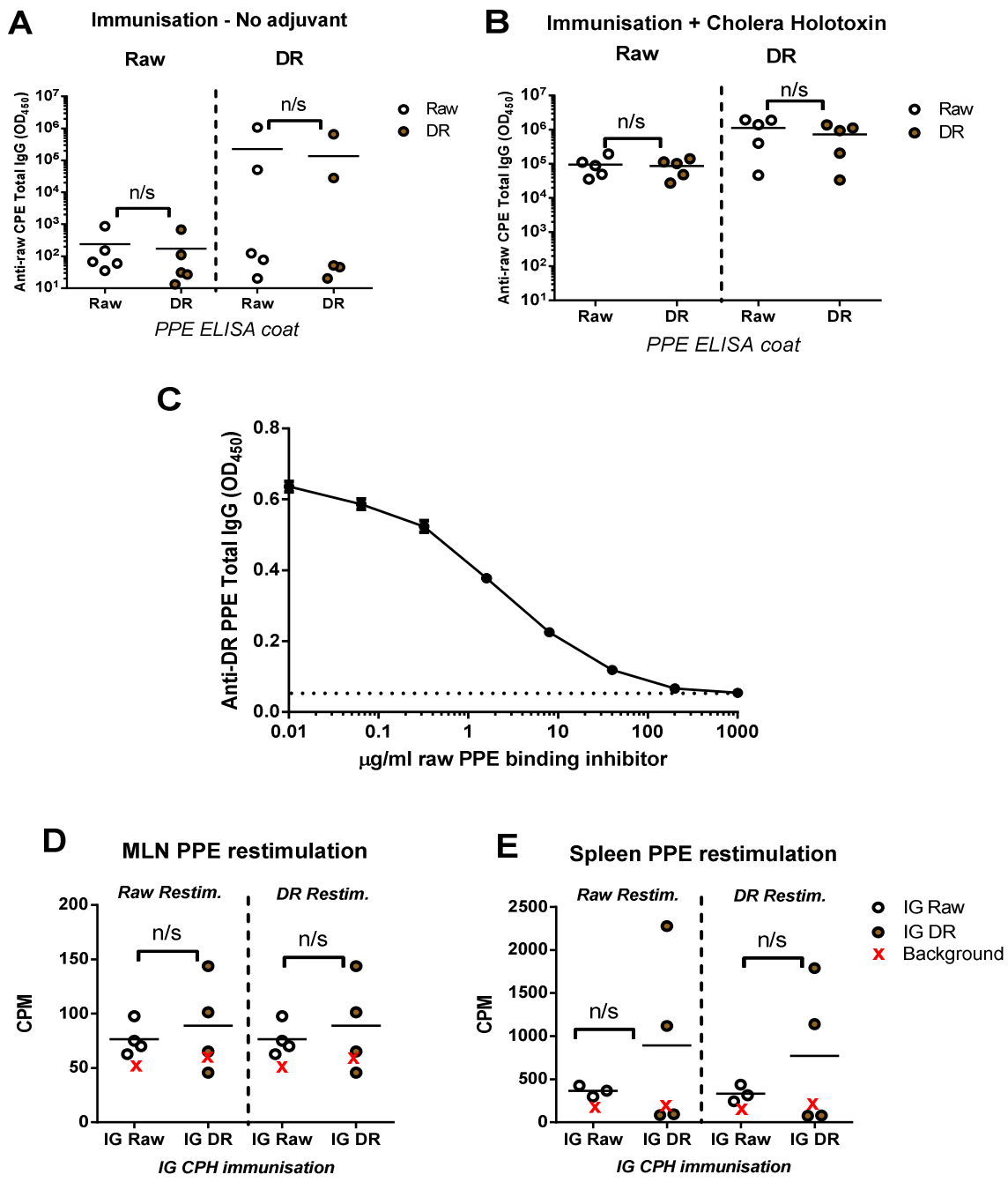


Figure 4.2 T-cell and serum immunoglobulin responses to raw and DR IG CPH, without Cholera Holotoxin. **(A-B)** Anti-peanut total serum IgG responses to raw and DR PPE at week 22 post-prime, **(A)** with raw and **(B)** with DR PPE. N = 5, representative of 3 independent experiments. **(C)** Competition ELISA for pooled DR-immunised IG serum binding of raw PPE in solution against DR PPE coating onto ELISA wells. **(D-E)** T-cell proliferation responses to restimulation with raw or DR PPE at week 22 post-prime, **(D)** in the MLN; **(E)** in the spleen. N = 4, representative of 3 independent experiments. n/s = non-significant; Mann-Whitney *U*-test.

4.5.2 DR gastrointestinal responses show a T_h2 bias but weak IgE production

The antibody responses generated by DR CPH exhibited a characteristic T_h2 bias which is a prerequisite for the induction of allergy. Responding mice produced significantly greater quantities of IgG1 relative to IgG2a, a balance which became especially evident when serum samples were titrated (Figure 4.3A).

Despite this bias, however, IgE production was weak in comparison, being present only at very low levels in serum (Figure 4.3B), and failing to elicit strong mast cell degranulation when used to arm Rat Basophilic leukaemia cells in an assay for IgE functional activity (Figure 4.3C). These findings are consistent with the health of the mice during the study. Mice were monitored for allergic symptoms before, immediately and several hours after feedings and challenges, according to the diagnostic criteria, but none were observed (data not shown). Nevertheless, the small amounts of functional IgE which were produced were predominantly found in mice immunised with DR CPH (Figure 4.3B-C).

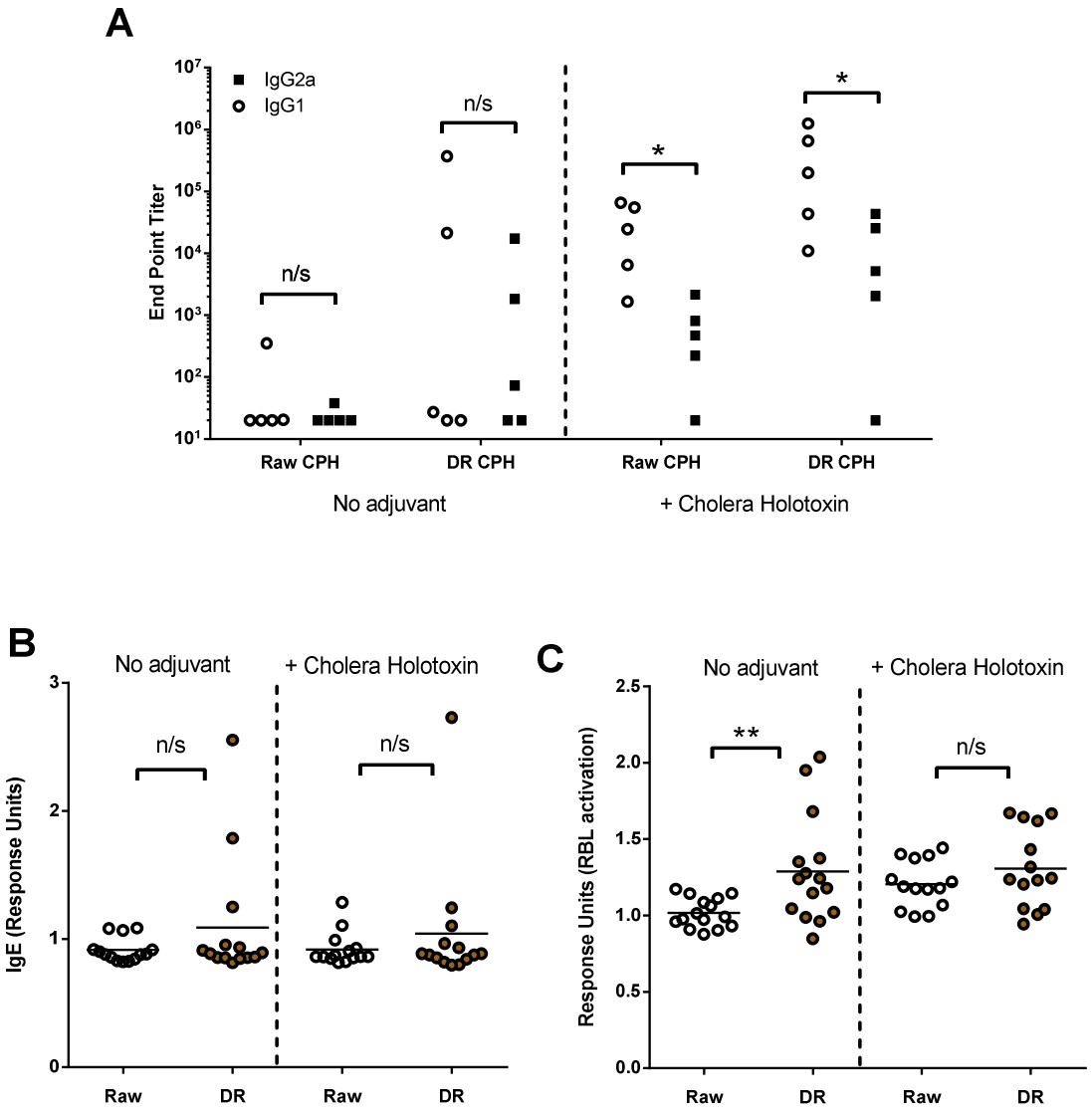


Figure 4.3 T_h2 skewing of antibody responses to IG peanut allergen. **(A)** Comparison of binding to raw PPE by IgG1 (T_h2) and IgG2a (T_h1) in IG-immunised mouse sera at week 12 post-prime. N = 5, representative of 3 independent experiments. **(B)** Serum raw-PPE specific IgE peanut at week 12 post-prime, with and without Cholera Holotoxin as an adjuvant. N = 14, 3 pooled independent experiments. **(C)** Raw PPE-specific RBL cell degranulation after arming with IG-immunised mouse IgE serum at week 12 post-prime, with and without Cholera Holotoxin as an adjuvant. N = 14, 3 pooled independent experiments. * p = ≤ 0.05; n/s = non-significant; Mann-Whitney *U*-test.

4.5.3 Subcutaneous priming with DR but not raw PPE primes robust T_h2 and functional IgE responses to subsequent GI exposure

Whilst the IG route of sensitisation is clinically important, gastrointestinal (GI) exposure contains the confounding factors of relative digestion rate and bioavailability within the gut, which may interfere with direct comparison of the immunogenic potential of raw and DR allergens. To dissect this aspect of immunisation, a subcutaneous model was also used, in which female Balb/c mice were injected with small quantities of endotoxin-depleted soluble peanut protein from raw or DR peanuts (Figure 4.4A), then subsequently boosted IG with feeding of DR CPH.

Although raw PPE was strongly immunogenic in its own right when administered in this manner, the key finding of the IG model was reproduced – DR PPE was more immunogenic than raw, although in the SC model, this effect was even more pronounced, attaining a clear trend immediately after the initial priming, and retaining this even after subsequent feeding challenges with dry-roasted CPH (Figure 4.4B). After multiple feedings, this effect attained statistical significance (Figure 4.4C).

Consistent with the findings of the IG model, antibody responses were directed equally against raw and DR PPE (Figure 4.5A), suggesting that, contrary to some other known allergens, the increased immunogenicity of DR PPE is not primarily based on the formation of novel epitopes. A competition ELISA carried out between raw and DR PPE using these sera from sensitised mice supported this contention, as raw PPE was able to wholly compete for the binding capacity of DR, confirming negligible involvement of neoepitopes (Figure 4.5B). Antigen restimulation of T-cells harvested from the mesenteric lymph nodes of the DR PPE-immunised groups indicated a robust and reproducibly greater proliferation of T-cells

upon restimulation than mice receiving raw PPE, an effect which, as with the ELISAs, applied equally to restimulation with raw or DR antigen (Figure 4.5C). Responses of a smaller magnitude were also observed upon restimulation of T-cells harvested from the spleens of the same mice, although without a significant difference between raw and DR-immunised mice (Figure 4.5D).

The SC model also showed an equivalent T_h2 bias to that observed for the IG route, with some responders showing IgG1 production more than an order of magnitude larger than IgG2a (Figure 4.6A). In contrast to the IG model, large quantities of IgE were produced, at much greater quantities for DR than for raw PPE (Figure 4.6B). Furthermore, this IgE was shown to be highly functional, as it was able to efficiently degranulate RBL mast cells even at low serum dilutions (Figure 4.6C). T-cell cytokine production assays carried out after PPE restimulation showed significant increases in T_h2 cytokine marker product for DR as opposed to raw PPE sensitisation, whereas the levels of T_h1 cytokines were found to be unchanged (Figure 4.6D).

Despite IgE production being dramatically higher after SC immunisation than was observed for IG immunisation, SC-immunised mice did not display any symptoms of allergic responses during and after orally receiving CPH (data not shown).

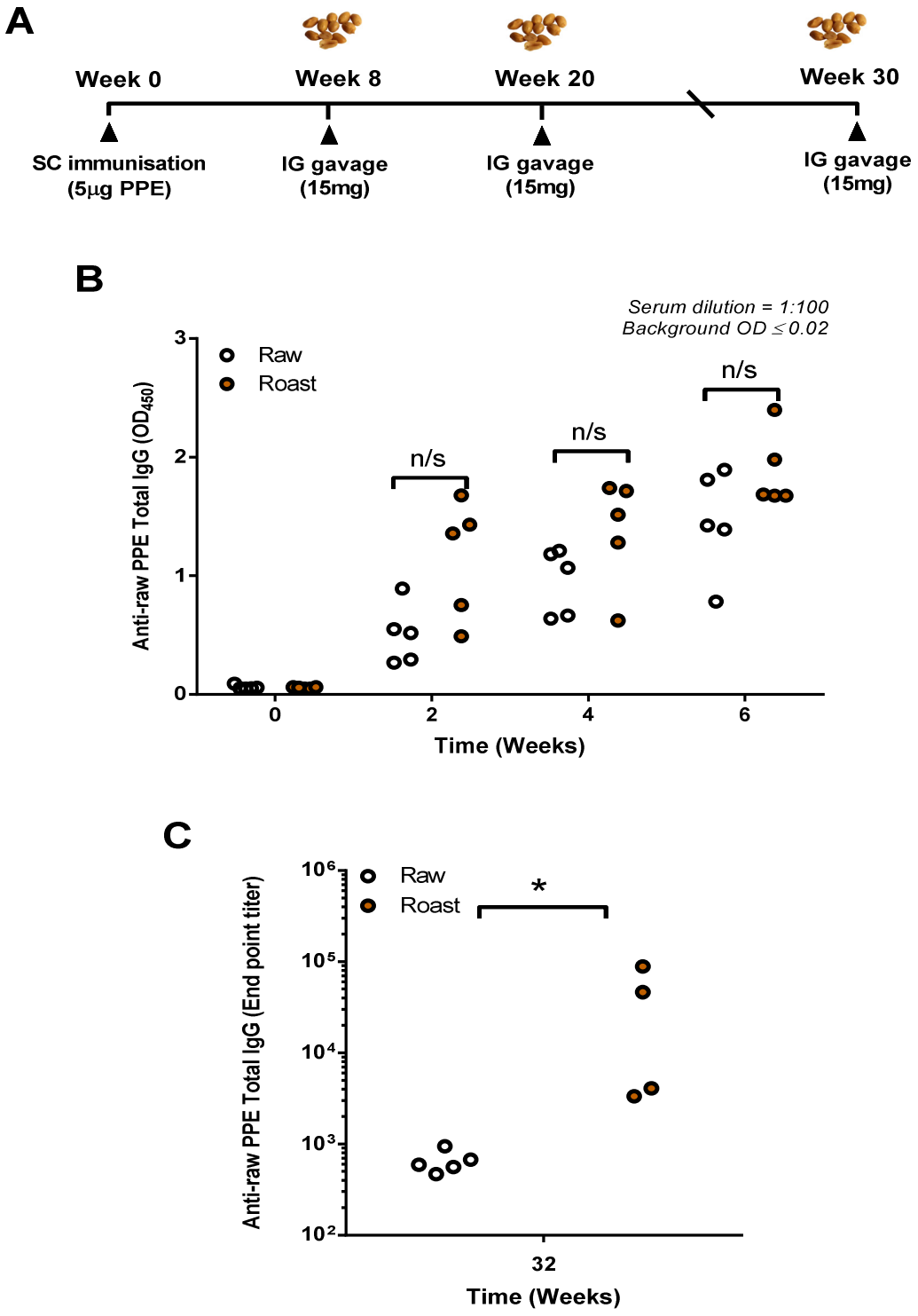


Figure 4.4 Subcutaneous immunogenicity of PPE in female Balb/c mice. **(A)** Schema of immunisation protocol. **(B)** Time course of peanut-specific total serum IgG responses after priming. **(C)** Anti-peanut total serum IgG responses two weeks after the final challenge. N = 5, representative of 3 independent experiments. * $p = \leq 0.05$; n/s = non-significant; Mann-Whitney *U*-test.

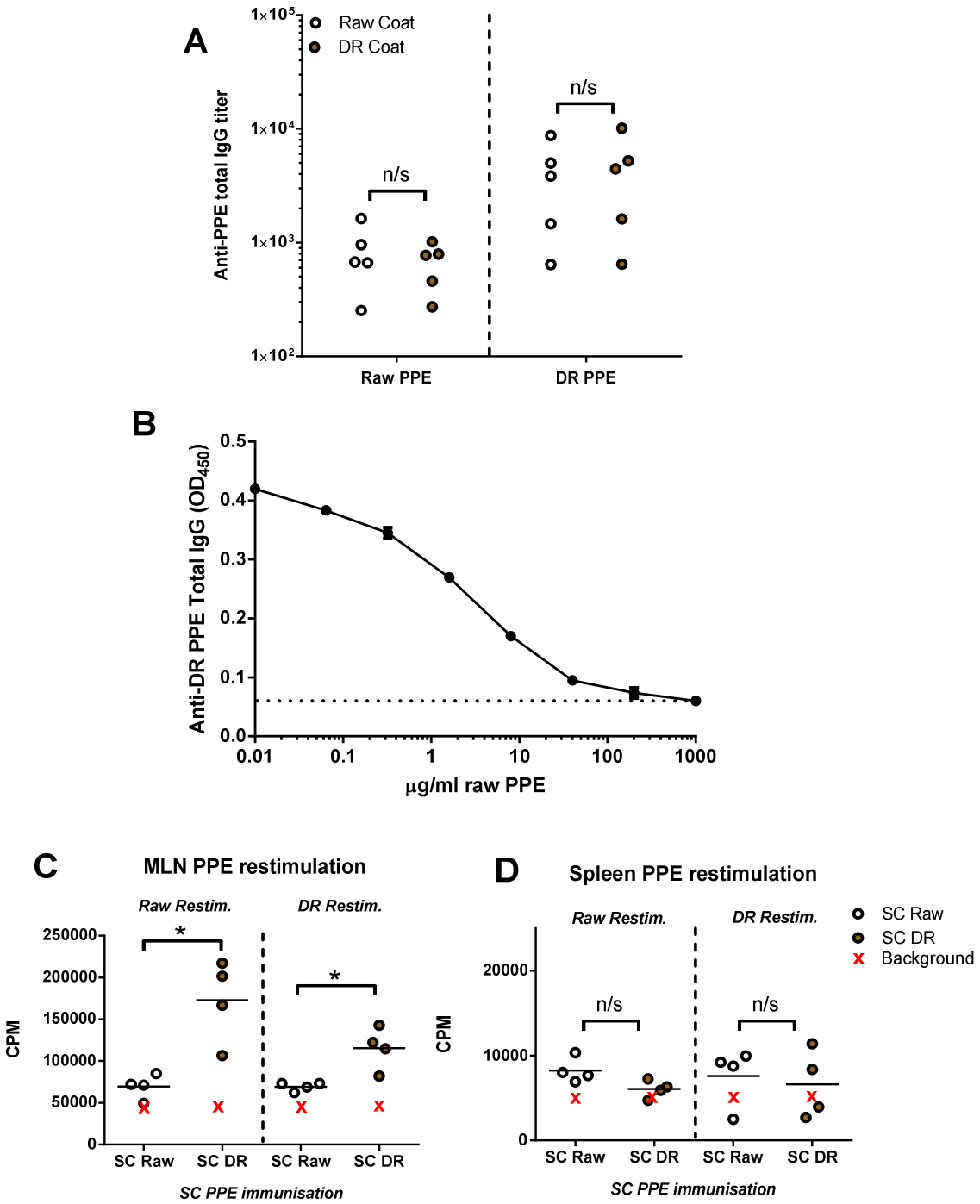


Figure 4.5 T-cell and serum immunoglobulin responses to raw and DR SC peanut allergen. **(A)** Anti-peanut total serum IgG responses to raw and DR PPE two weeks after the first challenge. N = 5, representative of 3 independent experiments. **(B)** Competition ELISA for pooled DR-immunised SC serum binding of raw PPE in solution against DR PPE coating onto ELISA wells. **(C-D)** T-cell proliferation responses to restimulation with raw or DR PPE at week 32 post-prime, **(C)** in the MLN; **(D)** in the spleen. N = 4, representative of 3 independent experiments. * $p = \leq 0.05$; n/s = non-significant; Mann-Whitney *U* -test.

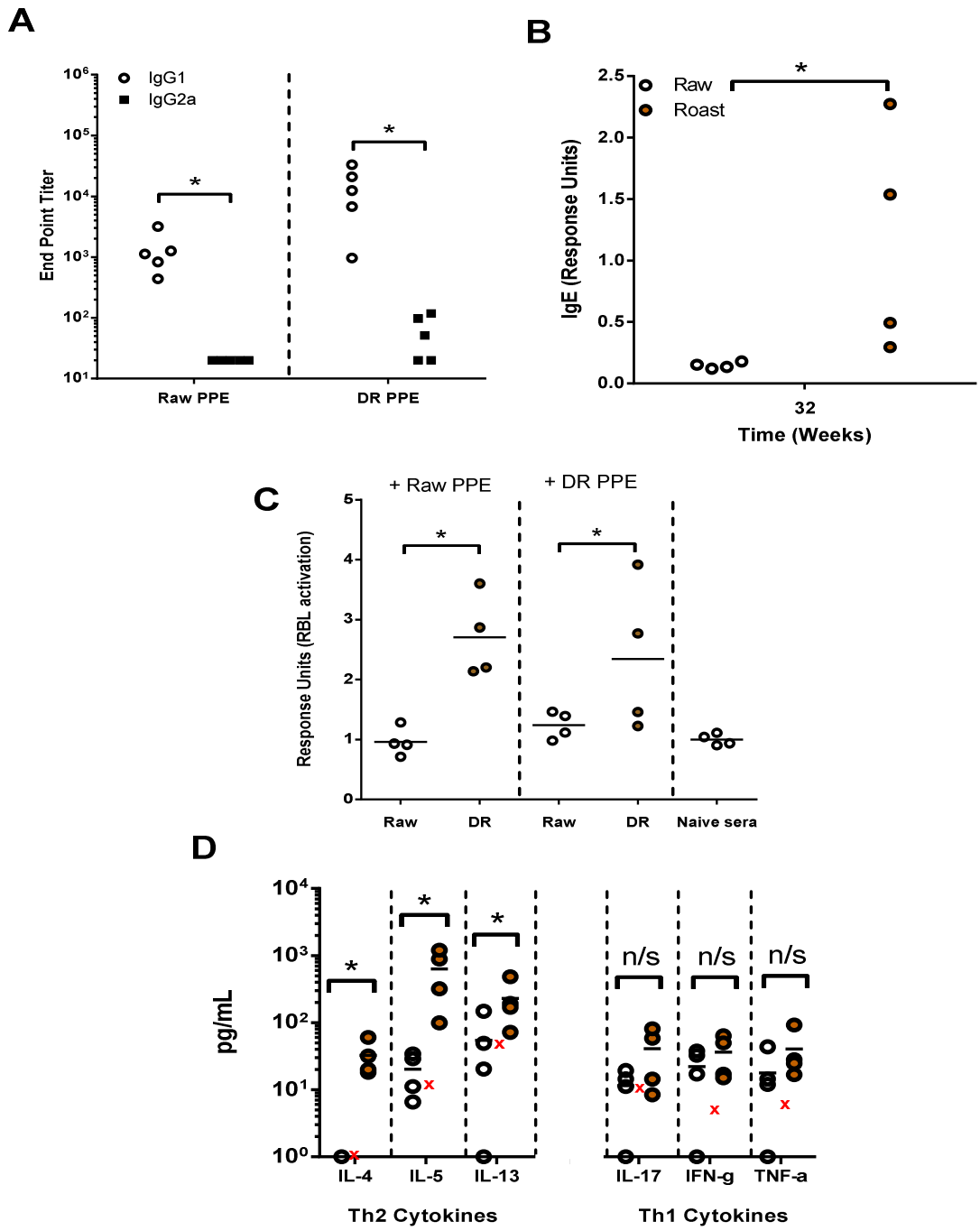


Figure 4.6 T_h2 skewing of antibody responses in SC-immunised mice. **(A)** Comparison of binding to raw PPE by IgG1 (T_h2) and IgG2a (T_h1) in sera at week 6 post-prime. N = 5, representative of 3 independent experiments. **(B)** Serum raw-PPE specific IgE peanut at week 32 post-prime. N = 4, representative of 3 independent experiments. **(C)** Raw PPE-specific RBL cell degranulation after arming with SC-immunised mouse IgE serum at two weeks after final challenge, stimulated by raw and DR PPE. N = 4, representative of 3 independent experiments. **(D)** Profile of T-cell cytokine secretions after restimulation with raw PPE, at two weeks after final challenge. N = 4, representative of 3 independent experiments. * $p = \leq 0.05$; n/s = non-significant; Mann-Whitney *U*-test.

4.5.4 DR peanut demonstrates enhanced immunogenicity and T_h2 bias even in a T_h1-biased mouse strain, C57Bl/6

Because all the preceding experiments had been conducted in a T_h2-responsive strain, Balb/c, it was of interest to investigate the effect dry-roasting would have in a strain of mice with a bias towards T_h1-type responses. The results were striking – when raw and DR PPE were administered SC, DR PPE was shown to be more immunogenic and produced a T_h2 bias in C57Bl/6 mice similar to that observed in Balb/c mice (Figure 4.7 A-B).

As might have been predicted by the relatively low responses even in susceptible Balb/c mice, the IG route elicited weak responses from C57Bl/6 mice. Without adjuvant, no detectable responses were produced (data not shown), even when the dose was increased to the maximum concentration that the viscosity of CPH would allow to be gavaged. In contrast, the addition of CT elicited detectable (though weak) serum IgG and IgE responses (Figure 4.7 C-D).

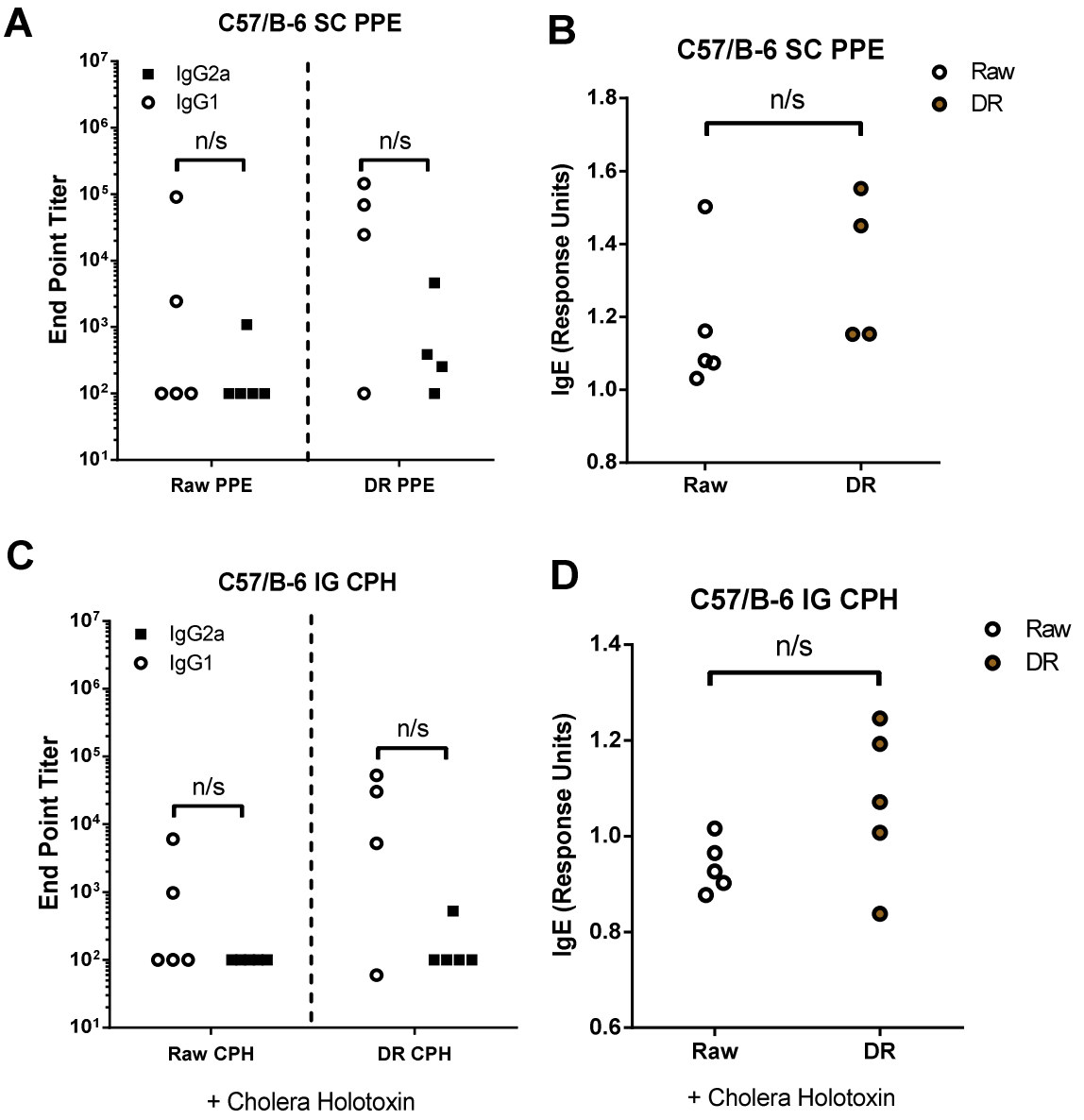


Figure 4.7 Immunogenicity and T_h2 response skewing of **(A-B)** SC-immunised PPE and **(C-D)** IG-immunised CPH in C57Bl/6 mice. N = 5. n/s = non-significant; Mann-Whitney *U* -test

4.5.5 Purified DR Ara h 1 shows a similar immunogenicity to complete DR PPE

The combined data from IG and SC models of sensitisation provide strong evidence that DR peanut is more immunogenic. However, it remains an open question whether this is a synergistic effect of multiple components of PPE and CPH, or whether it can be ascribed to changes in individual allergens. To begin the approach to answering this question, purified, endotoxin-depleted raw and DR Ara h 1 were administered to mice, using the same protocol of subcutaneous priming followed by subsequent IG challenge that had been successfully used for PPE (Figure 4.8A).

The observed effects were remarkably similar to that seen for total PPE, with raw Ara h 1 being strongly immunogenic in its own right, a property further enhanced by dry-roasting (Figure 4.8 B). The difference in magnitude between raw and DR-primed responses became statistically significant after IG challenge, and this trend was retained even after repeated challenges (Figure 4.8C). A bias in favour of T_h2 antibody complement was also detected, with IgG1 production predominating (Figure 4.9A) and higher levels of IgE in DR-Ara h 1 immunised mice (Figure 4.9B).

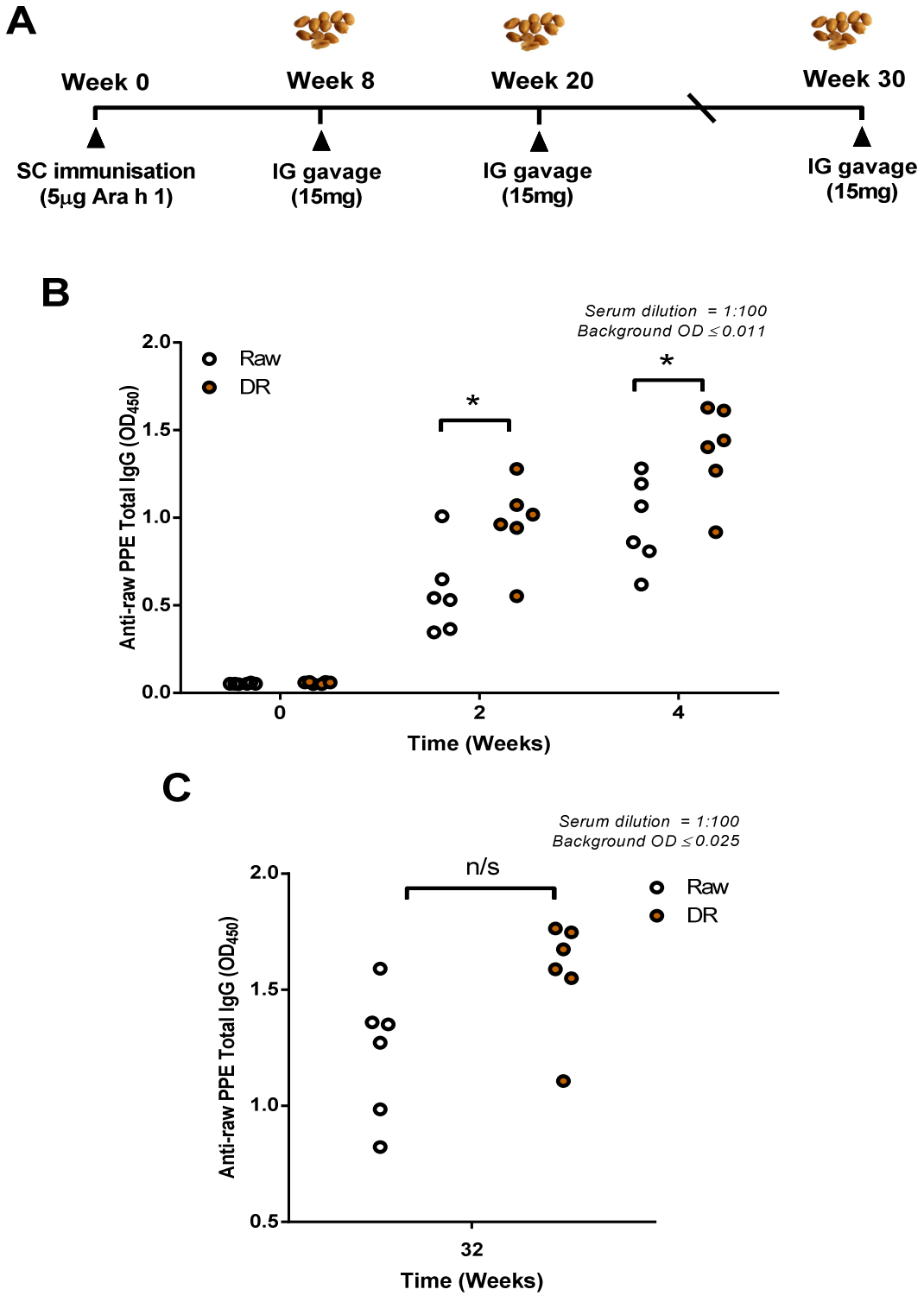


Figure 4.8 Subcutaneous immunogenicity of Ara h 1 in female Balb/c mice. **(A)** Schema of immunisation protocol. **(B)** Time course of peanut-specific total serum IgG responses after priming. **(C)** Anti-peanut total serum IgG responses two weeks after the final challenge. N = 5, representative of 3 independent experiments. N = 5. * p = ≤ 0.05; n/s = non-significant; Mann-Whitney *U*-test.

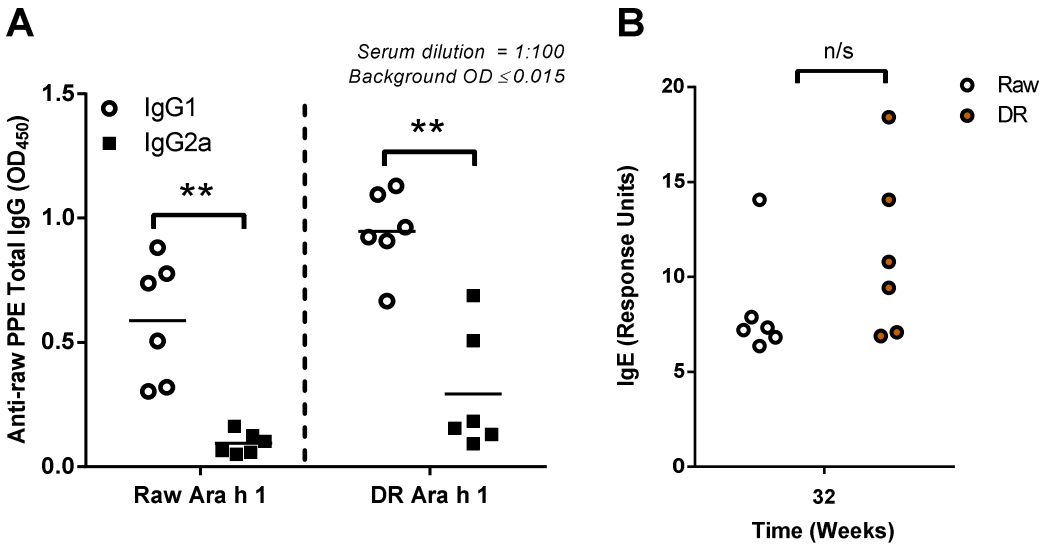


Figure 4.9 T_h2 skewing of antibody responses to SC immunised Ara h 1. **(A)** Comparison of binding to raw PPE by IgG1 (T_h2) and IgG2a (T_h1) in Ara h 1-immunised mouse sera at week 12 post-prime. **(B)** Serum raw-PPE specific IgE peanut at week 12 post-prime. N = 6, representative of 3 independent experiments. *** $p = \leq 0.001$; n/s = non-significant; Mann-Whitney *U* -test

4.5.6 Epicutaneous sensitisation can reproduce the differences observed in subcutaneous immunisation

Because I had analysed in some depth one chief contender for the route of sensitisation in humans, oral exposure, it would have been incomplete not to have investigated its chief rival – the theory that sensitisation occurs through damage to the skin. Using a model of epicutaneous sensitisation developed by the Artis Group of the University of Pennsylvania, I worked together in collaboration with Dr Mario Noti to probe the effects of dry-roasting on this model of peanut allergenicity.

Groups of female Balb/c mice were sensitised daily with raw or DR PPE mixed with either mc903 as an exfoliating agent to irritate the skin, or with ethanol alone as a carrier control, and subsequently challenged IG (figure 4.10A). Results were entirely consistent with previous observations from IG and SC models of peanut immunisation. DR PPE elicited higher systemic IgG responses than raw, both with and without exfoliating agent (Figure 4.10B). These responses were T_h2-skewed, and included increased (though still weak) serum IgE production (Figure 4.10C).

Additionally, histological analysis of intestinal tissue from mice immunised according to this protocol revealed other signs of allergenicity. Jejunal sections displayed signs of inflammation and eosinophil infiltration (Supplementary Figure B2A). DR peanut sensitisation was also found to cause a statistically significant increase in eosinophilic infiltration into the duodenum relative to raw (Supplementary Figure B2B).

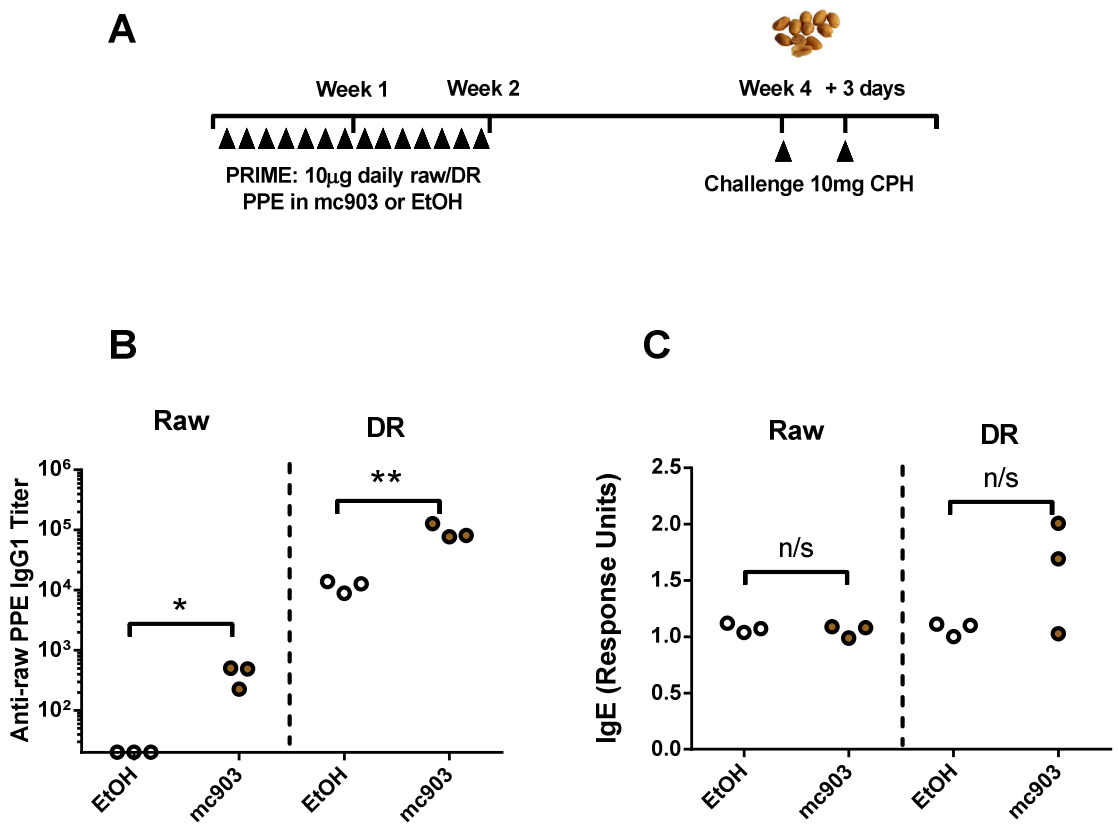


Figure 4.10 Immunogenicity of raw and DR PPE in an epicutaneous Balb/c model of peanut allergic sensitisation. **(A)** Schematic of the sensitisation protocol. **(B)** Serum peanut-specific IgG1 titres, post-2nd challenge. **(C)** Serum peanut-specific IgE responses, post-2nd challenge. * $p = \leq 0.05$; ** $p = \leq 0.01$; n/s = non-significant; Student's *T*-test.

4.5.7 T_h2 priming effects of DR peanut can be prevented by chemical reduction

With the thesis that dry-roasting can enhance peanut immunogenicity now strongly attested by data from IG, SC and EC sensitisation models, the next logical question was naturally which of the many chemical adducts formed during roasting was responsible. The most promising of these classes, RC formation, is also one of the easiest to test, as they are readily removed and reduced to carboxylic acids by the action of a suitably strong reducing agent.

Accordingly, both IG and SC models were attempted, with DR CPH, DR PPE and purified DR Ara h 1 being chemically reduced before administration to Balb/c mice (Figure 4.11 A and C). Proteins were checked for signs of degradation as a result of reduction and were found to be intact (Figure 4.11B). These reduced samples were administered to mice concurrently with one experimental repeat of the IG CPH (Sections 5.5.1-2), SC PPE (Section 4.5.4) and SC Ara h 1 (Section 4.5.6) immunisations already shown. Upon IG immunisation and subsequent feeding, DR-reduced CPH was significantly less immunogenic than DR in terms of serum IgG responses, but the IgE responses were too low to detect a significant difference (Figure 4.12 A and B).

Similarly, IgG responses were significantly lowered by reduction in the SC PPE model, but not Ara h 1 alone (Figures 4.12 C-D and 4.13 A-B). Furthermore the degree of class switching to IgE trended towards being lower both for DR-reduced PPE (Figure 4.12 D) and DR-reduced Ara h 1 (Figure 4.13B). Taken together, these results suggest a partial role of RC groups in peanut allergenicity.

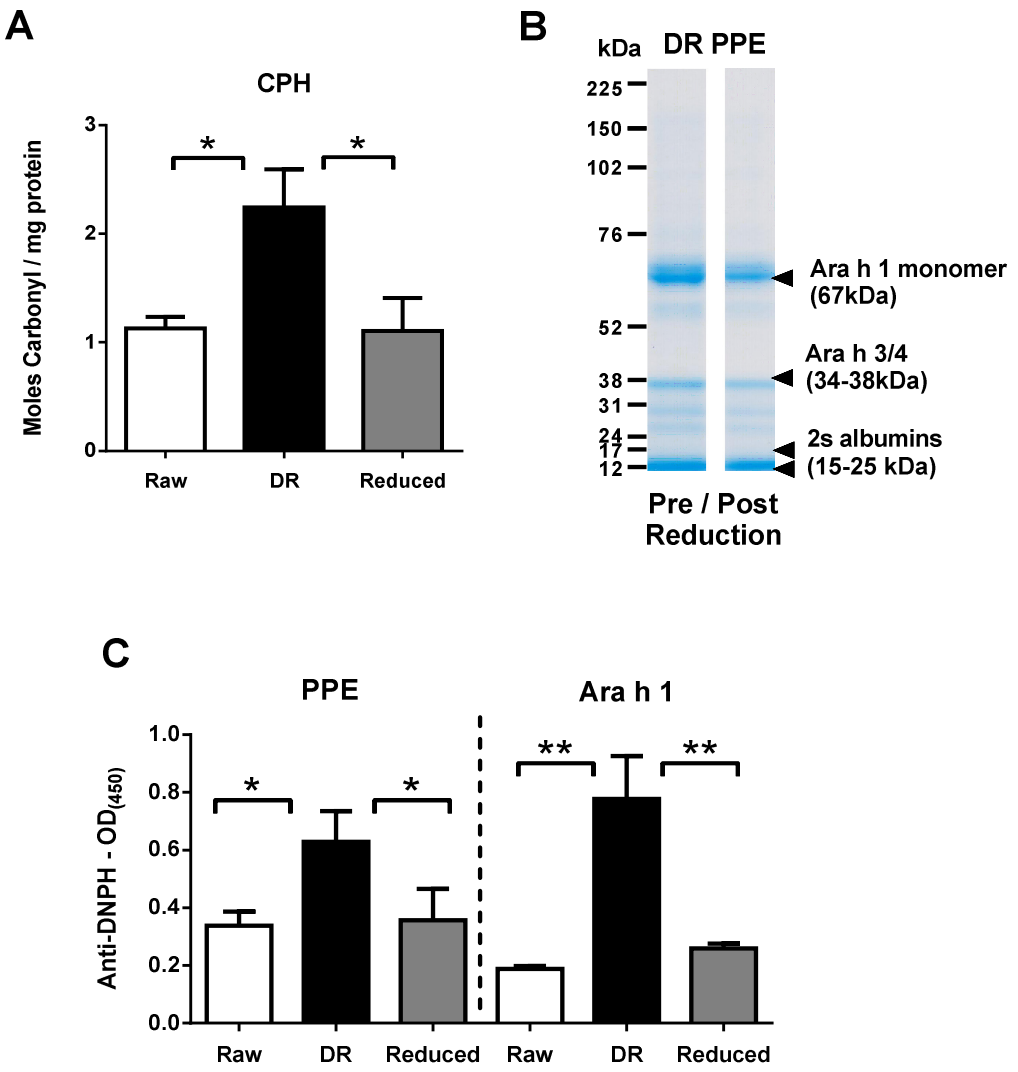


Figure 4.11 Reduction with sodium borohydride of peanut preparations for immunisation. Removal of RC adducts is measured by loss of DNPH reactivity, as measured by either **(A)** colorimetric carbonyl assay for CPH, **(B)** SDS-PAGE of reduced PPE showing intact proteins after reduction; or **(C)** anti-DNPH carbonyl ELISA for PPE and Ara h 1 (background OD ≤ 0.12). * p = ≤ 0.05; ** p = ≤ 0.01; Student's *T*-test.

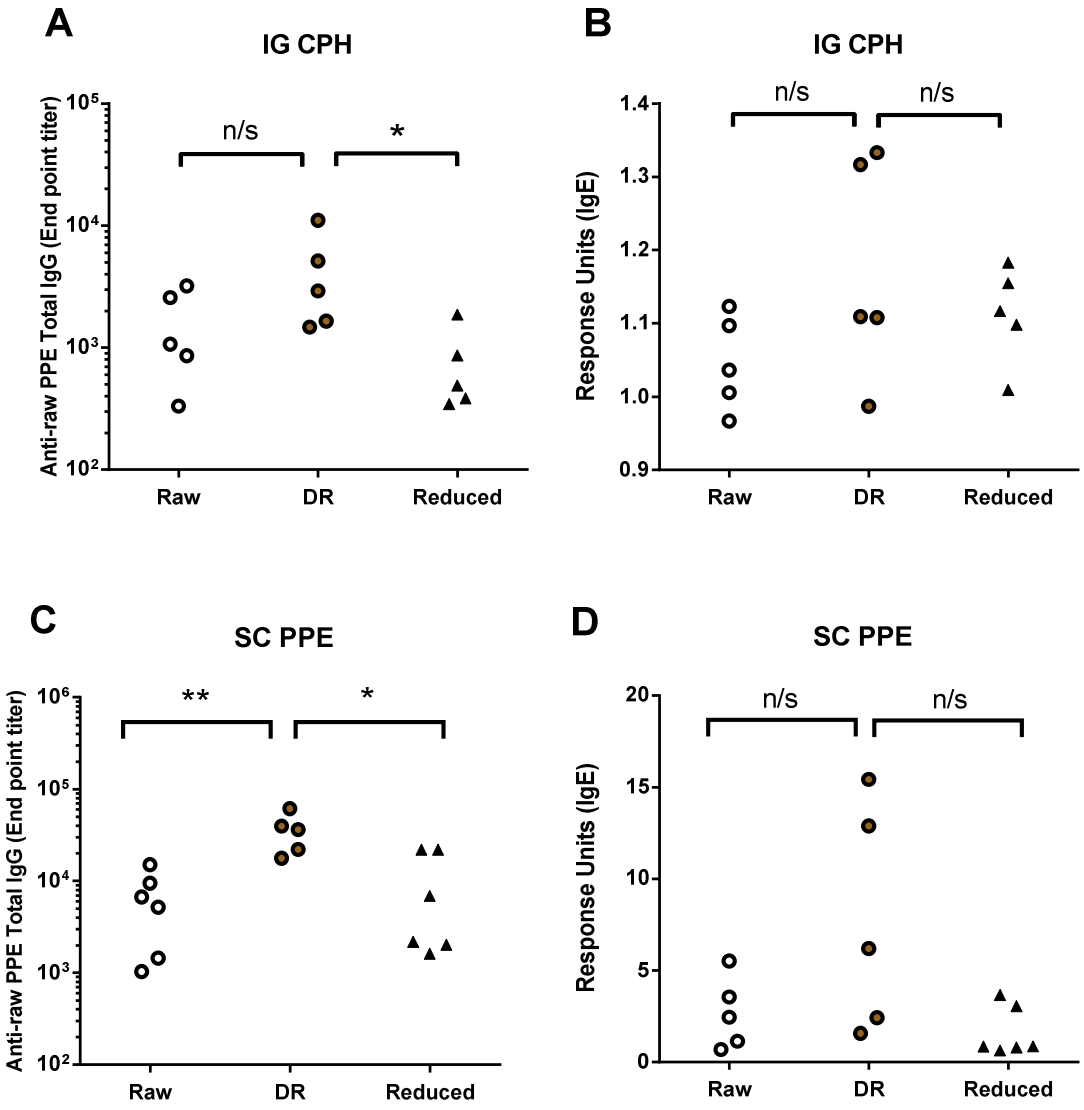


Figure 4.12 Comparison of the immunogenicity of raw, DR and reduced-DR CPH immunised IG and PPE immunised SC in Balb/c mice. **(A)** IG-CPH immunised peanut-specific total IgG levels, 12 weeks post-prime. **(B)** IG-CPH immunised peanut-specific IgE levels, 12 weeks post-prime. **(C)** SC-PPE immunised peanut-specific total IgG levels, 12 weeks post-prime. **(D)** SC-PPE immunised peanut-specific IgE levels, 12 weeks post-prime. * $p = \leq 0.05$; n/s = non-significant; Mann-Whitney *U*-test.

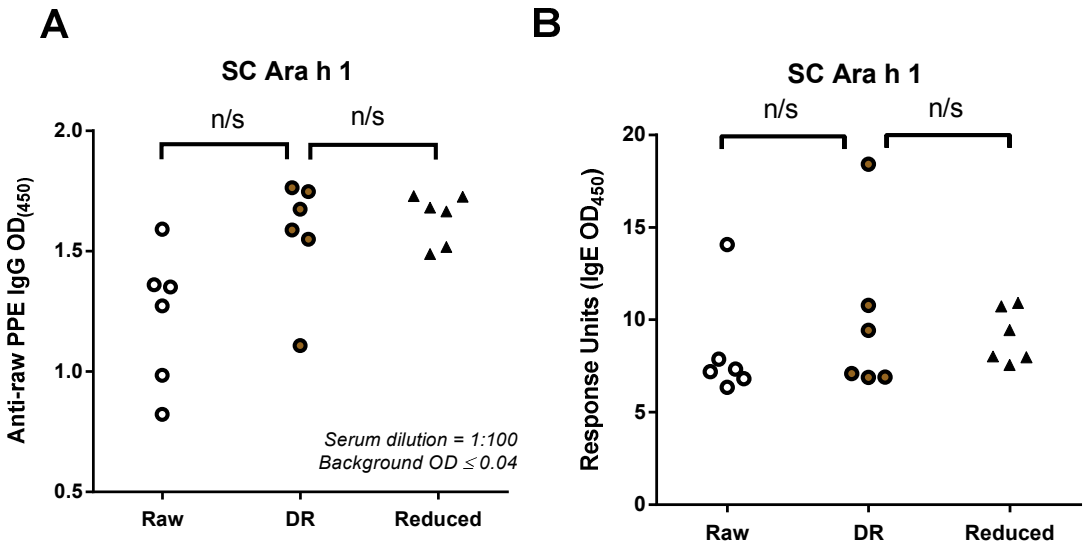


Figure 4.13 Comparison of the immunogenicity of raw, DR and reduced-DR purified Ara h 1 immunised SC in Balb/c mice. **(A)** Peanut-specific total IgG levels, 12 weeks post-prime. **(B)** Peanut-specific IgE levels, 12 weeks post-prime. * $p = \leq 0.05$; n/s = non-significant; Mann-Whitney *U*-test.

4.6 Discussion

These data demonstrate for the first time an enhanced immunogenicity and apparent T_h2 skewing of DR peanut relative to raw in multiple murine models of peanut allergy. Enhanced immune responses to DR were observed in IG, SC and EC models of sensitisation, indicating that these effects are manifested regardless of administration route, and in two different strains of mice, indicating that these effects are not reliant upon the specific genetic background of Balb/c mice. Additionally, an apparent bias towards T_h2 responses was demonstrated across all models, although this observation must be qualified due to baseline levels of murine IgG1 expression being significantly higher than those of IgG2a. These observations could therefore be strengthened by a comparison with other immunised antigens, ideally non-allergenic homologues of the peanut allergens, in order to rule out the possibility that such an apparent skewing effect is not simply due to higher baseline IgG1 production. Nevertheless, enhanced production of the T_h2 marker of specific IgE was also observed across multiple models, lending credence to the T_h2 skewing interpretation. However, as well as these similarities, differences were also observed between different administration routes, providing valuable information about the immunogenic potency of DR peanut in mice, and perhaps also by extension in humans.

One particularly striking feature of these data is the high degree of heterogeneity exhibited by mice within the same experimental groups. Some mice not only responded more strongly than others, but some, especially in the IG immunisations, barely responded at all. Whilst a limited degree of heterogeneity is to be expected even among inbred strains of laboratory mice, their observed range of responses appears to resemble allergy-susceptible populations of humans. Even among cohorts with strong known risk factors for allergy,

development of symptoms seems to be a stochastic process which cannot be predicted with certainty. Although in mouse strains such as Balb/c, individual genetic variation has been rendered minimal by systematic inbreeding (especially when factors such as diet, sex and age are controlled for) a corresponding lack of variation between individuals in life history, epigenetics and microbiota cannot simply be assumed. Research in both human cohorts and animal models suggests that these may have significant effects on allergy susceptibility (Lovinsky-Desir and Miller 2012). Therefore, possible explanations for this heterogeneity include not only sources of experimental variation, such as varying degrees of mucosal abrasion during gavage, but also individual health history of mice prior to inclusion in the experimental groups.

Unlike previous studies, here IG immunisation of mice used either only mild adjuvantation, or none whatsoever. Yet, higher responses to DR CPH were observed for both. Although some researchers recommend avoidance of peanut consumption in infancy as a prophylactic measure against developing peanut allergy (Zeiger 2003), the results from IG immunisation of mice do not support the contention that DR peanut is a potent allergen via the gastroinestinal route of exposure. Although low serum IgE does not necessarily prove that IgE is not being produced (Sampson and Ho 1997; Helm and Burks 2000), this interpretation is consistent with the weak responses to T-cell restimulation of splenocytes, indicative of poor systemic sensitisation.

It appears that whilst T_h2 biased responses were induced in the IG model of sensitisation, even in the presence of mucosal adjuvant this was insufficient to cause an efficient transition from IgG1 to IgE, enabling full allergic activity (Poulsen and Hummelshoj 2007). Although in more robust animal models of allergic anaphylaxis, greater IgE production and

full anaphylactic symptoms were observed (Adel-Patient, Bernard et al. 2005) (Song, Qu et al. 2010), I believe that these are unlikely to be representative of physiological exposure to dietary food proteins. However, a broader role for the gut-associated immune tissues should by no means be dismissed, as their importance has been demonstrated by the boosted responses of mice challenged orally with CPH after SC sensitisation, in agreement with previous studies using other food allergens (Dearman, Caddick et al. 2001). The questions of what additional factors can cause such weak initial responses to pass over the "threshold" into full-blown food allergy are of critical importance for future research.

The results observed in EC and SC models show convincingly that even when peanut proteins are administered in the absence of confounding factors influencing IG immunogenicity, such as digestibility and bioavailability, DR peanut proteins are robustly more immunogenic and T_h2-biasing than raw. This effect was observed both in sera, with high IgG1 and functional IgE production, and in T-cell responses to antigen restimulation, which showed both greater proliferation for DR-immunised T-cells but also a greater bias towards T_h2 cytokine production. This result was not due to increased aggregation of DR peanut protein, as prior native-PAGE of the PPE samples used had indicated that DR PPE contained fewer high molecular weight oligomers than raw. Subsequent IG exposure after initial immunisation increased serum immunoglobulin responses, as well as producing strong, T_h2-biased local responses in the mesenteric lymph nodes draining the gut. These findings are on the whole most consistent with the hypothesis that the primary route of peanut sensitisation is via the skin (Wang and Sampson 2009), especially when the skin is damaged or irritated, as observed in human eczema, which is often identified as a prior

stage in the “atopic march” towards the development of food allergy (Leung, Boguniewicz et al. 2004) and asthma (Spergel 2010).

The enhanced immunogenicity and T_h2 skewing effect of DR peanut products was particularly effective through the SC route of exposure, and furthermore, this effect is not only a synergistic property of the protein mixture in PPE, but can be reproduced by the single peanut allergen Ara h 1. Highly purified DR Ara h 1, containing fewer aggregates and oligomers than its raw counterpart, was able to elicit significantly higher T_h2-biased serum and T-cell responses than raw. An additional aspect of these findings is they demonstrate that raw Ara h 1 is potently immunogenic in its own right, in agreement with other reports (Shin, Compadre et al. 1998; Shreffler, Castro et al. 2006; Blanc, Vissers et al. 2011), and that dry-roasting enhances, rather than initiates, this property of the allergen.

As for the underlying chemical modifications which are responsible for this effect, among many AGE modifications RC species have been the prime candidate for a T_h2-skewing cause in previous reports (Moghaddam, Olszewska et al. 2006; Moghaddam, Gartlan et al. 2011). With regards to the effect of the reduction of RC species on DR peanut immunogenicity, the results presented are wholly consistent with the predictions generated by the hypothesis that RC formation may be one of the contributory mechanisms of the increased immunogenicity of DR peanut protein, although they fall short of demonstrating it conclusively as a dominant factor. Reduced DR PPE did not produce IgE responses higher than those of raw in any of the experimental models used, and in both IG CPH and SC PPE immunisation, total serum IgG was significantly lowered as well. Care must be taken in interpreting these data, however, since whereas prior to reduction CPH and PPE were LPS-free, and every effort was taken to ensure that reduction was carried out under aseptic

conditions, it was not possible to obtain reducing agents completely depleted of LPS, allowing for the possibility that some contamination may inadvertently have been reintroduced during the reduction process.

In conclusion, there seems to be strong evidence to the hypothesis support that dry roasting of peanuts does increase allergenicity in murine models of sensitisation compared to its raw counterpart, and at least a proportion of the enhanced immunogenicity appears to be derived from RC adduction.

Chapter 5
In vitro studies of peanut allergens

5.1 Abstract

Roasted peanut proteins possess a heightened ability to initiate T_h2-biased immune responses relative to raw, but the mechanisms of this effect remain unclear. In particular, it remains to be seen whether peanut allergens exert their effects by directly activating dendritic cells (DCs), by enhanced uptake, increased or modified receptor binding, antigen presentation or a combination of all of these. DCs were directly stimulated by a full panel of peanut extracts and purified allergens, and were found not to be conventionally activated by any of them. In contrast, DR peanut allergens bound preferentially to DC surfaces over raw. To characterise this effect further, DC binding was blocked with various agents, and found to be partially inhibited by known scavenger receptor agonists. An ELISA for binding of candidate receptors revealed that RAGE and CD36 showed enhanced binding to dry-roasted antigens. In addition, DR, but not raw, peanut allergen successfully competed with acetylated LDL uptake, a known ligand of SRA and CD36. However, none of these effects could be completely blocked by known scavenger receptor ligands. Taken together, these results indicate that the enhanced immunogenicity of DR peanut allergens may in part be due to enhanced binding to APCs, which appears to be mediated by multiple scavenger receptors.

5.2 Introduction

In Chapters 3 and 4, I have presented a range of evidence from *in vivo* murine models of peanut immunogenicity, using three different administration routes, with and without the use of adjuvants. In all of these cases, DR peanut preparations were found to be more immunogenic than raw. Taken together, these data strongly support the hypothesis that dry-roasting can enhance the immunogenicity and allergenicity of peanut proteins. However, in order to discover why dry-roasting produces this difference, it is necessary to make more detailed mechanistic studies of the interaction of peanut proteins with these different systems.

Known immunogenicity enhancers, including AGEs, have been described to influence innate immunity in a variety of ways (Wills-Karp 2010). They may allow increased binding to known receptors or interaction with additional receptors, enabling preferential or more rapid uptake relative to the unmodified protein (Ilchmann, Burgdorf et al. 2010). Alternatively, they may directly activate antigen-presenting cells by signalling through PRR receptors, as has been documented for house dust mite allergens (Trompette, Divanovic et al. 2009), resulting in co-stimulation which enhances the subsequent response. Which of these is the case for peanut allergy is far from clear – indeed, they are not mutually incompatible. However, demonstrating either requires direct measurements of interactions with the archetypal antigen-presenting cells, Dendritic Cells (DCs).

5.2.1 Bone-Marrow Derived Dendritic Cells are a key model for antigen processing

DCs are a core component of the front line of immunity, as they form one of the most important bridges between innate and adaptive immune responses (Steinman 2012). Although sub-populations have additional, distinctive functions (Reizis, Colonna et al. 2011), they share one important role in common. They act as "professional" antigen-presenting cells, whose primary function is to sample their environment, processing and presenting peptides on their surface for recognition by cognate leucocytes (Steinman 2012). Extracted bone marrow may be induced to form DCs *in vitro,* to produce populations of bone-marrow derived DCs (BMDCs) (Roney 2013).

Using this model, it should first be possible to determine whether or not the dry-roasted peanut allergens are capable of directly activating DCs. DC activation results in the expression of numerous co-stimulatory molecules on the cell surface, which vary depending on whether the antigens and environment in question have stimulated the DC into co-ordinating a T_h1 or T_h2 response (Moser and Murphy 2000). These molecules may be used as markers of DC activation, and in particular, it would be instructive to investigate those markers whose expression is associated with the induction of T_h2-type responses specifically, such as CD86 (Tsuyuki, Tsuyuki et al. 1997) and OX40-ligand (Lane 2000).

An alternative possibility, which also requires testing, is that rather than activating the DCs directly, DR peanut allergens may either bind to DCs better or be taken up more rapidly by them. The uptake and binding interactions of proteins with cells may readily be followed by fluorescently labelling the proteins in question, which can then be used to detect the internalisation or surface binding of the attached allergens via flow cytometry. The

adsorption or endocytosis of the fluorescent allergens will produce a corresponding shift in the fluorescence intensity of the relevant populations of cells.

5.2.2 Candidate receptors of DR peanut antigens need to be tested

Once any differences in uptake or binding between raw and DR peanut proteins have been detected by this method, its mechanism can be further investigated, either by attempting to block candidate receptors or determining whether peanut allergens can compete against known receptor ligands. Owing to the heterogenous nature of the physico-chemical modifications induced by dry-roasting, many potential binding partners for DR allergens exist on the surfaces of DCs. Previous studies published in the literature mark out a few possibilities as particularly worthy of interest, including the glycan-binding integrin protein DC-SIGN (Shreffler, Castro et al. 2006), Scavenger Receptors A and CD36 (Ilchmann, Burgdorf et al. 2010), and the Receptor for Advanced Glycation End Products (RAGE) (Morbini, Villa et al. 2006). All four proteins are good candidates because their natural functions appear to be related to the clearance of damaged (and especially oxidised) proteins from the body. In this capacity, they have been linked to disease states associated with defective metabolism of oxidised proteins. It is therefore *prima facie* highly plausible that they may also play a role in the interactions of DR allergens with APCs.

5.2.3 Main receptor candidates for DR peanut binding

The Scavenger Receptors are a class of proteins found on many phagocytic cells, whose function is to assist these cells with the uptake of damaged or oxidised proteins, usually to clear them from circulation (Murphy, Tedbury et al. 2005). They are, however, also present on the surface of DCs, potentially increasing the uptake and presentation rate of damaged

proteins. SRA and CD36 are prototypical examples of Class A and Class B scavenger receptors respectively. These two families are structurally and mechanistically distinct from each other (Stephen, Freestone et al. 2010), and are only referred to by a common name owing to their shared discovery through their binding action to oxidised lipoprotein. However, this is not their only ligand – both classes are quite promiscuous, binding to a wide range of ligands including collagen, oxidised phospholipids and even unmodified lipoproteins (Murphy, Tedbury et al. 2005). Owing to the presence of many large oxidised adducts on DR peanut allergens, an interaction with at least one class of SR would not be surprising. Multiple publications have attested that an analogous interaction takes place with the AGE-modified egg allergen ovalbumin (Hilmenyuk, Bellinghausen et al. ; Ilchmann, Burgdorf et al. 2010).

LOX-1 (Oxidised Low-Density Lipoprotein Receptor) is another scavenger receptor discovered in similar circumstances, by virtue of its role in binding to oxidised lipoproteins (Kume, Moriwaki et al. 2000) during the deposition of atherosclerotic plaques (Kita, Kume et al. 2000). In contrast to SRA and CD36, LOX-1 is a member of the c-type lectin superfamily (Mehta, Chen et al. 2006). Its extensively documented interactions with oxidised proteins make it another likely candidate for DR peanut interaction (Twigg, Freestone et al. 2012).

DC-SIGN is a c-type lectin (van Die, van Vliet et al. 2003), but unlike the three scavenger receptors, which are united by their common ligand in the form of oxidised lipoprotein, DC-SIGN binds to mannose and fucose carbohydrate oligomers on the surface of proteins (van Liempt, Bank et al. 2006). These are not found in mammalian tissues, and can therefore act as a potential marker for the presence of pathogens (Takahashi, Ip et al. 2006). However, they are also common in plants, and given that Ara h 1 (Altmann 2007) and possibly also the

2s albumins (Barre, Borges et al. 2005) are glycosylated, and Ara h 1 contains fucose and mannose residues (van Ree, Cabanes-Macheteau et al. 2000), an interaction with DC-SIGN is highly likely. One publication went so far as to suggest that Ara h1 in its raw form could directly act as an autoadjuvant through binding to DC-SIGN (Shreffler, Castro et al. 2006).

The potential importance of RAGE is of particular interest because its ligands are the complex AGEs which are formed at the later stages of the Maillard Reaction (Harja, Bu et al. 2008), which also produces the reactive carbonyl residues which were used to measure the oxidation state of dry-roasted peanut (Thornalley 2005). Demonstrating a binding contribution from RAGE would be consistent with the hypothesised role of the Maillard Reaction in the immunogenicity of roasted peanuts.

5.3 Chapter Goals

In this chapter, the following experimental strategy will be pursued to investigate *in vitro* uptake, processing and binding of raw and DR peanut allergens:

1. Analyse the interactions of raw and DR peanut proteins, as well as individual raw and DR allergens, with DCs, to determine whether they can activate these cells directly by conventional mechanisms;
2. Determine whether roasting results in increased DC uptake or binding, by using fluorescently labelled peanut allergens to follow the binding of these proteins to DCs;
3. Look for evidence of increased selective binding to specific receptors, by repeating surface binding experiments in the presence of various blocking agents targeted at receptors suspected to be implicated in DR peanut processing;
4. Where applicable, directly test the involvement of receptors *in vitro,* either by measuring peanut allergen binding to candidate receptors in an ELISA format, or by assessing binding to receptor-expressing cell lines.

5.4 Methods used in this Chapter

The follow methods are described in full in the indicated sections of Chapter 2, which contains full protocols and descriptions for all materials and methods used in this project.

- Determination of peanut concentration by BCA **(2.3.2)**

- Endotoxin depletion of samples **(2.3.5)**
- TLR4 assay for endotoxin content **(Supplementary A3)**
- Thp-1 assay for PRR activation **(2.3.6)**
- Fluoresceination of proteins **(2.3.11)**

- Receptor binding ELISA **(2.9.1)**
- Derivation of Dendritic Cells **(2.9.2 and 3)**
- Dendritic Cell stimulation **(2.9.4)**
- Cell surface binding assay **(2.9.5)**
- Inhibition of Acetylated Low Density Lipoprotein uptake **(2.9.6)**
- Flow cytometry analysis of stained cell populations **(2.9.7)**
- Statistical Analyses **(2.10)**

5.5 Results

5.5.1 Peanut allergens do not directly stimulate DCs through conventional pathways

The possibility that DR peanut allergens could directly activate DCs through conventional activation mechanisms is an intriguing one, as it would require the peanut allergens to mimic the usual ligands of some of the same pattern recognition receptors (PRRs) normally responsible for detecting the presence of hostile pathogens. Because this possibility would produce the same detectable output as contamination of the sample by bacterial or other pathogen molecules such as LPS, great care had to be taken to ensure that these were not present. All samples were filter-sterilised after each stage of purification, and thereafter manipulated only in aseptic conditions. Furthermore, prior to use, liquid samples were rigorously detoxified using a commercial endotoxin removal kit. The resulting LPS-depleted samples were tested by exposure to cell reporter lines expressing a variety of PRRs (Thp-1 cell line), which reported low to negligible signals far below the responses elicited by even a very weak dose of LPS (Figure 5.1). In addition, to further test for the especially potent and near-ubiquitous PAMP molecule LPS, samples were tested on a cell line expressing TLR4, one of the primary natural receptors for LPS in the innate immune system. Perhaps due to the increased sensitivity of this reporter cell line, a small amount of activation was apparent, possibly indicating the presence of small residual quantities of LPS contamination (Supplementary Figure B3).

With confounding factors thus minimised, samples of raw and DR PPE and CPH were all assayed by overnight incubation with bone-marrow-derived DCs (BMDCs). BMDCs were derived using a combination of GM-CSF containing medium and IL-4 stimulation, and the

purity of the final product phenotype was determined by staining for two key markers of immature DCs – the high surface expression of CD11/c and low-to-intermediate levels of MHC class II expression (Figure 5.2A). BMDC samples were also inspected visually for a morphological confirmation of their identity (Figure 5.2B). All batches of BMDCs used for subsequent experiments throughout the rest of this Chapter were characterised according to the same criteria and found to be highly comparable to the example shown.

After incubation, the BMDCs were analysed by flow cytometry, and compared to unstimulated control populations of cells, as well as a positive control of BMDCs incubated with varying concentrations of LPS. Importantly, the surface marker CD40, which is strongly correlated with the action of LPS, was only expressed at a very low level in BMDCs stimulated by the samples, well below the lowest concentration of LPS used (5.3A). Neither CPH or PPE, nor the individual peanut allergens were found to significantly activate BMDCs, as measured by surface expression of the T_h2-specific costimulatory markers of DC activation, CD86 and OX40-ligand (5.3B), with the sole exception of DR 2s albumins, which produced a weak upregulation of CD86 expression.

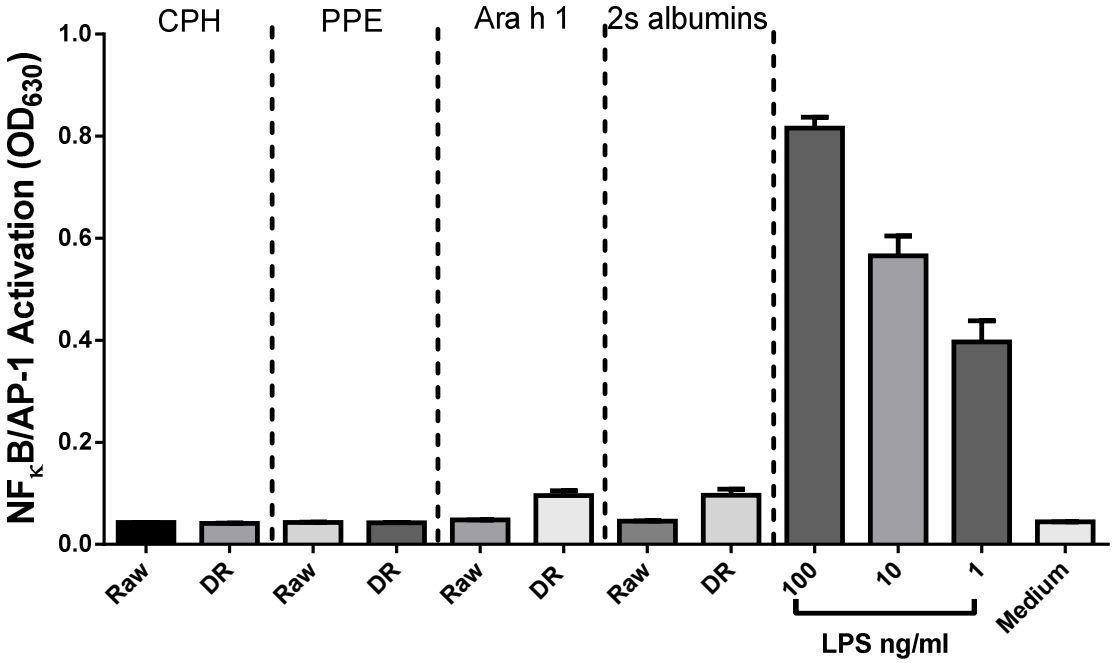


Figure 5.1 Testing of peanut preparations for bacterial endotoxin and other signs of microbial contamination, as measured by the secreted alkaline phosphatise (SEAP) activity of Thp-1 PRR cell reporter line, after 4 hours of reaction time. In this cell line SEAP is under the control of NFκB and AP-1 transcription factors.

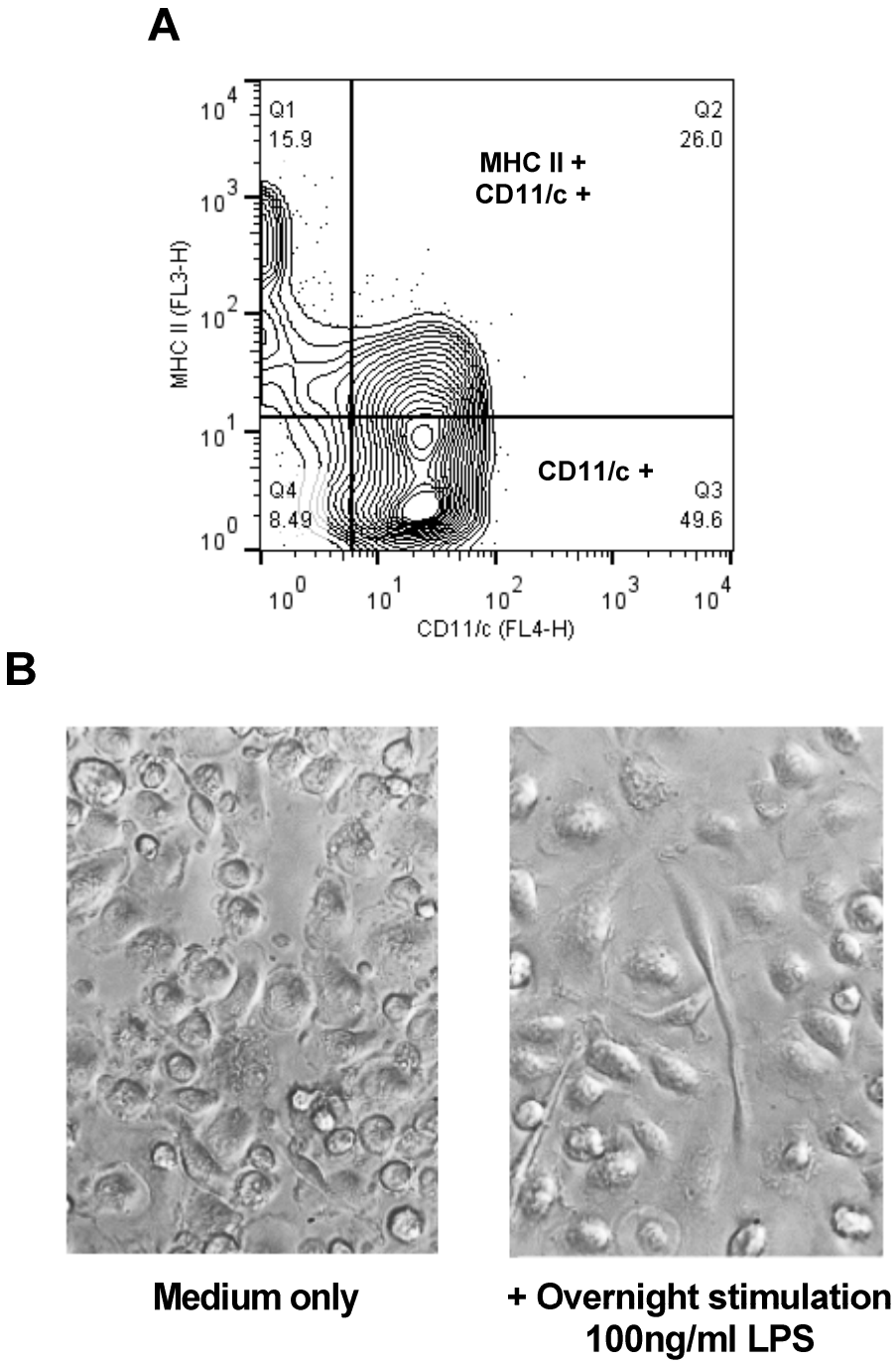


Figure 5.2 Phenotypic characterisation of bone-marrow derived dendritic cells. **(A)** MHC class II (FL3-H) and CD11/c (FL4-H) expression in the live cell gated population of BMDCS. **(B)** Morphology of BMDCs under a light microscope, before and after activation with LPS to a mature state (note the formation of long processes and loss of rounded shape).

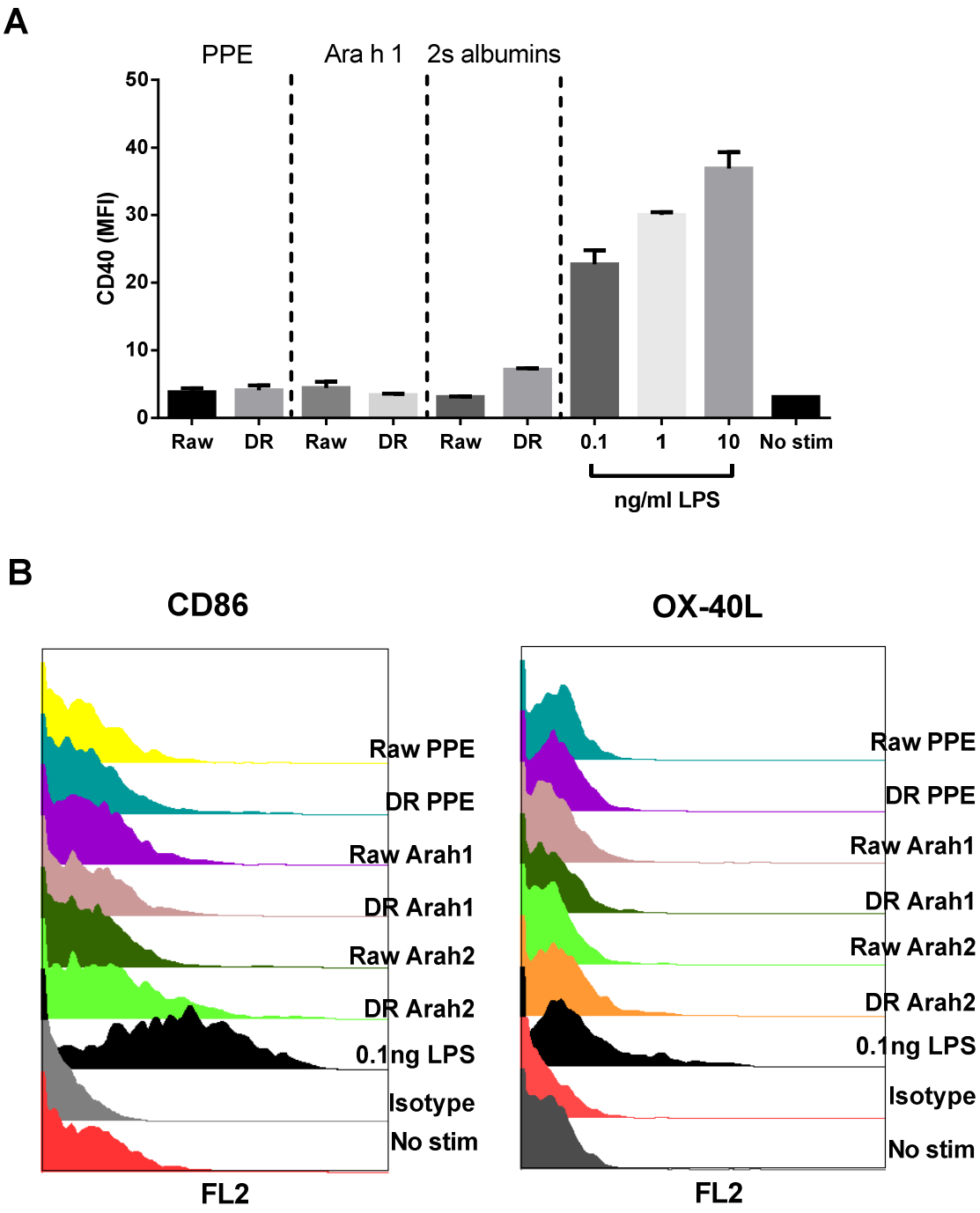


Figure 5.3 Flow cytometry luorescent antibody staining for DC activation markers of BMDC populations incubated overnight with raw and DR peanut allergens. **(A)** Bar chart of CD40 surface expression (FL1 channel, mean fluorescence intensity). **(B)** Histograms of cell surface expression of CD86 and OX-40 Ligand (mean fluorescence intensity, FL2 channel).

5.5.2 Roasted peanut allergens bind preferentially to DC surfaces

DC surface binding is an important early event of the encounters between an antigen and the immune system, as the recognition of antigens by DC receptors can be the first step towards their internalisation, degradation and eventual presentation. Hence, the degree of DC surface binding can determine the extent to which an antigen is preferentially taken out of solution by these "professional" antigen-presenting cells. A change in surface binding for DR peanut relative to raw peanut would be an important indication of increased receptor binding. Experimentally, however, it is important to distinguish this initial binding from other, later steps of antigen uptake, which can be influenced by numerous other factors such as the extracellular medium, and the presence of inflammatory agents such as LPS, even in minute quantities. Together these factors combine to determine the overall rate of uptake.

Fortunately, surface binding can be readily separated from subsequent steps *in vitro* by paralysing the cells with the use of drugs, such as Jasplakinolide or Cytochalasin B. These drugs interfere with the actin polymerisation of the cell cytoskeleton, either by blocking it directly (Cytochalasin B;(MacLean-Fletcher and Pollard 1980), or by stabilising actin filaments so that they cannot reform (Jasplakinolide; (Bubb, Spector et al. 2000). Both of these actions have the effect of preventing the formation of clathrin-coated pits and other endosomal structures effectively halting the uptake of material by the cell. It is therefore possible to use flow cytometry to measure the extent of binding of fluorescently labelled antigen to cells paralysed by these drugs, and to compare the binding rate of raw and DR allergens directly with no interference from the rate of antigen uptake.

Both DR Ara h 1 and the DR 2s albumins bound dramatically better to paralysed DC cell surfaces than their raw counterparts. Although DR antigen binding produced a much larger population of highly positive cells, these nevertheless only represented a minority of the total population, which did not experience an overall fluorescence shift indicative of global binding to the fluoresceinated allergens (Figure 5.4 A and B). The increased overall binding of DR peanut allergens appears to be driven mainly by a relatively small group of cells binding large amounts of antigen, a fact consistent with the observations of other groups who have investigated the uptake of AGE-adducted proteins by DCs (Ilchmann, Burgdorf et al. 2010; Moghaddam, Gartlan et al. 2011).

Accordingly, rather than expressing the results as mean fluorescence intensity, which could have been misleading in light of this fact, the sub-population of highly positive cells was gated and expressed as a percentage of the total cell population. Using this method, the increase in surface binding from raw to DR allergens was found to be statistically significant for both Ara h 1 and the 2s albumins, regardless of whether Jasplakinolide or Cytochalasin B was used to paralyse the cells (Figure 5.5A and B). Owing to the lower signal-to-noise ratio, Jasplakinolide was used for subsequent experiments. This provides a strong indicator that they are able either to bind more avidly to the same receptors as raw, or that they can interact with at least one additional receptor.

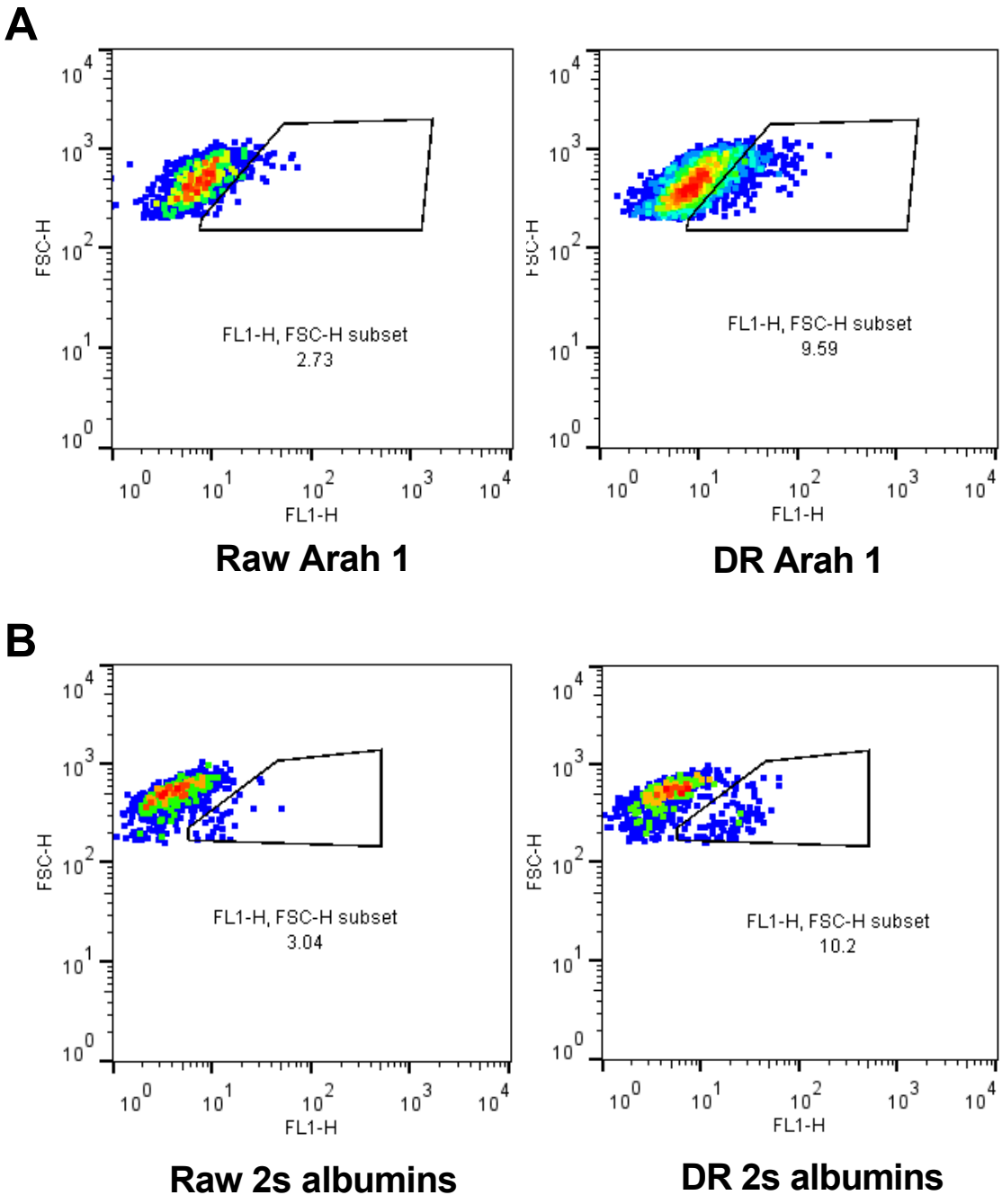


Figure 5.4 Gating strategy for the binding of raw and DR fluorescently-labelled peanut allergens to the surface of bone-marrow derived dendritic cells paralysed with Jasplakinolide. **(A)** Populations pulsed with Raw and DR Ara h 1. **(B)** Populations pulsed with Raw and DR 2s albumins.

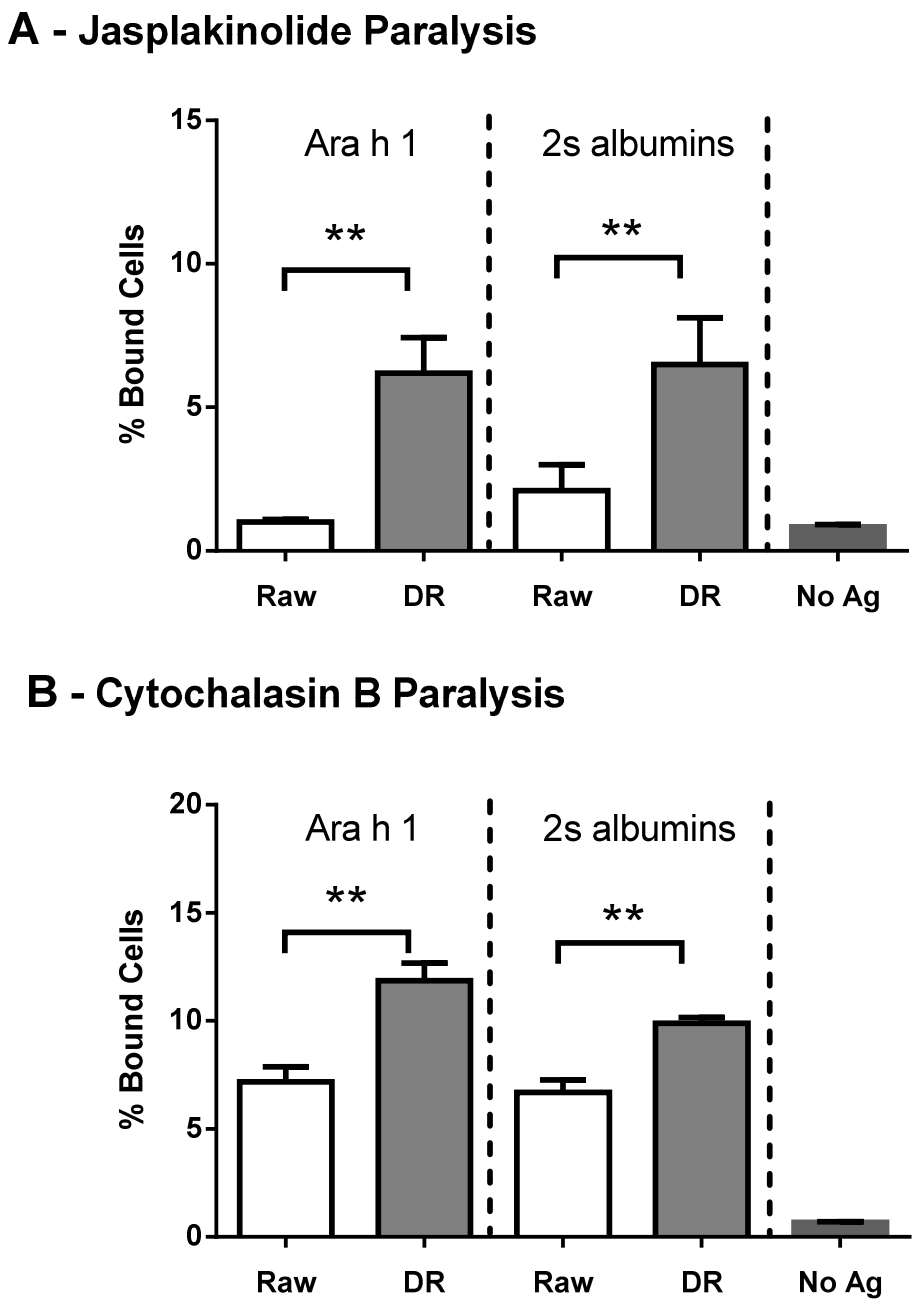


Figure 5.5 Cell surface binding of fluorescently labelled raw and DR peanut allergens to the surfaces of bone-marrow derived dendritic cells paralysed with **(A)** Jasplakinolide; and **(B)** Cytochalasin B. N = 3, representative of three independent experiments. ** $p = \leq 0.01$; Student's *T*-test.

5.5.3 DR Ara h 1 shows increased binding to several putative receptors

Despite its limitations as an indicator of receptor activity *in vivo*, the binding of soluble receptor chimera to immobilised antigen in ELISA format has been shown in numerous other studies to be a useful tool in the identification of binding partners for allergens. In the current case, DR Ara h 1 and 2s albumins were compared to their raw counterparts in terms of their binding to panel of innate immune receptors implicated in oxidised protein processing in prior literature – CD36, LOX-1, and RAGE. As a negative control, TLR3, a PRR which does not bind protein motifs, was also included.

Consistent with its enhanced DC surface binding, DR Ara h 1 exhibited enhanced binding to both RAGE and CD36, whilst binding to LOX-1 and the negative control TLR3 were unchanged (Figure 5.6). Similarly, roasted 2s albumins exhibited significant increases in binding to RAGE and CD36, but not LOX-1 or TLR3, relative to raw 2s albumins (Figure 5.6). However, the base level of binding for raw 2s albumins to CD36 and RAGE was nevertheless higher than that of raw Ara h 1, a fact for which I do not currently have an explanation but which is consistent with the widely reported thesis that Ara h 2 (and, by extension, its cross-reactive homologues) are the most important peanut allergens in human patients (Koppelman, Wensing et al. 2004; Flinterman, van Hoffen et al. 2007).

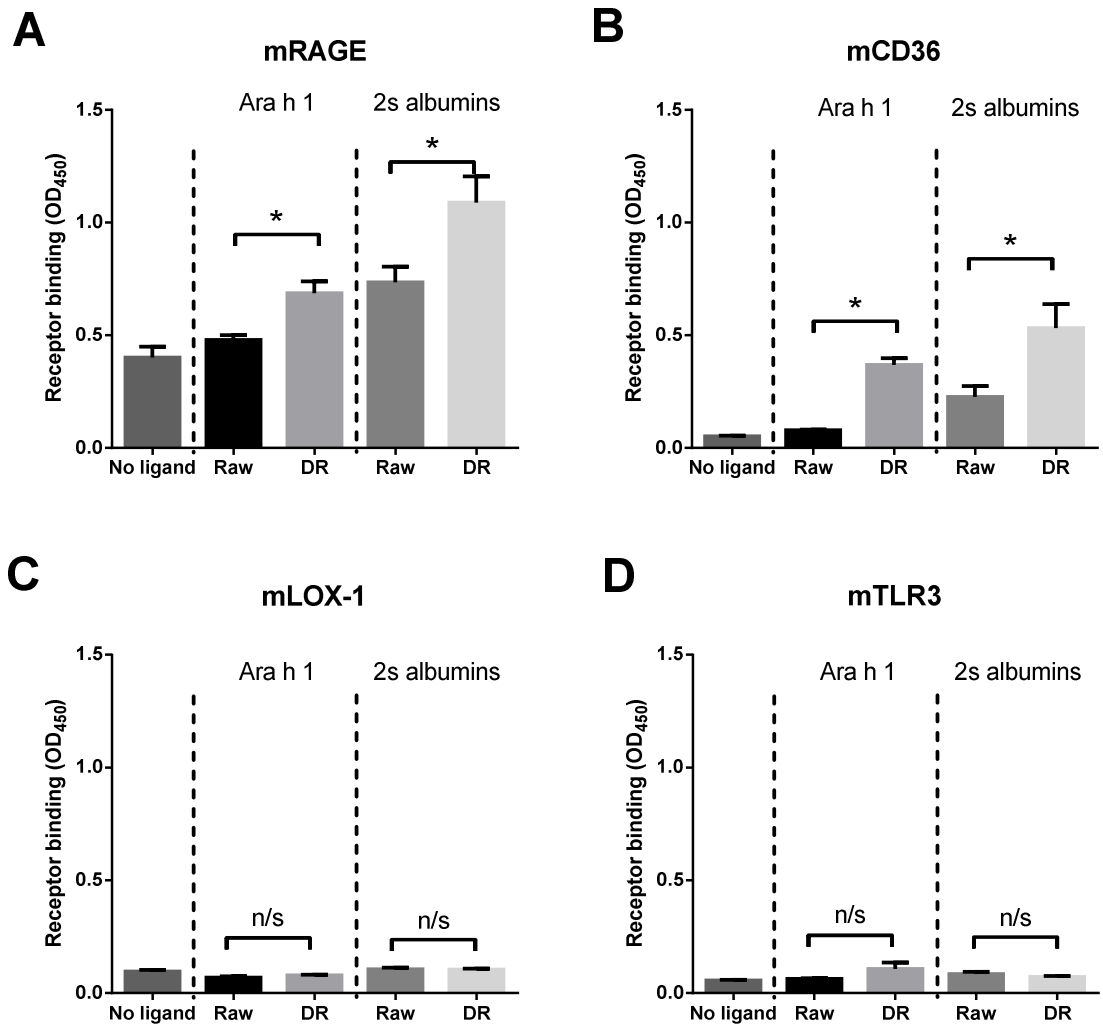


Figure 5.6 Receptor binding ELISA for raw and DR peanut allergens. **(A)** Binding to murine Receptor for Advanced Glycation End Products (mRAGE). **(B)** Binding to murine CD36. **(C)** Binding to murine Oxidised Low-Density-Lipoprotein receptor (mLOX-1). **(D)** Binding to murine Toll-Like Receptor 3 (mTLR3). N = 4, representative of four independent experiments. * $p = \leq 0.05$; n/s = non-significant; Student's *T*-test.

5.5.4 DR peanut allergen DC surface binding can be partially blocked by SR agonists

Returning to the *in vitro* model of DC surface binding, the contributions of individual scavenger receptors were further probed by repeating the experiment after pre-incubating the Jasplakinolide-paralysed DCs with known ligands of the receptors of interest at sufficient excess of concentration to saturate their receptors and prevent the binding of any other ligands, including the labelled peanut allergens. Effectiveness of inhibition was monitored by observing a decrease in the percentage of highly fluorescent, antigen-bound DCs which was observed in the total population, expressed as a percentage of the total binding without blocking agents.

Mannan, an oligosaccharide which blocks glycan-binding PRRs such as DC-SIGN (Geijtenbeek, Engering et al. 2002) and the mannose receptor (Shibata, Metzger et al. 1997), was the most potent inhibitor of DR Ara h 1 binding overall (Figure 5.7A), consistent with the previous report that mannose-binding receptor DC-SIGN is of particular importance as a receptor for Ara h 1 specifically (Shreffler, Castro et al. 2006). DR 2s albumin binding was also significantly inhibited, but to a lesser extent (Figure 5.7B).

Although blocking antibodies for both the SRA and CD36 receptors significantly reduced DR Ara h 1 binding, this effect was of a smaller magnitude than that of Mannan (Figure 5.7A). This effect was apparently not due to mutual redundancy, as a combination of both SRA and CD36 blocking antibodies did not produce a greater degree of inhibition. Similarly, even a combination of CD36 and SRA blocking antibodies did not significantly inhibit 2s albumin binding (Figure 5.7B).

In contrast, the broad-spectrum scavenger receptor ligand, malylated BSA (malBSA) (Abraham, Singh et al. 1995), which binds to many SRs besides SRA and CD36, was a more effective blocking agent for Ara h1 binding than both antibodies (Figure 5.7A), suggesting that other SRs may be involved as well. A very similar effect was observed for the inhibition of DR 2s albumin binding by malBSA, although this inhibition was somewhat less effective than that of Ara h 1 binding (Figure 5.7B). Finally, the RAGE blocking antibody was only partially effective at inhibiting cell surface binding of either Ara h 1 or the 2s albumins (Figure 5.7 A and B).

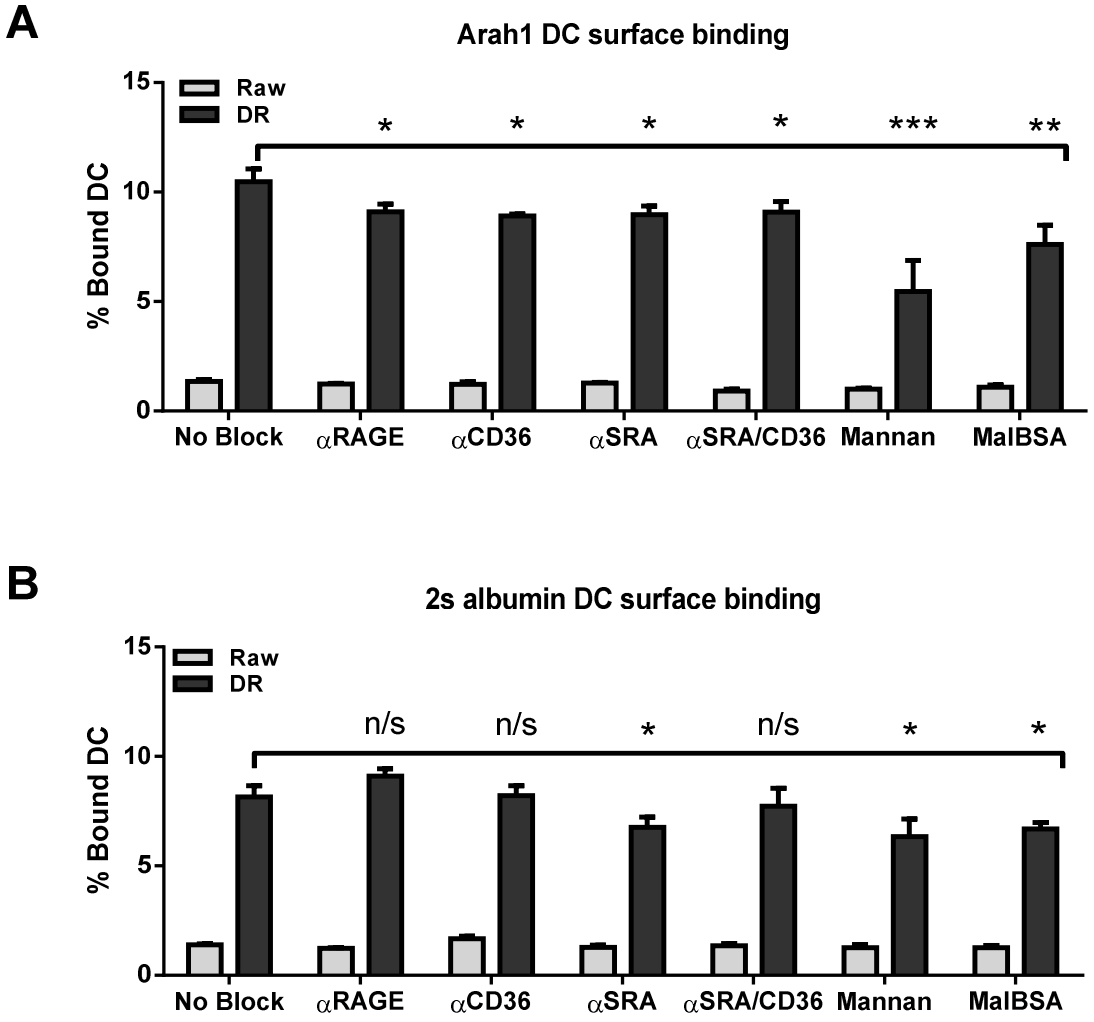


Figure 5.7 Inhibition of Jasplakinolide-paralysed DC surface binding of fluorescently labelled peanut allergens. **(A)** Cell surface binding of raw and DR Ara h 1. **(B)** Cell surface binding of raw and DR 2s peanut albumins. N = 3, representative of 3 independent experiments. * $p = \leq 0.05$; ** $p = \leq 0.01$; *** $p = \leq 0.001$; n/s = non-significant; Student's *T*-test

5.5.5 Roasted peanut allergens successfully compete against acetylated LDL uptake

Acetylated LDL is the original and most extensively characterised ligand of SRA and many other scavenger receptors, including CD36 (Greaves and Gordon 2009), and has been associated with the pathogenesis of atherosclerosis (Steinbrecher, Zhang et al. 1990; Aviram 1993), one of the pathologies in which protein oxidation products such as AGEs have been implicated (Harja, Bu et al. 2008). An ability to compete against, and prevent the uptake of, ac-LDL labelled with the fluorophore label Dil (ac-LDL-dil) (Figure 5.8A and B) would therefore be an important additional confirmation that these receptors are also important in mediating the enhanced binding of DR peanut allergens. As a positive control, maleylated BSA (malBSA), another proven scavenger receptor ligand (Abraham, Singh et al. 1995) and therefore efficient competitor for ac-LDL, was used as the benchmark for successful inhibition of ac-LDL-dil (Figure 5.8C).

I found that DR, but not raw, Ara h 1 was able to successfully compete for ac-LDL-dil uptake, significantly reducing the internalisation of this ligand (Figure 5.9). This property was not shared by total DR PPE at the concentrations tested. Neither the raw nor the DR 2s albumins significantly inhibited ac-LDL-dil uptake (Figure 5.9)

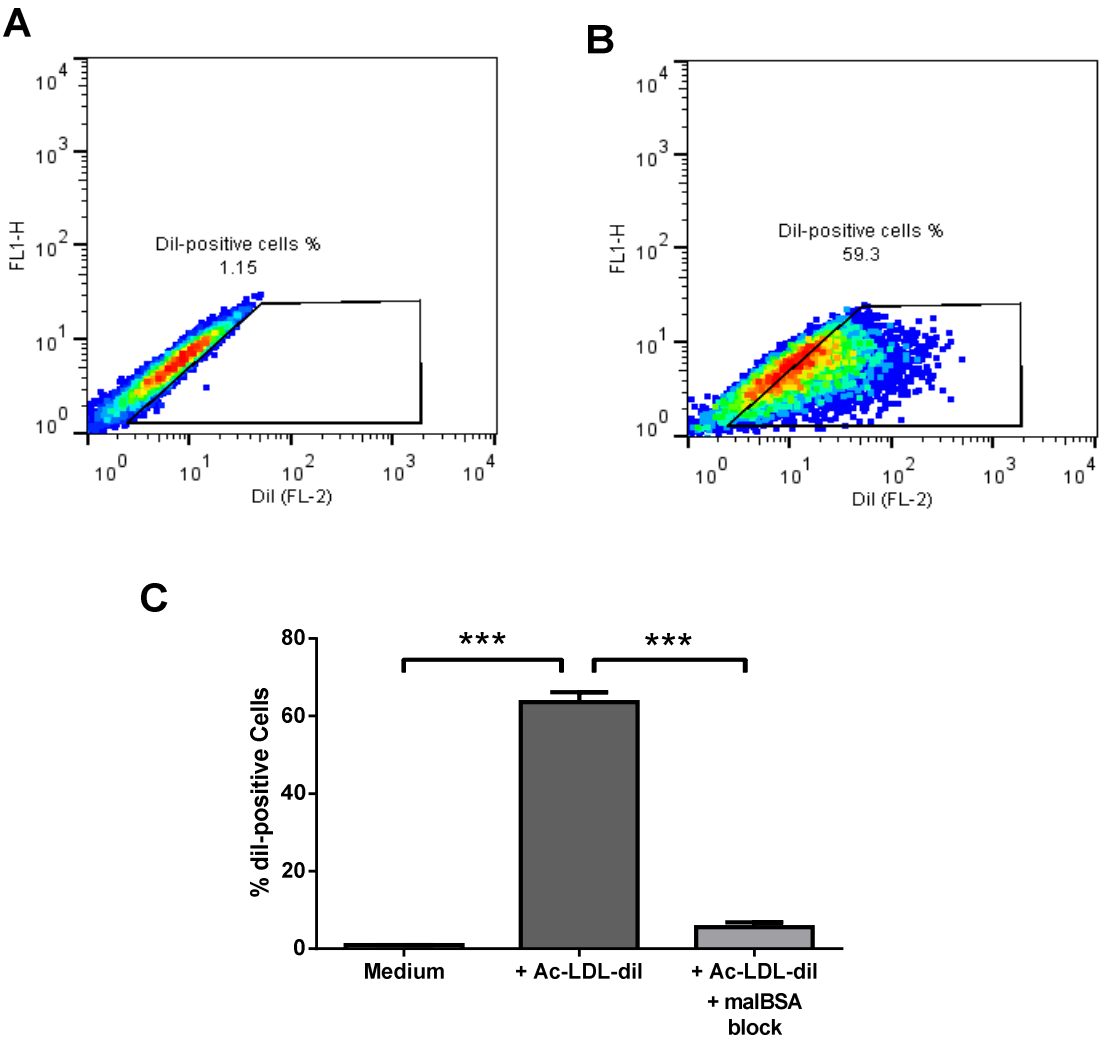


Figure 5.8 The internalisation by DCs of dil-lablled ac-LDL and its inhibition by malBSA. **(A)** Unstained DC population with illustrative gating strategy. **(B)** DC population pulsed with ac-LDL-dil (FL2 channel). **(C)** Bar chart of dil-positive cells as a percentage of total live cells. DCs pulsed with medium alone, ac-LDL-dil alone, or ac-LDL-dil after pre-incubation with malBSA. N = 3, representative of four independent experiments. *** $p = \leq 0.001$; n/s = non-significant; Student's *T*-test.

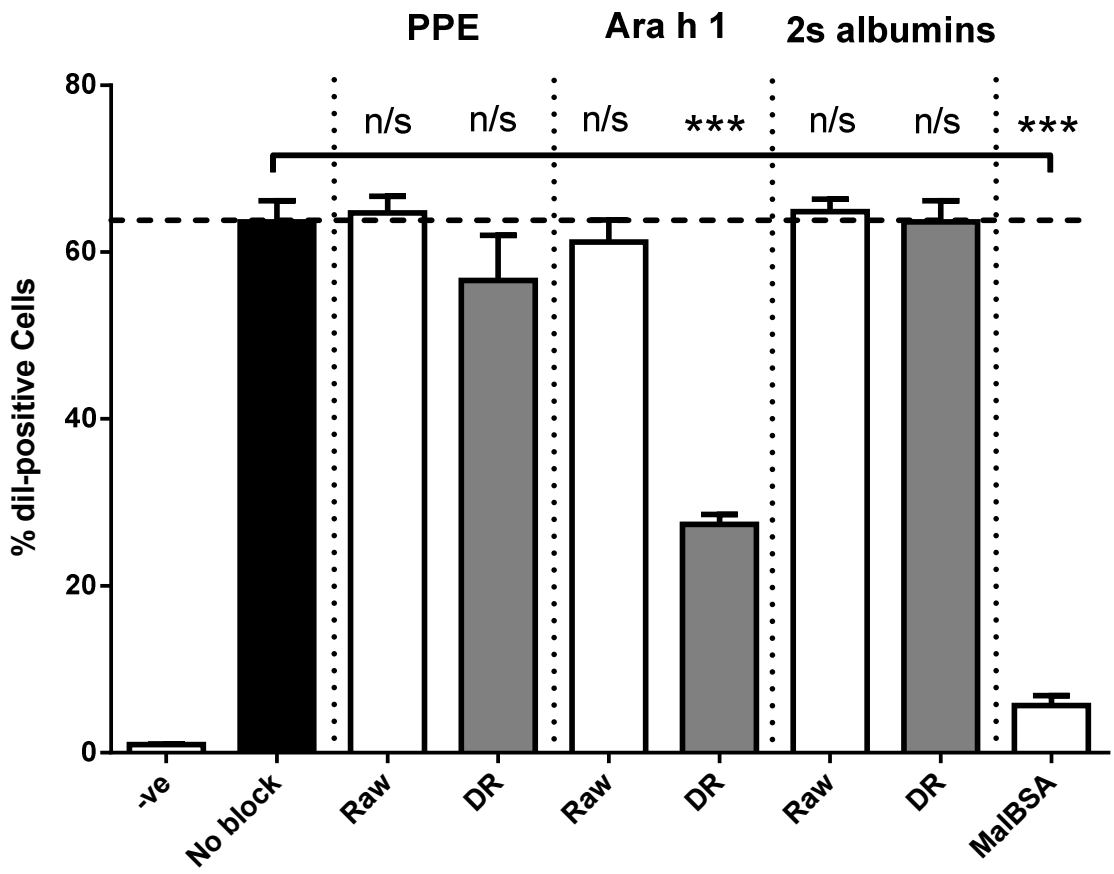


Figure 5.9 Inhibition of the uptake by DCs of ac-LDL-dil through pre-incubation with raw and DR peanut total PPE and purified raw and DR peanut allergens. Negative control: no ac-LDL-dil. Positive control: Inhibition by MalBSA. N = 3, representative of 3 independent experimental repeats. *** $p = \leq 0.001$; n/s = non-significant; Student's *T*-test.

5.6 Discussion

In this chapter, evidence has been presented that DR peanut allergens, neither together in CPH or PPE, nor individually, directly activate DCs. Only a very low level of microbial contamination was found, as measured by assays with PAMP reporter cell lines and commercial endotoxin detection. This was later corroborated by the lack of activation of DC exposed to peanut preparations. This renders very unlikely both the risk that the samples were significantly contaminated with microbial PAMPs, as well as the possibility that DR peanut allergens are more immunogenic via direct activation of DCs. In contrast, DR purified peanut allergens bind to DC surfaces more readily than their raw equivalents, a finding corroborated by increased binding in an ELISA format to known oxidised protein receptors CD36 and RAGE.

In passing, it should also be noted that in human patients, antigen exposure may often coincide with bacterial endotoxin contamination and other PAMPs. Indeed, coadministration of allergen and LPS can enhance allergenicity, as is seen in some models of asthmatic sensitisation (Duez, Gosset et al. 2006). Although the negative results from DC activation assays show that the enhanced binding of DR peanut allergens is independent from any association with LPS or other PAMPs, the possibility of a synergy between intrinsic enhanced allergen binding and LPS contamination *in vivo* should not be dismissed. Ongoing attempts to repeat DC binding experiments with the deliberate inclusion of measured quantities of LPS and other PAMPs are suggestive that a similar effect may be operative for oxidised proteins (unpublished personal communication, Sattentau Group).

The finding that raw peanut allergens only bound weakly to DCs, compared to a much stronger interaction with DR peanut allergens, provides support for the hypothesis, outlined in Chapter 2, that dry-roasting influences immunogenicity by altering the recognition of peanut allergens by the innate immune system. Although both DR Ara h 1 and the DR 2s albumins were found to bind more avidly to DC surfaces than their raw counterparts, the differing effects of distinct receptor blocking agents on these two classes of allergens are suggestive of some diversity in their respective binding mechanisms.

The effectiveness of the glycan receptor blocking agent Mannan at preventing the binding of DR Ara h 1 is not surprising, as this allergen is known to be glycosylated with glycans incorporating mannose residues (Altmann 2007) (van Ree, Cabanes-Macheteau et al. 2000) (Barre, Borges et al. 2005). However, Mannan was also effective at inhibiting DR Ara h 2 binding, despite the evidence for similar glycosylation in the 2s albumins being conflicting (Barre, Borges et al. 2005). Such an effect could potentially be due to Mannan's ability to also bind (and hence block) other lectin-like receptors on DC surfaces which would otherwise bind the charged AGE molecules found in DR peanut allergens.

Similarly, the more successful inhibition of ac-LDL uptake by DR Ara h 1, compared to no difference between raw and DR 2s albumins, is suggestive that scavenger receptors may be more important to DR Ara h 1 binding. Interestingly, the ability of purified DR Ara h 1 to interfere with ac-LDL uptake was not reflected in total DR PPE, which was not significantly different from raw PPE in this capacity. This apparent discrepancy is likely to be due to the relative abundance of Ara h 1 in PPE, as DR Ara h 1 is present at a lower concentration in the DR PPE than in its raw equivalent. The apparent significance of DR Ara h 1 in this capacity

(blocking ac-LDL alone, but not as part of PPE) is consistent with its recognition as a major peanut allergen (Chruszcz, Maleki et al. 2011).

Overall, however, the cell surface binding data in combination with receptor binding ELISA data strongly suggest a partial role for glycan receptors such as DC-SIGN, AGE receptors such as RAGE and scavenger receptors such as CD36 and SRA in mediating the enhanced binding of DR allergens. Similarly to most SR ligands (Pluddemann, Neyen et al. 2007) (Canton, Neculai et al. 2013), DR peanut proteins do not appear to ubiquitously bind to all SRs, as evidenced by the lack of detectable binding to the oxidised LDL receptor LOX-1 (Mehta, Chen et al. 2006).

Although not entirely conclusive, the data presented in this chapter are of considerable assistance for more sharply defining the problem of the mechanisms behind the enhanced immunogenicity of DR peanut allergens. In particular, they allow the ruling out of the hypothesis that DR peanut allergens directly activate DCs. Whilst the evidence is consistent with multiple receptors playing a role in enhanced DR peanut binding, it falls short of a clear demonstration, as it remains to be elucidated by future functional studies whether increased binding is translated into enhanced immunogenicity. Because specific receptor-blocking antibodies were markedly less effective than broader-spectrum blocking agents such as malBSA (Abraham, Singh et al. 1995), it appears likely either that other members of the ever-widening ranks of scavenger receptors (Stephen, Freestone et al. 2010) may play a role, or that oxidised proteins may bind in a manner which is unaffected by the blocking antibodies in question. Future work will need to address this diversity of apparent mechanisms by using combinations of blocking agents against broader panels of receptors, and, ideally, knockout strains of mice lacking individual receptors.

Regardless, it seems most likely that multiple receptors are involved in the uptake of peanut allergens, and upon roasting, new binding partners become involved, but to a partial extent and with a degree of mutual redundancy. This would be wholly consistent with the highly heterogenous nature of the modifications (O'Brien, Siraki et al. 2005) Supplementary Figure B4) which are incurred during dry-roasting which, even when created in reproducible relative proportions, contain a remarkable diversity of end products.

Chapter 6
Comparative immunogenicity of the peanut allergens

6.1 Abstract

In order to validate the applicability of murine models of peanut sensitisation to humans, experiments were conducted to examine the role of the peanut allergens identified as most important in allergic patients. PPE-immunised mice were found to produce a T_h2-biased antibody response primarily directed against Ara h 1, but not the 2s albumins or Ara h 3. IG immunised mice only showed weak T_h2 responses to Ara h 1, which may account for their weaker responses overall. Furthermore, large quantities of peanut-binding IgG2a (T_h1-related) antibodies were observed, even in un-immunised naïve mice. Due to the absence of peanut from the diet of the mice, this observation could not be explained by prior tolerisation. Subsequent Western blotting against soya protein (which was present in the diet) showed substantial cross-reactivity between soya and peanut 2s albumins, suggesting that the difference in immunogenicity between Ara h 1 and the 2s albumins was due to the contaminating factor of cross-reactive tolerisation to soya from the diet. This cross-tolerisation of soy-exposed mice was found to be so extensive that even when injected subcutaneously accompanied by adjuvant, purified 2s albumins were almost completely non-immunogenic compared to Ara h 1.

6.2 Introduction

Following the demonstration of enhanced immunogenicity of DR peanuts in murine models of peanut sensitisation, a primary concern was to ensure that the observed responses resembled those observed in human patients. Doing so would validate the relevance of the murine models for the investigation of peanut allergy, and would produce further evidence in support of translation to humans. One particularly important factor was to discover whether the relative reactivity of sensitised mouse sera to different peanut allergens matched the reactivity observed in patients. As was previously noted, allergens are classified as "major" or "minor" based on the percentage of patients who react to them (Chapman, Pomes et al. 2007), and thus an important indicator of the fidelity of any murine model of peanut allergy would be that it shares the same pattern of major and minor allergens as is observed among human patients.

Several research groups have addressed the question of the relative important of different peanut allergens based on relative reactivity to peanut-allergic patient serum, with Ara h 2 being frequently cited as the most important allergen in humans, although Ara h 6 is also believed to contribute to this due to its extensive homology and cross-reactivity to Ara h 2 (Koppelman, de Jong et al. 2005; Porterfield, Murray et al. 2009; Kulis, Chen et al. 2012; Chen, Wang et al. 2013). Ara h 1 is also considered an important peanut allergen in human patients (Shreffler, Castro et al. 2006; Vissers, Iwan et al. 2011), whereas other allergens such as Ara h 3/4 have historically been considered to be of lesser importance (Rabjohn, Helm et al. 1999), although some more recent studies have suggested that Ara h 3/4 may be more important than previously thought (Restani, Ballabio et al. 2005). The purification of

peanut allergens, in conjunction with the CPH and PPE derived from the same sources, allow the investigation of immunised mouse serum antibody responses by Western blotting and ELISA to determine whether or not my murine models of peanut sensitisation have accurately reproduced the relative importance of these peanut allergens as seen in humans.

These studies would be further complemented by a direct comparison of the immunogenicity of Ara h 1 and the 2s albumins in an established *in vivo* model of peanut immunogenicity. Although under ideal conditions the immunogenicity of individual peanut allergens would have been explored using the full range of administration routes used for total peanut protein preparations, the difficulty of isolating soluble peanut allergens of sufficient purity from dry-roasted peanuts (as elaborated in Chapter 3), rendered impractical the isolation of suitably large quantities of purified allergens. Accordingly, any direct *in vivo* studies of the immunogenicity of purified allergens were restricted to the SC immunisation route.

6.3 Chapter Goals

In this chapter, the following experimental strategy will be pursued to investigate the relative immunogenicity of the individual peanut allergens:

1. Examine the contribution of each individual allergen to systemic murine antibody responses to PPE and CPH through the use of anti-peanut serum Western blotting;
2. Place the Western blot studies in their proper context by investigating the background levels of peanut-reactive immunoglobulin in naive mice;
3. Compare the immunogenicity of Ara h 1 and the 2s peanut albumins (Ara h 2, 6 and 7) in a subcutaneous model of immunisation.

6.4 Methods used in this Chapter

The following methods were used in this Chapter to evaluate and compare the immunogenicity in mice of the peanut allergens:

- Protein gel electrophoresis and Western blotting (2.4);
- Subcutaneous immunisation (2.6.5);
- Peanut-specific immunoglobulin G ELISA (2.7.1);
- Peanut-specific immunoglobulin G Western blot (2.7.3).

6.5 Results

6.5.1 The three top human peanut allergens are represented in PPE responses

In order to determine whether the relative contributions of the different peanut allergens in my murine models of peanut sensitisation were equivalent to their human counterparts, mice were immunised SC with PPE adjuvanted with aluminium hydroxide (Lambrecht, Kool et al. 2009), and subsequently boosted IG with DR CPH, using the method described in Chapter 4 (for further details, please see Chapter 2, section 2.6.5). At week 32 the mice were sacrificed, their cardiac puncture sera were pooled, and a total IgG Western blot was performed against raw PPE.

Mouse IgG were seen to react strongly with all three of the most important human peanut allergens, Ara h 1, Ara h 3/4 and Ara h 2 (Figure 6.1), confirming that the same allergens are also dominant in the murine systemic responses.

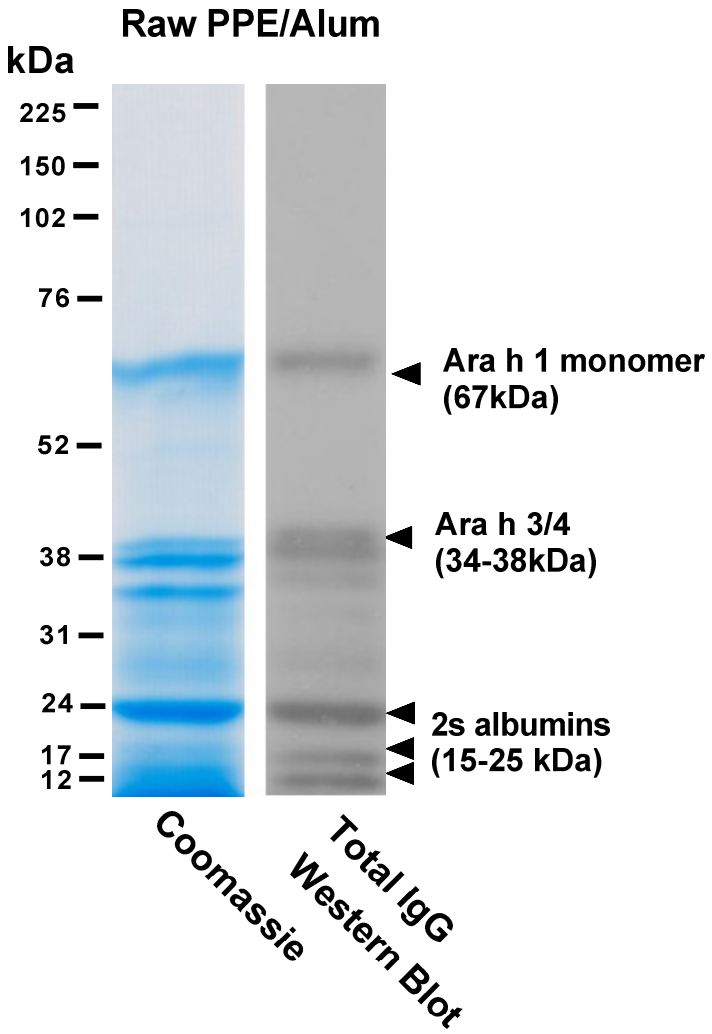


Figure 6.1 Total IgG serum Western blot of pooled sera (n = 4) from mice immunised with raw PPE adjuvanted with Alum. Coomassie stain of total protein for comparison.

6.5.2 Immune responses in PPE-immunised mice are primarily directed against Ara h 1

Because the total IgG Western blot was conducted under denaturing and reducing conditions, it only permits the detection of antibodies which bind to the linear epitopes of antigens determined by their amino acid sequence (Marnetto, Hellias et al. 2009). By contrast, ELISA can be conducted under near physiological conditions, allowing the detection of conformational epitopes determined by three-dimensional structure (Robotham, Xia et al. 2010), but has the disadvantage that it cannot be used to distinguish responses to individual proteins in a mixture. The purification of peanut allergens documented in Chapter 3 circumvented this difficulty, by allowing the coating of individual allergens on the ELISA plate.

Pooled sera from Balb/c mice immunised SC with raw PPE as described previously (Chapter 4, section 4.5.3) were used to probe an ELISA plate coated with either raw Ara h 1 or raw 2s albumins. Monoclonal antibodies against either Ara h 1 or Ara h 2 were used as positive and negative controls for the two different coating proteins, demonstrating that the two allergens bound to a comparable extent (Figure 6.2). Surprisingly, the vast majority of SC raw-PPE immunised mouse IgG1 was directed against Ara h 1, with only a small amount binding to the 2s albumins (Figure 6.2).

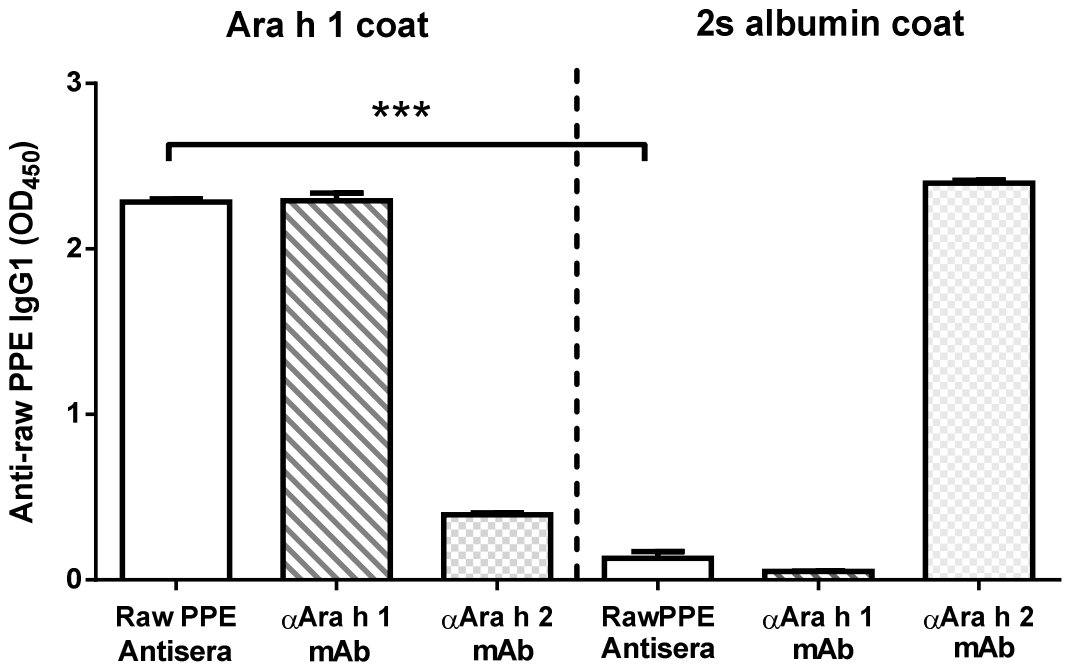


Figure 6.2 Detection of raw Ara h 1 or raw 2s albumins coated onto an ELISA plate by (1) SC raw + Alum PPE-immunised mouse serum (pooled from 4 animals); (2) monoclonal anti-Ara h 1 antibody; (2) monoclonal anti-Ara h 2 antibody. Antibodies diluted 1:10,000. Background OD ≤ 0.01. *** p = ≤ 0.001; Student's *T*-test

6.5.3 Serum western blotting indicates Ara h 2 binding in naive mice

Ara h 2 has frequently been described as the most important peanut allergen in producing the symptoms of human peanut-allergic patients (Kulis, Chen et al. 2012). In light of this, the apparent lack of 2s-albumin directed antibodies in mice immunised SC with PPE was unexpected. To further investigate this phenomenon, the same pooled sera were used in IgG1 and IgG2a serum Western blots, which allow the simultaneous evaluation of binding to multiple allergens. Keyhole Limpet Hemocyanin (KLH) was used as a negative binding control, due to its guaranteed absence from the murine diet.

IgG1 and IgG2a responses were compared between pooled sera of SC raw PPE-immunised Balb/c mice (Figure 6.3A) and the pooled sera of naïve Balb/c mice, which had never encountered peanut allergen (Figure 6.3B). KLH binding was negligible in all cases, confirming the peanut-specificity of the responses. For SC-immunised mice, IgG1 binding was more prominent overall, consistent with the previous serum ELISA data from this experiment, and as with the Ara h 1/2s albumin binding ELISA, the intensity of the Ara h 1 response was much greater than that of the 2s albumins (Figure 6.3A). However, IgG2a responses were completely absent against Arah1 and were entirely targeted to Ara h 2 and a protein of approximately 27kDA size, which previous publications suggest is most likely a fragment of Ara h 3/4 (Koppelman, Knol et al. 2003).

Even more striking, was that binding to both Ara h 2 and the Ara h 3/4 fragment was also observed in naïve mice which had never encountered peanut allergen (Figure 6.3B). This binding was balanced between IgG1 and IgG2a, with the IgG2a band intensity being similar to that seen for SC-immunised mice (Figure 6.3A).

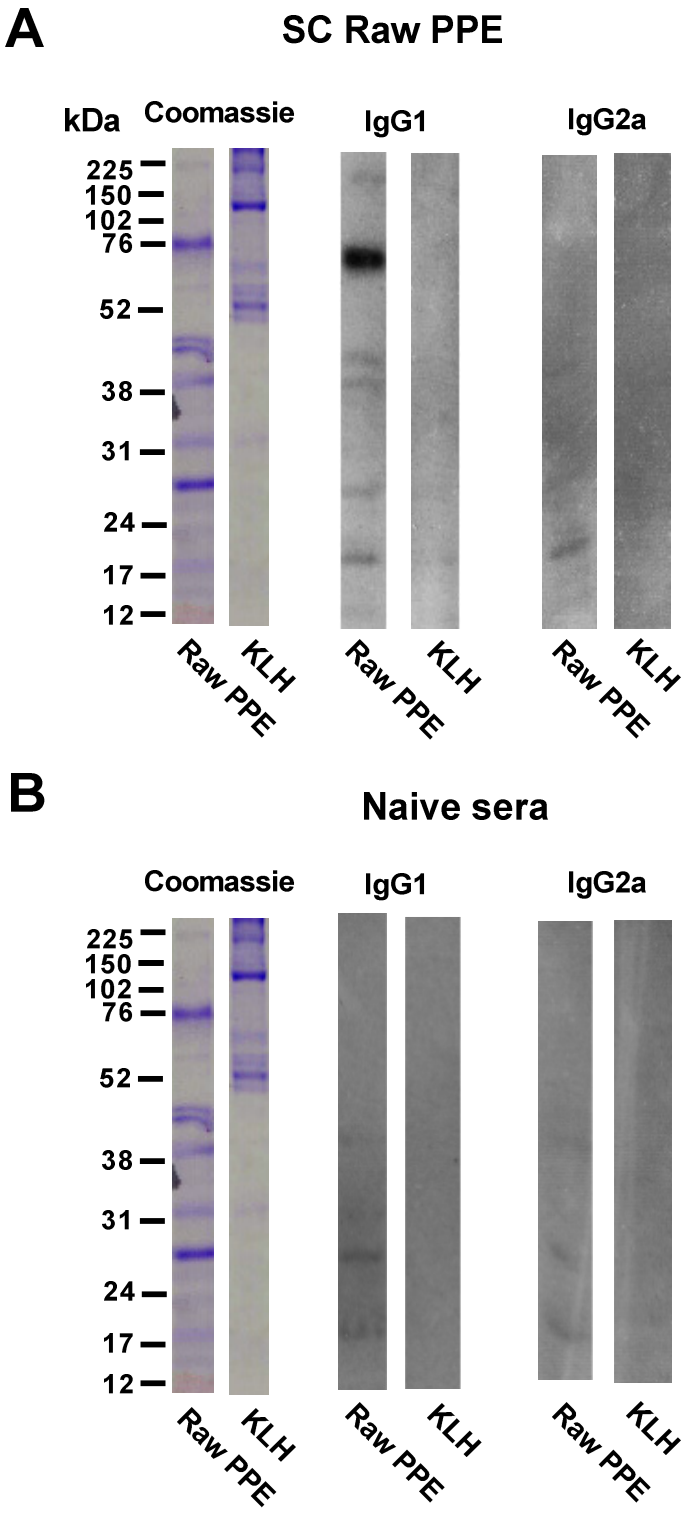


Figure 6.3 IgG1 and IgG2a Western blots of (A) mice immunised SC with raw PPE, (B) naïve mice without exposure to peanut allergens. Serum reactivity with raw PPE and KLH shown with Coomassie staining for comparison.

6.5.4 Anti-peanut IgG1 and IgG2a in naïve mice cross-reacts with soya allergens

Because the naïve Balb/c mice from which the positive Ara h 2 and Ara h 3/4 Western blots were prepared had been carefully maintained on a peanut-free diet for generations, the apparent presence of antibodies against peanut allergens was initially a mystery. The result was repeated multiple times using pooled sera from as broad a population of naïve Balb/c mice as possible, and was found to be ubiquitous across all mice from the experimental breeding population.

However, an examination of the mouse chow used as diet indicated that it contained protein and oil derived from soy, a plant phylogenetically closely related to peanut and containing homologous allergens (Sicherer, Sampson et al. 2000), and known to be involved in cross-reactivity to IgE from peanut-allergic human patients (Ballabio, Magni et al. 2010). Accordingly, a soya protein extract was prepared according to the same protocol used to derive PPE, and naïve sera reactivity was compared between PPE and soya extract, using the same serum Western blot method as before (Figure 6.4). Antibodies reacting with Ara h 2 were found to cross-react strongly to its soya homologue, Gly m 2s albumin (Moreno and Clemente 2008). Similarly, sera reactive with the Ara h 3/4 fragment also bound to a soya protein of equivalent weight, most likely a fragment of the Ara h 3 homologue (Beardslee, Zeece et al. 2000) glycinin soya allergen Gly m 6 (Holzhauser, Wackermann et al. 2009). No cross-reactivity was observed between Ara h 1 and its soya homologue Gly m 5 (Kroghsbo, Bogh et al. 2011).

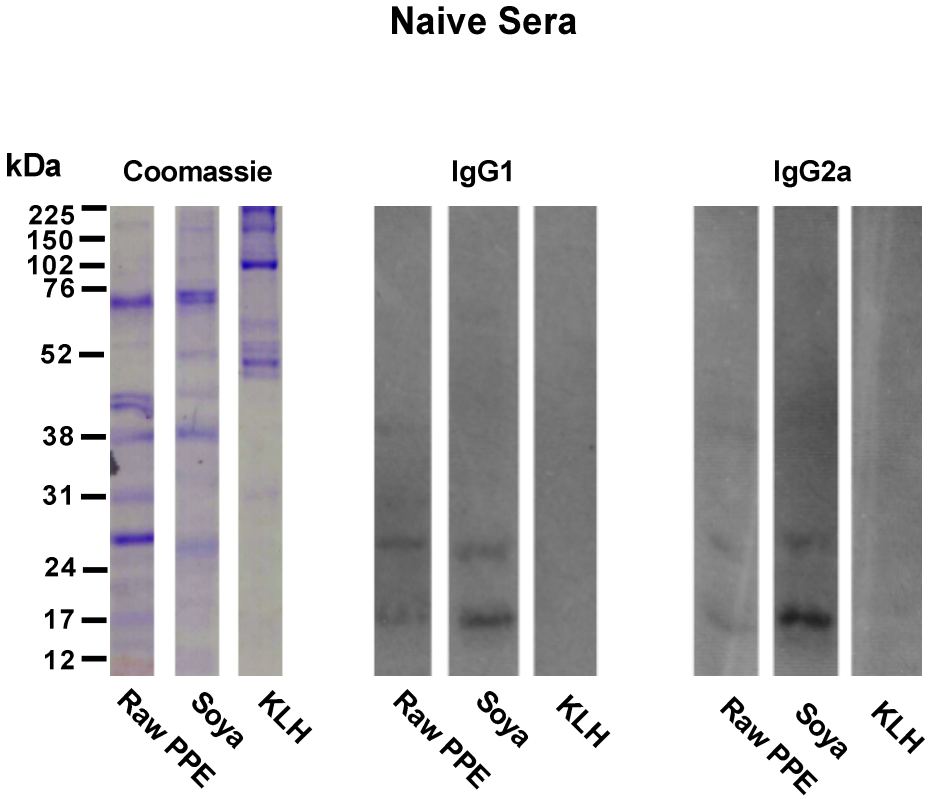


Figure 6.4 IgG1 and IgG2a Western blots of naïve mice without exposure to peanut allergens. Serum reactivity with raw PPE, soya and KLH shown with Coomassie staining for comparison.

6.5.5 IG-immunised mice lack robust anti-Ara h 1 responses compared to SC-immunised mice

With the "background" level of cross-reacting soya binding antibodies in naïve mice established, these were compared with the cross-reactivity of pooled serum antibodies in mice immunised either SC with raw PPE (Figure 6.5A) or IG with raw CPH co-adminstered with CT (Figure 6.5B). Whilst extensive IgG1 cross-reactivity to soya Gly m 2s albumin and the probable Gly m 6 fragment was observed for sera derived from both SC and IG immunisation routes, in neither case was any cross-reactivity to the soya Ara h 1 homologue Gly m 5 observed (Figures 6.5 A and B). Nor were responses detected to Ara h 1 in the IG immunised mice (Figure 6.5B). IgG2a responses to Ara h 2 and the Ara h 3/4 fragment (Figure 6.5 A and B) remained similar to those of naïve mice (Figure 6.4).

In order to determine whether this pattern of reactivity could also be observed for non-denatured epitopes by ELISA, the same sera from naïve (Figure 6.6A), SC-immunised (Figure 6.6B) and IG-immunised (Figure 6.6C) mice were compared in terms of their binding to total raw PPE, raw Ara h 1 and 2s albumins, and their cross-reactivity to soya extract. The majority of soya cross-reacting antibodies were observed to be IgG1 in all three groups of mice (Figure 6.6), although the relative reactivity in SC-immunised mice (Figure 6.6B) was much lower than either naïve (Figure 6.6A) or IG-immunised mice (Figure 6.6C). In all three groups (Figure 6.6) IgG2a responses to soy were low to undetectable. This is in contrast to the strong bands seen in Western blots, and indicates that the majority of the soya-binding antibodies are likely to be targeted to linear epitopes.

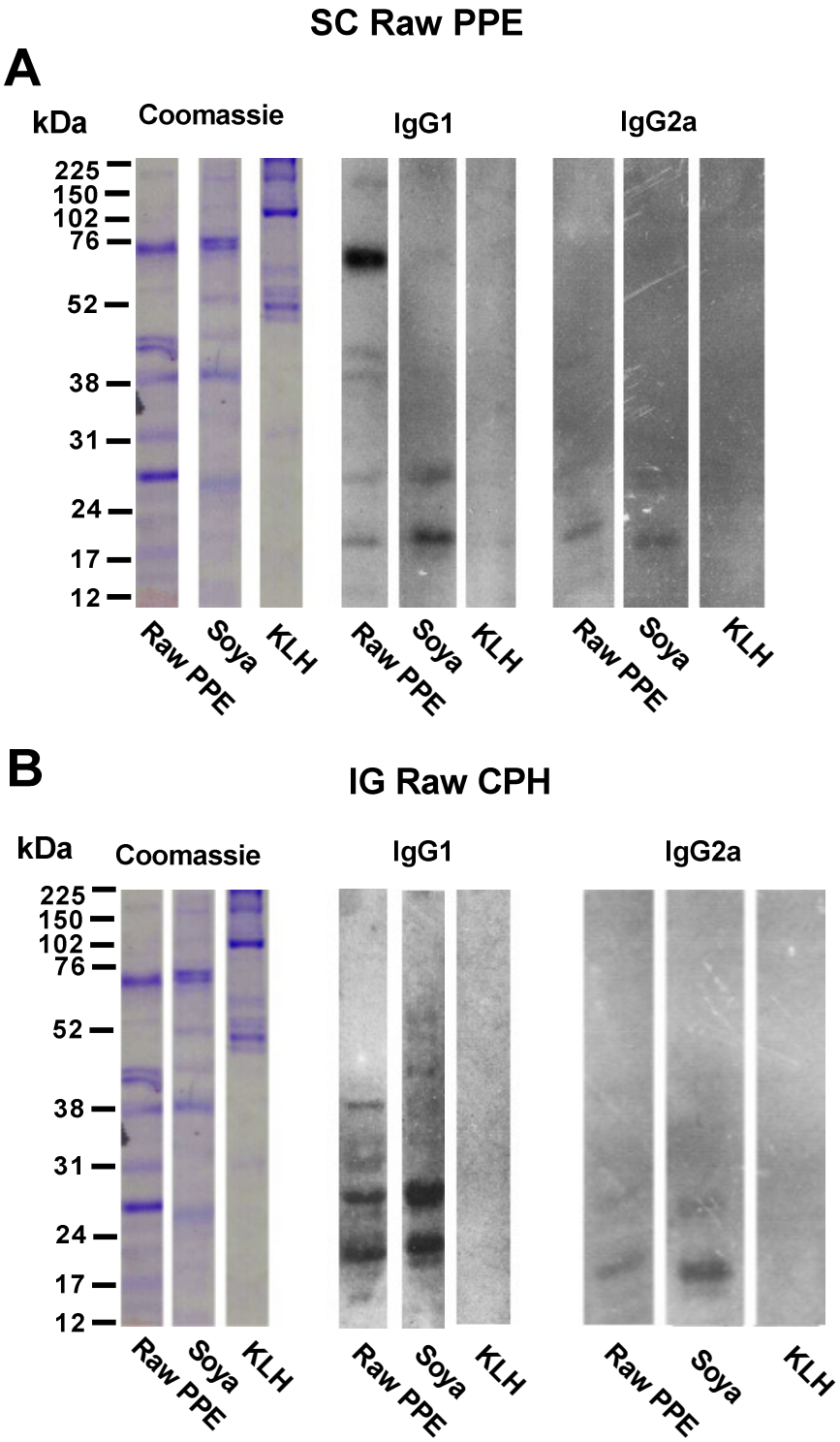


Figure 6.5 IgG1 and IgG2a Western blots of (A) mice immunised SC with raw PPE, (B) mice immunised IG with raw CPH + CT. Serum reactivity with raw PPE and KLH shown with Coomassie staining for comparison.

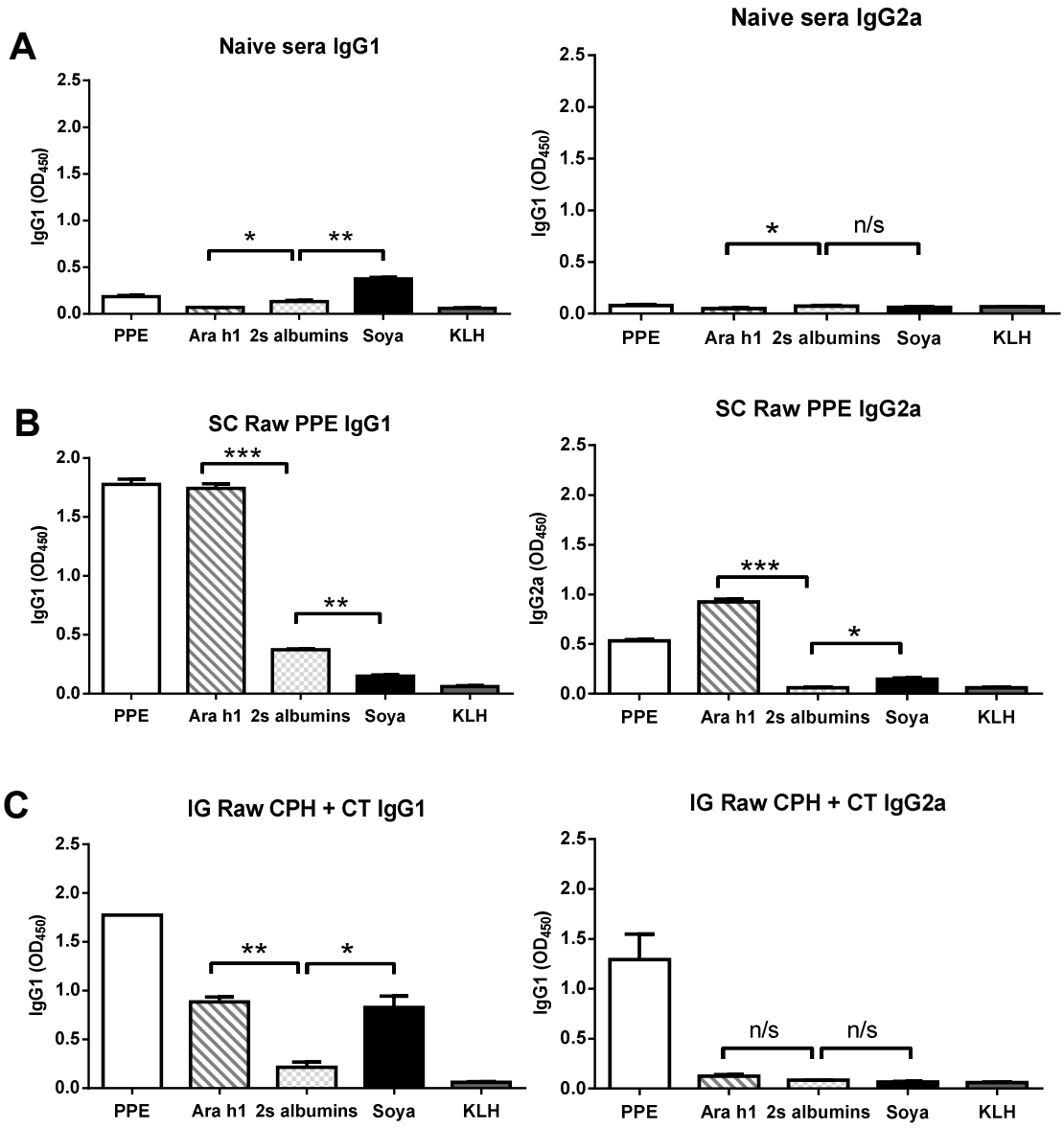


Figure 6.6 Serum reactivity against raw PPE, raw Ara h 1, raw 2s albumins, raw soya and KLH from naïve mice without exposure to peanut allergens **(A)**; mice immunised SC with raw PPE **(B)**; mice immunised IG with raw CPH + CT **(C)**. All serum dilutions = 1:100. All background optical densities ≤ 0.05 (IgG1) or ≤ 0.12 (IgG2a) * $p = \leq 0.05$; ** $p = \leq 0.01$; *** $p = \leq 0.001$; n/s = non-significant; Student's *T*-test

6.5.6 The 2s albumins are dramatically less immunogenic than Ara h 1 in soy-exposed mice

The substantially lower antibody responses to the 2s albumins in both IG and SC immunised mice relative to Ara h 1, taken together with the extensive presence of antibody cross-reactivity to soya proteins (Figures 6.5 and 6.6), gave rise to my hypothesis that prior dietary exposure to soya might have tolerised the mice against subsequent exposure to the 2s albumins, but not against Ara h 1. To test this hypothesis, groups of mice were immunised SC with either endotoxin-depleted raw Ara h 1 or raw 2s albumins with aluminium hydroxide adjuvant, using the method described previously. After six weeks, mice were boosted IG with DR CPH. Ara h 1 was clearly more immunogenic, with strong responses appearing by the second week, and this status was maintained throughout the duration of the experiment (Figure 6.7A). By contrast, responses to the 2s albumins remained weak until the sixth week (Figure 6.7A). Two weeks after the boosting with DR CPH, IgG1 (Figure 6.7B) and IgG2a (Figure 6.7B) levels were compared. 2s albumin responses were substantially less than those against Ara h1 for both isotypes, in striking contrast to reports of Ara h 2 immunogenicity in peanut-allergic human patients.

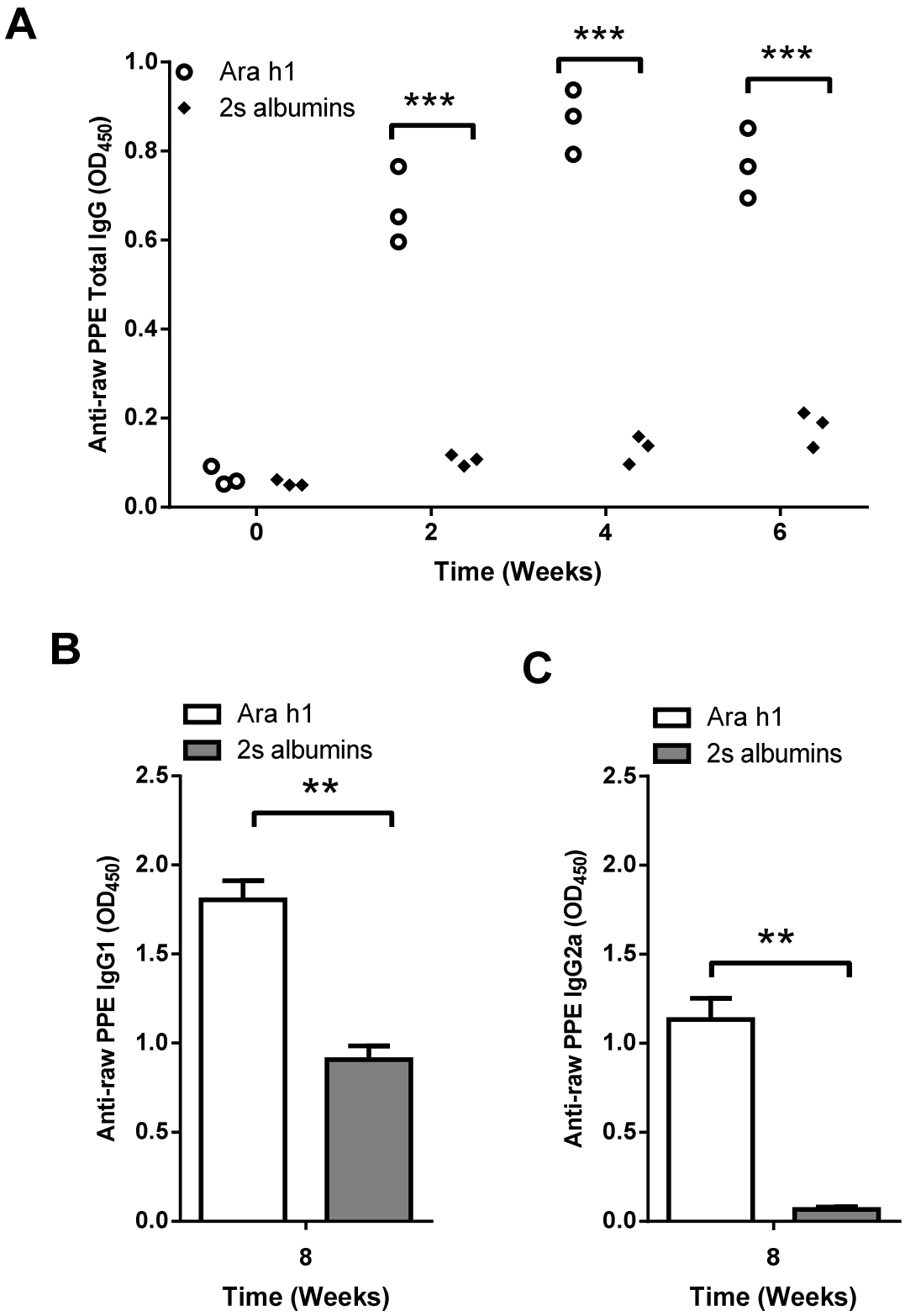


Figure 6.7 Immunogenicity of raw Ara h 1 and 2s albumins coadministered SC with aluminium hydroxide adjuvant. **(A)** Time course of serum total IgG following immunisation; **(B)** serum IgG1; **(C)** serum IgG2a at week 8. All serum dilutions = 1:100. All background optical densities ≤ 0.08 (total IgG), ≤ 0.05 (IgG1) or ≤ 0.12 (IgG2a) ** p = ≤ 0.01; *** p = ≤ 0.001; Student's *T*-test

6.6 Discussion

The results presented in this section began as an attempt to compare the antigenicity of peanut proteins between murine models and human patients, but quickly assumed an additional importance of their own due to the detection of peanut-binding antibodies in peanut-naïve Balb/c mice on a peanut-free diet. Whilst the relative dominance of Ara h 1 and Ara h 3/4 was consistent with reports from human patients, the lack of murine responses to the major human allergen Ara h 2 was surprising. Subsequent experiments revealed that this was not necessarily due to an absolute lower immunogenicity of 2s albumins in mice relative to humans. Rather, the evidence from immunisation with single allergens suggests that this phenomenon may well be due to cross-reactive tolerisation to soya proteins to which the mice had been exposed as part of their laboratory diet. Because the allergens were administered with aluminium hydroxide, a simple lack of 2s albumin immunogenicity would seem insufficient to explain the low responsiveness of these mice, since resistance to strongly adjuvanted antigens typically indicates suppression of responses by prior tolerance (Chapman, Emo et al. 2013). This interpretation is further supported by a comparison of Western blotting and ELISA data, which show that soya-binding antibodies in naïve mice appeared to bind primarily to linear rather than conformational epitopes, a phenomenon which is most consistent to their being raised against ingested soya proteins which were digested before encountering the immune system.

Such an interpretation is made more plausible by the findings of other research groups, which have demonstrated that purposeful tolerisation to soya allergens can be used to desensitise peanut-allergic mice (Pons, Ponnappan et al. 2004). Nevertheless the alternative possibility, that the relative importance of individual peanut allergens in mice is

intrinsically different from that in humans, cannot be completely ruled out on the basis of these observations alone, chiefly because they cannot distinguish between antibodies raised against peanut that cross-react with soy, and vice versa. In order to settle this matter definitively, it would be necessary to repeat these experiments in a population of mice whose diet excluded not only peanut antigens but also soya antigens, ideally by testing serum binding against mutually exclusive peanut and soya epitope constructs.

Cross-reactivity aside, these results are also significant because they demonstrate that the relative importance of the peanut allergens in serum immunoglobulin responses can be affected by the route of administration. Whilst both SC and IG immunised mice showed a clear IgG1 bias of antibodies against total PPE in ELISAs, Western blotting provides more information than these experiments because it allows the comparison of responses to individual allergens between IG and SC routes. As a result of these studies, detectable Ara h 1 responses were only found in SC immunised mice, but not in mice immunised IG. This would suggest, in light of the apparent resistance of this population of mice to sensitisation by 2s albumins, that the reason why SC immunisation produced more pronounced, and Ara h 1-dominated, responses than IG immunisation is that Ara h 1 availability to the immune system was greater via the SC route, possibly owing to its greater susceptibility to digestion when administered IG (Koppelman, Hefle et al. 2010).

In conclusion, these results demonstrate that the whilst the immune responses in the murine models of peanut allergy mirror the allergens most important for human peanut allergy, responses to individual allergens are highly affected by route of administration and diet. This finding is of potential relevance to the ongoing debate over the relationship between early exposure and subsequent development of allergic sensitisation.

Chapter 7
Summary of Conclusions and Future Perspectives

Throughout this project, the immunogenicity of raw and DR peanut has been explored using a variety of *in vitro* and *in vivo* model systems. These findings unambiguously support the hypothesis that DR peanut proteins are both more immunogenic than raw, and are able to skew these enhanced immune responses towards the T_h2-type responses associated with allergy. This effect was observed regardless of route of administration, although the intensity of responses differed between routes, with SC being strongest and IG weakest, and regardless of whether purified peanut allergens, total protein extract, or crude homogenate was analysed. There was evidence for T_h2 response skewing in both serum immunoglobulin responses, which were IgG1 and IgE-dominated, and in T-cell responses, which were stronger for DR peanuts and showed a bias towards T_h2 cytokines. This effect was also unchanged by the genetic background of the strain of mouse used, with both the T_h2-biased Balb/c mice and T_h1-biased C57Bl/6 mice exhibiting increased immunogenicity of DR peanut and T_h2 biased immunoglobulin production. Chemical reduction of the RC subpopulation of AGE modifications in DR peanut was found to reduce the otherwise enhanced immunogenicity and T_h2 bias of DR peanut extracts, in agreement with previous published reports for model antigens (Moghaddam, Olszewska et al. 2006; Moghaddam, Gartlan et al. 2011). Consistent with these observations, DR PPE exhibited enhanced surface binding to murine DCs and greater binding to and competition for the ligands of several innate

immunity receptors previously associated with AGE uptake and binding. Peanut proteins did not, however, canonically activate murine DCs.

Immunoglobulin responses to different peanut allergens were found to vary depending on the route of administration, with Ara h 1 being particularly important in SC immunised mice, and much less so in mice immunised IG. Regardless of the route of administration, these results were affected by cross-reactive serum responses to homologous proteins from soya in the mouse diet, which appeared to confer protection against sensitisation to the peanut 2s albumin allergens, but not to Ara h 1. Overall, these results clearly support the hypothesis that dry-roasting peanuts can induce chemical modifications to peanut proteins which enhance their immunogenicity and may play a key role in determining their allergenicity. However, they also highlight how this effect may interact with other causal factors, such as route of administration and the presence of homologous proteins in the diet.

Many questions arising from this study nevertheless remain to be answered. In particular, whilst the fact that the preparations used were essentially free of microbial contamination indicates that DR PPE's immunogenic and T_h2-skewing properties are intrinsic to the peanut proteins themselves, the mechanism or mechanisms by which this is effected remain unclear. It remains to be determined whether the observed increase in binding to DC receptors translates into increased antigen presentation to T cells, and whether this is necessary and sufficient to provoke strong subsequent T_h2-biased immune responses. It is still an open question whether or not this binding leads to increased uptake and presentation of DR peanut allergens, as has been observed in model systems of AGE and RC modifications (Allison and Fearon 2000; Moghaddam, Gartlan et al. 2011). An alternative

possibility, that DR peanut allergens can activate DCs by some non-canonical means by signalling indirectly through binding receptors (for example, through the association between CD36 and TLR4/6 heterodimer documented for oxidised LDL (Stewart, Stuart et al. 2010)) also cannot be discounted at this stage. Neither can the third possibility that both effects contribute to some degree, perhaps acting synergistically. Future research will need to address these outstanding questions by directly demonstrating increased uptake and presentation of DR peanut allergens.

Another aspect of my findings which deserves further investigation is the observation that whilst direct IG immunisation with DR CPH produces relatively weak T-cell responses locally in the MLN and systemically in the spleen, following an initial systemic SC immunisation, subsequent IG feeding can provoke robust local T-cell responses in the lymph nodes as well as smaller, but nevertheless strong systemic responses in the spleen. Consistent observations were also reported from the EC model of sensitisation, showing increased pathology and eosinophil infiltration in the gut of mice initially sensitised through the skin (Strid, Hourihane et al. 2005). Of equivalent interest is the finding that the relative importance of individual peanut allergens appeared to vary according to their route of administration. The increased responses to Ara h 1 in mice immunised SC, relative to those immunised IG, is consistent with the previously published observation that Ara h 1 may be more susceptible to digestion than the 2s albumins (Koppelman, Hefle et al. 2010), owing to Ara h 2's trypsin inhibitory properties (Maleki, Viquez et al. 2003). The bypassing of digestive processes by SC administration may protect Ara h 1 from digestion, accounting for its greater presence in immunoglobulin responses produced in this manner, and providing

further evidence that sensitisation to peanut allergens may not primarily occur through oral consumption in humans.

Taken together, these results are highly consistent with the hypothesis that initial sensitisation in human patients may take place through the skin, rather than through oral peanut intake, an observation supported by the fact that peanut-specific T-lymphocytes of peanut-allergic children predominantly display skin-homing markers (Chan, Turcanu et al. 2012), and a landmark study of Israeli children which, by contrast with previous received wisdom, suggested that oral peanut consumption in early childhood decreased the risk of allergy (Du Toit, Katz et al. 2008). A major 5-year study to confirm this finding is currently underway in Britain (Lack 2013), and it would be instructive to compare its eventual findings with murine experimental models – in particular, to determine whether prior GI exposure to peanuts lessens or worsens T_h2-biased responses to subsequently SC or EC administered DR allergens.

A related finding which is also of relevance to the ongoing debate over the role of early dietary exposure in allergy is the observation that cross-reactive tolerisation to soya 2s albumin allergens in mice could result in resistance to immunisation to their peanut homologues. In light of this, it is interesting to speculate whether the lower incidence of peanut allergy in Asian countries ascribed to differences in cooking methods (Beyer, Morrow et al. 2001) could also (at least in part) be explained by the greater prevalence of soya in the diet (Barrett 2006) of these same countries. Interestingly, a study which compared the serum IgE reactivity of Korean peanut-allergic patients to individual peanut allergens showed much less reactivity to Ara h 2 than has been reported in studies of Western patient groups (Kim, Lee et al. 2013), in whose diets soya is less common (Barrett

2006). This would be wholly consistent with the finding in this project that prior dietary exposure to soya protein appeared to result in tolerisation to the 2s albumin allergens, resulting in immunoglobulin responses being dominated by Ara h 1. If this finding can be substantiated by showing increased sensitivity to the 2s albumins in mice without soya in their diet, it could potentially be of direct relevance to proposed therapies which aim to use homologous soya proteins to desensitise allergic patients to peanut allergens (Pons, Ponnappan et al. 2004).

In light of the results documented in this thesis, it is interesting to speculate whether the apparent effect of dry-roasting and the Maillard Reaction on the immunogenicity of peanut proteins may also apply to other thermally processed foods (Maleki 2004). Although the thermal processing of peanut during dry-roasting is unusually intense, the Maillard Reaction itself is ubiquitous in cooking processes used for both allergenic and non-allergenic foodstuffs. It remains to be seen whether or not the exceptionally high allergenic potency of peanut is an anomaly caused by a very unfortunate chance combination of intrinsic protein structural characteristics and highly specific effects of certain cooking methods, or whether instead enhanced immunogenicity of allergens through the Maillard Reaction will emerge as part a broader paradigm linking food processing and allergenicity across many foods.

Appendix A
Supplementary Methods and Materials

The following methods describe experiments performed by the collaborators named as part of research collaborations between the Sattentau Group of the Sir William Dunn School of Pathology, University of Oxford, and other research groups. They are not included in the main Materials and Methods section because they were not performed by the thesis author, but have been included in this Appendix because of their direct relevance to the results described therein.

A1. Liberation and detection of Ara h 1 glycans

Liberation and detection of glycans on the surface of raw and DR Ara h 1 were performed by Dr Camille Bonomelli of the Scanlan Group, Department of Glycobiology, University of Oxford, as part of a collaboration. Oligosaccharides were released from glycoproteins with peptide-*N*-Glycosidase (PNGase) F from Coomassie blue-stained Nu-PAGE gels (Kuster, Wheeler et al. 1997). Excised bands were washed six times alternatively with acetonitrile and deionised water, and rehydrated with a 3000 Units/mL of PNGase F water solution. After incubation overnight at 37 ˚C, the enzymatically released *N*-linked glycans were eluted with water, and dried using a Concentrator Plus (Eppendorf).

Glycan samples were cleaned on a Nafion 117 membrane for an hour (Mohr, Bornsen et al. 1995), and mixed on a plate with the 2,5-dihydroxybenzoic acid (DHB) matrix (0.3 µL of a solution of 2,5-dihydroxybenzoic acid in acetonitrile:water (1:1, v:v)) and sodium chloride,

then allowed to dry at room temperature. The sample/matrix mixture was then recrystallized with ethanol. Desialylation was performed by heating samples at 80°C for 1 hr with 1% acetic acid. Dried samples were redissolved in water. Mass spectra were recording using a Shimazu AXIMA TOF2 MALDI TOF/TOF mass spectrometer (Kratos Analytical, Manchester, UK) according to the method described in Harvey *et al*. (Harvey, Royle et al. 2008) Glycan structures were assigned to peaks by reference to previously published literature on peanut allergen glycosylation.

A2. Quantification of eosinophils and histology in EC model of peanut sensitisation

Histological analysis of tissues and quantification of intestinal eosinophils from EC-sensitised Balb/c mice was performed by the Artis Group at the University of Pennsylvania, as part of a collaboration.

To identify eosinophils, lamina propria cells from the small intestine were isolated as described in Kim *et al* (Kim, Lee et al. 2013). Cells were processed into a single-cell suspension and then were incubated with Aqua Live/Dead Fixable Dye (Invitrogen) to remove dead cells, and stained with fluorochrome-conjugated mAbs specific for CD3ε, CD4, CD8, NK1.1, CD19, CD45 (eBioscience, CA, USA), and CD11c, CD5, and Siglec-F (BD Biosciences). All cells were run on a four-laser 14-color LSR II (BD Biosciences), and the data was analysed using FlowJo 8.7.1 (Tree Star, Ashland, USA). For the purposes of identification, eosinophils were defined as live, singlet, CD45+, CD3-CD4-CD5-CD8-NK1.1-CD11c-CD19-, SSC high, Siglec-F+ cells.

For histological analysis, after the removal of lamina propria from the small intestine, jejunum resections were fixed in 4% paraformaldehyde and embedded in paraffin, and 5 µm sections were cut and stained with hematoxylin and eosin (H&E).

A3. TLR4 Reporter Assay for Endotoxin Content

The TLR 4 reporter assay was performed by Dr Amin Moghaddam, of the Sir William Dunn School of Pathology, University of Oxford.

TLR4-transfected HEK-293T cell line (TLR4-HEK, Invitrogen, Carlsbad, USA), together with untransfected HEK-293T (HEK) cells (Invitrogen) as a negative control, were cultured in RPMI supplemented with 10% FCS. Stimulation of TLR4 in this cell line triggers the expression of IL-8, which was used as readout for endotoxin activity in samples. After harvesting, HEK and TLR4-HEK cells were transferred onto a 96-well plate at a density of 1million cells/ml, then stimulated with protein samples at a concentration of 20µg protein/ml. Purified LPS (Sigma) was employed as a positive control at concentrations of 100ng/ml, 10ng/ml and 1ng/ml. The resulting mixtures were incubated overnight, and supernatants were removed and either used immediately or frozen at -80°C for future use. Samples of supernatant were assayed for IL-8 content with a Legend-Max IL-8 ELISA kit (Biolegend, San Diego, USA).

Briefly, plates were coated overnight at 4°C with anti-IL-8 capture antibody, then after washing with PBST, blocked with a nonreactive protein solution provided by the manufacturer for 1 hour. Afterwards, wells were incubated for 2 hours at 37°C with TLR4-HEK or HEK control cell culture supernatant diluted 1 in 2 in blocking buffer, together with a range of recombinant IL-8 standards from 15.6 to 100pg/ml. After washing, wells were probed for 1 hour with anti-IL-8 biotinylated detection antibody, followed by 1 hour of

exposure to streptavidin-conjugated HRP. Wells were developed using Ultra-TMB substrate (Thermo Scientific, Rockford, USA) and read using an automatic plate reader (Molecular Devices, Sunnyvale, USA) at 450nm).

A4. Glycation of Hen Egg Lysozyme

Glycation and mass spectrum analysis of Hen Egg Lysozyme (HEL) was carried out by Dr Leo Kong, then of the Sir William Dunn School of Pathology, University of Oxford.

1 milligram of HEL (Sigma) was incubated with 1M glucose in PBS, pH7.4 (PAA) for 12 weeks, and then desalted extensively with a 3kDa molecular weight cutoff filter (Millipore). Unmodified and glucose-modified HEL were then analysed by intact protein mass spectrometry using an Orbitrap LTQ XL mass spectrometer (Thermo Scientific).

Appendix B
Supplementary Figures

The following figures are derived from data produced by the collaborators named as part of research collaborations between the Sattentau Group of the Sir William Dunn School of Pathology, University of Oxford, and other research groups. They are not included in the results sections in which they are referenced because they were not performed by the thesis author, but have been included in this Appendix because of their direct relevance to the results described therein.

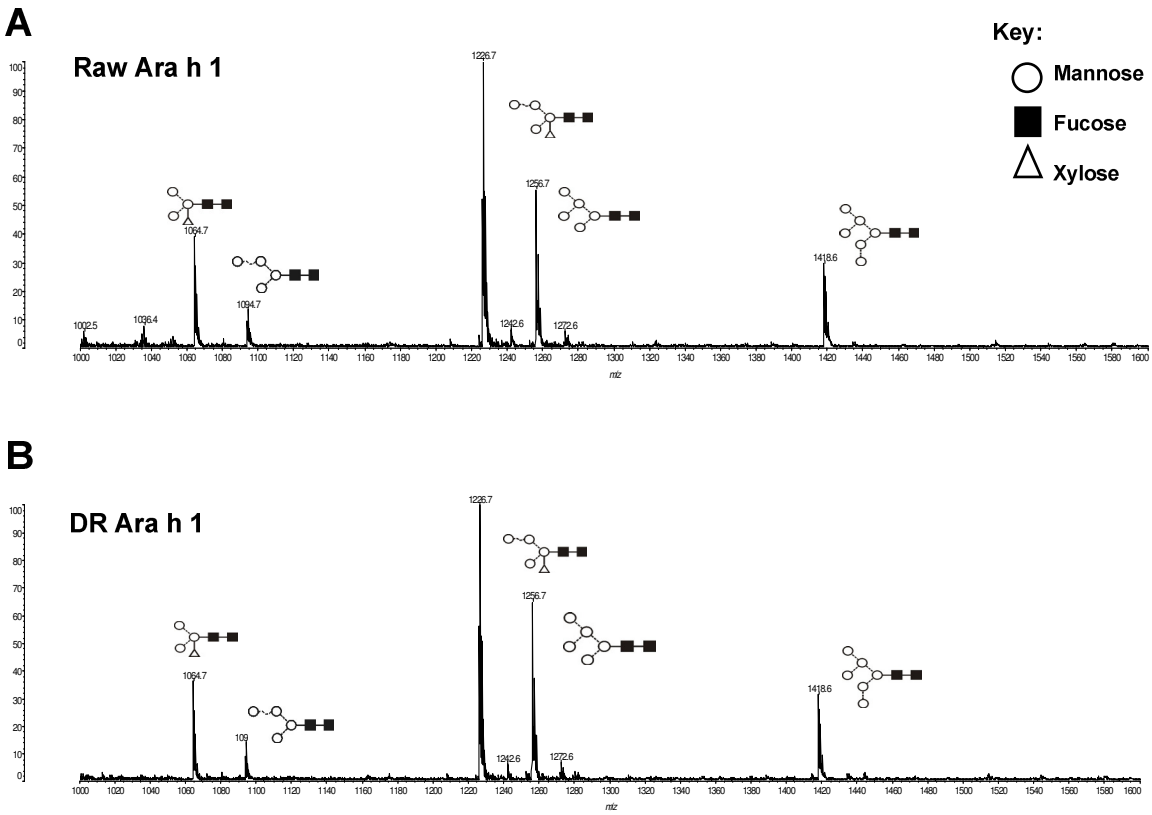
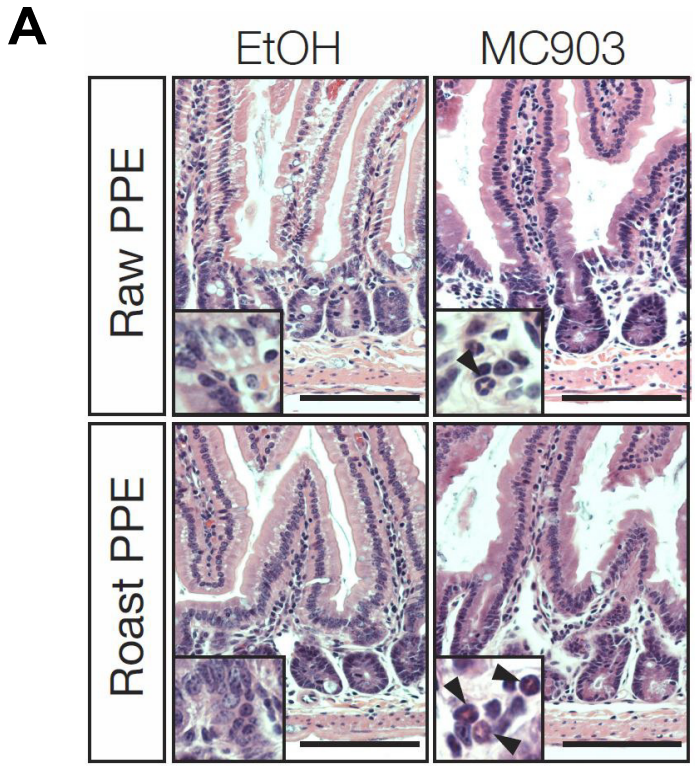


Figure B1. Protein surface glycan mass spectra of **(A)** raw and **(B)** DR Ara h 1, as determined by MALDI TOF/TOF spectrometry. Glycan structures assigned to peaks are depicted adjacent to their molecular masses. Data obtained by Dr Camille Bonomelli, of the Scanlan Group, Department of Glycobiology, University of Oxford.



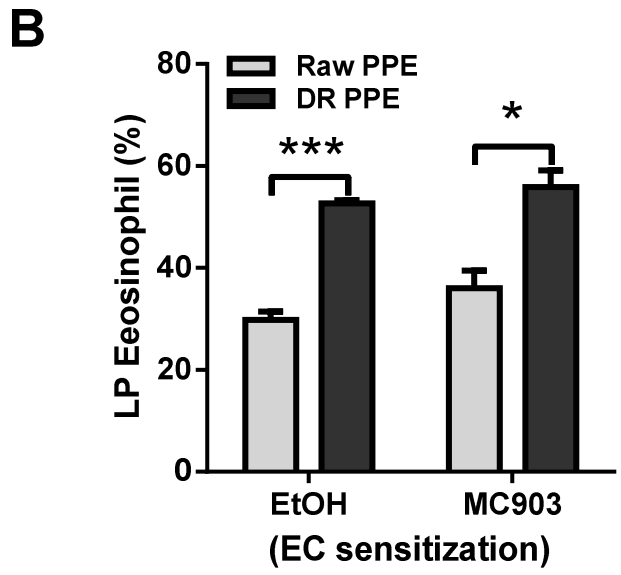


Figure B2. Histology of jejunum and eosinophil quantification in EC-sensitised Balb/c mice. **(A)** Histological sections of jejunum from mice sensitised EC with raw or DR PPE in the presence or absence of mc903 as an exfoliant. Arrows point to eosinophil infiltration. **(B)** Quantification of eosinophil infiltration into the lamina propria in the same individuals. N = 3, representative of 2 independent experiments. Data obtained by the Artis Group, University of Pennsylvania.

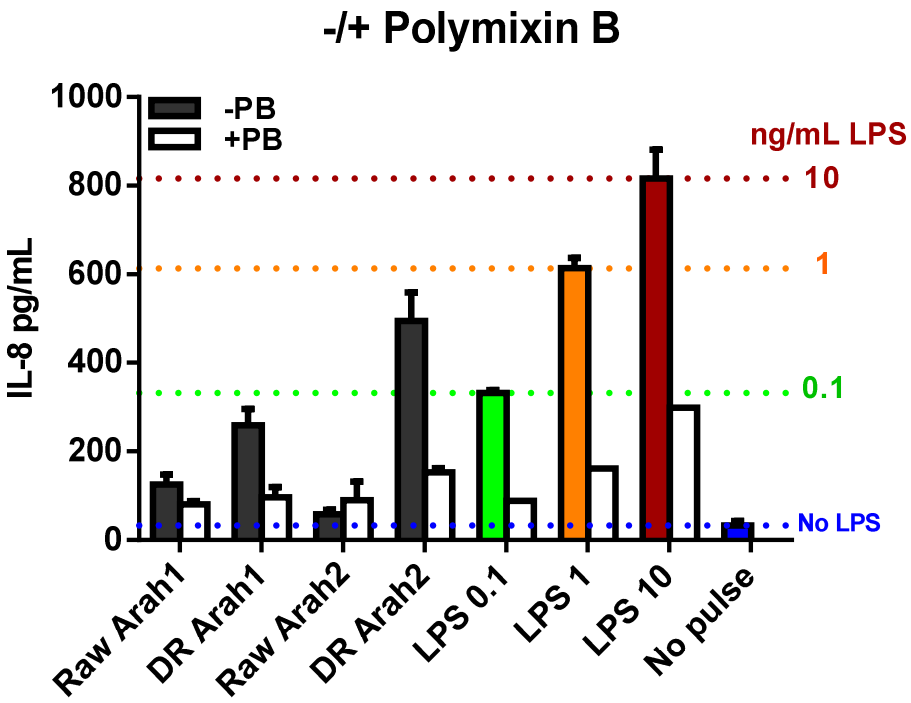
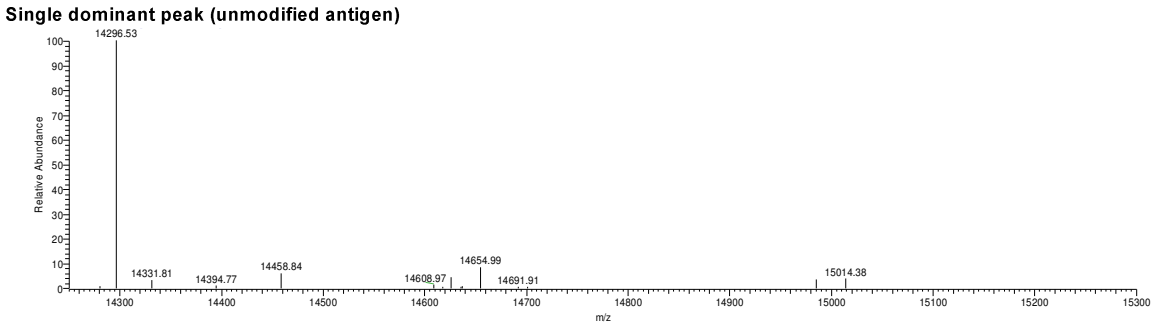


Figure B3. Testing of peanut preparations for bacterial endotoxin, as measured by IL-8 secretion from TLR4-expressing endotoxin cell reporter line, compared to untransfected control cells. N = 3, representative of four independent experiments. Data produced by Dr Amin Moghaddam of the Sir William Dunn School of Pathology, University of Oxford.

A - Unmodified HEL



B - Glycated HEL

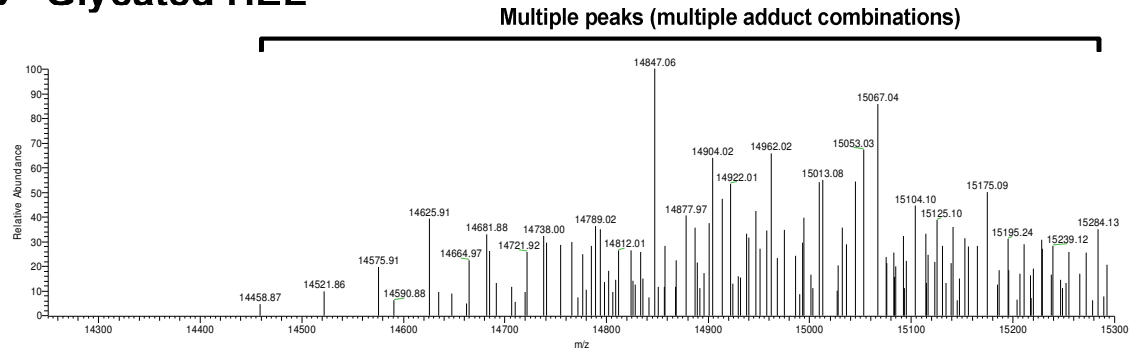


Figure B4. Intact protein mass spectra of Hen Egg Lysozyme in its native state **(A)** or glycated with glucose **(B)** to produce AGEs. Data produced by Dr Leo Kong, then of the Sir William Dunn School of Pathology, University of Oxford.

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