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The Role of Invariant Natural Killer T Cells and Lipids in the Development of Allergic Sensitisation

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Publications

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Communications in Congress

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COVID-19 Impact Statement

During the COVID-19 pandemic, our lab was closed for 6 months, which significantly impacted the progression of my PhD, due to the requirement of a tissue-culture facility to conduct my experiments. Upon re-opening of the lab, the pandemic continued to affect our research for months due to the delay of deliveries for essential materials. In addition to these experimental delays, being an Immunology lab, we were heavily involved in COVID-19 research throughout the pandemic. Since August 2020, I was involved in several COVID-19 research projects, with many different roles including the recruitment of participants across The University of Nottingham, completing a phlebotomy training course to aid research nurses in collecting blood samples from the large cohort, as well as conducting and analysing data from cell-based assays in the lab. I progressed with these COVID-19-related roles alongside my PhD studies, to this day. Our team was awarded a Vice-Chancellor's medal for these efforts during the pandemic.

Abstract

Introduction

IgE-mediated food allergies are increasing in prevalence, currently affecting 2.6% of infants in the UK. However, the mechanisms underpinning the first phase of IgE-mediated allergy, allergic sensitisation, are still not clear. Recently, the potential involvement of lipids in allergic sensitisation has been proposed, with reports that they can activate invariant natural killer T (iNKT) cells, to secrete Th1 and Th2 cytokines. However, the existing research in this area is limited and predominantly use murine models. Thus, this research developed a human *in vitro* method to study the role of lipids and iNKT cells in a model of allergic sensitisation. This method was applied to peanut allergy, one of the most common food allergies in children and adults. Thus, the total lipid fraction from peanuts (peanut oil) was utilised with and without the lipophilic peanut allergen, Ara h 8, to examine any influence on iNKT cell cytokine production, comparing between peanut-allergic and non-allergic individuals.

Methods

Due to low abundance of iNKT cells in human peripheral blood, iNKT cells were expanded over 14 days by stimulation with the glycolipid, α-Galactosylceramide (α-GalCer), which is a potent activator of iNKT cells. Autologous dendritic cells (DCs) were generated from monocytes and stimulated with either peanut oil, Ara h 8, or both peanut oil and Ara h 8. The expanded iNKT cells were then immunomagnetically isolated and co-cultured with autologous DCs to allow lipid and/or allergen presentation to iNKT cells. This co-culture was first optimised using α-GalCer-pulsed DCs before applying to peanut oil and Ara h 8. Th1 and Th2 iNKT cell cytokine expression was then measured during iNKT-DC co-culture by flow cytometry.

Results

Flow cytometry staining of iNKT cells from peanut-allergic and non-allergic subject's peripheral blood found a 5-fold higher iNKT cell population in peanut-allergic subjects compared to non-allergic subjects. The iNKT cells from both

subject groups were then successfully expanded, with iNKT cell populations increasing by 133-fold in peanut-allergic subjects and 122-fold in non-allergic subjects after 14 days of culture with α-GalCer. A shift in iNKT cell phenotype to CD4+ iNKT cells was observed in both subject groups after expansion. Also, DCs were successfully generated at high purities from monocytes, and imaging flow cytometry found the immature DCs can internalise lipids and allergens. Finally, iNKT cell co-culture with α-GalCer-pulsed DCs showed increases in iNKT cell production of IFNγ-only and IFNγ+IL4+ after 5 hours, confirming this *in vitro,* human, cell-based assay is functional. However, when the method was applied to peanut allergy, utilising peanut oil and Ara h 8, the results showed peanut oil and/or Ara h 8 did not have an influence on cytokine production by iNKT cells.

Conclusion

Overall, this study establishes a human model system where allergen-associated lipids can be assessed to determine whether they enhance iNKT cell Th2 cytokine secretion, shifting towards a state of allergic sensitisation. However, peanut oil had no effect on iNKT cell cytokine production. Future research could focus on a specific lipid class from peanut oil, such as the fatty acid oleic acid, to investigate any influence on iNKT cell cytokine production.

Main Hypothesis

It is hypothesised lipids influence allergic sensitisation to allergens by activating invariant natural killer T (iNKT) cells to secrete Th2 cytokines.

Objectives

- To explore the role of iNKT cells and lipid stimuli in the development of allergic sensitisation by developing a human, *in vitro*, cell-based assay.
- To characterise iNKT cell populations in allergic and non-allergic patient groups.

Abbreviations

α-GalCer	α-Galactosylceramide
APC	Antigen presenting cell
APE	Aqueous pollen extract
BEH	Behenic acid
BLA	α-lactalbumin
BLG	β-lactoglobulin
BSA	Bovine serum albumin
CD	Cluster of differentiation
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
Df	*Dermatophagoides farina*
ELISA	Enzyme-linked immunosorbent assay
FcϵR1	High affinity Fc receptor
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
HLA	Human leukocyte antigen
hmoDC	Human Monocyte-derived dendritic cell
HSA	Human serum albumin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN-γ	Interferon gamma
IL	Interleukin
iDC	Immature DC
iNKT	Invariant natural killer T cells
iTCR	Invariant T cell receptor
LAU	Lauric acid
LPC	Lysophosphatidylcholine
LPS	Bacterial lipopolysaccharide
MHC	Major Histocompatibility Complex
NFκB	Nuclear factor kappa B

NKT	Natural Killer T cell
nsLTP	Non-specific lipid-transfer protein
OLE	Oleic acid
OVA	Ovalbumin
PALM	Pollen-associated lipid mediator
PBA	Phosphate buffer albumin
PBS	Phosphate buffer saline
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PI	Propidium Iodine
PRR	Pattern recognition receptor
PG	Phosphatidylglycerol
PPE_1	Phytoprostanes E1
RPMI	Roswell Park Memorial Institute
STE	Stearic acid
Th1	T cell subset 1
Th2	T cell subset 2
TLR	Toll-like receptor
UFA	Unsaturated fatty acids

Chapter 1: General Introduction

1.1. Allergy

1.1.1. What is an allergy?

The term ‘allergy’ was first described by Clemens von Pirquet in 1906 as a result of his study investigating early skin reactions to the cowpox vaccination, where patients who had reactions to their initial inoculation then developed urticaria or asthma after their second dose (Bendiner 1981). Allergy, also known as hypersensitivity, can be defined as an inappropriate or exaggerated immune response to a non-self, innocuous substance. For instance, peanuts are usually a harmless substance, but in some individuals, exposure triggers an immune response. These substances are otherwise known as allergens. Hypersensitivity reactions can also lead to autoimmune diseases, where "self” substances drive the immune response, rather than “foreign” substances in allergy.

There are four main types of hypersensitivities which were first categorised by physicians Gell and Coombs in 1963 (Kaufmann 2019). Type I hypersensitivity is the most common form of allergy, characterised by the binding of allergen-specific IgE molecules to effector cells, such as mast cells and basophils, via a high affinity Fc receptor (FcεR1). Type I hypersensitivities can include allergies caused by aeroallergens (e.g. hay fever and allergic asthma), allergies caused by food allergens (e.g. peanut allergies) and allergies caused by topical allergens (e.g. atopic eczema). Allergies that are Type II-IV are less common, and involve other classes of antibodies or no antibodies. Type II hypersensitivity involves IgG and IgM to trigger antibody-dependant cell-mediated cytotoxicity, and can lead to allergy, such as allergic haemolytic anaemia. Type III hypersensitivity can also involve IgG and IgM and is characterised by antigen-antibody complexes in the bloodstream, which leads to allergic disease, such as allergic alveolitis. Finally, type IV is an effector T cell, macrophage, or other leukocyte-produced inflammatory response which

results in a delayed hypersensitivity reaction, leading to an allergy, such as contact dermatitis (Mak, Saunders, and Jett 2014b; Rajan 2003).

1.1.2. Prevalence of IgE-mediated Allergy

Type I hypersensitivities have increased over the decades, where the 'first wave' of the allergy epidemic surfaced more than 60 years ago. This saw IgE-mediated aeroallergen allergies become increasingly common, affecting 25- 50% of the population, varying between countries (D'Amato et al. 2007; Lacombe 2017; Sheehan et al. 2010). In recent decades, IgE-mediated food allergy prevalence has also rapidly increased, predominately in westernised countries, leading to scientists coining this the 'second wave' of the allergy epidemic (Loh and Tang 2018; Sheehan et al. 2010). There is also growing evidence for an increased prevalence in rapidly developing countries, such as China (Loh and Tang 2018). It is estimated that IgE-mediated food allergy affects up to 10% of children, and 6% of adults worldwide (Lee 2017; Osborne et al. 2011; Prescott et al. 2013; Waserman, Bégin, and Watson 2018) and that 38.7% of these children encounter severe IgE reactions, with 30.4% allergic to multiple foods; peanut, milk and shellfish being the highest (Gupta et al. 2011). In the UK, 4% of children under the age of 5 are diagnosed with challenge-proven food allergy (Loh and Tang 2018). Thus, IgE-mediated allergy is a widespread disease, increasing the importance to understand its underlying mechanisms and develop therapeutics.

1.1.3. Predisposition to IgE-mediated Allergy

Type I hypersensitivties can range from mild to life-threatening, thus it is important to predict the occurrence and severity of reactions to allergens (Pettersson et al. 2018). It is not completely understood why individuals develop Type 1 hypersensitivity, but it is accepted that a variety of factors such as genetics, the environment, the microbiome, and route of allergen exposure can all play a role in the development of allergic disease (**Fig. 1.1**). It is a

combination of these factors which could also explain why there are increases in the prevalence of allergic disease.

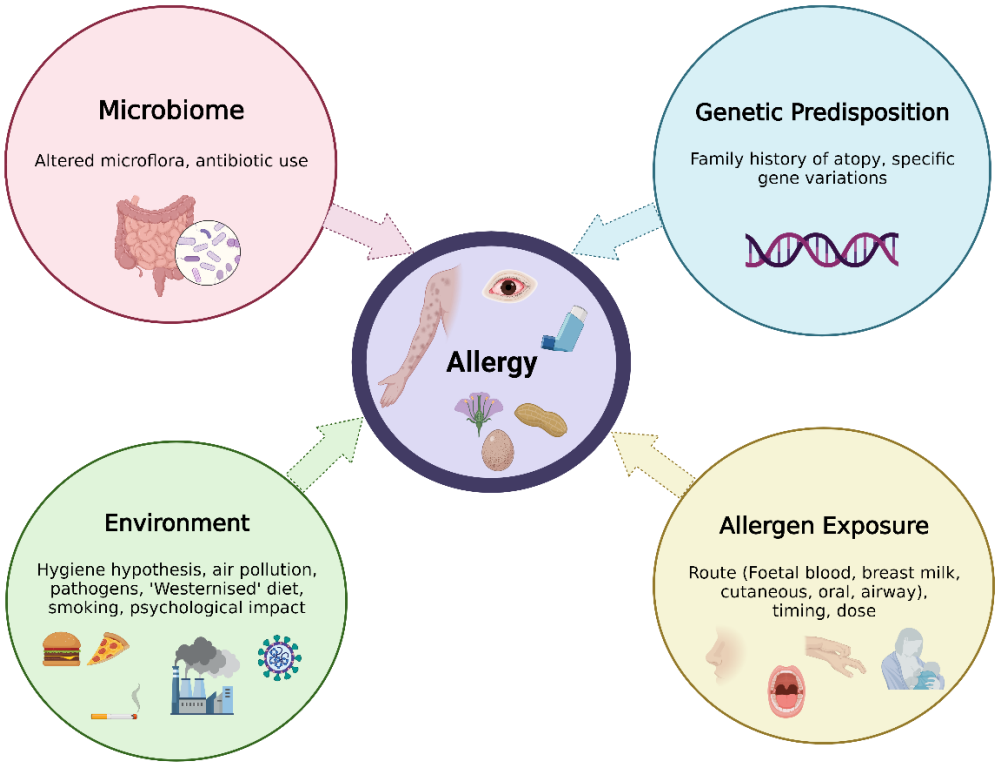


Figure 1. 1. Factors Impacting the Development of Allergy. The microbiome, genetics, environment, and route of allergen exposure can influence the development of IgE-mediated allergies. Such factors include a 'westernised' lifestyle, disruption of microbiota due to lack of microbial stimulation, antibiotic use, good hygiene, lack of contact with other children due to small families in urban environments, a family history of allergy, and sensitisation through the skin have all been shown to increase the risk of developing allergic disease. (Created using BioRender).

A family history of allergic disease is a key predictor of the development of IgE-mediated allergies. The very first study investigating the heritability of allergic disease found almost 50% of allergic individuals had a family history of similar allergies, compared to only approximately 15% in non-allergic individuals (Cooke and Veer Jr 1916). The heritability estimates for allergic disease vary, but there is evidence for rates as high as 95% for asthma, and 84%

for total IgE serum levels (Ober and Yao 2011). The introduction of Genome-wide association studies has allowed the identification of genes involved in complex disorders, such as allergic diseases (Portelli, Hodge, and Sayers 2015). The vast majority of genes identified in allergic disease were expressed in the skin, and less often in other tissues, suggesting genetic alterations to the skin barrier could facilitate the development of allergic sensitisation to allergens. The list of genes identified in allergic diseases are still incomplete, however, the top 10 genes identified thus far include: C11ORF30, STAT6, SLC25A46, HLADQB1/DQA1, IL1RL1/IL18R1, TLR1/6/10, LPP, MYC/PVT1, IL2/ADAD1, HLA-B (Kabesch and Tost 2020).

In addition to genetics, the environment can trigger allergy onset in genetically predisposed individuals. One environmental factor, named the hygiene hypothesis, suggests microbial stimulation in childhood can impact the development of allergies. The gastrointestinal (GI) tract and lungs are target sites for microbial stimulation during neonatal life. Epidemiological evidence has suggested that microbial exposure in early life correlates to the prevention of diseases, such as allergy and asthma in adulthood (Olszak et al. 2012), i.e. improved sanitation and hygiene are positively associated with allergy. Thus, the increase in public health measures in developed countries has reduced infectious disease and microbial exposure, in turn, promoting the development of allergies. Such measures include the introduction of vaccinations, decontamination of water, and pasteurisation and sterilisation of milk (Okada et al. 2010). Hence, a reduction in early-life exposures to pathogens due to vaccinations can explain increases in atopic individuals, as exposure to pathogens can help shift a developing immune system from a Th2-dominated type to a more defensive Th1 response (Pfefferle et al. 2021). Furthermore, recent research has suggested adjuvants accompanying inactivated vaccines could be responsible for the increase in allergies; increased administration of inactivated vaccines increased the likelihood of asthma and eczema development in infants (Yamamoto-Hanada et al. 2020). Thus, the effect of cumulative adjuvant exposure could increase the risk of allergy development.

The development of allergies has also been linked to a westernised lifestyle. Factors such as obesity, lack of exercise, a diet rich in processed foods, growing up with reduced contact to other children (e.g. an only child), and exposure to antibiotics are associated with decreased diversity of environmental microbiota and dysbiosis on the barrier organs. Rapidly developing countries have also seen rises in allergic disease, reinforcing the link between increased economic growth and changes to diet and lifestyle (Leung, Wong, and Tang 2018). This leads to altered thresholds for activation of the innate immune system and reduced tolerance, thus leading to the development of allergy later in life. Whereas, growing up in a rural environment with farm animals, a diverse diet rich in fibre, and early contact with other children has been shown to be beneficial to preventing allergy development in later life. These factors favour the development of highly diverse microbiota on the skin, respiratory mucosa, and GI tract, which are the body's barriers to external stimuli. This early interaction of commensal and environmental microbiota appears to be important in establishing a threshold for activating pattern recognition receptors (PRRs) and downstream signalling pathways, which then influences immune homeostasis and tolerance later in life (Gilles et al. 2018). A westernised lifestyle affects the intestinal epithelial cell metabolism which creates an environment which favours harmful microbes, such as *Escherichia coli* and *Salmonella*, and reduces symbiotic *Bacteroides, Prevotella, Desulfovibrio, and Lactobacillus* (Augustine et al. 2022). This microbial dysbiosis can also result in 'leaky gut syndrome', which is the increase in intenstinal permeability due to damage of epithelial cells by increased pathogenic bacteria, allowing intact allergens to reach the immune system (Usuda, Okamoto, and Wada 2021).

The route of allergen exposure can also impact the development of allergic sensitisation. Initially, exposure to allergens through the gut was suggested to cause allergic sensitisation to food allergens. Recently, there is evidence suggesting that allergen exposure through the skin can result in sensitisation, and oral exposure can in fact result in tolerance. This is further

reinforced by evidence suggesting environmental factors can cause damage to the skin barrier function, resulting in dysregulation, which leads to allergic sensitisation (Brough et al. 2020), as well as reinforced by the genome-wide association studies identifying skin-associated genes in allergic disease (Portelli, Hodge, and Sayers 2015). This phenomenon is coined the 'dual allergen exposure hypothesis', where oral exposure to food allergens results in tolerance, and exposure via damaged skin leads to allergy (Kulis et al. 2021). Oral tolerance occurs in the GI tract, where lymphoid tissues are able to discrimiate between self and non-self-antigens, thus only recognising harmful pathogens. Thus, oral tolerance refers to the state of immunological unresponsiveness to food allergens, the default response in healthy individuals which results in the protection from inappropriate immune responses to allergens (Tordesillas and Berin 2018). Impaired skin from conditions such as atopic dermatitis is well-established to be associated with peanut allergy development (Lack et al. 2003), compared to children with early and regular oral feeding of peanuts are associated with a dramatic decrease in peanut allergy incidence (Du Toit et al. 2015). This is supported by studies which have shown that individuals with atopic dermatitis had low skin and gut microbial diversity, and that this low microbial diversity preceded allergic sensitisation to food allergens (Augustine et al. 2022). Furthermore, recent studies have suggested the airways can actually be a route of food allergen sensitisation, as well as aeroallergens, with increased IL-33 production proving key in inducing allergic sensitisation in skin and the airways to food allergens (Brough et al. 2020; Smeekens et al. 2019). They highlight how intact protein allergens, such as from peanuts, are present in household dust, which is then inhaled by infants and leads to sensitisation, but only when exposed with dust mite allergens as adjuvants (Kulis et al. 2021). Thus, the route of the first allergen exposure is key in determining whether an allergen results in immune tolerance or allergic sensitisation.

1.1.4. Mechanisms of IgE-mediated Allergy

The mechanism of IgE-mediated allergy is currently understood to encompass two phases: allergic sensitisation (the induction phase) and elicitation of symptoms (the effector phase) (Lacombe 2017). **Figure 1.2** depicts the current understanding of the mechanisms of IgE allergy. The immune response to an allergen, such as house dust mite (HDM), peanut, or pollen begins with sensitisation, where the allergen makes contact with the epithelium for the very first time. The epithelial cells lining the airway, gut, nose, or skin constitute the first line of defence against allergens. They form a physical barrier as well as expressing various PRRs, which recognise the allergen and release cytokines to activate antigen presenting cells (APCs). For instance, Protease activated receptor 2 (PAR-2) on airway epithelial cells recognise HDM allergens and subsequently stimulate the secretion of cytokines to modulate DC behaviour (Salazar and Ghaemmaghami 2013b). The APCs in the epithelium lining of the airways, skin, or the GI tract, depending on the route of exposure, then internalises the allergen. The APC then migrates to local lymphoid organs to process and present the protein as peptides on Major Histocompatibility Complex (MHC) Class II molecules, which are found on the surface of APCs. These are recognised by T helper (Th) lymphocytes which, in addition to APC inflammatory cytokine production, are then activated to secrete IL-4 and IL-13 cytokines. It is the secretion of these cytokines which causes class-switching of B cells into allergen-specific antibody-secretory plasma cells. IL-4 and IL-13 induces class-switching from IgM isotype to IgE with co-stimulation involving CD40 and CD40 L. Germline gene transcription and DNA recombination then occur to result in the genetic rearrangement of IgE and ultimately the synthesis of allergen-specific IgE (Looney et al. 2016). The plasma cells then produce IgE specific to the allergen. These allergen-specific IgE antibodies can then diffuse locally and bind to the high affinity receptor for IgE (FcϵR1) and low- (FcεRII) affinity receptors on the surface of effector cells, mainly mast cells or basophils, via its Fc portion (Lee 2016). The high affinity receptors are responsible for most of the symptoms associated with allergy, whereas the low affinity receptors

(CD23) regulates the production of IgE from B cells and its transportation across the epithelial barrier (Li et al. 2006).

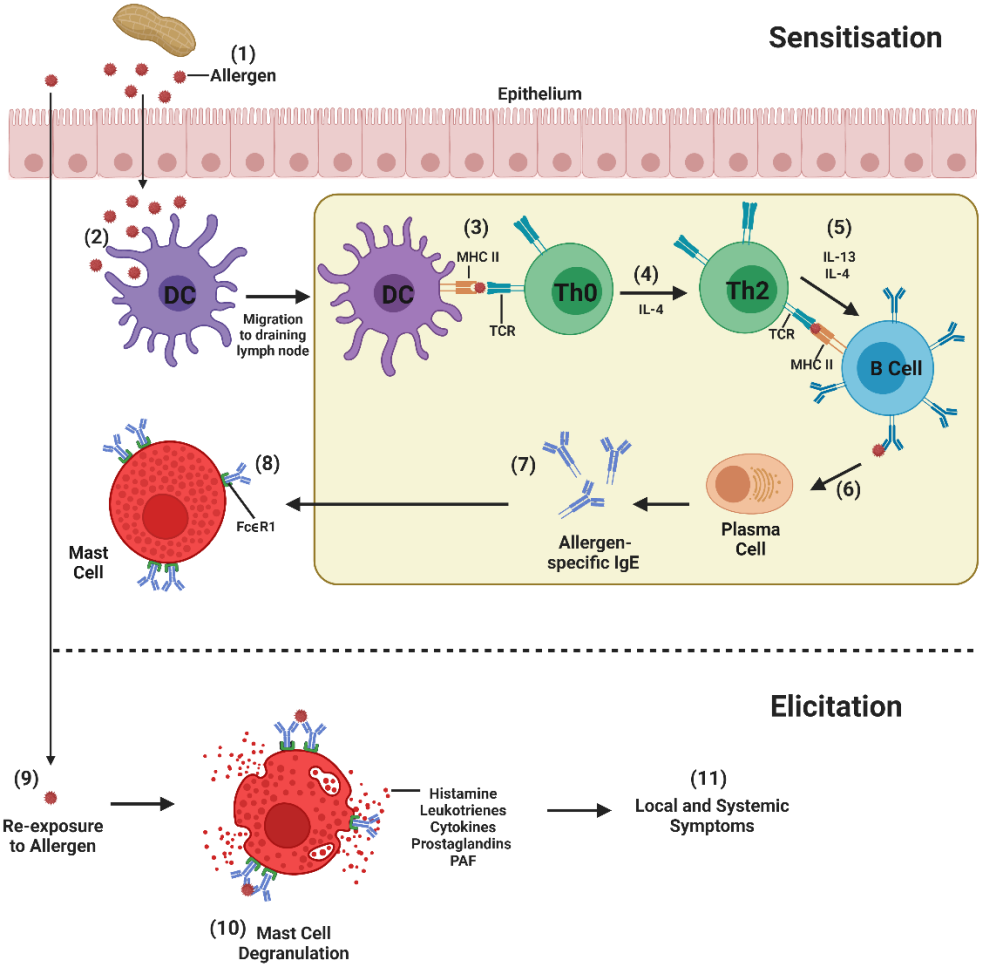


Figure 1. 2. The mechanism of IgE-mediated allergy. There are two phases to IgE-mediated allergy: allergic sensitisation and elicitation of symptoms. In the example of a peanut, (1) the allergen crosses the intestinal epithelium and is (2) internalised by APCs, such as DCs. The DC then processes the protein into peptide fragments and (3) presents it on its surface via MHC Class II molecules. The peanut allergen peptide and MHC II complex are presented to naïve CD4+ T cells, which (4) stimulates the production of IL-4 to differentiate into Th2 cells. The activated Th2 cells can then recognise more allergen-MHC II complexes on the surface of B cells, stimulating the release of cytokines IL-4 and IL-13. This promotes plasma cells to produce allergen-specific IgE antibodies. The IgE bind to FcεR1 receptors on effector cells, such as mast cells. If there is then further exposure to the peanut allergen, this triggers elicitation of symptoms. The allergen cross-links cell-bound IgE to which causes mast cell degranulation, releasing inflammatory mediators, such as histamine. It is the release of these mediators which results in the elicitation of symptoms. (Created using BioRender).

Upon further allergen exposure, known as the effector or elicitation phase, individuals present clinical symptoms due to the allergen cross-linking existing allergen-specific IgE bound to mast cells, via the Fab portion of IgE. IgE cross-linking on effector cells triggers a cascade of intracellular signaling pathways which modulates the immune response. Only a few receptors on mast cells need to be cross-linked for immediate hypersensitivity and it can take place in any areas of the body populated by IgE and mast cells e.g. respiratory tract, skin, GI tract, eyes, or it can even occur in the circulation with basophils which is what produces a systemic reaction (anaphylaxis) (Bax, Keeble et al. 2012). The signaling pathways induced after IgE cross-linking include the activation of tyrosine kinases which leads to the activation of multiple kinases, such as protein kinase C and various mitogen-activated protein kinases (MAPKs) (Méndez-Enríquez et al. 2022). These kinases then activate transcription factors, such as Nuclear factor kappa B (NFκB), which regulates inflammatory cytokine production, as well as activate lipases, such as phospholipase D which regulates degranulation, and finally phospholipase A, which regulates arachidonic acid metabolism. Arachidonic acid is the precursor for lipid mediators such as leukotrienes and prostaglandins (Méndez-Enríquez et al. 2022). Mast cells and basophils secrete leukotrienes, and can specifically secrete the slow-reacting but potent mediators of anaphylaxis, LTC_4 LTD_4 LTE_4, which is evident in severe allergic reactions (Gilroy and Bishop-Bailey 2019). In addition to cytokines and lipid mediators, activated effector cells, such as mast cells and basophils, can also secrete other mediators such as platelet activation factor (PAF) and chemokines (Broide 2001; Hamilton and Adkinson 2003). The mediators released from granules within the effector cells, such as histamine, heparin, and proteases, influence subsequent cellular responses in allergic inflammation to result in the rapid onset of symptoms. The physiological effects of histamine release include increased vascular permeability and smooth muscle contraction, resulting in symptoms such as swelling or difficulty in breathing. Prostaglandin release causes vasodilation and smooth muscle contraction. Cytokines IL-1 and TNF-α result in systemic anaphylaxis. IL-4 and

IL-13 secretion result in increased IgE production. PAF release causes platelet aggregation and degranulation. The overall effects of these inflammatory mediators include oedema, increased vascular permeability and vasodilation, leading to the clinical symptoms of itching, sneezing, bronchoconstriction, vomiting, and hives (Galli and Tsai 2012; He et al. 2013). Symptoms can occur rapidly but also at a later point, due to early and late-stage immune responses. The release of histamine, leukotrienes etc. result in the early onset of symptoms. However, hours later, mediators released during the early response begin to induce localised iflammation, called the late-phase response. The release of cytokines, such as TNF-α and IL-1 increase the expression of cell adhesion molecules on endothelial cells, resulting in the influx of eosinophils and neutrophils which cause inflammation.

Overall, the effector phase is powered by the high affinity of the IgE-FcεRI interaction, which helps orchestrate an aggressive and rapid immune response where the cross-linking of IgE leads to the degranulation of mast cells. This triggers the release of inflammatory molecules that drive the symptoms of an allergic reaction (Del Moral and Martínez-Naves 2017).

1.1.5. Treatment of IgE-mediated Allergy

Besides a serious impact on health, individuals with Type I hypersensitivity can experience a negative impact on their nutrition and social life (Cafarotti et al. 2023). Furthermore, due to the severity and unpredictability of some allergic reactions, the treatment of allergy is imperative. There is currently no cure for allergy, but there are an increasing number of methods for managing the disease. A classical approach for managing allergies is avoidance and rescue medications, such as epinephrine and antihistamines. For food allergy, this involves the strict avoidance of the triggering foods and educating the individual on using the rescue medicine. However, this method results in potential nutritional deficiences due to lack of diet diversity, especially in young children, and social restrictions e.g. eating out at restaurants (Salvilla et al. 2014).

In the 21st century, oral immunotherapy was introduced as a new treatment option for food allergy. It is a diesase-modifying therapy which involves a titrated oral admnistration of the triggering food at regular intervals to induce tolerance to the food (Arasi et al. 2018). This results in a higher threshold for the culrpit allergen to induce an allergic reaction. This has been succesfully applied to children with cow's milk, egg, and peanut allergy (Pajno et al. 2018). Allergen-specific immunotherapy (SIT) uses the same mechanisms to oral immunotherapy for food allergy, but instead the allergen is injected into the individual, which induces immunological tolerance and the induction of IgG4 blocking antibodies after repeated exposure to increasing amounts of allergen (Holgate and Polosa 2008). This has been effective in treating allergic rhinitis, venom allergy, drug allergy, and mild asthma. It is thought that T regulatory cells are the main responders to oral immunotherapy and SIT, as they can secrete inhibitory cytokines IL-10 and TGF-β which help cause clonal switching from Th2 cells to Tregs (Akdis and Akdis 2014). However, there is a safety risk factor for this immunotherapy, as individuals can suffer allergic reactions during each administration of the culprit allergen.

There is also an anti-IgE monoclonal antibody drug, Omalizumab, which has been clinically shown to increase reactivity threshold to food and aeroallergens. Omalizumab works by binding to the IgE constant region, Cε3, which then prevents the IgE binding to FcεRI on effector cells. It can also displace IgE from FcεRI and prevent cross-linking of IgE on effector cells (Cardet and Casale 2019). Thus, these methods interfere with the release of pro-inflammatory mediators and subsequent clincal manifestations. Etokimab which inhibits IL-33 production (Chinthrajah et al. 2019), Dupilumab which inhibits IL-4 production (Rial, Barroso, and Sastre 2019), and Mepolizumab which inhibits IL-5 production (Menzella et al. 2015), are other examples of monoclonal antibody drugs that have been developed for allergy treatment. However, monoclonal antibodies for treating allergy, especially food allergies, are still novel and require further large-cohort studies to investigate their efficacy (Manti et al. 2021).

For aeroallergies, antihistamines and inhaled corticosteroids are the main treatment options. Corticosteroids suppress cell-mediated inlammation in the airways by the inhibition of cytokines, chemokines, and adhesion molecules. Antihistamines, such as cetirizine, stop the pharmacological effects of histamine.

Despite the development of these therapies, the evaluation of treatment options among patients varies, and highlights the complex nature of allergic disease, and the need for personalised treatment options. Furthermore, without further understanding of the underlying mechanisms of allergic sensitisation, the development of diease-modifying therapies are limited.

1.2. Immune Cells in Allergic Sensitisation

1.2.1. Epithelial Cells

The epithelium is a membranous tissue lining the internal compartments of the majority of organs, acting as a physical barrier. It is selectively permeable due to the variety of receptors, transporters, and tight junctions, which regulate the movement of solutes across the epithelium (van Ree et al. 2014). Airway, skin, and intestinal epithelial cells express PRRs such as Toll-like Receptors (TLRs). These receptors enable the recognition of specific allergens, which activates epithelial cells to release an array of mediators, such as cytokines, to influence the function of nearby immune cells, such as DCs (Salazar and Ghaemmaghami 2013a). For instance, food allergens can cross the epithelial barrier by transcytosis, diffusion, or endocytosis (Schoos et al. 2020). Allergens with complex protease activity, such as Der p 1 from HDM, can cause damage and injury to epithelial cells. As a result of tissue injury and inflammation, epithelial cells can secrete cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (Noureddine, Chalubinski, and Wawrzyniak 2022). These cytokines can then influence the function of immune cells such as DCs, which can then influence allergic sensitisation. For instance, the production of TSLP can induce Th2 cell recruitment and polarization, whereas

IL-23 can induce Th2 cytokine production. Importantly, the cross-talk between epithelial cells and DCs has proven key in driving a Th2 response to allergens. The recognition of allergens by different PRRs on epithelial cells activates the secretion of chemokines which attract DCs, as well as epithelial cells secrete cytokines which can induce DC maturation (Pichavant et al. 2005). Furthermore, intraepithelial DCs can form tight junctions with epithelial cells which can facilitate allergen recognition and uptake.

1.2.2. Dendritic Cells

DCs have crucial roles in IgE-mediated allergy. Antigen recognition and uptake by innate immune cells is the first step in the process of allergic sensitisation, which determines the initiation of an adaptive immune response (Salazar and Ghaemmaghami 2013a). DCs patrol for antigens to internalise and are critical for initiation of the cellular and humoral response and protection from diseases or tumours. They bridge the innate and adaptive immune system. Upon exposure to allergens at the mucosal site, immature DCs (iDCs) take up allergens through endocytosis, pinocytosis, or phagocytosis, and then migrate to draining lymph nodes. The allergen is then processed and presented via MHC Class II molecules to T cell receptors, which activates the adaptive immune response. iDCs upregulate CCR-7 receptors in response to allergen encounters, which is the receptor for chemokines CCL21 and CCL19. These chemokines are expressed in afferent lymph endothelium and the T cell areas of lymph nodes, respectively, hence why DCs direct their responses to the draining lymph nodes (Cyster 1999). iDCs also express various C-type lectin receptors which determine their capacity for allergen uptake, including the mannose receptor and dendritic cell-specific intracellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN) (Emara et al. 2012). DCs are the most important APC in activating naïve T cells, which then differentiate into Th2 cells. This interaction between DCs and T cells occurs through a vast amount of interaction between adhesion molecules e.g. DC-SIGN and ICAM-3, as well as through co-stimulatory molecules such as CD80, CD86, and CD40 binding their respective ligands on T cells (Hammad et al. 2002). DCs then

control the strength and duration of T cell receptor stimulation, as well as the production of cytokines and ultimately the outcome of either tolerance, Th0, Th1, or Th2 (Lamiable et al. 2020). DCs can also produce cytokines to interact and direct other innate immune cells (Hole et al. 2019). DCs can also upregulate cell-surface markers such as CD40, CD80, CD86, and HLA-DR after exposure to allergens, as well as secrete cytokines such as IL-10 and IL-12. The upregulation of these markers, and release of cytokines can influence the response of the T cells (Ruiter and Shreffler 2012).

Human dendritic cells can be split into three subgroups: Classical (cDCs), plasmacytoid (pDCs), or monocyte-derived (moDCs) (Humeniuk, Dubiela, and Hoffmann-Sommergruber 2017). Human cDCs have a dendritic appearance, are found in both peripheral and lymphoid tissues, and can be further classified into type 1 and type 2 cDCs. Human Type 1 cDCs present antigens to CD8+ T cells, and have the capacity to promote Th1 differentiation through IL-12 and IFN-γ cytokine production, protecting against intracellular pathogens (Haniffa et al. 2012). Type-2 cDCs have been identified as the APC population which drive Th2 responses *in vivo,* potentially due to their high expression of tight junctions, which facilitate the internalisation of allergen. They are also essential for the initiation of CD4+ naïve T cell priming (Chow et al. 2016), and Th2 and Th17 differentiation (Plantinga et al. 2013b), thus are key DCs in allergy.

Plasmacytoid DCs have smooth surfaces and are located all over the body, and secrete predominantly type 1 IFN cytokines, which makes them crucial in antiviral immunity (Balan, Saxena, and Bhardwaj 2019). pDCs also promote regulatory T cell differentiation (Lewkowich et al. 2005) and infiltration into the tumour microenvironment, which has been shown to promote tumour growth (Aspord et al. 2013).

MoDCs are unlike other DCs in that they only become abundant during inflammation, and are found in both lymphoid and parenchymal tissues (León, López-Bravo, and Ardavín 2007). For instance, in allergic disease, monocytes can interact with $CD4^+$ T cells which results in the differentiation of monocytes into pro-inflammatory immunogenic moDCs (Zhang et al. 2020). MoDCs are

efficient at presentation of antigens to both $CD4^+$ and $CD8^+$ T cells, with the type of Th polarisation dependant on the inflammatory stimuli. MoDCs produce large amounts of cytokines and chemokines which are essential for the recruitment of Th2 cells after allergen exposure, and their subsequent activation (Plantinga et al. 2013b). For instance, a Th2 response after contact with dust mite allergens (Plantinga et al. 2013a). In contrast, moDCs have been shown to induce a Th1 response, such as after influenza infection (Nakano et al. 2009).

1.2.3. CD4+ helper T Cells

Once DCs have internalised and presented the allergen as peptides on their MHC II molecules, they can interact with CD4+ helper T cells in draining lymph nodes where T cells can then recognize the peptides and initiate a cascade of events leading to allergic sensitisation. Naïve CD4+ helper T cells can differentiate into subsets such as Th1, Th2, Th17, and T regulatory (Treg) cells depending on the influence of other immune cells and cytokines (Akdis et al. 2011). The differentiation of naïve T cells is dependent on the integration of several signals, including: TCR-mediated activation signal, co-stimulatory signals, cytokine and hormonal signals, and the composition of the extracellular matrix (Delespesse et al. 1997). The influence of the local cytokine milieu seems to exert the most important influence on T cell differentiation, with IL-4 predominantly inducing Th2 differentiation, and IL-12 inducing Th1 differentiation. It is well-established that the cellular source of IL-12 to activate Th1 differentiation is DCs or macrophages (Hsieh et al. 1993). However, the cellular source of IL-4 is less well-defined. Recent reports have suggested that NKT cells and basophils are primary sources of IL-4 production during Th2 differentiation (Yoshimoto 2018). During allergic sensitisation, the presentation of allergenic peptides to CD4+ T cells results in differentiation into Th2 cells. A high abundance of Th2 cells has been linked to the development of allergy (Akdis et al. 2004). Th2 cells can produce IL-4, IL-5, IL-9, IL-13 IL- 25, IL-31, and IL-33 cytokines, which promote an inflammatory immune response (Leffler, Stumbles, and Strickland 2018). On the other hand, in response to

intracellular pathogens, APCs can produce IL-12 and IFN-γ which leads to Th1 differentiation. Th1 cells secrete pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-2 which activate macrophages, NK cells, and CD8+ T cells to clear the pathogen (Berger 2000).

1.2.4. Regulatory T Cells

Treg cells are key in the maintenance of immune tolerance to allergens. As aforementioned, the dysregulation of Treg cells has been linked to the development of allergy. Treg cells are thus key in the maintenance of immune tolerance to allergens. Peripheral tolerance occurs when lymphocytes encounter innocuous allergens in peripheral tissues which results in immune suppression. Thus, failure in peripheral tolerance enables innocuous allergens to become a threat when there is no immune suppression (Romagnani 2006). Tregs are able to directly suppress Th2 cells and IgE-producing B cells to result in tolerance to allergens. The most studied subset of Tregs are natural Tregs which are CD4+CD25+Foxp3+ cells which produce IL-10 and Transforming Growth Factor-β (TGF-β). For example, DCs process food allergens and migrate to mesenteric lymph nodes where they secrete IL-10, TGF-beta which initiates naïve CD4+ T cell differentiation into Tregs. These Tregs can then suppress the activation of Th2 and type 2 innate lymphoid (ILC2) cells and their cytokine secretions (Ring et al. 2006). There are several mechanisms behind this suppression including the production of inhibitory cytokines IL-10 and TGF-β, as well as the down-modulation of CD80/CD86 expression on DCs which blocks allergen-specific Th2 cell immune responses (Noval Rivas and Chatila 2016). Furthermore, Tregs play an important role in the regulation of oral tolerance, as studies have shown that Tregs can suppress anaphylaxis and control Th2 immune responses, by the production of inhibitory cytokines such as IL-10, and inhibiting effector T cell proliferation which starves cells of IL-2. Hence why oral immunotherapy results in increased Treg cell numbers and function (Satitsuksanoa et al. 2018).

1.2.5. B Cells

B cells play a key role in the development of allergic sensitisation to allergens, primarily due to their unique ability to produce allergen-specific IgE antibodies. B cells are predominantly located in lymphoid tissues and form germinal centres with T cells and DCs (Leffler, Stumbles, and Strickland 2018). B cells with specific receptors for allergens can bind allergens directly or through follicular DCs (FDCs). The B cells can then present the allergenic peptides to CD4+ T cells to initiate T cell activation. CD40 molecules on B cells can also interact with CD40 ligands on activated CD4+ T cells, along with Th2 cytokines, to result in B cell activation (Iweala and Burks 2016).

In addition to Tregs, regulatory B cells (Bregs) also play a role in immune tolerance to allergens. Bregs can produce cytokines IL-10 and TGF-β which are anti-inflammatory, which then suppress inflammation and increase IgG4 antibodies in serum (Satitsuksanoa et al. 2021). This increase in IgG4 antibodies is observed in individuals gaining immune tolerance, which is observed during oral immunotherapy, the increasingly common treatment option for food allergies, where a small amount of the allergen source is eaten in increasingly large doses to desensitize the individual. Also, B cells can interact with Tregs to promote this IgG4 induction. Furthermore, Bregs also suppress DC maturation and induce Treg differentiation in the periphery (Rosser and Mauri 2015). Thus, B cells are fundamental for both allergic sensitisation and tolerance.

1.2.6. NKT Cells

In addition to the previously mentioned immune cells, NKT cells also play a role in IgE-mediated allergy. NKT cells are T lineage cells that share characteristics of both T cells and NK cells. Very limited NKT cell numbers are found in human blood, varying between 0.01% to 1% of the total lymphocytes in peripheral blood of healthy human donors. However, they comprise approximately 1% of lymphocytes in mice, thus why much research has utilised murine models to study NKT cells (Birkholz and Kronenberg 2015). Despite their limited abundance, they are found all over the body; peripheral blood, the

spleen, thymus, liver, bone marrow, and lymph nodes (Mak, Saunders, and Jett 2014a).

NKT cells were first described in 1983 (Godfrey et al. 2000). The key features characteristic of NKT cells include a heavily biased T-cell receptor (TCR) gene usage, CD1d restriction and high levels of cytokine production, particularly type 2 cytokines IL-4 and type 1 cytokine interferon gamma (IFN-γ). There are two major subtypes: type I and type II NKT cells (Girardi and Zajonc 2012). Type I, also known as invariant NKT (iNKT) cells, express an invariant TCRα chain, which is specific to a certain glycolipid presented by CD1d molecules, as well as some non-invariant TCRβ chains. Human iNKT cells express the invariant Va24-Ja18 TCRα chain preferentially coupled with a Vβ11 TCRβ chain (Sköld and Behar 2003). Type II NKT cells, in contrast, use diverse TCRα and β chains (Dhodapkar and Kumar 2017a). Type I iNKT cells are phenotypically CD4+CD8-, CD4-CD8- (DN), or CD4-CD8+ in a small subset.

iNKT cells are activated rapidly and exhibit both pro-inflammatory and immunoregulatory features, resulting in protective or harmful roles in numerous pathological states, such as microbial infection, autoimmune disease, allergic disease, and cancer (Macho-Fernandez and Brigl 2015). As mentioned, unlike conventional CD4+ T cells which recognise peptides presented by MHC Class II molecules on APCs, iNKT cells recognise glycolipids presented by MHC Class I-like CD1d molecules on APCs (Stock and Akbari 2008). iNKT cells are activated early on in an immune response, acting as rapid first line defence cells of the innate immune system, which links to the adaptive immune response (Mak, Saunders, and Jett 2014a). I*n vitro* studies have shown that CD4+ iNKT cells produce both Th1 and Th2-type cytokines, such as IFN-γ and IL-4, at a rate much faster than conventional naïve CD4+ T (Stock and Akbari 2008). Similarly to CD8+ conventional T cells, CD8+ and DN iNKT cells produce more Th1 cytokines (Brossay et al. 1998). Further to IFN- γ and IL-4, iNKT cells can also rapidly release copious amounts of other Th1 and Th2 cytokines, including IL-2, IL-9, IL-10, IL-13, IL-17, IL-21, and GM-CSF.

This differential effect of Th1 and Th2-type cytokine release and their ability to interact with other immune cells, gives iNKT immunomodulatory roles in disease, with powerful effects on αβ T cell differentiation and functions (Macho-Fernandez and Brigl 2015). **Figure 1.3** illustrates the effect of NKT cytokine release on other immune cells. Type 1 iNKT cells specifically play critical roles in local and systemic immune responses, with roles in tumour development, antimicrobial responses, and autoimmune and allergic disease (Macho-Fernandez and Brigl 2015).

During an infection with an lipopolysaccharide (LPS)-containing bacteria, NKT cells activate by the stimulation of DCs. DCs release a variety of cytokines and chemokines such as IL-12, which can potently stimulate NKT cells to produce Th1 cytokine IFN-γ (Van Kaer and Joyce 2005). It is the rapid release of vast amounts of cytokines which has led to the ability of iNKT cells to amplify adaptive immune responses, such as allergic sensitisation, as iNKT cells have been shown to enhance a Th2 immune response by the production of IL-4, such as in response to lipids derived from allergen sources (Mirotti et al. 2013b).

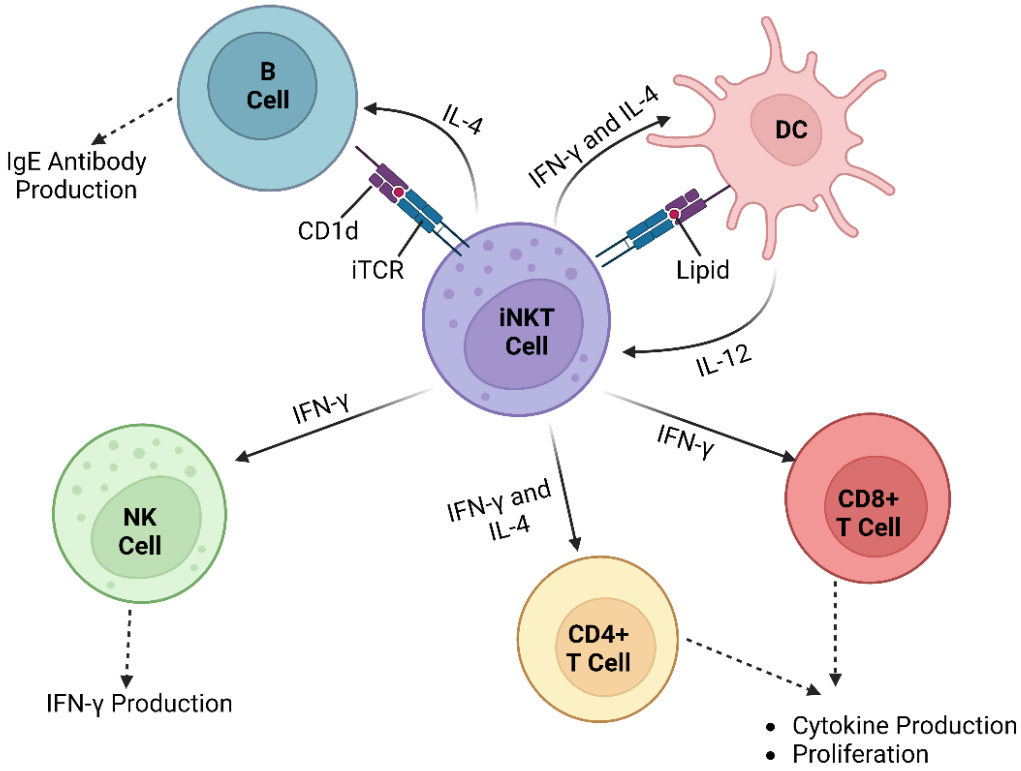


Figure 1. 3. Immunomodulatory effects of iNKT cells. iNKT cells can directly and indirectly modulate the function of other immune cells; DCs, T cells, B cells, and NK cells. The formation of a CD1d-lipid complex, recognised by iTCRs on iNKT cell surfaces, results in iNKT cell activation. This releases cytokines, predominantly IL-4 and IFN-γ, from iNKT cells, which can influence the activation and cytokine secretion of other immune cells. DCs release IL-12 which in combination or without the glycolipid presentation, can activate iNKTs to release IFN-γ. (Created using Biorender).

1.3. Allergens

Allergens are antigens which trigger immediate hypersensitivity reactions after penetrating biological barriers (Platts-Mills and Woodfolk 2011). Typically, allergens are glyco-proteins with a molecular mass ranging from 5 to 80 kDa (Humeniuk, Dubiela, and Hoffmann-Sommergruber 2017). There are four classes of allergens: indoor allergens such as HDM and mould, outdoor allergens such as grass pollen, plant/animal food allergens such as peanuts and cow's milk, and injected allergens such as bee venom (Karp 2010; Alcocer and Yman 2013).

The allergenicity of an allergen is determined by the ability for the allergen to elicit a Th2 response after DC stimulation, where DCs express a variety of surface receptors that recognise specific allergens (Galli, Tsai, and Piliponsky 2008). But, the question of "what makes an allergen an allergen?" has been long debated with no definitive answer. Much research has focused on proteolytic activity, resistance to pepsin degradation, glycosylation, route of exposure, dosage, exposure to allergen with agents that enhance or down-regulate sensitisation, and low molecular weight of the protein (Mullins et al. 2022).

Previous work at the University of Nottingham on the inhalant allergen from house dust mites (HDM), Der p 1, highlighted the role of proteolytic activity in the allergenicity of a protein. HDM is globally a common allergen, but also has a strong protease activity. The cysteine protease activity of Der p 1 was shown to enhance the total IgE and Der p 1–specific IgE production, compared to Der p 1 administered to mice with no cysteine proteolytic activity (Gough et al. 1999). The proteolytically active Der p 1 cleaved CD25 which lead to diminished Th1 cytokine production of IFN-γ, thus favouring a Th2 response. Specifically, Der p 1 cleavage of CD25 lead to impaired growth of Th1 cells. Furthermore, this was one of the first studies to show Der p 1 administration lead to inflammatory cell infiltration into the lungs (Gough et al. 2003; Gough, Sewell, and Shakib 2001). The role of carbohydrates has also been explored in major allergens, with glycosylation patterns being linked to allergenicity (Al-Ghouleh et al. 2012). DCs express PRRs such as C-type lectin receptors (CLRs) which recognise antigens, particularly glycoantigens. Mannosylation was found to be a key glycosylation pattern among some major allergens, such as Ara h 1, with a high mannan sugar content in these allergens. Thus mannose receptors were key in recognising and internalising allergens to initiate a Th2 response, such as for the major cat allergen Fel d 1 (Emara et al. 2011) and house dust mite allergens Der p 1 and Der p 2 (Royer et al. 2010).

In terms of food allergens, the abundance of food protein i.e. quantity of the food digested or quantity of protein in a food, can determine the

development of allergy. There is evidence for this in common allergens, such as Ara h 1 in peanuts, where this allergen constitutes a major proportion of the total protein in peanuts (Metcalfe et al. 1996). Resistance to denaturation and pepsin digestion is another factor. The more intact a protein is, the more likely it is to encounter cells of the gut immune system (Bannon 2004). Allergen stability in the gut is also affected by the structure of the allergen. For example, the disruption of disulphide bonds in food proteins leads to more digestion by pepsin and more binding of IgE epitopes due to its change in structure (Bannon 2004).

However, it is not fully understood what specific properties of proteins can predict their allergenicity. More recently, lipids have been suggested to enhance the allergenicity of proteins by shifting towards a Th2 response . Allergens also possess hydrophobic pockets or cavities which can have the capacity to bind ligands, which can then structurally change the allergen to influence interactions with DC receptors and influence Th2 responses (Mueller, Edwards, et al. 2010; Mueller, Gosavi, et al. 2010). There are several allergens in food and aeroallergen sources that have the capacity to interact with lipid-ligands through hydrophobic pockets/cavities or specialised domains, as shown in **Table 1.1** (Jappe et al. 2019a). These lipophilic allergens are clinically relevant, such as the major Brazil nut allergen, Ber e 1, or the major cow's milk allergen, Bos d 5, suggesting the adjuvant activity of lipids interacting with allergen structural features directly influence allergenicity. Moreover, oleosins are structural proteins found in vascular plant oil bodies and in plant cells which have been linked to severe allergic reactions, such as Ara h 10 and Ara h 11 from peanuts. They are integrated into the phospholipid membrane of oil bodies from oil-rich seeds and plant pollen, with a large hydrophobic domain bound to the oil bodies (Huang and Huang 2015). Despite the clear interactions between major allergens and lipids, the potential for lipids to act as adjuvants to the protein allergens has not been widely studied.

Table 1.1. Allergens associated with lipids. A list of allergens, their sources, and the mode of lipid-interaction. (Adapted from (Jappe et al. 2019a)).

Protein Family	Source	Allergen	Mode of lipid/ligand Interaction
Bet v 1 like	➢ Birch (*Betula verrucosa*) ➢ Peanut (*Arachis hypogaea*)	➢ Bet v 1 ➢ Ara h 8	➢ Binds ligands via hydrophobic pocket ➢ Binds ligands via hydrophobic pocket
Non-specific lipid transfer protein (nsLTP)	➢ Peach (*Prunus persica*) ➢ Peanut (*Arachis hypogaea*) ➢ Grape (*Vitas vinifera*)	➢ Pru p 3 ➢ Ara h 9 ➢ Vit v1	➢ Binds fatty acids in inner hydrophobic cavity ➢ Potentially binds lipids, phospholipids in inner hydrophobic cavity ➢ Binds phosphatidylcholine
Globulin	➢ Peanut (*Arachis hypogaea*) ➢ Mustard (*Sinapis alba*)	➢ Ara h 1 ➢ Sin a 2	➢ Interaction with phosphatidylglycerol vesicles ➢ Interaction with phosphatidylglycerol vesicles and mustard lipid
2S Albumin	➢ Brazil nut (*Bertholletia excelsa*)	➢ Ber e 1	➢ Lipid-binding hydrophobic cavity is assumed
Oleosins	➢ Peanut (*Arachis hypogaea*) ➢ Sesame (*Sesamum indicum*)	➢ Ara h 10, Ara h 11, Ara h 14, Ara h 15 ➢ Ses i 4, Ses i 5	➢ Bind phospholipids and lipids via hydrophobic domain creating an oil body ➢ Bind phospholipids and lipids via hydrophobic domain creating an oil body

Lipocalin	➢ Cow's milk (*Bos domesticus*) ➢ Dog (*Canis familiaris*)	➢ Bos d 5 ➢ Can f 6	➢ Carries hydrophobic molecules, phosphatidylcholine ➢ Binds LPS
Secreto-globulin	➢ Cat (*Felis domesticus*)	➢ Fel d 1	➢ Potentially binds TLR-ligands
Group 2 mite allergen	➢ House dust mite (*Dermatophagoides pteronyssinus*) ➢ House dust mite (*Dermatophagoides farinae*)	➢ Der p 2 ➢ Der f 2	➢ Binds LPS due to structural similarity with MD-2 ➢ Binds LPS due to structural similarity with MD-2
Group 5/7 mite allergen	➢ House dust mite (*Dermatophagoides pteronyssinus*)	➢ Der p5 ➢ Der p 7	➢ Hydrophobic cavities that might bind apolar ligands ➢ Hydrophobic cavities that might bind apolar ligands
Group 13 mite allergen	➢ House dust mite (*Dermatophagoides pteronyssinus*)	➢ Der p 13	➢ Selective binding of fatty acids in inner cavity
Group 14 mite allergen	➢ House dust mite (*Dermatophagoides pteronyssinus*)	➢ Der p 14	➢ Potential transporter of lipids
Group 21 mite allergen	➢ House dust mite (*Dermatophagoides pteronyssinus*)	➢ Der p 21	➢ Potentially binds lipids from house dust mite

1.4. Lipids

1.4.1. CD1d Presentation of Lipids

The international Lipid classification and Nomenclature Committee state there are 8 classes of lipids. Specific members from 5 of these classes (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids and saccharolipids) have been suggested to play a role in allergic sensitisation.

In contrast to proteins that are presented by MHC class II molecules, it is well-established that lipids are presented by CD1 molecules **(Figure 1.4)**.

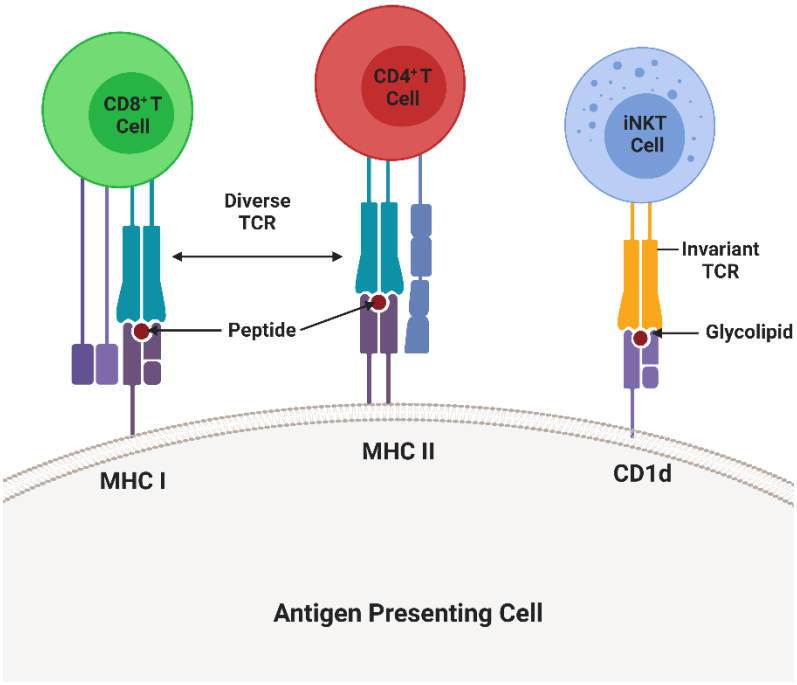


Figure 1.4. MHC I, MHC II, and CD1d antigen presentation to T cells. MHC I and MHC II class molecules present peptides to CD8+ and CD4+ T cells, respectively, via diverse T cell receptors (TCRs). CD1d molecules, similar to that of MHC I, present glycolipids to iNKT cells, via invariant TCRs. (Created using BioRender).

CD1 molecules are glycoproteins similar to MHC Class I molecules, comprising a heavy chain with three extracellular domains associated with β2 -microglobulin (β2 m). CD1 molecules are expressed on most APCs, including

DCs, macrophages and B cells. There are five isoforms of CD1; group 1 consists of CD1a-c, group 2 consists of CD1d, and group 3 consists of CD1e (Schiefner and Wilson 2009a). These 5 types of CD1 molecules are expressed in humans, whereas mice only express CD1d, however, CD1d molecules are highly conserved between humans and mice (Eckhardt and Bastian 2021).

Group 1 CD1 molecules present microbial lipids to clonally diverse T cells that mediate adaptive immunity. In contrast, the Group 2 CD1d molecule presents lipids to iNKT cells to stimulate a rapid innate immunity response. Group 3 CD1 molecules do not have an antigen presenting role, but are involved in lipid-processing (Barral and Brenner 2007).

1.4.2. Lipids in Allergen Sources

Lipids are small hydrophobic or amphipathic molecules (Fahy et al. 2009) that can be bound or co-delivered with allergenic proteins to the innate immune system. As shown in Table 1.1, lipids within an allergen source can be directly associated with allergenic proteins, as some proteins have the capacity to bind lipids through hydrophobic cavities, ionic, or hydrophobic bonds (Jappe et al. 2019b). These allergen-bound lipids can be termed protein-lipid complexes. There are several classes of allergenic proteins which have the ability to bind lipids, as well as lipid-ligands in the case of the lipid transfer protein (LTP) family. These include Bet v 1-like proteins, non-specific LTPs, 2S albumins, and oleosins (Jappe et al. 2019b). These proteins can bind various lipids and lipid-ligands, depending on their tertiary structure, including fatty acids, glycolipids, and phospholipids (Dubiela et al. 2019). This lipid-binding can then result in structural and biochemical changes to the protein, which alters the immune response provoked (Petersen et al. 2014b).

In contrast to directly binding allergens, lipids from an allergen source can also be co-delivered with the allergenic protein. The lipids can be present in pollen coats of plant allergen sources or in matrices of plant and animal foods. This includes pollen-associated lipid mediators (PALMs), which are bioactive lipids released from the pollen grain, or they can be present in the cell membranes, such as phospholipids (Gilles-Stein et al. 2016). These co-

delivered lipids can interact directly with immune cells to modulate the immune response (Traidl-Hoffmann et al. 2002). It is through allergenic protein-binding and activating immune cells that a variety of intrinsic lipids (lipids within an allergen source), have been shown to influence and promote allergic sensitization.

For instance, the common allergen pollen contains approximately 1-25% lipids, varying between plant species (Roulston and Cane 2000), with sphingolipids, such as glucosylceramides, estimated to compose up to 40% of the total lipids in pollen plasma membranes (Luttgeharm et al. 2015). Most of these lipids are saturated fatty acids with a high abundance of linoleic, arachidonic, stearic and palmitic acids (Luttgeharm et al. 2015; Roulston and Cane 2000). In contrast, unsaturated fatty acids, predominantly oleic acid, make up almost 50% of the total fat content in tree nuts. Like pollen, nut lipid content also varies between species, ranging from approximately 43 % in peanuts, to 66 % in macadamia nuts (**Table 1.2**) (Venkatachalam and Sathe 2006). There is also approximately 42-49% lipid content in the peanut allergen (Ros and Mataix 2006a; Venkatachalam and Sathe 2006). Peanut lipid content is high compared to other legumes, such as soy beans, which contain between 8.1-24% lipids (Medic, Atkinson, and Hurburgh Jr. 2014). Soy is less potent at driving allergic sensitisation, as shown in a clinical study where 0.25% of infants were sensitised to soy compared to 1.2% who were sensitised to peanuts (Cordle 2004). This study also suggested soy is a less potent allergen compared to peanuts, due to its high protein concentration threshold required for sensitisation i.e. ‘safe’ protein thresholds were determined as 0.1mg for peanut, and 400mg for soy (Cordle 2004).

Table 1.2. Chemical composition of nuts. The total lipid, protein, and sugar content in different edible nuts (Source: (Venkatachalam and Sathe 2006)).

Nut	Lipid (%)	Protein (%)	Sugars (%)
Almond	43.36	19.48	2.11
Brazil nut	66.71	13.93	0.69
Cashew nut	43.71	18.81	3.96
Hazelnut	61.46	14.08	1.41
Macadamia nut	66.16	8.40	1.36
Pecan	66.18	7.50	1.55
Pine nut	61.73	13.08	1.82
Pistachio	45.09	19.80	1.52
Walnut	64.50	13.46	2.06
Peanut	42.88	21.56	0.55

Also, the common allergens from House Dust Mite HDM (e.g. Der p 2, Der f 2), contains a myeloid differentiation (MD-2)-related lipid-recognition (ML) domain, which provides the capacity to bind lipids, such as bacterial LPS (Johannessen et al. 2005). Overall, this knowledge of lipids residing with proteins in allergenic substances contributes to the theory that they could also have a role in driving allergic sensitisation.

1.4.3. Mechanisms of Lipids Influencing Allergic Sensitisation

A systematic review of 19 papers examining the role of lipids in allergic sensitisation was conducted and published (Hopkins et al. 2022) **(Appendix A).** Several mechanisms were identified, which will now be presented.

1.4.3.1. CD1d-restricted iNKT Cell Activation

Lipids intrinsic to an allergen source and delivered to the immune system bound to allergenic proteins are termed lipid-ligands. This CD1d-iNKT cell mechanism is evident in the case of the lipid-ligand of Pru p 3 (from peach), in particular its lipid phytosphingosine tail, which was shown to activate murine-derived iNKT cells (determined by IL-2 secretion), through its lipid-ligand presentation on CD1d molecules (Tordesillas et al. 2017). Another study found the allergen protein, Ber e 1, failed to induce IgE production in sensitised mice when administered without its lipid fraction (Dearman, Alcocer, and Kimber 2007). When the lipid fraction was present, it acted as an adjuvant to IgE production. It was suggested the adjuvant activity of the lipid fraction could be due to its ligation of CD1d molecules (Dearman, Alcocer, and Kimber 2007). A subsequent study of Ber e 1 sensitization found the lipid fraction, named 'lipid C', induced the production of the Th2 cytokine IL-4 from iNKT cells to shift to allergic sensitization. They also found Ber e 1 can bind lipid C via a hydrophobic pocket, allowing the lipid-ligand to ligate CD1d molecules (Mirotti et al. 2013a). One study investigated milk and egg lipids, sphingomyelin and ceramide, respectively, in allergic sensitization (Jyonouchi et al. 2011). They established milk-sphingomyelin, but not egg-ceramide, can induce Th2-skewing of iNKT cells by presentation on human CD1d molecules. Unlike the aforementioned studies, this study also evaluated iNKT cell populations, revealing children with milk allergy had fewer iNKT cell numbers, but greater Th2 responses to milk-sphingomyelin than non-milk allergy controls.

As shown above with food allergies, lipids associated with aeroallergens have also been shown to influence allergic sensitization via CD1d-restricted iNKT cell activation. They have also demonstrated that lipids associated with aeroallergen sources are presented by CD1d molecules on APCs and

subsequently activate iNKT cells (Abos Gracia et al. 2017; Agea et al. 2005; Bansal, Gaur, and Arora 2016; González Roldán et al. 2019).

One study from the systematic review revealed PALMs primed DCs for the presentation of glycolipids to iNKT cells by CD1d upregulation (González Roldán et al. 2019). This supports findings from another study of olive pollen lipids (Abos Gracia et al. 2017), which established olive pollen lipids, but not aqueous pollen extracts (APEs), strongly activated human iNKT cells by increasing CD1d surface expression on iDCs and macrophages. All lipids analysed: polar lipids, diacylglycerols, free fatty acids, and triacylglycerol, were able to induce this increased CD1d expression. Despite altering the phenotype of iDCs, the olive pollen lipids did not alter their cytokine profile, but did induce secretion of IL-6 from macrophages, which further activated iNKT cells. Another study also found cypress pollen lipids were recognised by CD1d molecules (Agea et al. 2005). Furthermore, one study on cockroach allergy found the cockroach extract stimulated phospholipids to release lysophosphatidylcholine (LPC) and activate murine NKT cells, resulting in a Th2 shift. This NKT cell activation by LPC was inhibited when an anti-CD1d antibody was added (Bansal, Gaur, and Arora 2016).

1.4.3.2. Lipids Activate Immune Cells

In addition to an iNKT-CD1d mechanism of lipid-driven allergic sensitisation, the systematic review also highlighted the ability of lipids to directly activate immune cells to skew to allergic sensitisation. For example, one study established the mustard seed and peanut allergen proteins, Sin a 2 and Ara h 1 respectively, accompanied by mustard and peanut lipids, reduced human monocyte-derived dendritic cell (hmoDC) allergenic protein uptake potentially due to altered membrane composition (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). Reduced protein uptake favours a Th2 reaction, whereas higher doses of protein uptake results in tolerance (Wisniewski, Agrawal, and Woodfolk 2013). Another study discovered egg yolk lipids acted as a Th2-biasing adjuvant to egg white through

the upregulation of intestinal IL-33 by epithelial cells *in vitro*, which is crucial for DC activation and Th2 priming (Pablos-Tanarro et al. 2018). In addition to providing CD1d-iNKT activation evidence above, one study of the lipid-ligand of Pru p 3 (from peach) also established the lipid directly activated DCs as it matured human monocyte-derived DCs (Tordesillas et al. 2017).

Another study revealed evidence for and against lipids enhancing allergic sensitization (Palladino et al. 2018). This study, related to peanut sensitization, found the administration of peanut lipids alone resulted in increased production of the anti-inflammatory cytokine, IL-10, from keratinocytes, thus inhibiting a Th2-type response. Whereas, peanut lipids delivered with the peanut allergenic protein inhibited IL-10 production.

Another nut allergen study, also mentioned previously, found the allergenic protein, Ber e 1, failed to induce IgE production in sensitised mice when administered without its lipid fraction. It was only when the lipid fraction of the Brazil nut was present, the lipid acted as an adjuvant to IgE production. The total lipid fraction of the Brazil nut, including its composite sterols and polar lipids, all had marked adjuvant effects on IgE production. However, b-sitosterol and glycolipid-rich fractions had negligible impact on IgE production (Dearman, Alcocer, and Kimber 2007).

The studies in the systematic review investigating direct activation of immune cells by lipids focused on the role of PALMs in allergic sensitisation (Agea et al. 2005; Gilles et al. 2010; Gilles et al. 2009; Gutermuth et al. 2007; Oeder et al. 2015). Two human studies (Gilles et al. 2010; Gilles et al. 2009) highlighted aqueous birch pollen extracts (Bet.-APE)-derived PPE_1 modulated DC function and its cytokine production, specifically the inhibition of IL-12, preferentially inducing a Th2 response. Another study using a murine model of allergy found PPE_1 inhibited the LPS-induced production of IL-12 from DCs (Gutermuth et al. 2007), but when intranasally instilled with the egg allergen protein, Ovalbumin, PPE_1 inhibited Th2 polarization and cytokine release, suggesting lipids inhibit allergic sensitization. Another study also established PPE_1 and aqueous pollen extracts stimulated Th2-primed B cells to enhance IgE

production (Oeder et al. 2015). One study, previously mentioned for its evidence of CD1d recognition of PALMs, found the presence of PC and PE lipids from cypress pollen alone stimulated TCR $\alpha\beta^+$ $CD4^+$ T cell production of IL-4, enhancing a Th2 response (Agea et al. 2005).

Overall, these studies show PALMs can directly activate DCs, B cells, and $CD4^+$ T cells to shift to a Th2 response. Whereas, 1 study (Gutermuth et al. 2007) reports PALMs can promote and inhibit allergic sensitization.

1.4.3.3. Lipids Induce Conformational Changes of Allergens

Another mechanism identified in the systematic review was the influence of lipids on the structure of their associated allergenic proteins (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Hufnagl et al. 2018; Finkina et al. 2020a; Meng et al. 2020).

The digestibility of food proteins can determine whether the allergen is tolerated or becomes a sensitizing agent. High resistance to digestion in the gastrointestinal tract has been shown to increase the sensitization capacity of proteins (Pali-Schöll et al. 2018). Some studies suggest protein-lipid binding can influence allergenic protein structure which alters digestion of the allergen, and this can alter the sensitization capacity of allergens (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Finkina et al. 2020a; Meng et al. 2020). Another study found that, in addition to lipids intrinsic to an allergen source, allergenic proteins can also interact with membrane-bound lipids, such as phospholipids (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). This study, previously mentioned above as evidence for the direct activation of DCs, highlight that allergenic proteins can bind the membrane-bound lipids, phosphatidylglycerol (PG) vesicles, reducing their gastrointestinal degradation (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). Furthermore, the ability for proteins to bind PG vesicles was dependent on the pH conditions. In contrast, this was not the case for the mustard seed allergenic protein, Sin a 3, which is structurally different to

peanut allergens and was not affected by the presence of PG vesicles (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016).

Another study focused on the plant LTP, Len c 3 (Finkina et al. 2020a). It has been established that legumes contain a high level of lipids, composing mostly of unsaturated fatty acids (Grela and Günter 1995). The lentil allergenic protein, Len c 3, is highly stable to digestion. This study found Len c 3 binding of the unsaturated fatty acids: oleic acid (OLE), lauric acid (LAU), and stearic acid (STE), all reduced the rate of Len c 3 gastric degradation, apart from behenic acid (BEH) which did not alter degradation. Furthermore, OLE reduced Len c 3 degradation to 55% after 24 hours of simulated digestion, compared to 100% of Len c 3 degraded after 24 hours with no ligand. STE and OLE increased thermostability of Len c 3, whereas LAU and BEH only had a slight protective effect on the secondary structure. Despite these conformational changes, no lipid-ligand increased the IgE binding capacity of Len c 3.

In contrast, another study found protein-lipid binding did enhance the IgE-binding abilities of both whey proteins, α-lactalbumin (BLA) and β-lactoglobulin (BLG) (Meng et al. 2020). Whey proteins derived from cow's milk are widely used in the food industry due to their ability to emulsify, foam, and gelatinise food products (Lucey, Otter, and Horne 2017). These whey proteins also constitute the common allergenic proteins, α-lactalbumin (BLA) and β-lactoglobulin (BLG). Thus, the ability to reduce their allergenicity would be profitable to the food industry. The linear and conformational epitopes of proteins contribute towards the allergenicity of the allergen (Hochwallner et al. 2010). This study found BLA and BLG can bind C18 unsaturated fatty acids (UFA) to form protein-ligand complexes (Meng et al. 2020). This binding to the fatty acid resulted in the structural unfolding of BLG, where C18 UFA treatment induced a transition from a β-sheet to a random coil. Furthermore, BLA treatment with C18 UFA resulted in changes to tertiary structure. Therefore, this study suggests protein-lipid binding can alter allergenic protein structure which alters the allergenicity of the milk allergens.

In contrast, one study found intrinsic lipids do not alter allergenic protein structure and further stated they do not drive allergic sensitization (Hufnagl et al. 2018). Retinoic acid, found in cow's milk, had a high binding affinity for the common milk allergen protein, Bos d 5. This lipid did not alter the conformation of Bos d 5, and so not surprisingly did not alter its allergenicity or IgE binding in allergic children. Furthermore, the lipid-ligand supressed CD3+ CD4+ cell numbers which indicates an immunosuppressive effect on this population, which is pivotal in allergy induction.

Overall, these studies (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Finkina et al. 2020a; Meng et al. 2020) found lipids induced conformational changes of allergenic proteins which influenced allergic sensitisation, with 1 study suggesting that some lipids do not alter protein structure and thus allergenicity (Hufnagl et al. 2018).

1.4.3.4. Lipids Activate TLRs

Another mechanism of lipid-influenced allergic sensitisation is the direct activation of TLRs by lipids. One aeroallergen study investigated the lipid activation of TLRs (Satitsuksanoa et al. 2016). They report the HDM protein allergen, Der p 13, which is found in HDM faecal particles, has structural folds which bind lipids, and is highly selective for fatty acids. The lipid-ligand can then activate TLRs, such as TLR2, to stimulate inflammatory cytokines IL-8 and GM-CSF production in respiratory epithelial cells.

1.3.4.5. Summary of Lipids in Allergic Sensitisation

In summary, lipids can interact with allergenic proteins to influence the development of allergic sensitization. This protein-lipid interaction can result in reduced gastrointestinal degradation of the allergenic proteins through structural protein changes, the reduction of DC uptake of allergenic proteins to reduce immune tolerance, the regulation of Th2 cytokines, the enhancement of allergen-specific IgE, the activation of iNKT cells through CD1d ligation, and

finally, directly acting upon TLRs, epithelial cells, keratinocytes, and DCs. An overview of the lipid-driven mechanisms is illustrated in **Figure 1.5**.

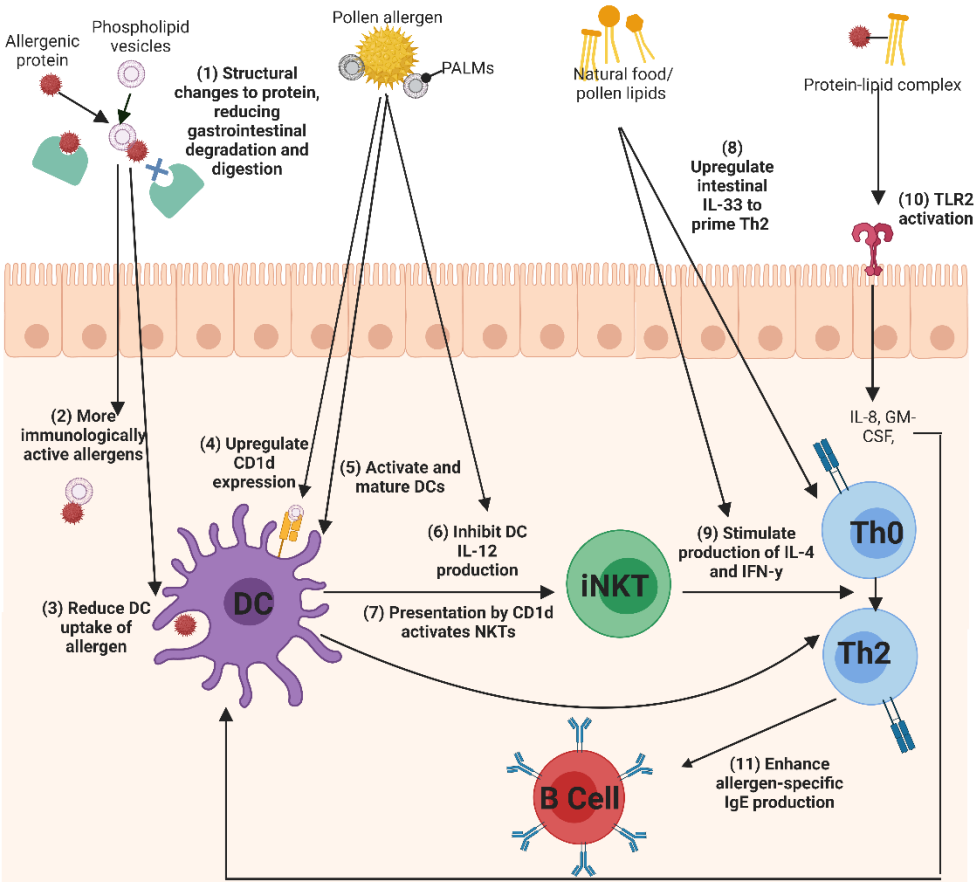


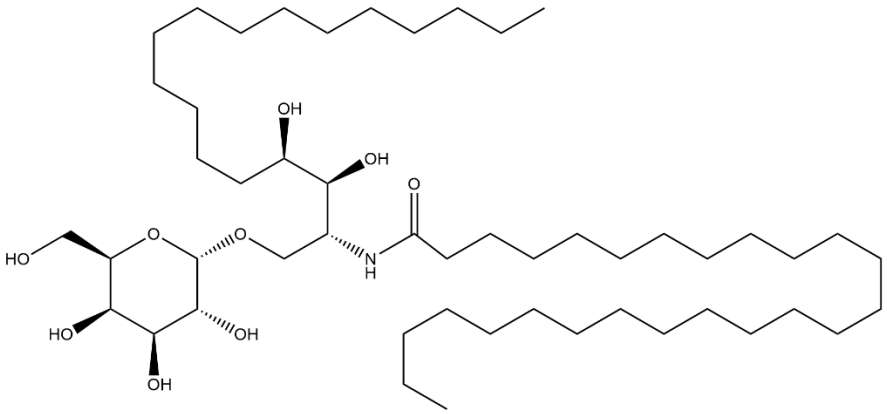
Figure 1.5. The mechanisms of lipids to influence allergic sensitization. (1) Phospholipids can bind allergens to reduced gastrointestinal degradation of the allergen, which (2) allows more immunologically active allergens to enter the immune system and can also (3) alter DC uptake of the allergen. Lipids, such as PALMs, can directly act upon DCs by (4) upregulating CD1d expression, (5) activating and maturing DCs, (6) and inhibiting I-12 production, which can all lead to the (7) activation of iNKT cells. Th0 cells could then be primed to Th2 cells by (8) IL-33 secretion from lipid-activated epithelial cells, or (9) by the secretion of IL-4 and IFN-γ cytokines from lipid-activated iNKT cells. (10) lipid-ligands can activate TLRs, such as TLR2, to initiate IL-8 and GM-CSF production, which in turn activates DCs. Finally, (11) lipids can also enhance the production of allergen-specific IgE from B cells. (Hopkins et al. 2022).

1.4.4. α-Galactosylceramide

To study the role of lipids and iNKT cells in allergic sensitisation, some studies utilise the lipid α-Galactosylceramide as a positive control, as it is the

most potent activator of iNKT cells. It is a well-established lipid involved in activating iNKT cells, but not diverse NKT cells (Dhodapkar and Kumar 2017a), and is a glycolipid derived from *Agelas mauritianus*, a marine sponge. Although it is actually likely derived from bacteria found within the marine sponge (Rampuria and Lang 2018). α-GalCer was subsequently modified for use in anti-cancer treatments, which lead to the discovery of iNKT cells. α-GalCer comprises an α-linked galactose, a phytosphingoid chain with 18 carbons, and 26 carbon acyl chain (**Fig. 1.6**) (Birkholz and Kronenberg 2015). Human CD1d molecules contains a hydrophobic groove which can accommodate the two alkyl chains of α-GalCer, resulting in the activation of iNKT cells (Koch et al. 2005).

α-GalCer has a strong anti-tumour activity and skews towards a Th0 reaction as it predominantly stimulates iNKT cells secretion of IL-4 and IFN-γ (Li et al. 2022).



KRN7000

Figure 1.6. Chemical structure of KRN7000 (alpha-galactosylceramide). α-GalCer comprises an α-linked galactose, a phytosphingoid chain with 18 carbons, and 26 carbon acyl chain. Created using ChemDraw.

1.5. Models of Allergic Sensitisation

Murine and human models of allergic sensitisation are widely utilised in current studies investigating lipids in allergic sensitisation, with approximately

half utilising human models and half utilising murine models (Hopkins et al. 2022). However, studies which solely rely on murine models should be interpreted with caution, due to differences to the human immune system and a lack of validated animal models. Once such difference is IL-10 produced by a Th2 response in mice is produced by a Th1 and Th2 response in humans (Mestas and Hughes 2004), unequal expression of FcεRI receptors on cell surfaces (Schülke and Albrecht 2019), induction of anaphylactic reactions independent of IgE, via IgG in mice, which is not relevant in human anaphylaxis (Finkelman 2007). Despite this, there are many similarities between mice and men, hence for the widespread use of murine models.

Murine models of allergy have an advantage of human models in respect to the fact the mice provide a whole system to investigate allergenic potency of substances, and test the potency/safety of novel therapeutic options, without risking the lives of human patients. They also have short generation time, are small in size, and easy to genetically manipulate. (Schülke and Albrecht 2019). Mice are often sensitised to the desired allergen via intranasal, intradermal, intraperitoneal, or oral routes. The existing murine food allergy models utilise food allergens such as milk, egg, and nuts. The allergens can be applied with an adjuvant to enhance allergic sensitisation to the allergen. Via the oral route, adjuvants include cholera toxin (CT), Staphylococcus enterotoxin B (SEB), or they can be adjuvant-free. Via the intraperitoneal route, allergens can be applied with aluminium hydroxide (Alum) or LPS. Intranasal route employs CT and LPS together. And the intradermal route tends to be without adjuvant (Schülke and Albrecht 2019).

In addition to not all CD1 molecules conserved between humans and mice, allergy in mice is not natural, thus, inducing sensitization to allergens is artificial and does not fully reflect the development of allergic sensitisation in humans.

Human tissue biopsies are a common model for studying allergy, as the sample contains the entire cell repertoire for analysis. For instance, bronchial

biopsies can be obtained from asthma patients and exposed to allergens in culture, and the allergic response produced is reflective of the asthmatic lung (Wood et al. 2013). Furthermore, gut biopsies can be obtained from food allergy patients and exposed to food allergens in order to measure the intestinal barrier function (Bischoff et al. 1997). However, biopsies are an invasive procedure, and hard to obtain a large number and size of sample.

Human co-culture systems are a minimally-invasive alternative which are more readily available. Unlike human tissue, it doesn't contain all the components involved in the allergic system, but a co-culture does allow multiple cell types to be incubated together and the interplay between cells investigated. For instance, DCs and iNKT cells can be isolated from human peripheral blood and co-cultured together. DC presentation of allergens and lipids to the iNKT cells can then be examined, and the subsequent cytokine response measured.

This is the model which this research has adopted to investigate the role of lipids and iNKT cells in allergic sensitisation as it allows the complex interactions between the two cell types to be measured in an environment which is easily manipulated, and the human blood samples easily sourced.

1.6. Flow Cytometry

Flow cytometry is a form of technology which allows the rapid multi-parametric analysis of single cells. It utilises lasers to produce scattered and fluorescent signals to detectors. The signals can then be converted into electronic signals by computer software and recorded (McKinnon 2018). Cells can then be analysed based on their size and any fluorescence by utilising fluorescent antibodies to tag intracellular and extracellular markers. This ability to measure intracellular and surface markers on potentially millions of cells in a single sample makes flow cytometry a powerful tool in not just immunology, but many other disciplines such as microbiology, drug discovery, and molecular biology.

Due to challenges with the spectral overlap between fluorochromes, a new type of flow cytometry, the spectral analyser, has recently been developed to solve this problem. **Figure 1.7** demonstrates the technique adopted by the spectral analyser. Essentially, the spectral analyser, first commercially launched by Sony Biotechnology in 2012, uses prisms and photomultiplier tubes (PMT) to collect and amplify light (Mitra-Kaushik et al. 2021). It uses multiple detectors to measure the full spectrum emission for every fluorophore utilising multiple lasers, which enables a more detailed analysis for each fluorophore. Furthermore, instead of compensation like conventional flow cytometry, spectral analysers utilise 'unmixing' which is a mathematical algorithm to identify each fluorophore (Niewold et al. 2020). This enables near-identical peak emissions to be distinguished and used together in a flow panel, allowing more parameters to be measured.

Spectral cytometry is key for this research because it allows in-depth multi-parameter analysis, only requiring a small volume of cells, which is essential when handling precious human samples. It also allows above 50 fluorophores to be detected, meaning over 50 antibodies can be utilised in one flow panel to provide in-depth phenotyping of multiple cell populations, while still only requiring a small amount of cells.

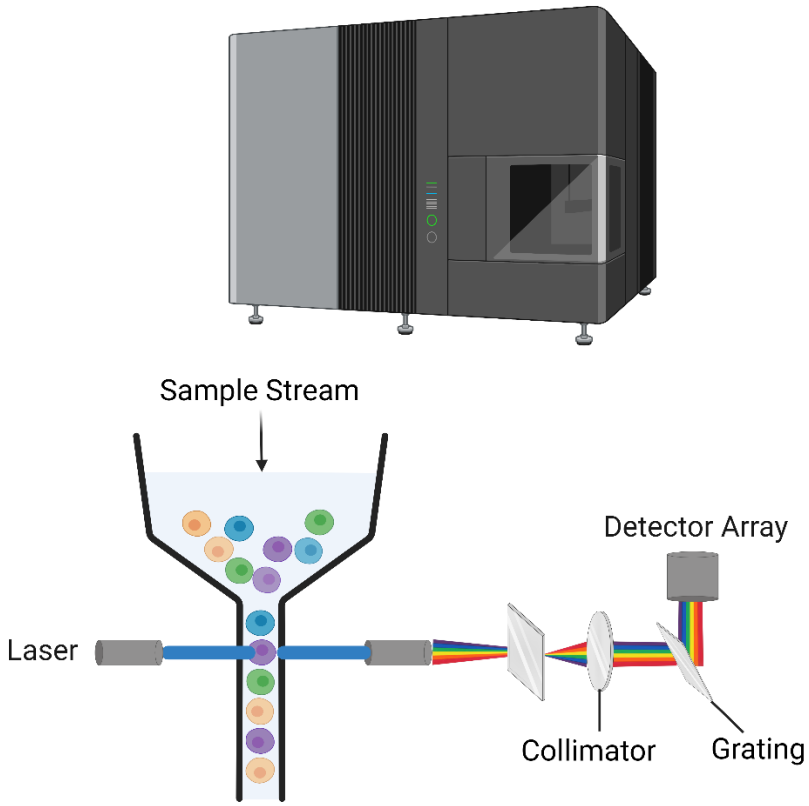


Figure 1.7. ID7000 Spectral Analyser. Compared to conventional flow cytometry, spectral cytometry uses gratings (prisms) to separate light and a collimating lens to parallelize and direct light linearly across an array of detectors. (Created using Biorender).

1.7. Applications to Industry

People are being introduced to new foods to help counteract the food insecurity problem, where almost 4.2 million people in the UK face food poverty (Brigid Francis-Devine 2022), leading to the development of new sustainable foods such as insect burgers and micro-protein mince. However, the introduction of novel proteins in these foods can pose health risks if the allergenicity of the food is not assessed. It is important to assess the risk of an allergic reaction and its severity. The current understanding of the events leading to allergic sensitisation are limited. Thus, Van Bilsen et al. created a sequence of events leading to allergic sensitisation based on existing research, and applied the adverse outcome pathway (AOP) (van Bilsen et al. 2017a). This identified several key events (KEs) which have the potential to be applied to a risk assessment of all proteinaceous materials. These key events include:

inflammatory responses at the epithelial level (KE1) and DC level (KE2, DC and macrophage migration (KE3), T cell priming, proliferation and polarisation (KE4), and finally B cell activation and class switching (KE5). Understanding the role of lipids in allergic sensitisation focuses on a specific aspect of KE4, involving T-cell priming, proliferation, and polarisation. Understanding these immune responses to certain proteins and their adjuvants (such as lipids) can help measure the allergenicity of the allergen, before introduction into developed food products. Thus, the cell-based research can then be used to build upon current immunogenicity assays, based on proteins, to incorporate lipids.

Furthermore, allergen source derived lipids, such as peanut oil, play a major role in not only the food industry, but also the cosmetic industry. Peanut oil is becoming an increasingly common ingredient in bath oils, soaps, and other cosmetics (Ring and Möhrenschlager 2007). As previously mentioned, the development of allergic sensitisation has been shown to occur via the skin, thus body cosmetics containing allergens and/or lipids may cause allergic sensitisation to certain allergens. Identifying whether lipids, such as peanut oil, can influence allergic sensitisation can subsequently help measure the risk of including certain lipids in cosmetics.

1.8. Study Aims

The present work aimed to develop a human, *in vitro*, cell-based assay to investigate the role of intrinsic lipids and invariant NKT cells in the development of allergic sensitisation. A co-culture model was developed to allow DC presentation of lipids with/without allergens to iNKT cell, and the subsequent cytokine secretion measured. Th1 or Th2 cytokine secretion thus indicated whether the lipid influenced allergic Sensitisation to the protein allergen. The developed assay was then applied to samples from patients with peanut allergies, where blood was obtained from peanut allergy participants and compared to non-allergic blood. It is hypothesised lipids influence allergic sensitisation to allergens by activating iNKT cells to secrete Th2 cytokines.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Culture Media

Roswell Park Memorial Institute (RPMI)-1640 Culture Medium containing 2mM L-glutamine (Cat no. R6504, Merck, UK) and supplemented with Penicilin (100 IU)-Streptomcyin (100µg/mL) (Cat no. P0781, Merck, UK), 10 mM HEPES (Cat no. H0887, Merck, UK), and 10% heat inactivated human AB serum (Cat no. H5667, Merck, UK) was used for cell cultures, unless otherwise stated.

2.1.2. Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood was collected in heparin-coated vacutainers (Cat no. 455051, Greiner Bio-One, UK). Histopaque (Cat no. 10771, SLS, UK) was used as a density gradient for PBMC isolation. Sterile Dulbecco's phosphate buffered saline (PBS) (Cat no. D8537, Merck, UK) was used to dilute blood 1:1 before layering on histopaque. SepMate^{TM} tubes (StemCell Technologies, UK) were purchased to increase efficiency of PBMC isolation; the tubes have a plastic insert which allows blood to be layered quicker than conventional PBMC isolation, as well as PBMCs can be poured off after centrifugation.

2.1.3. Immunomagnetic Cell Isolations

All immunomagnetic isolations of cells required a MACS Multistand (Cat no. 130-042-303, Miltenyi, UK) with either a MiniMACS separator (Cat no. 130-042-102, Miltenyi, UK) or a QuadroMACS separator (Cat no. 130-090-976). The isolation procedures also required a cell isolation buffer which constituted: 30% Human Serum Albumin solution (HSA) (Cat no. A9080, Merck, UK), 2mM Ethylenediaminetetraacetic acid (EDTA) (Merck, UK), and sterile PBS.

A $CD3^{+}CD56^{+}$ NKT Cell Isolation Kit (Cat no. 130-093-064, Miltenyi, UK) was utilised for a two-step isolation of human NKT cells. The kit constituted: CD3+CD56+ NKT Cell Biotin-Antibody human Cocktail, Anti-Biotin Microbeads, and CD56 Microbeads.

Invariant NKT cell immunomagnetic isolation required staining iNKT cells with a CD1d-α-GalCer R-PE tetramer (Cat no. D001-2C-G-1, Proimmune, UK), and then immunomagentically isolating the iNKT cells with anti-PE Microbeads (Cat no. 130-105-639, Miltenyi, UK).

$CD14^+$ monocyte immunomagnetic isolations required CD14 UltraPure Microbeads (Cat no. 130-118-906, Miltenyi, UK). GM-CSF and IL-4 (Miltenyi, UK) were then added to CD14 monocytes in culture to generate DCs.

2.1.4. Invariant NKT Cell Expansion

α-GalCer (100 ng/mL) (ABCAM, UK) was utilised as a positive control for activating and expanding iNKT cells, as it is the most potent activator. Recombinant human IL-2 (Cat no. 130-097-743, Miltenyi, UK) was also added to iNKT cell cultures to maintain viability. Cells were cultured in a 5% CO_2 incubator (Panasonic) set at 37 °C.

2.1.5. Flow Cytometry

A number of flow cytometers provided by The University of Nottingham's Flow Facility were utilised. Initial experiments consisted of fewer antibodies in the flow panel, thus, the BD Canto II (Beckman Coulter, UK) and Astrios (Beckman Coulter, UK) machines were utilised. 2nd year DC work utilised the Astrios flow cytometer, which allowed more antibodies in a panel to be measured. The ImageStreamX MkII (Luminex Coporation) was used for generating images of DCs. The ID7000 (Sony, UK) spectral cytometer was used at the latter end of the PhD, as the flow panel increased to 21 antibodies. This flow panel consisted of antibodies to primarily identify iNKT cells and DCs during their co-culture, and assess their cytokine production (**Table 2.1**). The brightness of antibodies refers to the intensity of the signal produced by each fluorophore, detected by the flow cytometer.

Table 2.1. 21-colour Flow Cytometry Panel. Antibodies utilised in the final assays with their fluorophore, brightness, the company sought from, and the concentration used.

Antibody	Fluorophore	Brightness	Company	Cat No.	Conc. Used (µg/mL)
CD14	BUV805	1	Bdbiosciences	612902	8
CD4	BUV395	2	Bdbiosciences	563550	8
HLA-DR	BUV496	2	Bdbiosciences	749866	8
CD80	BUV661	3	Bdbiosciences	741647	8
CD69	BUV737	3	Bdbiosciences	61281	8
TCR Vα24-Jα18	BUV563	3	Bdbiosciences	748830	4
Foxp3	BD R718	4	Bdbiosciences	566935	10
CD3	BV510	1	Biolegend	344828	3
CD19	BV570	1	Biolegend	302236	4
CD56	BV785	3	Biolegend	362550	4
CD209	APC Fire 750	3	Biolegend	330116	8
ZOMBIE	NIR	4	Biolegend	423106	1
IFN-y	BV650	2	Biolegend	502538	4
IL-4	BV605	3	Biolegend	500828	4
IL-10	PE Dazzle 594	5	Biolegend	501426	8
CD8	PERCP-VIO700	1	Miltenyi	130-110-682	4
VB11	VIOBLUE	2	Miltenyi	130-108-731	9
IL-5	APC	4	Miltenyi	130-117-205	2
IL-12	PEVio770	4	Miltenyi	130-103-677	1
CD1d tetramer	R-PE	5	Proimmune	D001-2C-G	2
CD25	AF532	3	Thermofisher	58-0259-42	2.5

Due to the large number of 'Brilliant Violet' fluorophores in the panel, a 'Brilliant Stain Buffer Plus' was purchased (Cat no. 568264, BDbiosciences, UK) to improve the spectral overlap of antibodies when running on the Spectral Cytometer.

Earlier work on NKT and DC optimisation experiments also required Annexin V and PI staining kit (Cat no. 130-092-052, Miltenyi, UK) for viability. The kits also needed an Annexin binding buffer (Cat no. 130-092-820, Miltenyi, UK) to help stain cells.

Phosphate buffer albumin (PBA) is required when washing cells during extracellular and intracellular staining. PBA was made in-house, consisting of PBS with 30% bovine serum albumin (BSA) (Cat no. A7284, Merck, UK) and 20% Sodium Azide (Cat no. S2002, Merck, UK). PBS was made using deionised water and PBS tablets (Cat no. BR0014G, Thermofisher, UK,). The solution was then sterile-filtered and stored at 4°C.

Fixation buffer was purchased from Biolegend (Cat no. 420801, UK) and permeabilisation buffer was purchased from Thermofisher (Cat no. 00-8333-56, UK) for fixing stained cells and permeabilising cells for intracellular staining, respectively.

A protein transport inhibitor cocktail 10x (Thermofisher, UK) was purchased for cytokine staining.

2.1.6. Lipids

In addition to α-GalCer (as above), a dansylated α-GalCer (Cat no. AG-CN2-0514-M001, Adipogen, UK) was also purchased for the imaging of lipid-uptake by DCs.

For peanut-allergy experiments, the initial experiments involved purchasing peanuts from a commercial supplier (Holland and Barrett, UK) and isolating the lipid fraction by the Folch method (Folch, Lees, and Sloane Stanley 1957). Due to concerns over the purity of the in-house peanut oil, a commercial peanut oil (Arachis Oil) (Handa Fine Chemicals, UK) was purchased for later

experiments with high purity. **Figure 2.1** details the chemical processing of the peanut oil to ensure high purity.

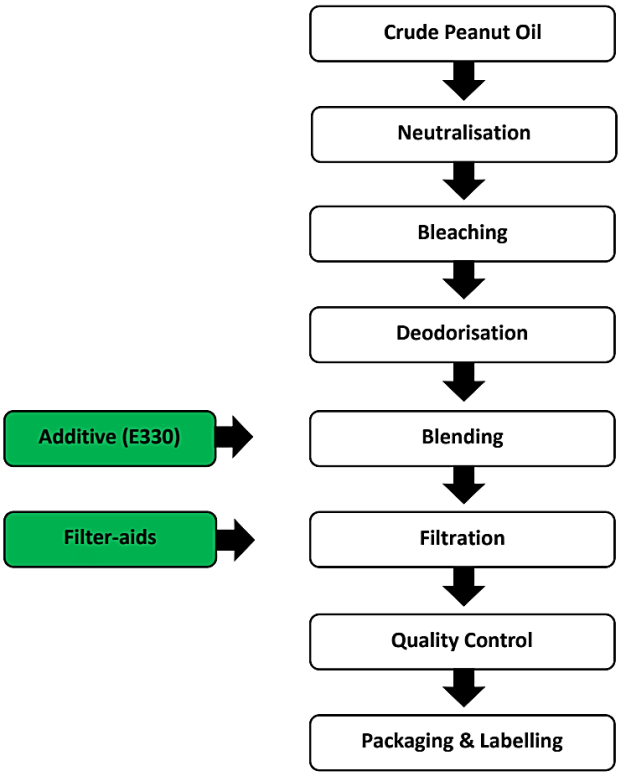


Figure 2.1. Flow Chart of Peanut Oil Processing. The manufacturing process of purified Arachis Oil to ensure any contaminating proteins are removed (Handa Fine Chemicals).

2.1.7. Allergens

Natural Ara h 2 (Cat no. NA-AH2-1) and Recombinant Ara h 8 (Cat no. RP-AH8-1) were purchased from Indoor Technologies (UK) for the Ara h 2- and Ara h 8-specific IgE ELISAs. Lotox Ara h 8 (Cat no. LTN-AH8), a low-endotoxin form of the allergen, was also purchased from Indoor Biotechnologies, UK, for the stimulation of DCs before co-culture with iNKT cells.

2.1.8. Total IgE ELISA

The Human IgE Uncoated ELISA Kit was purchased from Thermofisher Scientific (Cat. No. 88-50610-88).

2.1.9. Human Blood Samples

Prior to blood collection, healthy subjects were recruited by poster advertisement and informed consent was given. Healthy subjects had no history of allergies, were non-smokers, above the age of 18, and not a member of the department where the study was conducted. The study was approved by The University of Nottingham's Medical School Ethics Committee (Ref. 232-1902). Blood was obtained from the healthy human subjects at The Queen's Medical Centre (QMC), Nottingham, UK. The samples were transported to the lab for immediate use in cell assays. During the latter stages of my PhD, peanut allergy subjects were recruited through Cripp's Health Centre, Nottingham and samples were obtained for the study of peanut lipids in peanut allergy. Peanut allergic subjects were recruited under ethics approved by the NHS Health Research Authority Research Ethics Committee (Ref 21/SC/0183). These subjects must have had a history of IgE-mediated peanut allergy to be eligible, as well as being above the age of 18, non-smokers, and again, not a member of the research lab where the study is conducted. Further details regarding the peanut allergy subjects are detailed in Chapter 5: The Role of Peanut Lipids and iNKT cells in Allergic Sensitisation.

2.2. Methods

2.2.1. Cell Culture

2.2.1.1. Peripheral Blood Mononuclear Cell (PBMC) Isolation

To isolate PBMCs, 50mL of venous blood was obtained from non-allergic or peanut-allergic human volunteers using heparin blood collection tubes. 15mL of Histopaque was used as a density gradient and pipetted through the insert of a SepMate tube. The blood was diluted 1:1 with PBS + 2% human AB serum, and pipetted on top of the histopaque. The SepMate tubes were centrifuged at 1200g for 10 minutes, with the brake on. Then, as much supernatant as possible was removed with a Pasteur pipette before quickly tipping the PBMC layer into a fresh 50mL falcon tube and topping up to 50mL with PBS+2% human AB serum. The PBMCs were then washed for 8 minutes at

300g, with an acceleration of 3 and deceleration of 3 (3↑3↓), and then the media was tipped off and pellet re-suspended in another 50mL of PBS+2% human AB serum. The cell solution was centrifuged for another 8 minutes, at 200g, 3↑3↓, to ensure the removal of any platelets, before the supernatant was tipped off and the cell pellet re-suspended, ready for culture.

2.2.1.2. Cell Count

To culture cells at the correct concentration, cell counts were performed. At the earlier stages of my PhD, cell counts were performed manually by staining with trypan blue and counting the cells using a microscope; 20µL of cell suspension was mixed with 20µL of trypan Blue in a small Eppendorf (giving a dilution factor of 2). The coverslip was placed onto the haemocytometer and 10-20 µL of cell suspension/trypan Blue solution was pipetted into one side of the haemocytometer chamber. Cells in 3 out of 4 grids were counted and the cell concentration was calculated using the following equations:

Cells/mL = Average No. of cells counted x dilution factor x $1x10^4$

Total cells = Cells/mL x volume of cell suspension

At the later stages of my PhD, a Countess 3 Automated Cell Counter (Thermofisher, UK) was purchased. This required 10 µL of cell suspension and 10 µL of trypan blue to be mixed and inserted into a countess disposable slide. The slide was then inserted into the machine and the cell counts performed automatically.

2.2.2. NKT Cell Immunomagnetic Isolation

After isolation of PBMCs, NKT cells can then be isolated by negative selection of CD3+CD56+ cells, followed by positive selection of CD56 cells, leaving CD3+CD56+ NKT cells. Before NKT cell isolation, all MACS columns, MACS separators and MACS buffers were placed in the fridge to keep cool.

2.2.2.1. Magnetic labelling of non-$CD3^+CD56^+$ NKT Cells

The first step to isolate $CD3^+CD56^+$ NKT cells was to remove non-$CD3^+CD56^+$ cells, such as NK cells and monocytes. Firstly, the PBMCs were

mixed with 40 mL MACS buffer and centrifuged at 300g for 10 minutes, ↑ 3 ↓ 3. The supernatant was aspirated completely and re-suspended in 400 µL MACS buffer. 100 µL of $CD3^+CD56^+$ NKT Cell Biotin-Antibody Cocktail was then added to the cells and incubated at 4 ºC in the fridge for 10 minutes. This allows non-$CD3^+CD56^+$ cells to be labelled. The PBMCs were then mixed with 10 mL of MACS buffer and centrifuged again at 300g for 10 minutes. Next, the supernatant was aspirated completely and re-suspended in 400 µL of MACS buffer before being mixed with 100 µL Anti-Biotin Microbeads. These beads bind to any cells positive for the antibody cocktail. The cell solution was incubated in the fridge at 4 ºC for 15 minutes before adding 10 mL of MACS buffer and centrifuging at 300g for 10 minutes. Finally, the supernatant was aspirated completely and the cells were re-suspended in 500 µL of MACS buffer.

2.2.2.2. Depletion with LD Column

Once the PBMCs were labelled with the NKT antibody cocktail, the non-$CD3^+CD56^+$ cells were immunomagnetically isolated. The LD magnetic Column was placed on the MACS separator and rinsed with 2mL of MACS buffer. The cell suspension was then applied down the side of the column, waiting for the column to empty before being washed twice with 3 mL MACS buffer. The column retained all of the non-$CD3^+CD56^+$ cells, leaving unlabelled pre-enriched $CD3^+CD56^+$ NKT cells in the collection tube.

2.2.2.3. Magnetic labelling of CD3+CD56+ NKT cells

The pre-enriched $CD3^+CD56^+$ NKT cells were then directly labelled with CD56 microbeads to remove any cells negative for CD56, resulting in a pure population of NKT cells. Firstly, the cell suspension was centrifuged at 300g for 10 minutes, ↑3↓3. The supernatant was aspirated completely and re-suspended in 400 µL of MACS buffer before adding 100 µL of CD56 Microbeads and mixing. The cell solution was incubated in the fridge at 4 ºC for 15 minutes before 20mL of MACS buffer was added to the tube and centrifuged at 300g for 10 minutes, to remove any unbound beads. The supernatant was aspirated

completely by tipping off and re-suspended in 500 µL of MACS buffer, ready to be applied to the magnetic column.

2.2.2.4. Positive selection with MS column

Non-CD56 cells could then be removed by positive selection of $CD56^+$ cells. An MS column was placed on the MACS separator and rinsed with 500 µL of MACS buffer before applying the cell suspension to the column. The column was subsequently washed twice with 500 µL of MACS buffer, waiting for the column to empty before each wash. The column retains the NKT cells labelled with the CD56 microbeads, thus, the column was removed from the magnet and 1 mL of MACS buffer was pipetted into the column. Its contents was immediately plunged into a sterile tube. This tube contains the magnetically labelled $CD3^+$ $CD56^+$ NKT cells.

Figure 2.2 illustrates the overall process of NKT cell isolation.

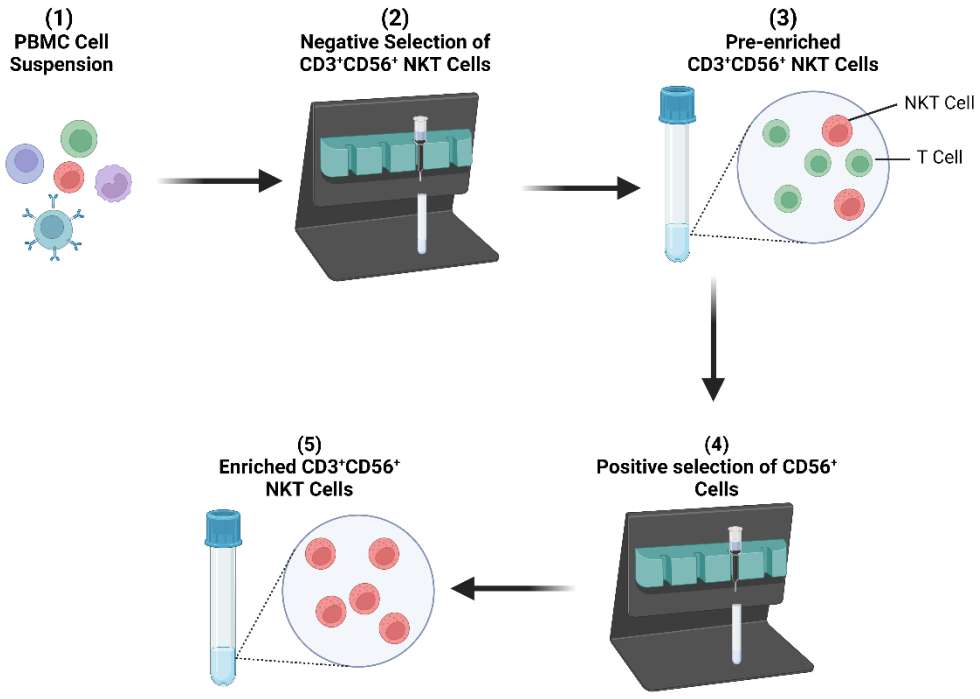


Figure 2.2. NKT cell immunomagnetic separation. (1) Human PBMCs were magnetically labelled with CD3+CD56+ Biotin-Antibody cocktail and Anti-Biotin Microbeads to indirectly label non-CD3+CD56+ cells. **(2)** The cells were applied to an LD column resulting in non-

CD3+CD56+ cells retained in the column, and a **(3)** flow-through fraction of pre-enriched CD3+CD56+ NKT cells. **(4)** This pre-enriched fraction was then directly magnetically labelled with CD56 Microbeads and applied to an MS column. **(5)** The column retained the labelled CD3+CD56+ NKT cells, which was then eluted. (Created using BioRender).

2.2.3. Invariant NKT Cell Expansion

Due to low cell numbers and poor viability after isolating NKT cells by immunomagnetic separation, a new method was developed. Invariant NKT cells were kept within the PBMC suspension and expanded with the glycolipid, α-GalCer, to increase cell numbers. Specifically, PBMCs were isolated from 50 mL human blood samples (as above) and cultured at $1x10^6$ cells/mL in RPMI supplemented with 10% human AB serum, in addition to 50 U/mL IL-2 and 100 ng/mL α-GalCer to stimulate iNKT cell expansion. α-GalCer was challenging to prepare, due to its lack of solubility in organic solutions. To solubilise the α-GalCer, DMSO was added to the α-GalCer to result in a 1mg/mL concentration. The solution was then heated to 80 °C for 3 minutes before sonicating at 25 kHz for 2 hours. Further dilutions were made using sterile PBS and stored at -20 °C before use. 0.1% DMSO was then used as a control during iNKT expansion to ensure the iNKT cell proliferation was due to α-GalCer, not the DMSO. The cells were then incubated at 37°C for up to 14 days in a 24-well flat-bottomed tissue-culture plate. This was later optimised to add the supplementation of 25 U/mL IL-2 every 4 days to maintain viability (**Fig. 2.3**).

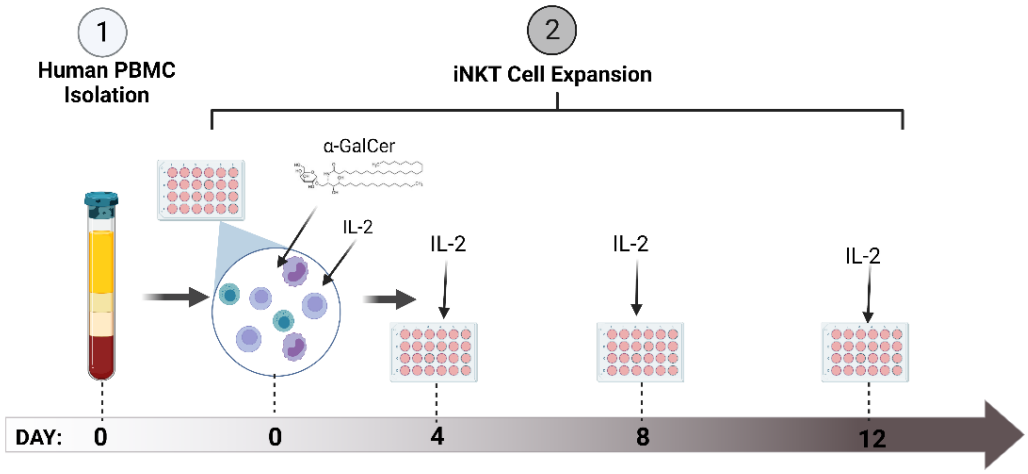


Figure 2.3. iNKT Cell Expansion. (1) Human PBMCs were isolated using SepMate tubes and density gradient centrifugation. **(2)** PBMCs were then cultured in the presence of the potent iNKT cell activator, α-GalCer, and IL-2 to maintain viability. The PBMCs were cultured at 37 °C for up to 14 days to allow iNKT cell expansion, re-stimulating with IL-2 every 4 days. (Created using BioRender).

To measure and characterise iNKT cells, PBMCs were stained for anti-CD1d-GalCer Tetramer, anti-CD3, anti-CD4, and anti-CD8, and analysed by flow cytometry. Anti-CD19 was used to exclude B cells from the analysis, as B cells can also bind to the CD1d-GalCer Tetramer. A blank-loaded tetramer (Proimmune, Oxford, UK) was used as a negative control. The CD1d-GalCer Tetramer works by binding to the invariant TCR on iNKT cells. All iNKT cells recognise α-GalCer-CD1d complexes, so this fluorescent tetramer can identify all iNKT cells, independent of whether the iNKT cells have been exposed to any other lipid stimuli.

2.2.4. Invariant NKT Cell Immunomagnetic Isolation

Expanded iNKT cells were immunomagnetically isolated for eventual co-culture with DCs. Before iNKT cell isolation, an MS MACS column, MACS separator and MACS buffer were placed in the fridge to keep cool. The iNKT cells were stained with 0.5 µL α-GalCer-loaded CD1d Tetramer R-PE per million cells for 30 minutes at 4°C. The cells were then mixed with 2 mL of MACS buffer

per 10 million cells and centrifuged at 300g for 10 minutes ↑3↓3. The supernatant was aspirated completely before tapping the tube to resuspend the pellet. The cells were then incubated with 20 µL of ultrapure anti-PE Microbeads per 10 million cells, for 15 minutes at 4°C to bind to any Tetramer PE-labelled iNKT cells. Again, the cells were then mixed with 2 mL of MACS buffer per 10 million cells and centrifuged at 300g for 10 minutes ↑3↓3. The iNKT cells should then be labelled with the magnetic beads.

Thus, the cell solution can then be positively selected by applying the cell solution to a MACS MS column. The MACS column was placed inside the MACS separator and magnetically attached to the stand. The column was rinsed prior to cell application with 500 µL of MACS buffer and then rinsed three times after the cell solution was applied by washing the column three times with 500 µL of MACS buffer. The column was rinsed only after the solution had completely emptied each time. The column then retains the labelled iNKT cells, so the column is finally removed from the magnet and 1mL of MACS buffer is added. Its contents is rapidly plunged into a sterile tube to collect the iNKT cells.

2.2.5. CD14+ Monocyte Isolation

PBMCs were firstly isolated from 50mL of venous blood (as above). Before monocyte isolation, an LS MACS column, MACS separator and MACS buffer were placed in the fridge to keep cool. PBMCs were placed in RPMI + 10% human AB serum and centrifuged at 300g for 5 minutes, ↑3↓3. The pellet was re-suspended in 80 µL MACS buffer and 20 µL of Ultrapure CD14 microbeads per 10 million cells, and incubated at 4°C for 15 minutes. The cells were then mixed with 20 mL of MACS buffer and centrifuged at 300g for 10 minutes. The supernatant was aspirated completely and the pellet was then re-suspended in 500 µL MACS buffer ready to be applied to an LS column. The column was first rinsed with 3mL of MACS buffer and the cell solution added down the side of the column. When the column had emptied, it was washed 3 times by adding 3mL of MACS buffer to the column, waiting for the solution to drip through entirely before adding the next 3mL of buffer. The column was

then removed from the magnetic separator. The column could then be eluted with 5mL of MACS buffer into a sterile falcon tube, containing the $CD14^+$ monocyte fraction.

2.2.6. DC Generation

Once $CD14^+$ monocytes had been isolated, the monocytes could be stimulated to generate immature DCs. See **Figure 2.4** for a schematic representation of DC isolation and generation. Firstly, 20 mL of RPMI + 10% human AB serum was added to the $CD14^+$ fraction to cushion the cells and then centrifuged at 300g for 5 minutes, ↑3↓3. The supernatant was aspirated completely by tipping off and then the pellet tapped to re-suspend. A cell count was conducted (as above) and the cells were re-suspended at $5x10^5$ cells/mL in RPMI + 10% human AB serum and added to wells of a 24 well flat-bottomed plate. 50 ng/mL GM-CSF and 20 ng/mL of human IL-4 was added to each well to stimulate the monocytes. The plate was placed in a 37°C CO_2 incubator for 3 days. On Day 3, 400µL of medium was removed from each well and subsequently replaced with 400µL fresh medium and supplemented with cytokines to achieve the same levels of IL-4 and GM-CSF as before. The cells were then returned to the incubator for a further 2 days, resulting in iDC generation. To ensure iDCs had been generated, some cells from wells were removed and extracellularly stained for markers: CD1d, CD14, CD40, CD83, CD80, CD86, HLA-DR, and CD209 (DC-Sign).

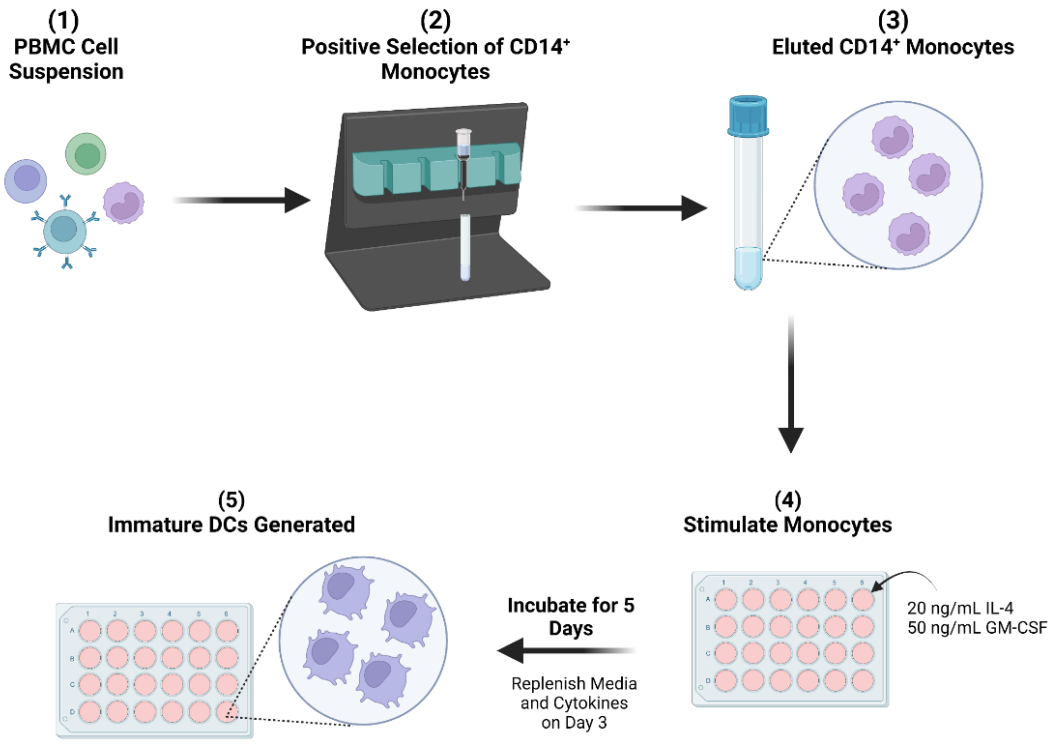


Figure 2.4. Isolation and Generation of Dendritic cells. (1) PBMCs were isolated from human peripheral blood and incubated with CD14 Microbeads before **(2)** $CD14^+$ cells were then positively isolated using immunomagnetic separation. **(3)** This $CD14^+$ positive fraction was eluted from the magnetic column and **(4)** stimulated with IL-4 and GM-CSF for 5 days to **(5)** generate immature DCs, replenishing the media and cytokines after 3 days of incubation. (Created using BioRender).

2.2.7. Stimulation of DCs with Lipids and Allergens

Once immature DCs had been generated, they could be stimulated with the lipid, α-GalCer, as a positive control, to determine the phenotype and any maturation of lipid-pulsed DCs. iDCs were stimulated with 100ng/mL of α-GalCer (reconstituted in DMSO) and replaced in the incubator for a further 24 hours to allow DC internalisation of the glycolipid. iDCs were also stimulated with 0.1 µL DMSO as a negative control. This volume was used as the α-GalCer is re-constituted in 0.1% DMSO. Thus, 0.1% (0.1µL) of DMSO was added to control wells. For peanut allergy experiments, the iDCs were also stimulated with 10 µg/mL of the peanut allergen Ara h 8 for 24 hours. To assess the role

of peanut lipids in allergic sensitisation, 1mg/mL of commercially sought peanut oil was used to stimulate DCs for 24 hours at 37°C. iDCs were also stimulated with both 10 μg/mL Ara h 8 and 1 mg/mL peanut oil for 24 hours to examine iNKT cell cytokine production when allergen and lipid are co-delivered.

2.2.8. iNKT-DC Co-culture

Once iNKT cells had been successfully expanded and isolated, and DCs were successfully isolated and iDCs generated, the two cell types could be co-cultured and cytokine production examined. **Figure 2.5** summaries the developed method leading to this DC:iNKT cell co-culture.

Specifically, both isolated iNKT cells and the stimulated DCs were counted and centrifuged with 10 mL of RPMI + 2% human AB serum for 5 minutes at 300g, ↑3↓3. The supernatants were aspirated completely by tipping off, and the cell pellets re-suspended. The iNKT cells were then added to a 96-well U-bottomed tissue-culture plate with either autologous α-GalCer-, DMSO-, peanut oil-, Ara h 8-, or both peanut oil and Ara h 8-pulsed DCs, at a ratio of 1:2 (DC:iNKTs). Due to the variability in cell numbers between subjects, the number of cells in each well differed between subjects, but the ratio of 1:2 was consistent, and the concentration of two cells together aimed for was 5 x 10^6 cells/mL. RPMI supplemented with 10% human AB serum was added to each well and mixed to result in a volume of 200 μL per well. At this point, 1X protein transport inhibitor cocktail was mixed in each well to allow cytokines to accumulate in the cell, ready for intracellular staining. The plate was then incubated for 5 hours at 37°C. Cells were then removed and stained for a 25-colour flow panel (**Table 2.1** in materials) to primarily examine cytokine production.

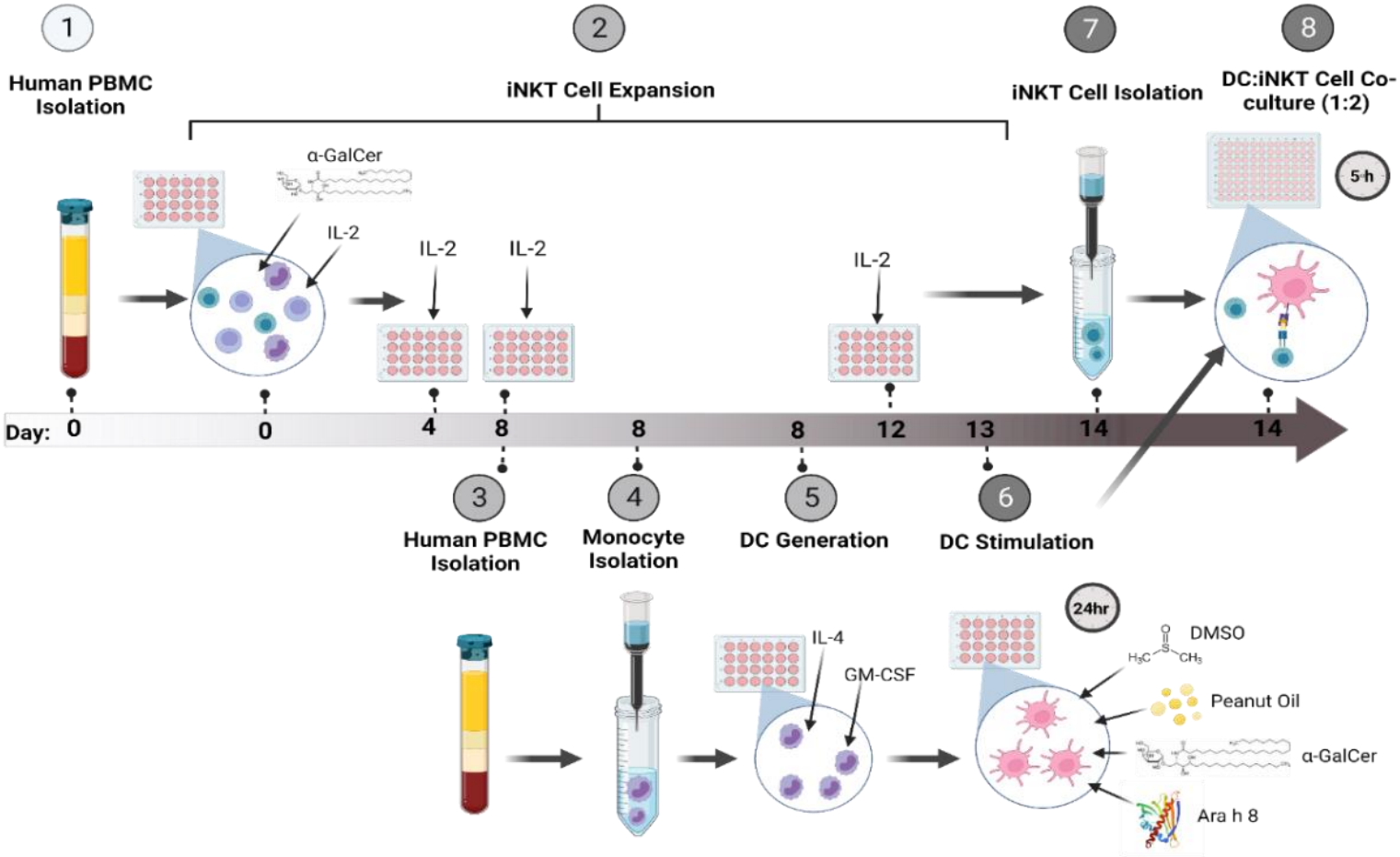

1
Human PBMC Isolation
2
iNKT Cell Expansion
7
iNKT Cell Isolation
8
DC:iNKT Cell Co-culture (1:2)
α-GalCer
IL-2
IL-2
IL-2
IL-2
5 h
Day: 0 0 4 8 8 8 12 13 14 14
3
Human PBMC Isolation
4
Monocyte Isolation
5
DC Generation
6
DC Stimulation
IL-4
GM-CSF
24hr
DMSO
Peanut Oil
α-GalCer
Ara h 8

Figure 2.5. A method for In vitro expansion of iNKT cells and subsequent co-culture with lipid-pulsed DCs. (1) PBMCs were isolated from whole human blood and **(2)** stimulated with the glycolipid, a-GalCer, and IL-2 to induce iNKT cell expansion. The cells were incubated for up to 14 days, **(3)** re-stimulating the culture with IL-2 every 4 days. **(4)** At Day 8 of iNKT expansion, blood was obtained from the same donor and PBMCs were isolated. **(5)** Monocytes were isolated using CD14+ immunomagnetic isolation. The monocytes were incubated with GM-CSF and IL-4 for 5 days to generate immature DCs (iDCs). **(6)** Once iDCs were generated, the DCs were pulsed with either α-GalCer, DMSO, Peanut Oil, Ara h 8, or both Peanut Oil and Ara h 8, and incubated for 24 hours to allow DC uptake of the lipids and/or allergens. **(7)** At Day 14 of iNKT cell expansion, iNKT cells were isolated from the PBMC culture by tagging the iNKT cells with a CD1d/a-GalCer tetramer, conjugated to a PE fluorophore. The iNKT cells could then be isolated by using anti-PE immunomagnetic isolation. **(8)** The isolated iNKT cells and the a-GalCer-pulsed DCs were then co-cultured together at a ratio of 1:2 (DC:iNKT) for up to 24 hours. Th1 and Th2 cytokine release was then measured and analysed by flow cytometry. (Created using BioRender).

2.2.9. Flow Cytometry

Cells were removed from culture and centrifuged before staining with fluorophore-conjugated antibodies. Zombie NIR dye was used to stain dead cells and exclude from analysis, unless otherwise stated. All antibodies were added at the manufacturer's recommended concentration. All analyses were performed using Kaluza or IDEAS software.

2.2.9.1. Extracellular Staining

Cells were harvested into FACS tubes, 1 mL of PBA added and then centrifuged at 300g, for 5 minutes. The supernatant was removed completely and the pellet was tapped to re-suspend. The appropriate antibodies were then added to the tube and incubated at 4ºC for 30 minutes. Then, 2mL of PBA was added and centrifuged for 5 minutes at 300g. The supernatant is completely removed and cell pellet is re-suspended. Finally, 200µL of fixation buffer (4% formaldehyde fix in 1X PBS) is added, and the samples were analysed by flow cytometry or stained for intracellular markers (see below). Kaluza software was used to interpret results.

2.2.9.2. Intracellular Cell Staining

Cytokines were stained for at 0-5 h of co-culture. Cells were cultured with (1X) protein transport inhibitor cocktail (ThermoFisher Scientific, UK) for 5 h at 37°C prior to harvesting. After staining surface markers (as above), the

cells were fixed with 500 µL of fixation buffer for 30 minutes or overnight at 4 °C. The cells were then centrifuged for 5 minutes at 300g (↑ 9, ↓ 9). The supernatant was aspirated and the tube was tapped to re-suspend. 2 mL of 1X permeabilisation buffer was added to the cells and incubated for 30 minutes at 4 °C. Again, the cells were centrifuged for 5 minutes at 300g (↑ 9, ↓ 9), before aspirating the supernatant completely and tapping to re-suspend. Fluorophore-conjugated antibodies IL-4, IFN-γ, IL-10, IL-5 and IL-12 were then added to the tube to stain the cells for 30 minutes at room temperature, in the dark. Finally, the cells were centrifuged for 5 minutes at 300g (↑ 9, ↓ 9), before aspirating the supernatant completely and tapping to re-suspend. The cells were then fixed with 200µL of fixation buffer and stored at 4 °C before they were analysed by flow cytometry. These cytokines, along with Foxp3, were also stained for at Day 0 and Day 14 of iNKT cell expansion, following the same procedure above.

2.2.9.3. Annexin V and PI staining

Annexin V and Propidium Iodide (PI) staining was used in early optimisation experiments to determine the viability of cells. 10x Annexin Binding Buffer was diluted to 1x using deionised water. The PBMCs were washed once in 1mL PBA and then the supernatant was aspirated. The cells were then washed once in 1mL Annexin binding buffer which is used to promote the binding of Annexin V conjugates. The supernatant was aspirated, and cells re-suspended in 50 µL of Annexin binding buffer. 5 µL of Annexin V (FITC) was added to each FACS tube and allowed to bind for 15 minutes at room temperature, in the dark. PBMCs were then centrifuged at 300g for 5 minutes with 1mL of binding buffer and then the supernatant was aspirated. Finally, the cells were re-suspended in 200 µL binding buffer and 0.33 µL of 1mg/mL PI staining solution. Samples were then analysed immediately.

2.2.10. IgE ELISAs

2.2.10.1. Total IgE ELISA

Healthy and allergic subject's plasma was isolated from peripheral blood samples and total IgE levels were measured by ELISA (ThermoFisher Scientific, UK), performed according to manufacturer's instructions.

Briefly, wells of a 96-well plate were coated with 100 µL of capture anti-human IgE, diluted 1:250 in coating buffer (1X PBS). The plate was sealed with aluminium tape and incubated at 4°C overnight. The plates were then washed twice with wash buffer (PBS-0.05% Tween-20), soaking for 1 minute between washes, and blocked with 250 µL of blocking buffer (PBS with 1% Tween-20 and 10% BSA) overnight at 4°C. After incubation, the blocking buffer was aspirated and the plate washed twice. Then, 100 µL of serially diluted human IgE standard was added to each well in duplicate, to produce a standard curve. Plasma samples were stored at 1:2 dilution in PBS, so samples were thawed and diluted 1:5 in Assay buffer (PBS with 1% Tween™ 20, 10% BSA) to result in a final 1:10 dilution. 100 µL of diluted sample was added to each well in duplicate, and assay buffer alone was used as blank. The plate was sealed and incubated at room temperate for 2 hours on a microplate shaker. After washing four times, 100 µL of detection buffer (HRP-conjugated anti-human IgE monoclonal antibody) was added to all wells and the plate was sealed again for 1 hour at room temperate, on a shaker. After washing four times, 100 µL of tetramethylbenzidine (TMB) substrate solution was added to all wells and incubated for 15 minutes at room temperature, in the dark. Finally, 100 µL of 1M sulphuric acid was added to stop the reaction, and the plate was read at 450 nm using a GloMax Discover Microplate Reader (Promega, US). A graph was then plotted showing the absorbance against the concentration of the standards. The unknown values of total IgE antibodies from healthy and allergic subjects were interpolated from the standard curve using a polynomial curve fit (GraphPad Prism 9.4.1 software).

2.2.10.2. Allergen-Specific IgE ELISA

In addition to total IgE quantification, allergen-specific IgE ELISAs were also conducted. Ara h 2 and Ara h 8-specfic IgE were quantified to verify the allergic status of the peanut-allergic subjects.

Based on previous work in the Fairclough Lab, 4 µg/mL of allergen was used to coat ELISA plates for allergen-specific IgE ELISAs. Thus, 4 µg/mL Ara h 2 and Ara h 8 (diluted in 1X PBS) were added to wells from two separate flat-bottomed 96 well plates and incubated overnight at room temperature. The plate was then washed three times with wash buffer (PBS-0.05% Tween-20) and blocked with 300µl of PBS with 1% Tween-20 and 10% BSA, for 1 hour. The plate was then washed 3 times before plasma samples diluted 1:5 were added to wells. A 1:5 dilution was chosen after trialling 1:2, 1:5, and 1:10 sample dilutions with peanut allergic and non-allergic plasma samples, with a 1:5 sample dilution giving the greatest absorbance. The plate was incubated for 2 hours at room temperature and then washed 3 times. 100 µL of detection buffer (HRP-conjugated anti-human IgE monoclonal antibody) was added to all wells and the plate was sealed again for 1 hour at room temperate, on a shaker. After washing four times, 100 µL of tetramethylbenzidine (TMB) substrate solution was added to all wells and incubated for 15 minutes at room temperature, in the dark. Finally, 100 µL of 1M sulphuric acid was added to stop the reaction, and the plate was read at 450 nm using a GloMax Discover Microplate Reader. The OD values for each sample were plotted on a graph where high absorbance indicates high allergen-specific IgE levels. (GraphPad Prism 9.4.1 software).

2.2.11. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 9.4.1. p-values <0.05 were considered significant for experiments. All data was analysed for normal distribution using the Kolmogorov–Smirnov test, before choosing a non-parametric or parametric statistical test. The majority of the tests used were Two-way ANOVA's and mixed effects analysis, involving Tukey's multiple comparison tests. Also, Pearson's R tests were conducted for correlations, with

unpaired t-tests to compare between the two variables. The tests used for specific data are detailed throughout the results sections.

Chapter 3: iNKT Cell Isolation, Expansion, and Characterisation

3.1. Introduction

Natural killer T cell are found in most areas of the body; peripheral blood, spleen, liver, thymus, bone marrow, and lymph nodes (Wu and Van Kaer 2011). NKT cells are phenotypically and functionally similar to natural killer (NK), such as the expression of CD56 cell surface marker and the secretion of IFN-γ (Krijgsman et al. 2019). NKT cells are both innate and adaptive immune cells due to their rapid response to stimuli (~2 hours) and the expression of a TCR which has undergone somatic rearrangement (Salio et al. 2014). There are two subsets of NKT cells, type 1 (invariant NKT cells) and type 2 (variant) NKT cells. Most iNKT cells express a TCRα chain (Vα24-Jα18 in humans) paired to a restricted set of TCRβ chains (Vβ11 in humans), and are powerfully activated by the glycolipid, α-GalCer. In comparison, variant NKT cells have a diverse, lesser-defined TCR repertoire, recognising non-α-GalCer lipids (Krijgsman, Hokland, and Kuppen 2018). Invariant NKT cells are the most-researched subtype, with known roles in autoimmune diseases, infectious diseases, cancer, and allergy (Exley, Dellabona, and Casorati 2021; Ahmadi et al. 2022). Specifically, iNKT cells are of interest to this research because they can recognise lipids from allergen sources. iNKT cells recognise glycolipids presented by a non-polymorphic MHC-I-like molecule, named CD1d (Beckman et al. 1994). Upon this recognition of a CD1d-lipid complex, iNKT cells can produce a variety of cytokines. These include Th17, Th2 and Th1-cell associated cytokines, but predominantly Th2 and Th1 cytokines IL-4 and IFN-γ, respectively. The type of cytokines released can be determined by the subset of iNKT cells, based on their expression of CD4 and/or CD8 cell surface markers (O'Reilly et al. 2011). The ability of iNKT cells to release cytokines in response to lipids from allergen sources has recently been reviewed (Hopkins et al. 2022), with the interaction of CD1d molecules on DCs deemed essential. It is the potent, rapid release of Th1 or Th2 cytokines in response to allergen-associated lipids which highlights iNKT cells as key immune regulatory cells in

diseases, including allergic sensitisation. In addition to cytokine secretion, the number of iNKT cells may be key in allergic sensitisation, as some studies suggest there are higher iNKT cell levels in asthma patients (Reynolds et al. 2009).

Very limited iNKT cell numbers are found in humans, comprising between 0.01% - 1% of the total lymphocytes in healthy human donors (Chan et al. 2013). In mice, they constitute approximately 1% of lymphocytes (Hammond et al. 2001), thus making them an easy model of iNKT cell study, but the relevance of mice models to human disease is disputed. It is thus not surprising that there is limited existing research in this area utilising human models (Hopkins et al. 2022).

It is well-established that allergen sources, such as peanuts, comprise not only proteins, but also other compounds, such as carbohydrates and lipids. Yet, the existing literature investigating lipids and iNKT cells in allergic sensitisation is limited, with even fewer studies utilising a human model of allergic sensitisation (Hopkins et al. 2022). Thus, this research aimed to develop a human model of lipids and iNKT cells in allergic sensitisation, replicating a key mechanism of iNKT cell activation: DC presentation of lipids to iNKT cells via CD1d molecules. In order to create a DC-iNKT cell co-culture model, iNKT cells must first be isolated from human PBMCs and characterised, and importantly, iNKT cell numbers measured.

Initial experiments presented here aimed to isolate general $CD3^+CD56^+$ NKT cells from human PBMCs and characterise the NKT cells. However, due to the limited number of NKT cells in human peripheral blood and lack of viability when cultured without the presence of other cells, this method needed to be optimised. Thus, subsequent experiments focussed on the NKT cell subtype, invariant NKT cells, as these cells can be stimulated with the potent iNKT cell activator, α-GalCer to induce iNKT cell expansion and subsequently increase iNKT cell numbers for eventual co-culture with DCs. Therefore, iNKT cell expansion was optimised, and the expanded iNKT cells were then immunomagnetically isolated and characterised by flow cytometry. These

steps were essential to ensure future co-cultures with lipid-stimulated DCs comprised a large yield of fully-characterised iNKT cells.

3.2. Materials and methods

3.2.1. NKT Cell Isolation and Characterisation

Full methods are found in section 2.2. Methods. Briefly, all experiments required human blood collection and the isolation of PBMCs by density gradient centrifugation (See 2.2.1.1. Peripheral Blood Mononuclear Cell (PBMC) Isolation). Initial NKT cell experiments aimed to isolate NKT cells from human PBMCs using magnetic labelling of $CD3^+CD56^+$ cells followed by negative selection of $CD3^+CD56^+$ cells and then positive selection of $CD56^+$ cells, leaving $CD3^+CD56^+$ NKT cells (See '2.2.2. NKT Cell Immunomagnetic Isolation' for more detail). The isolated NKT cells were then stained with fluorescent antibodies for flow cytometry (**Table 3.1**) to determine the purity of NKT cells throughout the isolation process, and identify any contaminating cells. To note, the concentration of antibody was not provided by Miltenyi, instead, the dilutions were provided.

To optimise the dilution of antibodies utilised for NKT isolation extracellular staining, titrations were performed. For antibodies diluted 1:50, CD3, CD4, CD8, CD14, CD19, CD25, CD56, and CD69, the manufacturer recommendation is 2µL of antibody per test. Thus, 1, 2, or 5 µL of diluted antibody was added to the cells. For antibodies diluted 1:11 (CD209), the manufacturer recommendation is 10 µL of antibody per test. Titrations of 5, 10 and 20 µL were performed. The titrations were performed according to the extracellular staining method detailed in Section 2.2.9.1. Extracellular Staining.

Table 3.1. NKT Cell Optimisation Antibodies. Antibodies utilised in NKT cell optimisation assays with their fluorophore and the company purchased from.

Antibody	Fluorophore	Company	Cat No.
CD3	PE Vio 770	Miltenyi	130-113-140
CD4	FITC	Miltenyi	130-113-791
CD8	PerCP Vio 700	Miltenyi	130-113-160
CD14	PerCP	Miltenyi	130-110-581
CD19	PE	Miltenyi	130-113-646
CD25	FITC	Miltenyi	130-113-283
CD56	APC	Miltenyi	130-100-698
CD69	PE	Miltenyi	130-112-613
CD209	APC	Miltenyi	130-109-649

Once NKT cells were successfully isolated from whole PBMCs, their viability in culture was assessed. This is essential as isolated NKT cells will be co-cultured with DCs isolated from the same blood sample. However, DCs have to be generated from isolated human monocytes for 6 days before NKT cell co-culture. Thus, initial experiments aimed to culture isolated NKT cells for 6 days while waiting for DCs to generate. NKT cells were cultured in RPMI + 10% human AB serum and viability was measured by flow cytometry, staining for Annexin V (apoptotic cells) and PI (necrotic cells) (See methods section 2.2.9.3.

Annexin V and PI staining for detail). Unstained controls were used to identify any background.

3.2.2. Invariant NKT Cell Expansion, Isolation and Characterisation

Initially, isolated human PBMCs were stimulated with 50 U/mL IL-2 and 100 ng/mL α-GalCer to stimulate iNKT cell expansion. 0.1% DMSO was used as a control, as the re-constituted α-GalCer contained 0.1% DMSO. The cells were then incubated at 37°C for up to 14 days. This method was later optimised to add the supplementation of 25 U/mL IL-2 every 4 days to maintain viability. See methods section 2.2.3. Invariant NKT Cell Expansion for more detail.

Expanded iNKT cells could then be immunomagnetically isolated for eventual co-culture with DCs, as detailed in methods section 2.2.4. Invariant NKT Cell Immunomagnetic Isolation. The iNKT cells were stained by flow cytometry with the α-GalCer-loaded CD1d Tetramer R-PE to label the iNKT cells. Anti-PE Microbeads were then added to the cell suspension before being added to a MACS column for immunomagnetic isolation.

To measure and characterise invariant NKT cells, PBMCs were stained for anti-CD1d-GalCer Tetramer (Proimmune, Oxford, UK), anti-CD3 (Cat no. 344828, Biolegend, UK), anti-CD4 (Cat no. 563550, BDbiosciences, UK), and anti-CD8 (Cat no. 130-110-682, Miltenyi, UK) and analysed by flow cytometry. Anti-CD19 (Cat no. 302236, Biolegend, UK) was used to exclude B cells from the analysis, as B cells can also bind to the CD1d-GalCer Tetramer. A blank-loaded CD1d tetramer (Cat no. D002-2A-G-2, Proimmune, UK) was used as a negative control for the GalCer-loaded tetramer to measure any non-specific binding to the tetramer. iNKT cell cytokine production was measured by staining for intracellular cytokines: IL-4 Brilliant Violet 605 (Cat no. 500828, Biolegend, UK), IFN-y Brilliant Violet 650 (Cat no. 502538, Biolegend, UK), IL-5 APC (Cat no. 130-117-205, Miltenyi, UK), and IL-10 PEDazzle594 (Cat no. 501426, Biolegend, UK). All antibodies were used according to the manufacturer's instructions.

3.3. Results

3.3.1. Optimisation of Antibody Volumes

This research project will be utilising flow cytometry to both phenotype cells and examine cellular function. It is thus important to confirm the antibody titration at the start of the study. As such, three volumes of antibody were tested on isolated PBMCs.

The antibodies optimised were chosen to characterise the cell types detailed in **Table 3.2**.

Table 3.2. Cell markers of Antibodies optimised. The cell-surface markers of antibodies optimised and the corresponding target cell to be identified.

Marker	**Target Cell**	**Marker**	**Target Cell**
CD3+CD56+	NKT cells	*CD19+*	B cells (B)
CD3-CD56+	NK cells (NK)	*CD14+*	Monocytes (Mo)
CD3-CD56-CD4+	Helper T cells (Th)	*CD209+*	Dendritic cells
CD3-CD56-CD8+	Cytotoxic T cells (Tc)		

Flow cytometry analysis of the antibody titrations using fresh PBMCs revealed that the manufacturer recommendation of 2 µL of antibody is optimal for six of the seven antibodies tested **(Figure 3.2b-h)** as there is little difference between fluorescence. However, for CD3 PE Vio 770 a 5 µl volume gives a stronger fluorescence than the recommended 2 µL **(Figure 3.2b).**

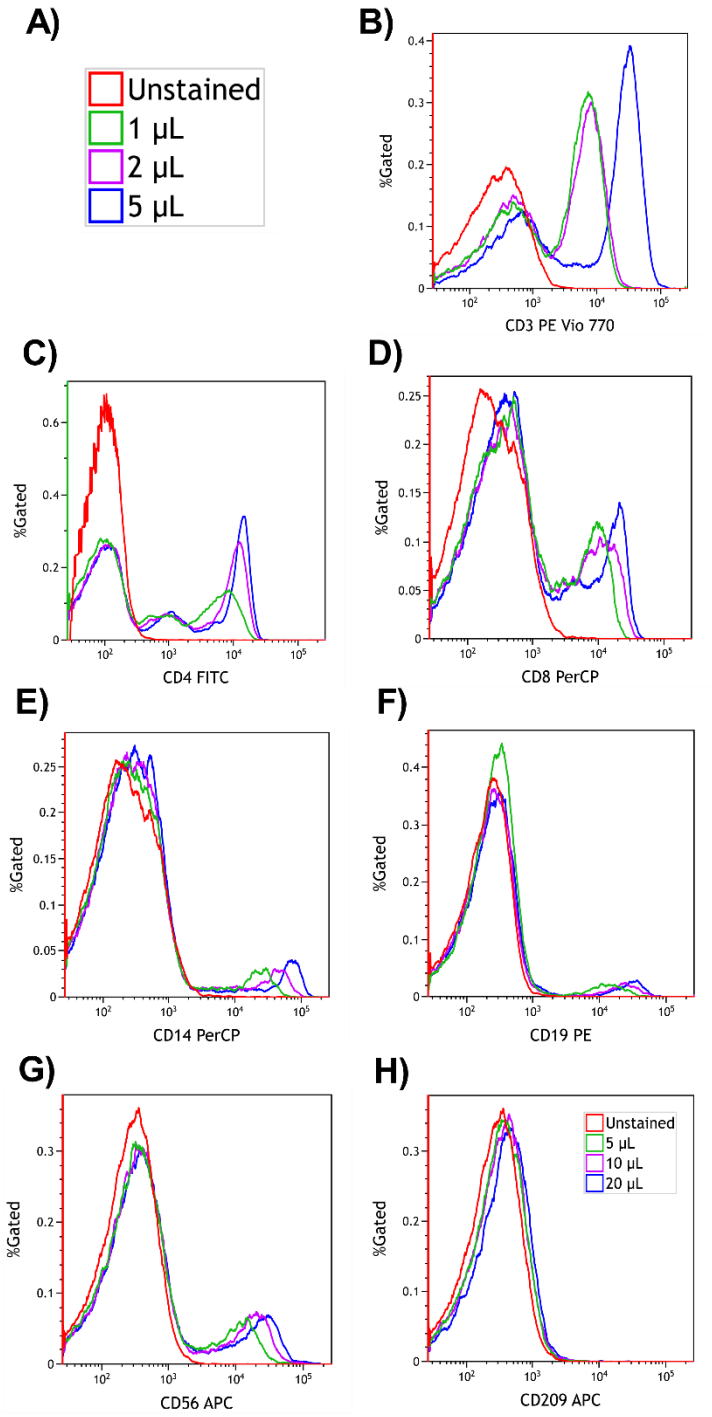


Figure 3.2. NKT Cell Antibody titrations. Antibody titrations were performed using fresh PBMCs to optimise the volume of antibody required for extracellular staining. (A) The legend for histograms B-G. 1, 2 and 5 µL titrations were performed for antibodies as follows - (B) CD3, (C) CD4, (D) CD8, (E) CD14, (F) CD19, and (G) CD56. 5, 10 and 20 µL titration was performed for CD209 APC in (**H**). Gating strategy: SSC/FSC dot plot to gate lymphocytes, then FSH/FSW dot plot to gate singlets.

This is reinforced by the calculation of stain indexes, presented in **Table 3.3**. The stain index is the ratio of the separation between the positive population and the negative population, divided by two times the standard deviation of the negative population. For all antibodies, the stain index increased with increased volume of antibody used. For CD3 PE Vio 770, the increase was most pronounced, as a stain index of 7.67 at 2 µL increased to 30.51 at 5 µL. This led to the use of 5 µL CD3 PE Vio 770 in the subsequent experiments. Despite higher stain indexes at the highest volume for the other antibodies too, these increases were not as pronounced, as seen in the histograms. Thus, considering costs, the manufacturer's recommendation was utilised in subsequent experiments.

Table 3.3. Stain Indexes for Antibody Dilutions. CD3, CD4, CD8, CD14, CD19, CD56, and CD209 antibody stain indexes were calculated to determine the optimal titration of antibody. This was calculated by the positive MFI minus the negative MFI, divided by two times the standard deviation of the negative population.

Antibody	Dilution (µL)	Stain Index	Antibody	Dilution (µL)	Stain Index
CD3 PE Vio 770	*1*	**6.90**	*CD19 PE*	*1*	**28.19**
	2	**7.67**		*2*	**49.51**
	5	**30.51**		*5*	**61.50**
CD4 FITC	*1*	**12.11**	*CD56 APC*	1	**17.36**
	2	**57.03**		*2*	**26.82**
	5	**73.59**		*5*	**33.66**
CD8 PerCP	*1*	**4.93**	*CD209 APC*	*5*	**0.040**
	2	**5.79**		*10*	**0.048**
	5	**8.29**		*20*	**0.072**
CD14 PerCP	*1*	**12.11**			
	2	**17.90**			
	5	**34.70**			

3.3.2. The Isolation of NKT cells from PBMCs

To enable the measurement of the effect of lipids on NKT cell function, it is important to be able to isolate a pure population of NKT cells from PBMCs. This could be done by flow cytometry, but this may have the disadvantage of activating the cells due to the antibodies used to select the cells of interest. Thus, this study tested immunomagnetic separation using both negative and positive selection so that there would be no activation of the cells, through binding to CD3 on the surface of the NKT cell.

Figure 3.3 shows exemplar flow cytometry density plots detailing the purity of NKT cells throughout the stages of NKT isolation, using CD3 and CD56 cell surface markers to identify NKT cells. In this sample, there is a starting NKT cell population of 8.65% (**Fig 3.3A**). Non-$CD3^+CD56^+$ cells are then depleted (**Fig 3.3B**), resulting in a pre-enriched NKT cell fraction containing 7.76% NKT cells (**Fig 3.3C**). Next, non-$CD56^+$ cells are depleted (**Fig 3.3D**), resulting in a final NKT cell purity of 90.08% (**Fig 3.3E**).

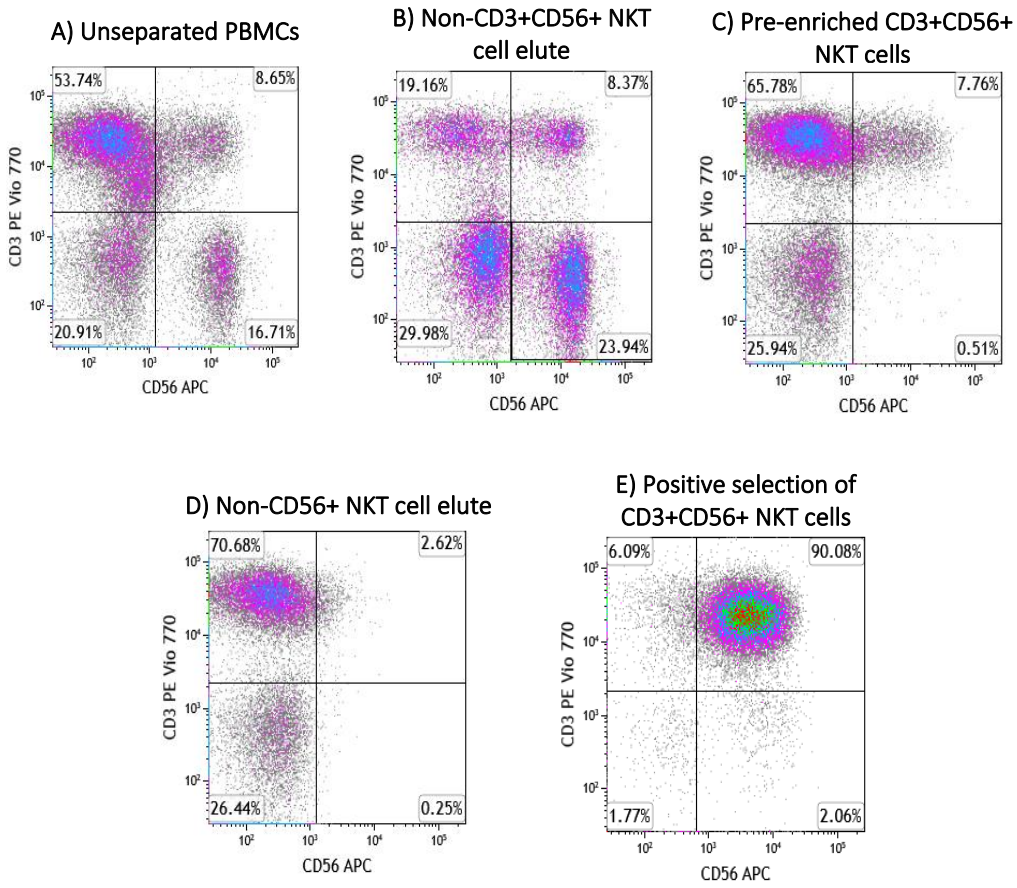


Figure 3.3. Representative Density Plots of NKT cell immunomagnetic separation. The density plots represent the purity of CD3+CD56+ NKT cells throughout the stages of NKT isolation. (A) illustrates a starting population of 8.65% NKT cells within PBMCs. (B) shows the percentage of NKT cells present in the non-CD3+CD56+ elute. (C) Displays the percentage of pre-enriched CD3+CD56+ NKT cells after isolation. (D) shows the percentage of NKT cells present in the non-CD56+ elute. Finally, (E) displays the final NKT population after CD56 positive isolation. N=1.

The NKT cell isolation protocol was repeated three times to measure the purity of NKT cells and examine activation status. The final average NKT cell purity across the three repeats was 83.95%, with most contamination from NK cells, T-helper cells (Th) and cytotoxic T cells (Tc) **(Fig. 3.4).** These graphs detail the number of each cell type present during the key stages of NKT isolation.

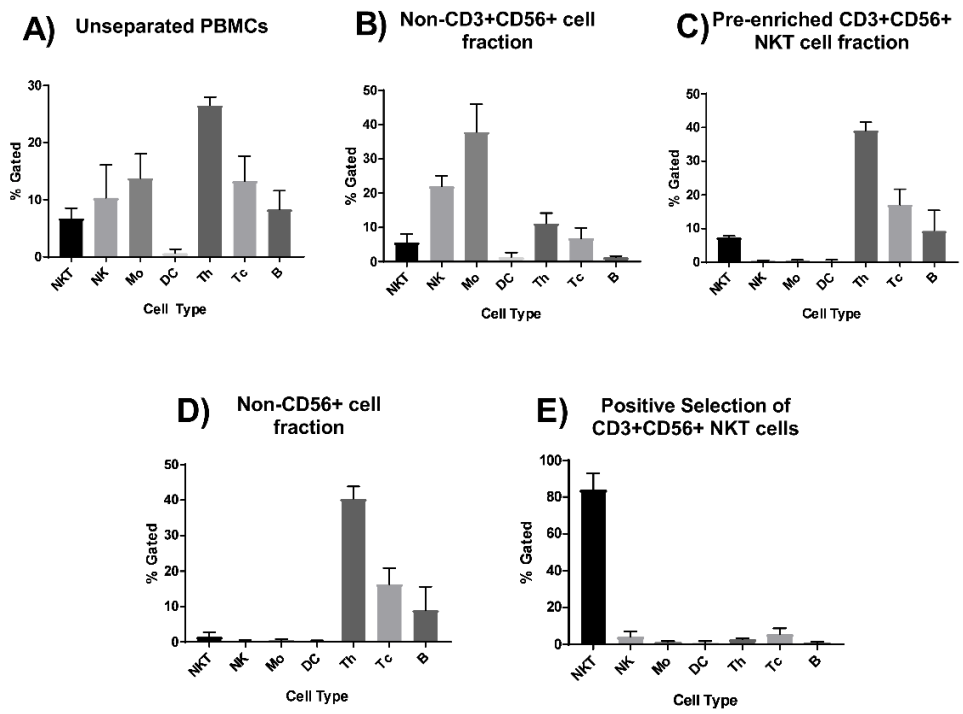


Figure 3.4. Mean and standard deviations of cell frequencies during NKT isolation. The graphs show mean cell frequencies across 3 NKT cell isolation data sets. It includes NKT cells, natural killer (NK) cells, monocytes (Mo), dendritic cells (DC), T-helper cells (Th), cytotoxic T cells (Tc), and B cells (B). All cells minus dead cells and debris were included in the analysis. (A) Mean cell frequencies initially present in PBMCs. (B) Mean cell frequencies in non-CD3+CD56+ cell fraction, after depletion of non-CD3+CD56+ cells. (C) Mean cell frequencies in the CD3+CD56+ fraction, after depletion of non-CD3+CD56+ cells. (D) Mean cell frequencies of non-CD56+ cells, after CD56+ positive selection. (E) Mean cell frequencies in CD56+ fraction, after CD56+ positive selection. N=3.

After NKT isolation, the activation of NKT cells throughout the process of immunomagnetic separation was assessed, with **Figure 3.5** showing representative flow plots. The non-CD56+ cell fraction stage was removed from these plots, as no NKT cells are present. A CD69 antibody to detect early activation of NKT cells, and a CD25 antibody to detect late activation of NKT cells were utilised (See Table 3.1 for antibody details). The plots show little/no activation of NKT cells throughout NKT cell isolation.

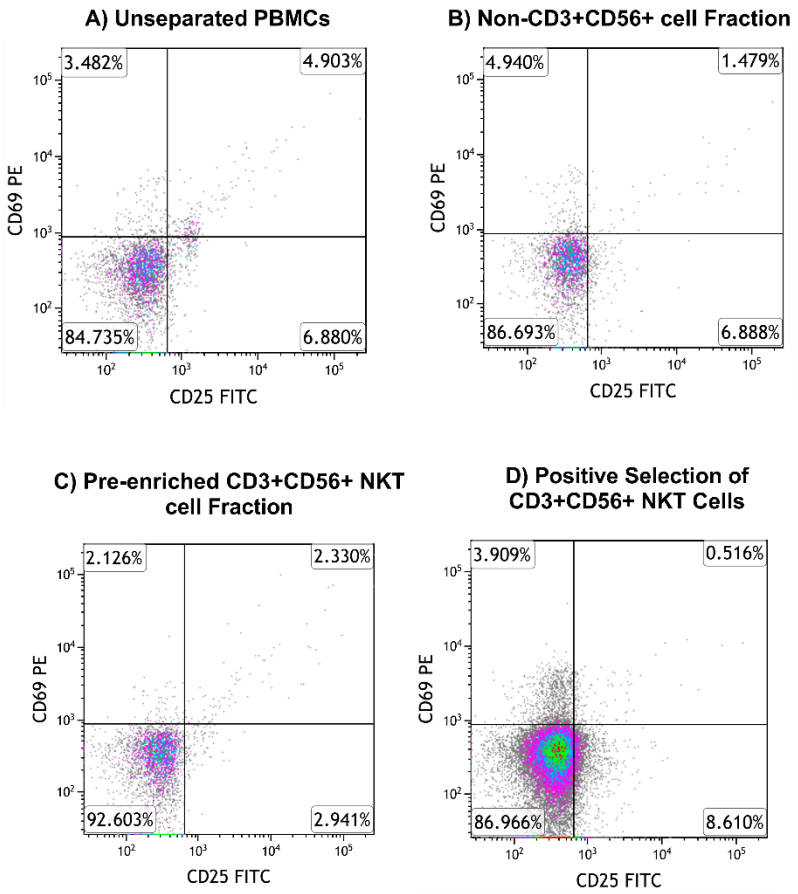


Figure 3.5. Activation of NKT cells during NKT cell isolation. CD3+CD56+ NKT cells were gated and analysed for any early activation (CD69+) or late activation (CD25+) throughout the stages of NKT isolation. Quadrants were set according to single colour controls. All dot plots show little/no activation over the course of NKT immunomagnetic separation. N=1.

This was replicated in 3 separate healthy donors (**Fig. 3.6**). The results are consistent with the representative dot plots, showing 89.23% (SD=6.96) of NKT cells were not activated before isolation. A two-way ANOVA revealed this was not significantly different from the end stage of NKT cell isolation, where 90.95% (SD=7.05, $p>0.05$) of NKT cells were not activated. This ensures the later co-culture experiments with DCs involves NKT cells that are inactivated before lipid exposure.

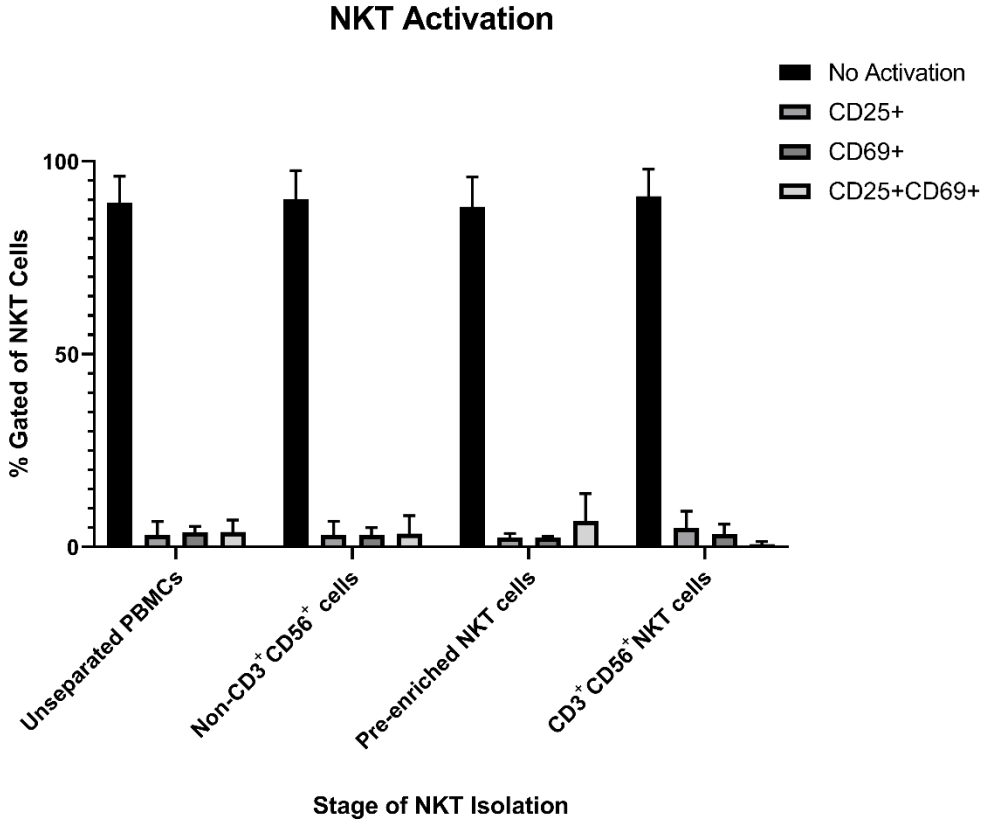


Figure 3.6. Average activation of NKT Cells during Isolation. CD3+CD56+ NKT cells were gated and analysed for any early activation (CD69+) or late activation (CD25+) throughout the stages of NKT isolation, across 3 healthy donors. Horizontal bars represent means, vertical bars represent standard deviation, N=3.

3.3.3. The Optimisation of NKT Cell Viability in Culture

Having identified that a pure population of NKT cells can be generated using immunomagnetic separation, and remain non-activated, the next step was to determine whether NKT cells could be maintained in culture for 6 days while DCs were being generated from human monocytes, to then be able to co-culture NKTs and DCs.

Initially, isolated NKTs were cultured in RPMI + 10% human AB serum. However, forward scatter/side scatter flow cytometry plots suggested the NKTs were dying within 24 hours and continued to die over 48 and 72 hours (**Figure 3.7**). This was identified by the NKT cell population decreasing in size, suggesting a decrease in viability.

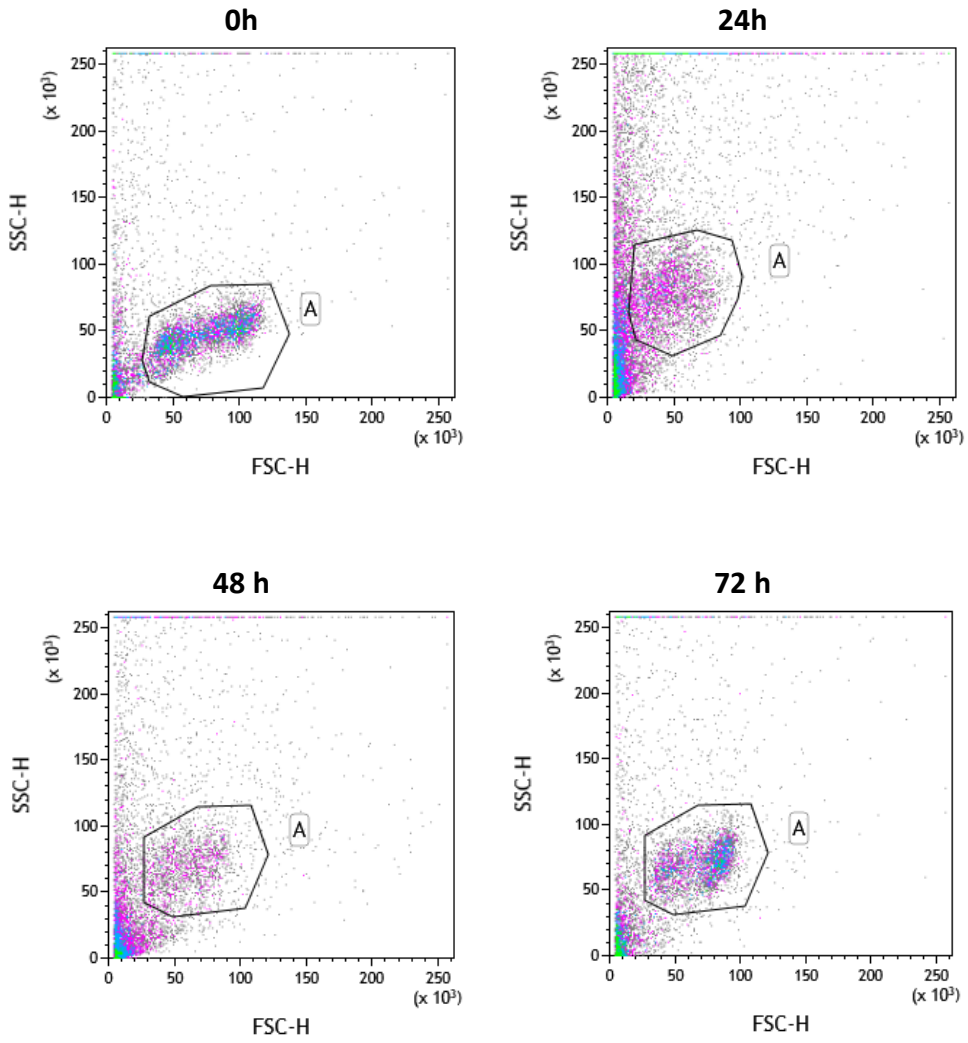


Figure 3.7. Viability of NKT cells over 72 hours (method 1). NKT cells were gated for using Forward scatter by side scatter. The NKT cell population at 24 hour intervals are shown. The dot plots show a shift in population to the left, suggesting the cells are dying. N=1.

Thus, the NKT cell culture method was altered to include the addition of 200 U/mL IL-2 to each well at 0hrs of culture to improve viability. Furthermore, Annexin V and PI staining was included to more accurately quantify the viability of the cells. After this second attempt, the Annexin V and PI staining showed the cells were still dying after 24hrs of culture (**Fig. 3.8**). A comparison of 0 hours and 24 hours show an increase in dead cells from 2.97% to 96.99%. Although, at 0 hours, 16.28% of cells were already in the early apoptosis stage.

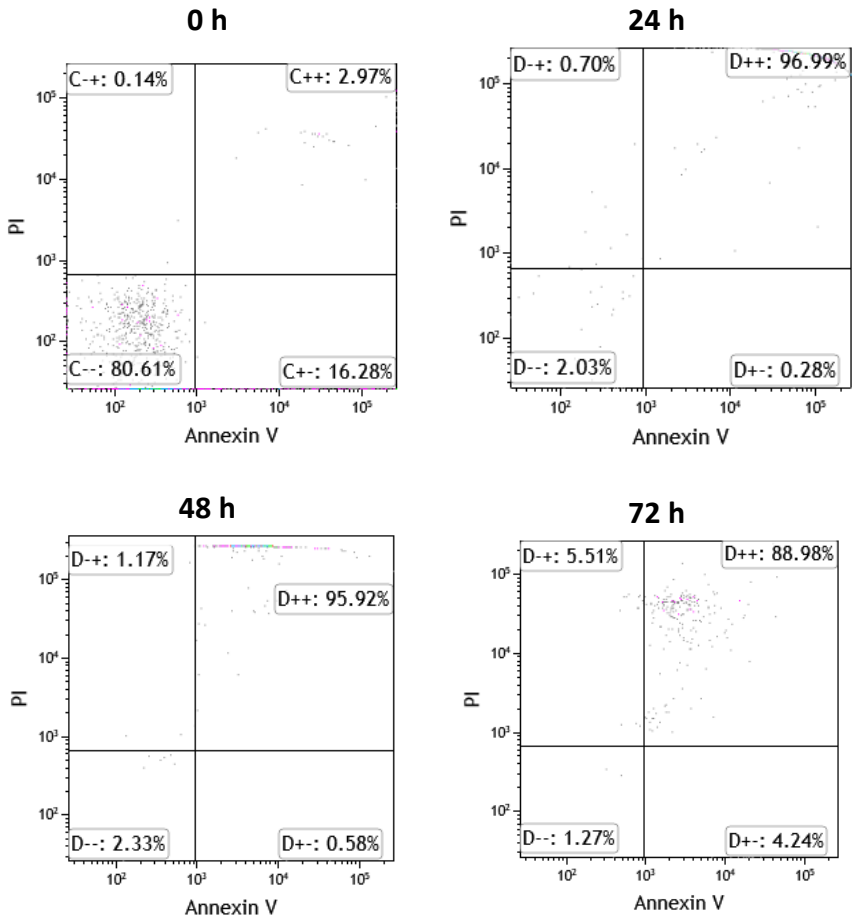


Figure 3.8. Viability of NKT cells over 72 hours (method 2). The dot plots presented show the viability of NKT cells over 72 hours, after the addition of IL-2 at 0 hours of culture. Annexin V and PI were stained for to determine cell viability. N=1.

This NKT cell culture method was repeated in 3 different donor samples (**Fig. 3.9**), which highlights the viability of NKT cells significantly decreased from a mean of 88.8% at 0 hours, to a mean of 15.5% after 24 hours of culture with RPMI +10% human AB serum and IL-2 ($p<0.001$, N=3).

Figure 3.9 suggests the cells were dying within the initial hours after NKT isolation. It was hypothesised this was a result of the lack of NKT cell numbers cultured, due to the low levels of NKT cells present in human peripheral blood. Thus, a new method was developed to increase NKT cell numbers and ultimately improve viability in culture.

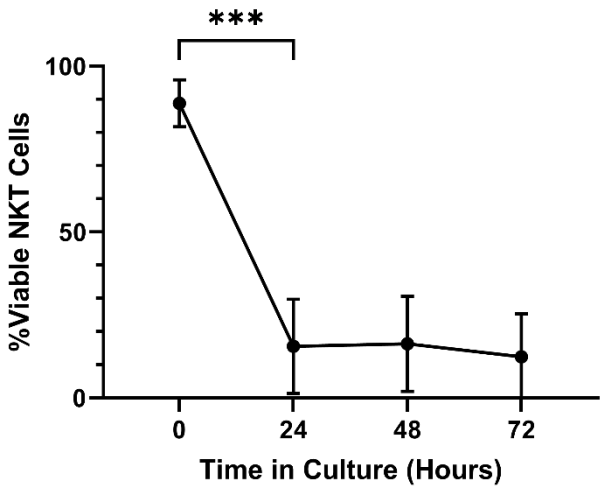


Figure 3.9. Viability of NKT Cells in Culture. Isolated NKT cells were cultured in RPMI + 10% human Ab serum and IL-2 for up to 72 hours. Annexin V and PI flow cytometry staining identified the percentage of viable cells at each 24-hour interval of culture. (***= p<0.001, vertical bars indicate standard deviation). N=3.

3.3.4. The Optimisation of Invariant NKT Cell Expansion

Due to low cell numbers and poor viability after isolating NKT cells immediately after PBMC isolation, a new method was developed to improve the yield and viability. Literature suggested the subtype of NKT cells, invariant NKT cells, can be expanded in culture utilising a potent activator of iNKT cells, α-GalCer (Schmid et al. 2018b) to improve the yield of iNKT cells. Thus, invariant NKT cells from non-allergic donors were stimulated with α-GalCer to increase cell numbers for subsequent co-culture with DCs. The gating strategy for identifying iNKT cells is presented in **Figure 3.10**, where cells were initially gated according to size (forward scatter) and granularity (side scatter) (Fig 3.10i), then single cells were selected (Fig 3.10ii), viable cells were gated (Fig 3.10iii), CD19 negative lymphocytes selected (Fig 3.10iv) and finally iNKT cells were gated for their expression of CD3 and the CD1d-aGalCer Tetramer (Fig 3.10v). Antibodies Vα24-Ja18 and Vβ11 were incorporated into the flow panel for an alternative method for identifying iNKT cells. However, as the tetramer was stained for 30 minutes before the addition of any other antibodies, we

realised the Vα24-Ja18 antibody could not bind to the iNKT cells and was thus not staining the cells. Thus, these iNKT markers could not be analysed by flow cytometry.

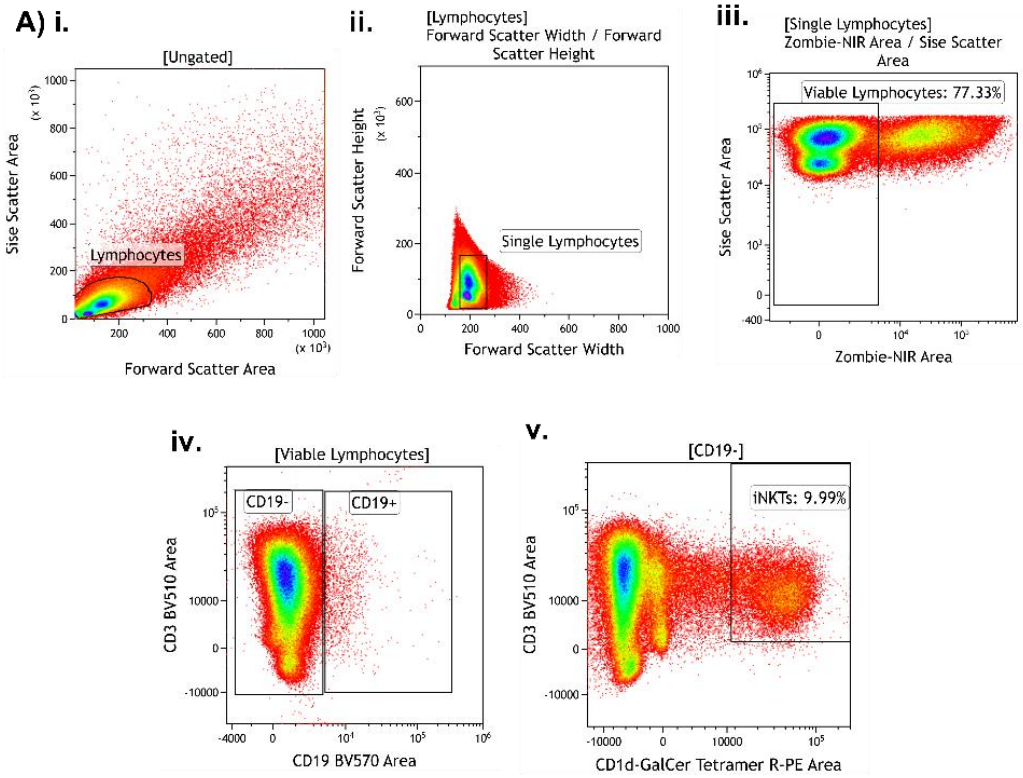


Figure 3.10. iNKT Cell gating strategy. (A) Gating strategy to measure iNKT cell cytokine release. (i) SSC/FSC dot plot to gate lymphocytes, (ii) FSH/FSW dot plot to gate singlets, (iii) Zombie NIR dot plot to gate on live lymphocytes, (iv) CD3/CD19 to exclude B cells from analysis, and (v) CD3/CD1d-GalCer Tetramer to gate on iNKT cells. The blank tetramer control is also gated using these steps, but plotting the blank tetramer against CD3 in part (v), using the same iNKT gate.

Based on previous literature, iNKT cells were stimulated with 50 U/mL IL-2 and 100ng/mL α-GalCer and cultured for 12 days to induce expansion (Brossay et al. 1998). Initially, the iNKT cell expansion experiments resulted in increases in the percentage of iNKT cells by day 12 of expansion (**Fig. 3.11a**). However, they lacked viability towards the end of the 12 day culture (**Fig. 3.11b**), resulting in a drop in the final iNKT cell population.

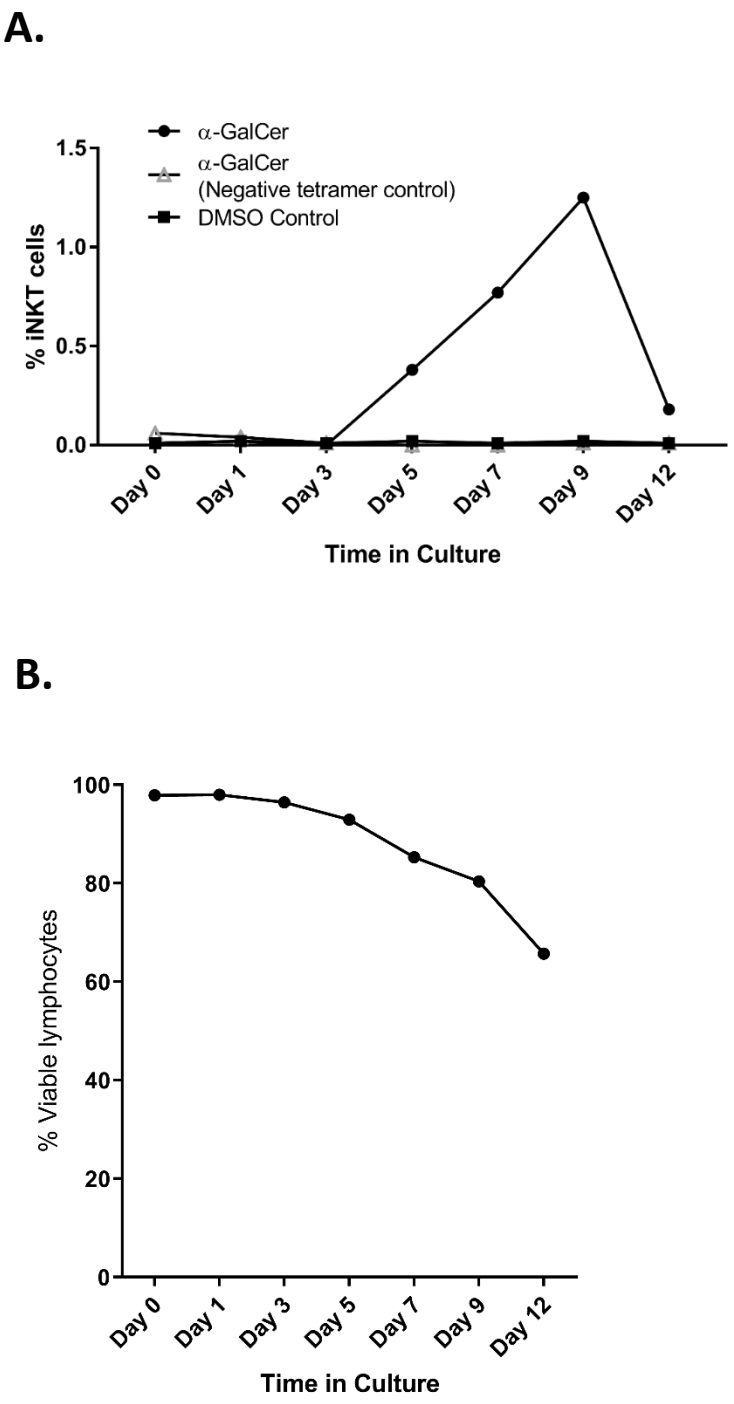


Figure 3.11. iNKT Cell Expansion and Viability. (A) The expansion of iNKT cells after stimulation with α-GalCer or the DMSO control, over 12 days of culture. A negative (blank) tetramer control was also stained for to determine any non-specific tetramer binding, which could give a false-positive number of iNKT cells. (B) The percentage of viable lymphocytes during iNKT cell expansion. N=1.

Thus, three different conditions were tested to optimise the viability and expansion of iNKT cells; the cells were stimulated with 50 U/mL IL-2 and 100 ng/mL α-GalCer on Day 0 of culture and then either no re-stimulation throughout culture, re-stimulation with 50 U/mL IL-2 every 4 days, or re-stimulation with 50 U/mL IL-2 and 100 ng/mL α-GalCer on Day 7 of culture (**Fig. 3.12**). The re-stimulation with IL-2 was based upon T cell expansion experiments often requiring IL-2 to improve proliferation (Ghaffari et al. 2021). The re-stimulation of α-GalCer on Day 7 of culture was tested based on this method utilised in existing literature (Schmid et al. 2018a). The culture was also increased from 12 to 14 days to allow a higher percentage of iNKT cells to develop, and because other studies tend to expand for longer than 12 days (Schmid et al. 2018a).

The results indicate stimulation with IL-2 every 4 days, resulted in the highest viability by Day 14 of culture, and maintained a high level of iNKT cells. No re-stimulation resulted in the lowest viability of lymphocytes. Thus, experiments going forward added IL-2 every 4 days to maintain viability of the iNKT cells.

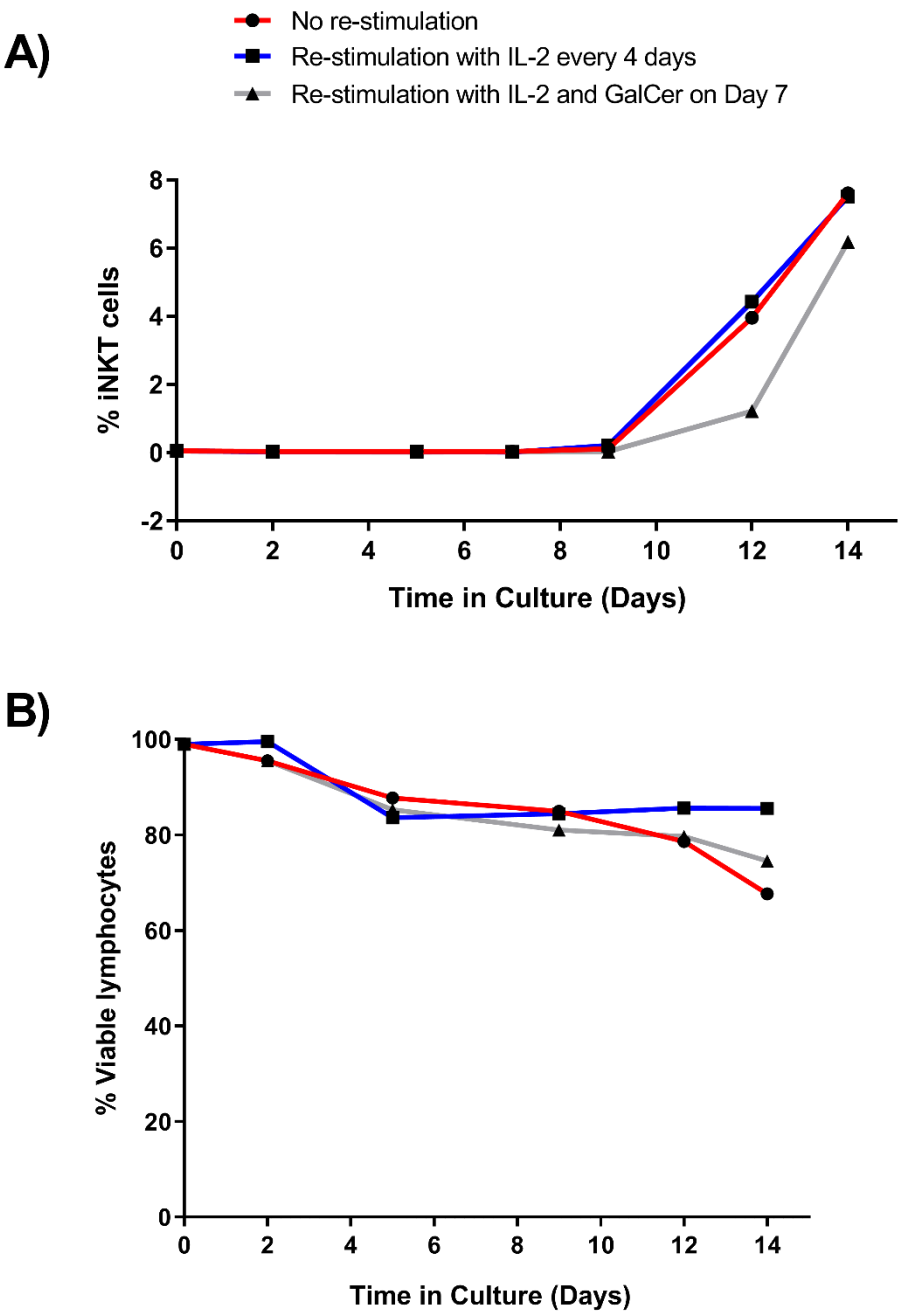


Figure 3.12. Optimisation of iNKT Cell Expansion. PBMCs were stimulated with IL-2 and α-GalCer on Day 0 of culture and then either (red) no re-stimulation throughout culture, (blue) re-stimulation with IL-2 every 4 days, or (grey) re-stimulation with IL-2 and α-GalCer on Day 7 of culture. The percentage of iNKT cells developed over the 14 day culture in each condition is shown (A) and the viability of the corresponding PBMC culture (B). N=1.

Once iNKT cell expansion had been optimised, the method was repeated in different blood donors. Zombie NIR viability staining by flow cytometry, presented in **Figure 3.13,** showed 99.2% (n=7, SD=0.78) of α-GalCer-stimulated lymphocytes were viable at Day 0 of culture, decreasing to 84.1% (n=7, SD=5.88) by Day 14 of culture. DMSO-stimulated lymphocytes were slightly less viable at Day 14 of culture, with 79.9% (n=7, SD=7.36) of cells viable.

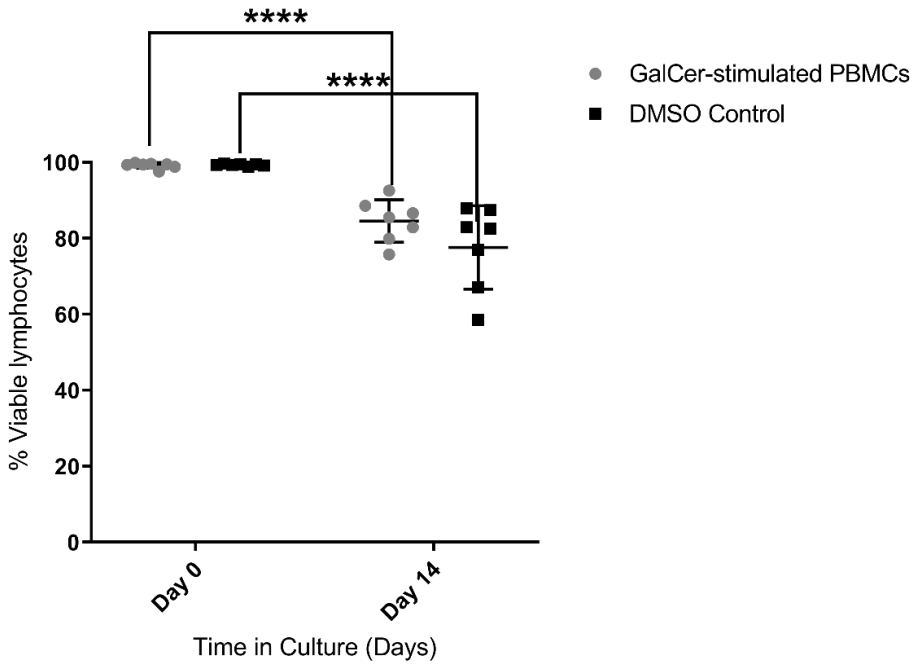


Figure 3.13. Viability of lymphocytes at Day 0 and Day 14 of iNKT cell expansion. PBMCs were stained with Zombie NIR dye and analysed by flow cytometry to determine the viability of lymphocytes at 0 hours and Day 14 of iNKT cell expansion. (**** $p<0.0001$, vertical bars indicate standard deviation, horizontal bars indicate the mean).

After viability was assessed, the effectiveness of iNKT cell expansion was examined. As shown in **Figure 3.14,** iNKT cell populations can be defined by the expression of cell-surface markers CD3 and α-GalCer-loaded CD1d tetramer. The PBMCs were cultured with either the DMSO control or α-GalCer, and then stained with CD3 and the α-GalCer-loaded CD1d tetramer to identify iNKT cells. The DMSO control was used as α-GalCer was reconstituted in DMSO. Some of the α-GalCer-stimulated cells were alternatively stained with a blank tetramer control to identify any non-specific binding of the tetramer, which

may then overestimate the number of iNKT cells present. To clarify, the α-GalCer-loaded CD1d tetramer works by binding to the invariant TCR on iNKT cells. Thus, all iNKT cells should recognise this α-GalCer-CD1d complex, which allows the identification and quantification of the iNKT cells in all conditions. The blank-loaded CD1d tetramer should not be recognised by iNKT cells as they will only bind to glycolipids presented by CD1d molecules.

At Day 0 of culture, the iNKT cell population in this representative sample was 0.42% of lymphocytes, which expanded to 16.77% by Day 14 of culture with α-GalCer. DMSO-stimulated PBMCs showed no expansion of iNKT cells by Day 14, constituting 0.47% of $CD19^-$ lymphocytes. The blank-loaded tetramer was used to detect any false positive binding of cells to the tetramer. This negative tetramer control also showed little expansion (0.80%) by day 14.

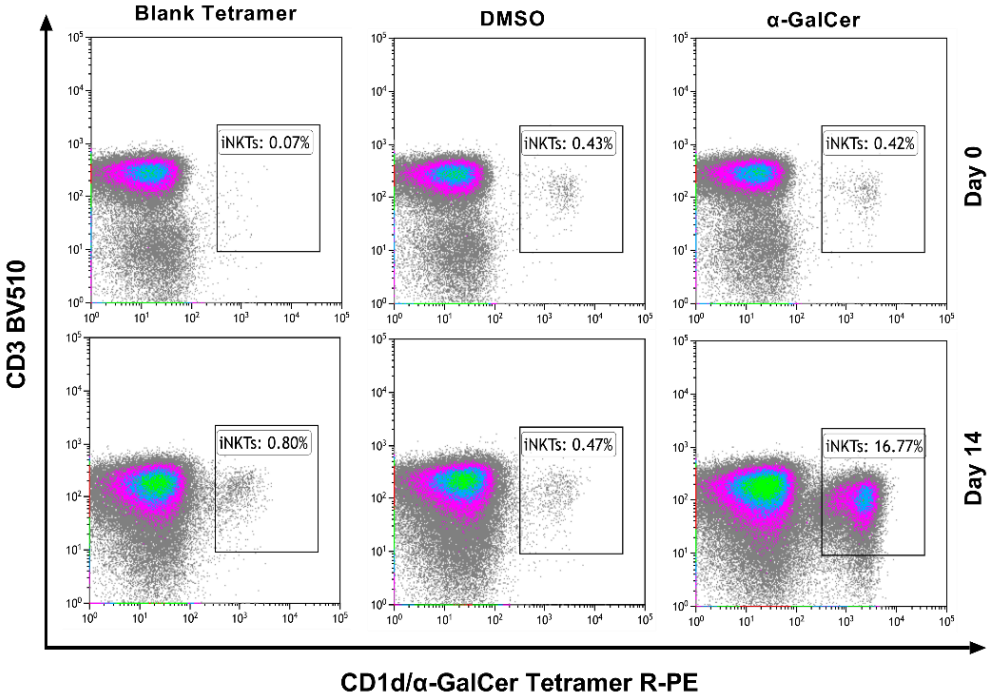


Figure 3.14. Density Plots of iNKT Cell Expansion. Representative dot plots indicating the percentage of iNKT cells cultured with either α-GalCer (right), DMSO control (middle), or stained with the CD1d tetramer control (left), at days 0 and 14 of culture. All events were gated on live, CD19 negative lymphocytes. iNKT cells were identified by expression of CD3 and staining with the α-GalCer-loaded CD1d tetramer.

Figure 3.15A demonstrates iNKT cell expansion across 9 healthy donors. The iNKT cells constituted 0.69% (SD=0.13) of $CD19^-$ lymphocytes before iNKT cell expansion. After 14 days of stimulation with α-GalCer and IL-2, the percentage of iNKT cells increased to 8.20% (SD=5.02), with all donor PBMCs showing some degree of iNKT cell proliferation. A two-way ANOVA revealed this Day 14 iNKT cell population was significantly different to the DMSO control of 0.51% iNKT cells (SD=1.30, $p<0.0001$) and the Negative GalCer Tetramer control of 0.21% iNKT cells (SD=0.28, $p<0.0001$). In line with the increasing percentage of iNKT cells after 14 days of expansion with α-GalCer, **Figure 3.15B** illustrates the increase in the relative number of iNKT cells. The starting mean cell count of 0.29 $x10^5$ (range 545 – 2.1 $x10^5$) iNKT cells increased to 1.91$x10^6$ by Day 14 of culture with α-GalCer, with a range of 1.92$x10^5$ to 5.71$x10^6$**.** This was significantly different to the DMSO control which only resulted in a mean of 6.63 $x10^5$ iNKT cells by Day 14 (range 1,473- 2.33 $x10^5$, $p<0.001$).

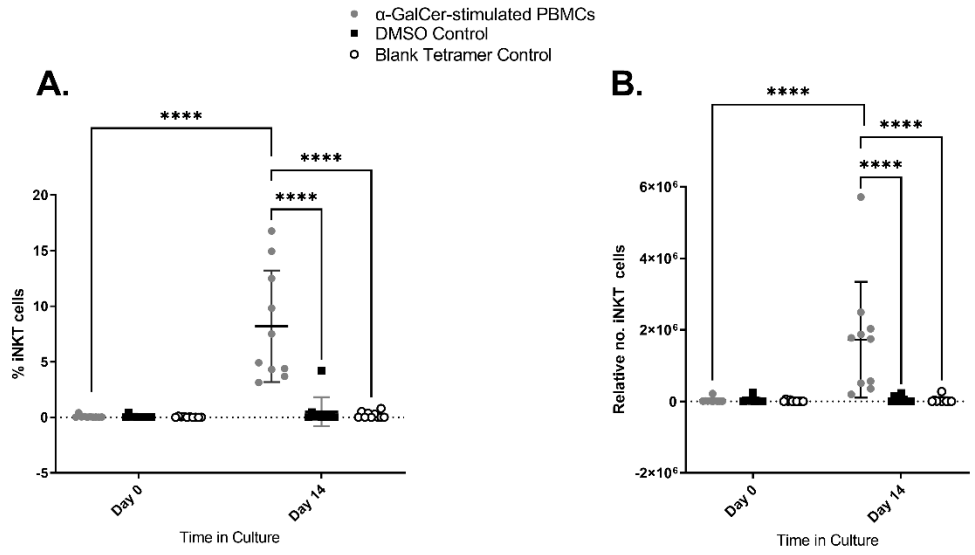


Figure 3.15. Mean iNKT Cell Expansion and Viability. (A) Average iNKT cell percentages (n=9) at Day 0 and Day 14 of expansion with α-GalCer, DMSO, or stained with the negative tetramer control. (B) Representative iNKT cell numbers at Day 0 and Day 14 of expansion with α-GalCer or the DMSO control. (**** $p<0.0001$, vertical bars indicate standard deviation, horizontal bars indicate the mean).

3.3.5. The Isolation of Invariant NKT Cells from PBMCs

To enable co-culture of the expanded iNKT cells with DCs in future experiments, after 14 days of expansion within a whole PBMC culture, the iNKT cells were isolated by immunomagnetic separation. Using the α-GalCer-loaded CD1d PE tetramer, iNKT cells were fluorescently tagged. Anti-PE microbeads were then added to bind the tetramer positive iNKT cells and then applied to a magnetic column for separation. **Figure 3.16** shows a representative example of iNKT cell isolation, highlighting an iNKT cell population of 16.77% before isolation. After immunomagnetic isolation, the resulting iNKT cell purity is 89.03%.

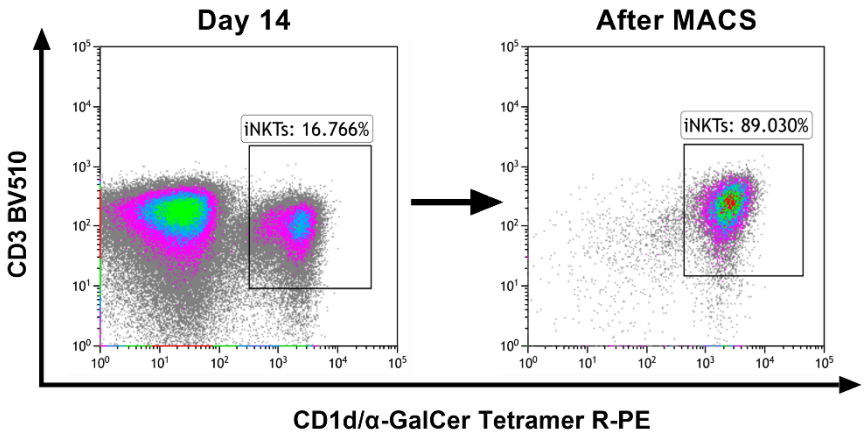


Figure 3.16. Invariant NKT Cell Immunomagnetic Isolation. Representative dot plots showing the purification of iNKT cells after 14 days of cell culture, by immunomagnetic isolation. CD3 and CD1d-GalCer-loaded Tetramer staining was used to identify iNKT cell populations by flow cytometry, before isolation (left) and after MACS immunomagnetic isolation (right). N=1.

Across 5 healthy donors, there was a mean expansion of 9.45% (SD=5.10) after 14 days of culture. After immunomagnetic isolation, there was an average purity of 89.66% (SD=3.13).

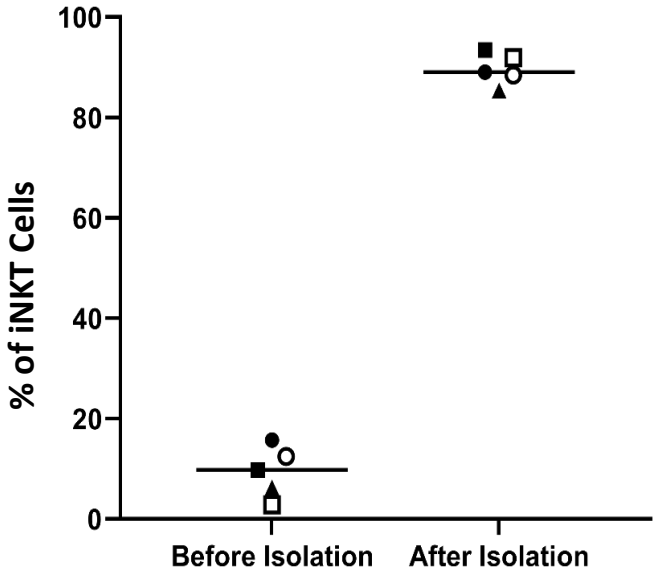


Figure 3.17. Average Invariant NKT Cell Purity after Immunomagnetic Isolation. A graph to show the purification of iNKT cells after 14 days of cell culture, by immunomagnetic isolation. The percentage of CD19- lymphocytes which were identified as iNKT cells at Day 14 of iNKT cell expansion (before isolation) and after iNKT cell immunomagnetic isolation are presented, across 5 healthy donors, depicted by different symbols. Horizontal bars indicate group means, N=5.

3.3.6. Characterisation of Invariant NKT Cells

Once iNKT cells had been successfully expanded and isolated, iNKT cells could be characterised further before later co-culture experiments with DCs. Flow cytometry analysis of iNKT cells at day 0 and day 14 of expansion with α-GalCer highlight a change in iNKT cell phenotype (**Fig. 3.18a**). The phenotype of iNKT cells shifted from predominantly double negative (CD4-CD8-) iNKT cells, to predominantly CD4+CD8- iNKT cells. **Figure 3.18b** demonstrates on average, at Day 0 of expansion, 64.3% of iNKT cells were CD4-CD8-, and 25.3% of iNKT cells were CD4+CD8-. By day 14 of expansion with α-GalCer, the percentage of CD8+ iNKT cells did not change significantly, whereas the percentage of CD4+CD8- iNKT cells significantly increased to 77.3% ($n=5$, $p<0.0001$), and the percentage of CD4-CD8- iNKT cells significantly decreased to 16.2%. The DMSO

control-stimulated iNKTs did not show any significant differences in phenotype between Day 0 and Day 14 of culture (data not shown).

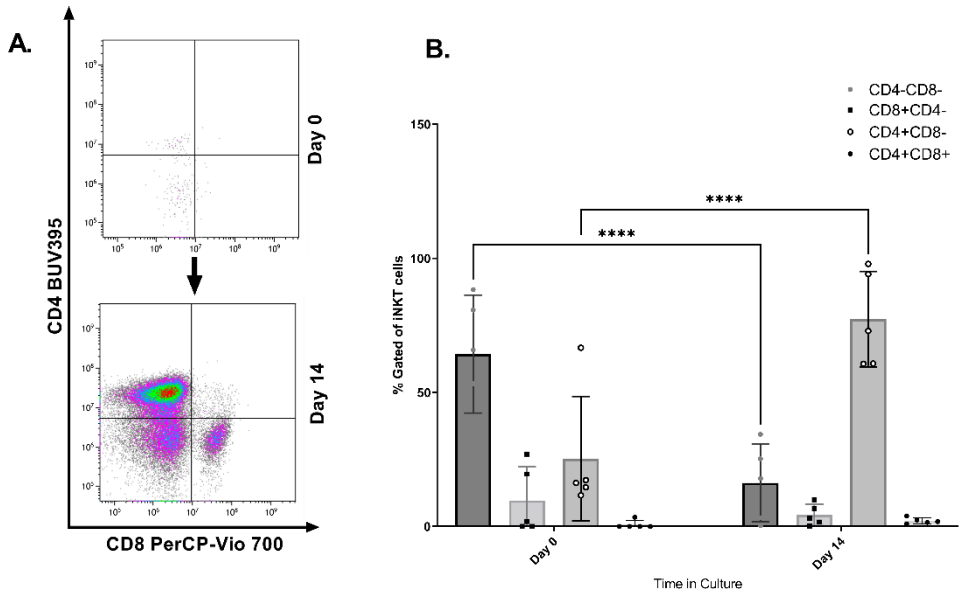


Figure 3.18. iNKT cell phenotype at Day 0 and Day 14 of Expansion. (A) Representative density plots indicating iNKT cell phenotype at Day 0 and Day 14 of iNKT cell expansion with α-GalCer, determined by CD4 and CD8 expression. (B) Average percentage of iNKT cells expressing CD4 and CD8 and Day 0 and Day 14 of expansion with α-GalCer. (Horizontal bars represent group means, vertical bars represent SD, **** p <0.0001*).*

Before isolated iNKT cells are co-cultured with DCs, the cytokine profile of the iNKT cells also needed to be determined. Thus, cytokines were measured by multicolour flow cytometry analysis (**Fig. 3.19**. At Day 0 of expansion, a mean of 10.88 % of iNKT cells secreted IL-4 (n=4, SD=11.41) and 2.98% (n=4, SD=4.77) of cells secreted IFN-γ. By Day 14 of α-GalCer-induced expansion of iNKT cells, the percentage of iNKT cells secreting IL-4 had significantly increased to 80.02% (n=4, SD=7.52, p<0.0001). IFN-γ-secreting iNKT cells had also increased to 19.63%, but this was not statistically significant (n=4, SD=28.29). IL-5 and IL-10 cytokine production was also measured by flow cytometry, however there was minimal differences in the percentage of cells secreting the cytokines between Day 0 and Day 14 of iNKT cell expansion; IL-5 production increased from a mean of 3.88% (n=4, SD=4.77) to 7.13% (n=4, SD=14.19), and IL-10 production

decreased from 5.90% (n=4, SD=4.86) at Day 0 to 1.06% at Day 14 (n=4, SD=0.90).

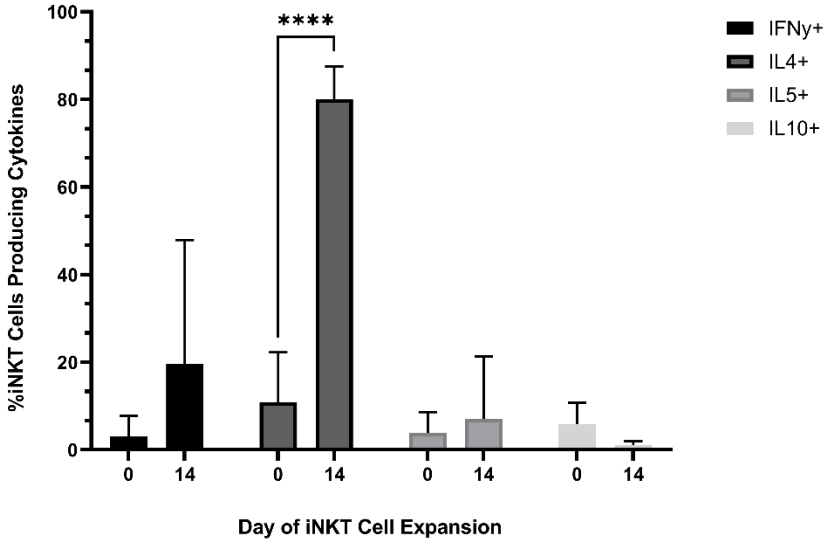


Figure 3.19. Percentage of iNKT Cells Producing Cytokines Before and After Expansion. PBMCs were stained for intracellular cytokine production at Day 0 and Day 14 of iNKT cell expansion. iNKT cells were then gated by flow cytometry and their cytokine production measured. Cytokines measured included IFN-γ, IL-4, IL-5, and IL-10. (**** $p<0.0001$, Vertical bars indicate standard deviation).

3.4. Discussion

NKT cells have recently been suggested to play a role in allergic sensitisation, due to their ability to rapidly release cytokines, resulting in the skewing of a Th1 or Th2 immune response. NKT cells can release these cytokines after recognition of lipid stimuli via their invariant TCRs, where the lipid is presented by DCs. Focussing on invariant NKT cells, this research aimed to model the presentation of lipids by DCs to activate iNKT cells, and measure the subsequent cytokine response. This is the mechanism replicated in this study, utilising the lipid α-GalCer to activate iNKT cells. To develop this human model of allergic sensitisation, NKT cells needed to be isolated from human peripheral blood, ready for co-culture with lipid-pulsed DCs.

The results presented highlight the difficulties in isolating NKT cells from human PBMCs, without prior expansion to increase the population of NKT cells. CD3+CD56+ NKT cells were successfully isolated at high purities from PBMCs using immunomagnetic isolation. However, once placed into culture, the NKT cell viability rapidly declined within the first 24 hours of culture. This posed an issue for later experiments. The low numbers of NKT cells in human peripheral blood could explain why viability was poor, as small numbers of NKT cells were cultured, lacking stimulation from other cells within a PBMC culture to maintain viability. This is reinforced by the numerous NKT and iNKT studies which analyse the cells within a PBMC culture, rather than isolating the specific cell type beforehand (Balasko, Graydon, and Fowke 2021; Schmid et al. 2018a; Watarai et al. 2008).

Research into Leukaemia treatments highlighted invariant NKT cells could be expanded in a whole PBMC culture, before their isolation, with the lipid α-GalCer (Brossay et al. 1998; Schmid et al. 2018b). Thus, this method was adapted to expand iNKT cells with α-GalCer for 14 days to yield a higher number of iNKT cells, and also improve iNKT cell viability.

The iNKT cell expansion initially had poor viability by Day 12 of expansion, thus the method was optimised to supplement the culture with IL-

2 every 4 days to maintain viability. This improved viability of iNKT cells, and the expansion results indicate an 80-fold expansion after 2 weeks of culture. This is supported by previous research which observed similar results (Gadola et al. 2002; Schmid et al. 2018b). Importantly, the DMSO control did not induce any iNKT cell proliferation, supporting the notion that lipids activate iNKT cells.

Once the iNKT cells had been expanded with α-GalCer and viability was acceptable, the iNKT cells were immunomagnetically isolated, consistently achieving high purities, supported by other research utilising this method of iNKT cell isolation (Park et al. 2019; Wilson et al. 2003). A high purity of iNKT cells was needed to minimise contamination from other cells when the iNKT cells are co-cultured with DCs. Thus, the presence of other cell types will influence the cytokine production of the DCs and iNKT cells, whereas we want to show the direct relationship between the two cell types. The iNKT cells were then characterised. iNKT cells are phenotypically $CD4^+CD8^-$, $CD4^-CD8^-$ (DN), or $CD4^-CD8^+$ in a small subset (Dhodapkar and Kumar 2017a). Flow cytometry analysis of culture-expanded iNKT cells highlighted DN iNKTs are most prominent in PBMCs of healthy subjects, but there is preferential expansion of $CD4^+$ iNKT cells, which have tolerogenic properties, such as preventing certain autoimmune diseases. This is consistent with previous literature, which also identified $CD4^+$ iNKT cells expanded more readily than DN and CD8+ iNKT cells (O'Reilly et al. 2011; Schmid et al. 2018b). However, DN and CD8+ iNKT subsets predominantly secrete Th1 cytokines and display cytotoxic properties, whereas CD4+ iNKT subsets exhibit both Th1 and Th2 phenotypes (O'Reilly et al. 2011). Thus, the preferential expansion of CD4+ iNKT cells could produce a bias in the cytokines produced during co-culture with lipid-pulsed DCs.

Furthermore, the cytokine profile of iNKT cells was assessed by flow cytometry to establish any cytokine production before their subsequent co-culture with autologous DCs, and to also determine the effect of the lipid α-GalCer on iNKT cell cytokine production. The analysis revealed high amounts of IL-4 production and some IFN-γ after iNKT cell expansion. This is expected as the iNKT cells are being potently activated by the α-GalCer, and has been

shown in previous research (Schmid et al. 2018a). Thus, any further cytokine secretion when iNKT cells are co-cultured with lipid-stimulated DCs will be measured relative to the amount of cytokines produced at Day 14 of expansion.

Here we show, by utilising the glycolipid, α-GalCer, the most potent activator of iNKT cells, that α-GalCer can induce robust expansion of human iNKT cells over the course of 14 days. Having shown we have viable iNKT cells we then wanted to optimise techniques to generate dendritic cells.

Chapter 4: Optimisation of Dendritic cell Techniques

4.1. Introduction

DCs are heterogeneous, antigen presenting cells which have crucial roles in IgE-mediated allergy. Monocyte-derived DCs (moDCs) become abundant during inflammation, and are efficient at presentation of allergens to both $CD4^+$ and $CD8^+$ T cells, (Plantinga et al. 2013a). During allergic sensitisation, DCs, such as moDCs, are exposed to allergens at the mucosal site, where the DC internalises the allergens through endocytosis, pinocytosis, or phagocytosis, and then migrate to draining lymph nodes. The allergen is then processed via endolysosomal compartments and presented as peptides via MHC Class II molecules to T cell receptors, which activates naïve T cells to differentiate and subsequently prompt the adaptive immune response (Salazar and Ghaemmaghami 2013a). Furthermore, DCs can also produce cytokines after allergen internalisation to interact and direct other innate immune cells, such as IL-10 and IL-12 (Hole et al. 2019). DCs can also upregulate cell-surface markers such as CD40, CD80, CD86, and HLA-DR after exposure to allergens. The upregulation of these markers, and release of cytokines can influence the response of the T cells (Ruiter and Shreffler 2012) and ultimately determine whether an individual is sensitised to an allergen or not.

In contrast to proteins that are presented by MHC class II molecules, it is well-established that lipids are presented by CD1 molecules. There has been particular interest in CD1d molecules as they present glycolipids to a specific group of T lymphocytes, called invariant natural killer T cells, which are powerful immune regulators. CD1d molecules are similar to MHC Class I molecules, comprising a heavy chain with three extracellular domains associated with β2 -microglobulin (β2 m) (Schiefner and Wilson 2009a). Studies within a recent systematic review suggests that lipids from allergen sources can be presented by these CD1d molecules on DCs to iNKT cells, to cause a shift towards allergic sensitisation, by stimulating Th2 cytokine secretion (Hopkins et al. 2022).

Due to the pivotal role of DCs, in particular its CD1d molecule, in presenting lipids to iNKT cells during allergic sensitisation, we aim to investigate the role of lipids, moDCs and iNKT cells in allergic sensitisation. We hypothesise that lipids influence a Th2 response, but they can only achieve this when co-delivered with a protein, thus acting as adjuvants to a Th2 response. Before this mechanism can be modelled *in vitro*, DC methods needed to be optimised.

In vitro DC research often utilise moDCs as they are similar in physiology, morphology, and function to conventional myeloid dendritic cells. MoDCs can be generated from peripheral blood, by stimulating CD14+ monocytes with IL-4 and GM-CSF and incubating for 5 days (Balan, Saxena, and Bhardwaj 2019). Immunomagnetic isolation of the CD14+ monocytes from human peripheral blood was adopted as this results in a high purity and homogeneous sample of isolated monocytes, compared to other techniques, such as adherence-mediated purification on tissue culture dishes. It is also a quick method which is essential as it minimises the time the monocytes are exposed to non-physiological conditions (Posch, Lass-Flörl, and Wilflingseder 2016).

Thus, the moDC methods optimised included the isolation of monocytes from human PBMCs, the generation of DCs from monocytes, and stimulation of iDCs with proteins and lipids. Research has shown that the internalisation of lipids and/or allergens by DCs can alter the phenotype and cytokine profile of the DCs (Abos Gracia et al. 2017; Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, et al. 2016). Thus, the moDCs were also characterised to determine any upregulation of maturation markers after stimulation with proteins and lipids. Techniques to maintain CD1d expression on DCs were also optimised, as this is the molecule of lipid presentation, found on DCs. Finally, the internalisation of lipids and proteins by DCs was analysed by imaging flow cytometry.

4.2. Materials and Methods

4.2.1. PBMC Isolation

50mL of blood was obtained from healthy human volunteers and diluted 1:1 with PBS + 2% FBS. This was then layered on top of Histopaque in SepMate tubes. The SepMate tubes were centrifuged at 1200g for 10 minutes, with the brake on. Then, as much supernatant as possible was removed before tipping the PBMC layer into a fresh tube and topping up with PBS + 2% FBS. The PBMCs were then washed for 8 minutes, twice, to ensure the removal of any platelets. See section 2.2.1.1. Peripheral Blood Mononuclear Cell (PBMC) Isolation for more detail.

4.2.2. CD14+ monocyte Isolation

PBMCs were mixed with 80µl of MACS buffer (per 10^7 cells) and magnetically labelled with 20µl CD14 microbeads and incubated at 4°C for 15 minutes. The cells were then washed with MACS buffer and centrifuged at 300g for 10 minutes. The pellet was then re-suspended in 500µl MACS buffer to then be applied to an LS column. The column was then washed 3 times with 3mL MACS buffer and eluted into a sterile tube with 5mL of buffer, to obtain the CD14+ fraction. See section 2.2.5. CD14+ Monocyte Isolation for more detail.

4.2.3. DC Generation

Once $CD14^+$ monocytes had been isolated, the monocytes were stimulated to generate iDCs. The monocytes were re-suspended in RPMI + 10% FBS and cultured in a 24 well flat-bottomed tissue culture-treated plate at 5 $x10^5$ cells/mL. 50 ng/mL GM-CSF and 20 ng/mL of human IL-4 was added to each well to initiate DC generation. The plate was placed in an incubator for 5 days, replenishing media and cytokines on Day 3. See methods section 2.2.6. DC Generation for more detail.

To ensure immature DCs had been generated, some cells from wells were removed and extracellularly stained for: CD1d, CD14, CD40, CD83, CD80, CD86, HLA-DR, and CD209 (DC-SIGN) (All purchased from Miltenyi Biotec, UK).

To establish any maturation of DCs with allergens and/or lipids, some iDCs were first stimulated with 100 ng/mL LPS (Cat no. L2630, Sigma, UK) for 24 hours, which is a classic method of maturation. To analyse the phenotype of DCs when stimulated with a protein, the well-established Ovalbumin (OVA), which is an egg protein allergen, was utilised. Thus, iDCs were stimulated with 100ng/mL of Cy5-conjugated OVA (Cat no. O-34784, Thermofisher, UK) or 100 ng/mL α-GalCer (glycolipid) to look at lipid-pulsed DC phenotypes, and then replaced in the incubator for 24 hours. LPS, OVA, and α-GalCer-stimulated DCs were then harvested from the wells and stained with the same antibodies (as above) as iDCs to measure changes in maturation markers.

Wherever foetal bovine serum (FBS) has been mentioned in these methods, later experiments replaced 10% FBS with 10% human AB serum to adhere to Unilever guidelines, where the use of animal-derived materials must be kept to an absolute minimum. The experiments affected by this change are clarified in the results.

4.2.1. Flow Cytometry

The antibodies utilised for assessing DC maturation are presented in **Table 4.1.**

Table 4.1. Flow Cytometry Antibodies for DC Maturation Markers. The marker, fluorophore, company purchased from, and catalogue number for the antibodies utilised in the flow cytometry staining of DCs.

Cell Marker	Fluorophore	Company	Cat no.
CD1d	PE	Miltenyi Biotech	130-099-982
CD14	PerCP	Miltenyi Biotech	130-110-581
CD19	PE	Miltenyi Biotech	130-113-646
CD40	PE VIO 770	Miltenyi Biotech	130-110-948
CD80	APC	Miltenyi Biotech	130-117-719
CD83	PE	Miltenyi Biotech	130-110-503
CD86	FITC	Miltenyi Biotech	130-116-159
CD209	APC	Miltenyi Biotech	130-124-257
CD209	PE VIO 770	Miltenyi Biotech	130-109-650
HLA-DR	FITC	Miltenyi Biotech	130-111-788
HLA-DR	APC	Miltenyi Biotech	130-111-790
Isotype Control	APC	Miltenyi Biotech	130-113-446
Isotype Control	PE	Miltenyi Biotech	130-113-450
Isotype Control	PE Vio 770	Miltenyi Biotech	130-113-452
Isotype Control	FITC	Miltenyi Biotech	130-113-449
Isotype Control	PerCP	Miltenyi Biotech	130-114-562

4.2.1.1. Extracellular Staining

For flow cytometry staining of extracellular markers, cells were harvested into FACS tubes with 1 mL of PBA and centrifuged at 300g, for 5 minutes. The supernatant was removed completely and the pellet was tapped to re-suspend. The appropriate antibodies were then added to the tube containing 50 µL of PBA, and incubated at 4ºC for 30 minutes before centrifuging at 300g for 5 minutes to remove any unbound antibodies. The supernatant is completely removed and cell pellet is re-suspended in fixation buffer before analysis by flow cytometry. Kaluza software was used to interpret results. More detail is found in section 2.2.9.1. Extracellular Staining.

4.2.1.2. Viability Staining

To stain for dead cells, Annexin V and Propidium Iodide (PI) staining was used in early experiments, before replacing with Zombie NIR. Briefly, PBMCs or DCs were centrifuged at 300g for 5 minutes in PBA and then the supernatant was aspirated. This wash was then repeated with 1mL Annexin binding buffer to prime the cells. The supernatant was aspirated, and cells re-suspended Annexin binding buffer and 5 µL of Annexin V for 15 minutes at room temperature, in the dark. PBMCs or DCs were then centrifuged at 300g for 5 minutes with binding buffer and then the supernatant was aspirated, before re-suspended in 200 µL binding buffer and 0.33 µL of 1mg/mL PI staining solution. Samples were then analysed immediately. See section 2.2.9.3. Annexin V and PI staining for more detail.

4.2.1.3. Imaging DC Internalisation of OVA and α-GalCer

ImageStream analysis of OVA-stimulated DCs and α-GalCer-stimulated DCs was performed to visualise whether the DCs had internalised the protein allergen or lipids. For OVA internalisation, the Ovalbumin used to stimulate DCs was purchased conjugated to a Cy5 fluorophore to analyse internalisation. The OVA-stimulated DCs were also stained for HLA-DR to identify DCs. For α-GalCer DC internalisation, 100ng/mL of a fluorescent derivative of α-GalCer, namely dansylated α-GalCer (Adipogen, UK) was used to stimulate iDCs for 24 hours.

The DCs were also stained for CD1d. All cells were analysed on ImageStream MkII and then interpreted on IDEAS software.

4.2.3. Statistical Analyses

A repeated-measures One-Way ANOVA was conducted with Dunnett's multiple comparisons test to compare CD1d expression at 0 hours of culture to 24 and 48 hours of culture (N=4). Another repeated-measures One-Way ANOVA was conducted with Dunnett's multiple comparisons test to compare immature DC expression of CD1d to LPS, OVA, DMSO, and α-GalCer stimulated DC expression (N=3).

4.3. Results

4.3.1. Immunomagnetic Isolation of $CD14^+$ Monocytes

To test the purity of immunomagnetically isolated $CD14^+$ monocytes, cells at all stages of separation were analysed by flow cytometry. The gating strategy for identifying PBMCs is presented in **Figure 4.1** where PBMCs are identified by plotting size and granularity (**Ai**), and then doublets are removed by plotting forward scatter height by forward scatter width (**Aii**).

Figure 4.1B shows representative histograms of CD14 expression at the different stages of $CD14^+$ isolation using anti-CD14 PerCP. The figure shows there is a starting population of 11.70% $CD14^+$ monocytes in PBMCs from a 50mL venous blood sample (**Fig. 4.1Bi**). After immunomagnetic separation, only 1.29% of $CD14^+$ cells were present in the negative fraction (**Fig. 4.1Bii**). In the final CD14 positive fraction, there were 96.23% $CD14^+$ monocytes (**Fig. 1Biii**).

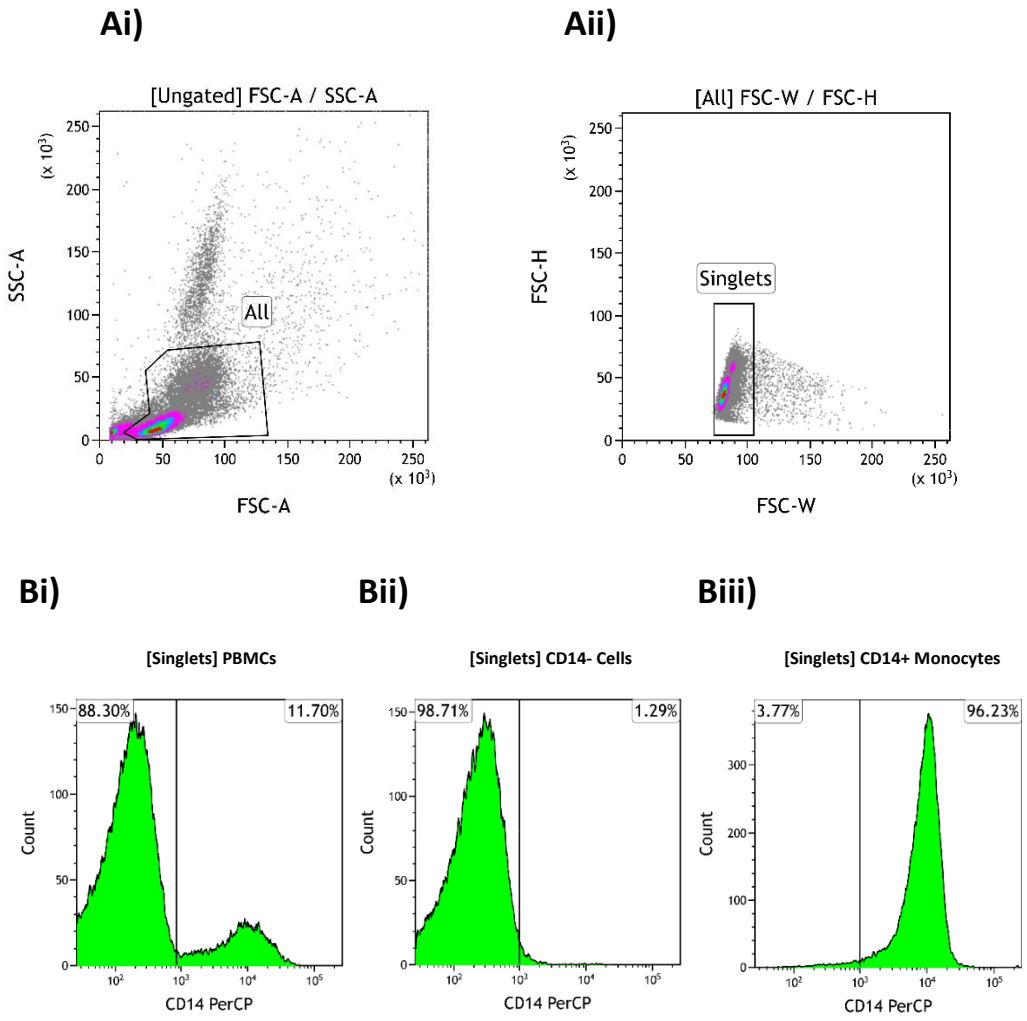


Figure 4.1. Immunomagnetic Isolation of CD14$^+$ monocytes. Monocytes were isolated using CD14 microbeads to produce a pure population of CD14$^+$ cells. To gate PBMCs and measure CD14 expression, (Ai) Cells were plotted for forward scatter by side scatter before gating PBMCs to exclude debris/dead cells and granulocytes. (Aii) The PBMCs were then gated to remove any double-cells by plotting forward scatter height by forward scatter width. Flow cytometry histograms of cells staining positive and negative for CD14 are presented. Representative histograms show (Bi) CD14$^{+/-}$ cells at PBMC stage. (Bii) CD14$^{+/-}$ cells present in the negative 'flow through' fraction, after immunomagnetic isolation. (Biii) the final positive fraction of 96.23% CD14$^+$ monocytes. (N=1).

Flow cytometry analysis of the size and granularity of the final CD14 positive fraction demonstrates the 3.77% of contamination in this sample was likely from lymphocytes (2.79%) (**Fig. 4.2**).

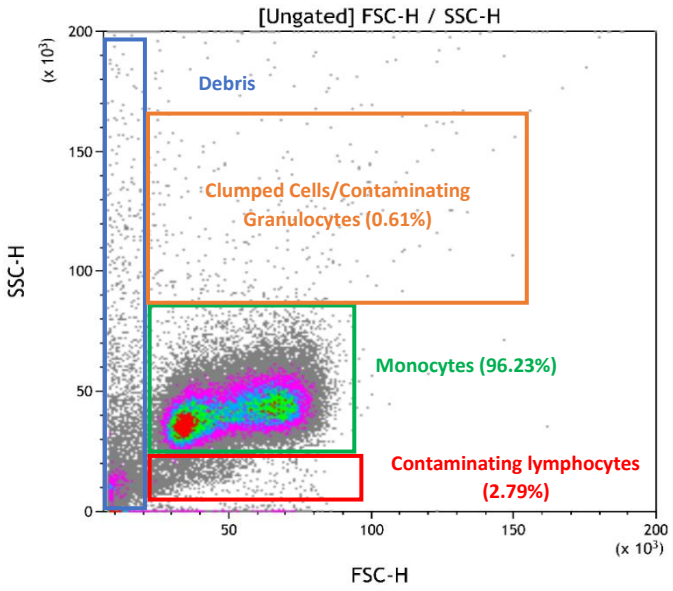


Figure 4.2. Density Plot of CD14⁺ Fraction. Flow cytometry analysis of CD14⁺ fraction after immunomagnetic separation, categorised by cell type. Debris was excluded from analysis (blue). Immunomagnetic isolation of CD14⁺ monocytes resulted in a high purity of 96.23% monocytes (green), with 2.79% contaminating lymphocytes (red), and 0.61% clumped cells/contaminating granulocytes (orange). (N=1).

This method of CD14⁺ immunomagnetic isolation was replicated with different blood donors. **Figure 4.3** indicates the isolation experiments achieved an average CD14⁺ purity of 93.86% (N=9, SD=3.30).

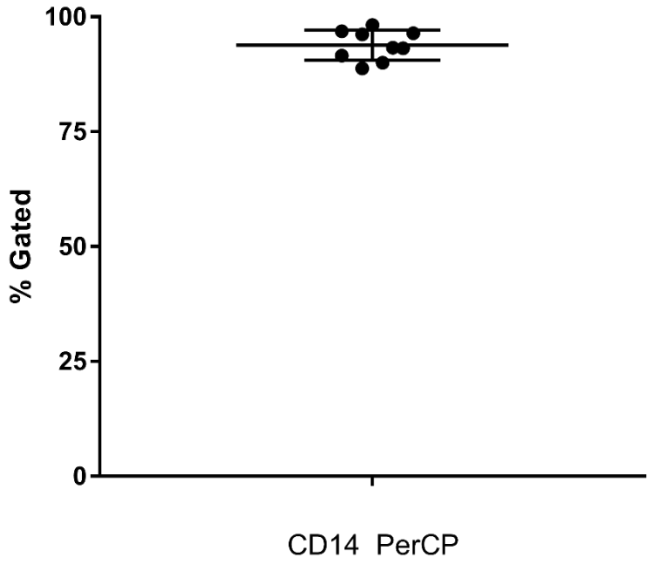


Figure 4.3. Average purity of CD14+ monocytes. The final average purity of $CD14^+$ monocytes after immunomagnetic isolation from PBMCs, using CD14 PerCP to identify $CD14^+$ cells by flow cytometry. Data points represent results from 9 different human blood donors. The average purity after isolation was 93.86% (N=9, SD=3.30).

Overall, the data show monocytes can be successfully isolated from human PBMCs using immunomagnetic separation, consistently achieving high purities, and with only a small percentage of contaminating lymphocytes. This ensures DCs can subsequently be generated from the isolated monocytes, with low contamination from other cells.

4.3.2. DC Generation from $CD14^+$ Monocytes

Once $CD14^+$ monocyte isolation had been successfully carried out, the monocytes were stimulated to generate iDCs. After 5 days of culturing the monocytes with both GM-CSF and IL-4, iDCs were generated. iDCs were then stimulated with LPS to mature the DCs. The phenotypes of iDCs and LPS-stimulated DCs were measured using flow cytometry, staining for markers associated with DCs. The phenotypes could then be compared to OVA and α-GalCer stimulated DCs to determine any maturation by the protein or lipid. This method of generating DCs and stimulating with proteins or lipids was essential to ensure the experiment worked correctly, as future experiments will stimulate iDCs with peanut lipids and peanut allergens. The peanut allergy

route had not been decided at this stage, which is why egg ovalbumin and α-GalCer were utilised instead of peanut allergens and lipids.

4.3.2.1. Immature DC and LPS-matured DC Generation

To determine the iDC phenotype, flow cytometric analysis of iDCs was conducted. The gating strategy for selecting DCs is presented in **Figure 4.4**. The DCs were generated from isolated monocytes, so the gating plots show only the single cell type. The DCs were identified by plotting size and granularity, and then doublets are removed by plotting forward scatter height by forward scatter width.

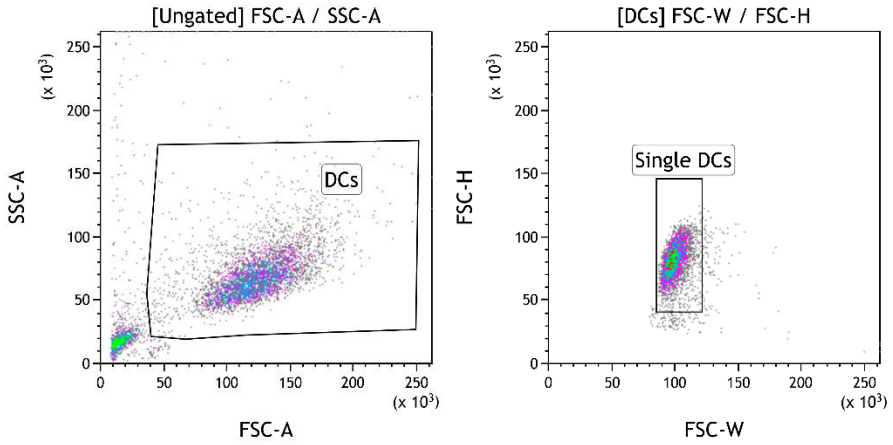


Figure 4.4. DC gating strategy. CD14+ monocytes were isolated and DCs generated**.** DCs were plotted for forward scatter by side scatter (left) before gating DCs to exclude debris/dead cells and granulocytes. The DCs were then gated to remove any double-cells (right) by plotting forward scatter height by forward scatter width.

The analysis indicated iDCs were positive for CD1d, CD40, CD80, CD209, and HLA-DR, compared to the isotype control (**Fig. 4.5)**. There was no expression of CD14, CD83 or CD86. This iDC phenotype is consistent with published literature (Andreae et al. 2002).

As expected, LPS-stimulated DCs exhibited increases in surface molecules associated with maturation: CD40, CD80, CD83, CD86 and HLA-DR,

suggesting the cells are matured. Furthermore, CD209 levels were unchanged with LPS-stimulation.

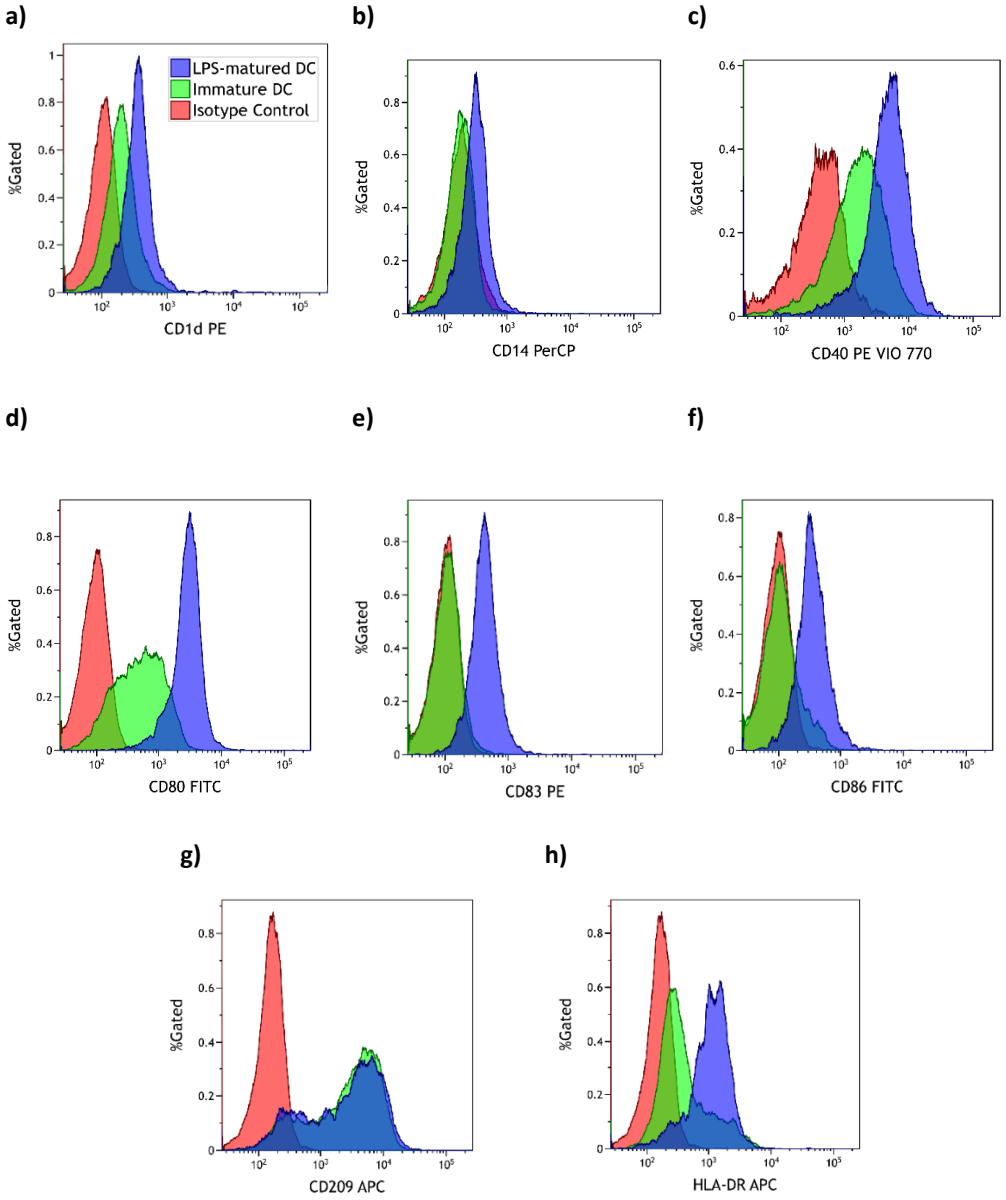


Figure 4.5. Phenotypes of iDCs and LPS-stimulated DCs. The flow cytometry histograms show the expression of DC markers CD1d, CD14, CD40, CD80, CD83, CD86, CD209, and HLA-DR (a-h) at Day 5 iDC stage (green) and Day 6 LPS-stimulated DCs (blue), compared with isotype controls (red). N=1.

This method of maturing DCs using LPS was replicated using 4 different healthy blood samples (**Figure 4.6**). Paired t-tests for each CD marker were performed to determine any significant differences between immature DCs and LPS-stimulated DCs. This demonstrates significant upregulation of CD80 in LPS-stimulated DCs compared to iDCs (p=0.006). The mean expression of CD80 increased to 13.46 rMFI on LPS-stimulated DCs, from 3.69 rMFI on iDCs. Mean CD86 expression was also significantly upregulated from 1.14 rMFI on iDCs to 2.84 rMFI on LPS-stimulated DCs (p=0.041). This upregulation of CD80 and CD86 indicates LPS stimulation successfully matured iDCs. However, the other key indicator of DC maturation, CD83, was not significantly upregulated from iDCs to LPS-stimulated DCs (p>0.05). In addition to the upregulation of CD80 and CD86, LPS-stimulated DCs also significantly upregulated CD40 (p=0.022) and HLA-DR (p=0.025). There were no significant differences between iDC and LPS-stimulated DC expression of CD1d and CD14 (p>0.05).

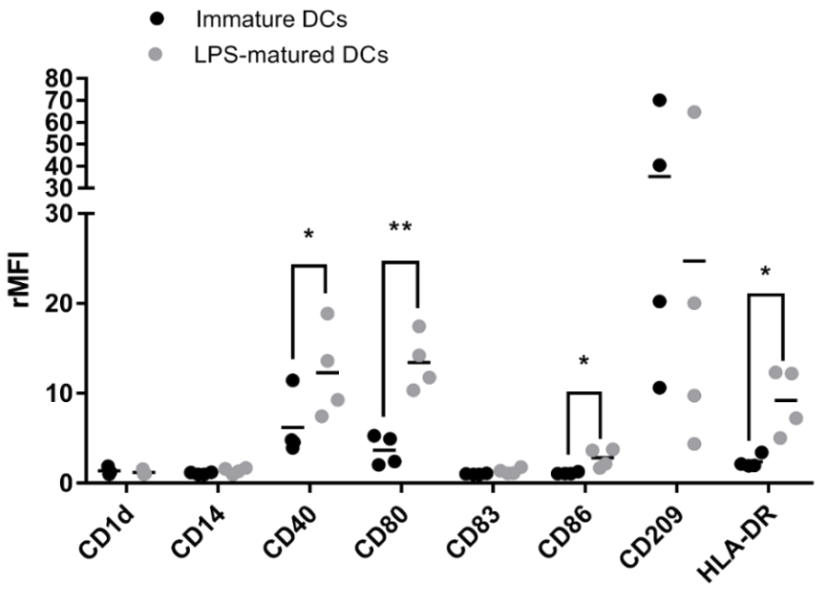


Figure 4.6. Effect of LPS-stimulation on DC Surface Markers. The relative MFI of CD1d, CD14, CD40, CD80, CD83, CD86, CD209, and HLA-DR were calculated for iDCs and LPS-stimulated DCs. The data points represent results from different blood samples (N=4), with horizontal bars representing group means. The data was analysed by paired t-tests for each marker, comparing LPS-stimulated DCs to immature DCs: **p<0.001, *p<0.05.

In conclusion, both iDCs and LPS-stimulated DCs express the expected DC markers. However, despite showing some CD80 upregulation this was not significantly upregulated, potentially due to the small sample size (n=4). However, there was significant upregulation of other maturation markers CD80 and CD86. Thus, this data concludes LPS-stimulated DCs were successfully matured. These results can now be used as a positive control for matured-DCs which can be compared with data from OVA and α-GalCer stimulated iDCs to determine whether they also stimulate DC maturation.

4.3.2.2. Ovalbumin-stimulated DCs

The egg protein, OVA, was then tested to see if it also induced any upregulation of DC surface markers. This was a proof of principle experiment for analysing DC phenotypes after stimulation with protein allergens. The OVA was conjugated with Cy5, thus any APC or PerCP antibody fluorophores used for phenotyping were avoided or replaced with suitable alternatives.

Figure 4.7 details the phenotype data for OVA-stimulated DCs, compared to iDCs and isotype controls for each marker. The figure shows similar shifts in marker expression to LPS-matured DCs, such as CD80, CD83, and CD86, although this is to a lesser extent compared to LPS-DCs.

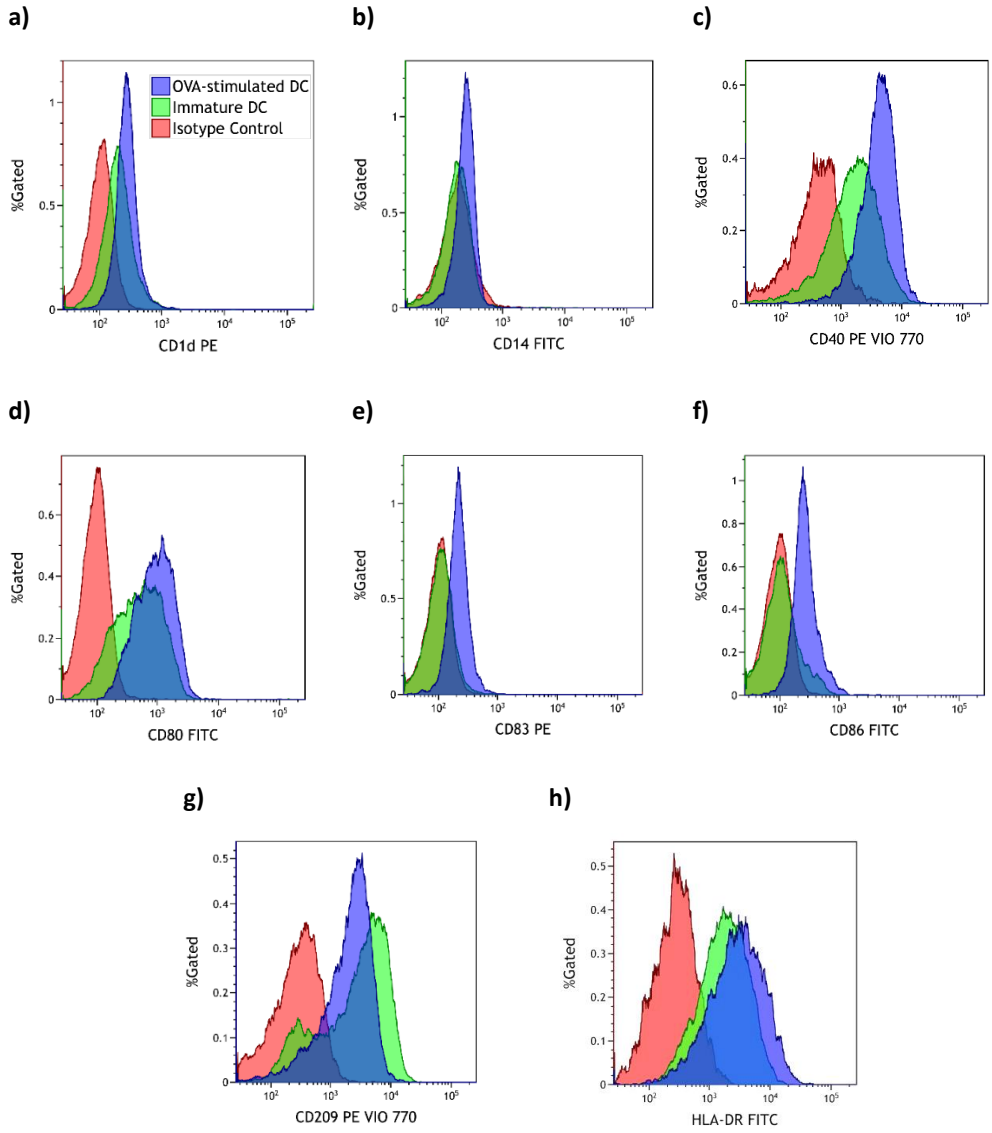


Figure 4.7. Phenotype of OVA-stimulated DCs. The histograms show the expression of DC markers CD1d, CD14, CD40, CD80, CD83, CD86, CD209, and HLA-DR (a-h) at Day 5 iDC stage (green) and Day 6 OVA-stimulated DCs (blue), compared with isotype controls (red). (N=1).

This method of OVA-stimulation to iDCs was repeated with different blood donors. Of these three repeats, data was available for markers CD1d, CD40, CD80, CD83, and CD86 as the majority of experiments were conducted prior to obtaining CD14, CD209 and HLA-DR antibodies with fluorophores appropriate for the use with the Cy5-conjugated OVA, so replicate data for these markers are not available. **Figure. 4.8** demonstrates the expression of

OVA-stimulated DC markers across the three experiments. The mean rMFI for CD1d, CD83 and CD86 were similar on iDCs (1.36, 1.05, 1.16) and OVA-stimulated DCs (1.40, 1.32, 1.54), respectively. CD40 expression increased from a mean of 6.66 rMFI (SD=4.2) on iDCs to 13.16 (SD=10.3) on OVA-stimulated DCs. Increases were also noted for CD80 from 4.23 (SD=1.6) on iDCs to 6.8 rMFI (SD=5.5) on OVA-stimulated DCs. Despite these increases, paired t-test analysis resulted in no significant differences between iDCs and OVA-stimulated DCs ($p>0.05$) for any marker expression, suggesting no significant upregulation of maturation markers.

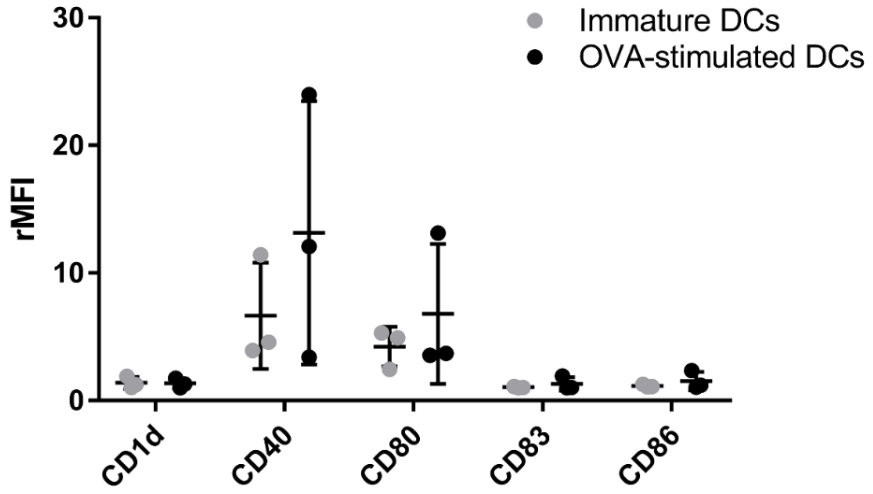


Figure 4.8. Average Expression Levels of OVA-stimulated DCs. The rMFI of CD1d, CD40, CD80, CD83, and CD86, were calculated for OVA-stimulated DCs and iDCs. The results of 3 different blood samples are shown by data points, with horizontal bars representing group means. Vertical bars represent standard deviation. Paired t-test analysis comparing iDC and OVA-stimulated DC marker expression was conducted: no significant difference found for any marker ($p>0.05$). (N=3).

Overall, the stimulation of OVA on iDCs resulted in weak upregulation of CD maturation markers compared to LPS-matured DCs. Despite some upregulation of key maturation markers, statistical analysis highlighted no significant upregulation from iDCs. Thus, OVA did not mature DCs after 24 hours of stimulation.

4.3.2.3. α-GalCer-stimulated DC Generation

After OVA-stimulated DCs had been phenotyped, DCs were stimulated with the lipid, α-GalCer, as a proof-of-principle to determine if lipids mature DCs or alter DC phenotype. This method loaded iDCs with 100ng/mL α-GalCer for 24 hours. iDCs were also stimulated with 100ng/mL of DMSO for 24 hours as a control, as α-GalCer was re-constituted in DMSO.

Figure 4.9 shows the flow cytometry histograms of markers for iDCs, α-GalCer-stimulated DCs and DMSO-stimulated DCs. The α-GalCer plots (blue) show shifts in CD1d, CD14, CD80, CD83, and CD86 expression, compared to iDCs. However, there is no differences between these α-GalCer plots and the DMSO control plots (purple), suggesting the shifts in expression are not a result of the lipid. Although, this does not apply to CD83 expression, where α-GalCer-stimulated DC expression was higher than the DMSO control. Although, when the relative MFI of these results are calculated, there are no significant differences to iDC expression, as shown in **Figure 4.10**.

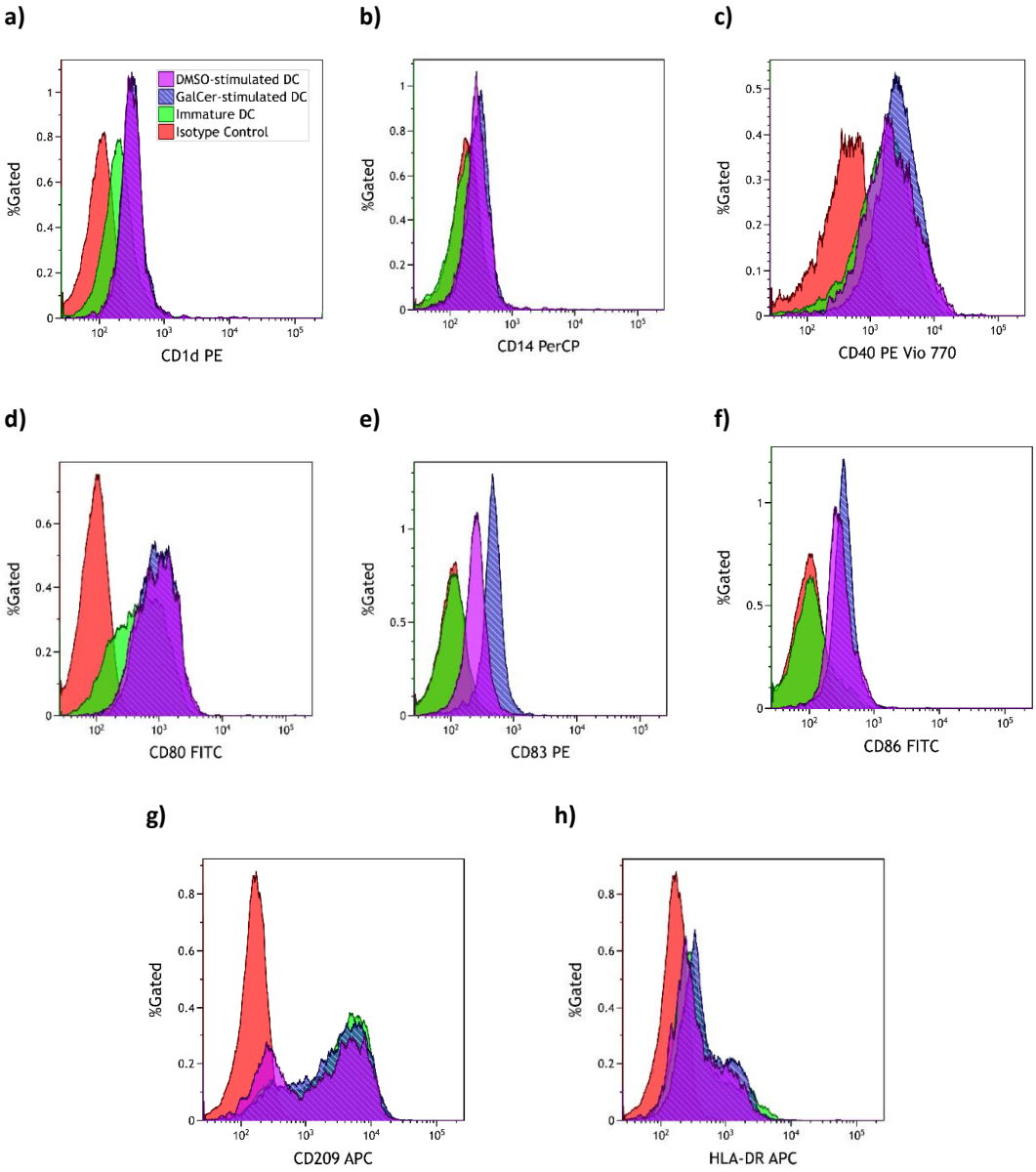


Figure 4.9. Phenotypes of α-GalCer- and DMSO-stimulated DCs. The flow cytometry histograms show the expression of DC markers CD1d, CD14, CD40, CD80, CD83, CD86, CD209, and HLA-DR (a-h) at Day 5 iDC stage (green) and Day 7 α-GalCer-stimulated DCs (blue) and DMSO-stimulated DCs (purple), compared with isotype controls (red). (N=1).

The stimulation of DCs using α-GalCer was replicated with 3 different blood samples (**Fig 4.10**). Despite the increase in CD83 expression in the histogram data above, replicate tests reveal the mean α-GalCer CD83 expression was 2.17 rMFi, compared to 1.00 rMFI in DMSO DCs. Two separate

paired t-tests were conducted: comparing iDC to α-GalCer and comparing iDC to DMSO marker expression. An unpaired t-test compared α-GalCer to DMSO marker expression. All three statistical test indicated no significant differences (p>0.05).

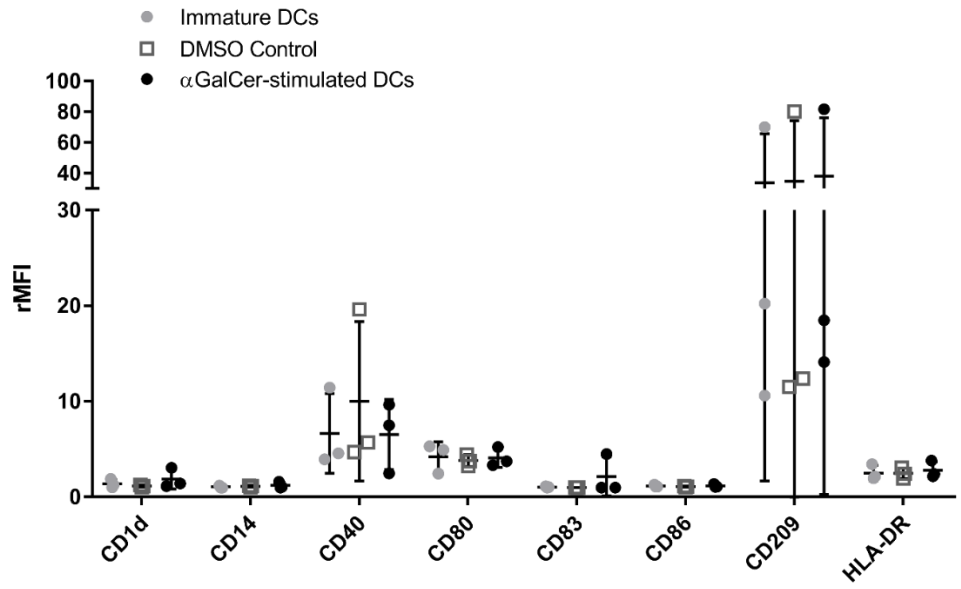


Figure 4.10. Average Expression Levels of surface markers on α-GalCer-and DMSO-stimulated DCs. The results of 3 experiments are shown, using 3 different blood samples. The rMFI of CD1d, CD14, CD40, CD80, CD83, CD86, CD209, and HLA-DR, was calculated for iDCs (grey circle) α-GalCer-stimulated DCs (black circle) and DMSO controls (square). Horizontal bars represent group means. Vertical bars represent standard deviation. Three t-test analyses were conducted: iDC compared to α-GalCer-stimulated DC marker expression, iDC compared to DMSO control marker expression, and α-GalCer-stimulated DC compared to DMSO control marker expression: no significant differences were observed for any marker in any of the 3 tests (p>0.05). (N=3).

This shows α-GalCer-stimulated DC phenotypes are not significantly different to DCs stimulated with DMSO. Crucially, it was hypothesised CD1d would be upregulated after stimulation with α-GalCer, based on literature from DC stimulation with other lipids (Abos Gracia et al. 2017), as this molecule presents lipids on the DC surface. However, no increases were observed. Overall, α-GalCer-stimulated DCs unexpectedly did not alter the phenotype compared to iDCs after 24 hours of stimulation.

This section has shown iDCs can be successfully generated and matured by LPS, exhibiting the expected phenotypes. Stimulation with the egg allergen, OVA, and glycolipid, α-GalCer, resulted in no significant upregulation of maturation markers. Looking at maturation data is one method to assess whether the protein or lipids influenced DC phenotype, thus this data may suggest the protein or lipids were not taken up by iDCs, or simply they were internalised but did not alter phenotype.

4.3.4. DC Internalisation of Proteins and Lipids

As internalisation of allergens and lipids is crucial for the iNKT-DC co-culture experiment, the internalisation of OVA and α-GalCer was investigated by imaging flow cytometry. **Figure 4.11** demonstrates OVA-stimulated DCs displayed internalisation of Cy5-conjugated OVA, with strong surface expression of HLA-DR. Although, it was expected that OVA would also be imaged near the cell surface, similar to existing research (Chiang et al. 2016). The presence of HLA-DR is important to highlight, as this is a marker for MHC II molecules, which presents proteins to T cells. This confirms the DCs are internalising the protein allergen ovalbumin, and that the method of stimulating DCs with the proteins is allowing DC internalisation.

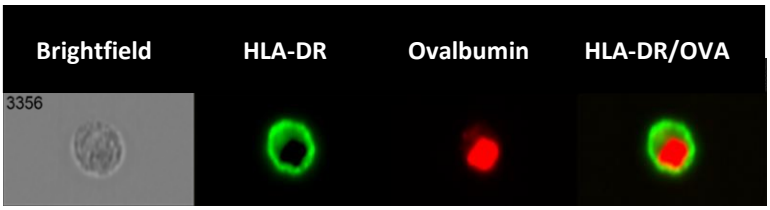


Figure 4.11. ImageStream Analysis of Ovalbumin Internalisation by DCs. OVA-pulsed DCs were analysed on the ImageStream MkII for HLA-DR (green) to identify the DCs, and the presence of Cy5-OVA (red). The brightfield of the DC is shown on the left. N=1.

Once OVA internalisation had been shown to be successful, α-GalCer internalisation was investigated. Unlike OVA, which was manufactured

conjugated to Cy5, α-GalCer was not conjugated to a fluorophore. There was difficulty in sourcing a fluorescent α-GalCer commercially, but, eventually, a 'Dansylated' α-GalCer was purchased, which is a fluorescent derivate of α-GalCer. DCs were cultured with dansylated α-GalCer and both conventional flow cytometry and imaging flow cytometry were utilised to determine if α-GalCer had been internalised. The conventional flow cytometry plots shown in **Figure 4.12A** indicates 78.9% of DCs were expressing CD1d, with 50.61% of DCs positive for dansylated α-GalCer and also positive for CD1d, the molecule crucial for presenting lipids. Imaging flow cytometry of this internalisation is presented in **Figure 4.12B,** where a-GalCer (purple) can be visualised inside of the DC. Thus, despite no effect on DC maturation, this imaging cytometry confirmed that α-GalCer had been internalised by DCs, and can be co-expressed with CD1d. This ensured that the lipid could then be presented by DCs to iNKTs, and its effect on iNKT cytokine production examined.

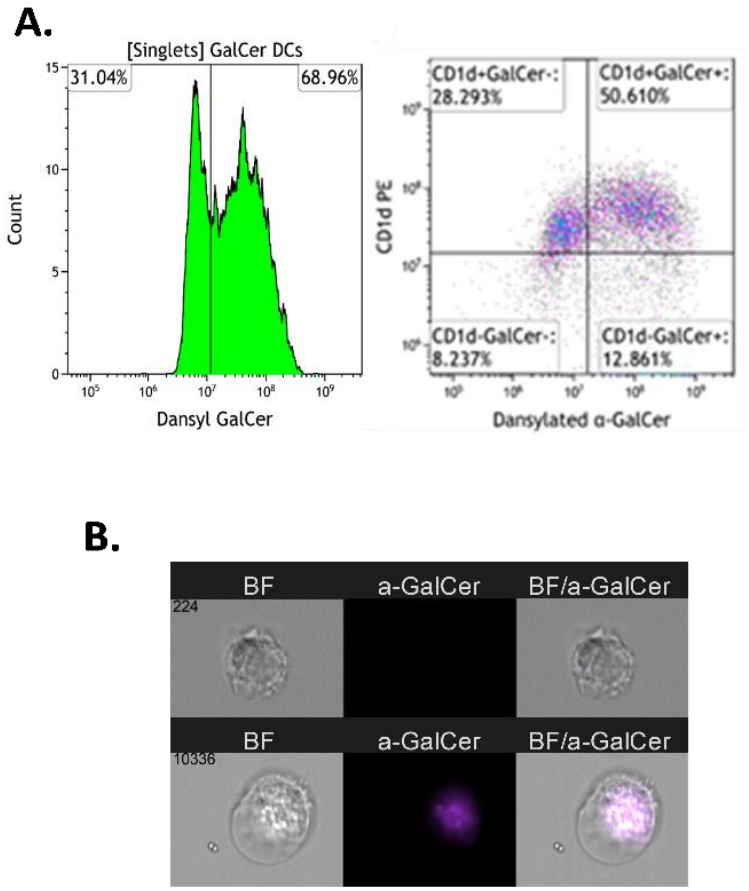


Figure 4.12. DC internalisation of the lipid α-GalCer. (A) Representative dot plot indicating percentage of DCs that have bound to the fluorescent dansylated α-GalCer. The dot plot also indicates the percentage of DCs that have bound α-GalCer, as well as express CD1d. (B) ImageStream data to show DC internalisation of α-GalCer (purple) in α-GalCer-pulsed DCs. The top bar indicates where the image is showing brightfield (BF) (left), showing α-GalCer channel only, or showing both BF and α-GalCer channels. N=1.

Overall, the investigations into DC internalisation of proteins were successful. ImageStream analysis revealed DC uptake of the protein OVA, with expression of MHC II. This provides a proof of concept that proteins can be taken up by iDCs and potentially presented on MHC II molecules *in vitro*. This method was then applied to examine DC uptake of the lipid α-GalCer. Once a fluorescent, dansylated, α-GalCer was utilised, internalisation of the lipid by

DCs was successfully imaged. Furthermore, DCs expressed CD1d, which was important to show as it is the molecule that presents lipids to iNKT cells.

4.3.3. CD1d Expression on DCs

Despite 78.9% of DCS expressing CD1d after dansylated α-GalCer-stimulation (**Fig. 12a**), its expression was lower than expected when evaluating the relative MFI of CD1d. CD1d expression on DCs is key to this research as it is the proposed route of lipid presentation to iNKT cells during allergic sensitisation. Throughout the generation of DCs, it was evident the rMFI of CD1d was consistently low, as illustrated in previous **Figures 4.5-4.10**. CD1d was also not upregulated on α-GalCer stimulated DCs, which was unexpected. Thus, despite a high percentage of DCs expressing CD1d, further experiments aimed to investigate the low rMFI of CD1d and determine if it could be increased to optimise lipid presentation to iNKTs in future experiments.

Initially, PBMCs were stained with the CD1d antibody to check the percentage of cells expressing CD1d before DCs were generated. **Figure 4.13b** indicates CD1d is expressed on 27.48% of PBMCs. CD14+ gating for monocytes highlighted the monocytes (purple) are the primary cells expressing CD1d, with a small percentage of lymphocyte gated cell (blue) also expressing CD1d, as shown in **Figure 4.13c**.

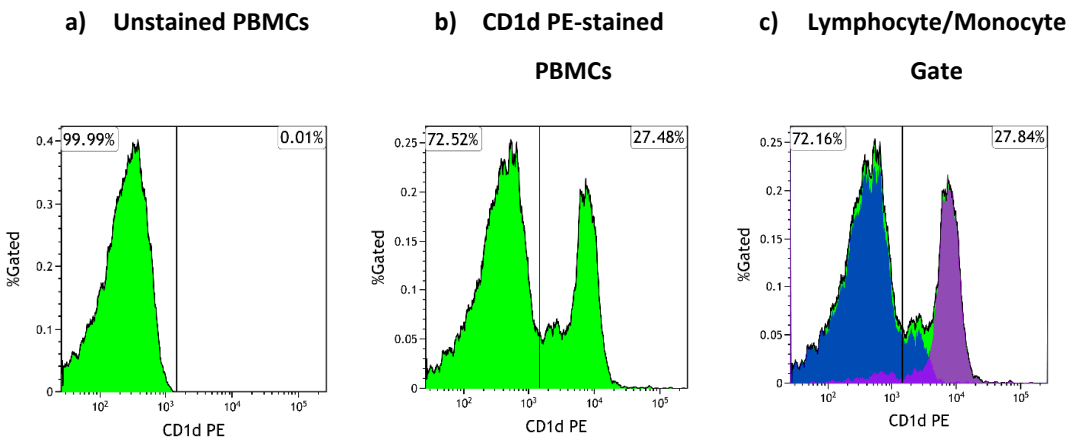


Figure 4.13. CD1d Expression on PBMCs. Flow cytometry analysis of CD1d expression on PBMCs. a) unstained human PBMCs used to determine CD1d^{+} gate setting. b) CD1d PE-

stained human PBMCs with 27.48% CD1d$^+$ cells. c) human PBMCs coded by cell type: lymphocytes (blue) and monocytes (purple). (n=1).

The number of cells expressing CD1d were then analysed during CD14 immunomagnetic isolation, to confirm this expression is maintained. PBMCs, the CD14$^-$ fraction of immunomagnetic separation, and the final CD14$^+$ fraction were stained for CD1d and analysed by flow cytometry (**Fig. 4.14).** The histograms display an initial population of 21.89% CD1d$^+$ cells in PBMCs. Only 6.89% of the CD14 negative fraction express CD1d$^+$ cells. The final CD14$^+$ fraction indicates 91.04% of the CD14$^+$ monocytes are CD1d$^+$. Thus, the majority of monocytes express CD1d.

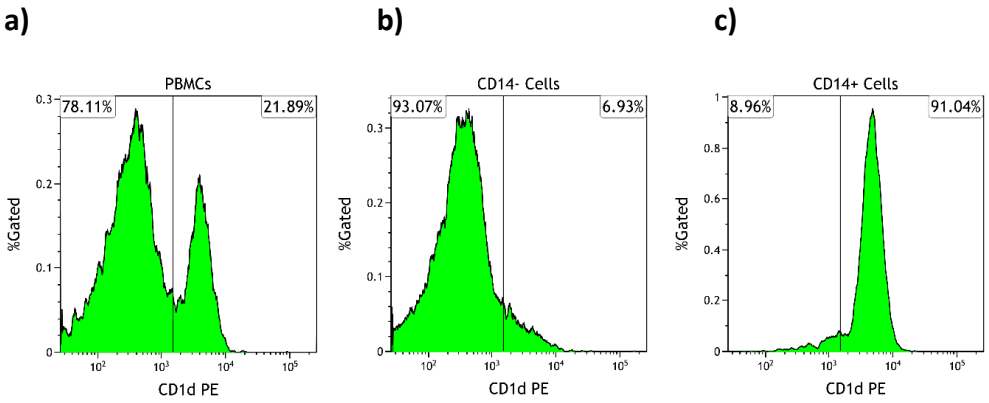


Figure 4.14. CD1d Expression throughout CD14+ immunomagnetic isolation. Flow cytometry analysis of CD1d expression on cells throughout the immunomagnetic separation of CD14 monocytes. a) CD1d expression on PBMCs. b) CD1d expression on CD14 negative fraction of immunomagnetic isolation. c) CD1d expression on the cells present in the final CD14 positive fraction. (N=1).

This demonstrates the number of monocytes expressing CD1d was high after immunomagnetic isolation, and that the low expression of CD1d seen in the stimulated DC graphs must be after the monocytes were placed in culture. Thus, CD1d expression was analysed by flow cytometry at ~24 hour intervals from the point of placing monocytes into culture with GM-CSF and IL-4. **Figure 4.15** shows CD1d expression decreased during culture, predominantly occurring within the first 22 hours of incubation. Furthermore, rMFI calculations at 0 hours of monocytes being placed in culture indicate the rMFI

of CD1d was 6.84, but decreased after 22 hours in culture to 3.29. CD1d rMFI continued to decrease at 48 hours in culture where the rMFI was 2.48.

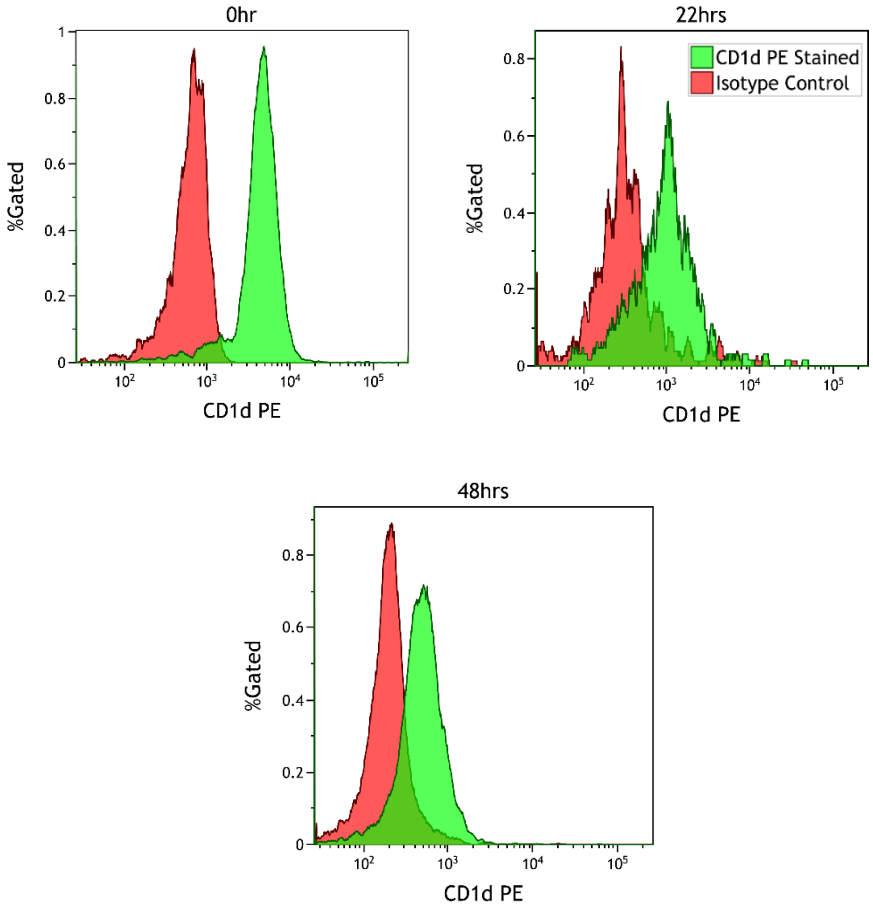


Figure 4.16. CD1d expression on monocytes at 24hr intervals during culture. Flow cytometry analysis of CD1d expression on monocytes while being cultured with GM-CSF and IL-4, at ~24 hour intervals. The isotype control (red) is plotted against the monocytes stained for CD1d PE (green). (N=1).

CD1d expression throughout culture was then analysed in 4 different human blood samples (**Figure 4.17**), with the significance of differences in CD1d expression at 0 hours of culture to 24 and 48 hours analysed by One-way ANOVA. The analysis revealed monocytes expressed an average of 7.10 rMFI (SD=1.04) at 0hrs. Supporting the histogram data above, monocytes were significantly downregulated after 24 hours in culture ($p=0.0002$), now expressing 2.43 rMFI of CD1d (SD=0.63). CD1d expression was even more significantly downregulated by 48 hours of culture ($p<0.0001$), where monocytes expressed a mean of 1.77 rMFI (SD=0.48).

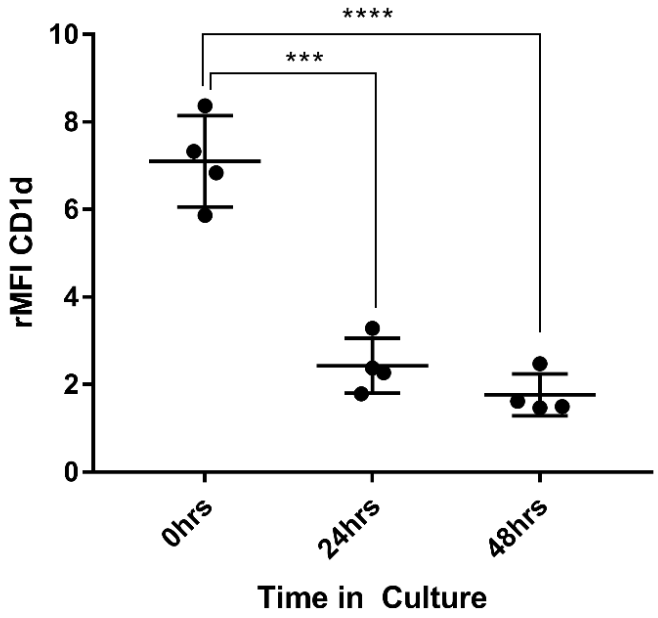


Figure 4.17. Average CD1d Expression during monocyte culture. CD1d expression on monocytes was analysed at 0hrs, 24hrs, and 48hrs of culture with IL-4 and GM-CSF. The rMFI values of CD1d from 4 different samples are depicted. Horizontal bars represent group means. Vertical bars represent standard deviation. RM One-way ANOVA with Dunnett's multiple comparison test compared CD1d expression at 0hrs to 24 and 48 hours in culture: ***p<0.001, ****p<0.0001. (N=4).

For completion, CD1d expression was analysed past 48 hours of culture to compare expression on iDCs and stimulated DCs. This confirms previous results of continued downregulation in iDCs and stimulated iDCs (**Fig. 4.18)** demonstrating there is further downregulation from a rMFI of 7.10 at 0 hours, to 1.77 rMFI at 48 hours of culture, to now 1.71 rMFI on iDCs. This downregulation continues when iDCs are stimulated with LPS (Mean= 1.44 rMFI), OVA (Mean =1.47 rMFI), α-GalCer (Mean= 1.36 rMFI) and the DMSO control (Mean=1.29 rMFI). A repeated-measures One-Way ANOVA was conducted with Dunnett's multiple comparisons test, comparing each stimulated DC to iDC expression, to determine if there was significant upregulation or downregulation. There were no significant differences ($p>0.05$). Thus, despite the continued trend of downregulation on stimulated DCs, this downregulation was not significant.

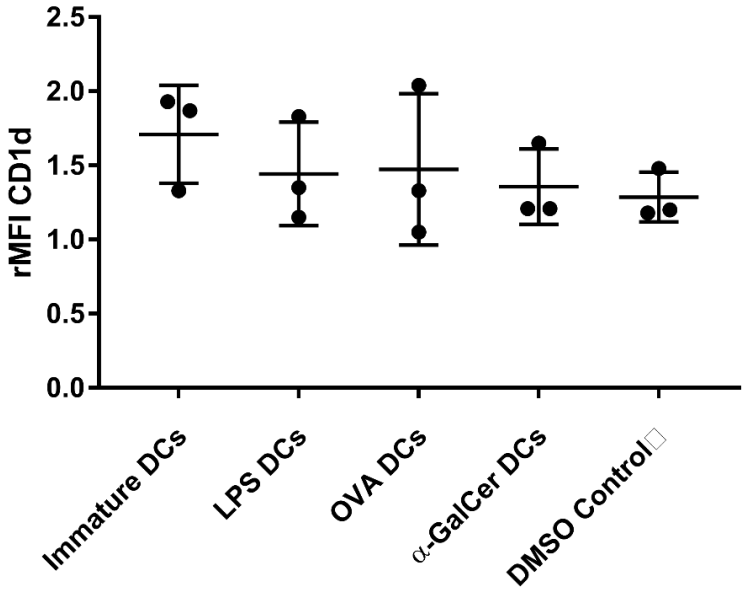


Figure 4.18. CD1d Expression of iDC, LPS-, OVA-, a-GalCer-, and DMSO-stimulated DCs. The average rMFI of CD1d on iDCs, LPS DCs, OVA DCs, a-GalCer DCs, and DMSO DCs. 3 different blood donors represented by data points. RM one-way ANOVA comparing iDCs CD1d expression to LPS-matured DCs, OVA-stimulated DCs, a-GalCer- stimulated DCs, and DMSO control DCs: no significant differences. (N=3).

The data demonstrates the majority of CD1d downregulation on DCs occurred within the first 24 hours of monocyte culture. Further downregulation continues throughout culture into iDCs and stimulated-DCs, but to a lesser extent than the first 24 hours of culture. It is possible that CD1d is downregulated due to components within the culture medium. We therefore aimed to alter the culture conditions for iDC generation to reduce this downregulation of CD1d, as this may enhance the presentation of lipids to iNKT cells during iNKT:DC co-culture.

4.3.4. The Influence of Media on CD1d Expression

In all aforementioned experiments, monocytes were cultured in RPMI supplemented with 10% FBS, along with cytokines GM-CSF and IL-4 to

stimulated DC generation. Literature has suggested FBS can contribute to downregulation of CD1d in culture ((Ronger-Savle et al. 2005), which could explain the downregulation of CD1d in the first 24 hours of culture. Furthermore, guidelines given by one of the funding bodies to this research, Unilever, stated non-animal products must be used wherever possible. Thus, different types of medias and supplements (predominantly human-derived) were analysed for their effect of CD1d expression.

The previously used culture media, RPMI media supplemented with 10% FBS, was used throughout these experiments as a comparison. The different medias analysed included RPMI supplemented with either 10% human AB serum, or 1% autologous plasma (AP). As well as the serum-free media AIM V, which was used without the addition of serum, or with the addition of 1% AP. As indicated in **Figure 4.19,** at 0hrs of culture, the rMFI of CD1d was 5.87. There is still downregulation of CD1d expression within 24 hours of culture, in all media conditions (**Figure 4.19b)**. After 48 hours in culture, the flow cytometry plots indicate CD1d expression is then further downregulated in all conditions (**Figure 4.19c)**. However, monocytes cultured in RPMI + 10% FBS exhibited lower CD1d expression than monocytes cultured in RPMI with 10% AB serum and AIM V with 1% AP. These two medias resulted in the least downregulation of CD1d at 48 hours of culture.

Also, an unstimulated DC control was tested to ensure the downregulation was not due to stimulation of monocytes with GM-CSF and IL-4. The results indicated the same downregulation as all other media conditions, suggesting the downregulation was not due to GM-CSF or IL-4.

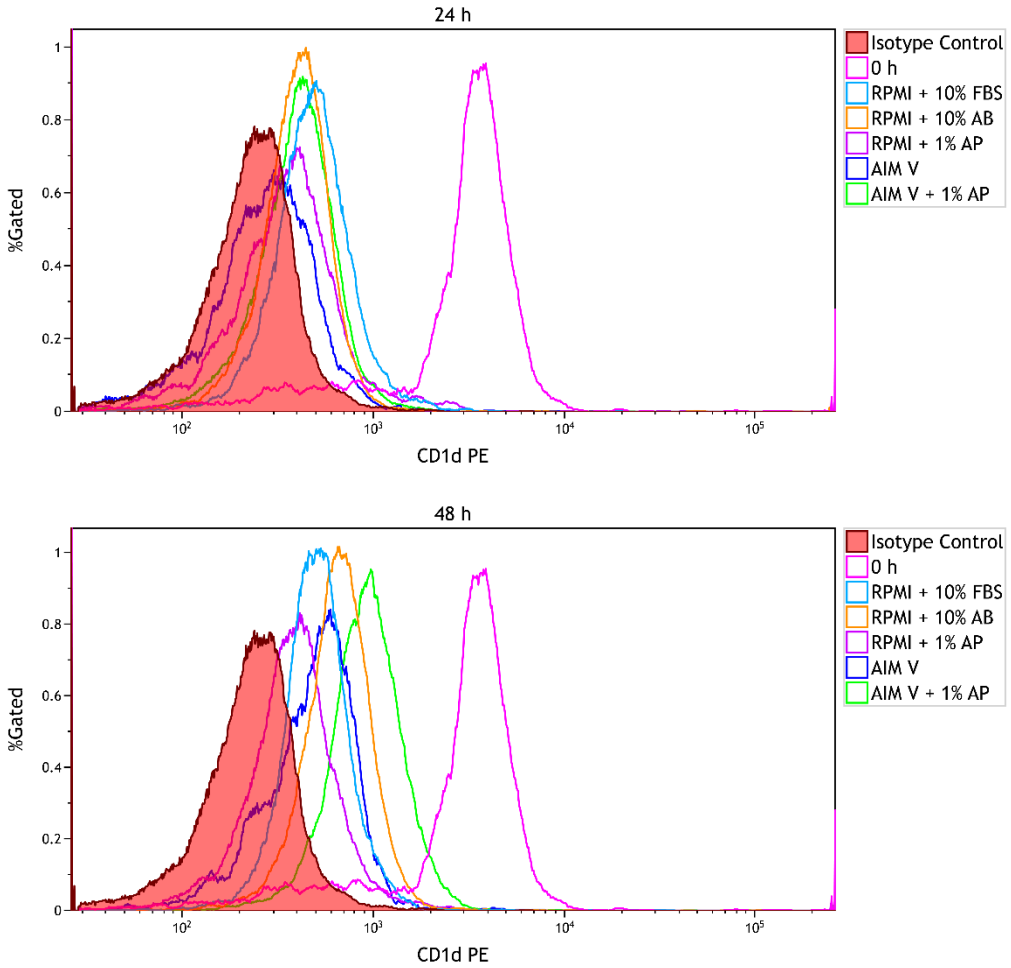


Figure 4.19. CD1d expression in different medias. Flow cytometry analysis of CD1d expression on monocytes cultured in different medias and supplemtents. (Top) 24 hours of culture; isotype control (red), 0 hours of monocyte culture (pink), and CD1d expression of monocytes cultured in RPMI + 10% FBS (light blue), RPMI + 10% AB serum (orange), RPMI + 1% AP (purple), AIM V (dark blue), and AIM V + 1% AP (green). c) 48 hours of culture; same key as b). (N=1).

This experiment was then replicated using different blood donors (**Fig. 4.20**). A One-Way ANOVA was conducted to compare the rMFI of CD1d in each media condition at 24 and 48 hours of culture, to the CD1d expression at 0 hours (before placed in culture), to determine which media resulted in the highest downregulation of CD1d expression. The average rMFI of CD1d at 0 hours was 7.58 (N=4). At 24 hours of culture, all conditions showed significant downregulation of CD1d compared to 0 hours of culture. RPMI supplemented with 10% FBS had an average 2.15 rMFI of CD1d (N=5, $p<0.0001$), and the rMFI of CD1d for RPMI supplemented with 10% AB was 2.06 (N=6, $p<0.0001$). RPMI

with 1% AP had a 1.89 rMFI of CD1d (N=3, p<0.0001) and AIM V with 1% AP had a 1.98 rMFI (N=4, P<0.05). AIM V alone had the lowest rMFI of 1.10 (N=3, p<0.0001). Thus, similar to results in the histograms above, the rMFI of CD1d at 24 hours was similar in each condition. This excludes AIM V with no supplement which had a lower CD1d rMFI than the other conditions at 24 hours of culture. Although, all results were significantly lower than CD1d expression before culture.

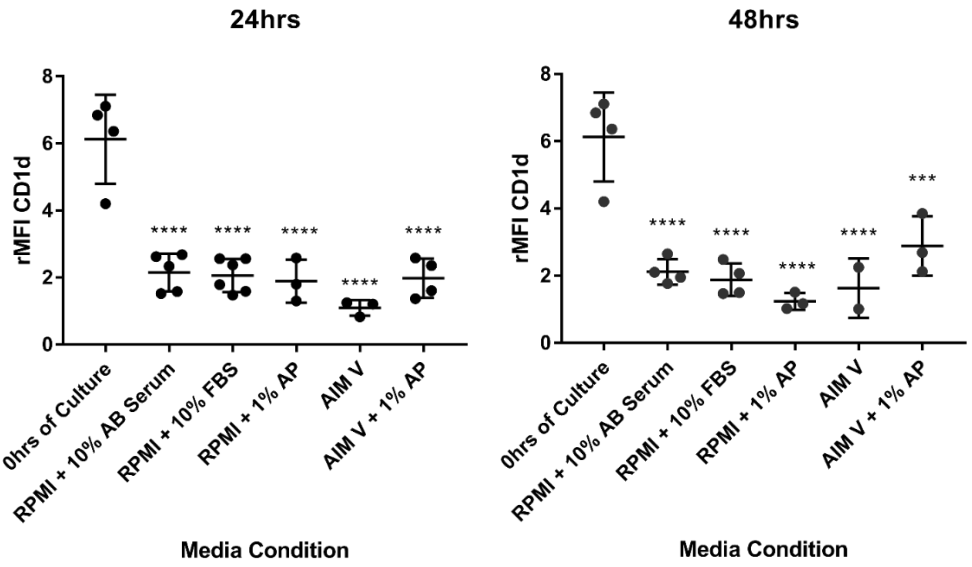


Figure 4.20. Average CD1d expression of monocytes in different medias. The average rMFI of CD1d for monocytes cultured in different medias and supplements, in the presence of GM-CSF and IL-4. Each data point represents results from a different donor. One-way ANOVA with Dunnett's multiple comparisons test of CD1d on monocytes at 0hrs of culture vs. CD1d of monocytes in each media condition at 24hrs and 48hrs of culture: ****p<0.0001, ***p<0.001.

At 48 hours in culture, the CD1d expression on monocytes in each condition began to differ. All monocytes still showed significantly lower CD1d expression compared to 0 hours of culture. The highest average rMFI of CD1d at 48 hours was on monocytes cultured with AIM V + 1% AP (rMFI 2.89, N=3), thus showing the least downregulation compared to 0 hours (p<0.001). The rMFI of monocytes cultured in RPMI supplemented with 10% AB serum expressed the next highest level of CD1d, with an average rMFI of CD1d 2.12

(N=4, p<0.0001). There was also significant downregulation of CD1d from 0 hours to 48 hours in the remaining conditions; RPMI + 10% FBS (rMFI=1.88, N=4, p<0.0001), RPMI + 1% AP (rMFI=1.23, N=3, p<0.0001), and AIM V with no supplement (rMFI=1.63, N=2, p<0.0001). These results indicate CD1d is significantly downregulated at 24 hours of culture and 48 hours of culture in all media conditions. However, AIM V + 1% AP and RPMI + 10% AB serum demonstrated the highest expression throughout.

In conclusion, the results show RPMI + 10% FBS does result in greater downregulation of CD1d than monocytes cultured with RPMI + 10% AB or AIM V + 1% AP. These two conditions resulted in the least downregulation of CD1d, with little differences in CD1d expression between the two. In addition to optimising CD1d expression, it was also important to determine which media maintained the highest viability of cells in culture. This was especially important to help distinguish between these two optimum medias for CD1d expression. This will ensure the best CD1d expression and the best yield of DCs for the DC:NKT co-culture in future experiments.

Thus, viability staining of the monocytes at 24 hour intervals during culture was conducted. **Figure 4.21** shows exemplar viability data for the different culture media tested. **Figure 4.21a** shows monocytes had a viability of 78.15% at 0 hours of culture. After 24 hours of culture, monocytes cultured in RPMI supplemented with 10% human AB serum (**Figure 4.21b**) had the highest number of viable cells (65.06%). AIM V + 1% AP cells (**Figure 4.21c)** had 50.26% viability, RPMI + 10% FBS cells (**Figure 4.21d**) had 45.35% viability, RPMI + 1% AP cells (**Figure 4.21e**) had 41.95% viability, and AIM V alone (**Figure 4.21f**) had the lowest viability of 26.67%. Therefore, at 24 hours of culture, RPMI + 10% AB serum was the optimum media for maintaining cell viability in this example.

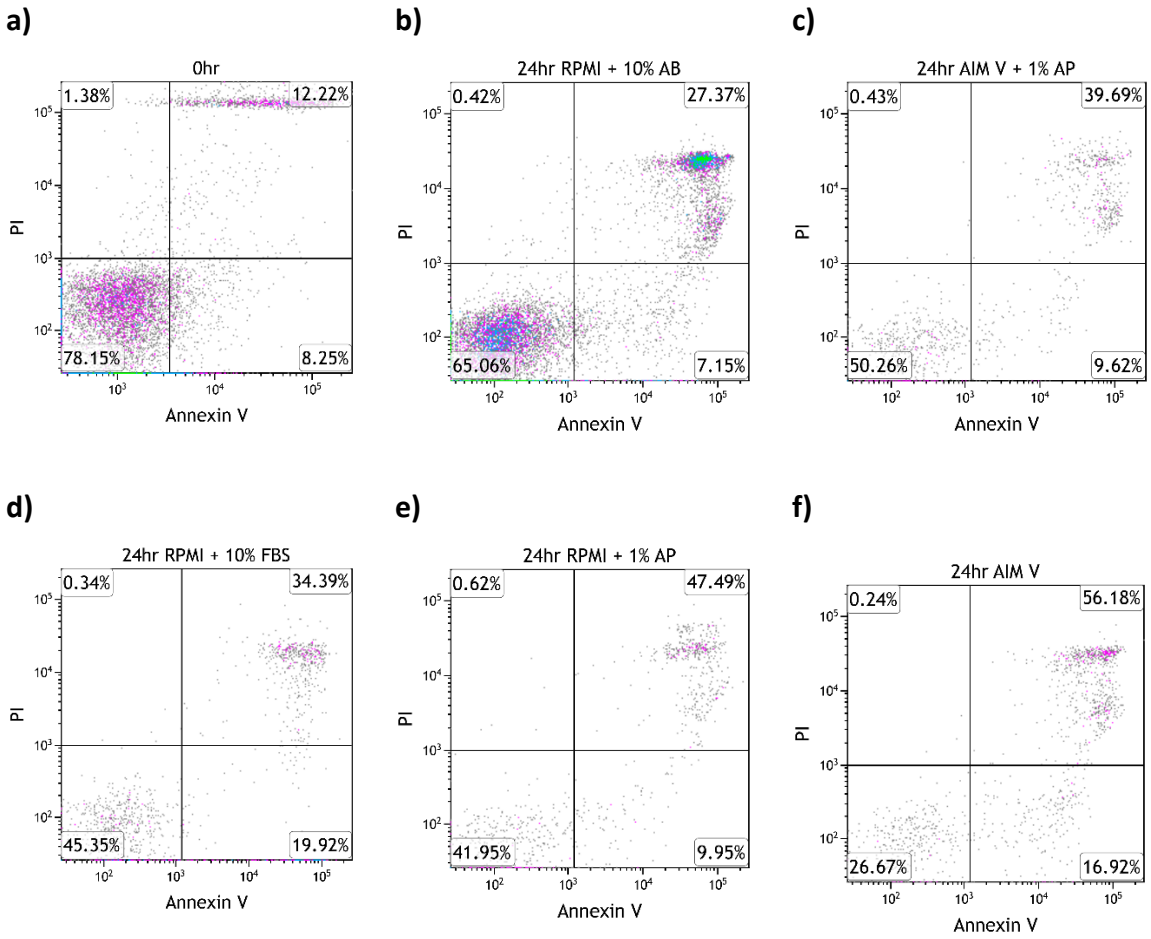


Figure 4.21. Viability staining of monocytes at 0 and 24 hours of culture in different medias. Annexin V and PI staining of cells was conducted at 24 hours of culture with different medias (a-f). The figure shows flow cytometry density plots with viable cells in the quadrant Annexin V negative and PI negative. Dead cells are found in the upper right quadrants, Annexin V positive, PI positive (N=1).

Figure 4.22 shows exemplar data after 48 hours of culture. Monocytes cultured in RPMI supplemented with 10% human AB serum (**Figure 4.22a**) had the highest number of viable cells again at 76.7%. AIM V + 1% AP cells (**Figure 4.22b**) had lower a lower viability of 56.63%. AIM V alone (**Figure 4.22c**) had 54.56% viability, RPMI + 10% FBS cells (**Figure 4.22d**) had 51.96% viability, and RPMI + 1% AP (**Figure 4.22e**) cells had the poorest cell viability of 48.29% in this example.

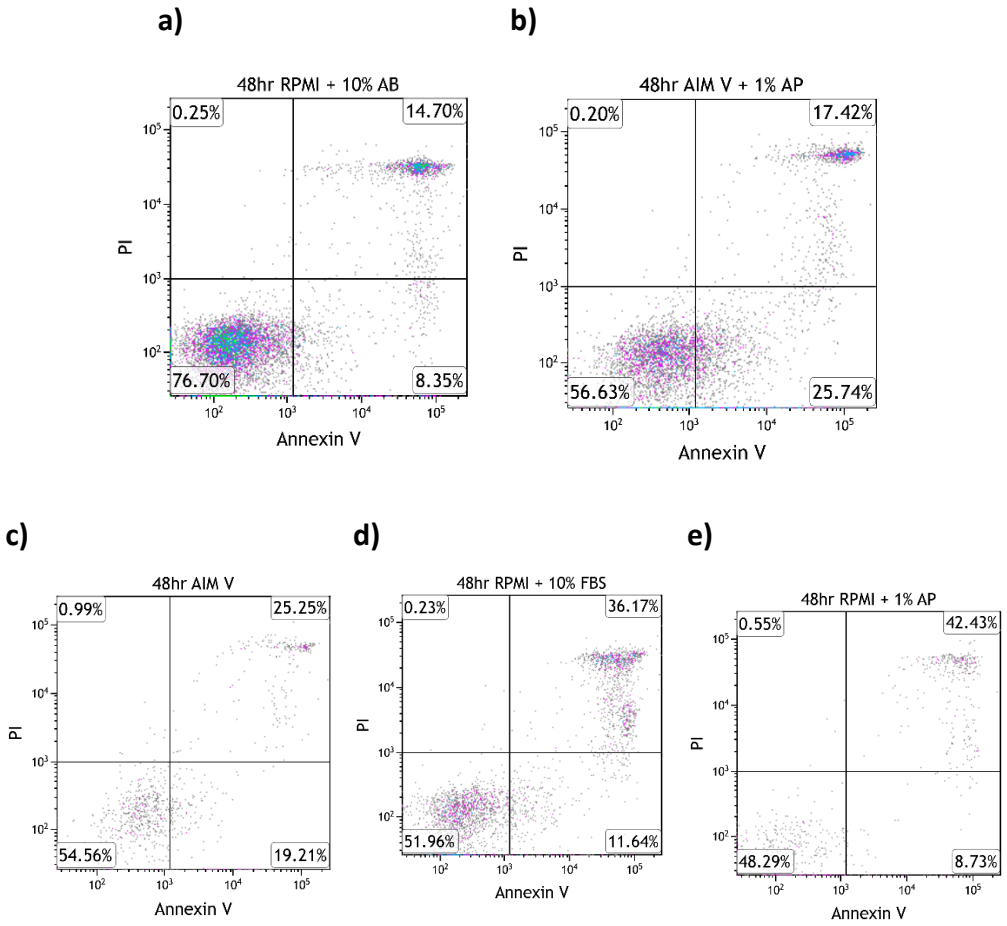


Figure 4.22. Viability staining at 48 hours in culture. Annexin V and PI staining of cells was conducted at 48 hours of culture with different medias (a-e). The figure shows flow cytometry density plots with viable cells in the quadrant Annexin V negative, PI negative. Dead cells are found in the upper right quadrants, Annexin V positive, PI positive. N=1.

Figure 4.23 represents repeated testing of the viability of monocytes cultured in the different medias, using different blood donors. The results show RPMI + 10% AB serum yielded the highest viability at an average of 67.95% at 24 hours in culture (N=6, SD=10.5), and 60.63% at 48 hours (N=2, SD=2.4). Cells cultured in RPMI + 10% FBS had the next highest viability of 56.47% at 24 hours (N=5, SD=14.5) and 55.37% at 48 hours (N=2, SD=23.4). AIM V + 1% AP then had an average viability of 52.62% at 24 hours (N=5, SD=12.2), and 36.36% at 48 hours (N=2, SD=3.0). Monocytes cultured in RPMI + 1% AP presented 47.99% viability at 24 hours (N=4, SD=19.4) and 24.86% at 48 hours of culture (N=2, SD=4.8). The media resulting in the lowest viability of cells was AIM V with no

supplement: 21.17% (N=3, SD=4.7) at 24 hours of culture, and 27.54% (N=2, SD=9.6) and 48 hours of culture.

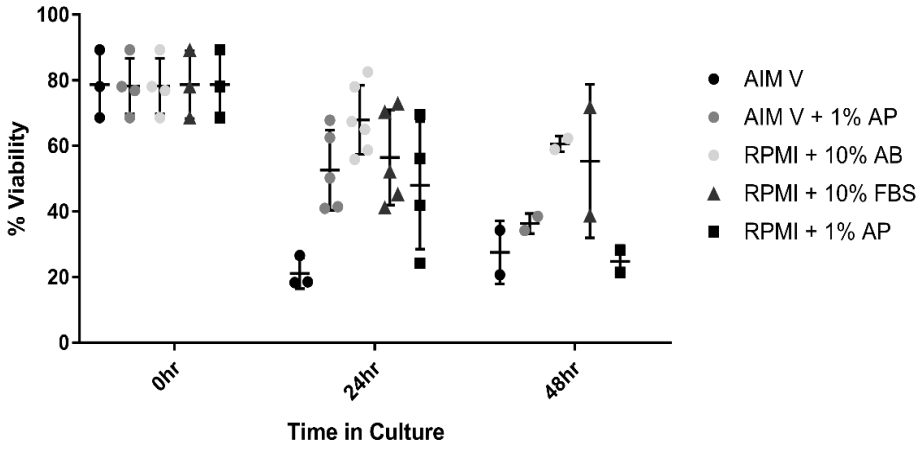


Figure 4.23. Viability of monocytes up to 48 hours of culture. The average viability of monocytes at 0, 24, and 48 hours in culture with different medias. Each data point represents a different blood sample. Horizontal bars represent group means. Vertical bars represent standard deviation.

The data so far suggest monocytes cultured in AIM V supplemented with 1% AP and RPMI supplemented with 10% AB consistently demonstrate the least downregulation of CD1d. Based on the viability data, it was decided to increase the autologous plasma supplement from AIM V + 1% AP to 2% AP to improve viability of cells. These two conditions were then used to examine CD1d expression for the full DC time course, as well as for LPS, OVA, DMSO, and α-GalCer-stimulated DCs. **Figure 4.24** shows there was then little difference in CD1d expression between the two conditions.

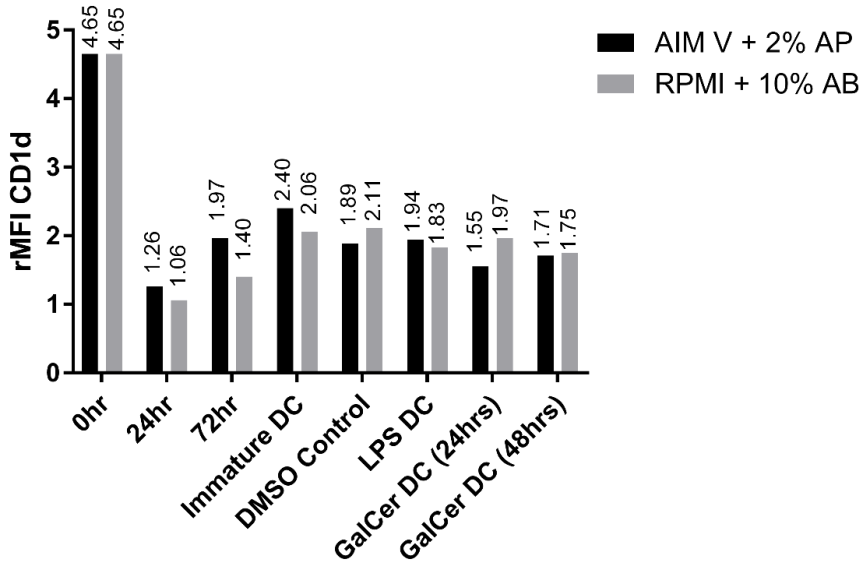


Figure 4.24. CD1d expression across DC time course in two different medias. A comparison of the rMFI of CD1d on monocytes cultured in either AIM V + 2% AP (black) or RPMI + 10% AB (grey), from 0 hours in culture, to mature DCs. (N=1).

Overall, culturing monocytes in RPMI supplemented with 10% AB serum resulted in the highest viability of cells. Combining the rMFI of CD1d and the viability data, it was decided RPMI supplemented with 10% human AB serum was the optimal culture medium for generating DCs for this study.

4.4. Discussion

DCs are critical for the presentation of allergens which pass through the epithelial barrier of mucosal surfaces (Hole et al. 2019). DCs then present the allergens to immune cells to initiate the development of allergic sensitisation. Recent research has shown DCs also present lipids to immune cells in allergic sensitisation, via CD1d molecules on its cell surface. The aim of these experiments were to successfully generate DCs for the subsequent experiments of DC-iNKT co-culture. Conditions for DC CD1d expression were also optimised and the internalisation of proteins and lipids were analysed to test the predicted mechanism of lipid presentation.

$CD14^+$ monocyte immunomagnetic isolation consistently resulted in high purities of between 89-98%. Lymphocytes were the main contaminant of the $CD14^+$ population, though this was expected using positive isolation of CD14 cells, as B cells also weakly express CD14 (Ziegler-Heitbrock et al. 1993) (Ziegler-Heitbrock et al.,1993). The contamination seen here is minimal compared to other methods, such as negative selection of CD14 monocytes, which results in high numbers of platelet contamination, reducing the final purity of monocytes to a maximum of 85% (Nielsen, Andersen, and Møller 2020).

Monocyte-derived iDCs were successfully generated using GM-CSF and IL-4, followed by the standard method of LPS to mature DCs. This was demonstrated by phenotypic analysis, where iDCs did not express CD14, but did express low levels of molecules such as CD1d, CD40, CD80, CD86, and HLA-DR, as well as high levels of CD209. This iDC phenotype is consistent with published literature (Andreae et al. 2002). LPS-matured DCs also expressed increases in the expected markers of DC maturation: CD80, CD83, and CD86. Although, CD83 was not significantly upregulated, potentially due to the small sample size which reduced the power of the analysis.

The egg protein, OVA, was then used to stimulate iDCs. These OVA-stimulated DCs also showed some increases in CD1d, CD40, CD80, CD83, CD86,

and HLA-DR, but none of these increases were statistically significant. This suggests the small sample size (n=3) was hindering significance, or that OVA simply did not mature DCs after 24 hours of culture. Studies which pulsed bone-marrow derived iDCs with OVA also found no significant increases in markers CD40, CD80, CD209, or MHC class II (Hao et al. 2007). It must be noted that the OVA was not purchased as endotoxin-free, so is likely contaminated with small amounts of LPS which may have influenced any increase in DC marker expression.

α-GalCer stimulation to iDCs resulted in no significant upregulation of maturation markers after 24 hours. Furthermore, findings from Nicol et al. (2011), which found α-GalCer-pulsed iDCs only matured when the DCs were cultured with iNKTs, which could indicate why the DCs were not matured in our experiments. This is further clarified by research which demonstrated NKT cells were essential for the maturation of DCs by α-GalCer, and without them, DCs did not show upregulation of maturation marker CD86 (Fujii et al. 2003). This contrasts to studies involving other lipids which found maturation markers did increase when stimulated with pollen lipids, without NKT cells present (Abós-Gracia et al. 2013).

As the maturation markers were not significantly upregulated in response to the allergen, OVA, or the lipid, α-GalCer, imaging cytometry analysis of their internalisation was conducted to confirm the DCs had in fact internalised the protein and lipid. OVA is taken up by DCs by receptor-mediated endocytosis, such as by mannose receptor-mediated endocytosis, to present to OVA-specific CD8+ T Cells (Burgdorf et al. 2010). This work demonstrated that Ovalbumin was successfully imaged to show internalisation by DCs. DCs which internalised OVA also expressed HLA-DR, which is important as it shows MHC class II molecules are present, which are the molecules which present proteins to T cells.

DC uptake of lipids is less understood. Different mechanisms have been described for the uptake of lipids, including phagocytosis and C-type lectins that can bind mannose residues (Barral and Brenner 2007). The fluorescently-

tagged α-GalCer (dansylated α-GalCer) was purchased and used to stimulate DCs. After 24 hours, approximately 50% of DCs had successfully internalised α-GalCer, as shown by imaging cytometry, and, importantly, this 50% were also expressing CD1d, the molecule which presents lipids.

The presence of CD1d on DCs is essential to present lipids to iNKT cells during allergic sensitisation. There was expression of CD1d on DCs, but rMFI analysis shows this is expression was low. We were concerned of the ability of DCs to present lipids to iNKT cells if the expression was low, thus, CD1d expression was further investigated. We found CD1d expression was high in PBMCs, but was downregulated by the iDC stage. The results show CD1d expression was not downregulated due to immunomagnetic separation or due to a poor antibody. It was discovered the downregulation of CD1d was occurring within 22 hours of monocyte culture with GM-CSF and IL-4. Flow cytometry data revealed this reduced expression was a result of media and supplement. The culture media used was RPMI supplemented with 10% FBS. However, literature highlights FBS can decrease CD1d expression on DCs, whereas other supplements, such as autologous plasma, does not (Gerlini et al. 2001).

Thus, five combinations of various medias and supplements were tested to optimise CD1d expression. RPMI supplemented with 10% human AB serum and AIM V supplemented with 2% autologous plasma resulted in the highest CD1d expression on DCs throughout culture. Surprisingly, the supplement-free medium AIM V resulted in the lowest CD1d expression. RPMI with AB serum also achieved the highest viability throughout monocyte culture, compared to all other medias. Thus, it was decided the optimum condition for monocytes was to be cultured in RPMI with 10% AB serum to generate DCs. This method results in least downregulation of CD1d, and maintains the highest percentage of viable cells.

Despite culture conditions being optimised, relative CD1d expression remained low. Similar research into stimulating human monocytes with pollen lipids suggest this low CD1d expression is expected and is sufficient to present

lipids to NKT cells (Abos Gracia et al. 2017). Importantly, they also found CD1d expression increased upon stimulation with pollen lipids after 48 hours. However, there is limited research on the effect of lipids on CD1d expression, thus, it was important to investigate whether a-GalCer supported findings from previous research and upregulated CD1d. Interestingly, when iDCs were stimulated with a-GalCer for 24 hours no upregulation of CD1d was observed. However, this may simply be due to the fact that different lipids have different effects on surface CD1d expression i.e. pollen lipids upregulate surface CD1d, but a-GalCer does not. Interestingly, lipid antigens which elicit a Th2 response when co-cultured with iNKT cells can directly bind surface CD1d instead of intracellular loading beforehand (Arora et al. 2016). α-GalCer produces Th1 and Th2 cytokines, so perhaps α-GalCer would bind CD1d molecules within the DCs endocytic compartments, which could explain why α-GalCer did not increase extracellular CD1d expression.

The present data show DCs were successfully generated from human-derived monocytes. The dendritic cells were also successfully matured by LPS, but maturation markers were not significantly up-regulated by OVA or α-GalCer. Importantly, CD1d expression on DCs was optimised by altering culture media, to reduce CD1d downregulation potentially caused by the use of FBS in media. The egg protein, OVA, and glycolipid, α-GalCer, were internalised by iDCs, as shown by imaging flow cytometry. Interestingly, α-GalCer did not upregulate CD1d expression on or within DCs, but 78.9% of DCs did express low level CD1d, as shown after dansylated GalCer internalisation, suggesting the CD1d-lipid system modelled should still work. Overall, iDC were generated and cultured with proteins and/or lipids. The next phase of optimisation experiments will test lipid and/or protein pulsed DCs co-cultured with iNKT cells and the resulting cytokine release will be observed.

Chapter 5: The Role of Peanut Lipids and iNKT cells in Allergic Sensitisation

5.1. Introduction

Peanuts are one of the most potent and prevalent allergen sources in developed countries, presenting a major cause of food allergy-induced fatalities in children and adults in the UK, due to the severity of allergic reactions, often causing anaphylaxis and occasionally death (Baseggio Conrado et al. 2021). The prevalence of peanut allergy in Western countries has doubled in the past decade, affecting around 1 in 50 individuals (Lieberman et al. 2021). The 'HealthNuts' study which utilised the gold-standard method of challenge-proven allergy to calculate the prevalence of peanut allergy, found that peanut allergy is the most prevalent food allergy in young children, despite decreasing from 3.1% at 1 year of age, to 1.9% at 4 years (Peters et al. 2017). And unlike other childhood allergies, such as milk and eggs, peanut allergy tends to persist into adulthood. Individuals often come into contact with peanuts through consumption, however, it's becoming increasingly common to use peanut oil in cosmetic products, allowing another route of contact with peanut allergens (Petersen et al. 2014b). Symptoms, such as anaphylaxis, can be triggered by miniscule amounts of peanut allergens, the threshold varying between peanut-allergic individuals, with the major peanut allergens being Ara h 1, Ara h 2, and Ara h 3 (Palladino and Breiteneder 2018). There are geographical differences between the prevalence of sensitisation to specific allergens, with individuals sensitised to Ara h 1 through to Ara h 15. But a study conducted across 11 European countries found Ara h 2 to be the major allergen which subjects were sensitised to (Ballmer-Weber et al. 2015), and it is thus conventional to test for Ara h 2-specific IgE to diagnose peanut allergy without the need for any other peanut allergens (Keet et al. 2021).

In addition to protein allergens, some research has shown the lipid content of allergen sources can promote or skew a Th0/Th1 non-allergic state, to a Th2-type allergic response (Abos Gracia et al. 2017; Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Mirotti et

al. 2013a; Pablos-Tanarro et al. 2018). Peanuts contain a high amount of fat content (~50%) which has been shown to comprise lipids associated to certain peanut allergens. For instance, the major peanut allergen, Ara h 1, has been shown to associate with phosphatidylglycerol vesicles (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, et al. 2016), as well as some minor peanut allergens, such as Ara h 8, have been identified as lipid-binding (Petersen et al. 2014b). Ara h 8 is the most well-established peanut allergen in terms of its ability to interact with lipids. Ara h 8 is a Bet v 1-like allergen, associated with mild allergic reactions and suggested to be responsible for oral allergy syndrome (OAS). OAS occurs due to sensitisation to airborne allergens, such as the Bet v 1 allergen from Birch pollen (Hurlburt et al. 2013), which then causes cross-reactivity when exposed to allergens such as Ara h 8, as it is a homolog to Bet v 1. This similar protein structure is thought to explain why some individuals sensitised to Bet v 1 then experience food allergies to peanuts. Existing, but limited, evidence has suggested Ara h 8 can bind fatty acids via a hydrophobic pocket, which can then interfere with allergen processing, such as by delaying its intestinal digestion (Petersen et al. 2014b). This can then enable an intact protein to be presented to the immune system, resulting in allergic sensitisation. IgE antibodies can then bind to epitopes (IgE binding sites) on the allergen's surface upon subsequent exposure to result in an allergic reaction (Pomés, Mueller, and Chruszcz 2020).

The literature investigating the role of lipids and lipophilic allergens is scarce. Although, some research has suggested the total lipid fraction from allergen sources can influence allergic sensitisation to the allergens (Abos Gracia et al. 2017; Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, et al. 2016). Thus, this research aimed to utilise the total lipid fraction from peanuts, and investigate whether this influences the cytokine production from iNKT cells.

It is hypothesised that lipids within an allergen function alongside the allergenic protein to cause further release of Th2 cytokines, leading to allergic

sensitisation. Thus, the total lipid fraction from peanuts with and without the presence of Ara h 8 will be analysed.

Previously, we have shown that iNKT cells can be expanded *in vitro* with the glycolipid α-GalCer to increase the number of cells. We have also shown that DCs can be generated from monocytes and internalise lipids and allergens. Thus, this chapter aims at co-culturing expanded iNKT cells with lipid and/or allergen pulsed DCs and subsequently measure any Th1 or Th2 iNKT cell cytokine production. The co-culture method was first optimised before applying to peanut lipids and peanut allergens, utilising cells from non-allergic and peanut-allergic individuals for comparison.

5.2. Materials and Methods

5.2.1. Subjects

Blood was obtained from 2 different groups: individuals with no allergies and individuals allergic to peanuts. Non-allergic and peanut-allergic subjects were recruited under ethics approved by the NHS Health Research Authority Research Ethics Committee (Ref 21/SC/0183). The blood samples were collected at Cripps Health Centre, Nottingham, by research nurses and then transported to the lab for immediate use in experiments. See Appendix B '**Appendix B: NHS HRA** REC Ethics Documents**'** for study documents relating to the ethics and recruitment of participants.

The eligibility criteria for both subject groups is highlighted in **Table 5.1**. The only difference in criteria between groups was non-allergic subjects had no history of IgE-mediated allergies, whereas peanut-allergic subjects had a clinical diagnosis of IgE-mediated allergy to peanuts.

Table 5.1. Study Eligibility criteria for Non-Allergic and Peanut-Allergy Subjects. Subjects were recruited based on their allergy status, age, smoker status, ability to attend the phlebotomy site and give informed consent. Individuals involved in any other research were excluded, as well as any students in the School of Life Science's Immunology department were excluded**.**

Inclusion criteria	Exclusion criteria
- No history of IgE-mediated allergy (Non-allergic Subjects). - Clinically diagnosed IgE-mediated allergy to peanuts (Peanut-Allergy Subjects)	Cigarette Smokers
Adults aged 18 or above	Are currently involved in other research.
Able to attend the QMC or Cripps Health Centre, Nottingham.	Are Students working in the University department where these studies are being performed.
Ability to give informed consent	

The demographics of the subjects recruited are displayed in **Table 5.2**. Information regarding the subject's age, sex, and form of allergy diagnosis (if applicable) were collected by questionnaire.

Table 5.2. Demographics of Subjects. The number of subjects, their age, sex, ethnicity, and route of allergy diagnosis, for non-allergic and peanut-allergic subjects.

	Non-Allergic	**Peanut-Allergic**
Number of Participants	6	6
Mean Age (Range)	(20-54)	(21-32)
Sex	Female: 4 Male: 2	Female: 4 Male: 2
Ethnicity	White British: 4 White Polish: 1 Latino: 1	White British: 5 Mixed Race: 1
Allergy Diagnosis	N/A	GP: 2 Skin-prick test: 4

5.2.2. Methods

5.2.2.1. IgE ELISAs

Plasma was isolated from subject blood samples and utilised in Total IgE ELISAs as well as allergen-specific IgE ELISAs. The total IgE ELISA was conducted according to the manufacturer's instructions (See section 2.2.10.1. Total IgE ELISA for full details). Ara h 2- and Ara h 8-specific ELISAs were developed in-house by coating 96-well plates with 4ug/mL of allergen. The sample dilution was then optimised by testing 1:2, 1:5, and 1:10 ratios. Full methods can be found in section 2.2.10.2. Allergen-Specific IgE ELISA.

5.2.2.2. Total Lipid extraction

Initial experiments involved purchasing peanuts from a commercial supplier (Holland and Barrett, UK). The total lipid fraction was then extracted from the nuts using the "Folch" method (Folch, Lees, and Sloane Stanley 1957). **Figure 5.1** illustrates the method. This involved 100g of peanut seeds blended in chloroform: methanol (2:1) before adding 0.9% Sodium Chloride solution and vortexing. The peanut solution was then centrifuged at 3000g for 10 minutes to allow the peanut oil solution to settle at the bottom of the tube. The peanut oil solution (lowest phase) could then be transferred to a new tube using a glass pipette, and more chloroform: methanol added. The solution was centrifuged again at 3000g for 10 minutes, and then the lowest phase transferred to a new tube. The solution was centrifuged again at 3000g for 10 minutes, but then this time removing the lowest phase with a syringe connected to a 0.45 µm PTFE filter to help remove any contaminating molecules. The peanut oil solution was then dried under nitrogen for 2 hours, until the chloroform: methanol had evaporated. This left the peanut oil only in the tube, which was re-dissolved in DMSO to create a 1 mg/mL solution, and stored at -80ºC.

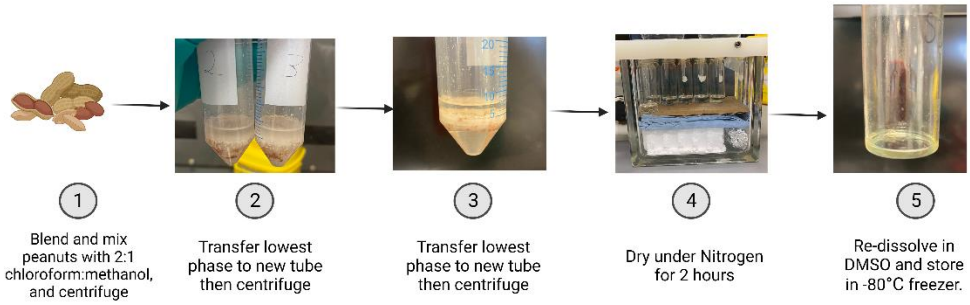


Figure 5.1. Total Lipid Extraction from Peanuts. (1) Peanut seeds were blended in 2:1 chloroform: methanol and centrifuged. (2) The lowest phase (peanut oil) was transferred to a new tube and centrifuged again with chloroform: methanol, (3) Again, the lowest phase was transferred to a new tube and centrifuged. (4) The peanut oil solution was then dried under nitrogen for up to 2 hours to remove any chloroform: methanol solution. (5) Pure peanut oil is left and re-dissolved in DMSO before storing at -80°C.

After isolating the peanut lipids, the aim was to quantify any contaminating proteins. However, due to the viscosity of the peanut oil, a BCA assay could not be conducted. Subsequently, peanut (Arachis) oil (Handa Fine Chemicals, UK) was purchased commercially, as the purity of the sample was guaranteed. Thus, this is the peanut oil utilised in the experiments. **Figure 2.1** in the Methods Chapter details the chemical processing of the peanut oil by the manufacturer to ensure high purity. Before use in assays, 1 mg/mL peanut oil was prepared by sonication for 1 hour at 25 kHz in 0.1% DMSO to help solubilize the lipid.

5.2.2.3. PBMC Isolation

PBMCs were isolated from non-allergic or peanut-allergic subject blood samples using density gradient centrifugation. Whole blood was layered onto Histopaque within SepMate^{TM} tubes and centrifuged at 1200g for 11 minutes, before removing the PBMC layer. See section 2.2.1.1. Peripheral Blood Mononuclear Cell (PBMC) Isolation for more detail.

5.2.2.4. iNKT Cell Expansion and Isolation

PBMCs were cultured with RPMI + 10% human AB serum and stimulated with either the DMSO control or 100 ng/mL of α-GalCer and then also 50 U/mL IL-2. The PBMCs were incubated for 14 days at 37 °C, re-stimulating with 20 U/mL IL-2 every 4 days. In a separate well, some PBMCs were stimulated with 1mg/mL peanut oil and 50 U/mL IL-2 to determine any expansion with the lipid (See section 2.2.3. Invariant NKT Cell Expansion). After 14 days, expanded iNKT cells were immunomagnetically isolated by staining the PBMCs with a CD1d-α-GalCer-loaded Tetramer, labelled with a PE fluorophore, for 30 minutes. The PBMCs could then be tagged with anti-PE Microbeads and applied to a magnetic column to retain any tetramer positive iNKT cells. See methods section 2.2.4. Invariant NKT Cell Immunomagnetic Isolation for more detail.

5.2.2.4. DC Generation

8 days after the first blood donation, a further blood sample was obtained from the same subject and PBMCs were isolated. $CD14^{+}$ monocytes were then immunomagnetically isolated from subject PBMCs and cultured in

RPMI + 10% human AB serum with 50ng/mL GM-CSF and 20 U/mL IL-4 for 5 days, replenishing media and cytokines after 3 days. See sections 2.2.5. CD14+ Monocyte Isolation and 2.2.6. DC Generation for further detail.

After 5 days of culture, immature DCs were generated. The iDCs were then stimulated for 24 hours with either 100 ng/mL α-GalCer, 0.1% DMSO control, 1 mg/mL Peanut Oil, 10 µg/mL Ara h 8, or both peanut oil and Ara h 8. See methods section 2.2.7. Stimulation of DCs with Lipids and Allergens for more detail.

5.2.2.5. Co-culture of DCs and iNKT cells

This co-culture method was first optimised to determine the correct timing of co-culture, by staining for IL-4 and IFN-γ cytokines at 8-hour intervals, for up to 24 hours.

Expanded iNKT cells were co-cultured with autologous DMSO, α-GalCer, peanut oil, Ara h 8, or both peanut oil and Ara h 8 DCs at a ratio of 1:2 (DC:iNKTs). The cells were cultured in RPMI supplemented with 10% human AB serum for up to 5 h. See section 2.2.8. iNKT-DC Co-culture for more detail.

An overview of the developed method for expanding iNKT cells and the subsequent co-culture with lipid and/or allergen pulsed DCs is presented in Chapter 2, Figure 2.5.

5.2.2.6. Flow Cytometry

PBMCs were stained using antibodies from the flow panel detailed in section 2.1.5. Flow Cytometry. Cells were removed from culture and centrifuged before staining with fluorophore-conjugated antibodies. Zombie NIR dye (Biolegend, UK) was used to stain dead cells and exclude from analysis. All cells were measured using BD FACS Canto II, ImageStream MkII (Amnis, UK) or ID700 Spectral flow cytometer (Sony, UK). All analyses were performed using Kaluza or IDEAS software. See section 2.2.9.1. Extracellular Staining for more detail.

Cytokines were stained for at 0-5 h and 24-29 h of culture. Cells were cultured with protein transport inhibitor cocktail (ThermoFisher Scientific, UK)

for 5 h at 37°C prior to harvesting. After staining surface markers, the cells were fixed and permeabilised, prior to staining for cytokines. Antibodies IL-4, IFN-y, IL-10, IL-5 and IL-12 were then stained for to measure cytokines and analysed by flow cytometry. See section 2.2.9.2. Intracellular Cell Staining for more detail.

5.2.2.7. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 9.3.1. All data was first analysed for normality. Normally-distributed data resulted in the use of two-way ANOVAs or mixed effects analyses. Non-normally distributed data was analysed by Mann-Whitney tests. Correlations were tested using Pearson's R coefficient. p-values <0.05 were considered significant for experiments.

5.3. Results

5.3.1. Optimisation of Sample Dilution for Allergen-specific ELISAs

Ara h 2 and Ara h 8-specific IgE ELISAs were conducted using plasma samples to confirm the peanut allergic subject's allergy. To optimise Ara h 2- and Ara h 8-specific IgE ELISAs, different dilutions of the plasma samples were added to the allergen-coated microtitre plates. A peanut-allergic sample was selected for this optimisation as they were expected to have high peanut allergen-specific IgE. A non-allergic sample was also utilised for comparison, and a blank well containing only Assay Buffer (PBS with 1% Tween™ 20, 10% BSA) was used as a negative control. The plasma samples were diluted 1:2, 1:5, and 1:10 in Assay Buffer and the ELISAs were performed. The blank was used to calculate the limit of detection (LoD) for both assays (mean absorbance of blank control multiplied by 2 x standard deviation of the blank), which determines the lowest detected absorbance. For Ara h 2-specific IgE, the blank controls had a mean of 0.115 (SD=0.013, N=4), and for Ara h 8-specific IgE, the blank controls had a mean of 0.141 (SD=0.050, N=4). Thus, the LoDs were 0.141 O.D and 0.241, for Ara h 2 and Ara h 8, respectively.

Figure 5.3 highlights the optimal sample dilution for both Ara h 2- and Ara h 8-specific ELISAs was a 1:5 dilution, as this resulted in the highest absorbance readings for the peanut-allergic sample. The mean absorbance was 0.886 O.D (SD=0.041) and 0.303 O.D (SD=0.098) for Ara h 2- and Ara h 8-specific IgE, respectively. For Ara h 8-specific IgE, the LoD was high due to the variation in blank absorbance. But, the 1:5 dilution still sits above the LoD for the peanut allergy subject, reinforcing this was the optimal dilution.

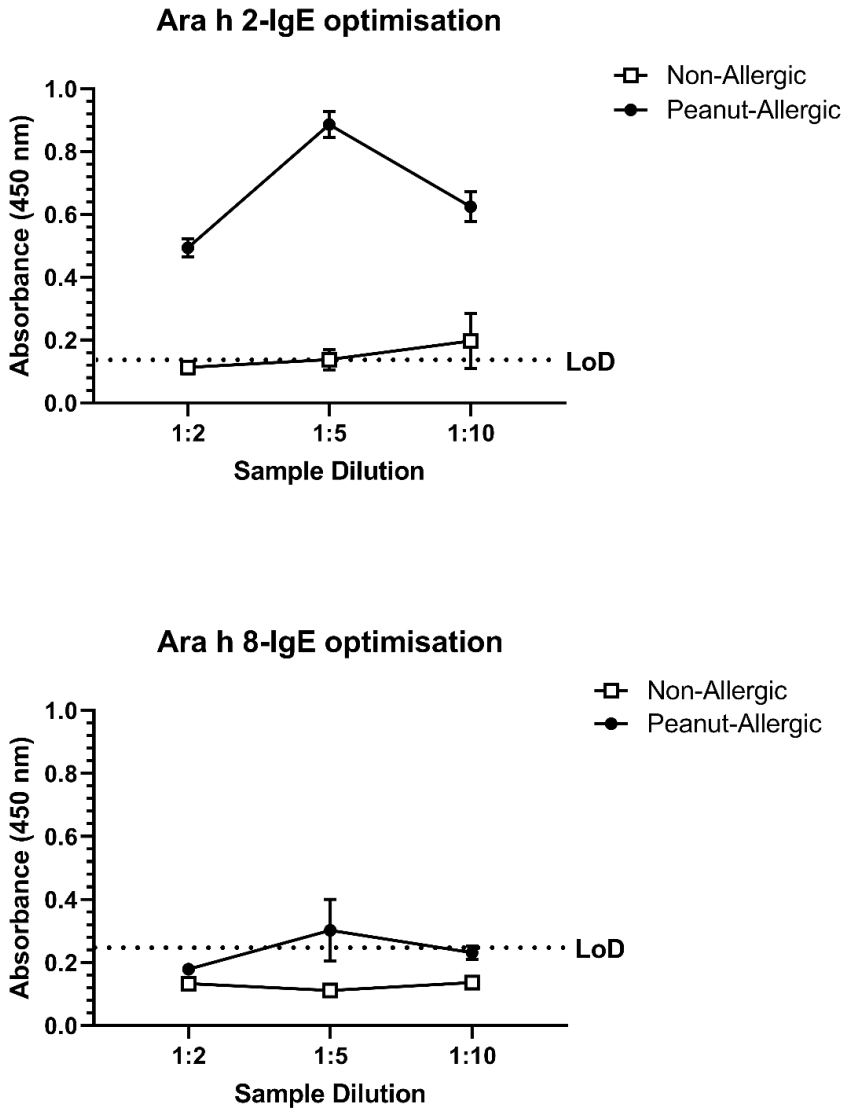


Figure 5.3. Optimisation of Sample Dilution for Allergen-specific ELISAs. Ara h- and Ara h 8-specific ELISAs were ran with peanut-allergic and non-allergic samples diluted 1:2, 1:5, or 1:10 in Assay Buffer (PBS with 1% Tween™ 20, 10% BSA). A blank (Assay buffer) was used as a negative control to calculate the limit of detection (LoD). The absorbance of the allergen-specific IgE signal was read at 450nm and compared between dilutions (N=1).

5.3.2. Total and Allergen-specific IgE Levels of Peanut-Allergic and Non-Allergic Subjects

In addition to peanut allergen-specific IgE, total IgE was also used as a tool to verify allergic status and confirm non-allergic subjects have low IgE levels. Ara h 2-specific IgE is one of the most common peanut allergens, often

used to diagnose peanut allergy in clinical settings, thus it was chosen for this ELISA to confirm peanut allergy in the allergic subjects. Also, Ara h 8-specific IgE was quantified as it is the lipophilic allergen which will be co-cultured with iNKT cells in subsequent experiments. Thus, it is of interest to decipher whether individuals with higher Ara h 8-specific IgE also produce more Th2 cytokines in response to the allergen during co-culture with iNKT cells. Thus, total, Ara h 2- and Ara h 8-specific IgE ELISAs were conducted utilising plasma from the 6 peanut-allergic subjects and 6 non-allergic subjects.

For analysis of the total IgE results, an IgE standard curve was first required to interpolate the concentration of the Total IgE in all samples. This was plotted and overlaid with the non-allergic and peanut-allergic sample absorbance's to estimate the IgE levels (**Fig. 5.4**). The LoD was also plotted, which was 0.163 O.D for total IgE. Normal serum IgE levels can vary between 5-500ng/mL, varying depending on atopic status (Thomas and Hales 2008). **Figure 5.4** shows all subject's total IgE sit above the LoD, with non-allergic subject's total IgE levels from plasma samples ranged from 7.36 ng/mL to 151.3 ng/mL, with a mean IgE level of 62.40 ng/mL (N=6, SD=62.0). The peanut-allergic subject's total IgE levels from plasma samples ranged from 53.42 ng/mL to 605.8 ng/mL, with a higher mean IgE level of 210.4 ng/mL (N=6, SD=214.9), compared to non-allergic controls. It is important to note that the graph shows two non-allergic individuals have higher total IgE than 3 of the peanut-allergic individuals, indicating they may have some undiagnosed allergies, or that the 3 peanut-allergic subjects have lower total IgE levels than expected. However, total IgE levels vary with age, sex, nationality, smoking status, alcohol intake, and the presence of other disease or parasitic infections (Baldacci, Omenaas, and Oryszczyn 2001). Thus, the variability in these results are expected, and so the total IgE levels cannot be used alone to confirm atopy of the subjects. But, in conjunction with clinical history and allergen-specific ELISAs, conclusions can be made.

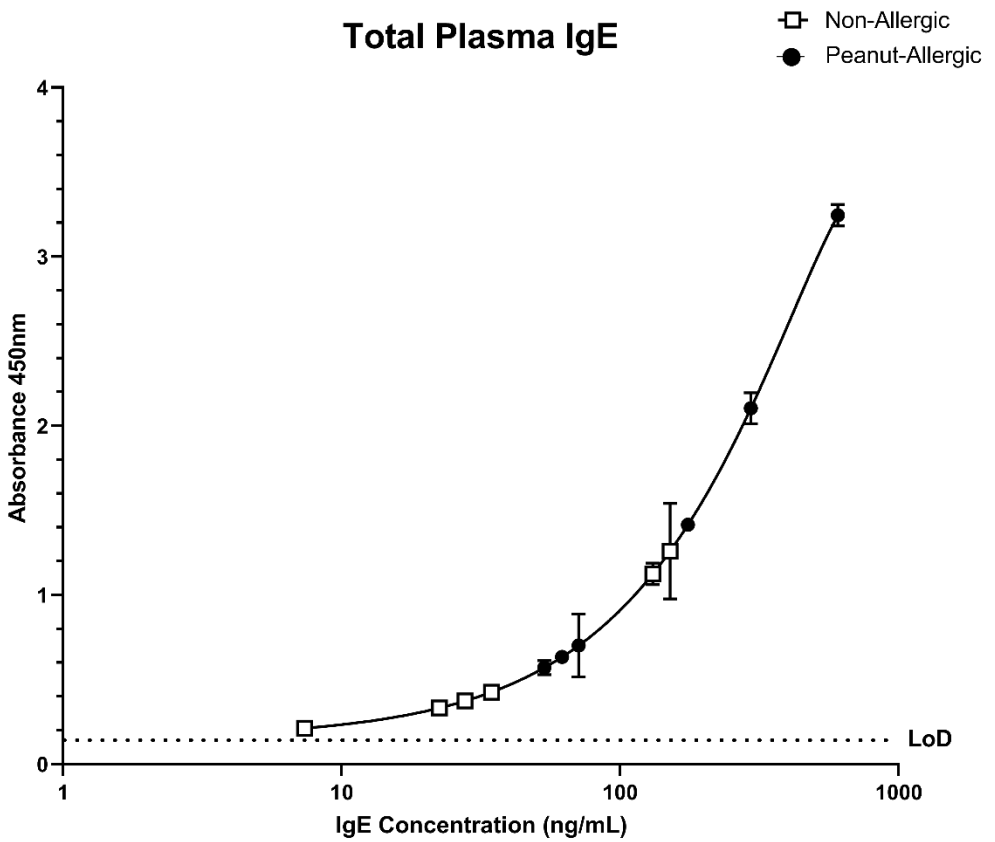


Figure 5.4. Total IgE Plasma Levels in Allergic and Non-Allergic subjects. An IgE standard curve was produced and total IgE absorbance values were plotted over the standard curve for non-allergic (N=6) and peanut-allergic subjects (N=6), to interpolate the total IgE concentration (ng/mL) in each sample. The Limit of total IgE detection is presented as a dotted line.

To test whether there was a significant difference in total IgE between subject groups, the data was first assessed for normality by producing a Kolmogorov-Smirnov test, which showed the data was not normally-distributed (KS=0.34, p=0.029). Thus, a non-parametric Mann-Whitney test was used and showed the median peanut-allergic total IgE (median= 123.2) was not significantly different to the non-allergic controls (median = 31.3) (*Mann-Whitney U* = 6, p=0.065, n=6).

Next, to use in conjunction with total IgE to determine allergy status, Ara h 2- and Ara h 8-specific IgE levels were analysed in both subject groups and presented in **Figure 5.5**. A standard curve could not be calculated for allergen-specific IgE as there was no allergen-specific IgE standard. Therefore,

the IgE absorbance is plotted, rather than concentration, for each subject group.

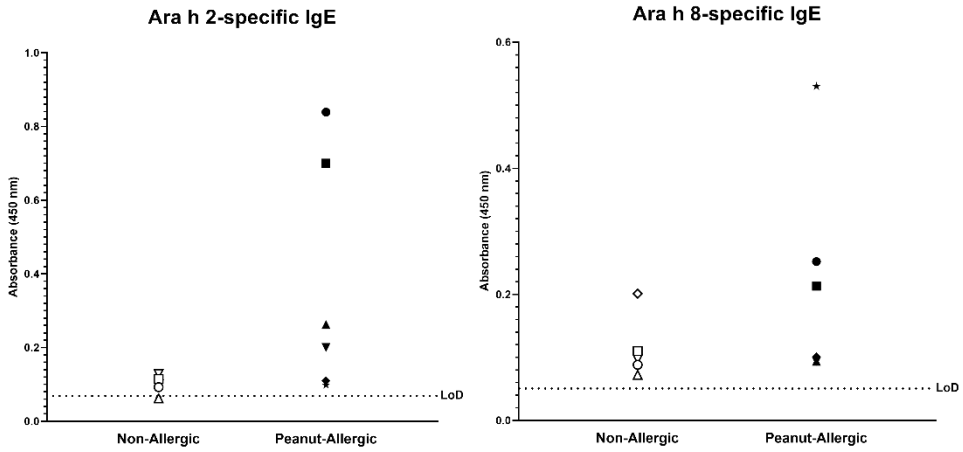


Figure 5.5. Peanut allergen-specific IgE Levels in Non-allergic and Peanut-allergic subjects. The absorbance values for Ara h 2- (left) and Ara h 8- (right) specific IgE levels from non-allergic and peanut-allergic subjects are presented (N=6 non-allergic, 6 peanut-allergic).

The results show Ara h 2-specific IgE ranged from 0.062 to 0.129 O.D. in non-allergic subjects (mean=0.102, SD=0.023, N=6). Compared to Ara h 2-specific IgE ranging from 0.099 to 0.839 O.D. in peanut-allergic subjects (mean=0.368, SD=0.320, N=6). Despite the higher mean of Ara h 2-IgE in peanut-allergic subjects, an unpaired t-test suggested this difference was not significant to non-allergic subjects ($t(10)=2.031$, $p=0.069$). Furthermore, the blank controls were lower and had less variation than during the sample dilution optimisation assays, thus, the Ara h 2-specific IgE LoD was 0.064, and the Ara h 8-specific IgE LoD was 0.051. For Ara h 2-specific IgE, there was thus one non-allergic sample below the LoD, so this sample was removed from further Ara h 2-IgE analysis. There were also 2 peanut-allergic samples with lower Ara h 2-specific IgE levels than non-allergic controls (black circle and star symbols on graph), suggesting they may be sensitised to other peanut allergens, instead of Ara h 2. This was confirmed in one of these subjects where they have high Ara h 8-specific IgE (black star symbol), confirming they have a peanut allergy.

Ara h 8-specific IgE produced lower OD values in both subject groups compared to Ara h 2-specific IgE. In non-allergic subjects, the Ara h 8-specific IgE ranged from 0.072 to 0.201 O.D. (mean=0.112, SD=0.045, N=6). The values were higher for peanut-allergic subjects, ranging from 0.095 to 0.530 O.D (mean=0.214, SD=0.169, N=6). Alike to Ara h 2, these differences between groups were also not significant, as the median of the non-allergic subject's Ara h 8–specific IgE (0.101 O.D.) was not significantly different from the median of peanut-allergic's (0.157 O.D.), as calculated by a Mann-Whitney test (U = 11, p=0.310). There were 2 subjects with lower Ara h 8-specific IgE compared to non-allergic controls (denoted by black diamond and triangle symbols). Importantly, one of these subjects had higher Ara h 2-specific IgE instead, confirming their peanut allergy. But the other subject had low Ara h 2 IgE as well as Ara h 8, suggesting they may be allergic to other peanut allergens, such as Ara h 1, as this subject has GP-confirmed allergies to peanuts, as well as cat dander, and pollen.

Next, the relationships between different IgE levels were measured; total IgE and Ara h 2-specific IgE (**Fig 5.6a**), total IgE and Ara h 8-specific IgE (**Fig. 5.6b**), and Ara h 2- and Ara h 8-specific IgE (**Fig 5.6c**) were analysed, as it was expected individuals with high total IgE would also have high peanut allergen-specific IgE. The graph shown in **Figure 5.6b** highlights a strong correlation between Total IgE and Ara h 8-specific IgE (R^2=0.830, p<0.0001). Thus, the higher the total IgE in peanut allergic subjects, the higher the Ara h 8-specific IgE levels. There were no significant correlations between total IgE and Ara h 2-IgE, or Ara h 2-IgE compared to Ara h 8-IgE.

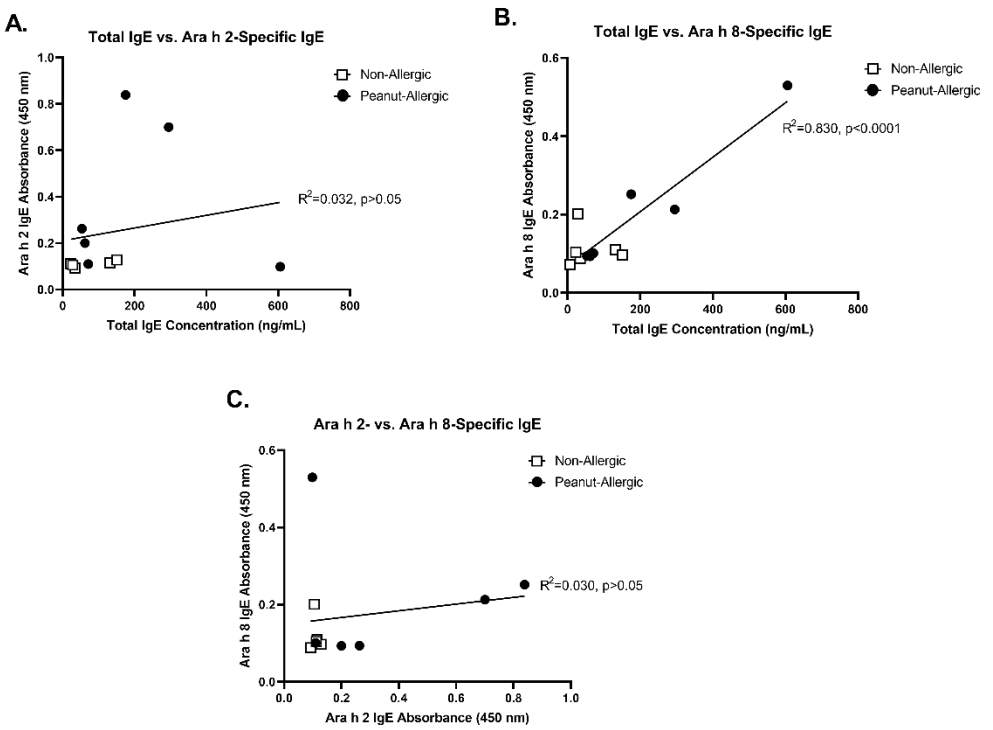


Figure 5.6. Correlations between subject Total IgE levels and allergen-specific IgE levels. Graphs showing Pearson's r correlations between (A) total IgE levels and Ara h 2-specific IgE, (B) total IgE and Ara h 8-specific IgE levels, and (C) Ara h 2- and Ara h 8-specific IgE levels, from both non-allergic and peanut-allergic subjects (N=6 non-allergic, 6 peanut-allergic).

Finally, relationships were also determined between the age or sex of subjects, and their IgE levels, as it is suggested these can impact their levels (Sadoway et al. 2015). However there were no significant correlations between age and total IgE (Fig 5.7Ai), Ara h 2-specific IgE (Fig 5.7Aii), or Ara h 8-specific IgE (Fig. 5.7Aiii). Furthermore, there were also no significant differences between male and female subjects in regards to their total IgE (Fig 5.7Bi), Ara h 2-specific IgE (Fig 5.7Bii), or Ara h 8-specific IgE (Fig 5.7Biii) levels ($p>0.05$, $n=6$).

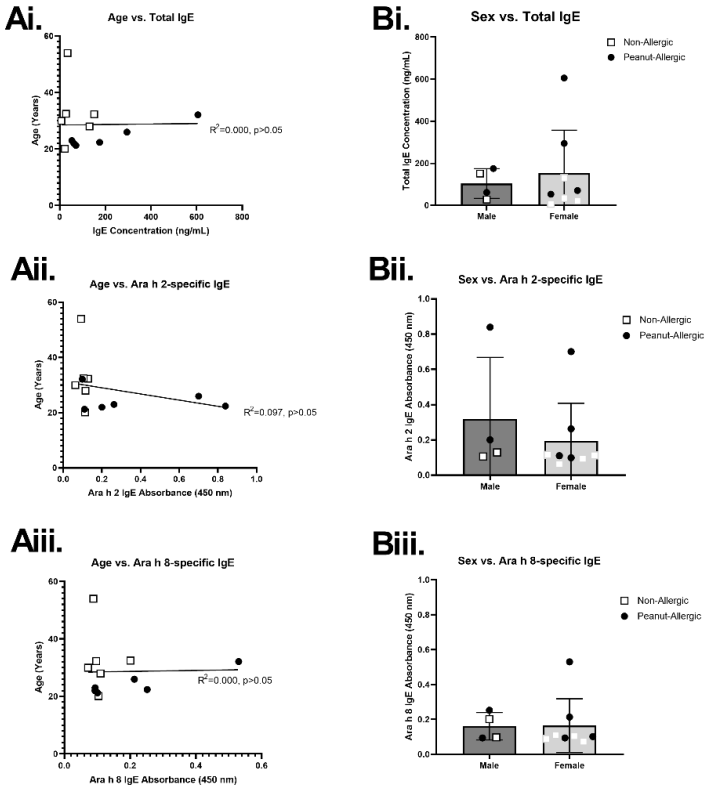


Figure 5.7. Age and Sex Correlations with IgE. The age (A) and sex (B) of non-allergic and peanut-allergic subjects were compared to total IgE levels (i), Ara h 2-specific IgE levels (ii), and Ara h 8-specific IgE levels (iii). Significant results are indicates with a p value below 0.05. Bar chart horizontal bars indicate mean, and vertical bars indicate standard deviation (n=6 non-allergic, 6 peanut-allergic).

Overall, the IgE measurements show peanut-allergic subjects had higher mean total, Ara h 2-, and Ara h 8- specific IgE levels, but were not significantly different to the non-allergic controls, potentially due to the small sample size. Despite the lack of statistical significance, the previous clinical diagnosis or positive skin-prick tests combined with the higher IgE levels here confirm they are peanut-allergic. Investigations into sex and age influences on IgE show no significant correlations. Although, there was a significant correlation between total IgE levels and Ara h 8 IgE levels, which is opposite to the expected results, as Ara h 8 sensitisation is often associated with milder peanut allergies, thus suggesting plasma total IgE would be lower.

5.3.3. iNKT Cell Expansion in Peanut Allergic Subjects

Before iNKT cells were co-cultured with autologous DCs, non-allergic and peanut-allergic donor iNKT cells were activated with the glycolipid, α-GalCer, to induce their expansion. iNKT cells were also stimulated with peanut oil to investigate whether this could also induce iNKT cell expansion. Previous literature suggested there are differences in iNKT cell populations between severe asthmatic subjects and healthy subjects (Antunes et al. 2018). Thus, the populations between subject groups were also compared for differences between numbers and phenotype.

As shown in **Figure 5.8,** iNKT cell populations can be defined by the expression of CD3 and α-GalCer-loaded CD1d tetramer. Initially, cells were gated according to size (forward scatter) and granularity (side scatter) (Fig 5.8Ai), then single cells were selected (Fig 5.8Aii), followed by gating on viable cells (Fig 5.8Aiii). B cells were excluded by selecting CD19- cells (Figure 5.8Aiv) as they can non-specifically bind to the tetramer. Then, iNKT cells were gated as being positive for both CD1d-aGalcer Tetramer and CD3 (Figure 5.8Av). The tetramer works by presenting cD1d-aGalCer complexes attached to a fluorophore, to iTCRs on iNKT cells.

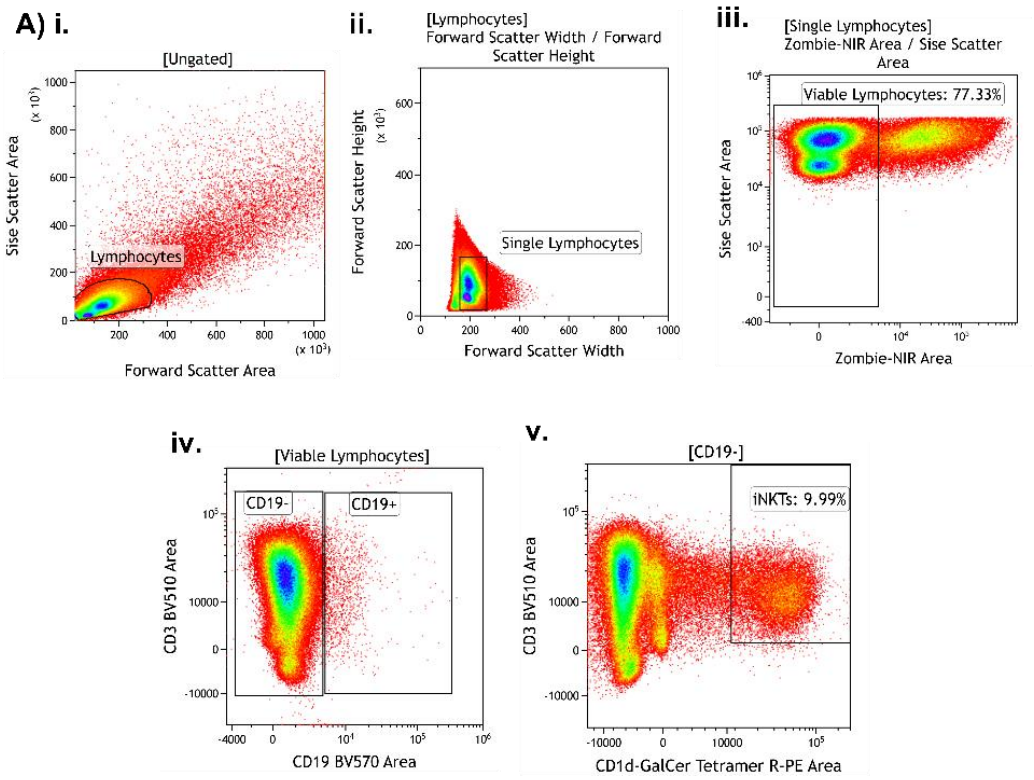


Figure 5.8. iNKT Cell gating strategy. (A) Gating strategy to measure iNKT cell cytokine release. (i) SSC/FSC dot plot to gate lymphocytes, (ii) FSH/FSW dot plot to gate singlets, (iii) Zombie NIR dot plot to gate on live lymphocytes, (iv) CD3/CD19 to exclude B cells from analysis, and (v) CD3/CD1d-GalCer Tetramer to gate on iNKT cells.

Figure 5.9 demonstrates the expansion of gated iNKT cells across 6 non-allergic subjects and 6 peanut-allergic subjects. In non-allergic subjects, the iNKT cells constituted a mean of 0.02% (SD=0.015) of $CD19^-$ lymphocytes before iNKT cell expansion, compared to a ~5-fold higher 0.11% (SD=0.106) in peanut-allergic subjects. Although, this difference was not significant. After 14 days of stimulation with α-GalCer and IL-2, the percentage of iNKT cells increased to 2.44% (SD=3.56) in non-allergic subjects. In peanut-allergy subjects, there was a significant increase in iNKT cell population, which was 6-fold greater than non-allergic controls, with a mean of 14.64% (SD=7.05, $P<0.0001$) after 14 days of stimulation. A two-way ANOVA revealed this difference in expanded iNKT cell population between subject groups was significant ($n=12$, $p<0.0001$).

Furthermore, the DMSO control resulted in no significant increase in iNKT cells, resulting in an iNKT cell population of 0.03% and 0.09% iNKT cells in non-allergic and peanut allergic subjects, respectively ($p>0.05$). The negative GalCer Tetramer control also showed minimal non-specific binding to the tetramer at Day 0 and Day 14 of expansion in both subject groups, with an average across subject groups of only 0.0195% (SD=0.012, N=4) of $CD19^-$ lymphocytes binding to the tetramer at Day 14 of expansion.

PBMCs stimulated with peanut oil showed no significant increases in iNKT cells after 14 days of culture in either subject group ($p>0.05$), suggesting the peanut oil may not be potent enough to activate iNKT cells.

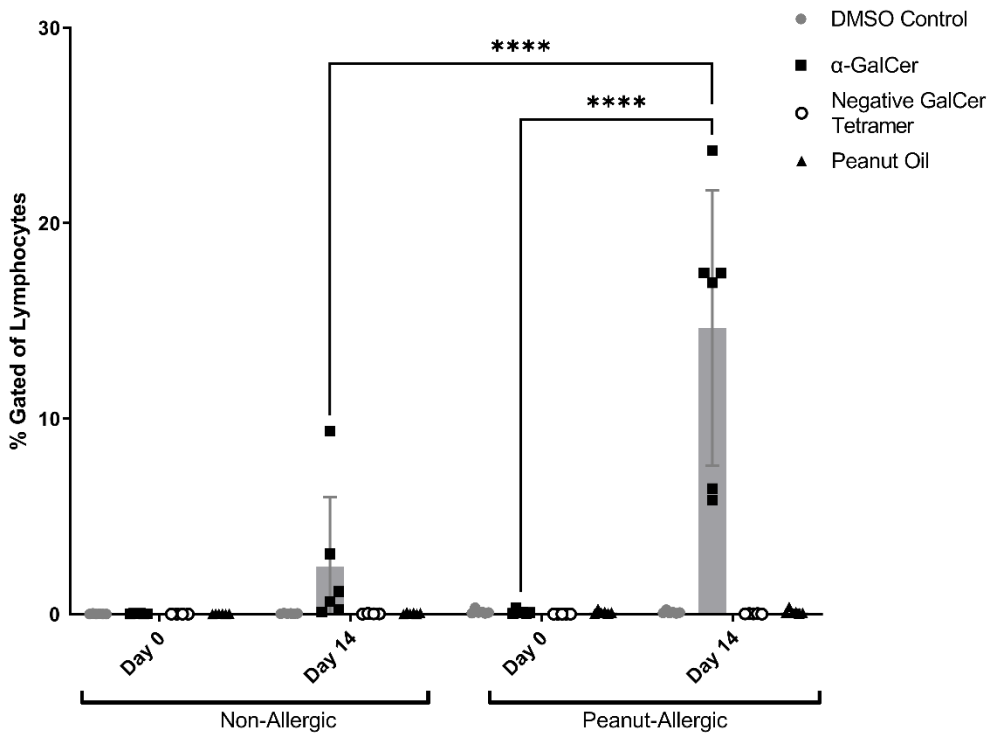


Figure 5.9. Mean iNKT Cell Expansion and Viability. (A) Average iNKT cell percentages at Day 0 and Day 14 of expansion with DMSO, α-GalCer, or stained with the negative tetramer control. (**** $p<0.0001$, vertical bars indicate standard deviation, horizontal bars indicate the mean, n=6 non-allergic, 6 peanut-allergic).

We also wanted to test whether there were any correlations between the percentage of iNKT cells present in subject peripheral blood and their IgE levels determined in the previous section, as some research has shown higher levels of iNKT cells are found in allergic individuals (Antunes et al. 2018.). **Figure 5.10.** portrays Pearson's r correlation tests between the percentage of iNKT cells in isolated PBMCs versus total IgE (**Fig 5.10a**), Ara h 2-specific IgE (**Fig. 5.10b**), and Ara h 8-specific IgE (**Fig 5.6c**), for subjects. The results show there are no significant correlations between iNKT cells in peripheral blood and total, Ara h 2, or Ara h –specific IgE levels ($p>0.05$), suggesting iNKT cell populations are independent of IgE abundance.

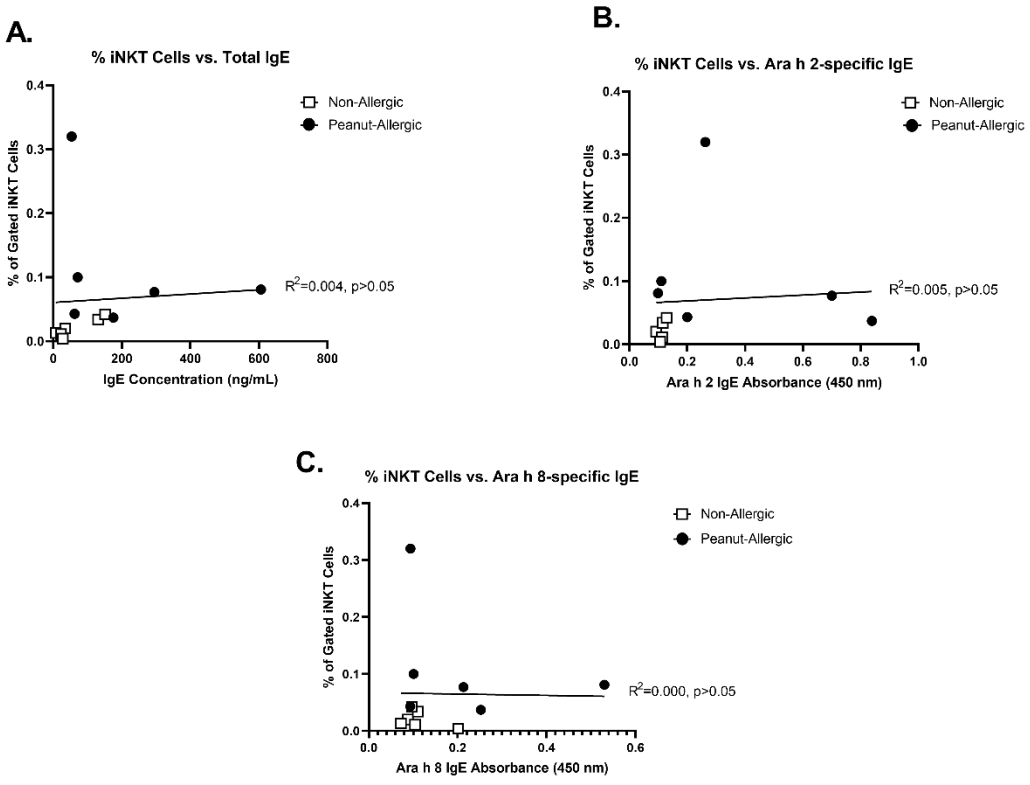


Figure 5.10. Correlations between the peripheral blood % of iNKT cells in subjects and IgE levels. Graphs showing Pearson's r correlations between the percentage of iNKT cells of non-allergic and peanut-allergic PBMCs with (A) total IgE levels, (B) Ara h 2-specific IgE levels, and (C) Ara h 8-specific IgE levels (n=6 non-allergic, 6 peanut-allergic).

Next, the percentage of iNKT cells after 14 days of iNKT cell expansion was analysed for any correlations with total IgE and allergen-specific IgE. **Figure 5.11** graphically represents the relationship between the percentage of iNKT cells after expansion with total IgE (**A**), Ara h 2-IgE (**B**), and Ara h 8-IgE (**C**). The graphs show some weak positive correlations, but they lack statistical significance ($p>0.05$).

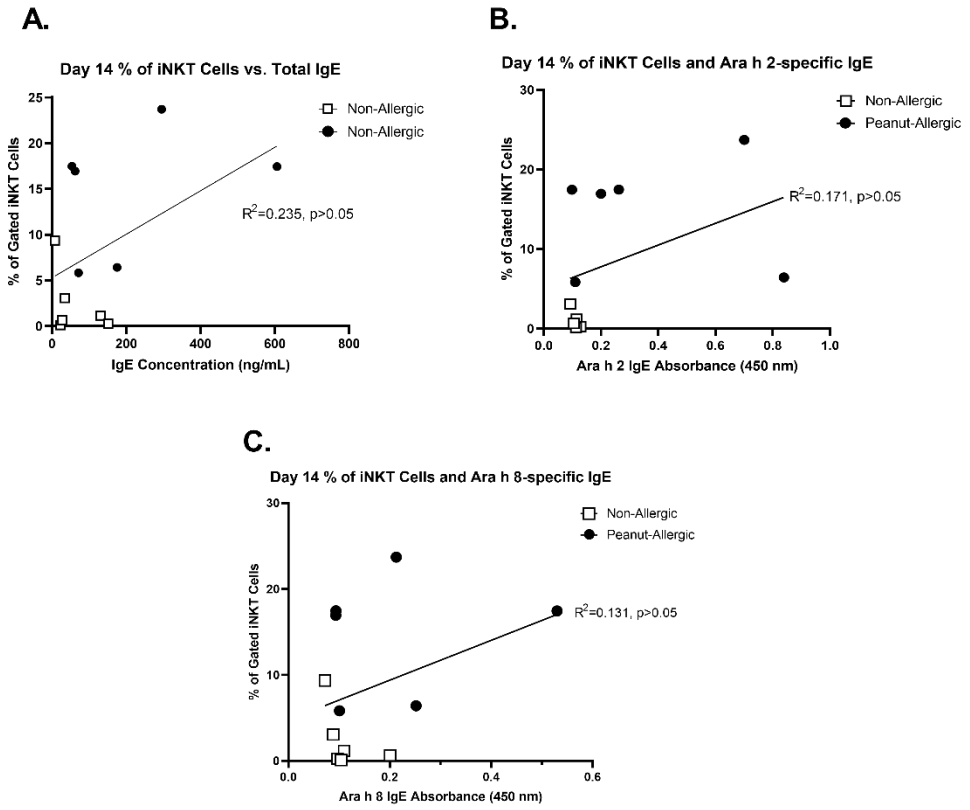


Figure 5.11. Correlations between the % of iNKT cells after expansion in subjects and IgE levels. Graphs showing Pearson's r correlations between the percentage of expanded iNKT cells of non-allergic and peanut-allergic PBMCs with (A) total IgE levels, (B) Ara h 2-specific IgE levels, and (C) Ara h 8-specific IgE levels (n=6 non-allergic, 6 peanut-allergic).

Also, iNKT cell numbers have been shown at higher frequencies in females, which then decline with age (Singh et al. 2022). Thus, the correlations between sex or age were also made with iNKT cells. **Figure 5.12Ai and 5.12Bi** highlight trends similar to previous research, where females have higher iNKT cell numbers than males (mean = 0.082 and 0.032 iNKT cells, respectively) and

a decline with age. However, these correlations were not significant. Furthermore, there were also no significant differences between sex or age and the % of iNKT cells after expansion with α-GalCer.

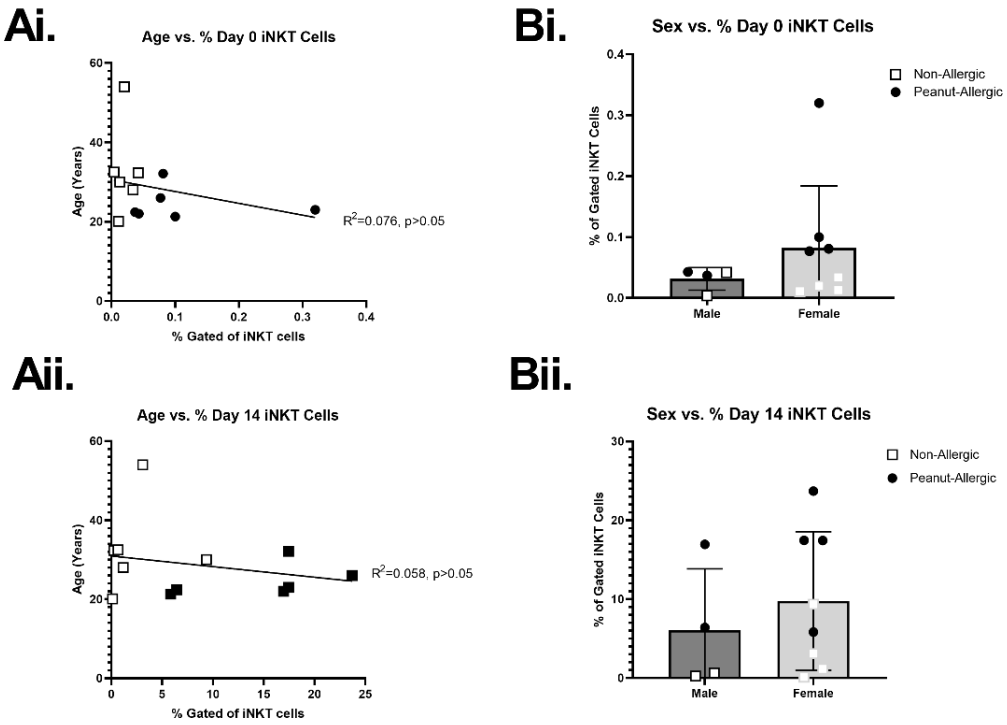


Figure 5.12. Age and Sex Correlations with % iNKT cells. The age (A) and sex (B) of non-allergic and peanut-allergic subjects were compared to (i) % iNKTs in peripheral blood and (ii) % iNKTs after expansion with α-GalCer. Significant results are indicates with a p value below 0.05. Bar chart horizontal bars indicate mean, and vertical bars indicate standard deviation (n=6 non-allergic, 6 peanut-allergic).

Next, the differences in iNKT cell phenotype between non-allergic and peanut-allergic subjects were then analysed by staining for CD4 and CD8 cell surface markers, as iNKT cells can be grouped by CD4/CD8 expression. These different iNKT cell subsets have different cytokine profiles, with CD4+ iNKT cells predominantly producing IL-4, and CD8+ iNKT cells predominantly producing IFN-γ (Schmid et al. 2018a). Flow cytometry analysis of iNKT cells at day 0 (**Fig. 5.13a**) show peanut-allergic subjects have a significantly higher number of double negative ($CD4^-CD8^-$) iNKT cells, compared to non-allergic controls ($p<0.01$), with a mean of 0.007% in non-allergic compared to 0.062% in peanut-

allergic subjects. After 14 days of iNKT cell expansion, the iNKT subsets DN, CD8+, and DP (double positive) significantly differed between subject groups. Again, the percentage of CD19- lymphocytes which were DN iNKT cells was significantly higher ($p<0.01$) in peanut-allergic subjects (mean=3.60%, SD=2.78) compared to non-allergic controls (mean=0.22%, SD=0.28). Also, the percentage of CD4+ iNKT cells was significantly greater ($p<0.0001$) in peanut-allergic subjects (mean=6.85%, SD=3.87) compared to non-allergic controls (mean=1.62%, SD=2.33). Despite a greater population of CD8+ iNKT cells in allergic individuals (mean=1.00%, SD=0.92), it was not significantly greater than the population in non-allergic individuals (mean=0.26%, SD=0.48). Some literature suggests there are no iNKT cells that are DP (Liu et al. 2008; Schmid et al. 2018a), but here we show that there is a small CD4+CD8+ iNKT cell population, supported by other research which identified a small DP population (Montoya et al. 2007). Although there were no significant differences between allergic and non-allergic individuals.

Figure 5.13c highlights differences between non-allergic iNKT cell populations before and after expansion. The graph demonstrates a significant increase in the percentage of iNKT cells which are CD4+ subset, after expansion with α-GalCer ($p<0.05$). **Figure 5.13d** highlights differences between peanut-allergic iNKT cell populations before and after expansion. Similarly to non-allergic controls, there was a significant increase in CD4+ subsets ($p<0.0001$), but there were also significant increases in DN and CD8+ populations ($p<0.05$). Thus, iNKT cells from peanut-allergic individuals began with a predominant DN subset before expansion, but this shifted to predominantly CD4+ subset after expansion.

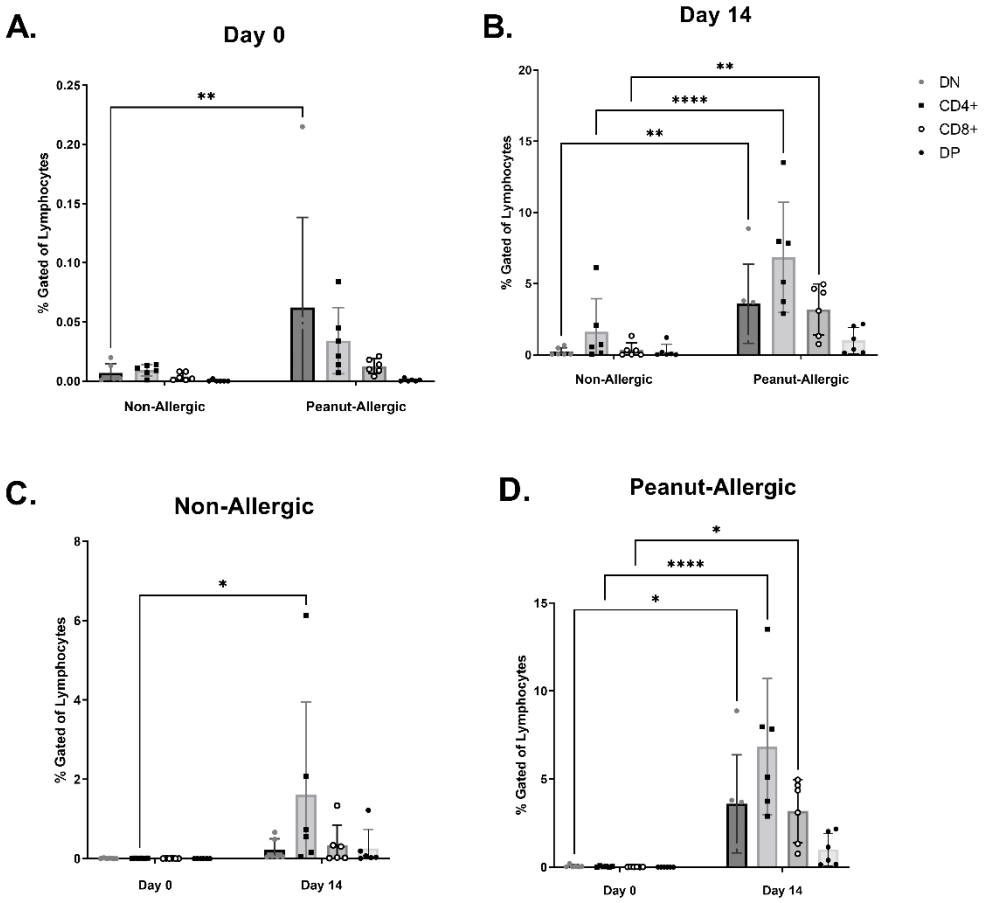


Figure 5.13. iNKT cell subsets before and after 14 days of expansion with α-GalCer. Flow cytometry analysis of iNKT cells allowed subsets to be identified by staining for CD4 and CD8 markers. Double negative (DN), CD4+ only, CD8+ only, and DP (double positive) subsets were quantified and compared between non-allergic and peanut-allergic subjects before and after iNKT cell expansion. (A) Non-allergic and peanut-allergic iNKT cell subsets before expansion (Day 0). (B) Non-allergic and peanut-allergic iNKT cell subsets after expansion (Day 14). (C) Non-allergic iNKT cells at day 0 and day 14 of expansion. (D) Peanut-allergic iNKT cells at day 0 and day 14 of expansion. (n=6 non-allergic, n=6 peanut-allergic, *p<0.05, **p<0.01, ****p<0.0001, vertical bars represent standard deviation, horizontal bars represent means).

Overall, this data shows iNKT cells are more abundant in peanut-allergic donors compared to non-allergic donors by 5-fold, although this was not statistically significant. But after expansion with α-GalCer for 14 days, peanut allergic subjects did have a significantly higher iNKT cell population than non-allergic subjects. Peanut-allergic subjects had predominantly DN iNKT cells in their peripheral blood, whereas non-allergic had predominantly CD4+. Then

expansion with α-GalCer induced a shift towards predominantly CD4+ iNKT cells in both subject groups.

5.3.4. Regulatory T Cells in Non-allergic and peanut-allergic Subjects

As well as identify iNKT cell populations within the PBMC culture, before and after expansion with lipids, Tregs were also stained for within the PBMC culture. Regulatory T cells can influence tolerance to allergens, thus we aimed to identify any differences in populations between non-allergic and peanut-allergic subjects. The abundance of Tregs at Day 0 and Day 14 of iNKT cell expansion was analysed, by incorporating CD25 and Foxp3 antibodies in the flow panel. CD25+Foxp3+ regulatory T cells were gated for from CD3+CD4+ T cells (**Fig 5.14**).

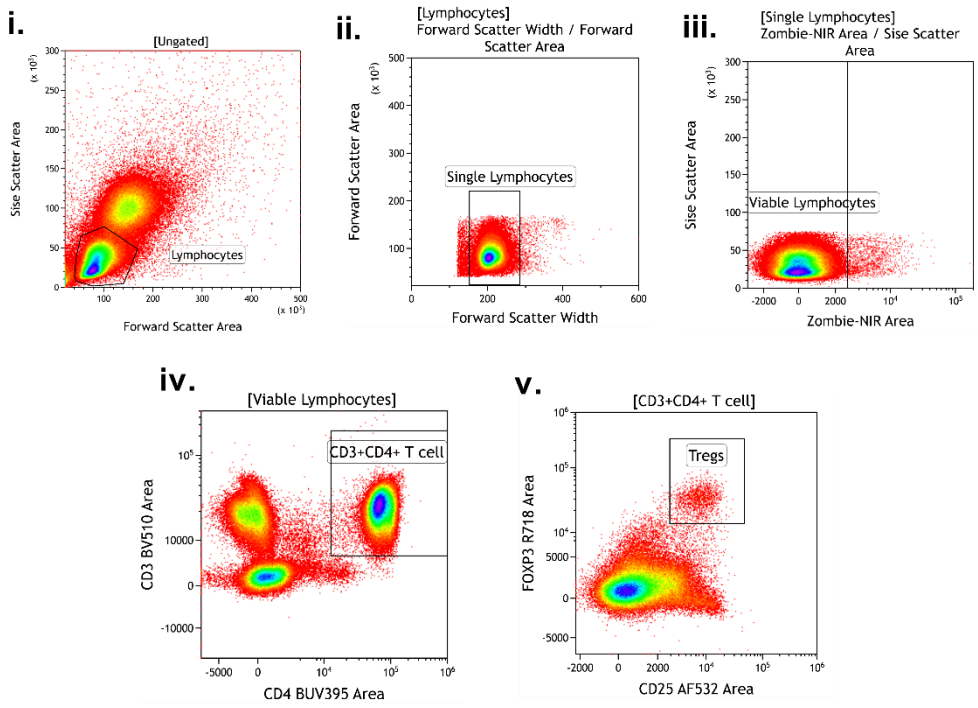


Figure 5.14. Regulatory T cell Gating. Gating strategy to phenotype Regulatory T cells. (i) SSC/FSC dot plot to gate lymphocytes, (ii) FSH/FSW dot plot to gate singlets, (iii) Zombie NIR dot plot to gate on live lymphocytes, (iv) CD3/CD4 to identify CD4+ T cells, (v) Foxp3/CD25 to identify regulatory T cells.

Figure 5.15 shows at Day 0 of culture, the Tregs were low in abundance, with a mean of 0.003% of CD4+ T cells in non-allergic subjects, and a slightly

higher mean of 0.007% in peanut-allergic subjects. After 14 days of culture, the Treg population increased in all conditions, with the smallest increases seen in peanut oil-stimulated cells. However, the peanut oil-stimulated Tregs from peanut-allergic subject's are significantly higher after 14 days of culture, compared to Tregs from non-allergic subjects ($p<0.05$, N=11).

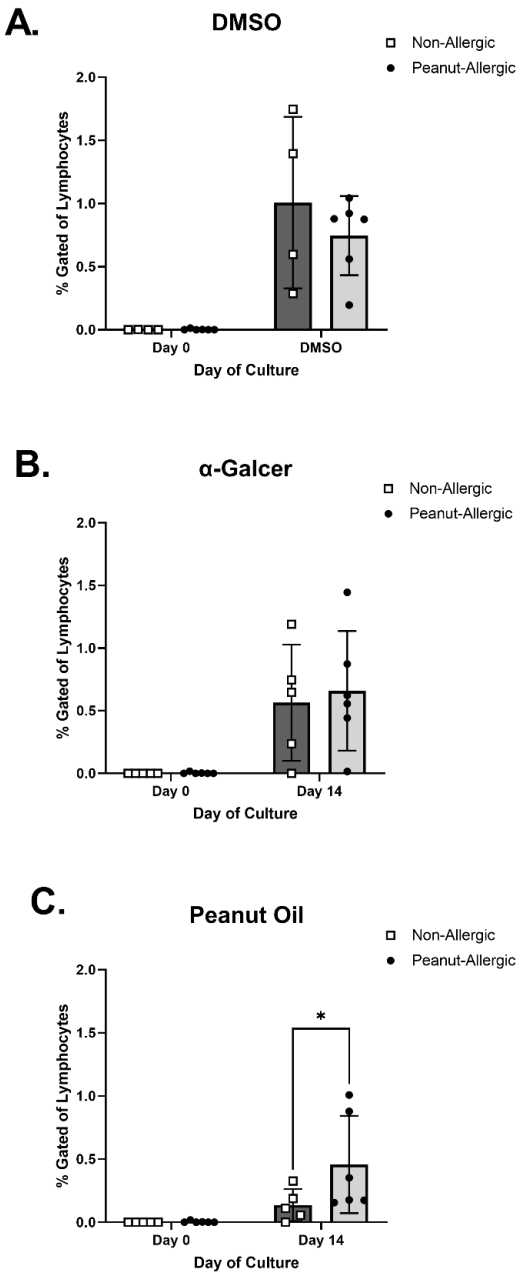


Figure 5.15. Regulatory T cells at Day 0 and Day 14 of culture. The percentage of lymphocytes which were regulatory T cells (CD4+CD25+Foxp3+) at Day 0 and Day 14 of iNKT cell expansion are shown. The PBMCs stimulated with either the DMSO control, α-GalCer, or peanut oil for 14

days are presented, from non-allergic and peanut-allergic subjects. Horizontal bars represent means, vertical bars represent standard deviation. (*p<0.05, n=11).

5.3.5. Optimisation of iNKT-DC Co-culture Incubation Time

We have shown iNKT cells can be expanded with the glycolipid α-GalCer to increase cell numbers. We have also shown moDCs can be generated and stimulated with allergens and lipids. Now, we co-cultured the expanded and isolated iNKT cells with stimulated moDCs to assess cytokine production by flow cytometry. Existing literature suggests a 2:1, iNKT:DC ratio for co-culture is optimal for evaluating cytokine production (Veinotte, Gebremeskel, and Johnston 2016), thus this ratio was adopted in this study.

The next variable to consider was the incubation length of the co-culture to measure cytokine production. Therefore, the cells were co-cultured for up to 24 hours to determine the optimal time to detect cytokine production. Healthy donor blood samples were obtained for these optimisation experiments.

The predominant iNKT cell cytokines, IL-4 and IFN-γ, were first stained at 8 hour intervals for up to 24 hours after DC co-culture. To stain for cytokine production by flow cytometry, cells were incubated for 5 hours with a protein transport inhibitor cocktail prior to cell harvest, thus the time points are each 5 hours in length. Thus, cytokine production was measured between 0-5 hours, 8-13 hours, 16-21 hours and 24-29 hours of co-culture. **Figure 5.16** shows exemplar plots from a non-allergic donor, highlighting the dampening of cytokine production over co-culture, with most cytokine production within 0-5 hours. The percentage of α-GalCer-stimulated iNKT cells producing IL-4 only was 88.27% at 0-5 h, decreasing to 77.31% by 24-29 hours of co-culture. Similarly, cells producing both IFN-γ and IL-4 decreased from 2.38% at 0-5 h to 1.52% at 24-29 h. Thus at 24-29 hours of co-culture, there was some IFN-γ and IL-4 production, but this was not as great as 0-5 hours.

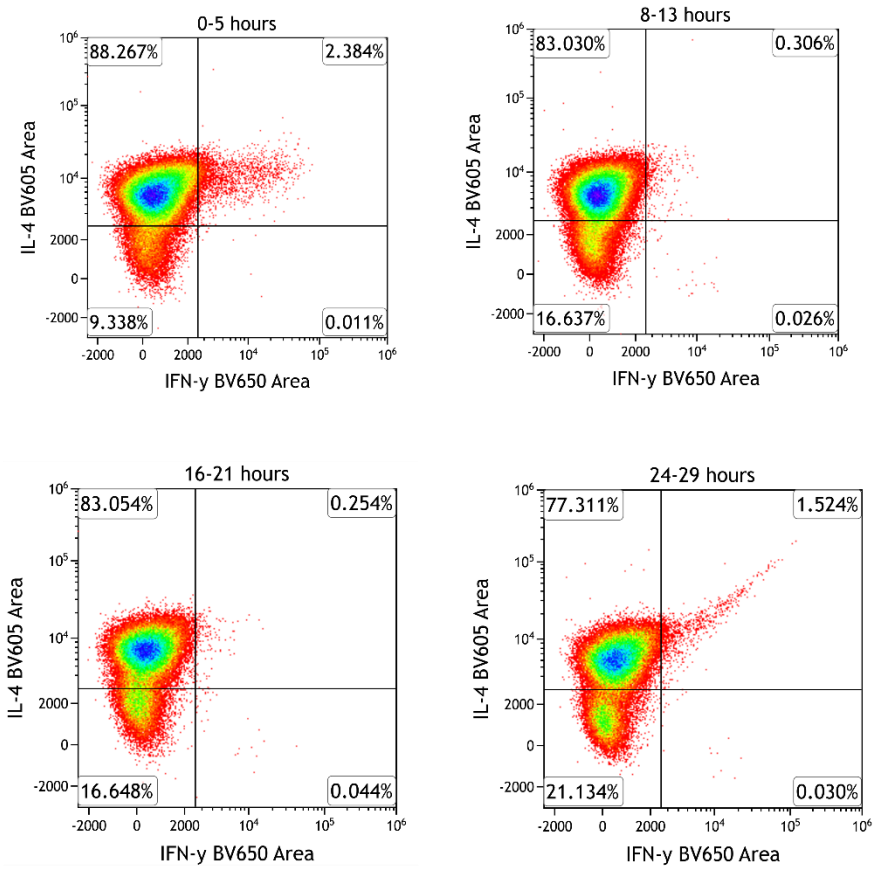


Figure 5.16. 8-hour interval cytokine staining during iNKT-DC Co-culture. iNKT cells and DCs were co-culture for up to 24 hours, staining for cytokines every 8 hours. The cells were incubated with a protein-transport inhibitor for 5 hours, hence why the time points are 5 hours in length i.e. 0-5 hours of cytokine production. IFN-γ and IL-4 were stained for by flow cytometry and plotted here as the percentage of iNKT cells secreting the representative cytokines. (n=1).

This experiment was replicated in 4 non-allergic donors, simplifying the experiment to just 0-5 h and 24-29 h of co-culture. In line with the exemplar plots, **Figure 5.17** demonstrates a significant decrease in the percentage of iNKT cells producing IFN-γ from 0-5 h to 24-29 h of co-culture with α-GalCer-stimulated DCs. This suggests 0-5 h is the optimal co-culture length to detect the rapidly produced IFN-γ. As all iNKT cells were expanded with α-GalCer before co-culture with α-GalCer-stimulated or DMSO-stimulated DCs, there was high levels of IL-4 produced before co-culture due to activation from expansion. Thus, the percentage of iNKT cells producing IL-4 was high before

co-culture, and then maintained high after co-culture with α-GalCer and DMSO stimulated DCs, at both 0-5 h and 24-29 h. Hence, there were no significant differences in IL-4 production between both time points in the α-GalCer or DMSO control ($p>0.05$).

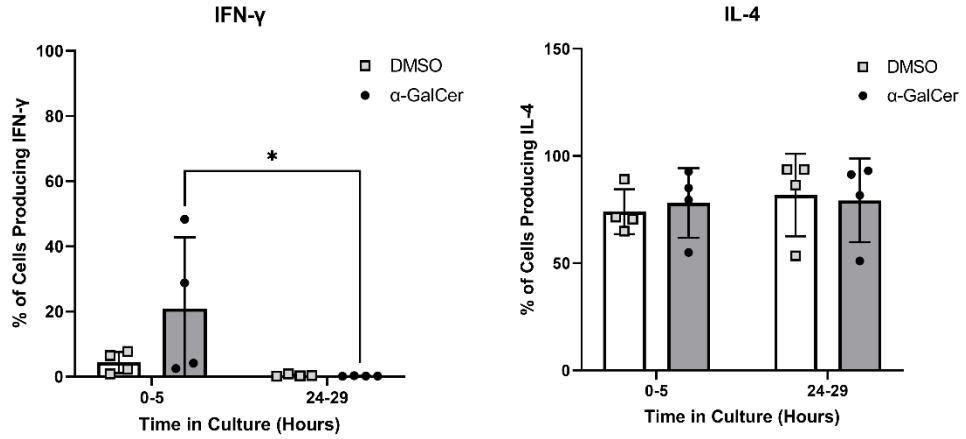


Figure 5.17. iNKT cell IFN-γ and IL-4 production at 0-5h and 24-29h of co-culture. The percentage of iNKT cells producing IFN-γ (left) and IL-4 (right) after 0-5 hours and 24-29 hours of DC-iNKT cell co-culture. DCs stimulated with either α-GalCer or the DMSO control and co-cultured with iNKT cells are shown. (*$p<0.05$, n=4, vertical bars indicate standard deviation).

Once the optimal timing of co-culture to enable measurement of iNKT cell cytokine production was established as 0-5 h, the method could be applied to non-allergic and peanut-allergic subject samples, to investigate whether peanut lipids influence allergic sensitisation.

5.3.6. DC Phenotyping after Stimulation with Peanut Oil and Ara h 8

To investigate the role of peanut lipids in allergic sensitisation, peanut lipids needed to be isolated from seeds. Initially, the total lipid fraction from peanut seeds was isolated following the Folch. method. However, due to concerns over the purity and complications with testing whether there were any contaminating proteins, a commercial peanut oil was sought with high purity. The fatty acid components of the purchased peanut oil is detailed in **Table 5.3**, with oleic acid as the most prominent (65.5%), followed by linoleic

acid (17%). The least abundant fatty acid was short-chain fatty acids with no double bonds (C16:0 below) (<0.1%).

Table 5.3. Peanut oil Fatty Acid Composition. The commercial peanut oil contained a variety of fatty acids, presented here with their carbon length and double bond number, along with their fatty acid class and percentage present in the peanut oil.

	Fatty Acid Class	**% of Peanut Oil**
C16:0 below	Fatty acid	<0.1
C16:0	Palmitic	7.9
C18:0	Stearic	2.4
C18:1	Oleic	65.5
C18:2	Linoleic	17.0
C18:3	Linolenic	0.2
C20:0	Arachidic	1.2
C20:1	Eicosenoic	1.7
C22:0	Behenic	2.5
C22:1	Erucic	0.2
C24:0	Lingnoceric	1.3

As this peanut oil was the total lipid fraction from peanuts, a high concentration (1mg/mL) was chosen to stimulate the DCs to allow any lipids low in abundance, such as the short-chain fatty acids, to bind CD1d molecules and be internalised. Of the very limited research on pulsing DCs with peanut

lipids, this concentration is in accordance with another study which also used 1mg/mL peanut lipids to stimulated human monocyte-derived DCs (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, et al. 2016).

In addition to peanut oil, DCs were stimulated with both peanut oil and Ara h 8, a lipid-binding peanut allergen, as it is hypothesised lipids work in conjunction with allergens to influence allergic sensitisation. Ara h 8 was also used alone to stimulate DCs, as a control for the lipid with allergen condition. DCs were also stimulated with α-GalCer as a positive control, and DMSO as a negative control. After 24 hours of DC stimulation with the lipids and/or allergens, the DCs were co-cultured with autologous iNKT cells, and the cells were phenotyped by flow cytometry after co-culture. The co-cultures were compared between non-allergic subjects and peanut-allergic subjects. **Figure 5.18.** presents the gating strategy for viable DCs, before DC markers and cytokines were analysed. The cells were gated according to size (forward scatter) and granularity (side scatter) (**Fig 5.18a**), then DCs were selected by gating for CD209 positive cells (**Fig 5.18b**) followed by selection of single cells (**Fig 5.18c**), and finally viable DCs were gated by selecting Zombie negative cells (**Fig. 5.18d**).

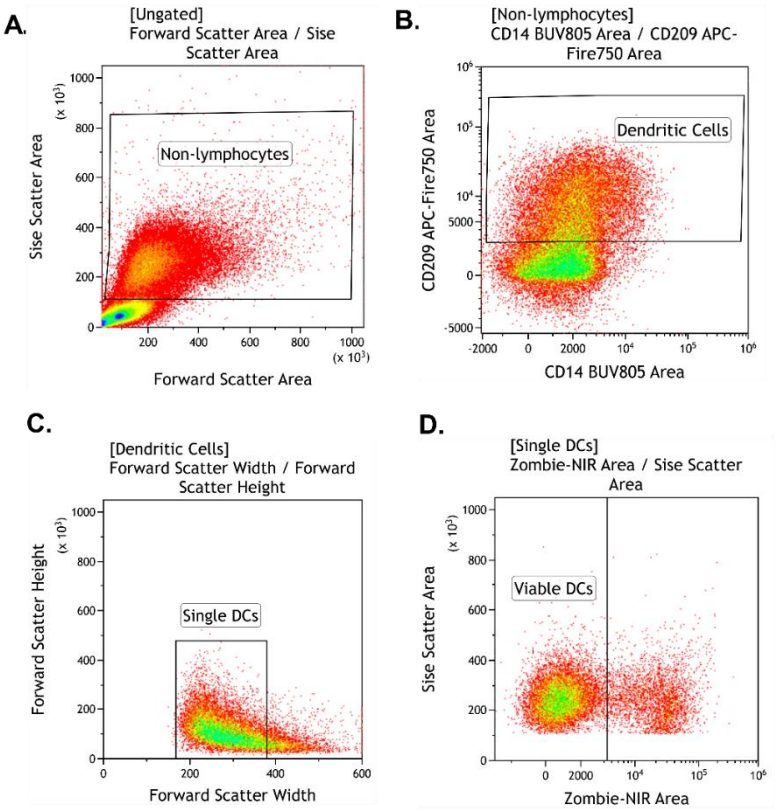


Figure 5.18. DC gating strategy. (A) non-lymphocytes were gated for by selecting for granularity. (B) DCs were then gated by selecting CD209 positive cells. (C) Any clumped DCs were excluded by selecting for single DCs. (D) finally, Zombie negative cells were gated for to select viable DCs.

It was important to phenotype the DCs in terms of their maturation markers and cytokine production, as these can influence the iNKT cell response during the co-culture. Before maturation markers and cytokine production was analysed, the viability of the DCs during co-culture was determined to decipher whether the peanut oil was toxic, due to its high concentration. **Figure 5.19** demonstrates the viability of peanut oil-stimulated DCs (mean= 72.78%) was not significantly lower than the negative control (mean= 73.26%), as determined by Tukey's multiple comparisons test ($p>0.05$). Viability was also consistent between the other conditions, with the lowest viability in Ara h 8-stimulated DCs (mean=64.49%).

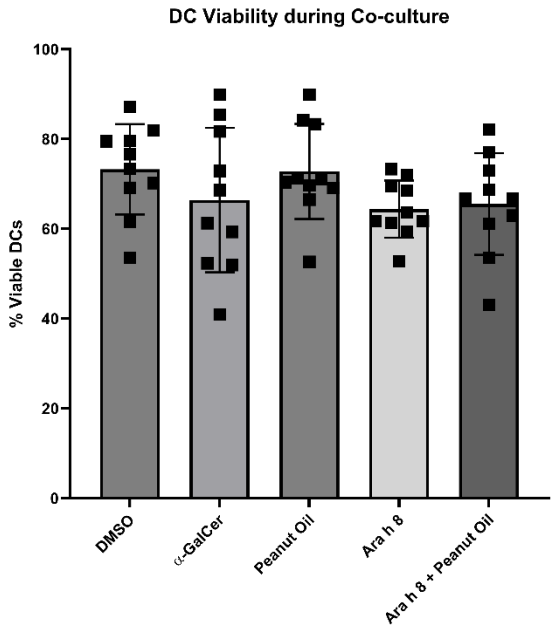


Figure 5.19. DC Viability during co-culture. The percentage of viable DCs were gated from DCs negative for Zombie viability dye. The percentage of viable DCs are presented for iNKT:DC co-cultures where DCs were stimulated with either the DMSO control, α-Galcer, peanut oil, Ara h 8, or both peanut oil + Ara h 8. N=12.

Next, DC markers expressed on the viable, stimulated DCs were examined, comparing between DCs from peanut-allergic subjects to non-allergic subjects. The aim of this was to understand if peanut oil had an effect on DC markers, suggesting an interaction between lipid and DCs. DC maturation marker presented in Chapter 4 showed there was upregulation of DC markers CD80 and HLA-DR in response to LPS. Thus, the investigation of peanut oil and/or Ara h 8 on DC markers was conducted to establish any similar results. Hence, DC maturation markers HLA-DR and CD80, as well as DC surface markers CD14 and CD209 were analysed. The inclusion of other maturation markers, such as CD40 and CD83, would be beneficial, but due to the already large flow panel design, the addition of even more antibodies would have been too costly.

Figure 5.20 demonstrates the percentage of DCs expressing each DC marker (**Ai-Aiv**) and the relative median fluorescence intensity for each marker (**Bi-Biv**). The rMFI was calculated by dividing the MFI of the cytokines by the unstained control. Flow cytometry analysis of DC markers show the

percentages of DCs in each condition producing CD80 and HLA-DR, as well as their rMFI, are not significantly different from the DMSO control ($p>0.05$), with large variances between samples. In addition, CD14 and CD209 expression were not significantly changed from the DMSO control. Based on the DC data from Chapter 4, despite LPS causing upregulation of these markers, α-GalCer did not. It is therefore not surprising that peanut oil also had no effect on DC maturation. Furthermore, a mixed effects analysis with Sidak's multiple comparisons test found there were no significant differences between DC maturation marker expression from non-allergic compared to peanut-allergic subjects ($p>0.05$).

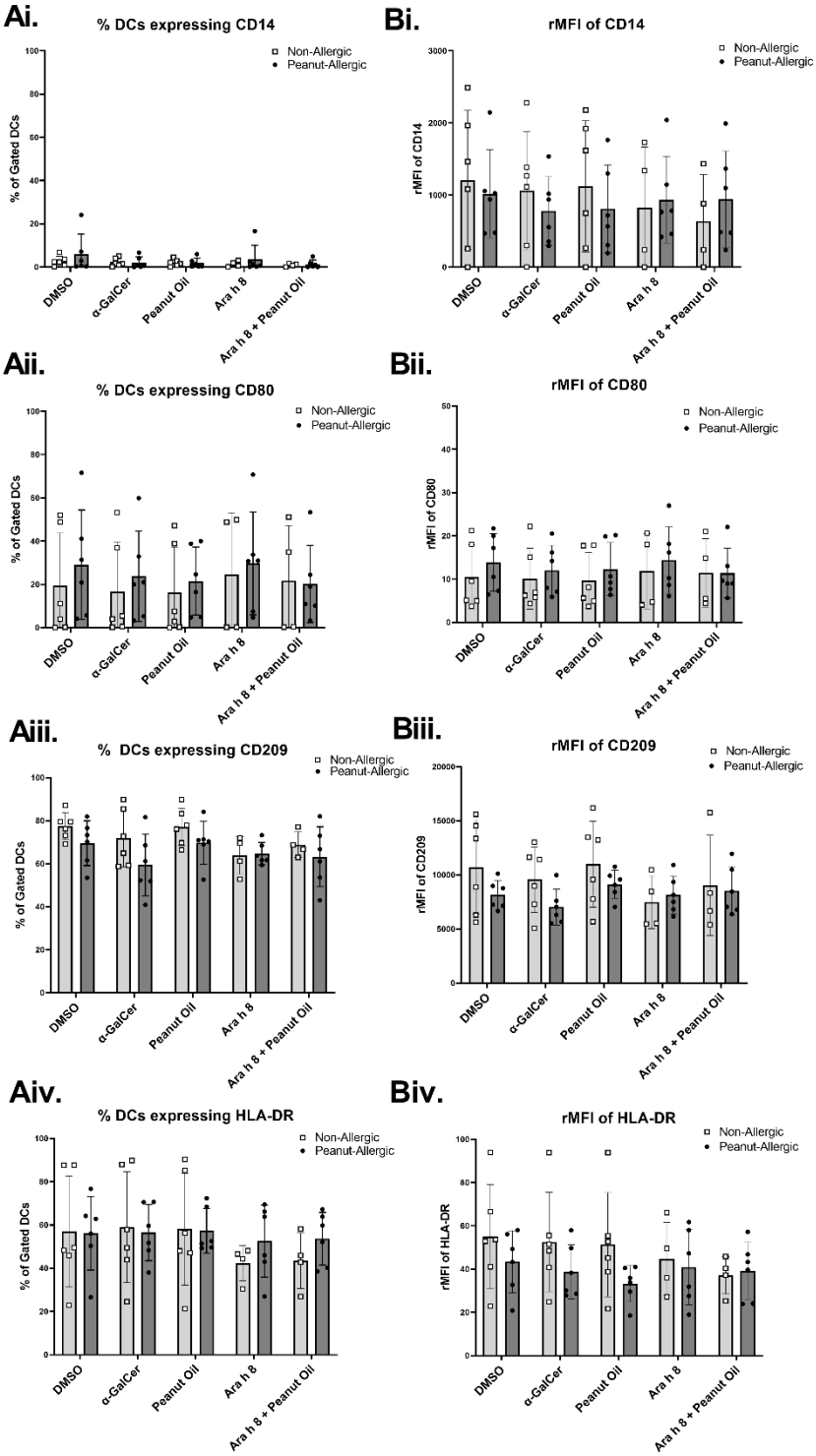


Figure 5.20. DC maturation in non-allergic and peanut-allergic subjects. DCs from non-allergic and peanut-allergic subjects were stimulated with either: DMSO control, α-GalCer, peanut oil, Ara h 8, or both peanut oil and Ara h 8, for 24 hours. The DCs were co-cultured with iNKT cells for 5 hours and the cells were stained for flow cytometry analysis. The relative MFI was then calculated for DC markers HLA-DR, CD80, CD14, and CD209. (n=6 non-allergic, 6 peanut-allergic).

Despite the lack of differences in DC marker expression between the stimulated DCs and the DMSO control, DC cytokine production was assessed by flow cytometry, as this could also influence the cytokines produced by iNKT cells. Cytokines IL-10 and IL-12 were identified by intracellular staining. The percentage of DCs producing each cytokine as well as the cytokines rMFI in each condition was analysed, with the results presented in **Figure 5.21**.

A mixed-effects analysis with Sidak's multiple comparisons suggested there was a significant difference between non-allergic and peanut-allergic individual's rMFI of IL-10 ($p<0.05$, $n=6$), with a mean difference of 3.33 rMFI. Also, the percentage of DCs producing IL-12 was raised in Ara h 8-stimulated DCs in both subject groups, although this was not significantly different. There were also no other significant results in terms of rMFI or the % of DCs producing the cytokine, between any condition or subject group.

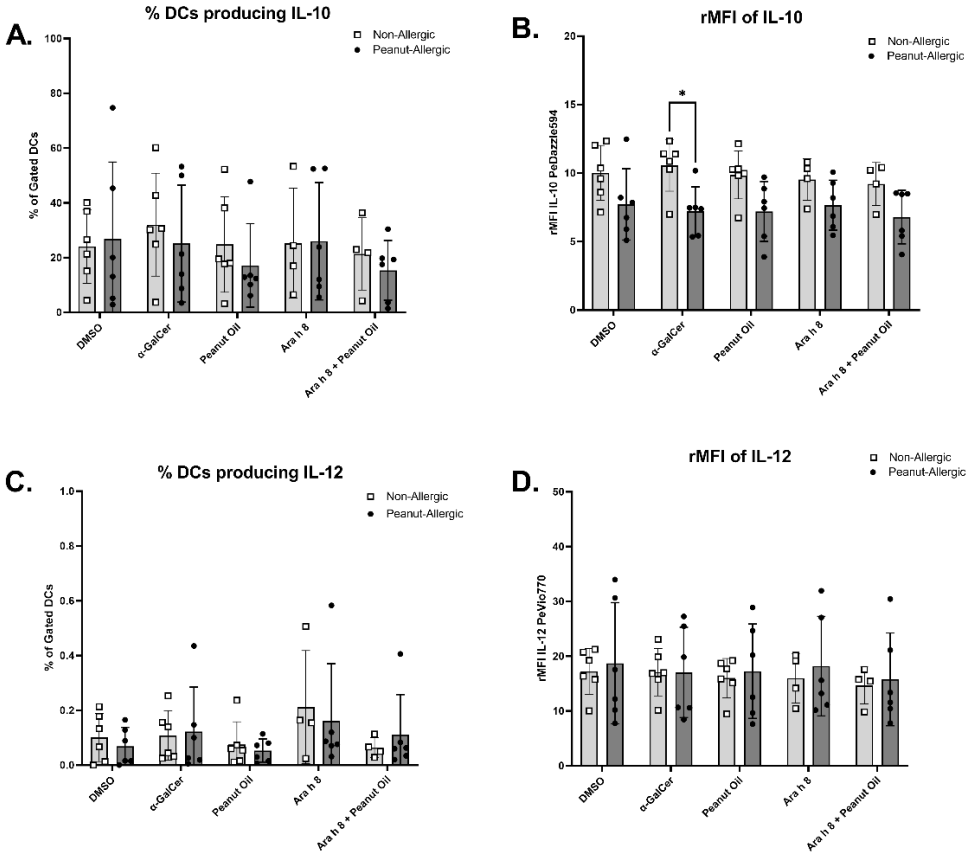


Figure 5.21. DC Cytokine Production during co-culture. The cytokine production from DCs stimulated with either the DMSO control, α-GalCer, peanut oil, Ara h 8, or both Ara h 8 and peanut oil, after 5 hours of co-culture with autologous iNKT cells. Cytokines were stained for and analysed by flow cytometry. The graphs represent (A) % DCs producing IL-10 (B), rMFI of IL-10, (C) % DCs producing IL-12, (D) rMFI of IL-12. All results are comparing DCs from non-allergic and peanut-allergic subjects. (*p<0.05, vertical bars represent standard deviation, n=6 non-allergic, 6 peanut-allergic).

Overall, the DCs stimulated with peanut oil, Ara h 8, or α-GalCer showed no differences in DC surface markers from the DCs stimulated with the DMSO control, suggesting they were not matured. Furthermore, non-allergic subjects had a significantly higher rMFI of IL-10 than peanut-allergic subjects in α-GalCer-stimulated DCs, suggesting they produced more Treg cytokines in response to the glycolipid. This trend was also seen in the other conditions, but was not significant. Despite slight increases in IL-12 production in Ara h 8-stimulated DCs, there were no significant changes in cytokine production or

percentage of DCs producing the cytokines, compared to the DMSO control. Furthermore, despite the lack of effect of peanut oil and Ara h 8 on DC phenotype and cytokine profile, this is similar to what was observed in Chapter 4, where fluorescent α-GalCer had no effect on DC phenotype. However, it was subsequently shown to be internalised by DCs through imaging flow cytometry. Thus, despite the lack of effect on DC phenotype, the DCs have still likely internalised the peanut oil and/or Ara h 8, and are presenting the lipids or proteins to the iNKT cells.

5.3.7. iNKT Cell Cytokine Production during Co-culture with Lipid-pulsed DCs

Following co-culture, Th1 and Th2 cytokine production can be measured to determine whether the lipid can enhance allergic sensitisation.

At Day 14 of expansion, the α-GalCer-expanded iNKT cells were immunomagnetically isolated and subsequently co-cultured with either DMSO-pulsed DCs, α-GalCer-pulsed DCs, peanut oil-pulsed DCs, Ara h 8-pulsed DCs, or DCs pulsed with both peanut oil and Ara h 8, for up to 5 hours, at a ratio of 2:1 (iNKTs-DCs). Before staining for cytokines, the cells were incubated with a protein transport inhibitor for 5 hours to allow the accumulation of cytokines within the iNKT cell. Thus the cytokines were measured at 0-5 hours of co-culture.

In the exemplar plots in **Figure 5.22**, representing a peanut-allergic individual, there was a decrease in the percentage of iNKT cells producing IL-4 only, across all conditions, with 81.80% IL4+ before co-culture, decreasing to as low as 34.57% in the peanut oil + Ara h 8-stimulated iNKTs. Furthermore, an increase in the percentage of iNKT cells producing both IL-4 and IFN-γ after 5 hours of co-culture with the positive control, α-GalCer-pulsed DCs was observed. There was also an increase in iNKT cells producing only IFN-γ in α-GalCer-pulsed DCs. However, the peanut oil, Ara h 8, and both peanut oil + Ara h 8 conditions exhibited no clear differences in IFN-γ production compared to the DMSO control. Furthermore, IL-10 and IL-5 cytokine staining show decreases in IL-10 production across all conditions.

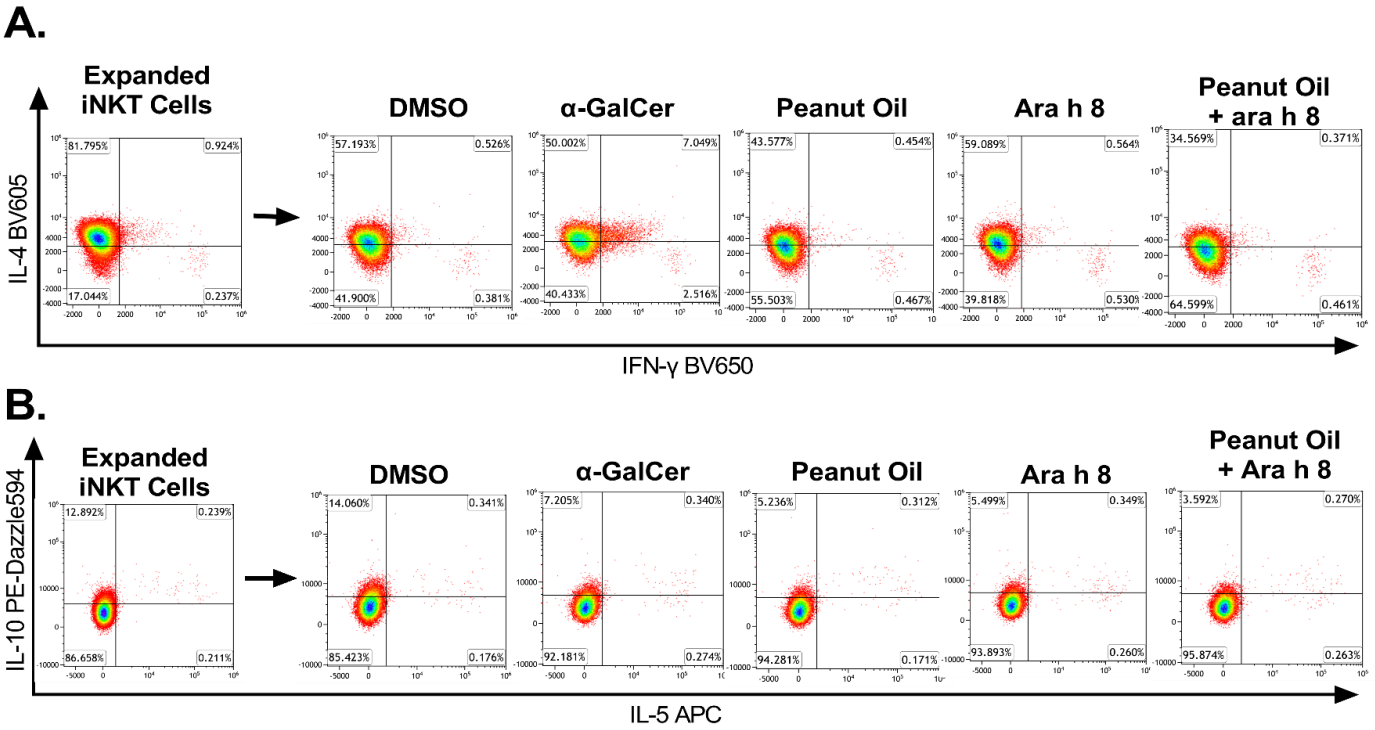


Figure 5.22. Exemplar Dot plots of iNKT Cell Cytokine Production. (A) Representative IL-4 and IFN-γ cytokine dot plots by flow cytometry, from a peanut allergic subject. The plots from left to right are iNKT cells after expansion but before co-culture, followed by co-culture with DMSO-pulsed DCs, α-GalCer-pulsed DCs, peanut oil-pulsed DCs, Ara h 8-pulsed DCs, and peanut oil + Ara h 8-pulsed DCs. (B) Representative IL-5 and IL-10 cytokine dot plots by flow cytometry, from a peanut allergic subject. The plots from left to right are iNKT cells after expansion but before co-culture, followed by co-culture with DMSO-pulsed DCs, α-GalCer-pulsed DCs, peanut oil-pulsed DCs, Ara h 8-pulsed DCs, and peanut oil + Ara h 8-pulsed DCs.

Analysing the iNKT cell cytokine production across all peanut-allergic and non-allergic subjects, **Figures 23Ai-Aiv** demonstrate changes in the percentage of iNKT cells producing total cytokines IFN-γ, IL-4, IL-5, and IL-10 from before co-culture (with backgrounds subtracted, i.e. minus the DMSO control). **Figures 23Bi-Biv** show the fold change in rMFI of each cytokine, compared to before co-culture, and background subtracted.

These graphs support the results from the representative dot plots; the % of iNKT cells producing IFN-γ increased in all conditions compared to before co-culture and after background levels were removed, increasing by as much as 33.12% in peanut-allergic iNKT cells in the α-GalCer condition. Peanut oil-stimulated iNKT cells showed a slightly lower increase in the percentage of iNKT cells producing IFN-γ compared to the α-GalCer condition, with a mean of 18.92% in non-allergic, and 15.58% in peanut-allergic subjects. iNKT cells in the Ara h 8 and peanut oil + Ara h 8 conditions showed lower and almost identical IFN-γ production levels. Furthermore, the rMFI of IFN-γ increased the most in response to α-GalCer-pulsed DCs, by a mean of 29.90% in peanut-allergic subjects and 15.22% in non-allergic subjects, although this was not statistically different. There were no significant differences in cytokine production between groups in any other condition.

The percentage of iNKT cells producing IL-4 decreased in all conditions, with no significant differences between subject groups ($p>0.05$). This decreased the most (by 39.52%) in Ara h 8-stimulated iNKTs from peanut-allergic subjects, and the least by α-GalCer non-allergic subjects (mean=14.37%). Although, the rMFI of IL-4 actually increased in all conditions, but only marginally, and this was highest in α-GalCer-stimulated non-allergic iNKT cells (mean = 0.38%).

The percentage of iNKT cells producing IL-5 during co-culture increased across all conditions, but its rMFI showed decreases, suggesting the number of iNKT cells producing IL-5 increased, but of those that were producing IL-5, the amount of IL-5 the cells produced decreased.

Also, there were large increases in the percentage of iNKT cells producing IL-10 across all conditions and subjects, with the highest mean increase of 26.71% in non-allergic α-GalCer-stimulated iNKT cells. The rMFI across all conditions also increased, but only in peanut-allergic subjects, suggesting they produced more IL-10 than non-allergic subjects.

Overall, all conditions show increases in the percentage of iNKT cells producing IFN-y, IL-5, and IL-10 compared to the DMSO control, and decreases in IL-4 production, although, these were not significantly different to the DMSO control. The cytokine profiles of non-allergic and peanut-allergic individuals also did not differ significantly ($p>0.05$, $n=6$). Importantly, the peanut oil induced similar trends to α-GalCer-stimulated iNKT cells, but the effect was not as great, and no different to the Ara h 8 only condition, suggesting the peanut oil is not having a significant effect on iNKT cell cytokine production.

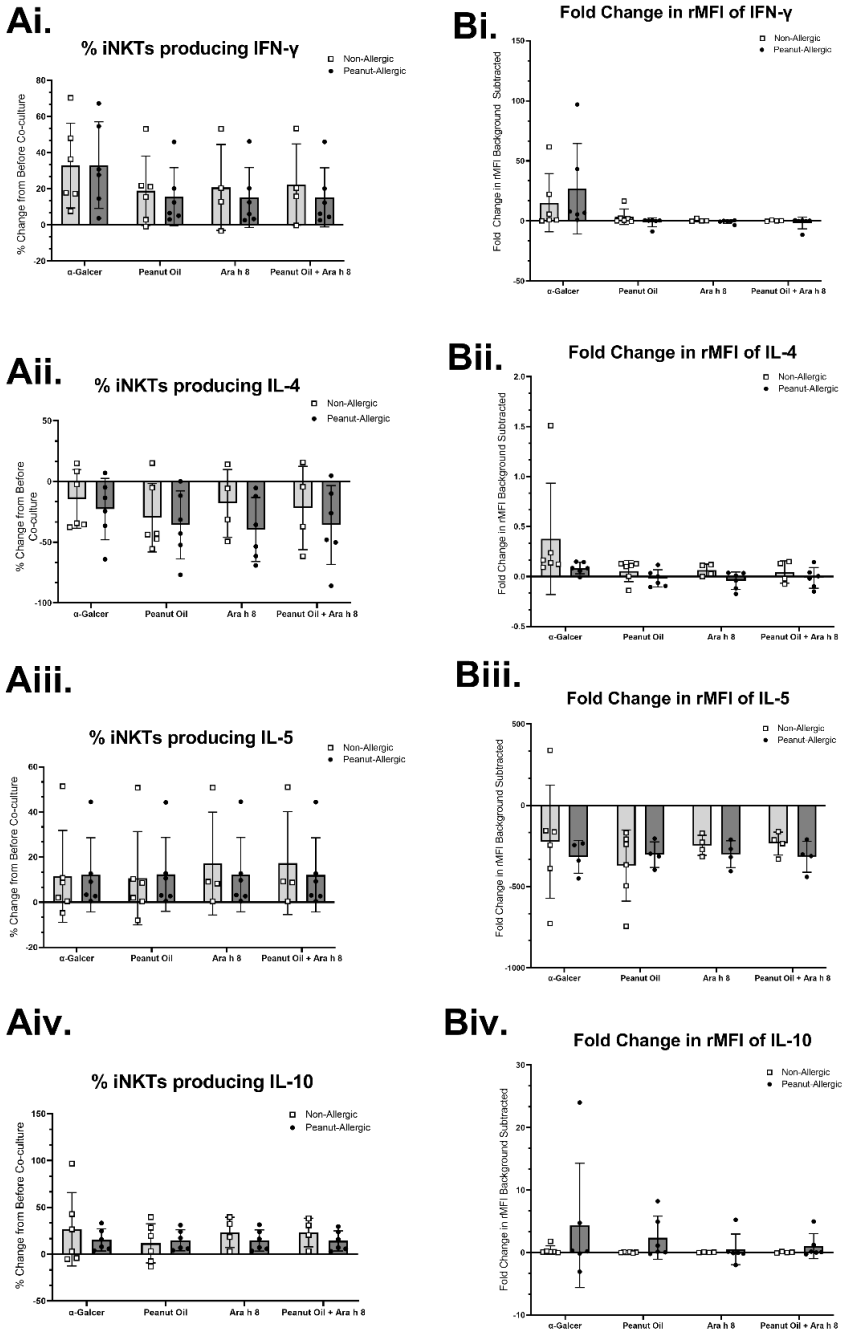


Figure 23. iNKT Cell cytokine production during co-culture with DCs. Non-allergic and peanut-allergic iNKT cell cytokine production was analysed after co-culture with either DMSO-pulsed DCs, α-GalCer-pulsed DCs, peanut oil-pulsed DCs, Ara h 8-pulsed DCs, or peanut oil + Ara h 8-pulsed DCs. The cytokines measured were IFN-γ, IL-4, IL-5, and IL-10. **Ai-Aiv** demonstrate changes in the percentage of iNKT cells producing cytokines from before co-culture, background subtracted (minus the DMSO control). **Bi-Biv** show the fold change in rMFI of each cytokine, compared to before co-culture, and background subtracted. Horizontal bars represent means, vertical bars represent standard deviation, n=6 non-allergic, 6 peanut-allergic).

After the total IFN-γ, IL-4, IL-5, and IL-10 cytokine production was analysed, next, the percentage of iNKT cells producing different combinations of the 4 cytokines was examined. iNKT cells were gated for as previously shown, and then a tree diagram produced in Kaluza software was used to obtain the percentages of iNKT cells producing IFN-γ, IL-4, IL-5, and IL-10 in combination (**Fig 5.24**).

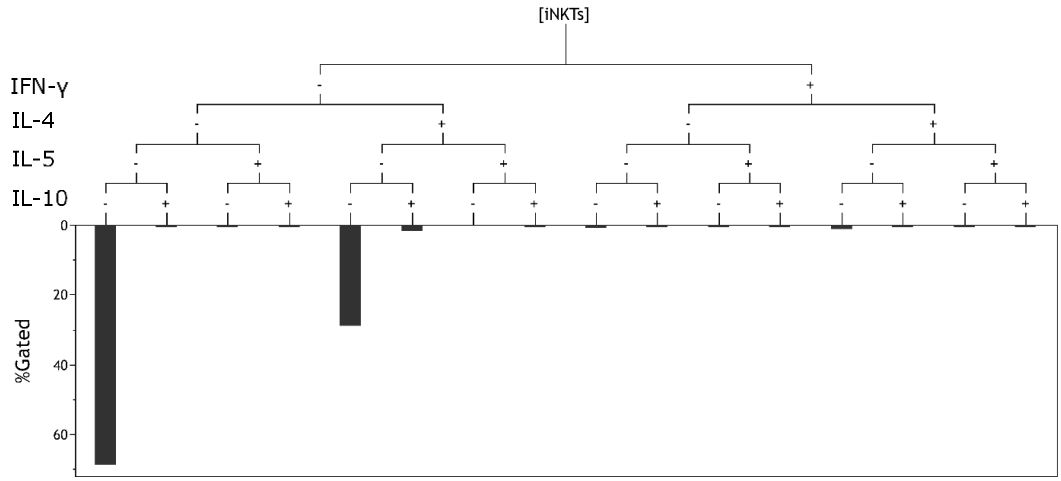


Figure 5.24. Tree Diagram for iNKT Cytokine Gating. iNKT cells were gated for and a tree diagram of the percentage of cytokines gated was made in Kaluza software. iNKT cells producing different combinations of IFN-γ, IL-4, IL-5, and IL-10 cytokines were measured. N=1.

A heat map of the percentage of iNKT cells producing these cytokine combinations at 0-5 hours of co-culture with DCs is shown in **Figure 5.25A**. The heat map illustrates the percentage of iNKT cells producing a combination of cytokines, relative to before being placed in culture with DCs (i.e. relative to day 14 of iNKT cell expansion) and minus the background (DMSO) for the 6 non-allergic and 6 peanut-allergic subjects. The iNKT cell cytokine combinations showing trends in the heat map were then selected and graphed below to further examine the cytokine production (**Fig 5.25B**).

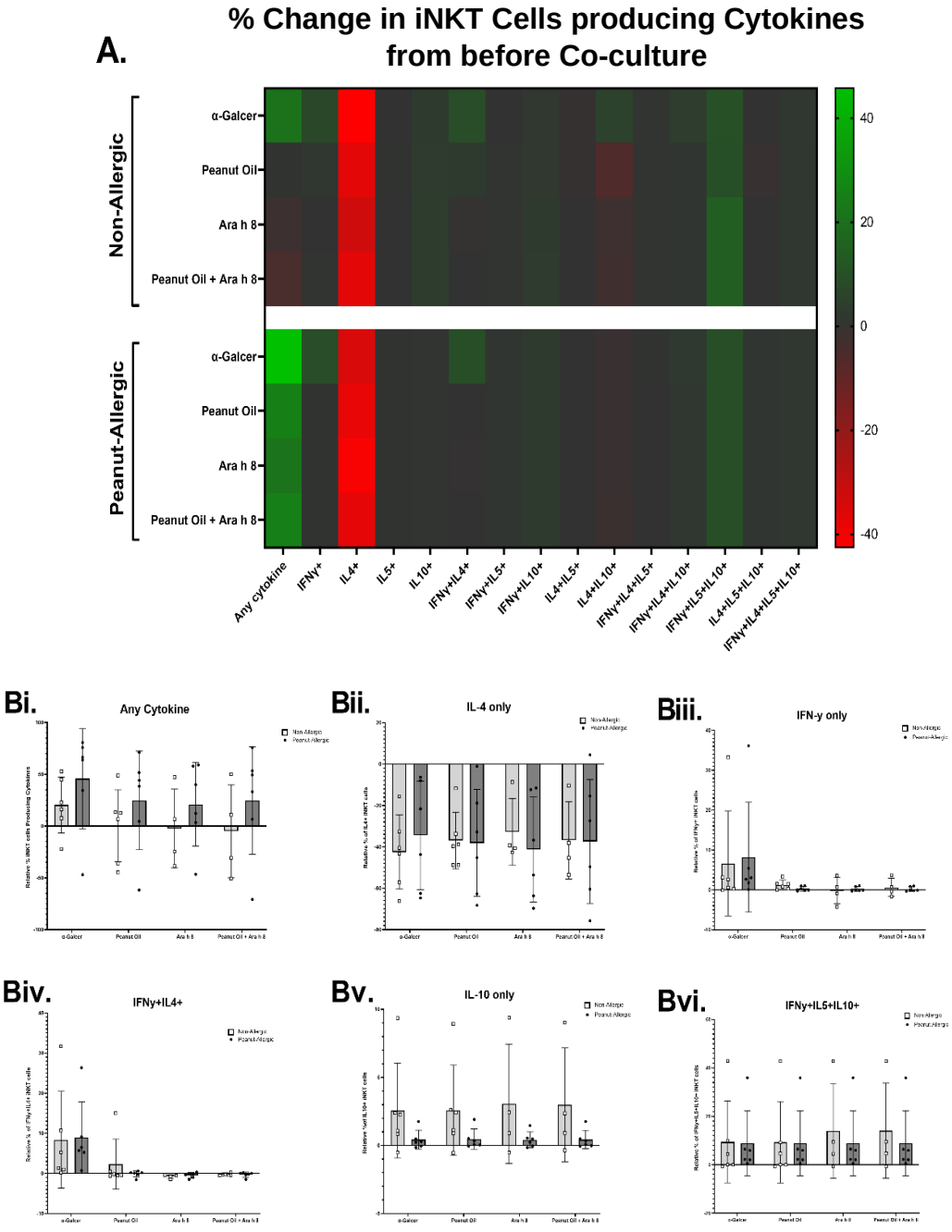
The heat map and graph 5.25Bi highlight peanut-allergic subjects are producing more total cytokines than non-allergic iNKT cells, across all conditions. In the peanut oil-stimulated co-culture, peanut allergic iNKT cells

produced a mean increase of 24.75%, compared to 0.19% in non-allergic subjects, although these results were highly variable between subjects (34.18% SD in non-allergic, and 47.60% SD in peanut-allergic subjects).

Another clear trend identified in the heat map is the large decrease in iNKT cells producing only IL-4, across all conditions, in both subject groups, as highlighted by the red data points in the heat map. Although again, this was not significant, as demonstrated in graph 5.25Bii. Despite the decrease in IL-4-only producing iNKT cells, there were notable increases in the percentage of iNKT cells producing both IFNγ+IL4+ in both subject groups, in response to α-GalCer, but not peanut oil, Ara h 8, or both peanut oil + Ara h 8. Graph 5.25Biii shows non-allergic and peanut-allergic subjects had a mean increase of 8.38% and 8.95%, respectively, regarding the % of iNKTs producing IFNγ+IL4+ in response to α-GalCer-pulsed DCs. This suggests IL-4-only producing iNKT cells before co-culture are now also producing IFN-γ after co-culture with α-GalCer-pulsed DCs, which would explain these changes in cytokine production.

The heat map also indicates the percentage of iNKT cells producing only IFN-γ increased in response to α-GalCer-pulsed DCs, in both subject groups. Graph 5.25Biv highlights this increase was by an average of 6.63% (non-allergic) and 8.20% (peanut-allergic) during the co-culture with α-GalCer-pulsed DCs, compared to iNKT cells before co-culture, minus the DMSO control, although this was not statistically significant. This increase in IFN-γ was not observed in iNKTs cultured with DCs pulsed with the peanut oil or Ara h 8, or both.

The percentage of iNKT cells producing IL-10-only increased more in non-allergic subjects, across all conditions. For instance, Graph 5.25Bv shows in peanut oil co-cultures, non-allergic iNKT cells producing IL-10-only increased by a mean of 2.58%, compared to 0.47% in peanut-allergic subjects. Although, these percentage increases of IL-10-only are marginal. Finally, the percentage of iNKT cells producing a combination of IFNγ, IL-5, and IL-10 increased in all conditions (Fig 5.25Bvi), such as by 9.32% in peanut oil-exposed non-allergic iNKTs, and 8.88% in peanut-allergic. But again, this was not statistically significant.



5.25. A heat map of iNKT cell cytokine production during co-culture with DCs. Non-allergic (top) and peanut-allergic (bottom) iNKT cells were stained for cytokines IFN-γ, IL-4, IL-5, IL-10, and IL-12 after co-culture with either DMSO-pulsed DCs, α-GalCer-pulsed DCs, peanut oil-pulsed DCs, Ara h 8-pulsed DCs, or peanut oil + Ara h 8-pulsed DCs. The percentage of iNKT cells producing different combinations of these cytokines are presented, with green indicating a high percentage, and red indicating a low percentage. The percentage of iNKTs producing the cytokines is relative to before co-culture. Calculated by dividing the % of iNKT cells producing the cytokines in each condition, by the % of iNKT cells producing the cytokines before co-culture.

Overall, this data indicates there were increases in IFNγ+ and IFNγ+IL4+ producing iNKT cells after co-culture with a-GalCer-pulsed DCs in both non-allergic and peanut-allergic subjects. The percentage of IFNγ+IL4+IL5+ iNKT cells increased, and the % of IL-4-only iNKT cells decreased across all conditions, for both subject groups. However, none of these changes were significantly different from the DMSO control. In conclusion, peanut oil with/without Ara h 8 did not have a significant effect on iNKT cell cytokine profile after 5 hours of culture.

Despite there being no significant influences of the peanut oil on iNKT cell cytokine production, the subsets of iNKT cells producing cytokines can next be identified for any differences between subjects. This would also help determine if it is a specific subset responsible for the increases in iNKT cells producing IFN-γ only, both IFN-γ and IL-4, and IFNγ+IL4+IL5+. **Figure 5.26** demonstrates the percentage of iNKT cell subsets producing no IFN-γ or IL-4 (A), IFN-γ only (B), IL-4 only (C), or both IFN-γ and IL-4 (D), in response to co-culture with α-GalCer-, peanut oil-, Ara h 8-, and both peanut oil + Ara h 8-pulsed DCs.

The results show iNKT cell subtypes can exhibit different cytokine profiles, suggesting the abundance of certain subtypes can determine whether a Th1 or Th2 response is produced. Specifically, CD4+ iNKTs were the main subtype producing no IFN-γ or IL-4 during all co-cultures, in both subject groups. CD4+ iNKTs constituted 50.71% (SD= 19.77) in non-allergic, and 42.58% in peanut-allergic (SD= 11.39) of the iNKTs producing neither of the cytokines in response to α-GalCer-DCs. The next most abundant iNKT phenotype was DN, with DN iNKTs constituting a mean of 35.61% (SD=22.96) in non-allergic subjects, and 30.73% (SD= 14.62) in α-GalCer co-cultures. CD8+ and DP iNKTs were least abundant in the no cytokine-producing iNKTs.

Of the iNKT cells producing IFN-γ-only, CD8+ iNKT cells were dominant in the peanut oil (48.47% in non-allergic, 59.94% in peanut-allergic), Ara h 8 (67.73% in non-allergic, and 65.79% in peanut-allergic), and both peanut oil + Ara h 8 conditions (mean=68.00% in non-allergic, 63.70% in peanut-allergic).

Whereas in α-GalCer co-cultures, CD4+ iNKT cells were just as prominent; 36.35% CD4+ and 37.67% in non-allergic subjects. DN iNKTs were in low abundance, and DP iNKTs the least.

For IL-4-only producing iNKTs, CD4+ iNKTs were prominent across conditions and subjects, with a mean of 54.41% in non-allergic, and 59.74% in peanut-allergic during α-GalCer co-cultures. A similar abundance of iNKT cell subtypes are shown in iNKT cells producing both IFN-γ and IL-4, as Graphs 5.21c-d also show a dominant CD4+ phenotype.

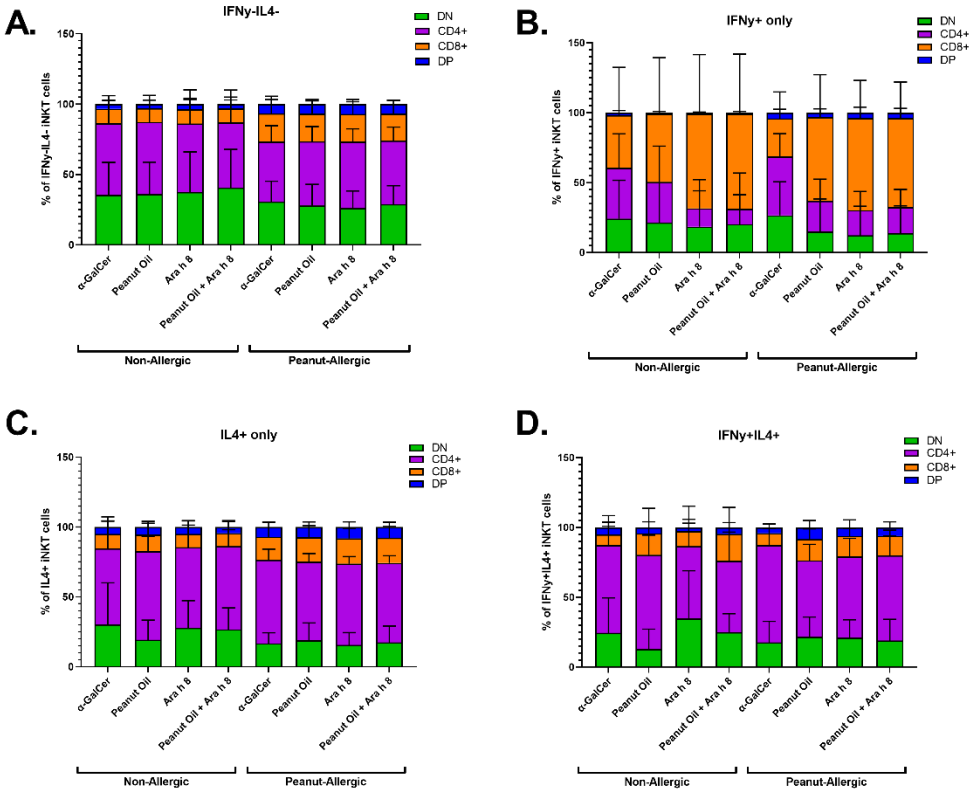


Figure 5.26. iNKT cell cytokine production by subtype. The relative MFI of IFN-γ and IL-4 cytokine production by each iNKT cell subtype in response to co-culture with α-GalCer-, peanut oil-, Ara h 8-, and both peanut oil + Ara h 8-pulsed DCs. iNKT cells from non-allergic (left) and peanut-allergic (right) subjects are presented. The results are relative to the MFI of cytokines before co-culture i.e. co-culture results were divided by before co-culture results.

These findings are in accordance with existing literature, which states that CD8+ iNKT cells produce mainly IFN-γ, and CD4+ INKT cells produce mainly IL-4 (Schmid et al. 2018a).

To ensure all avenues of iNKT cell cytokine production analysis had been explored, Clustering analysis was then performed using FlowJo software to analyse iNKT cell phenotypes between subjects. Single viable CD3/CD1d-aGalCer Tetramer iNKT cells were first gated in FlowJo before equal sampling of 5,000 events from each 6 non-allergic and 6 peanut-allergic subjects. FlowSOM clustering was first performed to obtain 30 meta-clusters which could then be presented on a tSNE plot. The percentage of each cluster which belonged to non-allergic or peanut-allergic was determined.

The clustering analysis was performed on iNKTs co-cultured with peanut oil-stimulated DCs, as analysing cytokine responses to this lipid is the main objective of this research. The analysis found 30 clusters present (Fig 5.27Ai-Aii) after analysing the iNKT cells with parameters CD1d-aGC tetramer, CD3, CD4, CD8, CD25, CD69, IFNγ, IL-4, IL-5, and IL-10. The DC and T cell markers were removed from analysis to allow for small iNKT cell populations to be identified. The expression of these markers across the clusters are presented in Figure 5.25B. To determine if there were any differences between iNKT phenotypes of non-allergic and peanut-allergic subjects, the percentage of subject iNKT cells present in each cluster was measured (Fig 5.27C). Figure 5.25D highlights the cluster ‘pop 6’ was significantly higher in non-allergic compared to peanut-allergic subjects ($p<0.05$, N=6). To identify the marker expression of this cluster (Fig. 5.25Ei), Cluster Explorer analysis was run (Fig. 5.25Eii) which found the population was CD3+/CD4-/CD8+/CD1d-Galcer-tetramer+/CD25+/CD69-/IL4+/IL5+/IL10+/IFNγ+. Interestingly, these are CD8+ iNKT cells with late activation that are producing all 4 cytokines.

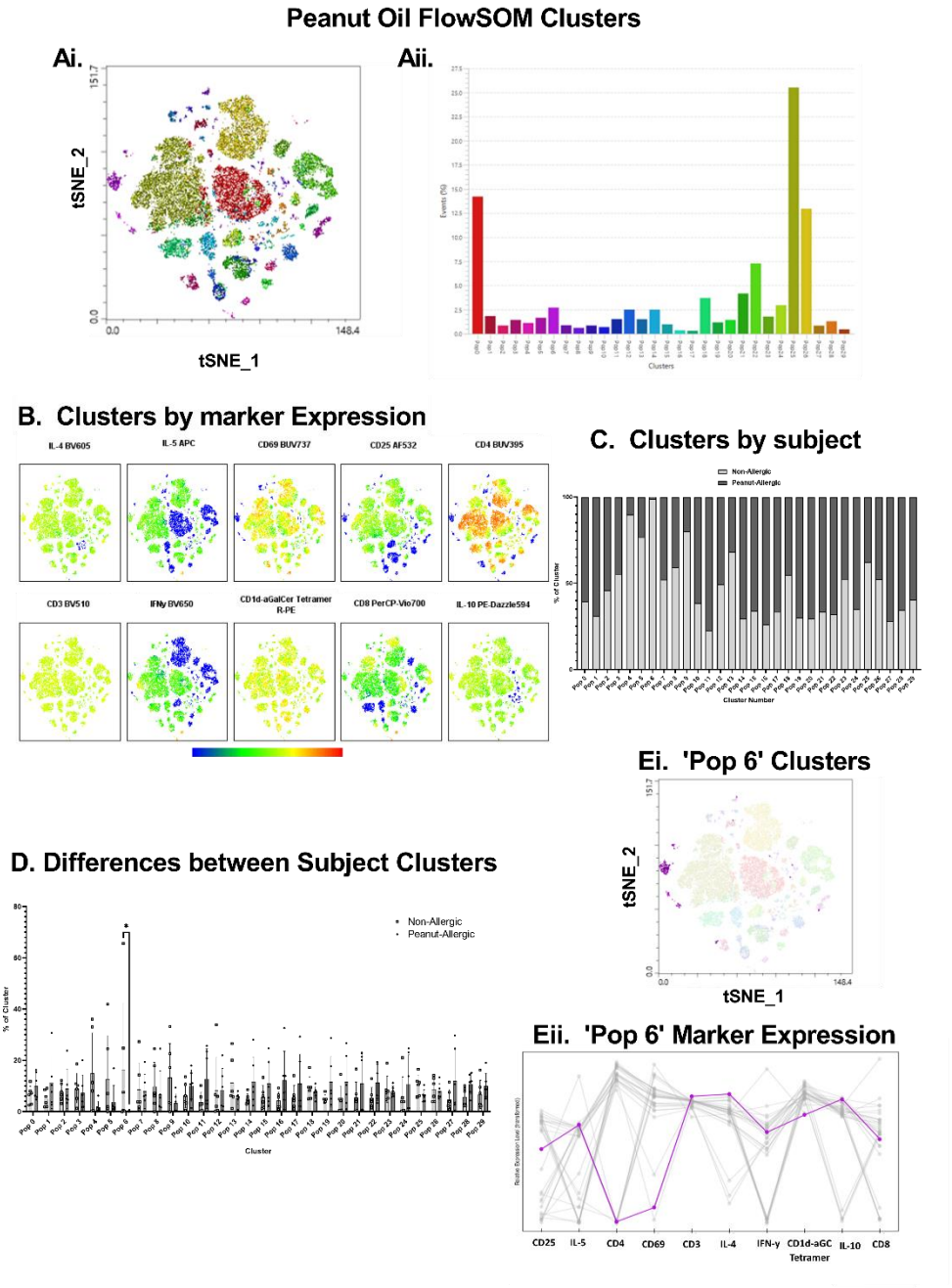


Figure 27. Peanut Oil iNKT Cell Clustering Analysis. (Ai) FlowSOM clusters from peanut oil-exposed iNKT cells presented as a tSNE plot. (Aii) FlowSOM clusters by % of events. (B) Heatmap of Clusters by marker expression. (C) % of cluster occupied by non-allergic or peanut-allergic iNKT cells. (D) Statistical analysis of differences between clusters of non-allergic and peanut-allergic subjects. (Ei) tSNE plot of 'pop 6' to show location of cluster. (Eii) Marker expression of 'pop 6'. *=$p<0.05$.

To determine if the phenotype of peanut oil-stimulated iNKT cells was different to the positive lipid control, α-GalCer, Clustering analysis was also conducted for iNKTs co-cultured with α-GalCer-stimulated DCs (**Fig. 5.28**). The analysis found 20 clusters, of which there were no significant differences in non-allergic and peanut-allergic iNKT cells. This highlights the non-allergic CD8+ iNKT cells producing all 4 cytokines population identified in the peanut oil clustering is unique to the peanut oil.

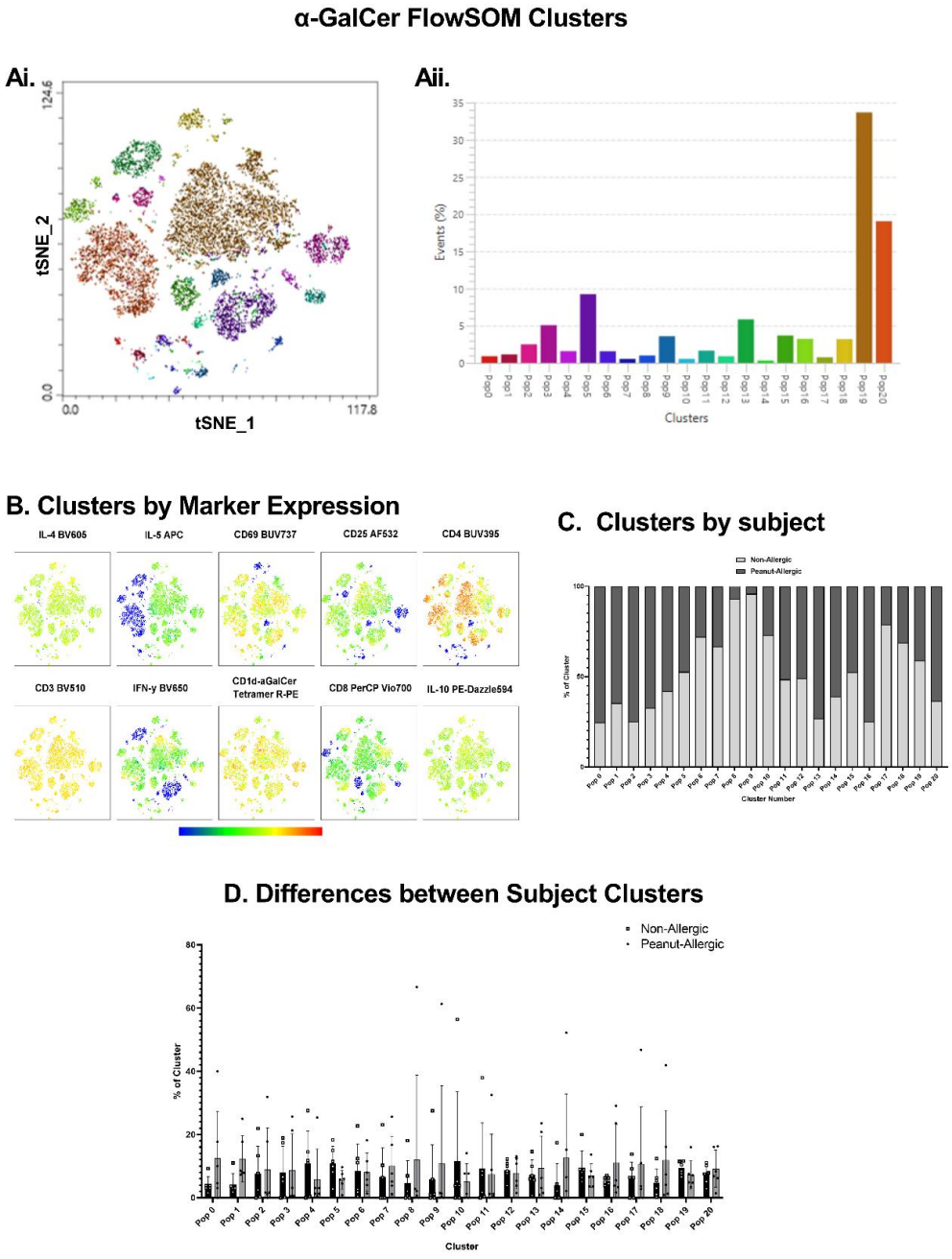


Figure 5.28. α-GalCer iNKT Cell Clustering Analysis. (Ai) FlowSOM clusters from peanut oil-exposed iNKT cells presented as a tSNE plot. (Aii) FlowSOM clusters by % of events. (B) Heatmap of Clusters by marker expression. (C) % of cluster occupied by non-allergic or peanut-allergic iNKT cells. (D) Statistical analysis of differences between clusters of non-allergic and peanut-allergic subjects.

5.4. Discussion

Here, we have developed an *in vitro* co-culture model of human iNKT cells and DCs, to investigate the role of lipids in allergic sensitisation. By utilising the glycolipid, α-GalCer, the most potent activator of iNKT cells, iNKT cells were expanded to increase cell numbers before co-culture with lipid and/or allergen-pulsed DCs. Focussing on peanut allergy, the total lipid fraction from peanuts was investigated for any influence on allergic sensitisation to the lipophilic peanut allergen, Ara h 8. It was hypothesised that either Ara h 8 would bind lipids from peanut oil and DCs would subsequently internalise the allergen-lipid complex, to then present the complex to iNKT cells, or alternatively the peanut lipids without the protein allergen could interact with iNKT cells, resulting in Th1 and/or Th2 cytokine production. Ultimately, the measure of this cytokine production would indicate whether peanut oil can influence allergic sensitisation to peanut allergens, such as Ara h 8. Furthermore, blood from peanut-allergic and non-allergic individuals were obtained to compare their iNKT cell phenotypes, as it is suggested allergic-individuals have different iNKT cell populations. The data highlights there are differences in iNKT cell numbers and phenotypes between allergic and non-allergic individuals, however the cytokine production in response to peanut oil and/or Ara h 8 was minimal in both subject groups, which will now be discussed.

Firstly, the subject's allergic status was confirmed, so total IgE, Ara h 2- and Ara h 8-specific IgE from plasma samples were measured by ELISA. Overall, the allergic subjects had a higher mean total IgE, Ara h 2-IgE, and Ara h 8-IgE compared to non-allergic subjects. Interestingly, total IgE was significantly correlated with Ara h 8-specific IgE levels which is opposite to what was expected, as Ara h 8-sensitivity is associated with milder allergy. Despite higher IgE means across peanut-allergic subjects, there were 3 non-allergic individuals with higher total IgE than peanut-allergic individuals. This questions whether these non-allergic controls were truly free of IgE-mediated allergies. However, there is variability in total IgE levels, which is why alone it is not used as a diagnostic tool for allergy (Holmes et al. 2016). Moreover, factors such as

alcohol consumption, helminth infection, obesity, and metabolic diseases can all increase total IgE levels (Carballo et al. 2021), thus without further investigations, the total IgE levels are hard to draw conclusions. Also, one peanut-allergic subject with low total IgE levels and low Ara h 2- and Ara h 8-specific IgE levels has GP-confirmed allergies to peanuts, cat dander, and pollen. Thus, this suggests that measuring IgE levels may not be an accurate representation of allergy status. This may be influenced by the half-life of allergen-specific IgE in the blood. As if this subject has not encountered any of the allergens for many years (as most people avoid peanuts who are allergic), then there is a chance the peanut allergen-specific IgE levels may be low. Furthermore, peanut allergy has been shown to be substantially lower than peanut sensitisation in children i.e. approximately 10% of children in the UK have peanut allergen-specific IgE, but only ~2% of these children have peanut allergy symptoms. Furthermore, only 12.2% of children sensitised to peanuts had detectable peanut specific IgE levels (Nicolaou et al. 2010).

There is debate in the literature as to whether iNKT cells are increased or decreased in allergic disease, with some literature suggesting children with milk allergies had fewer iNKT cell numbers (Jyonouchi et al. 2011), and some literature suggesting iNKT cells were increased in children with allergic asthma (Antunes et al. 2018.). Here, iNKT cell populations before and after expansion with α-GalCer were compared between peanut-allergic and non-allergic individuals. The data highlights there was a 5-fold higher percentage of lymphocytes which were iNKT cells in peanut allergic individuals compared to non-allergic individuals, before expansion, although this was not statistically significant, likely due to the small sample size. Thus supporting the notion that iNKT cells are increased in allergic disease. This is the first evidence of differences in iNKT cell differences in peanut-allergic versus non-allergic individuals. Furthermore, most research has focused on children's iNKT cell populations in allergy, whereas this research focused on adults over 18 years of age with allergy, which again, is the first study detailing these iNKT cell population differences in allergic adults. Furthermore, females had higher iNKT

cells, which declined with age which was supported by existing literature (Singh et al. 2022), but this was not significant and should be interpreted cautiously due to low iNKT cell numbers. Nonetheless, the increased percentage of iNKT cells in peanut-allergic individuals highlights its potential role in the pathogenesis of allergic disease.

Analysing iNKT cells after 14 days of expansion, there were clear increases in iNKT cell populations in both subject groups, however, the peanut-allergic subjects exhibited a significantly higher iNKT cell population by Day 14. This is potentially due to their higher starting iNKT cell population, as the fold-change in expansion was similar between subjects (122 in non-allergic, and 133 in peanut-allergic). Studies have adopted this method of iNKT cell expansion with α-GalCer before, but less so to study allergic disease. Of the available literature, one study stimulated iNKTs from children with food allergy with the lipid milk sphingomyelin, and found the iNKTs proliferated in response (Jyonouchi 2010). Thus, in addition to α-GalCer, some PBMCs were stimulated with peanut oil to decipher whether it could activate iNKT cells to proliferate. However, peanut oil-stimulated PBMCs displayed no iNKT cell expansion after 14 days. This suggests there may be a specificity for iNKT cell recognition of lipids, or because we utilised the whole lipid fraction rather than a specific lipid class, which could result in less abundance of the activating lipid.

Analysis of iNKT cell subtypes revealed CD4+ iNKT cells were the most prominent subtype after expansion in both groups. Before expansion, peanut-allergic subject's iNKT cells comprised mostly DN phenotype, so expansion with α-GalCer initiated a shift in phenotype to CD4+. Literature suggests DN and CD8+ iNKT subsets predominantly secrete Th1 cytokines and display cytotoxic properties, whereas CD4+ iNKT subsets exhibit both Th1 and Th2 phenotypes (O'Reilly et al. 2011). Thus, the preferential expansion of CD4+ iNKT cells could produce a bias in the cytokines produced during co-culture with lipid-pulsed DCs. This is reinforced from data in this study, where flow cytometry analysis revealed CD4+ iNKT cells produced the most IL-4 in response to α-GalCer, than other iNKT cell subsets, in peanut-allergic subjects. In comparison, DP iNKT cells

produced the most IL-4 in non-allergic subjects. This supports existing literature as it highlights CD4+ iNKT cells secret mostly Th2 cytokines, such as IL-4.

After optimisation assays revealed co-culturing iNKTs with DCs for 5 hours resulted in the strongest cytokine production, compared to 24 hours, the cytokines were measured from both cell types during 0-5 hours of co-culture. The rapid cytokine production is expected, as iNKT cells respond within hours to stimulation (Krovi and Gapin 2018). Before co-culture, the expanded iNKT cells predominantly produced IL-4, which is consistent with previous research (Sag et al. 2017). Then after co-culture, this data indicates there were increases in IFN-γ+ and IFN-γ+IL4+ producing iNKT cells after co-culture with a-GalCer pulsed DCs in both non-allergic and peanut-allergic subjects. The percentage of IFNγ+IL4+IL5+ iNKT cells also increased and IL-4-only iNKT cells decreased, across all conditions for both subject groups. Despite the clear trends in data, none of the cytokine production was changed significantly from the DMSO control, likely due to the small sample size. If this was repeated with a larger group, it may show that iNKT cells from both subject groups produced both Th1 and Th2 cytokines in response to the potent iNKT cell activator α-GalCer, but not in response to peanut oil and/or Ara h 8. A key finding was peanut-allergic iNKT cells produced more cytokines in response to all DC conditions, suggesting the iNKT cells are more readily activated. This is supported by research which showed despite children with milk allergy having fewer iNKT cells, they proliferated just as efficiently as non-allergic, and produced more Th2 cytokines (Jyonouchi et al. 2011). Clustering analysis of iNKT cells co-culture with peanut oil-pulsed DCs found one iNKT cell type was more abundant in non-allergic subjects, which was CD8+ iNKT cells with late activation that are producing IFN-γ, IL-4, IL-5, and IL-10 cytokines. This was unexpected as CD8+ iNKT cells primarily produce IFN-γ, but this indicates there are small CD8+ populations which produce just as much Th2 cytokines. Clustering analysis of iNKT cells co-cultured with α-GalCer-pulsed DCs showed no differences between subject groups, highlighting this small CD8+ iNKT cell population is specific to peanut-oil exposure.

As well as iNKT cells, DCs were also phenotyped by flow cytometry. Analysing DCs, the results showed no increase in maturation markers after 24 hours of stimulation with α-GalCer, peanut oil, or Ara h 8. A limitation of this is immature DCs were not stained for maturation markers before their stimulation with peanut oil or Ara h 8. However, the DMSO control should result in no change of DC marker expression, thus, comparing the results to this allows us to see any changes in marker expression. Furthermore, the results indicate similar DC cytokine production between all conditions and subject groups, apart from non-allergic α-GalCer-stimulated DCs that produced a significantly higher amount of IL-10 than peanut-allergic DCs. This is supported by existing research which found there were no differences in IL-12 production or DC marker expression by moDCs between non-allergic and allergic individuals (Bellinghausen et al. 2000). Overall, this suggests that the DCs aren't producing any cytokines in response to the stimulants which would influence iNKT cell cytokine production.

As Tregs can influence the immune tolerance of allergens, the Treg population before and after PBMC culture with α-GalCer or peanut oil for 14 days was analysed. The data highlights no significant differences in Treg populations between the two subject groups before culture. However, there was a significantly greater Treg population after culture with peanut oil in peanut-allergic subjects compared to non-allergic subjects. This is inconsistent with existing literature which suggests a higher proportion of Tregs results in tolerance of allergens (Liu et al. 2021).

Overall, the present data show peanut-allergic individuals exhibit higher iNKT cell numbers, with a DN phenotype, which shifts to a CD4+ iNKT cell phenotype after expansion with α-GalCer. The co-culture of iNKT cells with DC-pulsed peanut oil and/or Ara h 8 did not induce any significantly different cytokine production compared to the DMSO control, likely due to the small sample size. However, future research could focus on the most abundant fatty acid in peanut oil, oleic acid, and look for immune responses against this

specific lipid, as research has suggested it can influence allergic sensitisation to food allergens.

Chapter 6: General Discussion

IgE-mediated allergies are increasing in prevalence, yet the mechanisms underpinning how certain individuals become sensitised to allergens are not fully understood. Allergic sensitisation is the first phase of IgE-mediated allergy development, where individuals are exposed to an allergen for the very first time. This involves DC presentation of allergens to naïve T cells via MHC II molecules, resulting in Th2 differentiation and B cell production of allergen-specific IgE (Salazar and Ghaemmaghami 2013b). Various factors have been suggested to influence allergic sensitisation, such as the structure and functions of protein allergens (Gough et al. 2003; Mullins et al. 2022; Wildner et al. 2017). Although not everybody develops allergies to well-established allergens, highlighting a clear gap in the knowledge underlying allergic sensitisation. Recently, other immune cells have been proposed to influence allergic sensitisation, such as epithelial cells. Specifically, genetically-predisposed individuals have been shown to have impaired barrier function of epithelial cells, resulting in the increased absorption of allergens, leading to allergic sensitisation (Smallcombe et al. 2019). In addition, iNKT cells have also been shown to influence allergic sensitisation, through the rapid release of Th1 and Th2 cytokines in response to lipids associated with protein allergens. It is well-established that protein allergens are accompanied by other components, such as carbohydrates and lipids. But limited research has focused on these components and their influence on allergic sensitisation. A recent systematic review of the studies investigating the role of lipids in allergic sensitisation found that a common mechanism proposed was the presentation of lipids via CD1d molecules on DCs to invariant NKT cells, resulting in their activation and Th2 cytokine production, thus shifting to allergic sensitisation (Hopkins et al. 2022). Some research suggested the lipid alone can influence allergic sensitisation (Mirotti et al. 2013b), whereas a recent review suggested lipophilic allergens interact with lipids, and it is the combination of the two molecules which influences allergic sensitisation (Jappe et al. 2019a).

This existing, but limited, research into lipids in allergic sensitisation mainly utilised murine models due to the higher iNKT cell population compared to humans. Although, this poses concerns regarding the applicability of the results to develop human therapies to treat diseases, such as allergy. A recent systematic review found 8 studies investigating iNKT-CD1d interactions in allergic sensitisation, with 3 of these utilising murine models (Bansal, Gaur, and Arora 2016; González Roldán et al. 2019; Dearman, Alcocer, and Kimber 2007), 2 utilising both murine and human (Mirotti et al. 2013b; Tordesillas et al. 2017), and 3 utilising human models only (Agea et al. 2005; Abos Gracia et al. 2017; Jyonouchi et al. 2011). The 2 studies utilising both murine and human models to study allergic sensitization could be deemed important to show lipid effects in two model systems. However, studies solely recruiting murine models needs to be cautiously interpreted, as there are clear differences to the human immune system. One such difference is that cytokines, such as IL-10, produced by a Th2 response in mice are actually produced by a Treg, Th1 and Th2 response in humans (Mestas and Hughes 2004). In addition, inducing allergy in mice is artificial and does not fully reflect the development of allergic sensitisation in humans. Hence the need for more research in iNKT cells and lipids in allergic sensitisation, utilising human models.

The research presented here provides a human, *in vitro* model system for investigating the role of lipids, iNKT cells, and DCs in the development of allergic sensitisation. The model system was first developed and optimised before incorporating peanut lipids and allergens to investigate the role of lipids in peanut allergy. Peanut allergy is one of the most common food allergies in children, affecting approximately 2% of the population in Western countries and is often a life-long disease (Lieberman et al. 2021). Symptoms are often more severe than other food allergies, such as wheat and soy, often resulting in anaphylaxis, which is life-threatening. Thus, it is essential to further understand the mechanisms underlying peanut allergy to help develop treatments and prevent fatalities. Furthermore, peanuts comprise a high proportion of lipids, approximately 42-49% (Ros and Mataix 2006a;

Venkatachalam and Sathe 2006), which is higher than other legumes, such as soy beans, which contain between 8.1-24% lipids (Medic, Atkinson, and Hurburgh Jr. 2014) and are less potent at driving allergic sensitisation. Thus, the high lipid content of peanuts makes it a good choice to study the role of lipids in allergic sensitisation to peanut allergens. By utilising the total lipid fraction from peanuts, peanut oil, and the lipophilic peanut allergen, Ara h 8, the influence of peanut lipids with or without the presence of Ara h 8 was examined for any influence on iNKT cell cytokine production. The results were compared between peanut-allergic and non-allergic individuals, to assess whether there were differences in iNKT cell phenotypes and their response to lipids.

6.1. Higher iNKT Cells Numbers in Peanut-Allergic Subjects

Before cytokine production could be measured from iNKT cells co-cultured with peanut lipid and/or Ara h 8-pulsed DCs, iNKT cells first had to be expanded. This is due to the extremely low abundance of iNKT cells in human peripheral blood, possibly why the application of human iNKT cells in allergic sensitisation research is limited. The challenges of utilising human NKT cells were highlighted in the early stages of this research, where NKT cells were isolated directly from human PBMCs and the cell numbers were very low, resulting in poor viability in culture. Subsequent experiments focused on proliferating the iNKT cells, where the glycolipid, α-GalCer, the most potent activator of iNKT cells, was found to induce robust expansion of human iNKT cells over the course of 14 days, increasing the numbers of iNKT cells. Flow cytometry analysis of the iNKT cell populations in peripheral blood found there was a greater population of iNKT cells in peanut-allergic individuals, compared to non-allergic iNKT cells. Moreover, the peanut-allergic iNKT cells then expanded more readily than non-allergic iNKT cells, likely due to the higher starting iNKT cell population. To the best of my knowledge, this is a novel phenomenon which has not yet been shown in individuals with peanut allergy. There has been limited research into quantifying iNKT cell populations in allergic individuals, but the opinions are mixed, with some suggesting they are

decreased in milk-allergic children, but exhibit strong Th2 cytokine responses to lipids (Jyonouchi et al. 2011), and some suggesting they are increased in children with allergic asthma (Antunes et al. 2018.). Here we show there was a 5-fold higher percentage of iNKT cells from peripheral blood of peanut-allergic adults, compared to non-allergic adults, which supports the hypothesis that iNKT cells play a role in allergy. Furthermore, the expansion with α-GalCer highlight a preferential expansion of $CD4^+$ iNKT cell subtypes in both peanut-allergic and non-allergic subjects, which has been identified previously (Schmid et al. 2018a). $CD4^+$ iNKT cells are predominantly IL-4 producing cells which have tolerogenic properties as shown in studies where IL-4 producing iNKT cells prevent autoimmune diseases, such as encephalomyelitis (Miyamoto, Miyake, and Yamamura 2001). Although, in allergic sensitisation, the release of IL-4 promotes a Th2 immune response, thus the α-GalCer-expanded iNKT cells promote CD4+ phenotypes which then enhance a Th2 response.

6.2. DC Uptake of Lipids

To replicate iNKT cell activation during allergic sensitisation, DCs were generated and pulsed with peanut oil and the peanut allergen, Ara h 8, and later co-cultured with the expanded iNKT cells to allow DC presentation of the lipid and/or allergen to iNKT cells, via CD1d-iTCR interaction. Our results show DCs can be successfully generated from human monocytes and stimulated with Ara h 8 and peanut oil before subsequent co-culture with autologous iNKT cells. There were no effects on DC maturation markers or cytokine production after stimulation with peanut oil or Ara h 8. Thus, there is the question of whether the DCs internalized the peanut oil and/or Ara h 8, as we did not investigate this by imaging cytometry. Future studies could identify whether peanut oil is internalized by DCs, but the peanut oil would need to be fluorescently tagged, and as it contains a vast variety of lipids, this would be difficult unless focusing on a specific lipid class to tag. Furthermore, research has shown that lipids can reduce allergen uptake by human moDCs (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, et al. 2016). The reduction of allergen

uptake is associated with a reduction of tolerance and skewing to a Th2 response (Wisniewski, Agrawal, and Woodfolk 2013). Thus, fluorescently tagging the lipids and performing imaging cytometry of peanut lipids with and without Ara h 8 could also investigate this phenomenon. In addition, investigating Ara h 8 internalisation with or without the presence of lipids could also prove valuable in deciphering whether the protein and lipid are internalised and presented on CD1d molecules.

Chapter 4 details efforts in optimising CD1d expression on DCs, as these are the molecules which present lipids to iNKT cells. The percentage of immature DCs expressing CD1d was high, but the rMFI of CD1d was low. We found that culturing monocytes with FBS downregulated CD1d expression more than when monocytes were co-cultured with human AB serum. But its low expression was enough to present the lipid α-GalCer to iNKT cells, as indicated by their rapid cytokine production. We had hypothesized that CD1d expression would increase on DCs, based on previous research showing the total lipid fraction from olive pollen enhanced CD1d expression (Abos Gracia et al. 2017). But this was not observed in response to the positive control lipid, α-GalCer, or the peanut oil. This suggests there may be a specificity for CD1d upregulation due to structural features of the lipids. Although, other literature also suggests CD1d surface expression is low on lipid-stimulated DCs; glycolipids producing Th1 cytokines, as seen with α-GalCer producing IFNγ, require access to the lysosomal loading compartment found inside DCs to be processed before loading onto CD1d molecules (Keller, Freigang, & Lünemann, 2017), and thus intracellular CD1d may increase with α-GalCer stimulation.

6.3. Lipid-driven iNKT Cell Cytokine Production

The iNKT-DC co-culture model was first developed using the positive control, α-GalCer, where the α-GalCer-pulsed DCs stimulated increases in the percentage of iNKT cells producing IFN-γ and IL-4+IFNγ+, and decreases in IL-4 only iNKT cells, within the first 5 hours of co-culture. This reinforces the model

system developed works correctly. Despite these clear trends in the data, none of the cytokine production was changed significantly from the DMSO control, likely due to the small sample size. A power calculation was conducted using R software (calculation: pwr.anova.test(k=2,f=.27,sig.level=.05,power=.8) and found 55 individuals in each subject group would be required to reach statistical power. This highlights our sample size of 6 is insufficient. However, this sample size was a reflection of the timeframe and funding available for this PhD.

Furthermore, when the system was applied to peanut oil and/or Ara h 8, the cytokines produced by the iNKT cells showed little cytokine production in comparison to the positive control, α-GalCer. Despite no influence from the peanut oil and/or Ara h 8, a key finding was peanut-allergic iNKT cells produced more cytokines in response to all DC conditions, suggesting the iNKT cells are more readily activated. Clustering analysis of iNKT cells co-cultured with peanut oil-pulsed DCs found one iNKT cell type was more abundant in non-allergic subjects, which was CD8+ iNKT cells with late activation that are producing IFN-y, IL-4, IL-5, and IL-10 cytokines. This was unexpected as CD8+ iNKT cells primarily produce IFN-y, but this indicates there are CD8+ populations which produce just as much Th2 cytokines.

Here, we utilised the total lipid fraction from peanuts to investigate whether it can skew allergic sensitisation. Due to the lack of cytokine response observed, it could be questioned whether looking at specific lipids from peanuts would be optimal. The focus on the total lipid fraction from peanuts was due to research suggesting the total lipid fraction from peanut and pollen allergen sources can influence allergic sensitisation (Abos Gracia et al. 2017; Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, et al. 2016), and that this was thought as most biologically relevant, as individuals who eat a peanut will be exposed to the entire lipid fraction. However, as the effects of peanut oil on iNKT cell cytokine production were minimal, with no significant differences to the DMSO control, this could be a result of the insufficient concentration of specific lipids in the culture. As other studies focus

on a specific lipid's influence on allergic sensitisation, and have found they do enhance allergic sensitisation i.e. the lipid fraction 'C' enhances allergic sensitisation to Ber e 1 (Mirotti et al. 2013b). Thus, future research could focus on specific lipids from within peanut oil to investigate whether these show more significant results. In addition, the intense processing of the refined peanut oil to remove any proteins could result in altered bioactivity of the lipids present (Rigby et al. 2011), which could impact the effect of the peanut oil on the iNKT cell cytokine production.

Moreover, as seen in Table 5.3, oleic fatty acids are the most abundant fatty acid in peanuts, thus this could be a lipid of interest. Existing research has shown that oleic fatty acids can influence allergic sensitisation to the lentil allergen, Len c 3, where oleic acid bound Len c 3 and subsequently reduced gastric digestion of the allergen and increased its thermostability (Finkina et al. 2020b), enhancing the allergenicity of Len c 3. Furthermore, a high intake of the two major monounsaturated fatty acids, oleic and palmitoleic acid, has been linked with the development of hay fever (Trak-Fellermeier et al. 2004). Thus, narrowing down on the lipids utilised in culture could be a more focussed route in investigating lipids in allergic sensitisation.

6.4. Treg populations in Peanut-Allergic Subjects

The research presented here highlighted a significantly greater Treg population after culture with peanut oil in peanut-allergic subjects compared to non-allergic subjects. This is inconsistent with existing literature which suggests a higher proportion of Tregs results in tolerance of allergens (Liu et al. 2021). Although, these findings could be explained by the presence of oleic acid, which is a major fatty acid source in our peanut oil, has been shown to restore defects in Tregs suppressive function in autoimmune diseases (Pompura et al. 2021). Tregs rely on fatty acid β-oxidation (FAO)–driven oxidative phosphorylation (OXPHOS) for their differentiation and function. The study found Oleic acid amplifies Treg FAO-driven OXPHOS metabolism which

results in increased Treg suppressive function. This could explain why peanut-allergic Tregs increased compared to non-allergic subjects in response to peanut oil, as allergic individuals also have dysfunctions in Tregs (Lan et al. 2020), so the oleic acid could potentially be partially restoring this function.

6.5. General Limitations

A major impact on this research is the small number of peanut-allergic and non-allergic participants, as this has affected the significance of the results. Especially because the results are highly variable between donors. This was due to the delays in NHS ethics approval during the COVID-19 pandemic, delaying the recruitment of subjects and subsequently resulting in reduced participant numbers.

Another limitation is the potential issue in peanut oil solubility in the assays. A possibility for the lack of iNKT cell cytokine production in response to peanut oil with/without Ara h 8 may have been due to the difficulties in solubilising the peanut oil before its addition into culture media. Throughout this research, the importance in lipid preparation before use in the cell-based assays was essential. Lipids are well-known to be difficult to solubilise, as they are lipophilic. Thus, organic solvents, such as DMSO, were utilised for solubilising the lipids. Furthermore, once solubilised, they needed to be sonicated and heated to ensure full solubility. Ensuring each lipid sample is fully solubilised was essential before applying to the cells, as then there can be inconsistencies in the amount of lipid presented to cells. However, the extent to which the peanut oil was solubilised is unknown, thus, it may not have been internalised by DCs in culture.

The iNKT cytokine measurement could also be affected by the fact the iNKT cells are activated and thus producing copious amounts of IL-4 before co-culture with DCs, due to the expansion with α-GalCer and IL-2. Although, because the cytokine production results were calculated relative to any

cytokine production before co-culture, this helps identify what cytokine production is as a result of the co-culture.

The measurement of total IgE as a tool to verify allergic status is flawed by the variability in total IgE levels, which is why alone it is not used as a diagnostic tool for allergy (Holmes et al. 2016). Total IgE varies due to factors such as alcohol consumption, helminth infection, obesity, and metabolic diseases (Carballo et al. 2021). Hence why allergen-specific IgE ELISAs were performed to use in conjunction with total IgE results to confirm allergic status. Notably, there was one peanut-allergic subject with low allergen-specific IgE levels, but if this subject has not encountered any of the allergens for many years (as most people avoid peanuts who are allergic), then there is a chance the peanut allergen-specific IgE levels may be low. Hence, allergen-specific IgE results may be influenced by the half-life of allergen-specific IgE in the blood.

6.6. Applications

With the increasing prevalence of allergic disease, the development of new treatments is in demand. Treating allergies with desensitization is the current treatment for food allergies, but this often results in allergic reactions during the sensitisation process. Understanding the underlying mechanisms of allergic sensitisation could lead to the development of new treatments and prevention approaches to type 1 hypersensitivity. This research has shown that iNKT cells do play a role in allergic sensitisation, highlighted by their increased abundance in peanut-allergic individuals. Thus, this contributes to the better-understanding of allergic sensitisation, and subsequently future studies could investigate these cell types further as potential therapeutic targets.

Despite peanut oil not influencing iNKT cell cytokine production, other lipids from allergen sources have been shown to influence allergic sensitisation (Hopkins et al. 2022). Companies are constantly developing new foods which require safety assessments of any novel foods. Therefore, the application of lipids in toxicological assays to determine the allergenicity of products is

essential, as the lipid fraction from an allergy source could increase the allergenicity of products. Thus the incorporation of lipids into the assessment is key.

6.7. Concluding Remarks

Overall, this study established an *in vitro*, human model system where allergen-associated lipids can be assessed to determine whether they enhance iNKT cell Th2 cytokine secretion, shifting towards a state of allergic sensitisation. Human DC and iNKT cell techniques were successfully optimised before co-culturing the two cell types and measuring cytokine production. Applying the developed assay to peanut allergic subjects, flow cytometry staining of iNKT cells found a 5-fold higher iNKT cell population in peanut-allergic subjects compared to non-allergic subjects. The iNKT cells from both subject groups were then successfully expanded, with iNKT cell populations increasing by 133-fold in peanut-allergic subjects and 122-fold in non-allergic subjects after 14 days of culture with α-GalCer. A shift in iNKT cell phenotype to CD4+ iNKT cells was observed in both subject groups after expansion. Finally, iNKT cell co-culture with α-GalCer-pulsed DCs showed increases in iNKT cell production of IFNγ-only and IFNγ+IL4+ after 5 hours, confirming this *in vitro,* human, cell-based assay is functional. However, when the method was applied to peanut allergy, utilising peanut oil and Ara h 8, the results showed peanut oil and/or Ara h 8 did not have an influence on cytokine production by iNKT cells, potentially due to poor lipid solubility. Despite the lack of cytokine production in response to the peanut oil, this assay system can be replicated using alternative allergen-associated lipids, such as peanut-associated fatty acids, to determine whether they stimulate iNKT cells to secrete Th2 cytokines, and thus shift towards allergic sensitisation of protein allergens. Importantly, based on this research and the current literature, it is evident that the role of lipids in allergic sensitisation varies depending on the lipid examined. Thus, focusing on identifying the specific lipids capable of skewing towards Th2

immune responses and characterize any interaction with lipophilic allergens is essential.

REFERENCES

Abós-Gracia, B., M. G. del Moral, J. López-Relaño, V. Viana-Huete, L. Castro, M. Villalba and E. Martínez-Naves (2013). "Olea europaea pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells." J Allergy Clin Immunol **131**(5): 1393-1399.e1395.

Abos Gracia, B., J. López Relaño, A. Revilla, L. Castro, M. Villalba, B. Martín Adrados, J. R. Regueiro, E. Fernández-Malavé, E. Martínez Naves and M. Gómez Del Moral (2017). "Human Invariant Natural Killer T Cells Respond to Antigen-Presenting Cells Exposed to Lipids from Olea europaea Pollen." Int Arch Allergy Immunol **173**(1): 12-22.

Agea, E., A. Russano, O. Bistoni, R. Mannucci, I. Nicoletti, L. Corazzi, A. D. Postle, G. De Libero, S. A. Porcelli and F. Spinozzi (2005). "Human CD1-restricted T cell recognition of lipids from pollens." Journal of Experimental Medicine **202**(2): 295-308.

Ahmadi, A., Z. Fallah Vastani, M. Abounoori, M. Azizi, A. Labani-Motlagh, S. Mami and S. Mami (2022). "The role of NK and NKT cells in the pathogenesis and improvement of multiple sclerosis following disease-modifying therapies." Health Science Reports **5**(1): e489.

Akdis, M., S. Burgler, R. Crameri, T. Eiwegger, H. Fujita, E. Gomez, S. Klunker, N. Meyer, L. O'Mahony, O. Palomares, C. Rhyner, N. Ouaked, A. Schaffartzik, W. Van De Veen, S. Zeller, M. Zimmermann and C. A. Akdis (2011). "Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases." J Allergy Clin Immunol **127**(3): 701-721 e701-770.

Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, C. Kegel, R. Disch, C. B. Schmidt-Weber, K. Blaser and C. A. Akdis (2004). "Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells." J Exp Med **199**(11): 1567-1575.

Al-Ghouleh, A., R. Johal, I. K. Sharquie, M. Emara, H. Harrington, F. Shakib and A. M. Ghaemmaghami (2012). "The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent." PLoS One **7**(3): e33929.

Angelina, A., S. Sirvent, C. Palladino, A. Vereda, J. Cuesta-Herranz, T. Eiwegger, R. Rodriguez, H. Breiteneder, M. Villalba and O. Palomares (2016). "The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity." Allergy **71**(9): 1284-1294.

Angelina, A., S. Sirvent, C. Palladino, A. Vereda, J. Cuesta-Herranz, T. Eiwegger, R. Rodríguez, H. Breiteneder, M. Villalba and O. Palomares (2016). "The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity." Allergy **71**(9): 1284-1294.

Antunes, L., A. P. Duarte de Souza, P. D. de Araújo, L. A. Pinto, M. H. Jones, R. T. Stein and P. M. Pitrez (2018). "iNKT cells are increased in children with severe therapy-resistant asthma." Allergologia et Immunopathologia **46**(2): 175-180.

Augustine, T., M. Kumar, S. Al Khodor and N. van Panhuys (2022). "Microbial Dysbiosis Tunes the Immune Response Towards Allergic Disease Outcomes." Clinical Reviews in Allergy & Immunology.

Balan, S., M. Saxena and N. Bhardwaj (2019). "Dendritic cell subsets and locations." Int Rev Cell Mol Biol **348**: 1-68.

Ballmer-Weber, B. K., J. Lidholm, M. Fernández-Rivas, S. Seneviratne, K.-M. Hanschmann, L. Vogel, P. Bures, P. Fritsche, C. Summers, A. C. Knulst, T.-M. Le, I. Reig, N. G. Papadopoulos, A. Sinaniotis, S. Belohlavkova, T. Popov, T. Kralimarkova, F. de Blay, A. Purohit, M. Clausen, M. Jedrzejczak-Czechowcz, M. L. Kowalski, R. Asero, R. Dubakiene, L. Barreales, E. N. Clare Mills, R. van Ree and S. Vieths (2015). "IgE recognition patterns in peanut allergy are age dependent: perspectives of the EuroPrevall study." Allergy **70**(4): 391-407.

Bannon, G. A. (2004). "What makes a food protein an allergen?" Curr Allergy Asthma Rep **4**(1): 43-46.

Bansal, P., S. N. Gaur and N. Arora (2016). "Lysophosphatidylcholine plays critical role in allergic airway disease manifestation." Sci Rep **6**: 27430.

Barral, D. C. and M. B. Brenner (2007). "CD1 antigen presentation: how it works." Nature Reviews Immunology **7**(12): 929-941.

Baseggio Conrado, A., D. Ierodiakonou, M. H. Gowland, R. J. Boyle and P. J. Turner (2021). "Food anaphylaxis in the United Kingdom: analysis of national data, 1998-2018." BMJ **372**: n251.

Bashir, M. E. H., J. H. Lui, R. Palnivelu, R. M. Naclerio and D. Preuss (2013). "Pollen Lipidomics: Lipid Profiling Exposes a Notable Diversity in 22 Allergenic Pollen and Potential Biomarkers of the Allergic Immune Response." PLOS ONE **8**(2): e57566.

Beckman, E. M., S. A. Porcelli, C. T. Morita, S. M. Behar, S. T. Furlong and M. B. Brenner (1994). "Recognition of a lipid antigen by CD1-restricted αβ+ T cells." Nature **372**(6507): 691-694.

Bendiner, E. (1981). "Baron von Pirquet: the aristocrat who discovered and defined allergy." Hospital practice (Office ed.) **16**(10): 137, 141, 144 passim.

Berin, M. C. and W. G. Shreffler (2008). "T_H2 adjuvants: Implications for food allergy." Journal of Allergy and Clinical Immunology **121**(6): 1311-1320.

Birkholz, A. M. and M. Kronenberg (2015). "Antigen specificity of invariant natural killer T-cells." Biomedical Journal **38**(6): 470-483.

Bischoff, S. C., J. Mayer, J. Wedemeyer, P. N. Meier, G. Zeck-Kapp, B. Wedi, A. Kapp, Y. Cetin, M. Gebel and M. P. Manns (1997). "Colonoscopic allergen provocation (COLAP): a new diagnostic approach for gastrointestinal food allergy." Gut **40**(6): 745-753.

Broide, D. H. (2001). "Molecular and cellular mechanisms of allergic disease." J Allergy Clin Immunol **108**(2 Suppl): S65-71.

Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona and M. Kronenberg (1998). "CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution." J Exp Med **188**(8): 1521-1528.

Brough, H. A., K. C. Nadeau, S. B. Sindher, S. S. Alkotob, S. Chan, H. T. Bahnson, D. Y. M. Leung and G. Lack (2020). "Epicutaneous sensitization in the development of food allergy: What is the evidence and how can this be prevented?" Allergy **75**(9): 2185-2205.

Bublin, M., T. Eiwegger and H. Breiteneder (2014). "Do lipids influence the allergic sensitization process?" The Journal of allergy and clinical immunology **134**(3): 521-529.

Caza, T. and S. Landas (2015). "Functional and Phenotypic Plasticity of CD4⁺ T Cell Subsets." BioMed Research International **2015**: 521957.

Chan, A. C., E. Leeansyah, A. Cochrane, Y. d' Udekem d' Acoz, D. Mittag, L. C. Harrison, D. I. Godfrey and S. P. Berzins (2013). "Ex-vivo analysis of human Natural Killer T cells demonstrates heterogeneity between tissues and within established CD4+ and CD4− subsets." Clinical & Experimental Immunology **172**(1): 129-137.

Chow, K. V., A. M. Lew, R. M. Sutherland and Y. Zhan (2016). "Monocyte-Derived Dendritic Cells Promote Th Polarization, whereas Conventional Dendritic Cells Promote Th Proliferation." The Journal of Immunology **196**(2): 624-636.

Cooke, R. A. and A. V. Veer Jr (1916). "Human sensitization." The Journal of Immunology **1**(3): 201-305.

Cordle, C. T. (2004). "Soy protein allergy: incidence and relative severity." J Nutr **134**(5): 1213s-1219s.

Cyster, J. G. (1999). "Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs." J Exp Med **189**(3): 447-450.

D'Amato, G., L. Cecchi, S. Bonini, C. Nunes, I. Annesi-Maesano, H. Behrendt, G. Liccardi, T. Popov and P. van Cauwenberge (2007). "Allergenic pollen and pollen allergy in Europe." Allergy **62**(9): 976-990.

Dearman, R. J., M. J. C. Alcocer and I. Kimber (2007). "Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1." Clinical and Experimental Allergy **37**(4): 582-591.

Del Moral, M. G. and E. Martínez-Naves (2017). "The Role of Lipids in Development of Allergic Responses." Immune network **17**(3): 133-143.

Dhodapkar, M. V. and V. Kumar (2017). "Type II NKT Cells and Their Emerging Role in Health and Disease." The Journal of Immunology **198**(3): 1015-1021.

Dhodapkar, M. V. and V. Kumar (2017). "Type II NKT Cells and Their Emerging Role in Health and Disease." J Immunol **198**(3): 1015-1021.

Du Toit, G., G. Roberts, P. H. Sayre, H. T. Bahnson, S. Radulovic, A. F. Santos, H. A. Brough, D. Phippard, M. Basting, M. Feeney, V. Turcanu, M. L. Sever, M. Gomez Lorenzo, M. Plaut and G. Lack (2015). "Randomized Trial of Peanut Consumption in Infants at Risk for Peanut Allergy." New England Journal of Medicine **372**(9): 803-813.

Dubiela, P., R. Del Conte, F. Cantini, T. Borowski, R. Aina, C. Radauer, M. Bublin, K. Hoffmann-Sommergruber and S. Alessandri (2019). "Impact of lipid binding on the tertiary structure and allergenic potential of Jug r 3, the non-specific lipid transfer protein from walnut." Scientific Reports **9**(1): 2007.

Exley, M. A., P. Dellabona and G. Casorati (2021). "Exploiting CD1-restricted T cells for clinical benefit." Mol Immunol **132**: 126-131.

Fahy, E., S. Subramaniam, R. C. Murphy, M. Nishijima, C. R. H. Raetz, T. Shimizu, F. Spener, G. van Meer, M. J. O. Wakelam and E. A. Dennis (2009). "Update of the LIPID MAPS comprehensive classification system for lipids." Journal of lipid research **50 Suppl**(Suppl): S9-S14.

Finkelman, F. D. (2007). "Anaphylaxis: lessons from mouse models." J Allergy Clin Immunol **120**(3): 506-515; quiz 516-507.

Finkina, E. I., D. N. Melnikova, I. V. Bogdanov, N. S. Matveevskaya, A. A. Ignatova, I. Y. Toropygin and T. V. Ovchinnikova (2020). "Impact of Different Lipid Ligands on the Stability and IgE-Binding Capacity of the Lentil Allergen Len c 3." Biomolecules **10**(12).

Folch, J., M. Lees and G. H. Sloane Stanley (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**(1): 497-509.

Foo, A. C., P. M. Thompson, S. Arora, E. F. DeRose, L. Perera and G. A. Mueller (2019). "Influence of Hydrophobic Cargo Binding on the Structure, Stability, and Allergenicity of the Cockroach Allergen Bla g 1." Journal of Allergy and Clinical Immunology **143**(2 Supplement): AB213.

Furman, D., B. P. Hejblum, N. Simon, V. Jojic, C. L. Dekker, R. Thiébaut, R. J. Tibshirani and M. M. Davis (2014). "Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination." Proc Natl Acad Sci U S A **111**(2): 869-874.

Gadola, S. D., N. Dulphy, M. Salio and V. Cerundolo (2002). "Vα24-JαQ-Independent, CD1d-Restricted Recognition of α-Galactosylceramide by Human CD4⁺ and CD8αβ⁺ T Lymphocytes." The Journal of Immunology **168**(11): 5514-5520.

Galli, S. J. and M. Tsai (2012). "IgE and mast cells in allergic disease." Nat Med **18**(5): 693-704.

Garrido-Arandia, M., L. Tordesillas, N. Cubells, V. Esteban, W. Barcik, L. O'Mahony, L. F. Pacios and A. Diaz-Perales (2018). "The ligand of the major peach allergen Pru P 3 is presented to iNKT cells." Allergy: European Journal of Allergy and Clinical Immunology **73**(Supplement 105): 781.

Gepp, B., D. Ackerbauer, N. Lengger, F. Gruber, M. Mildner and H. Breiteneder (2014). "The major birch pollen allergen Bet v 1 binds lipids from birch and grass pollen but not from peanuts." Allergy: European Journal of Allergy and Clinical Immunology **69**(SUPPL. 99): 464.

Gilles-Stein, S., I. Beck, A. Chaker, M. Bas, M. McIntyre, L. Cifuentes, A. Petersen, J. Gutermuth, C. Schmidt-Weber, H. Behrendt and C. Traidl-Hoffmann (2016). "Pollen derived low molecular compounds enhance the human allergen specific immune response in vivo." Clinical and Experimental Allergy.

Gilles, S., C. Akdis, R. Lauener, P. Schmid-Grendelmeier, T. Bieber, G. Schäppi and C. Traidl-Hoffmann (2018). "The role of environmental factors in allergy: A critical reappraisal." Exp Dermatol **27**(11): 1193-1200.

Gilles, S., I. Beck, A. Chaker, M. McIntyre, L. Cifuentes, H. Bier, A. Petersen, J. Ring, H. Behrendt, C. Schmidt-Weber and C. Traidl-Hoffmann (2014). "Effects of pollen-derived non-protein substances on the allergic immune response in vivo: Roles of adenosine, PALMs and neuroreceptors." Experimental Dermatology **23**(3): e3.

Gilles, S., D. Jacoby, C. Blume, M. J. Mueller, T. Jakob, H. Behrendt, K. Schaekel and C. Traidl-Hoffmann (2010). "Pollen-derived low-molecular weight factors inhibit 6-sulfo LacNAc + dendritic cells' capacity to induce T-helper type 1 responses." Clinical and Experimental Allergy **40**(2): 269-278.

Gilles, S., V. Mariani, M. Bryce, M. J. Mueller, J. Ring, T. Jakob, S. Pastore, H. Behrendt and C. Traidl-Hoffmann (2009). "Pollen-derived E1-phytoprostanes signal via PPAR-gamma and NF-kappaB-dependent mechanisms." J Immunol **182**(11): 6653-6658.

Girardi, E. and D. M. Zajonc (2012). "Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells." Immunological reviews **250**(1): 167-179.

Godfrey, D. I., K. J. Hammond, L. D. Poulton, M. J. Smyth and A. G. Baxter (2000). "NKT cells: facts, functions and fallacies." Immunol Today **21**(11): 573-583.

González Roldán, N., R. Engel, S. Düpow, K. Jakob, F. Koops, Z. Orinska, C. Vigor, C. Oger, J. M. Galano, T. Durand, U. Jappe and K. A. Duda (2019). "Lipid Mediators From Timothy Grass Pollen Contribute to the Effector Phase of Allergy and Prime Dendritic Cells for Glycolipid Presentation." Front Immunol **10**: 974.

Gough, L., E. Campbell, D. Bayley, G. Van Heeke and F. Shakib (2003). "Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model." Clin Exp Allergy **33**(8): 1159-1163.

Gough, L., H. F. Sewell and F. Shakib (2001). "The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen." Clin Exp Allergy **31**(10): 1594-1598.

Grela, E. R. and K. D. Günter (1995). "Fatty acid composition and tocopherol content of some legume seeds." Animal Feed Science and Technology **52**(3): 325-331.

Guilliams, M., F. Ginhoux, C. Jakubzick, S. H. Naik, N. Onai, B. U. Schraml, E. Segura, R. Tussiwand and S. Yona (2014). "Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny." Nat Rev Immunol **14**(8): 571-578.

Gupta, R. S., E. E. Springston, M. R. Warrier, B. Smith, R. Kumar, J. Pongracic and J. L. Holl (2011). "The prevalence, severity, and distribution of childhood food allergy in the United States." Pediatrics **128**(1): e9-17.

Gutermuth, J., M. Bewersdorff, C. Traidl-Hoffmann, J. Ring, M. J. Mueller, H. Behrendt and T. Jakob (2007). "Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses in vivo." Journal of Allergy and Clinical Immunology **120**(2): 293-299.

Hamilton, R. G. and N. F. Adkinson, Jr. (2003). "23. Clinical laboratory assessment of IgE-dependent hypersensitivity." J Allergy Clin Immunol **111**(2 Suppl): S687-701.

Hammad, H., B. N. Lambrecht, P. Pochard, P. Gosset, P. Marquillies, A.-B. Tonnel and J. l. Pestel (2002). "Monocyte-Derived Dendritic Cells Induce a House Dust Mite-Specific Th2 Allergic Inflammation in the Lung of Humanized SCID Mice: Involvement of CCR7." The Journal of Immunology **169**(3): 1524-1534.

Hammond, K. J. L., D. G. Pellicci, L. D. Poulton, O. V. Naidenko, A. A. Scalzo, A. G. Baxter and D. I. Godfrey (2001). "CD1d-Restricted NKT Cells: An Interstrain Comparison." The Journal of Immunology **167**(3): 1164-1173.

He, S. H., H. Y. Zhang, X. N. Zeng, D. Chen and P. C. Yang (2013). "Mast cells and basophils are essential for allergies: mechanisms of allergic inflammation and a proposed procedure for diagnosis." Acta Pharmacol Sin **34**(10): 1270-1283.

Hochwallner, H., U. Schulmeister, I. Swoboda, M. Focke-Tejkl, V. Civaj, N. Balic, M. Nystrand, A. Härlin, J. Thalhamer, S. Scheiblhofer, W. Keller, T. Pavkov, D. Zafred, B. Niggemann, S. Quirce, A. Mari, G. Pauli, C. Ebner, N. G. Papadopoulos, N. G. Papadopoulos, U. Herz, E. A. F. van Tol, R. Valenta and S. Spitzauer (2010). "Visualization of clustered IgE epitopes on alpha-lactalbumin." The Journal of allergy and clinical immunology **125**(6): 1279-1285.e1279.

Hole, C. R., C. M. L. Wager, N. Castro-Lopez, A. Campuzano, H. Cai, K. L. Wozniak, Y. Wang and F. L. Wormley (2019). "Induction of memory-like dendritic cell responses in vivo." Nature Communications **10**(1): 2955.

Hopkins, G. V., S. Cochrane, D. Onion and L. C. Fairclough (2022). "The Role of Lipids in Allergic Sensitization: A Systematic Review." Front Mol Biosci **9**: 832330.

Hopkins, G. V., S. Cochrane, D. Onion and L. C. Fairclough (2022). "The Role of Lipids in Allergic Sensitization: A Systematic Review." Frontiers in Molecular Biosciences **9**.

Hufnagl, K., D. Ghosh, S. Wagner, A. Fiocchi, L. Dahdah, R. Bianchini, N. Braun, R. Steinborn, M. Hofer, M. Blaschitz, G. A. Roth, G. Hofstetter, F. Roth-Walter, L. F. Pacios and E. Jensen-Jarolim (2018). "Retinoic acid prevents immunogenicity of milk lipocalin Bos d 5 through binding to its immunodominant T-cell epitope." Scientific Reports **8**: 12.

Humeniuk, P., P. Dubiela and K. Hoffmann-Sommergruber (2017). "Dendritic Cells and Their Role in Allergy: Uptake, Proteolytic Processing and Presentation of Allergens." Int J Mol Sci **18**(7).

Humeniuk, P., S. Geiselhart, C. Battin, T. Webb, P. Steinberger, W. Paster and K. Hoffmann-Sommergruber (2019). "A jurkat based NFkappaB-EGFP INKT reporter cell line to evaluate the interaction of food-derived lipids with INKT cell receptors." Allergy: European Journal of Allergy and Clinical Immunology **74**(Supplement 106): 602.

Hurlburt, B. K., L. R. Offermann, J. K. McBride, K. A. Majorek, S. J. Maleki and M. Chruszcz (2013). "Structure and Function of the Peanut Panallergen Ara h 8 *." Journal of Biological Chemistry **288**(52): 36890-36901.

Iweala, O. I. and A. W. Burks (2016). "Food Allergy: Our Evolving Understanding of Its Pathogenesis, Prevention, and Treatment." Curr Allergy Asthma Rep **16**(5): 37.

Iweala, O. I., P. B. Savage and S. P. Commins (2018). "A Role for CD1d-restricted invariant natural killer T cells and glycolipids in alpha-gal allergy." Journal of Allergy and Clinical Immunology **141**(2 Supplement 1): AB288.

Jappe, U., C. Schwager, A. B. Schromm, N. González Roldán, K. Stein, H. Heine and K. A. Duda (2019). "Lipophilic Allergens, Different Modes of Allergen-Lipid Interaction and Their Impact on Asthma and Allergy." Frontiers in Immunology **10**(122).

Johannessen, B. R., L. K. Skov, J. S. Kastrup, O. Kristensen, C. Bolwig, J. N. Larsen, M. Spangfort, K. Lund and M. Gajhede (2005). "Structure of the house dust mite allergen Der f 2: implications for function and molecular basis of IgE cross-reactivity." FEBS Lett **579**(5): 1208-1212.

Jyonouchi, S., V. Abraham, J. S. Orange, J. M. Spergel, L. Gober, E. Dudek, R. Saltzman, K. E. Nichols and A. Cianferoni (2011). "Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin." J Allergy Clin Immunol **128**(1): 102-109.e113.

Kabesch, M. and J. Tost (2020). "Recent findings in the genetics and epigenetics of asthma and allergy." Seminars in Immunopathology **42**.

Kaufmann, S. H. E. (2019). "Immunology's Coming of Age." Frontiers in Immunology **10**.

Koch, M., V. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Besra, R. R. Schmidt, E. Y. Jones and V. Cerundolo (2005). "The crystal structure of human CD1d with and without alpha-galactosylceramide." Nat Immunol **6**(8): 819-826.

Krijgsman, D., N. L. de Vries, A. Skovbo, M. N. Andersen, M. Swets, E. Bastiaannet, A. L. Vahrmeijer, C. J. H. van de Velde, M. H. M. Heemskerk, M. Hokland and P. J. K. Kuppen (2019). "Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile." Cancer Immunology, Immunotherapy **68**(6): 1011-1024.

Krijgsman, D., M. Hokland and P. J. K. Kuppen (2018). "The Role of Natural Killer T Cells in Cancer—A Phenotypical and Functional Approach." Frontiers in Immunology **9**.

Krovi, S. H. and L. Gapin (2018). "Invariant Natural Killer T Cell Subsets—More Than Just Developmental Intermediates." Frontiers in Immunology **9**.

Kulis, M. D., J. M. Smeekens, R. M. Immormino and T. P. Moran (2021). "The airway as a route of sensitization to peanut: An update to the dual allergen exposure hypothesis." Journal of Allergy and Clinical Immunology **148**(3): 689-693.

Lack, G., D. Fox, K. Northstone and J. Golding (2003). "Factors Associated with the Development of Peanut Allergy in Childhood." New England Journal of Medicine **348**(11): 977-985.

Lacombe, S. (2017). "British Society of Immunology: Allergy Policy Briefing." from https://www.immunology.org/policy-and-public-affairs/briefings-and-position-statements/allergy.

Lamiable, O., J. U. Mayer, L. Munoz-Erazo and F. Ronchese (2020). "Dendritic cells in Th2 immune responses and allergic sensitization." Immunology & Cell Biology **98**(10): 807-818.

Lee, J.-B. (2016). "Regulation of IgE-Mediated Food Allergy by IL-9 Producing Mucosal Mast Cells and Type 2 Innate Lymphoid Cells." Immune network **16**(4): 211-218.

Lee, S. (2017). "IgE-mediated food allergies in children: prevalence, triggers, and management." Korean journal of pediatrics **60**(4): 99-105.

Leffler, J., P. A. Stumbles and D. H. Strickland (2018). "Immunological Processes Driving IgE Sensitisation and Disease Development in Males and Females." International Journal of Molecular Sciences **19**(6): 1554.

León, B., M. López-Bravo and C. Ardavín (2007). "Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania." Immunity **26**(4): 519-531.

Leung, A. S. Y., G. W. K. Wong and M. L. K. Tang (2018). "Food allergy in the developing world." J Allergy Clin Immunol **141**(1): 76-78.e71.

Li, H., A. Nowak-Wegrzyn, Z. Charlop-Powers, W. Shreffler, M. Chehade, S. Thomas, G. Roda, S. Dahan, K. Sperber and M. C. Berin (2006). "Transcytosis of IgE-antigen complexes by CD23a in human intestinal epithelial cells and its role in food allergy." Gastroenterology **131**(1): 47-58.

Loh, W. and M. L. K. Tang (2018). "The Epidemiology of Food Allergy in the Global Context." International journal of environmental research and public health **15**(9): 2043.

Looney, T. J., J. Y. Lee, K. M. Roskin, R. A. Hoh, J. King, J. Glanville, Y. Liu, T. D. Pham, C. L. Dekker, M. M. Davis and S. D. Boyd (2016). "Human B-cell isotype switching origins of IgE." J Allergy Clin Immunol **137**(2): 579-586.e577.

Lucey, J. A., D. Otter and D. S. Horne (2017). "A 100-Year Review: Progress on the chemistry of milk and its components." J Dairy Sci **100**(12): 9916-9932.

Luttgeharm, K. D., A. N. Kimberlin, R. E. Cahoon, R. L. Cerny, J. A. Napier, J. E. Markham and E. B. Cahoon (2015). "Sphingolipid metabolism is strikingly different between pollen and leaf in Arabidopsis as revealed by compositional and gene expression profiling." Phytochemistry **115**: 121-129.

Macho-Fernandez, E. and M. Brigl (2015). "The Extended Family of CD1d-Restricted NKT Cells: Sifting through a Mixed Bag of TCRs, Antigens, and Functions." Frontiers in Immunology **6**.

Mak, T. W., M. E. Saunders and B. D. Jett (2014). Chapter 11 - NK, γδ T and NKT Cells. Primer to the Immune Response (Second Edition). T. W. Mak, M. E. Saunders and B. D. Jett. Boston, Academic Cell**:** 247-268.

Mak, T. W., M. E. Saunders and B. D. Jett (2014). Chapter 18 - Immune Hypersensitivity. Primer to the Immune Response (Second Edition). T. W. Mak, M. E. Saunders and B. D. Jett. Boston, Academic Cell**:** 487-516.

Mallevaey, T., A. J. Clarke, J. P. Scott-Browne, M. H. Young, L. C. Roisman, D. G. Pellicci, O. Patel, J. P. Vivian, J. L. Matsuda, J. McCluskey, D. I. Godfrey, P. Marrack, J. Rossjohn and L. Gapin (2011). "A molecular basis for NKT cell recognition of CD1d-self-antigen." Immunity **34**(3): 315-326.

McKinnon, K. M. (2018). "Flow Cytometry: An Overview." Curr Protoc Immunol **120**: 5.1.1-5.1.11.

Medic, J., C. Atkinson and C. R. Hurburgh Jr. (2014). "Current Knowledge in Soybean Composition." Journal of the American Oil Chemists' Society **91**(3): 363-384.

Méndez-Enríquez, E., M. Salomonsson, J. Eriksson, C. Janson, A. Malinovschi, M. E. Sellin and J. Hallgren (2022). "IgE cross-linking induces activation of human and mouse mast cell progenitors." Journal of Allergy and Clinical Immunology **149**(4): 1458-1463.

Meng, X., Z. Zeng, J. Gao, P. Tong, Y. Wu, X. Li and H. Chen (2020). "Conformational changes in bovine α-lactalbumin and β-lactoglobulin evoked by interaction with C18 unsaturated fatty acids provide insights into increased allergic potential." Food Funct **11**(10): 9240-9251.

Mestas, J. and C. C. Hughes (2004). "Of mice and not men: differences between mouse and human immunology." J Immunol **172**(5): 2731-2738.

Metcalfe, D. D., J. D. Astwood, R. Townsend, H. A. Sampson, S. L. Taylor and R. L. Fuchs (1996). "Assessment of the allergenic potential of foods derived from genetically engineered crop plants." Crit Rev Food Sci Nutr **36 Suppl**: S165-186.

Miles, E. A. and P. C. Calder (2017). "Can Early Omega-3 Fatty Acid Exposure Reduce Risk of Childhood Allergic Disease?" Nutrients **9**(7).

Mirotti, L., E. Florsheim, L. Rundqvist, G. Larsson, F. Spinozzi, M. Leite-De-Moraes, M. Russo and M. Alcocer (2013). "Lipids are required for the development of Brazil nut allergy: The role of mouse and human iNKT cells." Allergy: European Journal of Allergy and Clinical Immunology **68**(1): 74-83.

Mirotti, L., E. Florsheim, L. Rundqvist, G. Larsson, F. Spinozzi, M. Leite-de-Moraes, M. Russo and M. Alcocer (2013). "Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells." Allergy **68**(1): 74-83.

Moser, M. and K. M. Murphy (2000). "Dendritic cell regulation of TH1-TH2 development." Nature Immunology **1**(3): 199-205.

Nakano, H., K. L. Lin, M. Yanagita, C. Charbonneau, D. N. Cook, T. Kakiuchi and M. D. Gunn (2009). "Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses." Nat Immunol **10**(4): 394-402.

Neefjes, J., M. L. M. Jongsma, P. Paul and O. Bakke (2011). "Towards a systems understanding of MHC class I and MHC class II antigen presentation." Nature Reviews Immunology **11**(12): 823-836.

Noureddine, N., M. Chalubinski and P. Wawrzyniak (2022). "The Role of Defective Epithelial Barriers in Allergic Lung Disease and Asthma Development." J Asthma Allergy **15**: 487-504.

Noval Rivas, M. and T. A. Chatila (2016). "Regulatory T cells in allergic diseases." Journal of Allergy and Clinical Immunology **138**(3): 639-652.

O'Reilly, V., S. G. Zeng, G. Bricard, A. Atzberger, A. E. Hogan, J. Jackson, C. Feighery, S. A. Porcelli and D. G. Doherty (2011). "Distinct and overlapping effector functions of expanded human CD4+, CD8α+ and CD4-CD8α- invariant natural killer T cells." PloS one **6**(12): e28648-e28648.

Ober, C. and T. C. Yao (2011). "The genetics of asthma and allergic disease: a 21st century perspective." Immunol Rev **242**(1): 10-30.

Oeder, S., F. Alessandrini, O. F. Wirz, A. Braun, M. Wimmer, U. Frank, M. Hauser, J. Durner, F. Ferreira, D. Ernst, M. Mempel, S. Gilles, J. T. M. Buters, H. Behrendt, C. Traidl-Hoffmann, C. Schmidt-Weber, M. Akdis and J. Gutermuth (2015). "Pollen-derived nonallergenic substances enhance Th2-induced IgE production in B cells." Allergy **70**(11): 1450-1460.

Okada, H., C. Kuhn, H. Feillet and J.-F. Bach (2010). "The 'hygiene hypothesis' for autoimmune and allergic diseases: an update." Clinical & Experimental Immunology **160**(1): 1-9.

Olszak, T., D. An, S. Zeissig, M. P. Vera, J. Richter, A. Franke, J. N. Glickman, R. Siebert, R. M. Baron, D. L. Kasper and R. S. Blumberg (2012). "Microbial exposure during early life has persistent effects on natural killer T cell function." Science **336**(6080): 489-493.

Osborne, N. J., J. J. Koplin, P. E. Martin, L. C. Gurrin, A. J. Lowe, M. C. Matheson, A. L. Ponsonby, M. Wake, M. L. Tang, S. C. Dharmage and K. J. Allen (2011). "Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants." J Allergy Clin Immunol **127**(3): 668-676.e661-662.

Pablos-Tanarro, A., D. Lozano-Ojalvo, M. Martinez-Blanco, E. Molina and R. Lopez-Fandino (2018). "Egg Yolk Provides Th2 Adjuvant Stimuli and Promotes

Sensitization to Egg White Allergens in BALB/c Mice." Molecular Nutrition & Food Research **62**(13): 11.

Pali-Schöll, I., E. Untersmayr, M. Klems and E. Jensen-Jarolim (2018). "The Effect of Digestion and Digestibility on Allergenicity of Food." Nutrients **10**(9).

Palladino, C. and H. Breiteneder (2018). "Peanut allergens." Molecular Immunology **100**: 58-70.

Palladino, C., B. Gepp, A. Angelina, S. Sirvent, C. Radauer, N. Lengger, T. Eiwegger and O. Palomares (2016). "The interplay of Ara h 1 and peanut lipids in the allergic sensitization process." Allergy: European Journal of Allergy and Clinical Immunology **71**(Supplement 102): 624-625.

Palladino, C., M. S. Narzt, M. Bublin, M. Schreiner, P. Humeniuk, M. Gschwandtner, C. Hafner, W. Hemmer, K. Hoffmann-Sommergruber, M. Mildner, O. Palomares, F. Gruber and H. Breiteneder (2018). "Peanut lipids display potential adjuvanticity by triggering a pro-inflammatory response in human keratinocytes." Allergy **73**(8): 1746-1749.

Perez Rodriguez, L., M. Martinez Blanco, E. Molina, R. Lopez Fandino and D. Lozano-Ojalvo (2019). "Egg yolk acts as adjuvant activating innate immune responses to egg white allergens in BALB/C MICE." Allergy: European Journal of Allergy and Clinical Immunology **74**(Supplement 106): 109.

Peters, R. L., J. J. Koplin, L. C. Gurrin, S. C. Dharmage, M. Wake, A.-L. Ponsonby, M. L. K. Tang, A. J. Lowe, M. Matheson, T. Dwyer and K. J. Allen (2017). "The prevalence of food allergy and other allergic diseases in early childhood in a population-based study: HealthNuts age 4-year follow-up." Journal of Allergy and Clinical Immunology **140**(1): 145-153.e148.

Petersen, A., S. Rennert, S. Kull, W. M. Becker, H. Notbohm, T. Goldmann and U. Jappe (2014). "Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8." Biological Chemistry **395**(2): 239-250.

Pettersson, M. E., G. H. Koppelman, B. M. J. Flokstra-de Blok, B. J. Kollen and A. E. J. Dubois (2018). "Prediction of the severity of allergic reactions to foods." Allergy **73**(7): 1532-1540.

Pfefferle, P. I., C. U. Keber, R. M. Cohen and H. Garn (2021). "The Hygiene Hypothesis – Learning From but Not Living in the Past." Frontiers in Immunology **12**.

Pichavant, M., A.-S. Charbonnier, S. Taront, A. Brichet, B. Wallaert, J. Pestel, A.-B. Tonnel and P. Gosset (2005). "Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment." Journal of Allergy and Clinical Immunology **115**(4): 771-778.

Plantinga, M., M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, H. Hammad and B. N. Lambrecht (2013). "Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen." Immunity **38**(2): 322-335.

Portelli, M. A., E. Hodge and I. Sayers (2015). "Genetic risk factors for the development of allergic disease identified by genome-wide association." Clin Exp Allergy **45**(1): 21-31.

Prescott, S. L., R. Pawankar, K. J. Allen, D. E. Campbell, J. Sinn, A. Fiocchi, M. Ebisawa, H. A. Sampson, K. Beyer and B. W. Lee (2013). "A global survey of changing patterns of food allergy burden in children." World Allergy Organ J **6**(1): 21.

Radzikowska, U., A. O. Rinaldi, Z. Çelebi Sözener, D. Karaguzel, M. Wojcik, K. Cypryk, M. Akdis, C. A. Akdis and M. Sokolowska (2019). "The Influence of Dietary Fatty Acids on Immune Responses." Nutrients **11**(12): 2990.

Rajan, T. V. (2003). "The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation." Trends Immunol **24**(7): 376-379.

Rampuria, P. and M. L. Lang (2018). Chapter 5 - Regulation of Humoral Immunity by CD1d-Restricted Natural Killer T Cells. Immunology. M. A. Hayat, Academic Press**:** 55-73.

Reynolds, C., J. Barkans, P. Clark, H. Kariyawasam, D. Altmann, B. Kay and R. Boyton (2009). "Natural killer T cells in bronchial biopsies from human allergen challenge model of allergic asthma." J Allergy Clin Immunol **124**(4): 860-862; author reply 862.

Ring, S., S. C. Schäfer, K. Mahnke, H.-A. Lehr and A. H. Enk (2006). "CD4+CD25+ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue." European Journal of Immunology **36**(11): 2981-2992.

Romagnani, S. (2006). "Immunological tolerance and autoimmunity." Intern Emerg Med **1**(3): 187-196.

Roper, R. L., D. H. Conrad, D. M. Brown, G. L. Warner and R. P. Phipps (1990). "Prostaglandin E2 promotes IL-4-induced IgE and IgG1 synthesis." The Journal of Immunology **145**(8): 2644-2651.

Ros, E. and J. Mataix (2006). "Fatty acid composition of nuts--implications for cardiovascular health." Br J Nutr **96 Suppl 2**: S29-35.

Ros, E. and J. Mataix (2006). "Fatty acid composition of nuts – implications for cardiovascular health." British Journal of Nutrition **96**(S2): S29-S35.

Rosser, Elizabeth C. and C. Mauri (2015). "Regulatory B Cells: Origin, Phenotype, and Function." Immunity **42**(4): 607-612.

Roulston, T. H. and J. H. Cane (2000). "Pollen nutritional content and digestibility for animals." Plant Systematics and Evolution **222**(1/4): 187-209.

Rudolph, M. G., R. L. Stanfield and I. A. Wilson (2006). "HOW TCRS BIND MHCS, PEPTIDES, AND CORECEPTORS." Annual Review of Immunology **24**(1): 419-466.

Ruiter, B. and W. G. Shreffler (2012). "The role of dendritic cells in food allergy." Journal of Allergy and Clinical Immunology **129**(4): 921-928.

Ruysschaert, J.-M. and C. Lonez (2015). "Role of lipid microdomains in TLR-mediated signalling." Biochimica et Biophysica Acta (BBA) - Biomembranes **1848**(9): 1860-1867.

Sag, D., M. Özkan, M. Kronenberg and G. Wingender (2017). "Improved Detection of Cytokines Produced by Invariant NKT Cells." Scientific Reports **7**(1): 16607.

Salazar, F. and A. Ghaemmaghami (2013). "Allergen Recognition by Innate Immune Cells: Critical Role of Dendritic and Epithelial Cells." Frontiers in Immunology **4**.

Salazar, F. and A. M. Ghaemmaghami (2013). "Allergen recognition by innate immune cells: critical role of dendritic and epithelial cells." Front Immunol **4**: 356.

Salio, M., J. D. Silk, E. Y. Jones and V. Cerundolo (2014). "Biology of CD1- and MR1-Restricted T Cells." Annual Review of Immunology **32**(1): 323-366.

Salo, P. M., S. J. Arbes, Jr., R. Jaramillo, A. Calatroni, C. H. Weir, M. L. Sever, J. A. Hoppin, K. M. Rose, A. H. Liu, P. J. Gergen, H. E. Mitchell and D. C. Zeldin (2014). "Prevalence of allergic sensitization in the United States: Results from the National Health and Nutrition Examination Survey (NHANES) 2005-2006." Journal of Allergy and Clinical Immunology **134**(2): 350-359.

Satitsuksanoa, P., K. Jansen, A. Głobińska, W. van de Veen and M. Akdis (2018). "Regulatory Immune Mechanisms in Tolerance to Food Allergy." Frontiers in Immunology **9**.

Satitsuksanoa, P., M. Kennedy, D. Gilis, M. Le Mignon, N. Suratannon, W. T. Soh, J. Wongpiyabovorn, P. Chatchatee, M. Vangveravong, T. Rerkpattanapipat, A. Sangasapaviliya, S. Piboonpocanun, E. Nony, K. Ruxrungtham, A. Jacquet and t. M. A. R. C. s. team (2016). "The minor house dust mite allergen Der p 13 is a fatty acid-binding protein and an activator of a TLR2-mediated innate immune response." Allergy **71**(10): 1425-1434.

Schiefner, A. and I. A. Wilson (2009). "Presentation of lipid antigens by CD1 glycoproteins." Curr Pharm Des **15**(28): 3311-3317.

Schiefner, A. and I. A. Wilson (2009). "Presentation of lipid antigens by CD1 glycoproteins." Current pharmaceutical design **15**(28): 3311-3317.

Schmid, H., C. Schneidawind, S. Jahnke, F. Kettemann, K.-A. Secker, S. Duerr-Stoerzer, H. Keppeler, L. Kanz, P. B. Savage and D. Schneidawind (2018). "Culture-Expanded Human Invariant Natural Killer T Cells Suppress T-Cell Alloreactivity and Eradicate Leukemia." Frontiers in immunology **9**: 1817-1817.

Schoos, A.-M., D. Bullens, B. Chawes, J. Costa, L. De Vlieger, A. Dunn Galvin, M. Epstein, J. Garssen, C. Hilger, K. Knipping, A. Kuehn, D. Mijakoski, D. Munblit, N. Nekliudov, C. Ozdemir, K. Patient, D. Peroni, S. Stoleski, E. Stylianou and K. Verhoeckx (2020). "Immunological Outcomes of Allergen-Specific Immunotherapy in Food Allergy." Frontiers in Immunology **11**: 2736.

Schülke, S. and M. Albrecht (2019). "Mouse Models for Food Allergies: Where Do We Stand?" Cells **8**(6).

Sheehan, W. J., P. A. Rangsithienchai, S. N. Baxi, A. Gardynski, A. Bharmanee, E. Israel and W. Phipatanakul (2010). "Age-specific prevalence of outdoor and indoor aeroallergen sensitization in Boston." Clin Pediatr (Phila) **49**(6): 579-585.

Sköld, M. and S. M. Behar (2003). "Role of CD1d-restricted NKT cells in microbial immunity." Infection and immunity **71**(10): 5447-5455.

Smeekens, J. M., R. M. Immormino, P. A. Balogh, S. H. Randell, M. D. Kulis and T. P. Moran (2019). "Indoor dust acts as an adjuvant to promote sensitization to peanut through the airway." Clinical & Experimental Allergy **49**(11): 1500-1511.

Smole, U., N. Balazs, Y. Sobanov, C. Radauer, M. Bublin, K. Hoffmann-Sommergruber, E. Jensen-Jarolim, D. Mechtcheriakova and H. Breiteneder (2011). "Lipid raft mediated uptake of the major birch pollen allergen Bet v 1.0101 activates Th2 polarising signaling pathways in dendritic cells of allergic donors." Allergy: European Journal of Allergy and Clinical Immunology **66**(SUPPL. 94): 54.

Stock, P. and O. Akbari (2008). "Recent advances in the role of NKT cells in allergic diseases and asthma." Current allergy and asthma reports **8**: 165-170.

Thomas, W. and B. Hales (2008). "Immune Responses to Inhalant Allergens." World Allergy Organization Journal **1**: 89-95.

Tordesillas, L., N. Cubells-Baeza, C. Gomez-Casado, C. Berin, V. Esteban, W. Barcik, L. O'Mahony, C. Ramirez, L. F. Pacios, M. Garrido-Arandia and A. Diaz-Perales (2017). "Mechanisms underlying induction of allergic sensitization by Pru p 3." Clinical and Experimental Allergy **47**(11): 1398-1408.

Traidl-Hoffmann, C., A. Kasche, T. Jakob, M. Huger, S. Plötz, I. Feussner, J. Ring and H. Behrendt (2002). "Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes." Journal of Allergy and Clinical Immunology **109**(5): 831-838.

Usuda, H., T. Okamoto and K. Wada (2021). "Leaky Gut: Effect of Dietary Fiber and Fats on Microbiome and Intestinal Barrier." International Journal of Molecular Sciences **22**(14): 7613.

van Bilsen, J. H. M., E. Sienkiewicz-Szłapka, D. Lozano-Ojalvo, L. E. M. Willemsen, C. M. Antunes, E. Molina, J. J. Smit, B. Wróblewska, H. J. Wichers, E. F. Knol, G. S. Ladics, R. H. H. Pieters, S. Denery-Papini, Y. M. Vissers, S. L. Bavaro, C. Larré, K. C. M. Verhoeckx and E. L. Roggen (2017) "Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins." Clinical and translational allergy **7**, 13 DOI: 10.1186/s13601-017-0152-0.

van Bilsen, J. H. M., E. Sienkiewicz-Szłapka, D. Lozano-Ojalvo, L. E. M. Willemsen, C. M. Antunes, E. Molina, J. J. Smit, B. Wróblewska, H. J. Wichers, E. F. Knol, G. S. Ladics, R. H. H. Pieters, S. Denery-Papini, Y. M. Vissers, S. L. Bavaro, C. Larré, K. C. M. Verhoeckx and E. L. Roggen (2017). "Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins." Clin Transl Allergy **7**: 13.

Van Kaer, L. and S. Joyce (2005). "Innate Immunity: NKT Cells in the Spotlight." Current Biology **15**(11): R429-R431.

van Ree, R., L. Hummelshøj, M. Plantinga, L. K. Poulsen and E. Swindle (2014). "Allergic sensitization: host-immune factors." Clinical and Translational Allergy **4**(1): 12.

Venkatachalam, M. and S. K. Sathe (2006). "Chemical Composition of Selected Edible Nut Seeds." Journal of Agricultural and Food Chemistry **54**(13): 4705-4714.

Venter, C., R. W. Meyer, B. I. Nwaru, C. Roduit, E. Untersmayr, K. Adel-Patient, I. Agache, C. Agostoni, C. A. Akdis, S. C. Bischoff, G. du Toit, M. Feeney, R. Frei, H. Garn, M. Greenhawt, K. Hoffmann-Sommergruber, N. Lunjani, K. Maslin, C. Mills, A. Muraro, I. Pali-Schöll, L. K. Poulson, I. Reese, H. Renz, G. C. Roberts, P. Smith, S. Smolinska, M. Sokolowska, C. Stanton, B. Vlieg-Boerstra and L. O'Mahony (2019). "EAACI position paper: Influence of dietary fatty acids on asthma, food allergy, and atopic dermatitis." Allergy **74**(8): 1429-1444.

Waidyatillake, N. T., S. C. Dharmage, K. J. Allen, C. J. Lodge, J. A. Simpson, G. Bowatte, M. J. Abramson and A. J. Lowe (2018). "Association of breast milk fatty acids with allergic disease outcomes-A systematic review." Allergy **73**(2): 295-312.

Waserman, S., P. Bégin and W. Watson (2018). "IgE-mediated food allergy." Allergy, Asthma & Clinical Immunology **14**(2): 55.

Wisniewski, J., R. Agrawal and J. A. Woodfolk (2013). "Mechanisms of tolerance induction in allergic disease: integrating current and emerging concepts." Clin Exp Allergy **43**(2): 164-176.

Wood, R. A., S. H. Sicherer, A. W. Burks, A. Grishin, A. K. Henning, R. Lindblad, D. Stablein and H. A. Sampson (2013). "A phase 1 study of heat/phenol-killed, E. coli-encapsulated, recombinant modified peanut proteins Ara h 1, Ara h 2, and Ara h 3 (EMP-123) for the treatment of peanut allergy." Allergy **68**(6): 803-808.

World-Allergy-Organization (2011). White Book on Allergy. United Kingdom.

Wu, L. and L. Van Kaer (2011). "Natural killer T cells in health and disease." Front Biosci (Schol Ed) **3**(1): 236-251.

Zhang, X., P. Zheng, T. R. Prestwood, H. Zhang, Y. Carmi, L. L. Tolentino, N. Wu, O. Choi, D. A. Winer, S. Strober, E.-S. Kang, M. N. Alonso and E. G. Engleman (2020). "Human Regulatory Dendritic Cells Develop From Monocytes in Response to Signals From Regulatory and Helper T Cells." Frontiers in Immunology **11**.

Appendix

Appendix A: Published Systematic Review

The Role of Lipids in Allergic Sensitization: A Systematic Review

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Abstract

Background

Immunoglobulin E (IgE)-mediated allergies are increasing in prevalence, with IgE-mediated food allergies currently affecting up to 10% of children and 6% of adults worldwide. The mechanisms underpinning the first phase of IgE-mediated allergy, allergic sensitization, are still not clear. Recently, the potential involvement of lipids in allergic sensitization has been proposed, with reports that they can bind allergenic proteins and act on immune cells to skew to a T helper type 2 (Th2) response.

Objectives

The objective of this systematic review is to determine if there is strong evidence for the role of lipids in allergic sensitization.

Methods

19 studies were reviewed, 10 of which were relevant to lipids in allergic sensitization to food allergens, 9 relevant to lipids in aeroallergen sensitization.

Results

The results provide strong evidence for the role of lipids in allergies. Intrinsic lipids from allergen sources can interact with allergenic proteins to predominantly enhance but also inhibit allergic sensitization through various mechanisms. Proposed mechanisms included reducing the gastrointestinal degradation of allergenic proteins by altering protein structure, reducing dendritic cell (DC) uptake of allergenic proteins to reduce immune tolerance, regulating Th2 cytokines, activating invariant natural killer T (iNKT) cells through CD1d presentation, and directly acting upon toll-like receptors (TLRs), epithelial cells, keratinocytes, and DCs.

Conclusion

The current literature suggests intrinsic lipids are key influencers of allergic sensitization. Further research utilising human relevant *in vitro* models and clinical studies are needed to give a reliable account of the role of lipids in allergic sensitization.

INTRODUCTION

For over 50 years, there has been a substantial, worldwide increase in the prevalence of allergic disease (World-Allergy-Organization 2011). IgE-mediated allergies are among those increasing in prevalence; globally, IgE-mediated sensitization to environmental allergens (e.g. pollen) affect up to 40% of individuals (World-Allergy-Organization 2011), and IgE-mediated food sensitization affects up to 10% of children, and 6% of adults (Lee 2017; Osborne et al. 2011; Prescott et al. 2013; Waserman, Bégin, and Watson 2018). In addition, the most recent statistics from the National Health and Nutrition Examination Survey (NHANES) report up to 44.6% of United States children were sensitized to at least 1 environmental or food allergen source (Salo et al. 2014). Allergic sensitization, the first phase of IgE allergy development, is central to the development of atopic disease. Yet, the underpinning mechanisms of allergic sensitization have not been fully elucidated (van Bilsen et al. 2017b). Further research to gain additional insight into these mechanisms is crucial to fully comprehend the pathogenesis of allergic disease, which could consequently drive the development of new treatments.

The mechanisms by which proteins from within an allergen source (allergenic proteins) drive allergic sensitization have been explored in more detail, compared to the limited research into the role of associated molecules. Allergenic proteins are derived from a variety of allergen sources, such as peanuts, house dust mites (HDM), and pollen. Indeed, allergenic sources are composed of proteins that are accompanied by other compounds, including carbohydrates and lipids. For instance, the major allergen source, peanut, contains a high abundance of lipid, approximately 49% (Ros and Mataix 2006b). Despite evidence for the high abundance of lipids in various allergen sources, few studies have explored the role of these compounds in allergic sensitization, including their ability to interact with allergenic proteins.

Lipids are small hydrophobic or amphipathic molecules (Fahy et al. 2009) that can be bound or co-delivered with allergenic proteins to the innate immune system. Lipids within an allergen source can be directly associated with allergenic proteins, as some proteins have the capacity to bind lipids

through hydrophobic cavities, ionic, or hydrophobic bonds (Jappe et al. 2019b). These allergen-bound lipids can be termed protein-lipid complexes. There are several classes of allergenic proteins which have the ability to bind lipids, as well as lipid-ligands in the case of the lipid transfer protein (LTP) family. These include Bet v 1-like proteins, non-specific LTPs, 2S albumins, and oleosins (Jappe et al. 2019b). These proteins can bind various lipids and lipid-ligands, depending on their tertiary structure, including fatty acids, glycolipids, and phospholipids (Dubiela et al. 2019). This lipid-binding can then result in structural and biochemical changes to the protein, which alters the immune response provoked (Petersen et al. 2014a). In contrast to directly binding allergens, lipids from an allergen source can also be co-delivered with the allergenic protein. The lipids can be present in pollen coats of plant allergen sources or in matrices of plant and animal foods. This includes pollen-associated lipid mediators (PALMs) which are bioactive lipids released from the pollen grain, or they can be present in the cell membranes, such as phospholipids (Gilles-Stein et al. 2016). These co-delivered lipids can interact directly with immune cells to modulate the immune response (Traidl-Hoffmann et al. 2002). It is through allergenic protein-binding and activating immune cells that a variety of intrinsic lipids (lipids within an allergen source), have been shown to influence and promote allergic sensitization.

Indeed, there are numerous papers examining the relationship between intrinsic lipids and allergic sensitization, as discussed in previous reviews (Bublin, Eiwegger, and Breiteneder 2014; Del Moral and Martínez-Naves 2017). Though this is limited, and there has thus far been no systematic review and synthesis of the available studies. Hence, the aim of this systematic review, believed to be the first on this topic, will appraise all existing literature on the interaction of allergen source-derived lipids with allergenic proteins and cells of the immune system, to influence a Th2 response in IgE-mediated food allergies and aeroallergies. This will contribute to the understanding of the mechanisms underpinning allergic sensitization, as well as provide insight into the different study designs to enable further, much needed research.

METHODS

Search Strategy

Articles were sought from three databases: PubMed, Web of Science, and EMBASE. One item of grey literature was found via Wiley Online Library. Each database was filtered by selecting for articles published in English language as well as excluding reviews.

See **Supplement 1** for full search terms. The key terms used were as follows: (i) lipid terms “lipid”, “fatty acid”, “lipid-binding”, “PALM” and (ii) allergy terms “allergy”, “allergies”, “allergen”, “pollen”, “IgE”, “sensitization”, “Th2”. Certain terms were specifically excluded from the search to remove irrelevant results: “pain”, “asthma”, “AHR”, “contact”, “n-3”, “n-6”, “maternal”, “predict”, “prevent”, “dermatitis”, “cross-reactivity”, “profile”, “diagnostic”.

A PRISMA 2009 flow diagram, detailing the process of this systematic review, is shown in **Figure 1.** The search was conducted on the 18th August 2021 using the terms above, yielding a total of 2607 articles; PubMed (1806), Web of Science (369), EMBASE (632), and 1 further article was found using Wiley Online Library. Duplicates were then removed using EndNote software. The remaining titles and abstracts were scanned for relevance to the role of lipids in allergic sensitization. The scanning process was validated by an independent reviewer.

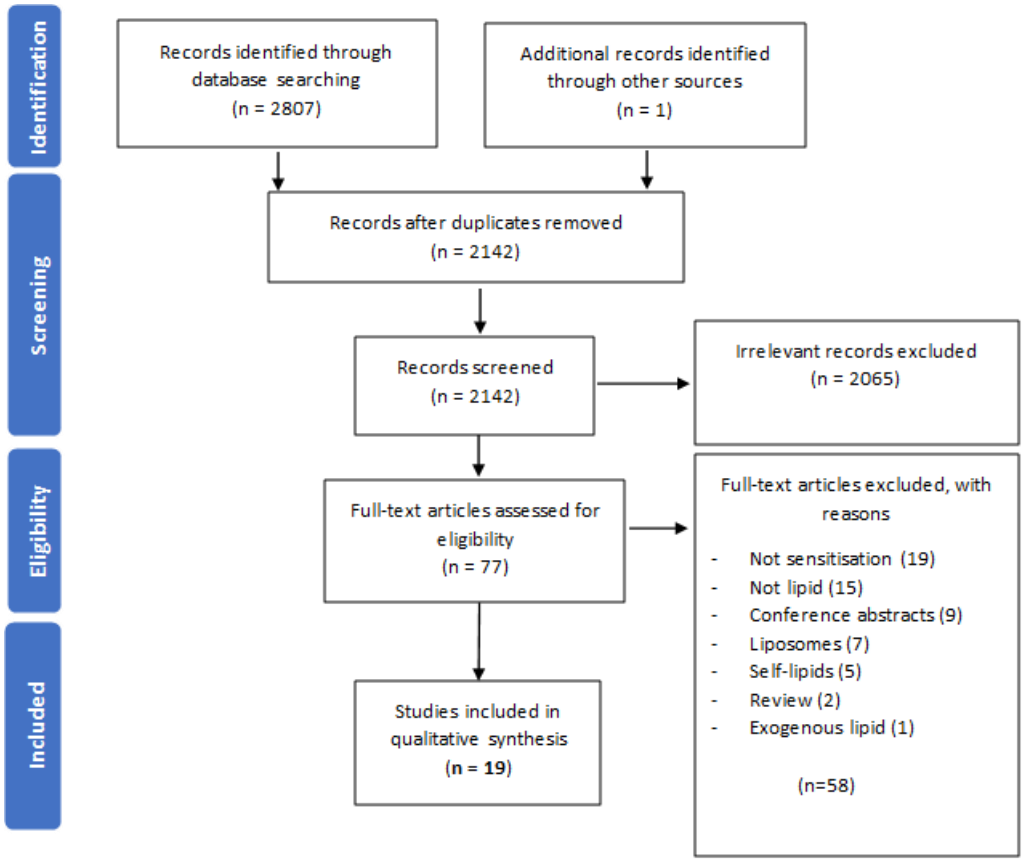

Figure 1. A PRISMA 2009 flow chart detailing the process of study selection. Publications were sought from 3 databases, duplicates removed, records screened for relevance, full-texts of the remaining articles evaluated for their eligibility, and the remaining studies were grouped into 3 different categories. This review focuses on the 19 studies investigating the role of lipids in sensitization.

Inclusion and Exclusion Criteria

Full texts were then assessed for eligibility in the analysis. The full study eligibility criteria is shown in **Table 1**. In brief, the focus of this review will be on the role of natural lipids found within an allergen source, such as protein-lipid complexes and membrane-bound lipids. The focus is on natural, intrinsic lipids, as the aim of this review is to capture what mechanisms of lipid-influenced allergic sensitization can arise from simply ingesting or inhaling certain allergen sources. Thus, this review excludes any lipids from an exogenous source e.g. microbial lipids, as well as self-lipids (e.g. digestive lipids), as these lipids are not found within an allergen source. The role of omega 3 and omega 6 fatty acids in allergic sensitization were also excluded as there are already many existing systematic reviews, reviews, and position papers within this area (Miles and Calder 2017; Radzikowska et al. 2019; Venter et al. 2019; Waidyatillake et al. 2018). There were several papers regarding the use of liposomes to capture allergens for drug delivery in allergy treatment. Again, these articles were excluded as they were not natural lipids from an allergen source and did not investigate allergic sensitization.

Table 1. The inclusion and exclusion criteria used to determine article eligibility for this systematic review.

Inclusion	Exclusion
- IgE-mediated food allergy	- Non-IgE-mediated allergies
- IgE-mediate inhalant allergy	- Lipids in asthma
- Intrinsic lipids	- Lipids in the elicitation phase
- Allergic sensitisation	- Lipids in the prevention/protection of allergic sensitisation
- English language	- Non-lipids
- Clinical data	- n-3 or n-6 fatty acids
- Experimental data	- Exogenous lipids e.g. Microbial lipids
- Healthy subjects	- Allergen-encaptured Liposomes for drug delivery
- Allergic subjects	- Self-lipids e.g. digestive emulsion lipids, cholesterol
- Human models	- Non-English language publications
- Animal models	- Conference abstracts
-	- Reviews

Data Extraction

This search resulted in 19 papers which provide data on lipids influencing allergic sensitization. The 19 eligible papers were analysed and the findings synthesised. Data on study design, the subject model, and findings were reviewed.

To quantify the robustness of studies included in this review, the papers were scored based on criteria set in **Table 2**. Quality assessment scores for each study were determined by the criteria: sample size, the definition of control, representation of the sample, models of allergic sensitization utilised, the robustness of the model, the methods used to prepare lipids, and the characterization of lipid. Scores from each category were summed and divided by the highest possible total score to calculate study quality scores. This scoring system aims to give insight into the designs adopted by current research in this area, where lower-scored studies are not less reliable, but are lacking in characteristics which strengthen the results of the study, such as a small sample size.

Table 2. Reasoning for study 'quality' scores. Quality assessment scores for each study were determined by the criteria: sample size, the definition of control, representation of the sample, models of allergic sensitization utilised, the robustness of the model, the methods used to prepare lipids, and the characterization of lipid. The overall score was calculated by the sum of each category, divided by the highest possible total score.

Category	Reasoning for scores
Sample size	- Evidence of statistical power calculation to determine the study sample size **(2)** - No evidence of power calculation, but sample size was stated **(1)** - No power calculation AND sample size was NOT stated **(0)**
Defined controls	- Inclusion of healthy controls **(1)** - No healthy controls **(0)**
Representative sample	- 1 point for each of the following: At least 1/3 of each gender **(1)** A range of age groups **(1)** Inclusion of subjects allergic to the allergen of study **(1)** - Unclear sample details **(0)** If both human and murine models utilised, the model with the greater representative sample score will be used
Model	- Human (2) - Murine **(1)** - Unclear **(0)** - If both human and murine models are utilised, the study will be awarded the higher mark of **(2)**
Robustness of model	- Animal models: Allergic animals were immunised by intraperitoneal injection/epicutaneous administration **(1)** Allergic animals were immunised by intraperitoneal injection/epicutaneous administration AND had specific IgE to allergen OR positive allergen challenge. (2) - Human models: Allergic participants were sought from a clinical setting **(1)** Allergic participants were sought from a clinical setting AND had a positive skin prick testing to allergen, had specific IgE to allergen, or had a positive allergen challenge **(2)** - Unclear allergic subject definitions **(0)** - If both human AND murine models were utilised, the model with the greater robustness score will be awarded
Lipid preparation	- The study used commercially sourced lipids (2) - The study performed extraction of the lipid from the allergen source **(1)** - The study performed extraction of the lipid from the allergen source AND further purification of lipid (2) - The study used commercially sourced lipids AND extracted/purified lipids from an allergen source (2) - Unclear **(0)**
Lipid characterisation	- The exact lipids responsible for the outcomes were defined e.g. polar lipids, fatty acids, phospholipids. (2) - The lipids responsible for the outcome were not well defined e.g. simply "pollen lipids" or "aqueous pollen extract" or "peanut lipids." **(1)** - The lipids used were not defined e.g. "lipids" **(0)**
Overall quality score	The combined score for the categories above divided by the highest possible score of 14

Quality assessment scores for each study were determined by the criteria: sample size, the definition of control, representation of the sample, models of allergic sensitization utilised, the robustness of the model, the methods used to prepare lipids, and the characterization of lipid. The overall score was calculated by the sum of each category, divided by the highest possible total score.

RESULTS

The 19 relevant papers included in this systematic review, (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Palladino et al. 2018; Dearman, Alcocer, and Kimber 2007; Mirotti et al. 2013a; Tordesillas et al. 2017; Hufnagl et al. 2018; Jyonouchi et al. 2011; Finkina et al. 2020a; Meng et al. 2020; Pablos-Tanarro et al. 2018; Agea et al. 2005; Abos Gracia et al. 2017; Gilles et al. 2009; Gilles et al. 2010; Oeder et al. 2015; González Roldán et al. 2019; Gutermuth et al. 2007; Bansal, Gaur, and Arora 2016; Satitsuksanoa et al. 2016) which are directly relevant to the role of lipids in allergic sensitization, illustrate the majority of studies were conducted within the last decade. All papers were published within the last 15 years, with 58% published in the last 5 years (2016-2021).

Of the 19 studies reporting a relationship between lipids and allergic sensitization, 16 solely report lipids enhance allergic sensitization (Abos Gracia et al. 2017; Agea et al. 2005; Angelina, Sirvent, Palladino, Vereda, Cuesta-

Herranz, Eiwegger, Rodriguez, et al. 2016; Bansal, Gaur, and Arora 2016; Dearman, Alcocer, and Kimber 2007; Gilles et al. 2010; Gilles et al. 2009; González Roldán et al. 2019; Jyonouchi et al. 2011; Mirotti et al. 2013a; Oeder et al. 2015; Pablos-Tanarro et al. 2018; Tordesillas et al. 2017; Satitsuksanoa et al. 2016; Finkina et al. 2020a; Meng et al. 2020), 2 studies report lipids can both enhance and inhibit allergic sensitization (Gutermuth et al. 2007; Palladino et al. 2018), and 1 study observed lipids inhibited allergic sensitization (Hufnagl et al. 2018).

The results of the systematic review have been split into two sections; lipids associated to food allergens and lipids associated to aeroallergens.

Intrinsic Lipids in Driving Food Allergies

Of the 19 articles, 10 of these discuss the role of lipids in allergic sensitization to food allergenic proteins (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Dearman, Alcocer, and Kimber 2007; Hufnagl et al. 2018; Jyonouchi et al. 2011; Mirotti et al. 2013a; Pablos-Tanarro et al. 2018; Palladino et al. 2018; Tordesillas et al. 2017; Finkina et al. 2020a; Meng et al. 2020). Nine out of the 10 papers found lipids can shift or enhance allergic sensitization of food allergenic proteins (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Dearman, Alcocer, and Kimber 2007; Jyonouchi et al. 2011; Mirotti et al. 2013a; Pablos-Tanarro et al. 2018; Palladino et al. 2018; Tordesillas et al. 2017; Finkina et al. 2020a; Meng et al. 2020). **Table 3a** summaries the methods and outcomes for these food allergy studies (N.B. Only factors of the articles that were relevant to the role of lipids in allergic sensitization were included in the table).

These 10 studies identify 3 main mechanisms of food-derived lipids influencing allergic sensitization: the activation of iNKT cells through CD1d molecules, direct activation of immune cells, and the induction of conformational changes to allergenic proteins. The evidence for these mechanisms will now presented in further detail.

Table 3a. A summary of the primary articles discussed, relevant to lipids in food allergies. Key details of each food allergy study are presented, along with whether the study provides evidence for the role of lipids driving or inhibiting allergic sensitization.

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Angelina [23	2016	Sin a 2 and Ara h 1	Phospholipids, peanut and mustard lipids	Dendritic cells	Human	Human sera from patients allergic to mustard or peanuts were collected. Allergen-lipid binding was assessed by SDS-PAGE and spectroscopic binding assays. The ability of dendritic cells (DCs) to capture and uptake peanut/mustard allergens, with or without lipids, was measured by flow cytometry and confocal microscopy, along with cytokine levels.	Sin a 2 and Ara h 1 bound phosphatidylglycerol acid and intrinsic lipids, resulting in resistance to gastrointestinal digestion, reduced uptake by DCs, retained Immunoglobulin E (IgE) reactivity of allergen, increased IL-1B levels and increased protection from microsomal degradation.	Enhances allergic sensitisation
Palladino [24]	2018	Ara h 1, Ara h 2	Peanut lipids	Keratinocytes	Human	Human keratinocytes were exposed to peanut lipids with or without the major peanut allergens, Ara h 1 or Ara h 2 and their cytokine release measured by enzyme-linked immunosorbent assay (ELISA).	Peanut lipids with or without allergen stimulated human keratinocytes to increase production of GM-CSF. Peanut lipids alone increased IL-10 secretion from keratinocytes. Whereas peanut lipids with allergen inhibited IL-10 secretion.	Enhances and inhibits allergic sensitisation
Dearman [25]	2007	Ber e 1	Brazil nut lipids	N/A	Murine	Female BALB/c mice immunised with Ber e 1, combined with and without natural brazil nut lipids. Serum samples were analysed for Ber e 1-specific IgE and IgG in assays.	Ber e 1 with total lipid fraction produced significant adjuvant effects on Immunoglobulin G (IgG) and IgE. Natural Ber e 1 containing endogenous lipids also produced IgG and IgE antibody.	Enhances allergic sensitisation

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Mirotti [26]	2013	Ber e 1	Brazil nut lipids	iNKT cells	Murine and human	Female BALB/c mice were sensitised to Ber e 1 and specific lipid fractions, followed by IgE measurements by ELISA and passive cutaneous anaphylaxis (PCA). Lipid-binding of Ber e 1 was measured using fluorescent probes and NMR. In vitro production of IL-4 was measured by flow cytometry and ELISA.	Lipid fraction (lipid C) interacted with Ber e 1 via a lipid-binding site to induce Ber-specific IgE. iNKT-deficient mice produced lower levels of IgE than wild type. *In vitro*, Ber/lipid C-stimulated murine iNKT cells produced IL-4 but not IFN-γ in a CD1d dose-dependent manner.	Enhances allergic sensitisation
Tordesillas [27]	2017	Pru p 3	Pru p 3 lipid-ligand: Phytosphingosine tail	iNKT cells, epithelial cells, and MoDCS	Murine and human	*In vitro* cultures of human moDCs, PBMC, epithelial and murine DN32.D3, and invariant natural killer T (iNKT) hybridoma cell lines were incubated with the Pru p 3 lipid-ligand extracted from peach peel extract. Cells were assessed for maturation, IgE production, and cytokine production.	The lipid-ligand of Pru p 3 induced the maturation of moDCs. It induced higher levels of IgE than Pru p 3 alone. The immunological capacity of the Pru p 3 ligand was mediated by CD1d and was able to activate murine iNKTs.	Enhances allergic sensitisation
Hufnagl [28]	2018	Milk lipocalin Bos d 5	Retinoic acid	T cell	Human	*In vitro* and in *silico* retinoic acid (RA)-Bos d 5 binding assays were performed. PBMCs stimulated with Bos d 5 and T cells were assessed by flow cytometry and their cytokine release.	Bos d 5 has high binding affinity to retinoic. RA-bound Bos d 5 decreased CD3+CD4+ cell types and supressed IL-10,IL-13 and IFN-γ production. This reduced the immunogenicity of Bos d 5 and its allergenicity.	Inhibits allergic sensitisation

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Jyonouchi [29]	2011	Milk and egg allergens	Cow's milk-sphingomyelin, hen's egg-ceramide	iNKT cells	Human	PBMCs from children with cow's milk or hen's egg allergy, and healthy controls were incubated with α-GalCer, cow's milk–sphingomyelin, or hen's egg–ceramide. iNKTs were quantified, and their cytokine production and proliferation were assessed. Human CD1d tetramers loaded with milk-sphingomyelin or egg-ceramide were used to determine food-sphingolipid binding to the iNKT-T cell receptor (TCR).	Milk-sphingomyelin, but not egg-ceramide, engaged the iNKT-TCR and induced iNKT proliferation and T-helper 2 (Th2)-type IL-4 secretion. Children with food allergy had significantly fewer peripheral blood iNKTs which exhibited a greater Th2 response to α-GalCer and milk sphingomyelin compared to iNKTs of healthy controls.	Enhances allergic sensitisation
Finkina [30]	2020	Len c 3	Fatty acids: oleic C18:1 (OLE), lauric acid C12:0 (LAU), stearic C18:0 (STE), and behenic C22:0 (BEH)	N/A	Human	Circular dichroism spectroscopy was used to assess the influence of the selected Fatty acids on thermostability of rLen c 3. Gastrointestinal degradation of Len c 3 was simulated and characterised by RP-HPLC and SDS-PAGE. Allergen-specific IgE ELISAs were conducted to determine IgE binding abilities of Len c 3 with lipid-ligands.	The binding of OLE, LAU, and STE all reduced the rate of Len c 3 gastric degradation, apart from BEH. STE and OLE increased thermostability of Len c 3, whereas LAU and BEH had only a slight protective effect on the secondary structure. No lipid-ligand affected IgE binding capacity of Len c 3.	Enhances allergic sensitisation

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Meng **[31]**	2020	α-lactalbumin (BLA) and β-lactoglobulin (BLG)	C18 unsaturated fatty acid (UFA)	N/A	Human	The secondary and tertiary structures of BLA and BLG after treatment with C18 UFAs were characterized by circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy, and ANS fluorescence spectroscopy. Potential allergenicity was determined by Inhibition IgE ELISAs with milk-allergic patients' sera.	The binding of whey allergens to C18 UFAs resulted in the unfolding of BLA and BLG protein structures. This change in structure resulted in the enhanced IgE binding ability of BLA and BLG.	Enhances allergic sensitisation
Pablos-Tanarro **[32]**	2018	Egg	Egg yolk lipids	Intestinal epithelial cells, Dendritic cells	Murine and human	Female BALB/c mice were orally sensitised to egg white and egg yolk with/without adjuvant or intraperitoneally without adjuvant. In vitro assays assessed human epithelial and dendritic cell functions.	Egg yolk produced Th2-biasing effects through the upregulation of intestinal IL-33 expression. Egg yolk also favoured Th2 polarisation during DC presentation of allergens to T cells.	Enhances allergic sensitisation

CD1d-restricted iNKT Cell Activation

Four of the 10 food allergy studies report lipid presentation by CD1d molecules (Dearman, Alcocer, and Kimber 2007; Jyonouchi et al. 2011; Mirotti et al. 2013a; Tordesillas et al. 2017), with 3 of these also reporting the activation of iNKT cells (Jyonouchi et al. 2011; Mirotti et al. 2013a; Tordesillas et al. 2017).

Intrinsic lipids can be delivered to the immune system bound to allergenic proteins. This CD1d-iNKT cell mechanism is evident in the case of the lipid-ligand of Pru p 3 (from peach), in particular its lipid phytosphingosine tail, which was shown to activate murine-derived iNKT cells (determined by IL-2 secretion), through its lipid-ligand presentation on CD1d molecules (Tordesillas et al. 2017). Another study found the allergen protein, Ber e 1, failed to induce IgE production in sensitised mice when administered without its lipid fraction (Dearman, Alcocer, and Kimber 2007). When the lipid fraction was present, it acted as an adjuvant to IgE production. It was suggested the adjuvant activity of the lipid fraction could be due to its ligation of CD1d molecules (Dearman, Alcocer, and Kimber 2007). A subsequent study of Ber e 1 sensitization found the lipid fraction, named 'lipid C', induced the production of the Th2 cytokine IL-4 from iNKT cells to shift to allergic sensitization. They also found Ber e 1 can bind 'lipid C' via a hydrophobic pocket, allowing the protein-lipid complex to ligate CD1d molecules (Mirotti et al. 2013a). One study investigated milk and egg lipids, sphingomyelin and ceramide, respectively, in allergic sensitization (Jyonouchi et al. 2011). They established milk-sphingomyelin, but not egg-ceramide, can induce Th2-skewing of iNKT cells by presentation on human CD1d molecules. Unlike the aforementioned studies, this study also evaluated iNKT cell populations, revealing children with milk allergy had fewer iNKT cell numbers, but greater Th2 responses to milk-sphingomyelin than the iNKT cells of non-milk allergy controls.

Overall, all 4 studies report some lipids do promote allergic sensitization through CD1d presentation on DCs and subsequent activation of CD1d-restricted iNKT cells. The quality of these studies were assessed and the calculated scores were similar, with 2 out of 4 studies scoring 0.86 (Tordesillas et al. 2017; Mirotti et al. 2013a), one study scored 0.79 (Jyonouchi et al. 2011), and the final study was awarded a lower score of 0.64 (Dearman, Alcocer, and Kimber 2007) (**Table 3b).** Notably, this study used murine models only, which contributed to its lower score.

Table 3b. A summary of the quality of each food allergy study included in this systematic review. Studies were scored out of 1 for sample quality and methodological quality. Only aspects of each study relevant to the role of lipids in allergic sensitization were scored.

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size***	***Defined Controls***	***Representative Sample***	***Model***	***Robustness of Model***	***Lipid Preparation***	***Lipid Characterisation***	***Overall Quality Score***
	(n/2)	***(n/1)***	***(n/3)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/1)***
Angelina (2016) [23]	Unknown (0)	Yes (1)	Unknown (0)	Human (2)	Allergic samples sought from allergy unit within a hospital (1)	Phospholipids commercially sought and passed through an extruder. Mustard/peanut lipids extracted from source and purified. (2)	Phospholipids: Phosphatidylglycerol, Phosphatidylcholine. Mustard/peanut lipids. (1.5)	**0.54**
Palladino (2018) [24]	Unclear, at least 3 (1)	Yes (1)	Unknown (0)	Human (2)	Unknown (0)	Peanut lipids extracted and purified. (2)	Peanut lipids (1)	**0.50**

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size***	***Defined Controls***	***Representative Sample***	***Model***	***Robustness of Model***	***Lipid Preparation***	***Lipid Characterisation***	***Overall Quality Score***
	(n/2)	***(n/1)***	***(n/3)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/1)***
Dearman (2007) [25]	"groups of 5 mice" (1)	Yes (1)	Female BALB/c mice, 8-12 weeks old, allergic subjects (2)	Murine (1)	Mice sensitised by intraperitoneal (i.p.) injection of allergen and total IgE (not allergen specific) measured (1)	Total lipids extracted from Brazil nuts and purified. Lipids were then separated into classes by chromatography. (2)	Brazil nut b-sitosterol, total lipid fraction, sterols, free fatty acids, polar lipids (2)	**0.71**
Mirotti (2013) [26]	Unknown mice numbers, 4 humans. (1)	Yes (1)	Female BALB/c mice, 8-12 weeks old, allergic subjects. Unknown human participant characteristics. (2)	Human and Murine (2)	Mice sensitised by ip injection of allergen and total IgE (not specific) measured. Human allergic subjects selected by positive skin prick tests to brazil nut/walnut/peanut (2)	Lipids extracted and purified from brazil nut. (2)	Brazil nut 'Lipid C': mainly triglycerides, sterylglycosides, Phosphatidylethanolamine, PC, phosphatidic acid, and a sulphonated di-galacto lipid (2)	**0.86**

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size*** ***(n/2)***	***Defined Controls*** ***(n/1)***	***Representative Sample*** ***(n/3)***	***Model*** ***(n/2)***	***Robustness of Model*** ***(n/2)***	***Lipid Preparation*** ***(n/2)***	***Lipid Characterisation*** ***(n/2)***	***Overall Quality Score*** ***(n/1)***
Tordesillas (2017) [27]	Unclear, at least 8 (1)	Yes (1)	Female 6-8 week old C3H/HeOuJ mice, unknown human subject details, Allergic subjects (2)	Human and murine (2)	Mice sensitised by epicutaneous administration and specific IgE measured. Unknown human sample details. (2)	Lipid-ligand extracted from peach peel and separated by chromatography. (2)	Pru p 3 lipid-ligand (phytosphingosine tail) (2)	**0.86**
Hufnagl (2018) [28]	29 allergic, (1)	Yes (1)	Children only, allergic subject included (1)	Human (2)	Allergic/healthy participants defined by positive/negative oral allergen challenge to milk, respectively. (2)	Lipid sought commercially. (2)	All-trans retinoic acid (2)	**0.79**
Jyonouchi (2011) [29]	27 (1)	Yes (1)	23 males and 4 females, children only, allergic subjects included (1)	Human (2)	Allergic participants had a positive skin prick test and/or presence of specific IgE, positive food challenge and clinical stability on a diet excluding milk and/or egg.	Lipids commercially sought. (2)	Cow's milk–sphingomyelin, or hen's egg–ceramide (2)	**0.79**

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size***	***Defined Controls***	***Representative Sample***	***Model***	***Robustness of Model***	***Lipid Preparation***	***Lipid Characterisation***	***Overall Quality Score***
	(n/2)	***(n/1)***	***(n/3)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/1)***
Finkina (2020) [30]	10 human sera samples (1)	Yes (1)	Unknown (0)	Human (2)	Allergic samples obtained from a clinical diagnostic centre at a research institute. (1)	Lipids commercially sought. (2)	Fatty acids: oleic C18:1 (OLE), lauric acid C12:0 (LAU), stearic C18:0 (STE), and behenic C22:0 (BEH) (2)	**0.64**
Meng (2020) [31]	10 human sera samples (1)	Yes (1)	7 male, 3 females, and a range of age groups. Allergic subjects included. (2)	Human (2)	Allergic samples sought from patients at a hospital (1)	Lipids commercially sought. (2)	C18 unsaturated fatty acids from: oleic acid (OA), linoleic acid (LA), c9, t11-conjugated linoleic acid (CLA), α-linolenic acid (ALA), and γ-linolenic acid (GLA). (2)	**0.79**

	Sample Quality			***Methodological Quality***				
First author (Year) [reference]	***Sample Size*** ***(n/2)***	***Defined Controls*** ***(n/1)***	***Representative Sample*** ***(n/3)***	***Model*** ***(n/2)***	***Robustness of Model*** ***(n/2)***	***Lipid Preparation*** ***(n/2)***	***Lipid Characterisation*** ***(n/2)***	***Overall Quality Score*** ***(n/1)***
Pablos-Tanarro (2018) [32]	Unclear, at least 4 humans (1)	Yes (1)	Female 6-week old BALC/c mice, allergic subjects (2)	Human and murine (2)	Oral or ip. injection sensitisation and allergen-specific IgE measurement. Unknown human sample details. (2)	Egg yolk separated from egg white. (1)	Egg yolk lipids (1)	**0.71**

Lipids Activate Immune Cells

Five of the 10 food allergy studies investigated the role of lipids in directly activating immune cells (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Dearman, Alcocer, and Kimber 2007; Pablos-Tanarro et al. 2018; Palladino et al. 2018; Tordesillas et al. 2017).

Three of the 5 studies found lipids do enhance allergic sensitisation. One such study established the mustard seed and peanut allergen proteins, Sin a 2 and Ara h 1 respectively, accompanied by lipids derived from mustard and peanuts, reduced human monocyte-derived dendritic cell (hmoDC) allergenic protein uptake (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). Reduced protein uptake favours a Th2 reaction, whereas higher doses of protein uptake results in tolerance (Wisniewski, Agrawal, and Woodfolk 2013). Another study discovered egg yolk lipids acted as a Th2-biasing adjuvant to egg white through the upregulation of intestinal IL-33 by epithelial cells *in vitro,* which is crucial for DC activation and Th2 priming (Pablos-Tanarro et al. 2018). In addition to providing CD1d-iNKT activation evidence above, one study of the lipid-ligand of Pru p 3 (from peach) also established the lipid directly activated DCs as it matured human monocyte-derived DCs (Tordesillas et al. 2017).

One of the 5 studies revealed evidence for and against lipids enhancing allergic sensitization (Palladino et al. 2018). This study, related to peanut sensitization, found the administration of peanut lipids alone resulted in increased production of the anti-inflammatory cytokine, IL-10, from keratinocytes, thus inhibiting a Th2-type response. Whereas, peanut lipids delivered with the peanut allergenic protein inhibited IL-10 production.

Another nut allergen source study, also mentioned previously, found the allergenic protein, Ber e 1, failed to induce IgE production in sensitised mice when administered without its lipid fraction. It was only when the lipid fraction of the Brazil nut was present, the lipid acted as an adjuvant to IgE production. The total lipid fraction of the Brazil nut, including its composite sterols and polar lipids, all had marked adjuvant effects on IgE production. However, b-sitosterol and glycolipid-rich fractions had negligible impact on IgE production (Dearman, Alcocer, and Kimber 2007).

As shown in **Table 3b**, the 4 studies solely stating lipids enhance allergic sensitization received quality scores of 0.54 (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016), 0.64 (Dearman,

Alcocer, and Kimber 2007), 0.71 (Pablos-Tanarro et al. 2018) and 0.86 (Tordesillas et al. 2017).The study stating lipids may inhibit allergic sensitization received the lowest score of all the included food allergy studies, receiving a score of 0.50 (Palladino et al. 2018). This study mainly lost points due to lack of reporting sample characteristics such as sample size, gender, age, and how they defined their allergic and healthy cohort.

Lipids Induce Conformational Changes of Allergens

Four out of the 8 food allergen studies measured the influence of lipids on the structure of their associated allergenic proteins (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Hufnagl et al. 2018; Finkina et al. 2020a; Meng et al. 2020).

The digestibility of food proteins can determine whether the protein is tolerated or becomes a sensitizing agent. High resistance to digestion in the gastrointestinal tract has been shown to increase the sensitization capacity of proteins (Pali-Schöll et al. 2018). Three of these studies suggest protein-lipid binding can influence allergenic protein structure to alter digestion, and thus alter the sensitization capacity of the allergenic protein (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Finkina et al. 2020a; Meng et al. 2020). One study found that, in addition to lipids intrinsic to an allergen source, the peanut allergenic proteins, Sin a 2 and Ara h 1, can also interact with membrane-bound lipids (lipids derived from the cell membrane), such as phospholipids (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). This study, previously mentioned above as evidence for the direct activation of DCs, highlights that Sin a 2 and Ara h 1 can bind phosphatidylglycerol (PG) vesicles, reducing their gastrointestinal degradation (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). Furthermore, the ability for proteins to bind PG vesicles was dependent on the pH conditions; at pH 2.0, the phospholipids increased α-helix in Sin a 2 and β-sheet in Ara h 1, thus enhancing the content of allergenic protein secondary structure. In contrast, this was not the case for the mustard seed allergenic protein, Sin a 3, which is structurally different to peanut allergenic proteins, which highlighted it's structure and digestion was not affected by the presence of PG vesicles (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016).

Another study focused on the plant lipid transfer protein (LTP), Len c 3 (Finkina et al. 2020a). It has been established that legumes contain a high level of lipids, composing mostly of unsaturated fatty acids (Grela and Günter 1995). The lentil allergenic protein, Len c 3, is highly stable to digestion. This study

found Len c 3 binding of the unsaturated fatty acids: oleic acid (OLE), lauric acid (LAU), and stearic acid (STE), all reduced the rate of Len c 3 gastric degradation, apart from behenic acid (BEH) which did not alter degradation. Furthermore, OLE reduced Len c 3 degradation to 55% after 24 hours of simulated digestion, compared to 100% of Len c 3 degraded after 24 hours with no ligand. STE and OLE increased thermostability of Len c 3, while increasing the content of α-helices. Whereas, LAU and BEH only had a slight protective effect on the secondary structure. Despite these conformational changes, no lipid-ligand increased the IgE binding capacity of Len c 3.

In contrast, another study found protein-lipid binding did enhance the IgE-binding abilities of both whey proteins, α-lactalbumin (BLA) and β-lactoglobulin (BLG) (Meng et al. 2020). Whey proteins derived from cow's milk are widely used in the food industry due to their ability to emulsify, foam, and gelatinise food products (Lucey, Otter, and Horne 2017). These whey proteins also constitute the common allergenic proteins, α-lactalbumin (BLA) and β-lactoglobulin (BLG). Thus, the ability to reduce their allergenicity would be profitable to the food industry. The linear and conformational epitopes of proteins contribute towards the allergenicity of the allergen (Hochwallner et al. 2010). This study found BLA and BLG can bind C18 unsaturated fatty acids (UFA) to form protein-ligand complexes (Meng et al. 2020). This binding to the fatty acid resulted in the structural unfolding of BLG, where C18 UFA treatment induced a transition from a β-sheet to a random coil. Furthermore, BLA treatment with C18 UFA resulted in changes to tertiary structure. Therefore, this study suggests protein-lipid binding can alter allergenic protein structure which alters the allergenicity of the milk allergens.

In contrast, one study found intrinsic lipids do not alter allergenic protein structure and further stated they do not drive allergic sensitization (Hufnagl et al. 2018). Retinoic acid, found in cow's milk, had a high binding affinity for the common milk allergen protein, Bos d 5. This lipid did not alter the conformation of Bos d 5, and so not surprisingly did not alter its allergenicity or IgE binding in allergic children. Furthermore, the protein-lipid complexes supressed CD3+ CD4+ cell numbers which indicates an immunosuppressive effect on this population, which is pivotal in allergy induction.

Overall, 3 out of the 4 studies (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Finkina et al. 2020a; Meng et al. 2020) found lipids induced conformational changes of allergenic proteins which influenced allergic sensitisation, with 1 study suggesting that some lipids do not alter protein structure and thus allergenicity (Hufnagl et al. 2018). These studies highlight that different lipids, even those from the same class, have

different effects on the structure of allergenic proteins. Furthermore, the quality assessment for these studies (**Table 3b)** was mixed, with scores of 0.54 (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016), 0.64 (Finkina et al. 2020a), and 0.79 (Meng et al. 2020; Hufnagl et al. 2018). Notably, one of the highest scoring studies stated retinoic acid does not promote allergic sensitization to milk allergens (Hufnagl et al. 2018), gaining points as it is one of the only studies of the review which utilised a large, well-defined cohort of human patients.

Summary of Lipids in Food Allergies

Seven out of the 10 papers discussed how lipid-allergenic protein binding influences allergic sensitization (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Dearman, Alcocer, and Kimber 2007; Hufnagl et al. 2018; Mirotti et al. 2013a; Tordesillas et al. 2017; Finkina et al. 2020a; Meng et al. 2020), with 1 also studying membrane-bound lipids (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). The final 3 papers studied the impact of lipids directly on immune cells (Hufnagl et al. 2018; Jyonouchi et al. 2011; Palladino et al. 2018). The proposed mechanisms of lipids promoting allergic sensitization include activating iNKT cells through CD1d presentation, resulting in the upregulation of Th2 cytokines. Lipids also directly activate immune cells such as DCs to modulate activation and its allergenic protein uptake. There was also evidence for lipids inducing conformational changes to the protein to result in reduced gastrointestinal degradation of the protein, which shifts towards a Th2 response. Although, 2 out of the 10 papers on food allergies provided limited data showing that lipids can supress allergic sensitization to allergens when the lipids were delivered without the allergenic protein (Palladino et al. 2018) and by supressing $CD3^{+}CD4^{+}$ T cell populations (Hufnagl et al. 2018).

Intrinsic Lipids in Aeroallergies

Nine out of the total 19 studies examined the role of intrinsic lipids in allergic sensitization to aeroallergens (Abos Gracia et al. 2017; Agea et al. 2005; Bansal, Gaur, and Arora 2016; Gilles et al. 2010; Gilles et al. 2009; González Roldán et al. 2019; Gutermuth et al. 2007; Oeder et al. 2015; Satitsuksanoa et al. 2016). All 9 studies reported the lipids do enhance allergic sensitization (Abos Gracia et al. 2017; Agea et al. 2005; Bansal, Gaur, and Arora 2016; Gilles et al. 2010; Gilles et al. 2009; González Roldán et al. 2019; Gutermuth et al. 2007; Oeder et al. 2015; Satitsuksanoa et al. 2016). Although, 1 of these studies

highlight lipids can also inhibit a Th2 response (Gutermuth et al. 2007). **Table 4a** outlines the methods and outcomes of these studies.

Table 4a. A summary of the primary articles discussed, relevant to lipids in aeroallergies. Key details of each aeroallergy study are presented, along with whether the study provides evidence for the role of lipids driving or inhibiting allergic sensitization.

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Agea [33]	2005	Cypress pollen	PALMs: phosphatidylc-holine (PC), phosphatidyle-thanolamine (PE)	CD4+ T Cells, Dendritic cells	Human	T cell lines from cypress pollen-sensitive individuals were pulsed with cypress pollen lipids and cytokine responses were measured by enzyme-linked immunosorbent assay (ELISA). DC capture of pollen grains were assessed in the presence of anti-CD1d and anti-CD1a and analysed by confocal imaging.	PC and PE pollen lipids stimulated the proliferation of T cells from cypress-sensitive subjects and required CD1a+ and CD1d+ antigen presenting cells for lipid recognition. The responding T cells secreted both IL-4 and IFN-y.	Enhances allergic sensitisation
Abos Gracia [34]	2017	Olea	Olive pollen lipids (polar lipids, diaglycerolds, triaglycerols, free fatty acids)	iNKT cells, macrophages, and dendritic cells	Human	Invariant natural killer T (iNKT) cells, macrophages, and DCs were obtained from healthy blood donors, using flow cytometry to determine phenotype and cytotoxic killing assay to determine iNKT cell activation.	iDCs and macrophages exposed to total olive pollen lipids showed increased CD1d surface expression which resulted in the strong activation of iNKT cells.	Enhances allergic sensitisation
Gilles [35]	2009	Birch pollen	E1 phytoprostan-es (PPE1)	Dendritic cells (DCs)	Human	Analysed the role of PPE1 in regulating DC function and analysed its effect on NF-kappa-B signalling. DC phenotype was measured by flow cytometry and cytokine release by ELISA	PPE1 enhanced Th2 polarisation by modulating DC function via PPAR dependent pathways which inhibited NF kappa B activation, thus reducing DC IL-12 production.	Enhances allergic sensitisation

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Gilles [36]	2010	Birch pollen	Aqueous birch pollen extracts, PPE1	Dendritic cells (slanDCs), T cells	Human	SlanDCs were stimulated with aqueous birch pollen extracts, with or without lipopolysaccharide (LPS). DC phenotype was measured by flow cytometry and cytokine release by ELISA.	PPE1 inhibited secretion of LPS-produced IL-12 p70 and IL-6. SlanDCs exposed to aqueous pollen extracts were impaired in eliciting an IFN-gamma response in naive CD4+ T cells.	Enhances allergic sensitisation
Oeder [37]	2015	Ragweed, birch, grass, or pine pollen	Aqueous pollen extracts (APEs), PPE1	B Cells	Murine and Human	B cells from murine splenocytes and from blood samples of healthy donors were incubated under Th2-like conditions with APEs or its constituents. Secreted total IgE was quantified by ELISA. B cell proliferation was measured by CFSE staining.	PPE1 and Pollen extracts from various plant species enhanced Th2-induced production of total IgE and priming of B cells.	Enhances allergic sensitisation
Gonzalez [38]	2019	Timothy grass pollen	Aqueous pollen extracts PALMs	Dendritic cells	Murine	Bone marrow-derived DCs (BMDCs) were analysed by flow cytometry for changes in the expression of surface CD1d, in response APE stimulation. CD1d−/− BMDCs were used to rule out non-specific CD1d staining.	Surface expression of CD1d on BMDCs was significantly increased in APE stimulated BMDCs.	Enhances allergic sensitisation
Gutermuth [39]	2007	Ovalbumin	Bet APE, PPE1	Dendritic cells	Murine	In vitro T cell responses to ovalbumin were measured or in vivo ova-specific CD4 T cells were transferred into mice. Mice were then challenged with ovalbumin with or without the presence of Bet APE/PPE1. Cytokines measured by ELISA.	PPE1 Inhibited LPS-induced IL-12p70 production of DCs. Bet APEs with allergen increased Th2 differentiation, whereas PPE1 and PPF1 inhibited TH2 proliferation and cytokine release.	Enhances and inhibits allergic sensitisation

First Author [Ref]	Year	Allergen(s)	Lipid(s)	Cells Responding	Model	Method	Outcome	Effect on allergic sensitisation
Bansal [40]	2016	Cockroach extract	Lysophosphati-dylcholine (LPC)	NKT cells	Murine	Mice were sensitised to cockroach extract and LPC production was blocked by sPLA2. Anti-CD1d was also used to block CD1d. Bronchoalveolar lavage fluid (BALF) was collected and cytokine release measured by ELISA. Flow cytometry identified NKT populations.	Cockroach extract activated phospholipids which secrete LPC. sPLA2 inhibition blocked LPC production which inhibited CD1d-restricted NKT cell activation. IL-4 and IL-5 secretion was blocked when LPC was inhibited.	Enhances allergic sensitisation
Satitsuksanoa [41]	2016	Der p 13	Fatty acid	Epithelial cells	Human	rDer p 13 ligand binding capacity was analysed by fluorescence-based lipid-binding assays, and *in silico* structural prediction. Cytokine release by sandwich ELISAs and epithelial activation assays were conducted.	Der p 13 contained a potential binding site highly selective for hydrophobic ligands and can bind fatty acids. It triggered IL-8 and GM-CSF secretion in respiratory epithelial cells through a TLR2-, MyD88-, NF-kB-, and MAPK-dependent signalling pathway.	Enhances allergic sensitisation

The 9 papers exploring the role of aeroallergen source-derived lipids can be grouped into 3 main mechanisms, two of which are similar to the food allergen studies; the activation of iNKT cells through CD1d molecules, and the direct activation of immune cells. The final mechanism reported was the activation of TLRs.

CD1d-iNKT Cell Activation

As shown above with food allergies, lipids associated with aeroallergens have also been shown to influence allergic sensitization via CD1d-restricted iNKT cell activation. Four of the 9 aeroallergy studies described lipids associated with aeroallergen sources were shown to be presented by CD1d molecules on APCs and subsequently activated iNKT cells (Abos Gracia et al. 2017; Agea et al. 2005; Bansal, Gaur, and Arora 2016; González Roldán et al. 2019).

One study revealed PALMs primed DCs for the presentation of glycolipids to iNKT cells by CD1d upregulation (González Roldán et al. 2019). This supports findings from another study of olive pollen lipids (Abos Gracia et al. 2017), which established olive pollen lipids, but not aqueous pollen extracts (APEs), strongly activated human iNKT cells by increasing CD1d surface expression on iDCs and macrophages. All lipids analysed: polar lipids, diacylglycerols, free fatty acids, and triacylglycerol, were able to induce this increased CD1d expression. Despite altering the phenotype of iDCs, the olive pollen lipids did not alter their cytokine profile, but did induce secretion of IL-6 from macrophages, which further activated iNKT cells. Another study also found cypress pollen lipids were recognised by CD1d molecules (Agea et al. 2005). Furthermore, one study on cockroach allergy found the cockroach extract stimulated phospholipids to release lysophosphatidylcholine (LPC) and activate murine NKT cells, resulting in a Th2 shift. This NKT cell activation by LPC was inhibited when an anti-CD1d antibody was added (Bansal, Gaur, and Arora 2016).

Overall, all 4 studies report lipids do promote allergic sensitization by presentation on CD1d molecules and activation of iNKT cells. Two of these studies scored 0.64 (Bansal, Gaur, and Arora 2016) and 0.93 (Agea et al. 2005), with the latter scoring the highest in this review (**Table 4b**). This study utilised a larger sample size, involving a well-defined human cohort. However, another 2 were some of the lower scores of this review studies; 0.43 (González Roldán et al. 2019) and 0.50 (Abos Gracia et al. 2017), primarily losing points due to lack of reporting sample features such as sample size, age of the participants, and defining their healthy and allergic cohorts.

Table 4b. A summary of the quality of each aeroallergy study included in this systematic review. Studies were scored out of 1 for sample quality and methodological quality. Only aspects of each study relevant to the role of lipids in allergic sensitization were scored.

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size***	***Defined Controls***	***Representative Sample***	***Model***	***Robustness of Model***	***Lipid Preparation***	***Lipid Characterisation***	***Overall Quality Score***
	(n/2)	***(n/1)***	***(n/3)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/2)***	***(n/1)***
***Abos Gracia (2017)* [34]**	Unknown (0)	Yes (1)	Unknown (0)	Human (2)	Unknown (0)	Lipid extracted from olive pollen grains and purified. (2)	Polar lipids, diacylglycerols, free fatty acids, triacylglycerols (2)	**0.50**
Agea (2005) [33]	14 (1)	Yes (1)	6 males, 8 females, 19-45 yr olds, allergic subjects (3)	Human (2)	Allergic subjects defined by clinical history of rhinoconjunctivitis and/or asthma, as well as positive skin prick tests and serum specific IgE levels (2)	Phospholipids commercially sought and prepared in absolute ethanol. And lipids extracted from cypress pollen and purified. (2)	Phospholipids: PC, PE (2)	**0.93**

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size*** ***(n/2)***	***Defined Controls*** ***(n/1)***	***Representative Sample*** ***(n/3)***	***Model*** ***(n/2)***	***Robustness of Model*** ***(n/2)***	***Lipid Preparation*** ***(n/2)***	***Lipid Characterisation*** ***(n/2)***	***Overall Quality Score*** ***(n/1)***
Bansal (2016) [40]	Unknown (0)	Yes (1)	Female BALB/c mice, 4-6 weeks old, allergic subjects (2)	Murine (1)	Mice sensitised by intraperitoneal (i.p.) injection (1)	LPC commercially sought (2)	LPC (2)	**0.64**
Gilles (2009) [35]	Unknown (0)	Yes (1)	18-46 year olds, NO allergic subjects (1)	Human (2)	Healthy volunteers were screened for IgE against common allergens, and refrained from medication 2 weeks prior to blood sampling. (2)	Phytoprostanes extracted and purified from linoleic acid. (2)	Phytoprostanes PPE1 (2)	**0.64**

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size*** ***(n/2)***	***Defined Controls*** ***(n/1)***	***Representative Sample*** ***(n/3)***	***Model*** ***(n/2)***	***Robustness of Model*** ***(n/2)***	***Lipid Preparation*** ***(n/2)***	***Lipid Characterisation*** ***(n/2)***	***Overall Quality Score*** ***(n/1)***
Gilles (2010) [36]	Unknown (0)	Yes (1)	20-51 year olds, allergic subjects (2)	Human (2)	All subjects defined by total IgE serum levels. Allergic subjects had positive IgE against allergen, and a positive history of allergic rhinitis. All subjects refrained from medication for 15 days before blood donation. (2)	Phytoprostanes extracted and purified from linoleic acid. (2)	Phytoprostanes PPE1 and PPF1 (2)	**0.79**
Gonzalez (2019) [38]	Unknown (0)	Yes (1)	Unknown, no allergic subjects (0)	Murine (1)	Unknown (0)	PALMs extracted from APEs and filtered/purified. (2)	APE, PALMs PPE1 and PPF1 (2)	**0.43**

	Sample Quality			*Methodological Quality*				
First author (Year) [reference]	***Sample Size*** ***(n/2)***	***Defined Controls*** ***(n/1)***	***Representative Sample*** ***(n/3)***	***Model*** ***(n/2)***	***Robustness of Model*** ***(n/2)***	***Lipid Preparation*** ***(n/2)***	***Lipid Characterisation*** ***(n/2)***	***Overall Quality Score*** ***(n/1)***
Gutermuth (2007) [39]	Unknown (0)	Yes (1)	Unknown (0)	Murine (1)	Unknown (0)	Phytoprostanes extracted and purified from linoleic acid. APEs filtered from pollen grains. (2)	Bet-APE, PPE1 and PPF1 (2)	**0.43**
Oeder (2015) [37]	Unknown (0)	Yes (1)	Female C57BL/6 and BALB/c mice, 6-10 week-old, allergic participants (2)	Human and Murine (2)	Mice sensitised by i.p. injection and total IgE measured (not allergen-specific IgE) (1)	Pollen grains commercially sought then filtered to obtain protein-free APEs. (2)	Amb-APE, PPE1 (2)	**0.71**
Satitsuksanoa (2016) [41]	Unknown (0)	Yes (1)	Unknown (0)	Human (2)	Unknown (0)	Lipids commercially sought (2)	Cis-parinaric acid (2)	**0.50**

Lipids Activate TLRs

One of the 9 aeroallergen studies investigated the lipid activation of TLRs (Satitsuksanoa et al. 2016). This study suggests the HDM protein allergen, Der p 13, has certain structural features, allowing highly selective lipid-binding of fatty acids.

The protein-lipid complex can then activate Toll like receptors (TLRs), such as TLR2, to stimulate inflammatory cytokines IL-8 and GM-CSF production in respiratory epithelial cells. This study concludes lipids enhance allergic sensitization, however, the study was awarded a below-average quality score of 0.50 (Satitsuksanoa et al. 2016) (**Table 4b**). Again, this was mostly due to a lack of reporting sample sizes and not defining their cohorts.

Immune Cell Activation

All 5 studies reporting aeroallergen lipids can directly activate immune cells focused on PALMs (Agea et al. 2005; Gilles et al. 2010; Gilles et al. 2009; Gutermuth et al. 2007; Oeder et al. 2015). Two human studies (Gilles et al. 2010; Gilles et al. 2009) highlighted aqueous birch pollen extracts (Bet.-APE)-derived PPE_1 modulated DC function and its cytokine production, specifically the inhibition of IL-12, preferentially inducing a Th2 response. Another study using a murine model of allergy found PPE_1 inhibited the LPS-induced production of IL-12 from DCs (Gutermuth et al. 2007), but when intranasally instilled with the egg allergen protein, Ovalbumin, PPE_1 inhibited Th2 polarization and cytokine release, suggesting lipids inhibit allergic sensitization. Another study also established PPE_1 and aqueous pollen extracts stimulated Th2-primed B cells to enhance IgE production (Oeder et al. 2015). One study, previously mentioned for its evidence of CD1d recognition of PALMs, found the presence of PC and PE lipids from cypress pollen alone stimulated TCR $\alpha\beta^+$ $CD4^+$ T cell production of IL-4, enhancing a Th2 response (Agea et al. 2005).

Overall, 4 studies show PALMs can directly activate DCs, B cells, and $CD4^+$ T cells to shift to a Th2 response. Whereas, 1 study (Gutermuth et al. 2007) reports PALMs can promote and inhibit allergic sensitization. The quality assessment (**Table 4b**) highlights 3 of the studies which suggested lipids promote allergic sensitization were above average; 0.71 (Oeder et al. 2015), 0.79 (Gilles et al. 2010), and 0.93 (Agea et al. 2005). However, 1 study (Gilles et al. 2009), as well as the study reporting lipids both promote and inhibit allergic sensitization (Gutermuth et al. 2007), scored below average; 0.64 (Gilles et al. 2009) and 0.43 (Gutermuth et al. 2007). As before, this was mostly due to lack of reporting sample sizes, how representative the sample was, and how robust the model was.

Summary of Lipids in Aeroallergies

The mechanisms proposed include aeroallergens proteins can bind to lipids and activate TLRs to shift to a Th2 response. PALMs, notably $PPE_{1,}$ can act upon DCs to modulate its subsequent cytokine release to favour allergic sensitization. Similar to food allergenic lipids, aeroallergen lipids can also activate iNKT cells through CD1d presentation.

DISCUSSION

Allergens from food, pollen, and insect faecal particles are delivered to the immune system in association with a variety of immunomodulatory components, such as lipids. This systematic review captures growing evidence for the role of lipids in allergic sensitization. In summary, lipids can interact with allergenic proteins to influence the development of allergic sensitization. This protein-lipid interaction resulted in reduced gastrointestinal degradation of the allergenic proteins through structural protein changes, the reduction of DC uptake of allergenic proteins to reduce immune tolerance, the regulation of Th2 cytokines, the enhancement of allergen-specific IgE, the activation of iNKT cells through CD1d ligation, and finally, directly acting upon TLRs, epithelial cells, keratinocytes, and DCs. **Figure 2** summarises the main mechanisms identified in this review of how lipids influence allergic sensitization.

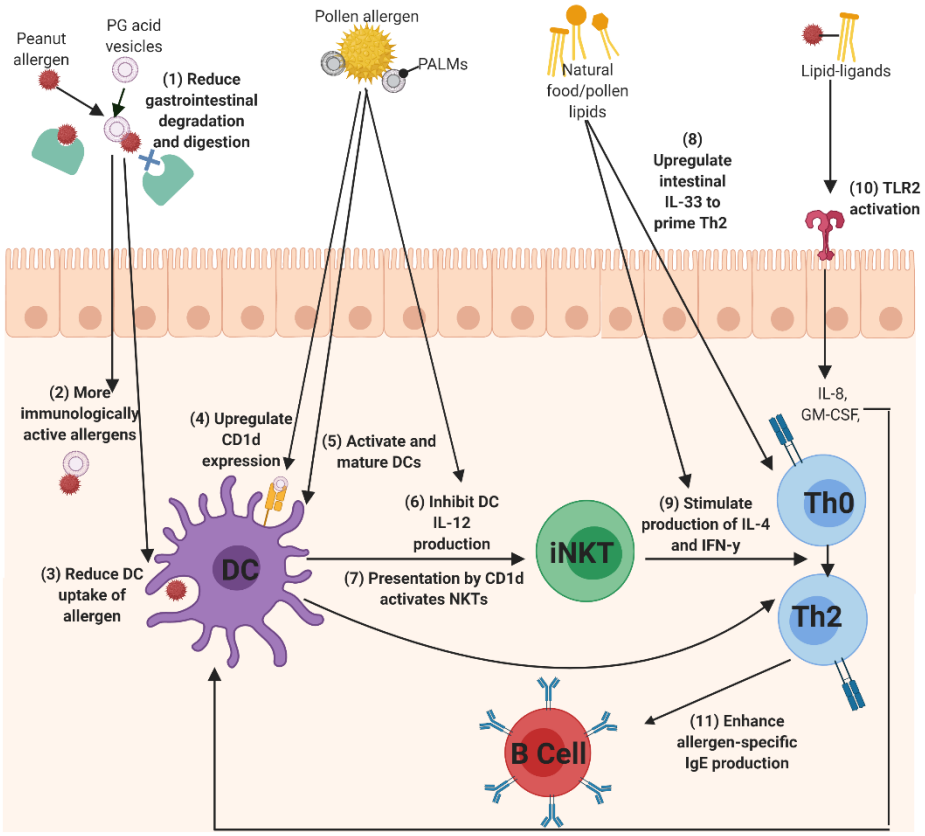

Figure 2. The mechanisms of lipids to influence allergic sensitization. (1) Phospholipids can bind allergens to reduced gastrointestinal degradation of the allergen, which (2) allows more immunologically active allergens to enter the immune system and can also (3) alter DC uptake of the allergen. Lipids, such as PALMs, can directly act upon DCs by (4) upregulating CD1d expression, (5) activating and maturing DCs, (6) and inhibiting I-12 production, which can all lead to the (7) activation of iNKT cells. Th0 cells could then be primed to Th2 cells by (8) IL-33 secretion from lipid-activated epithelial cells, or (9) by the secretion of IL-4 and IFN-γ cytokines from lipid-activated iNKT cells. (10) protein-lipid complexes can activate TLRs, such as TLR2, to initiate IL-8 and GM-CSF production, which in turn activates DCs. Finally, (11) lipids can also enhance the production of allergen-specific IgE from B cells. (Created using Biorender.com).

The results of this systematic review show 18 out of 19 (Abos Gracia et al. 2017; Agea et al. 2005; Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Bansal, Gaur, and Arora 2016; Dearman, Alcocer, and Kimber 2007; Gilles et al. 2010; Gilles et al. 2009; González Roldán et al. 2019; Jyonouchi et al. 2011; Mirotti et al. 2013a; Oeder et al. 2015; Pablos-Tanarro et al. 2018; Tordesillas et al. 2017; Satitsuksanoa et al. 2016; Finkina et al. 2020a; Meng et al. 2020; Palladino et al. 2018) studies reported lipids can enhance allergic sensitization, revealing a strong weight of evidence towards the role of lipids in driving a Th2-type response. Although, two of these studies also report lipids can inhibit allergic sensitization (Gutermuth et al. 2007; Palladino et al. 2018). And one paper in this review solely suggests lipids inhibit allergic sensitization (Hufnagl et al. 2018).

For the 95% of studies in the systematic review reporting lipids can enhance allergic sensitization, a key finding was the ability for lipids intrinsic to food and inhalant allergen sources to promote allergic sensitisation via the activation of CD1d-restricted iNKT cells, with 47% of studies reporting this mechanism. During allergic sensitization, in contrast to proteins that are presented by MHC class II molecules, it is well-established that lipids are presented by CD1 molecules (Schiefner and Wilson 2009b). There has been particular interest in CD1d molecules as they present glycolipids to a specific group of T lymphocytes, called invariant natural killer T (iNKT) cells, which are powerful immune regulators (Dhodapkar and Kumar 2017b). This data supports existing knowledge of the presentation of lipids by CD1d molecules on APCs, and the subsequent recognition by iNKT cells, to result in the release of Th2-skewing cytokines. As all 8 studies which reported this mechanism enhances allergic sensitization, it provides a strong weight of evidence for CD1d-restricted iNKT cells in the role of enhancing, or in some cases initiating, allergic sensitization. On another note, not all lipids tested were successful in activating iNKT cells, which potentially indicates a specificity for iNKT cells to recognise CD1d-lipid complexes. Some research suggests this specificity could be due to the structure of the lipid, specifically its head group, as self-lipids with larger head groups decreased or prevented interaction with iNKT-TCRs (Mallevaey et al. 2011).

Another key mechanism was the ability for lipids to directly and indirectly activate cells of the immune system, such as DCs, NKT cells, T cells, keratinocytes, epithelial cells and B cells, with 53% of studies reporting this mechanism. The studies highlighted the ability for certain food allergen-associated lipids to reduce human moDC protein uptake (Angelina, Sirvent,

Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). High doses of protein uptake leads to the induction of tolerance, whereas low doses of allergenic protein uptake favours a Th2 response (Wisniewski, Agrawal, and Woodfolk 2013). Hence the reduction of Sin a 2 and Ara h 1 capture by human moDCs reduced the allergen tolerance and thus shifted to/enhanced the development of allergic sensitization. Interestingly, the administration of peanut lipids accompanied with peanut allergenic protein inhibited the production of the anti-inflammatory cytokine, IL-10, from keratinocytes (Palladino et al. 2018). Thus, the co-delivery of peanut lipids with peanut protein promotes an inflammatory state, favouring a Th2 response. Aeroallergen studies focused on the role of PALMs in allergic sensitization. PALMs are hypothesised to induce and enhance allergic sensitization. Pollen grains co-release allergenic proteins and PALMs when stimulated with water, which can then interact to form protein-lipid complexes (Bashir et al. 2013). Once released by the pollen grain, PALMs can then interact with pollen-exposed human epithelia (Gilles et al. 2009). The 5 studies investigating PALMs explored their ability to act upon DCs, B cells and T cells. For instance, one study found PALMs induced CD1d upregulation on murine DCs. This is likely a result of preparing the cell for lipid presentation to iNKT cells, which once activated, can release Th2 cytokines (González Roldán et al. 2019). Similarly, another study using a murine model of allergy found the PALM, $PPE_{1,}$ inhibited the LPS-induced production of IL-12 from DCs (Gutermuth et al. 2007). Importantly, IL-12 production promotes a Th1 response, and in its absence, a Th2 response is favoured (Moser and Murphy 2000). Thus, this explains why the inhibition of IL-12 noted in three studies resulted in a Th2 shift.

Four studies suggested lipids can act as an adjuvant to IgE production during allergic sensitization. The PALM, $PPE_{1,}$ is the main lipid component of birch and ambrosia APEs (González Roldán et al. 2019). PPE_1 is structurally similar to endogenous prostaglandins, which have also been reported to stimulate IgE production from B cells (Roper et al. 1990), hence this could explain the structure of PALMs determines its adjuvant activity. Lipids administered alone shifted towards a Th2 reaction, (Palladino et al. 2018). This is similar to another study who found the lipid fraction of the Brazil nut allergenic protein, Ber e 1, was essential to stimulate an IgE response (Mirotti et al. 2013a). Another study found Brazil nut sterols and polar lipids all had marked adjuvant effects on IgE production. However, other Brazil nut lipids, β-sitosterol and glycolipid-rich fractions, did not impact IgE (Dearman, Alcocer, and Kimber 2007). Thus, it is important to note that there is some specificity for lipids driving allergic sensitization, potentially determined by structural qualities.

Another mechanism, proposed by one aeroallergy study, suggested the Der p 13 lipid-ligand can activate TLR2 to stimulate inflammatory cytokines in epithelial cells (Satitsuksanoa et al. 2016). This was the only paper in the systematic review stating this lipid-induced effect. Although, this mechanism is endorsed by a recent review which explained other lipids, such as membrane-bound lipids, can also influence TLR activity (Ruysschaert and Lonez 2015).

The final mechanism reported in this systematic review regarding lipids enhancing allergic sensitization was lipid-induced conformational changes to allergenic proteins, which enhanced allergenicity. One study highlighting this mechanism found phospholipid-binding resulted in reduced gastrointestinal degradation of the peanut allergenic protein (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). This resistance to degradation allows immunologically active protein allergens to reach the gut immune system and trigger allergic sensitization by presentation to DCs, and also trigger the effector phase upon further exposure. Another 2 studies investigating the lipid-protein binding of lentil allergens (Finkina et al. 2020a) and milk allergens (Meng et al. 2020) supported these findings, adding that lipid-binding enhances thermostability of allergenic proteins during digestion. In contrast, this was not the case in the mustard seed allergenic protein, Sin a 3, which is structurally different to peanut allergen proteins, and was not affected by the presence of PG vesicles (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016). As aforementioned, this infers allergen structure may determine the interaction with lipid membranes, affecting DC uptake of the protein. Overall, the ability for lipids to favour allergic sensitization through altering the structure of its associated allergenic proteins is well supported. However, it must be noted that different lipids have different effects on the secondary structures of allergenic proteins, even those proteins which are from the same class and structurally similar. A recent review on protein-lipid binding supports lipids in inducing conformational changes to the allergenic protein and the subsequent altered allergenic properties, as well as highlights the different structural effects induced by different lipids (Jappe et al. 2019b).

The three studies with reports that lipids can inhibit the development of allergic sensitization investigated peanut lipids (Palladino et al. 2018), retinoic acid from milk (Hufnagl et al. 2018), and PPE_1 from birch pollen (Gutermuth et al. 2007). Although, the study of peanut lipids had the third lowest quality score of all 19 studies, thus, the findings should be interpreted with caution. In contrast, the study into the role of retinoic acid in allergic sensitisation was awarded a higher than average quality score of 0.86. The proposed mechanisms of lipid-inhibited allergic sensitization include the

inhibition of Th2 cytokine secretion (Gutermuth et al. 2007; Hufnagl et al. 2018) and the upregulation of Th1 cytokine secretion (Palladino et al. 2018). The study that solely states lipids inhibit allergic sensitization reported that stimulation with retinoic acid bound to Bos d 5 milk allergenic protein supressed IL-10 and IL-13 cytokine release. Thus, this study suggests Bos d 5 loading of retinoic acid supresses a Th2 response and its allergenicity. This Bos d 5 loading of retinoic acid correlated with reduced lysosomal digestion of the protein allergen. Despite this study inferring some lipids do not promote allergic sensitization, it still provided clear evidence for the formation of protein allergen-lipid binding, which is a key phenomenon that highlights allergenic proteins are co-delivered to the immune system with other compounds, such as lipids. The study on birch pollen allergy also reported lipids can inhibit allergic sensitization when the lipid PPE_1 was delivered with an allergenic protein, as its complex inhibited Th2 polarization and cytokine release (Gutermuth et al. 2007). Again, suggesting lipids inhibit allergic sensitization when accompanied by its associated protein allergen. The findings of these two studies contrasts with another study reporting lipids can inhibit allergic sensitization, as it stated the accompaniment of peanut lipids without its associated allergenic protein actually stimulated Th1 cytokines, IL-10, to be released (Palladino et al. 2018), and suggesting lipids can inhibit allergic sensitization alone. Overall, the three studies stating lipids can inhibit allergic sensitization put importance on the effect of delivering lipids accompanied by allergenic proteins to the immune system, with two studies implying lipids co-delivered with allergenic proteins inhibits a Th2 response, and one study contrasting to state lipids without allergenic proteins actually inhibit a Th2 response.

A major criticism of the studies examined is the quality assessment scores, as many of the included studies were low. The aim of this scoring system was to highlight the robustness of existing research in this area. Low-scoring studies are not to be deemed unreliable, but reflect the need for further research which has adopted specific characteristics, such as larger sample sizes and human model systems. The majority of these studies lost points due to lack of reporting sample sizes and defining the cohort of samples. Indeed, only 53% of studies published data on sample size. Of this data, only 2 studies (Hufnagl et al. 2018; Jyonouchi et al. 2011) employed a cohort of above 20. This limits the power of the study to detect associations and highlights the need for more studies to report sample sizes. Furthermore, no statistical power calculations to determine sample size were evident throughout the papers.

In contrast, all studies (excluding one (Pablos-Tanarro et al. 2018)) received high scores for the preparation of their lipid, where they either

commercially sought lipids or provided detailed methods for the extraction and purification of lipids. Most studies also scored highly for characterisation of the lipid used. Although, several studies lacked clarification on the type of lipids they utilised (Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodriguez, et al. 2016; Pablos-Tanarro et al. 2018; Palladino et al. 2018), simply stating 'peanut lipids or 'egg lipids'. The range of lipids encapsulated in an allergen source is wide, thus, the lack of specificity then poses difficulty in drawing conclusions to which lipid promotes or inhibits a Th2 response.

This systematic review included studies utilising human and murine models, with 47% of the studies using murine models of allergic sensitization. The use of mice to study allergic sensitization could be deemed important in addition to human data. However, studies solely recruiting murine models needs to be cautiously interpreted, taking into consideration differences to the human immune system and a lack of validated animal models. For instance, mice only express CD1d receptors on DCs, and cytokines, such as IL-10, produced by a Th2 response in mice are produced by a Th1 and Th2 response in humans (Mestas and Hughes 2004). Furthermore, allergy in mice is not natural, thus, inducing sensitization to allergens is artificial and does not fully reflect the development of allergic sensitisation in humans. Based on this information, murine models were scored lower in the quality assessment due to the potentially reduced human relevance.

General limitations of this systematic review include the lack of evaluation for differences in lipid metabolism between males and females. It is evident that gender was not considered in most studies, but evidence suggests the lipid metabolism differences between genders could lead to differences in immune responses (Furman et al. 2014). Thus, future studies should consider this factor. Furthermore, during the article search stage of this systematic, there were many relevant papers excluded from this review as they were conference abstracts, rather than peer-reviewed publications. For instance, there was a collection of studies relevant to non-specific lipid transfer proteins (nsLTPs), but these were only available as conference abstracts (Foo et al. 2019; Garrido-Arandia et al. 2018; Gepp et al. 2014; Gilles et al. 2014; Humeniuk et al. 2019; Iweala, Savage, and Commins 2018; Palladino et al. 2016; Perez Rodriguez et al. 2019; Smole et al. 2011). Once, or if, these conference abstracts have been published as articles, an updated review would be beneficial.

Future research of lipids in allergic sensitization could allow pathomechanistic insights, leading to the development of new treatments and prevention approaches to type 1 hypersensitivity, during this allergy epidemic. It is evident that the effect lipids have on allergic sensitization differs depending on the lipid and protein class. Thus, future research is needed to identify the

specific lipids involved in enhancing Th2 pathways, and to characterize their potential interaction with allergenic proteins. Ultimately, this research highlights that it is the combination of components from the allergen source which promote allergic sensitization. Hence, it is key that these components are studied together and their combined effects on the immune system measured.

This research also highlights the importance of whole allergen source extracts used in allergy diagnostics, such as skin-prick testing. As using purified allergenic proteins, without the lipid cargo present, may result in false-negative responses, due to some research suggesting the lipid fraction must be present in order to trigger a Th2 response. Furthermore, establishing factors which enhance allergic sensitization are essential to identify potential food allergens as part of food safety assessment processes. Especially as novel foods are constantly being introduced to consumers to counteract the food insecurity problem, the importance of assessing the allergenicity of food proteins in key. The incorporation of lipids into current immunogenicity assays could therefore provide critical evidence to the assessment of protein allergenicity.

Ultimately, this systematic review concludes lipids intrinsic to an allergen source can act as immune adjuvants, through the various mechanisms discussed. Adjuvants are defined as substances which have the capacity to enhance the immune response to an allergen (Berin and Shreffler 2008). Thus, it could be speculated that the allergenicity of protein allergens could be determined by the presence of the lipids. However, due to the limited number of papers available for this systematic review, further research is essential to validate these findings, before the results can be applied elsewhere. Overall, there is a consensus that lipids do promote allergic sensitization.

ABBREVIATIONS

APE	Aqueous pollen extract
BEH	Behenic acid
BLA	α-lactalbumin
BLG	β-lactoglobulin
CD1	Cluster of differentiation 1
DC	Dendritic cell
Df	*Dermatophagoides farina*
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
hmoDC	Human Monocyte-derived dendritic cell
IgE	Immunoglobulin E
IFN- γ	Interferon gamma
IL	Interleukin
iNKT	invariant natural killer T cells
LAU	Lauric acid
LPC	Lysophosphatidylcholine
LPS	Bacterial lipopolysaccharide
MHC	Major Histocompatibility Complex
nsLTP	Non-specific lipid-transfer protein
OLE	Oleic acid
PALM	Pollen-associated lipid mediator
PG	Phosphatidylglycerol
PPE_1	Phytoprostanes E1
STE	Stearic acid
Th1	T cell subset 1
Th2	T cell subset 2
TLR	Toll-like receptor
UFA	Unsaturated fatty acids

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References

Abós-Gracia, B., M. G. del Moral, J. López-Relaño, V. Viana-Huete, L. Castro, M. Villalba, and E. Martínez-Naves. 2013. 'Olea europaea pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells', *J Allergy Clin Immunol*, 131: 1393-9.e5.

Abos Gracia, B., J. López Relaño, A. Revilla, L. Castro, M. Villalba, B. Martín Adrados, J. R. Regueiro, E. Fernández-Malavé, E. Martínez Naves, and M. Gómez Del Moral. 2017. 'Human Invariant Natural Killer T Cells Respond to Antigen-Presenting Cells Exposed to Lipids from Olea europaea Pollen', *Int Arch Allergy Immunol*, 173: 12-22.

Agea, E., A. Russano, O. Bistoni, R. Mannucci, I. Nicoletti, L. Corazzi, A. D. Postle, G. De Libero, S. A. Porcelli, and F. Spinozzi. 2005. 'Human CD1-restricted T cell recognition of lipids from pollens', *Journal of Experimental Medicine*, 202: 295-308.

Ahmadi, Alireza, Zahra Fallah Vastani, Mahdi Abounoori, Mahdieh Azizi, Alireza Labani-Motlagh, Sajad Mami, and Sanaz Mami. 2022. 'The role of NK and NKT cells in the pathogenesis and improvement of multiple sclerosis following disease-modifying therapies', *Health Science Reports*, 5: e489.

Akdis, M., and C. A. Akdis. 2014. 'Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens', *J Allergy Clin Immunol*, 133: 621-31.

Akdis, M., S. Burgler, R. Crameri, T. Eiwegger, H. Fujita, E. Gomez, S. Klunker, N. Meyer, L. O'Mahony, O. Palomares, C. Rhyner, N. Ouaked, A. Schaffartzik, W. Van De Veen, S. Zeller, M. Zimmermann, and C. A. Akdis. 2011. 'Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases', *J Allergy Clin Immunol*, 127: 701-21 e1-70.

Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, C. Kegel, R. Disch, C. B. Schmidt-Weber, K. Blaser, and C. A. Akdis. 2004. 'Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells', *J Exp Med*, 199: 1567-75.

Al-Ghouleh, A., R. Johal, I. K. Sharquie, M. Emara, H. Harrington, F. Shakib, and A. M. Ghaemmaghami. 2012. 'The glycosylation pattern of common allergens: the

recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent', *PLoS One*, 7: e33929.

Alcocer, Marcos, and Lars Yman. 2013. 'Allergy.' in David Wild (ed.), *The Immunoassay Handbook* (Elsevier Ltd).

Andreae, S., F. Piras, N. Burdin, and F. Triebel. 2002. 'Maturation and activation of dendritic cells induced by lymphocyte activation gene-3 (CD223)', *J Immunol*, 168: 3874-80.

Angelina, Sirvent, Palladino, Vereda, Cuesta-Herranz, Eiwegger, Rodríguez, Breiteneder, Villalba, and Palomares. 2016. 'The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity', *Allergy*, 71: 1284-94.

Angelina, A., S. Sirvent, C. Palladino, A. Vereda, J. Cuesta-Herranz, T. Eiwegger, R. Rodriguez, H. Breiteneder, M. Villalba, and O. Palomares. 2016. 'The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity', *Allergy*, 71: 1284-94.

Antunes, L., A. P. Duarte de Souza, P. D. de Araújo, L. A. Pinto, M. H. Jones, R. T. Stein, and P. M. Pitrez. 2018. 'iNKT cells are increased in children with severe therapy-resistant asthma', *Allergologia et Immunopathologia*, 46: 175-80.

Arasi, Stefania, Lucia Caminiti, Giuseppe Crisafulli, and Giovanni Battista Pajno. 2018. 'A general strategy for de novo immunotherapy design: the active treatment of food allergy', *Expert Review of Clinical Immunology*, 14: 665-71.

Arora, P., S. S. Kharkwal, T. W. Ng, S. Kunnath-Velayudhan, N. K. Saini, C. T. Johndrow, Y. T. Chang, G. S. Besra, and S. A. Porcelli. 2016. '"Endocytic pH regulates cell surface localization of glycolipid antigen loaded CD1d complexes"', *Chem Phys Lipids*, 194: 49-57.

Aspord, C., M. T. Leccia, J. Charles, and J. Plumas. 2013. 'Plasmacytoid dendritic cells support melanoma progression by promoting Th2 and regulatory immunity through OX40L and ICOSL', *Cancer Immunol Res*, 1: 402-15.

Augustine, Tracy, Manoj Kumar, Souhaila Al Khodor, and Nicholas van Panhuys. 2022. 'Microbial Dysbiosis Tunes the Immune Response Towards Allergic Disease Outcomes', *Clinical Reviews in Allergy & Immunology*.

Balan, S., M. Saxena, and N. Bhardwaj. 2019. 'Dendritic cell subsets and locations', *Int Rev Cell Mol Biol*, 348: 1-68.

Balasko, Allison, Colin Graydon, and Keith R. Fowke. 2021. 'Novel in vitro invariant natural killer T cell functional assays', *Journal of Immunological Methods*, 499: 113171.

Baldacci, S., E. Omenaas, and M.P. Oryszczyn. 2001. 'Allergy markers in respiratory epidemiology', *European Respiratory Journal*, 17: 773-90.

Ballmer-Weber, B. K., J. Lidholm, M. Fernández-Rivas, S. Seneviratne, K.-M. Hanschmann, L. Vogel, P. Bures, P. Fritsche, C. Summers, A. C. Knulst, T.-M. Le, I. Reig, N. G. Papadopoulos, A. Sinaniotis, S. Belohlavkova, T. Popov, T. Kralimarkova, F. de Blay, A. Purohit, M. Clausen, M. Jedrzejczak-Czechowcz, M. L. Kowalski, R. Asero, R. Dubakiene, L. Barreales, E. N. Clare Mills, R. van Ree, and S. Vieths. 2015. 'IgE recognition patterns in peanut allergy are age dependent: perspectives of the EuroPrevall study', *Allergy*, 70: 391-407.

Bannon, G. A. 2004. 'What makes a food protein an allergen?', *Curr Allergy Asthma Rep*, 4: 43-6.

Bansal, P., S. N. Gaur, and N. Arora. 2016. 'Lysophosphatidylcholine plays critical role in allergic airway disease manifestation', *Sci Rep*, 6: 27430.

Barral, Duarte C., and Michael B. Brenner. 2007. 'CD1 antigen presentation: how it works', *Nature Reviews Immunology*, 7: 929-41.

Baseggio Conrado, Alessia, Despo Ierodiakonou, M Hazel Gowland, Robert J Boyle, and Paul J Turner. 2021. 'Food anaphylaxis in the United Kingdom: analysis of national data, 1998-2018', *BMJ*, 372: n251.

Bashir, Mohamed Elfatih H., Jan Hsi Lui, Ravishankar Palnivelu, Robert M. Naclerio, and Daphne Preuss. 2013. 'Pollen Lipidomics: Lipid Profiling Exposes a Notable Diversity in 22 Allergenic Pollen and Potential Biomarkers of the Allergic Immune Response', *PLoS ONE*, 8: e57566.

Beckman, Evan M., Steven A. Porcelli, Craig T. Morita, Samuel M. Behar, Stephen T. Furlong, and Michael B. Brenner. 1994. 'Recognition of a lipid antigen by CD1-restricted αβ+ T cells', *Nature*, 372: 691-94.

Bellinghausen, I., U. Brand, J. Knop, and J. Saloga. 2000. 'Comparison of allergen-stimulated dendritic cells from atopic and nonatopic donors dissecting their effect on autologous naive and memory T helper cells of such donors', *J Allergy Clin Immunol*, 105: 988-96.

Bendiner, E. 1981. 'Baron von Pirquet: the aristocrat who discovered and defined allergy', *Hospital practice (Office ed.)*, 16: 137, 41, 44 passim.

Berger, A. 2000. 'Th1 and Th2 responses: what are they?', *BMJ*, 321: 424.

Berin, M. Cecilia, and Wayne G. Shreffler. 2008. 'T_H2 adjuvants: Implications for food allergy', *Journal of Allergy and Clinical Immunology*, 121: 1311-20.

Birkholz, Alysia M., and Mitchell Kronenberg. 2015. 'Antigen specificity of invariant natural killer T-cells', *Biomedical Journal*, 38: 470-83.

Bischoff, S. C., J. Mayer, J. Wedemeyer, P. N. Meier, G. Zeck-Kapp, B. Wedi, A. Kapp, Y. Cetin, M. Gebel, and M. P. Manns. 1997. 'Colonoscopic allergen provocation (COLAP): a new diagnostic approach for gastrointestinal food allergy', *Gut*, 40: 745-53.

Brigid Francis-Devine, Shadi Danechi, Yago Zayed, Aleksandra Gorb, Xameerah Malik. 2022. "Food poverty: Households, food banks and free school meals." In.: UK House of Commons.

Broide, D. H. 2001. 'Molecular and cellular mechanisms of allergic disease', *J Allergy Clin Immunol*, 108: S65-71.

Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. 'CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution', *J Exp Med*, 188: 1521-8.

Brough, Helen A., Kari C. Nadeau, Sayantani B. Sindher, Shifaa S. Alkotob, Susan Chan, Henry T. Bahnson, Donald Y. M. Leung, and Gideon Lack. 2020. 'Epicutaneous

sensitization in the development of food allergy: What is the evidence and how can this be prevented?', *Allergy*, 75: 2185-205.

Bublin, Merima, Thomas Eiwegger, and Heimo Breiteneder. 2014. 'Do lipids influence the allergic sensitization process?', *J Allergy Clin Immunol*, 134: 521-29.

Burgdorf, Sven, Verena Schuette, Verena Semmling, Katharina Hochheiser, Veronika Lukacs-Kornek, Percy Knolle, and Christian Kurts. 2010. 'Steady-state cross-presentation of OVA is mannose receptor-dependent but inhibitable by collagen fragments', *Proceedings of the National Academy of Sciences of the United States of America*, 107: E48-9; author reply E50.

Cafarotti, Arianna, Mattia Giovannini, Philippe Begìn, Helen A. Brough, and Stefania Arasi. 2023. 'Management of IgE-mediated food allergy in the 21st century', *Clinical & Experimental Allergy*, 53: 25-38.

Carballo, I., M. Alonso-Sampedro, E. Gonzalez-Conde, J. Sanchez-Castro, C. Vidal, F. Gude, and A. Gonzalez-Quintela. 2021. 'Factors Influencing Total Serum IgE in Adults: The Role of Obesity and Related Metabolic Disorders', *International Archives of Allergy and Immunology*, 182: 220-28.

Cardet, Juan Carlos, and Thomas B. Casale. 2019. 'New insights into the utility of omalizumab', *Journal of Allergy and Clinical Immunology*, 143: 923-26.e1.

Chan, A. C., E. Leeansyah, A. Cochrane, Y. d' Udekem d' Acoz, D. Mittag, L. C. Harrison, D. I. Godfrey, and S. P. Berzins. 2013. 'Ex-vivo analysis of human Natural Killer T cells demonstrates heterogeneity between tissues and within established CD4+ and CD4− subsets', *Clinical & Experimental Immunology*, 172: 129-37.

Chiang, Meng-Chieh, Kirsteen M. Tullett, Yoke Seng Lee, Adi Idris, Yitian Ding, Kylie J. McDonald, Andrew Kassianos, Ingrid M. Leal Rojas, Varinder Jeet, Mireille H. Lahoud, and Kristen J. Radford. 2016. 'Differential uptake and cross-presentation of soluble and necrotic cell antigen by human DC subsets', *European Journal of Immunology*, 46: 329-39.

Chinthrajah, S., S. Cao, C. Liu, S. C. Lyu, S. B. Sindher, A. Long, V. Sampath, D. Petroni, M. Londei, and K. C. Nadeau. 2019. 'Phase 2a randomized, placebo-controlled study of anti-IL-33 in peanut allergy', *JCI Insight*, 4.

Chow, Kevin V., Andrew M. Lew, Robyn M. Sutherland, and Yifan Zhan. 2016. 'Monocyte-Derived Dendritic Cells Promote Th Polarization, whereas Conventional Dendritic Cells Promote Th Proliferation', *The Journal of Immunology*, 196: 624-36.

Cooke, Robert A, and Albert Vander Veer Jr. 1916. 'Human sensitization', *The Journal of Immunology*, 1: 201-305.

Cordle, C. T. 2004. 'Soy protein allergy: incidence and relative severity', *J Nutr*, 134: 1213s-19s.

Cyster, J. G. 1999. 'Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs', *J Exp Med*, 189: 447-50.

D'Amato, G., L. Cecchi, S. Bonini, C. Nunes, I. Annesi-Maesano, H. Behrendt, G. Liccardi, T. Popov, and P. van Cauwenberge. 2007. 'Allergenic pollen and pollen allergy in Europe', *Allergy*, 62: 976-90.

Dearman, R. J., M. J. C. Alcocer, and I. Kimber. 2007. 'Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1', *Clinical and Experimental Allergy*, 37: 582-91.

Del Moral, Manuel Gómez, and Eduardo Martínez-Naves. 2017. 'The Role of Lipids in Development of Allergic Responses', *Immune network*, 17: 133-43.

Delespesse, Guy, Yusei Ohshima, Uno Shu, Liang-Peng Yang, Christian Demeure, Chang-You Wu, Dae-Gyoo Byun, and Marika Sarfati. 1997. 'Differentiation of naive human CD4 T cells into TH2/TH1 effectors', *Allergology International*, 46: 63-72.

Dhodapkar, M. V., and V. Kumar. 2017a. 'Type II NKT Cells and Their Emerging Role in Health and Disease', *J Immunol*, 198: 1015-21.

Dhodapkar, Madhav V., and Vipin Kumar. 2017b. 'Type II NKT Cells and Their Emerging Role in Health and Disease', *The Journal of Immunology*, 198: 1015-21.

Du Toit, George, Graham Roberts, Peter H. Sayre, Henry T. Bahnson, Suzana Radulovic, Alexandra F. Santos, Helen A. Brough, Deborah Phippard, Monica Basting, Mary Feeney, Victor Turcanu, Michelle L. Sever, Margarita Gomez Lorenzo, Marshall Plaut, and Gideon Lack. 2015. 'Randomized Trial of Peanut Consumption in Infants at Risk for Peanut Allergy', *New England Journal of Medicine*, 372: 803-13.

Dubiela, Pawel, Rebecca Del Conte, Francesca Cantini, Tomasz Borowski, Roberta Aina, Christian Radauer, Merima Bublin, Karin Hoffmann-Sommergruber, and Stefano Alessandri. 2019. 'Impact of lipid binding on the tertiary structure and allergenic potential of Jug r 3, the non-specific lipid transfer protein from walnut', *Scientific reports*, 9: 2007.

Eckhardt, E., and M. Bastian. 2021. 'Animal models for human group 1 CD1 protein function', *Mol Immunol*, 130: 159-63.

Emara, M., P. J. Royer, Z. Abbas, H. F. Sewell, G. G. Mohamed, S. Singh, S. Peel, J. Fox, F. Shakib, L. Martinez-Pomares, and A. M. Ghaemmaghami. 2011. 'Recognition of the major cat allergen Fel d 1 through the cysteine-rich domain of the mannose receptor determines its allergenicity', *J Biol Chem*, 286: 13033-40.

Emara, M., P. J. Royer, J. Mahdavi, F. Shakib, and A. M. Ghaemmaghami. 2012. 'Retagging identifies dendritic cell-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN) protein as a novel receptor for a major allergen from house dust mite', *J Biol Chem*, 287: 5756-63.

Exley, M. A., P. Dellabona, and G. Casorati. 2021. 'Exploiting CD1-restricted T cells for clinical benefit', *Mol Immunol*, 132: 126-31.

Fahy, Eoin, Shankar Subramaniam, Robert C. Murphy, Masahiro Nishijima, Christian R. H. Raetz, Takao Shimizu, Friedrich Spener, Gerrit van Meer, Michael J. O. Wakelam, and Edward A. Dennis. 2009. 'Update of the LIPID MAPS comprehensive classification system for lipids', *Journal of lipid research*, 50 Suppl: S9-S14.

Finkelman, F. D. 2007. 'Anaphylaxis: lessons from mouse models', *J Allergy Clin Immunol*, 120: 506-15; quiz 16-7.

Finkina, E. I., D. N. Melnikova, I. V. Bogdanov, N. S. Matveevskaya, A. A. Ignatova, I. Y. Toropygin, and T. V. Ovchinnikova. 2020a. 'Impact of Different Lipid Ligands

on the Stability and IgE-Binding Capacity of the Lentil Allergen Len c 3', *Biomolecules*, 10.

Finkina, Ekaterina I., Daria N. Melnikova, Ivan V. Bogdanov, Natalia S. Matveevskaya, Anastasia A. Ignatova, Ilia Y. Toropygin, and Tatiana V. Ovchinnikova. 2020b. 'Impact of Different Lipid Ligands on the Stability and IgE-Binding Capacity of the Lentil Allergen Len c 3', *Biomolecules*, 10: 1668.

Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. 'A simple method for the isolation and purification of total lipides from animal tissues', *J Biol Chem*, 226: 497-509.

Foo, A. C., P. M. Thompson, S. Arora, E. F. DeRose, L. Perera, and G. A. Mueller. 2019. 'Influence of Hydrophobic Cargo Binding on the Structure, Stability, and Allergenicity of the Cockroach Allergen Bla g 1', *Journal of Allergy and Clinical Immunology*, 143: AB213.

Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. 'Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein', *J Exp Med*, 198: 267-79.

Furman, D., B. P. Hejblum, N. Simon, V. Jojic, C. L. Dekker, R. Thiébaut, R. J. Tibshirani, and M. M. Davis. 2014. 'Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination', *Proc Natl Acad Sci U S A*, 111: 869-74.

Gadola, Stephan D., Nicolas Dulphy, Mariolina Salio, and Vincenzo Cerundolo. 2002. 'Vα24-JαQ-Independent, CD1d-Restricted Recognition of α-Galactosylceramide by Human CD4⁺ and CD8αβ⁺ T Lymphocytes', *The Journal of Immunology*, 168: 5514-20.

Galli, S. J., and M. Tsai. 2012. 'IgE and mast cells in allergic disease', *Nat Med*, 18: 693-704.

Galli, S. J., M. Tsai, and A. M. Piliponsky. 2008. 'The development of allergic inflammation', *Nature*, 454: 445-54.

Garrido-Arandia, M., L. Tordesillas, N. Cubells, V. Esteban, W. Barcik, L. O'Mahony, L. F. Pacios, and A. Diaz-Perales. 2018. 'The ligand of the major peach allergen Pru P 3 is presented to iNKT cells', *Allergy: European Journal of Allergy and Clinical Immunology*, 73: 781.

Gepp, B., D. Ackerbauer, N. Lengger, F. Gruber, M. Mildner, and H. Breiteneder. 2014. 'The major birch pollen allergen Bet v 1 binds lipids from birch and grass pollen but not from peanuts', *Allergy: European Journal of Allergy and Clinical Immunology*, 69: 464.

Gerlini, G., H. P. Hefti, M. Kleinhans, B. J. Nickoloff, G. Burg, and F. O. Nestle. 2001. 'Cd1d is expressed on dermal dendritic cells and monocyte-derived dendritic cells', *J Invest Dermatol*, 117: 576-82.

Ghaffari, Sasan, Monireh Torabi-Rahvar, Sajjad Aghayan, Zahra Jabbarpour, Kobra Moradzadeh, Azadeh Omidkhoda, and Naser Ahmadbeigi. 2021. 'Optimizing interleukin-2 concentration, seeding density and bead-to-cell ratio of T-cell expansion for adoptive immunotherapy', *BMC Immunology*, 22: 43.

Gilles-Stein, S., I. Beck, A. Chaker, M. Bas, M. McIntyre, L. Cifuentes, A. Petersen, J. Gutermuth, C. Schmidt-Weber, H. Behrendt, and C. Traidl-Hoffmann. 2016. 'Pollen derived low molecular compounds enhance the human allergen specific immune response in vivo', *Clinical and Experimental Allergy*.

Gilles, S., C. Akdis, R. Lauener, P. Schmid-Grendelmeier, T. Bieber, G. Schäppi, and C. Traidl-Hoffmann. 2018. 'The role of environmental factors in allergy: A critical reappraisal', *Exp Dermatol*, 27: 1193-200.

Gilles, S., I. Beck, A. Chaker, M. McIntyre, L. Cifuentes, H. Bier, A. Petersen, J. Ring, H. Behrendt, C. Schmidt-Weber, and C. Traidl-Hoffmann. 2014. 'Effects of pollen-derived non-protein substances on the allergic immune response in vivo: Roles of adenosine, PALMs and neuroreceptors', *Experimental Dermatology*, 23: e3.

Gilles, S., D. Jacoby, C. Blume, M. J. Mueller, T. Jakob, H. Behrendt, K. Schaekel, and C. Traidl-Hoffmann. 2010. 'Pollen-derived low-molecular weight factors inhibit 6-sulfo LacNAc + dendritic cells' capacity to induce T-helper type 1 responses', *Clinical and Experimental Allergy*, 40: 269-78.

Gilles, S., V. Mariani, M. Bryce, M. J. Mueller, J. Ring, T. Jakob, S. Pastore, H. Behrendt, and C. Traidl-Hoffmann. 2009. 'Pollen-derived E1-phytoprostanes signal via PPAR-gamma and NF-kappaB-dependent mechanisms', *J Immunol*, 182: 6653-8.

Gilroy, Derek W., and David Bishop-Bailey. 2019. 'Lipid mediators in immune regulation and resolution', *British Journal of Pharmacology*, 176: 1009-23.

Girardi, Enrico, and Dirk M. Zajonc. 2012. 'Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells', *Immunological reviews*, 250: 167-79.

Godfrey, D. I., K. J. Hammond, L. D. Poulton, M. J. Smyth, and A. G. Baxter. 2000. 'NKT cells: facts, functions and fallacies', *Immunol Today*, 21: 573-83.

González Roldán, N., R. Engel, S. Düpow, K. Jakob, F. Koops, Z. Orinska, C. Vigor, C. Oger, J. M. Galano, T. Durand, U. Jappe, and K. A. Duda. 2019. 'Lipid Mediators From Timothy Grass Pollen Contribute to the Effector Phase of Allergy and Prime Dendritic Cells for Glycolipid Presentation', *Front Immunol*, 10: 974.

Gough, L., E. Campbell, D. Bayley, G. Van Heeke, and F. Shakib. 2003. 'Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model', *Clin Exp Allergy*, 33: 1159-63.

Gough, L., O. Schulz, H. F. Sewell, and F. Shakib. 1999. 'The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response', *J Exp Med*, 190: 1897-902.

Gough, L., H. F. Sewell, and F. Shakib. 2001. 'The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen', *Clin Exp Allergy*, 31: 1594-8.

Grela, E. R., and K. D. Günter. 1995. 'Fatty acid composition and tocopherol content of some legume seeds', *Animal Feed Science and Technology*, 52: 325-31.

Gupta, R. S., E. E. Springston, M. R. Warrier, B. Smith, R. Kumar, J. Pongracic, and J. L. Holl. 2011. 'The prevalence, severity, and distribution of childhood food allergy in the United States', *Pediatrics*, 128: e9-17.

Gutermuth, J., M. Bewersdorff, C. Traidl-Hoffmann, J. Ring, M. J. Mueller, H. Behrendt, and T. Jakob. 2007. 'Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses in vivo', *Journal of Allergy and Clinical Immunology*, 120: 293-99.

Hamilton, R. G., and N. F. Adkinson, Jr. 2003. '23. Clinical laboratory assessment of IgE-dependent hypersensitivity', *J Allergy Clin Immunol*, 111: S687-701.

Hammad, Hamida, Bart N. Lambrecht, Pierre Pochard, Philippe Gosset, Philippe Marquillies, André-Bernard Tonnel, and Joël Pestel. 2002. 'Monocyte-Derived Dendritic Cells Induce a House Dust Mite-Specific Th2 Allergic Inflammation in the Lung of Humanized SCID Mice: Involvement of CCR7', *The Journal of Immunology*, 169: 1524-34.

Hammond, Kirsten J. L., Daniel G. Pellicci, Lynn D. Poulton, Olga V. Naidenko, Anthony A. Scalzo, Alan G. Baxter, and Dale I. Godfrey. 2001. 'CD1d-Restricted NKT Cells: An Interstrain Comparison', *The Journal of Immunology*, 167: 1164-73.

Haniffa, M., A. Shin, V. Bigley, N. McGovern, P. Teo, P. See, P. S. Wasan, X. N. Wang, F. Malinarich, B. Malleret, A. Larbi, P. Tan, H. Zhao, M. Poidinger, S. Pagan, S. Cookson, R. Dickinson, I. Dimmick, R. F. Jarrett, L. Renia, J. Tam, C. Song, J. Connolly, J. K. Chan, A. Gehring, A. Bertoletti, M. Collin, and F. Ginhoux. 2012. 'Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells', *Immunity*, 37: 60-73.

Hao, Siguo, Ou Bai, Fang Li, Jinying Yuan, Suzanne Laferte, and Jim Xiang. 2007. 'Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity', *Immunology*, 120: 90-102.

He, S. H., H. Y. Zhang, X. N. Zeng, D. Chen, and P. C. Yang. 2013. 'Mast cells and basophils are essential for allergies: mechanisms of allergic inflammation and a proposed procedure for diagnosis', *Acta Pharmacol Sin*, 34: 1270-83.

Hochwallner, Heidrun, Ulrike Schulmeister, Ines Swoboda, Margit Focke-Tejkl, Vera Civaj, Nadja Balic, Mats Nystrand, Annika Härlin, Josef Thalhamer, Sandra Scheiblhofer, Walter Keller, Tea Pavkov, Domen Zafred, Bodo Niggemann, Santiago Quirce, Adriano Mari, Gabrielle Pauli, Christof Ebner, Nikolaos G. Papadopoulos, Nikolaos G. Papadopoulos, Udo Herz, Eric A. F. van Tol, Rudolf Valenta, and Susanne Spitzauer. 2010. 'Visualization of clustered IgE epitopes on alpha-lactalbumin', *J Allergy Clin Immunol*, 125: 1279-85.e9.

Hole, Camaron R., Chrissy M. Leopold Wager, Natalia Castro-Lopez, Althea Campuzano, Hong Cai, Karen L. Wozniak, Yufeng Wang, and Floyd L. Wormley. 2019. 'Induction of memory-like dendritic cell responses in vivo', *Nature Communications*, 10: 2955.

Holgate, Stephen T., and Riccardo Polosa. 2008. 'Treatment strategies for allergy and asthma', *Nature Reviews Immunology*, 8: 218-30.

Holmes, L.J., D. Ryan, G.A. Tavernier, and R.M. Niven. 2016. 'A clinically observed variability in serum total IgE in patients being considered for omalizumab therapy', *European Respiratory Journal*, 48: PA4101.

Hopkins, Georgina V., Stella Cochrane, David Onion, and Lucy C. Fairclough. 2022. 'The Role of Lipids in Allergic Sensitization: A Systematic Review', *Frontiers in Molecular Biosciences*, 9.

Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. 'Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages', *Science*, 260: 547-9.

Huang, M. D., and A. H. Huang. 2015. 'Bioinformatics Reveal Five Lineages of Oleosins and the Mechanism of Lineage Evolution Related to Structure/Function from Green Algae to Seed Plants', *Plant Physiol*, 169: 453-70.

Hufnagl, K., D. Ghosh, S. Wagner, A. Fiocchi, L. Dahdah, R. Bianchini, N. Braun, R. Steinborn, M. Hofer, M. Blaschitz, G. A. Roth, G. Hofstetter, F. Roth-Walter, L. F. Pacios, and E. Jensen-Jarolim. 2018. 'Retinoic acid prevents immunogenicity of milk lipocalin Bos d 5 through binding to its immunodominant T-cell epitope', *Scientific reports*, 8: 12.

Humeniuk, P., P. Dubiela, and K. Hoffmann-Sommergruber. 2017. 'Dendritic Cells and Their Role in Allergy: Uptake, Proteolytic Processing and Presentation of Allergens', *Int J Mol Sci*, 18.

Humeniuk, P., S. Geiselhart, C. Battin, T. Webb, P. Steinberger, W. Paster, and K. Hoffmann-Sommergruber. 2019. 'A jurkat based NFkappaB-EGFP INKT reporter cell line to evaluate the interaction of food-derived lipids with INKT cell receptors', *Allergy: European Journal of Allergy and Clinical Immunology*, 74: 602.

Hurlburt, Barry K., Lesa R. Offermann, Jane K. McBride, Karolina A. Majorek, Soheila J. Maleki, and Maksymilian Chruszcz. 2013. 'Structure and Function of the Peanut Panallergen Ara h 8 *', *Journal of Biological Chemistry*, 288: 36890-901.

Iweala, O. I., and A. W. Burks. 2016. 'Food Allergy: Our Evolving Understanding of Its Pathogenesis, Prevention, and Treatment', *Curr Allergy Asthma Rep*, 16: 37.

Iweala, O. I., P. B. Savage, and S. P. Commins. 2018. 'A Role for CD1d-restricted invariant natural killer T cells and glycolipids in alpha-gal allergy', *Journal of Allergy and Clinical Immunology*, 141: AB288.

Jappe, Uta, Christian Schwager, Andra B. Schromm, Nestor González Roldán, Karina Stein, Holger Heine, and Katarzyna A. Duda. 2019a. 'Lipophilic Allergens, Different Modes of Allergen-Lipid Interaction and Their Impact on Asthma and Allergy', *Frontiers in Immunology*, 10.

———. 2019b. 'Lipophilic Allergens, Different Modes of Allergen-Lipid Interaction and Their Impact on Asthma and Allergy', *Frontiers in Immunology*, 10.

Johannessen, B. R., L. K. Skov, J. S. Kastrup, O. Kristensen, C. Bolwig, J. N. Larsen, M. Spangfort, K. Lund, and M. Gajhede. 2005. 'Structure of the house dust mite allergen Der f 2: implications for function and molecular basis of IgE cross-reactivity', *FEBS Lett*, 579: 1208-12.

Jyonouchi, S. 2010. 'Inkt Cell Proliferation To Food-derived Sphingolipids In Food Allergic Vs Non-food Allergic Children', *Journal of Allergy and Clinical Immunology*, 125: AB224.

Jyonouchi, S., V. Abraham, J. S. Orange, J. M. Spergel, L. Gober, E. Dudek, R. Saltzman, K. E. Nichols, and A. Cianferoni. 2011. 'Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin', *J Allergy Clin Immunol*, 128: 102-09.e13.

Kabesch, Michael, and Jörg Tost. 2020. 'Recent findings in the genetics and epigenetics of asthma and allergy', *Seminars in Immunopathology*, 42.

Karp, C. L. 2010. 'Guilt by intimate association: what makes an allergen an allergen?', *J Allergy Clin Immunol*, 125: 955-60; quiz 61-2.

Kaufmann, Stefan H. E. 2019. 'Immunology's Coming of Age', *Frontiers in Immunology*, 10.

Keet, Corinne, Mihaela Plesa, Daria Szelag, Wayne Shreffler, Robert Wood, Joan Dunlop, Roger Peng, Jennifer Dantzer, Robert G. Hamilton, Alkis Togias, and Michael Pistiner. 2021. 'Ara h 2–specific IgE is superior to whole peanut extract–based serology or skin prick test for diagnosis of peanut allergy in infancy', *Journal of Allergy and Clinical Immunology*, 147: 977-83.e2.

Koch, M., V. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Besra, R. R. Schmidt, E. Y. Jones, and V. Cerundolo. 2005. 'The crystal structure of human CD1d with and without alpha-galactosylceramide', *Nat Immunol*, 6: 819-26.

Krijgsman, Daniëlle, Natasja L. de Vries, Anni Skovbo, Morten N. Andersen, Marloes Swets, Esther Bastiaannet, Alexander L. Vahrmeijer, Cornelis J. H. van de Velde, Mirjam H. M. Heemskerk, Marianne Hokland, and Peter J. K. Kuppen. 2019. 'Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile', *Cancer Immunology, Immunotherapy*, 68: 1011-24.

Krijgsman, Daniëlle, Marianne Hokland, and Peter J. K. Kuppen. 2018. 'The Role of Natural Killer T Cells in Cancer—A Phenotypical and Functional Approach', *Frontiers in Immunology*, 9.

Krovi, S. Harsha, and Laurent Gapin. 2018. 'Invariant Natural Killer T Cell Subsets—More Than Just Developmental Intermediates', *Frontiers in Immunology*, 9.

Kulis, Michael D., Johanna M. Smeekens, Robert M. Immormino, and Timothy P. Moran. 2021. 'The airway as a route of sensitization to peanut: An update to the dual allergen exposure hypothesis', *Journal of Allergy and Clinical Immunology*, 148: 689-93.

Lack, Gideon, Deborah Fox, Kate Northstone, and Jean Golding. 2003. 'Factors Associated with the Development of Peanut Allergy in Childhood', *New England Journal of Medicine*, 348: 977-85.

Lacombe, Shannon. 2017. 'British Society of Immunology: Allergy Policy Briefing'. https://www.immunology.org/policy-and-public-affairs/briefings-and-position-statements/allergy.

Lamiable, Olivier, Johannes U Mayer, Luis Munoz-Erazo, and Franca Ronchese. 2020. 'Dendritic cells in Th2 immune responses and allergic sensitization', *Immunology & Cell Biology*, 98: 807-18.

Lan, Feng, Nan Zhang, Claus Bachert, and Luo Zhang. 2020. 'Stability of regulatory T cells in T helper 2–biased allergic airway diseases', *Allergy*, 75: 1918-26.

Lee, Jee-Boong. 2016. 'Regulation of IgE-Mediated Food Allergy by IL-9 Producing Mucosal Mast Cells and Type 2 Innate Lymphoid Cells', *Immune network*, 16: 211-18.

Lee, Sooyoung. 2017. 'IgE-mediated food allergies in children: prevalence, triggers, and management', *Korean journal of pediatrics*, 60: 99-105.

Leffler, Jonatan, Philip A. Stumbles, and Deborah H. Strickland. 2018. 'Immunological Processes Driving IgE Sensitisation and Disease Development in Males and Females', *Int J Mol Sci*, 19: 1554.

León, B., M. López-Bravo, and C. Ardavín. 2007. 'Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania', *Immunity*, 26: 519-31.

Leung, A. S. Y., G. W. K. Wong, and M. L. K. Tang. 2018. 'Food allergy in the developing world', *J Allergy Clin Immunol*, 141: 76-78.e1.

Lewkowich , Ian P., Nancy S. Herman , Kathleen W. Schleifer , Matthew P. Dance , Brian L. Chen , Krista M. Dienger , Alyssa A. Sproles , Jaimin S. Shah , Jörg Köhl , Yasmine Belkaid , and Marsha Wills-Karp 2005. 'CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function', *Journal of Experimental Medicine*, 202: 1549-61.

Li, H., A. Nowak-Wegrzyn, Z. Charlop-Powers, W. Shreffler, M. Chehade, S. Thomas, G. Roda, S. Dahan, K. Sperber, and M. C. Berin. 2006. 'Transcytosis of IgE-antigen complexes by CD23a in human intestinal epithelial cells and its role in food allergy', *Gastroenterology*, 131: 47-58.

Li, Y. R., Y. Zhou, M. Wilson, A. Kramer, R. Hon, Y. Zhu, Y. Fang, and L. Yang. 2022. 'Tumor-Localized Administration of α-GalCer to Recruit Invariant Natural Killer T Cells and Enhance Their Antitumor Activity against Solid Tumors', *Int J Mol Sci*, 23.

Lieberman, Jay A., Ruchi S Gupta, Rebecca C. Knibb, Tmirah Haselkorn, Stephen Tilles, Douglas P. Mack, and Guillaume Pouessel. 2021. 'The global burden of illness of peanut allergy: A comprehensive literature review', *Allergy*, 76: 1367-84.

Liu, Guirong, Manman Liu, Junjuan Wang, Yao Mou, and Huilian Che. 2021. 'The Role of Regulatory T Cells in Epicutaneous Immunotherapy for Food Allergy', *Frontiers in Immunology*, 12.

Liu, T. Y., Y. Uemura, M. Suzuki, Y. Narita, S. Hirata, H. Ohyama, O. Ishihara, and S. Matsushita. 2008. 'Distinct subsets of human invariant NKT cells differentially regulate T helper responses via dendritic cells', *Eur J Immunol*, 38: 1012-23.

Loh, Wenyin, and Mimi L. K. Tang. 2018. 'The Epidemiology of Food Allergy in the Global Context', *International journal of environmental research and public health*, 15: 2043.

Looney, T. J., J. Y. Lee, K. M. Roskin, R. A. Hoh, J. King, J. Glanville, Y. Liu, T. D. Pham, C. L. Dekker, M. M. Davis, and S. D. Boyd. 2016. 'Human B-cell isotype switching origins of IgE', *J Allergy Clin Immunol*, 137: 579-86.e7.

Lucey, J. A., D. Otter, and D. S. Horne. 2017. 'A 100-Year Review: Progress on the chemistry of milk and its components', *J Dairy Sci*, 100: 9916-32.

Luttgeharm, K. D., A. N. Kimberlin, R. E. Cahoon, R. L. Cerny, J. A. Napier, J. E. Markham, and E. B. Cahoon. 2015. 'Sphingolipid metabolism is strikingly different between pollen and leaf in Arabidopsis as revealed by compositional and gene expression profiling', *Phytochemistry*, 115: 121-9.

Macho-Fernandez, Elodie, and Manfred Brigl. 2015. 'The Extended Family of CD1d-Restricted NKT Cells: Sifting through a Mixed Bag of TCRs, Antigens, and Functions', *Frontiers in Immunology*, 6.

Mak, Tak W., Mary E. Saunders, and Bradley D. Jett. 2014a. 'Chapter 11 - NK, γδ T and NKT Cells.' in Tak W. Mak, Mary E. Saunders and Bradley D. Jett (eds.), *Primer to the Immune Response (Second Edition)* (Academic Cell: Boston).

———. 2014b. 'Chapter 18 - Immune Hypersensitivity.' in Tak W. Mak, Mary E. Saunders and Bradley D. Jett (eds.), *Primer to the Immune Response (Second Edition)* (Academic Cell: Boston).

Mallevaey, Thierry, Andrew J. Clarke, James P. Scott-Browne, Mary H. Young, Laila C. Roisman, Daniel G. Pellicci, Onisha Patel, Julian P. Vivian, Jennifer L. Matsuda, James McCluskey, Dale I. Godfrey, Philippa Marrack, Jamie Rossjohn, and Laurent Gapin. 2011. 'A molecular basis for NKT cell recognition of CD1d-self-antigen', *Immunity*, 34: 315-26.

Manti, S., G. Pecora, F. Patanè, A. Giallongo, G. F. Parisi, M. Papale, A. Licari, G. L. Marseglia, and S. Leonardi. 2021. 'Monoclonal Antibodies in Treating Food Allergy: A New Therapeutic Horizon', *Nutrients*, 13.

McKinnon, K. M. 2018. 'Flow Cytometry: An Overview', *Curr Protoc Immunol*, 120: 5.1.1-5.1.11.

Medic, Jelena, Christine Atkinson, and Charles R. Hurburgh Jr. 2014. 'Current Knowledge in Soybean Composition', *Journal of the American Oil Chemists' Society*, 91: 363-84.

Méndez-Enríquez, Erika, Maya Salomonsson, Jens Eriksson, Christer Janson, Andrei Malinovschi, Mikael E. Sellin, and Jenny Hallgren. 2022. 'IgE cross-linking induces activation of human and mouse mast cell progenitors', *Journal of Allergy and Clinical Immunology*, 149: 1458-63.

Meng, X., Z. Zeng, J. Gao, P. Tong, Y. Wu, X. Li, and H. Chen. 2020. 'Conformational changes in bovine α-lactalbumin and β-lactoglobulin evoked by interaction with C18 unsaturated fatty acids provide insights into increased allergic potential', *Food Funct*, 11: 9240-51.

Menzella, F., M. Lusuardi, C. Galeone, S. Taddei, and L. Zucchi. 2015. 'Profile of anti-IL-5 mAb mepolizumab in the treatment of severe refractory asthma and hypereosinophilic diseases', *J Asthma Allergy*, 8: 105-14.

Mestas, J., and C. C. Hughes. 2004. 'Of mice and not men: differences between mouse and human immunology', *J Immunol*, 172: 2731-8.

Metcalfe, D. D., J. D. Astwood, R. Townsend, H. A. Sampson, S. L. Taylor, and R. L. Fuchs. 1996. 'Assessment of the allergenic potential of foods derived from genetically engineered crop plants', *Crit Rev Food Sci Nutr*, 36 Suppl: S165-86.

Miles, E. A., and P. C. Calder. 2017. 'Can Early Omega-3 Fatty Acid Exposure Reduce Risk of Childhood Allergic Disease?', *Nutrients*, 9.

Mirotti, L., E. Florsheim, L. Rundqvist, G. Larsson, F. Spinozzi, M. Leite-De-Moraes, M. Russo, and M. Alcocer. 2013a. 'Lipids are required for the development of Brazil nut allergy: The role of mouse and human iNKT cells', *Allergy: European Journal of Allergy and Clinical Immunology*, 68: 74-83.

———. 2013b. 'Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells', *Allergy*, 68: 74-83.

Mitra-Kaushik, Shibani, Anita Mehta-Damani, Jennifer J. Stewart, Cherie Green, Virginia Litwin, and Christèle Gonneau. 2021. 'The Evolution of Single-Cell Analysis and Utility in Drug Development', *The AAPS Journal*, 23: 98.

Miyamoto, Katsuichi, Sachiko Miyake, and Takashi Yamamura. 2001. 'A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells', *Nature*, 413: 531-34.

Montoya, C. J., D. Pollard, J. Martinson, K. Kumari, C. Wasserfall, C. B. Mulder, M. T. Rugeles, M. A. Atkinson, A. L. Landay, and S. B. Wilson. 2007. 'Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11', *Immunology*, 122: 1-14.

Moser, M., and K. M. Murphy. 2000. 'Dendritic cell regulation of TH1-TH2 development', *Nature Immunology*, 1: 199-205.

Mueller, G. A., L. L. Edwards, J. J. Aloor, M. B. Fessler, J. Glesner, A. Pomes, M. D. Chapman, R. E. London, and L. C. Pedersen. 2010. 'The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins', *J Allergy Clin Immunol*, 125: 909-17 e4.

Mueller, G. A., R. A. Gosavi, J. M. Krahn, L. L. Edwards, M. J. Cuneo, J. Glesner, A. Pomes, M. D. Chapman, R. E. London, and L. C. Pedersen. 2010. 'Der p 5 crystal structure provides insight into the group 5 dust mite allergens', *J Biol Chem*, 285: 25394-401.

Mullins, E., J. L. Bresson, T. Dalmay, I. C. Dewhurst, M. M. Epstein, L. George Firbank, P. Guerche, J. Hejatko, H. Naegeli, F. Nogué, N. Rostoks, J. J. Sánchez Serrano, G. Savoini, E. Veromann, F. Veronesi, A. Fernandez Dumont, and F. J. Moreno. 2022. 'Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology', *Efsa j*, 20: e07044.

Nakano, H., K. L. Lin, M. Yanagita, C. Charbonneau, D. N. Cook, T. Kakiuchi, and M. D. Gunn. 2009. 'Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses', *Nat Immunol*, 10: 394-402.

Nicolaou, Nicolaos, Maryam Poorafshar, Clare Murray, Angela Simpson, Henric Winell, Gina Kerry, Annika Härlin, Ashley Woodcock, Staffan Ahlstedt, and Adnan Custovic. 2010. 'Allergy or tolerance in children sensitized to peanut: Prevalence and differentiation using component-resolved diagnostics', *Journal of Allergy and Clinical Immunology*, 125: 191-97.e13.

Nielsen, M. C., M. N. Andersen, and H. J. Møller. 2020. 'Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages in vitro', *Immunology*, 159: 63-74.

Niewold, Paula, Thomas Myles Ashhurst, Adrian Lloyd Smith, and Nicholas Jonathan Cole King. 2020. 'Evaluating spectral cytometry for immune profiling in viral disease', *Cytometry Part A*, 97: 1165-79.

Noureddine, N., M. Chalubinski, and P. Wawrzyniak. 2022. 'The Role of Defective Epithelial Barriers in Allergic Lung Disease and Asthma Development', *J Asthma Allergy*, 15: 487-504.

Noval Rivas, Magali, and Talal A. Chatila. 2016. 'Regulatory T cells in allergic diseases', *Journal of Allergy and Clinical Immunology*, 138: 639-52.

O'Reilly, Vincent, Shijuan G. Zeng, Gabriel Bricard, Ann Atzberger, Andrew E. Hogan, John Jackson, Conleth Feighery, Steven A. Porcelli, and Derek G. Doherty. 2011. 'Distinct and overlapping effector functions of expanded human CD4+, CD8α+ and CD4-CD8α- invariant natural killer T cells', *PloS one*, 6: e28648-e48.

Ober, C., and T. C. Yao. 2011. 'The genetics of asthma and allergic disease: a 21st century perspective', *Immunological reviews*, 242: 10-30.

Oeder, S., F. Alessandrini, O. F. Wirz, A. Braun, M. Wimmer, U. Frank, M. Hauser, J. Durner, F. Ferreira, D. Ernst, M. Mempel, S. Gilles, J. T. M. Buters, H. Behrendt, C. Traidl-Hoffmann, C. Schmidt-Weber, M. Akdis, and J. Gutermuth. 2015. 'Pollen-derived nonallergenic substances enhance Th2-induced IgE production in B cells', *Allergy*, 70: 1450-60.

Okada, H., C. Kuhn, H. Feillet, and J.-F. Bach. 2010. 'The 'hygiene hypothesis' for autoimmune and allergic diseases: an update', *Clinical & Experimental Immunology*, 160: 1-9.

Olszak, T., D. An, S. Zeissig, M. P. Vera, J. Richter, A. Franke, J. N. Glickman, R. Siebert, R. M. Baron, D. L. Kasper, and R. S. Blumberg. 2012. 'Microbial exposure during early life has persistent effects on natural killer T cell function', *Science*, 336: 489-93.

Osborne, N. J., J. J. Koplin, P. E. Martin, L. C. Gurrin, A. J. Lowe, M. C. Matheson, A. L. Ponsonby, M. Wake, M. L. Tang, S. C. Dharmage, and K. J. Allen. 2011. 'Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants', *J Allergy Clin Immunol*, 127: 668-76.e1-2.

Pablos-Tanarro, A., D. Lozano-Ojalvo, M. Martinez-Blanco, E. Molina, and R. Lopez-Fandino. 2018. 'Egg Yolk Provides Th2 Adjuvant Stimuli and Promotes Sensitization to Egg White Allergens in BALB/c Mice', *Molecular nutrition & food research*, 62: 11.

Pajno, G. B., M. Fernandez-Rivas, S. Arasi, G. Roberts, C. A. Akdis, M. Alvaro-Lozano, K. Beyer, C. Bindslev-Jensen, W. Burks, M. Ebisawa, P. Eigenmann, E. Knol, K. C. Nadeau, L. K. Poulsen, R. van Ree, A. F. Santos, G. du Toit, S. Dhami, U. Nurmatov, Y. Boloh, M. Makela, L. O'Mahony, N. Papadopoulos, C. Sackesen, I. Agache, E. Angier, S. Halken, M. Jutel, S. Lau, O. Pfaar, D. Ryan, G. Sturm, E.-M. Varga, R. G. van Wijk, A. Sheikh, A. Muraro, and EAACI Allergen Immunotherapy Guidelines Group. 2018. 'EAACI Guidelines on allergen immunotherapy: IgE-mediated food allergy', *Allergy*, 73: 799-815.

Pali-Schöll, I., E. Untersmayr, M. Klems, and E. Jensen-Jarolim. 2018. 'The Effect of Digestion and Digestibility on Allergenicity of Food', *Nutrients*, 10.

Palladino, C., B. Gepp, A. Angelina, S. Sirvent, C. Radauer, N. Lengger, T. Eiwegger, and O. Palomares. 2016. 'The interplay of Ara h 1 and peanut lipids in the allergic sensitization process', *Allergy: European Journal of Allergy and Clinical Immunology*, 71: 624-25.

Palladino, C., M. S. Narzt, M. Bublin, M. Schreiner, P. Humeniuk, M. Gschwandtner, C. Hafner, W. Hemmer, K. Hoffmann-Sommergruber, M. Mildner, O. Palomares, F. Gruber, and H. Breiteneder. 2018. 'Peanut lipids display potential adjuvanticity by triggering a pro-inflammatory response in human keratinocytes', *Allergy*, 73: 1746-49.

Palladino, Chiara, and Heimo Breiteneder. 2018. 'Peanut allergens', *Molecular Immunology*, 100: 58-70.

Park, J. Y., J. Kwon, E. Y. Kim, J. Fink, H. K. Kim, and J. H. Park. 2019. 'CD24(+) Cell Depletion Permits Effective Enrichment of Thymic iNKT Cells While Preserving Their Subset Composition', *Immune network*, 19: e14.

Perez Rodriguez, L., M. Martinez Blanco, E. Molina, R. Lopez Fandino, and D. Lozano-Ojalvo. 2019. 'Egg yolk acts as adjuvant activating innate immune responses to egg white allergens in BALB/C MICE', *Allergy: European Journal of Allergy and Clinical Immunology*, 74: 109.

Peters, Rachel L., Jennifer J. Koplin, Lyle C. Gurrin, Shyamali C. Dharmage, Melissa Wake, Anne-Louise Ponsonby, Mimi L. K. Tang, Adrian J. Lowe, Melanie Matheson, Terence Dwyer, and Katrina J. Allen. 2017. 'The prevalence of food allergy and other allergic diseases in early childhood in a population-based study: HealthNuts age 4-year follow-up', *Journal of Allergy and Clinical Immunology*, 140: 145-53.e8.

Petersen, A., S. Rennert, S. Kull, W. M. Becker, H. Notbohm, T. Goldmann, and U. Jappe. 2014a. 'Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8', *Biological Chemistry*, 395: 239-50.

———. 2014b. 'Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8', *Biol Chem*, 395: 239-50.

Pettersson, M. E., G. H. Koppelman, B. M. J. Flokstra-de Blok, B. J. Kollen, and A. E. J. Dubois. 2018. 'Prediction of the severity of allergic reactions to foods', *Allergy*, 73: 1532-40.

Pfefferle, Petra I., Corinna U. Keber, Robert M. Cohen, and Holger Garn. 2021. 'The Hygiene Hypothesis – Learning From but Not Living in the Past', *Frontiers in Immunology*, 12.

Pichavant, Muriel, Anne-Sophie Charbonnier, Solenne Taront, Anne Brichet, Benoît Wallaert, Joel Pestel, André-Bernard Tonnel, and Philippe Gosset. 2005. 'Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment', *Journal of Allergy and Clinical Immunology*, 115: 771-78.

Plantinga, M., M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, H. Hammad, and B. N. Lambrecht. 2013a. 'Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen', *Immunity*, 38: 322-35.

Plantinga, Maud, Martin Guilliams, Manon Vanheerswynghels, Kim Deswarte, Filipe Branco-Madeira, Wendy Toussaint, Leen Vanhoutte, Katrijn Neyt, Nigel Killeen, Bernard Malissen, Hamida Hammad, and Bart N Lambrecht. 2013b. 'Conventional and Monocyte-Derived CD11b⁺ Dendritic Cells

Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen', *Immunity*, 38: 322-35.

Platts-Mills, T. A., and J. A. Woodfolk. 2011. 'Allergens and their role in the allergic immune response', *Immunol Rev*, 242: 51-68.

Pomés, Anna, Geoffrey A. Mueller, and Maksymilian Chruszcz. 2020. 'Structural Aspects of the Allergen-Antibody Interaction', *Frontiers in Immunology*, 11.

Pompura, Saige L., Allon Wagner, Alexandra Kitz, Jacob LaPerche, Nir Yosef, Margarita Dominguez-Villar, and David A. Hafler. 2021. 'Oleic acid restores suppressive defects in tissue-resident FOXP3 Tregs from patients with multiple sclerosis', *The Journal of Clinical Investigation*, 131.

Portelli, M. A., E. Hodge, and I. Sayers. 2015. 'Genetic risk factors for the development of allergic disease identified by genome-wide association', *Clin Exp Allergy*, 45: 21-31.

Posch, W., C. Lass-Flörl, and D. Wilflingseder. 2016. 'Generation of Human Monocyte-derived Dendritic Cells from Whole Blood', *J Vis Exp*.

Prescott, S. L., R. Pawankar, K. J. Allen, D. E. Campbell, JKh Sinn, A. Fiocchi, M. Ebisawa, H. A. Sampson, K. Beyer, and B. W. Lee. 2013. 'A global survey of changing patterns of food allergy burden in children', *World Allergy Organ J*, 6: 21.

Radzikowska, Urszula, Arturo O. Rinaldi, Zeynep Çelebi Sözener, Dilara Karaguzel, Marzena Wojcik, Katarzyna Cypryk, Mübeccel Akdis, Cezmi A. Akdis, and Milena Sokolowska. 2019. 'The Influence of Dietary Fatty Acids on Immune Responses', *Nutrients*, 11: 2990.

Rajan, T. V. 2003. 'The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation', *Trends Immunol*, 24: 376-9.

Rampuria, Pragya, and Mark L. Lang. 2018. 'Chapter 5 - Regulation of Humoral Immunity by CD1d-Restricted Natural Killer T Cells.' in M. A. Hayat (ed.), *Immunology* (Academic Press).

Reynolds, C., J. Barkans, P. Clark, H. Kariyawasam, D. Altmann, B. Kay, and R. Boyton. 2009. 'Natural killer T cells in bronchial biopsies from human allergen challenge model of allergic asthma', *J Allergy Clin Immunol*, 124: 860-2; author reply 62.

Rial, M. J., B. Barroso, and J. Sastre. 2019. 'Dupilumab for treatment of food allergy', *J Allergy Clin Immunol Pract*, 7: 673-74.

Rigby, N. M., A. I. Sancho, L. J. Salt, R. Foxall, S. Taylor, A. Raczynski, S. A. Cochrane, R. W. Crevel, and E. N. Mills. 2011. 'Quantification and partial characterization of the residual protein in fully and partially refined commercial soybean oils', *J Agric Food Chem*, 59: 1752-9.

Ring, J, and M Möhrenschlager. 2007. 'Allergy to peanut oil – clinically relevant?', *Journal of the European Academy of Dermatology and Venereology*, 21: 452-55.

Ring, Sabine, Stephan C. Schäfer, Karsten Mahnke, Hans-Anton Lehr, and Alexander H. Enk. 2006. 'CD4+CD25+ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue', *European Journal of Immunology*, 36: 2981-92.

Romagnani, S. 2006. 'Immunological tolerance and autoimmunity', *Intern Emerg Med*, 1: 187-96.

Ronger-Savle, S., J. Valladeau, A. Claudy, D. Schmitt, J. Peguet-Navarro, C. Dezutter-Dambuyant, L. Thomas, and D. Jullien. 2005. 'TGFβ Inhibits CD1d Expression on Dendritic Cells', *Journal of Investigative Dermatology*, 124: 116-18.

Roper, R L, D H Conrad, D M Brown, G L Warner, and R P Phipps. 1990. 'Prostaglandin E2 promotes IL-4-induced IgE and IgG1 synthesis', *The Journal of Immunology*, 145: 2644-51.

Ros, E., and J. Mataix. 2006a. 'Fatty acid composition of nuts--implications for cardiovascular health', *Br J Nutr*, 96 Suppl 2: S29-35.

Ros, Emilio, and José Mataix. 2006b. 'Fatty acid composition of nuts – implications for cardiovascular health', *British Journal of Nutrition*, 96: S29-S35.

Rosser, Elizabeth C, and Claudia Mauri. 2015. 'Regulatory B Cells: Origin, Phenotype, and Function', *Immunity*, 42: 607-12.

Roulston, T. H., and J. H. Cane. 2000. 'Pollen nutritional content and digestibility for animals', *Plant Systematics and Evolution*, 222: 187-209.

Royer, P. J., M. Emara, C. Yang, A. Al-Ghouleh, P. Tighe, N. Jones, H. F. Sewell, F. Shakib, L. Martinez-Pomares, and A. M. Ghaemmaghami. 2010. 'The mannose receptor mediates the uptake of diverse native allergens by dendritic cells and determines allergen-induced T cell polarization through modulation of IDO activity', *J Immunol*, 185: 1522-31.

Ruiter, Bert, and Wayne G. Shreffler. 2012. 'The role of dendritic cells in food allergy', *Journal of Allergy and Clinical Immunology*, 129: 921-28.

Ruysschaert, Jean-Marie, and Caroline Lonez. 2015. 'Role of lipid microdomains in TLR-mediated signalling', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1848: 1860-67.

Sadoway, Tara, Justin Buck, Anne Marie Salapatek, and Piyush Patel. 2015. 'Age and Sex Are Important Considerations for Studies Involving Reactivity to Allergenic Stimuli', *Journal of Allergy and Clinical Immunology*, 135: AB134.

Sag, Duygu, Müge Özkan, Mitchell Kronenberg, and Gerhard Wingender. 2017. 'Improved Detection of Cytokines Produced by Invariant NKT Cells', *Scientific Reports*, 7: 16607.

Salazar, F., and A. M. Ghaemmaghami. 2013a. 'Allergen recognition by innate immune cells: critical role of dendritic and epithelial cells', *Front Immunol*, 4: 356.

Salazar, Fabian, and Amir Ghaemmaghami. 2013b. 'Allergen Recognition by Innate Immune Cells: Critical Role of Dendritic and Epithelial Cells', *Frontiers in Immunology*, 4.

Salio, Mariolina, Jonathan D. Silk, E. Yvonne Jones, and Vincenzo Cerundolo. 2014. 'Biology of CD1- and MR1-Restricted T Cells', *Annual Review of Immunology*, 32: 323-66.

Salo, Päivi M., Samuel J. Arbes, Jr., Renee Jaramillo, Agustin Calatroni, Charles H. Weir, Michelle L. Sever, Jane A. Hoppin, Kathryn M. Rose, Andrew H. Liu, Peter J. Gergen, Herman E. Mitchell, and Darryl C. Zeldin. 2014. 'Prevalence of allergic

sensitization in the United States: Results from the National Health and Nutrition Examination Survey (NHANES) 2005-2006', *Journal of Allergy and Clinical Immunology*, 134: 350-59.

Salvilla, S. A., A. E. Dubois, B. M. Flokstra-de Blok, S. S. Panesar, A. Worth, S. Patel, A. Muraro, S. Halken, K. Hoffmann-Sommergruber, A. DunnGalvin, J. O. Hourihane, L. Regent, N. W. de Jong, G. Roberts, and A. Sheikh. 2014. 'Disease-specific health-related quality of life instruments for IgE-mediated food allergy', *Allergy*, 69: 834-44.

Satitsuksanoa, P., M. Kennedy, D. Gilis, M. Le Mignon, N. Suratannon, W. T. Soh, J. Wongpiyabovorn, P. Chatchatee, M. Vangveravong, T. Rerkpattanapipat, A. Sangasapaviliya, S. Piboonpocanun, E. Nony, K. Ruxrungtham, A. Jacquet, and the Mite Allergy Research Cohort study team. 2016. 'The minor house dust mite allergen Der p 13 is a fatty acid-binding protein and an activator of a TLR2-mediated innate immune response', *Allergy*, 71: 1425-34.

Satitsuksanoa, Pattraporn, Monique Daanje, Mübeccel Akdis, Scott D. Boyd, and Willem van de Veen. 2021. 'Biology and dynamics of B cells in the context of IgE-mediated food allergy', *Allergy*, 76: 1707-17.

Satitsuksanoa, Pattraporn, Kirstin Jansen, Anna Głobińska, Willem van de Veen, and Mübeccel Akdis. 2018. 'Regulatory Immune Mechanisms in Tolerance to Food Allergy', *Frontiers in Immunology*, 9.

Schiefner, A., and I. A. Wilson. 2009a. 'Presentation of lipid antigens by CD1 glycoproteins', *Curr Pharm Des*, 15: 3311-7.

Schiefner, André, and Ian A. Wilson. 2009b. 'Presentation of lipid antigens by CD1 glycoproteins', *Current pharmaceutical design*, 15: 3311-17.

Schmid, Hannes, Corina Schneidawind, Simona Jahnke, Felix Kettemann, Kathy-Ann Secker, Silke Duerr-Stoerzer, Hildegard Keppeler, Lothar Kanz, Paul B. Savage, and Dominik Schneidawind. 2018a. 'Culture-Expanded Human Invariant Natural Killer T Cells Suppress T-Cell Alloreactivity and Eradicate Leukemia', *Frontiers in Immunology*, 9.

———. 2018b. 'Culture-Expanded Human Invariant Natural Killer T Cells Suppress T-Cell Alloreactivity and Eradicate Leukemia', *Frontiers in immunology*, 9: 1817-17.

Schoos, Ann-Marie, Dominique Bullens, Bo Chawes, Joana Costa, Liselot De Vlieger, Audrey Dunn Galvin, Michelle Epstein, J. Garssen, Christiane Hilger, Karen Knipping, Annette Kuehn, Dragan Mijakoski, Daniel Munblit, Nikita Nekliudov, Cevdet Ozdemir, Karine Patient, Diego Peroni, Sasho Stoleski, Eva Stylianou, and Kitty Verhoeckx. 2020. 'Immunological Outcomes of Allergen-Specific Immunotherapy in Food Allergy', *Frontiers in Immunology*, 11: 2736.

Schülke, S., and M. Albrecht. 2019. 'Mouse Models for Food Allergies: Where Do We Stand?', *Cells*, 8.

Sheehan, W. J., P. A. Rangsithienchai, S. N. Baxi, A. Gardynski, A. Bharmanee, E. Israel, and W. Phipatanakul. 2010. 'Age-specific prevalence of outdoor and indoor aeroallergen sensitization in Boston', *Clin Pediatr (Phila)*, 49: 579-85.

Singh, Parvind, Marianna Szaraz-Szeles, Zoltan Mezei, Sandor Barath, and Zsuzsanna Hevessy. 2022. 'Gender-dependent frequency of unconventional T cells in a healthy adult Caucasian population: A combinational study of invariant NKT

cells, γδ T cells, and mucosa-associated invariant T cells', *Journal of Leukocyte Biology*, 112: 1155-65.

Sköld, Markus, and Samuel M. Behar. 2003. 'Role of CD1d-restricted NKT cells in microbial immunity', *Infection and immunity*, 71: 5447-55.

Smallcombe, Carrie C., Debra T. Linfield, Terri J. Harford, Vladimir Bokun, Andrei I. Ivanov, Giovanni Piedimonte, and Fariba Rezaee. 2019. 'Disruption of the airway epithelial barrier in a murine model of respiratory syncytial virus infection', *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 316: L358-L68.

Smeekens, Johanna M., Robert M. Immormino, Peter A. Balogh, Scott H. Randell, Michael D. Kulis, and Timothy P. Moran. 2019. 'Indoor dust acts as an adjuvant to promote sensitization to peanut through the airway', *Clinical & Experimental Allergy*, 49: 1500-11.

Smole, U., N. Balazs, Y. Sobanov, C. Radauer, M. Bublin, K. Hoffmann-Sommergruber, E. Jensen-Jarolim, D. Mechtcheriakova, and H. Breiteneder. 2011. 'Lipid raft mediated uptake of the major birch pollen allergen Bet v 1.0101 activates Th2 polarising signaling pathways in dendritic cells of allergic donors', *Allergy: European Journal of Allergy and Clinical Immunology*, 66: 54.

Stock, Philippe, and Omid Akbari. 2008. 'Recent advances in the role of NKT cells in allergic diseases and asthma', *Curr Allergy Asthma Rep*, 8: 165-70.

Thomas, Wayne, and Belinda Hales. 2008. 'Immune Responses to Inhalant Allergens', *World Allergy Organization Journal*, 1: 89-95.

Tordesillas, L., and M. C. Berin. 2018. 'Mechanisms of Oral Tolerance', *Clin Rev Allergy Immunol*, 55: 107-17.

Tordesillas, L., N. Cubells-Baeza, C. Gomez-Casado, C. Berin, V. Esteban, W. Barcik, L. O'Mahony, C. Ramirez, L. F. Pacios, M. Garrido-Arandia, and A. Diaz-Perales. 2017. 'Mechanisms underlying induction of allergic sensitization by Pru p 3', *Clinical and Experimental Allergy*, 47: 1398-408.

Traidl-Hoffmann, Claudia, Anna Kasche, Thilo Jakob, Michael Huger, Sabine Plötz, Ivo Feussner, Johannes Ring, and Heidrun Behrendt. 2002. 'Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes', *Journal of Allergy and Clinical Immunology*, 109: 831-38.

Trak-Fellermeier, M.A., S. Brasche, G. Winkler, B. Koletzko, and J. Heinrich. 2004. 'Food and fatty acid intake and atopic disease in adults', *European Respiratory Journal*, 23: 575-82.

Usuda, Haruki, Takayuki Okamoto, and Koichiro Wada. 2021. 'Leaky Gut: Effect of Dietary Fiber and Fats on Microbiome and Intestinal Barrier', *Int J Mol Sci*, 22: 7613.

van Bilsen, J. H. M., E. Sienkiewicz-Szłapka, D. Lozano-Ojalvo, L. E. M. Willemsen, C. M. Antunes, E. Molina, J. J. Smit, B. Wróblewska, H. J. Wichers, E. F. Knol, G. S. Ladics, R. H. H. Pieters, S. Denery-Papini, Y. M. Vissers, S. L. Bavaro, C. Larré, K. C. M. Verhoeckx, and E. L. Roggen. 2017a. 'Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins', *Clin Transl Allergy*, 7: 13.

van Bilsen, Jolanda H. M., Edyta Sienkiewicz-Szłapka, Daniel Lozano-Ojalvo, Linette E. M. Willemsen, Celia M. Antunes, Elena Molina, Joost J. Smit, Barbara Wróblewska, Harry J. Wichers, Edward F. Knol, Gregory S. Ladics, Raymond H. H. Pieters, Sandra Denery-Papini, Yvonne M. Vissers, Simona L. Bavaro, Colette Larré, Kitty C. M. Verhoeckx, and Erwin L. Roggen. 2017b. "Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins." In *Clinical and Translational Allergy*, 13.

Van Kaer, Luc, and Sebastian Joyce. 2005. 'Innate Immunity: NKT Cells in the Spotlight', *Current Biology*, 15: R429-R31.

van Ree, Ronald, Lone Hummelshøj, Maud Plantinga, Lars K. Poulsen, and Emily Swindle. 2014. 'Allergic sensitization: host-immune factors', *Clin Transl Allergy*, 4: 12.

Veinotte, Linnea, Simon Gebremeskel, and Brent Johnston. 2016. 'CXCL16-positive dendritic cells enhance invariant natural killer T cell-dependent IFNγ production and tumor control', *OncoImmunology*, 5: e1160979.

Venkatachalam, Mahesh, and Shridhar K. Sathe. 2006. 'Chemical Composition of Selected Edible Nut Seeds', *Journal of Agricultural and Food Chemistry*, 54: 4705-14.

Venter, C., R. W. Meyer, B. I. Nwaru, C. Roduit, E. Untersmayr, K. Adel-Patient, I. Agache, C. Agostoni, C. A. Akdis, S. C. Bischoff, G. du Toit, M. Feeney, R. Frei, H. Garn, M. Greenhawt, K. Hoffmann-Sommergruber, N. Lunjani, K. Maslin, C. Mills, A. Muraro, I. Pali-Schöll, L. K. Poulson, I. Reese, H. Renz, G. C. Roberts, P. Smith, S. Smolinska, M. Sokolowska, C. Stanton, B. Vlieg-Boerstra, and L. O'Mahony. 2019. 'EAACI position paper: Influence of dietary fatty acids on asthma, food allergy, and atopic dermatitis', *Allergy*, 74: 1429-44.

Waidyatillake, N. T., S. C. Dharmage, K. J. Allen, C. J. Lodge, J. A. Simpson, G. Bowatte, M. J. Abramson, and A. J. Lowe. 2018. 'Association of breast milk fatty acids with allergic disease outcomes-A systematic review', *Allergy*, 73: 295-312.

Waserman, Susan, Philippe Bégin, and Wade Watson. 2018. 'IgE-mediated food allergy', *Allergy, Asthma & Clinical Immunology*, 14: 55.

Watarai, Hiroshi, Ryusuke Nakagawa, Miyuki Omori-Miyake, Nyambayar Dashtsoodol, and Masaru Taniguchi. 2008. 'Methods for detection, isolation and culture of mouse and human invariant NKT cells', *Nature Protocols*, 3: 70-78.

Wildner, Sabrina, Brigitta Elsässer, Teresa Stemeseder, Peter Briza, Wai Tuck Soh, Mayte Villalba, Jonas Lidholm, Hans Brandstetter, and Gabriele Gadermaier. 2017. 'Endolysosomal Degradation of Allergenic Ole e 1-Like Proteins: Analysis of Proteolytic Cleavage Sites Revealing T Cell Epitope-Containing Peptides', *Int J Mol Sci*, 18: 1780.

Wilson, Michael T., Cecilia Johansson, Danyvid Olivares-Villagómez, Avneesh K. Singh, Aleksandar K. Stanic, Chyung-Ru Wang, Sebastian Joyce, Mary Jo Wick, and Luc Van Kaer. 2003. 'The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion', *Proceedings of the National Academy of Sciences*, 100: 10913-18.

Wisniewski, J., R. Agrawal, and J. A. Woodfolk. 2013. 'Mechanisms of tolerance induction in allergic disease: integrating current and emerging concepts', *Clin Exp Allergy*, 43: 164-76.

Wood, R. A., S. H. Sicherer, A. W. Burks, A. Grishin, A. K. Henning, R. Lindblad, D. Stablein, and H. A. Sampson. 2013. 'A phase 1 study of heat/phenol-killed, E. coli-encapsulated, recombinant modified peanut proteins Ara h 1, Ara h 2, and Ara h 3 (EMP-123) for the treatment of peanut allergy', *Allergy*, 68: 803-8.

World-Allergy-Organization. 2011. *White Book on Allergy* (United Kingdom).

Wu, L., and L. Van Kaer. 2011. 'Natural killer T cells in health and disease', *Front Biosci (Schol Ed)*, 3: 236-51.

Yamamoto-Hanada, Kiwako, Kyongsun Pak, Mayako Saito-Abe, Limin Yang, Miori Sato, Hidetoshi Mezawa, Hatoko Sasaki, Minaho Nishizato, Mizuho Konishi, Kazue Ishitsuka, Kenji Matsumoto, Hirohisa Saito, Yukihiro Ohya, Shin Yamazaki, Yukihiro Ohya, Reiko Kishi, Nobuo Yaegashi, Koichi Hashimoto, Chisato Mori, Shuichi Ito, Zentaro Yamagata, Hidekuni Inadera, Michihiro Kamijima, Takeo Nakayama, Hiroyasu Iso, Masayuki Shima, Youichi Kurozawa, Narufumi Suganuma, Koichi Kusuhara, Takahiko Katoh, Environment Japan, and Group Children's Study. 2020. 'Cumulative inactivated vaccine exposure and allergy development among children: a birth cohort from Japan', *Environmental Health and Preventive Medicine*, 25: 27.

Yoshimoto, T. 2018. 'The Hunt for the Source of Primary Interleukin-4: How We Discovered That Natural Killer T Cells and Basophils Determine T Helper Type 2 Cell Differentiation In Vivo', *Front Immunol*, 9: 716.

Zhang, Xiangyue, Pingping Zheng, Tyler R. Prestwood, Hong Zhang, Yaron Carmi, Lorna L. Tolentino, Nancy Wu, Okmi Choi, Daniel A. Winer, Samuel Strober, Eun-Suk Kang, Michael N. Alonso, and Edgar G. Engleman. 2020. 'Human Regulatory Dendritic Cells Develop From Monocytes in Response to Signals From Regulatory and Helper T Cells', *Frontiers in Immunology*, 11.

Ziegler-Heitbrock, H. W., G. Fingerle, M. Ströbel, W. Schraut, F. Stelter, C. Schütt, B. Passlick, and A. Pforte. 1993. 'The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages', *Eur J Immunol*, 23: 2053-8.

SUPPLEMENTARY MATERIAL

Supplement 1. Full search terms used in PubMed, Web of Science, and EMBASE.

PubMed was searched with terms: (((((lipid OR fatty acid OR lipid-binding OR PALM)) AND (allergy OR allergies OR allergen* OR pollen)) AND (Immunoglobulin E OR IgE OR sensitisation OR sensitization OR Th2)) NOT pain NOT asthma NOT AHR NOT contact NOT (n-3 OR n-6) NOT maternal NOT (predict* OR prevent*) NOT dermatitis NOT cross-reactivity NOT profile NOT diagnos*).

EMBASE was searched with the terms: (((lipid or fatty acid or lipid-binding or PALM) and (allergy or allergies or allergen* or pollen) and (Immunoglobulin E or IgE or sensitisation or sensitization or Th2)) not pain not asthma not AHR not contact not (n-3 or n-6) not maternal not (predict* or prevent*) not dermatitis not cross-reactivity not profile not diagnos*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word].

Web of Science used the search terms: (((((TS=(lipid OR fatty acid OR lipid-binding OR
PALM)) AND TS=(allergy OR allergies OR allergen* OR pollen)) AND TS=(Immunoglobulin E OR IgE OR sensitisation OR sensitization OR Th2)) NOT TS=(pain) NOT TS=(asthma) NOT TS=(AHR) NOT TS=(contact) NOT TS=(n-3 OR n-6) NOT TS=(maternal) NOT TS=(predict* OR prevent*) NOT TS=(dermatitis) NOT TS=(cross-reactivity) NOT TS=(profile) NOT TS=(diagnos*))) *AND* LANGUAGE: (English) *AND* DOCUMENT TYPES: (Article)

Appendix B: NHS HRA REC Ethics Documents

Bi. Study Protocol

SYNOPSIS:

Title	The Role of NKT Cells and Lipid Stimuli in the Development of Allergic Sensitisation.
Short title	Lipids in allergic sensitisation
Chief Investigator	Dr Lucy Fairclough
Objectives	• To characterise immune cell profiles, particularly of the 'Natural Killer T Cells' (NKT Cells), of human blood donors with allergies, compared to human blood donors with no allergies. • To establish whether NKT Cells produce an immune response found in allergy (Th2 cytokine response) when stimulated with lipids that are associated to allergens. • To determine whether lipid-activated NKT cells stimulate other key immune cells in allergy, particularly T- Cells (promote Th2 differentiation of naive T cells).
Trial Configuration	Single Centre
Setting	Primary and Secondary care
Sample size estimate	The aim is to recruit 15 allergic subjects. 15 healthy participants will be recruited through existing ethics. This sample size is based upon previous studies of this nature.
Number of participants	30
Eligibility criteria	Patients with clinically diagnosed IgE-mediated food allergies of interest to the study, non-smokers, aged 18 or above, able to attend Cripps Health Centre or the Queen's medical Centre, and are not currently involved in other research. Volunteers will not be Students working in the University department where these studies are being performed, neither should they be Unilever employees, according to Unilever Ethics Standard Guidelines.
Description of interventions	50 mL of blood take from participants arm on two separate occasions.

Duration of study	Until all samples are collected and analysed (Approximately 01/07/2021 – 01/12/2022). 30 minutes per participant.
Randomisation and blinding	Only the research team will have access to subject medical records and personal data. The data will be link-anonymised so no subject is identifiable by the data transferred to the research team.
Outcome measures	• NKT cell Th1 and Th2 cytokine responses to lipid stimuli. • NKT cell phenotyping by flow cytometry. • NKT cell proliferation in response to lipid stimuli.
Statistical methods	All statistical analysis will be carried out using Prism or SPSS.

Abbreviations

AE	Adverse Event
BBSRC	Biotechnology and Biological Sciences Research Council
CI	Chief Investigator overall
CRF	Case Report Form
DC	Dendritic Cell
DMC	Data Monitoring Committee
GCP	Good Clinical Practice
ICF	Informed Consent Form
IgE	Immunoglobulin E
NHS	National Health Service
NKT	Natural Killer T cells
PI	Principal Investigator at a local centre
PIS	Participant Information Sheet
QMC	Queen’s Medical Centre
REC	Research Ethics Committee
R&D	Research and Development department
SAE	Serious Adverse Event
SPSS	Statistical Package for the Social Sciences
Th1	Type 1 T helper Cell
Th2	Type 2 T helper Cell

TRIAL / STUDY BACKGROUND INFORMATION AND RATIONALE

Immunoglobulin E (IgE)-mediated allergies are overreactions of the immune system in response to harmless substances, such as peanuts, milk, soy, and grass, which result in the production of IgE antibodies. These antibodies trigger certain cells to release chemicals, which cause an allergic reaction. The symptoms of an IgE-mediated allergy typically appear within minutes, including swelling of the throat, nasal congestion, skin rashes, and anaphylaxis (a severe and possibly life-threatening systemic allergic response).

The prevalence of Immunoglobulin E (IgE)-mediated allergies is increasing globally. This poses a major public health concern as the mechanisms underpinning allergic sensitisation (the first phase in the development of IgE-mediated allergies) are currently not understood, making it difficult to manage the disease. Recent research has suggested lipids (including fats and oils) could play a role in allergic sensitisation, through their recognition by a specific cell type in the body (Mirotti et al., 2013).

This research aims to build upon recent work at the UoN (Fairclough et al., 2008) by developing techniques to isolate and characterise a type of cell found in human blood, NKT cells. The lipid-activation profiles of these NKT cells in allergic and non-allergic subjects will be characterised. Additionally, the role of NKT cells and lipid stimuli in the development of allergic sensitisation will be investigated using in vitro human cell assays. This work will help understand the mechanisms behind allergic sensitisation and has the potential to be used in the future to test if a substance might drive an allergic response.

Ethics approval is needed to allow allergic subject recruitment into the study, specifically subjects who have IgE-mediated food allergies. Experiments with NKT cells from this allergic population will allow a comparison to non-allergic NKT cells (recruited using existing ethics). We are proposing to obtain 50 mL of blood from allergic subjects on two separate occasions, 8 days apart. The blood will be taken by a trained phlebotomist at Cripps Health Centre or the QMC, so we do not envisage any adverse reactions to participants other than mild bruising at the site of blood taking and possibly feeling faint.

TRIAL / STUDY OBJECTIVES AND PURPOSE

PURPOSE

The prevalence of Immunoglobulin E (IgE)-mediated allergies is increasing globally. This poses a major public health concern as the mechanisms underpinning allergic sensitisation (the first phase in the development of IgE-mediated allergies) are currently not understood, making it difficult to manage the disease. Allergenic substances are composed of proteins accompanied by other components, such as carbohydrates and lipids. Proteins are the target for IgE, however, lipids have been the focus of much recent research as it has been found they can promote or skew the Th2 type allergic response. Therefore it is imperative to understand the role lipids have in the sensitisation phase of allergy such that it can be avoided or effectively treated and we are able to study this with an already established laboratory assay that can incorporate lipids. Critical to the research therefore is the comparison of non-allergic and clinical documented allergic individuals.

PRIMARY OBJECTIVE

To investigate the role of lipids in the development of IgE-mediated allergic sensitisation.

SECONDARY OBJECTIVES

To characterise immune cell profiles particularly of the 'Natural Killer T Cells' (NKT Cells) of human blood donors with allergies, compared to human blood donors with no allergies.

To establish whether NKT Cells produce an immune response found in allergy (Th2 cytokine response) when are stimulated with lipids that are associated to allergens.

To determine whether lipid-activated Natural Killer Cells stimulate other key immune cells in allergy particularly T- Cells (promote Th2 differentiation of naive T cells).

TRIAL / STUDY DESIGN

TRIAL / STUDY CONFIGURATION

A single centre study where subjects will be recruited and their blood taken at Cripps Health Centre or the QMC Hospital. The blood will then be transported to the Life Sciences building at The University of Nottingham for all lab and statistical analyses.

Primary endpoint

The measurement of NKT cell Th1 and Th2 cytokine responses to food allergen-associated lipid stimuli (e.g. peanut lipids).

Secondary endpoint

The participant's NKT cells will be phenotyped by flow cytometry to distinguish differences in NKT cells to healthy controls.

The NKT cells will also be measured for their proliferation in response to allergen-associated lipid stimuli, in comparison to healthy controls.

Stopping rules and discontinuation

If the trained phlebotomist is struggling to obtain blood from the participants arm, the participant can reschedule their appointment to try again another day. If the issue persists, their participation will be discontinued.

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT

Study Duration: Enrolment will begin when the ethics application has been approved. We are hoping this is by July 2021. The research team will then begin to recruit participants immediately and the study will commence up to December 2022, when the student's PhD funding ends. Thus, the study will last around 1.5 years.

Participant Duration: The participant will be required to give blood on two occasions, lasting approximately 15 minutes each time, totalling 30 minutes for the whole study.

The end of the study will be when the last blood is taken from the last participant.

SELECTION AND WITHDRAWAL OF PARTICIPANTS

Recruitment

Participants will be recruited from the Queen's Medical Centre allergy clinics or Cripps Health Centre. The initial approach will be from a member of the subjects usual care team (a member of the research team). The research team will identify eligible

subjects from their NHS database and text eligible subjects to enquire about their interest in the study. The text will include a member of the research team's email address for subjects to contact for further information and declare whether they want to take part.

Subjects will also be recruited at the end of a subjects clinic appointment. In this case, a member of the research team will verbally ask if they are interested in the study and ask whether they fit the eligibility criteria.

Any subjects that are interested and respond to the recruitment text or the verbal invitation, will then be emailed the participant information sheet,, along with the study eligibility criteria, so the subjects can self-screen before agreeing to take part..

If the subject fits the eligibility criteria and has read the participant information sheet, the research team will respond to arrange a date for them to attend the clinic. The subjects will also be given the opportunity to ask any questions via email or telephone before attending the clinic, and during their clinic appointment.

During their clinic appointment, the subject will be met by a member of the research team who will discuss the information sheet and consent form, followed by the participant giving consent by signing the written consent form. A written questionnaire will also be filled in by the participant to confirm their eligibility. The research team will then escort them to the phlebotomy suite where their blood will be taken by a trained phlebotomist.

The clinical investigator, research team, or their nominee, e.g. a colleague from the participant's usual care team, will inform the participant or their nominated representative (other individual or other body with appropriate jurisdiction), of all aspects pertaining to participation in the study.

It will be explained to the potential participant that entry into the trial is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

Eligibility criteria

Inclusion criteria

- Clinically diagnosed IgE-mediated allergy to the food allergen of interest

- Adults aged 18 or above
- Able to attend the QMC or Cripps Health Centre, Nottingham.
- Ability to give informed consent

Exclusion criteria

- Smokers
- Are currently involved in other research.
- Are Students working in the University department where these studies are being performed.

Expected duration of participant participation

Study participants will be participating in the study for 30 minutes.

Removal of participants from therapy or assessments/Participant Withdrawal

The participants are free to withdraw at any time and without giving a reason. If they withdraw, the research team will no longer collect any information from them, but they will keep any existing participant information. If subjects withdraw before giving both samples of blood required for the study, more participants will be recruited to reach the projected sample size.

Participants may be withdrawn from the trial either at their own request or at the discretion of the Investigator. The participants will be made aware that this will not affect their future care. Participants will be made aware (via the information sheet and consent form) that should they withdraw the data collected to date cannot be erased and may still be used in the final analysis.

Informed consent

All participants will provide written informed consent on the day of their first blood donation. The Informed Consent Form will be signed and dated by the participant in the research team's clinic, before they enter the study and give blood. The research team will explain the details of the trial and provide a Participant Information Sheet, ensuring that the participant has sufficient time to consider participating or not. The research team will answer any questions that the participant has concerning study participation.

Informed consent will be collected from each participant before they undergo any interventions (including physical examination and history taking) related to the study.

One copy of this will be kept by the participant, one will be kept by the research team, and a third will be retained in the subjects hospital records.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the study, continuing consent will be obtained using an amended Consent form which will be signed by the participant.

TRIAL / STUDY TREATMENT AND REGIMEN

The research team will identify subjects with the desired food allergies using their NHS patient database. The research team will then text potential participants to enquire about their interest in the study. The text will state they have been identified as a subject with an allergy of interest to the research team. Subjects will also be recruited at the end of a subjects clinic appointment. In this case, the research team will verbally ask if they are interested in the study and ask whether they fit the eligibility criteria. Any subjects who reply to say they are interested and eligible will then be contacted to arrange a date and time most suitable for them to travel to the clinic to give their first and second blood donations. Participants will be informed that it is essential for them to give their second blood donation exactly 8 days after their first donation, due to the experimental requirements.

The participants will then arrive at Cripps Health Centre or the QMC for their first blood donation appointment. The participant will meet the research team within Cripps Health Centre or the allergy clinic. Here, the research team will talk through the Participant Information Sheet and Consent form and answer any questions the participant may have. The participant will then be asked to sign the consent form. The participant will also confirm their eligibility by filling in a questionnaire. The questionnaire includes eligibility questions such as: do you smoke, do you have an IgE-mediated food allergy, as well as personal/demographic questions such as: what is your age, sex, ethnicity, what medications do you take. These details will be used to draw comparisons between different demographics during data analysis.

The participant will then be taken into a room where a trained phlebotomist will take their blood. The participant will then return home. The student research team member will transport the blood from phlebotomy to their lab in the Life Sciences building on University Park for experiments.

Eight days after the first blood donation, the participant will return to the allergy clinic at the QMC or Cripps Health Centre for their final 50 mL blood donation. Again, a

trained phlebotomist will take their blood in the room. After this donation, an inconvenience allowance of £25 will be sent to the University's finance department for processing into the participant's bank account.

The participant will then return home and will not be required to participate further. The participants will be given the research team's contact details if they have any questions or wish to withdraw from the study etc. The student research team member will then transport the blood from phlebotomy to their lab again in the Life Sciences building.

Compliance

Participants are required to have their blood taken by a trained professional. If on the day they do not wish for their blood to be taken, their appointment can be a rearranged or they can be withdrawn from the study if they do not want to continue.

Criteria for terminating trial

Due to this being a study where only 50 mL of blood will be taken from participants, twice, we do not envisage any issues resulting in the termination of the study.

TRANSPORT AND STORAGE OF THE TISSUES

Samples will be stored in a linked anonymised format and labelled using a combination of study reference, unique study identifier and cross referenced with location code numbers to permit accurate linkage to study data and the consent form. The master database will be held by Miss Stephanie Pearson in a password encrypted file.

The analysis of samples will take place at the University of Nottingham within the Department of Life Sciences.

Blood samples will be collected at the phlebotomy suite of the QMC or Cripps Health Centre and transferred, by foot, to the research team’s lab within the Life Sciences building, by a member of the research team. All blood transport boxes will contain a complete inventory of all samples.

Plasma samples will be derived from the participant’s blood and will be stored in aliquots at -80 degrees centigrade in a locked freezer within the research team’s lab for up to 5 years after the study has ended, until required in the experiments.

Once analysis has taken place, any sample that is left over will be disposed of in accordance with the Human Tissue Act, 2004.

LABORATORY ANALYSES

The PhD student of the research team, Miss G Hopkins, will utilise the first blood donation to carry out Natural Killer T (NKT) cell expansion experiments in the lab. Briefly, this involves Peripheral blood mononuclear cell (PBMC) isolation from the blood and stimulation with either an allergen, an allergen-associated lipid, or both allergen and lipid. The PBMCs will then be cultured for up to 14 days in a 37 °C incubator. NKT cells are extremely rare in the blood, thus this step will allow the lipid to activate the proliferation of NKT cells to result in a greater NKT cell population, enabling the use of NKT cells in the subsequent stages of the experiments.

Plasma will also be isolated from this blood sample and frozen for subsequent total IgE ELISAs.

8 days after the first blood donation, the participant will return to the phlebotomy suite at QMC or Cripps Health Centre for their final 50 mL blood donation. This second lot of blood will be used for isolating monocytes from the blood and stimulating them with cytokines to generate dendritic cells (DCs) after 6 days of stimulation.

These DCs will then be mixed with the NKT cells that were generated from the first blood sample. The cell mixture will be stimulated with either a food allergen, a food allergen-associated lipid, or both allergen and lipid. It is hypothesised that the DCs will internalise the lipid and/or allergen and then present it to the NKT cells. This will cause the NKT cell to release Th1 or Th2 cytokines. Th2 cytokine release indicates allergic sensitisation to the food allergen. Th1 cytokine release indicates no allergic sensitisation.

Spectral flow cytometry will be used to analyse the effect of the allergens and/or lipids on the ability of the cells to release Th1 or Th2 cytokines. It is hypothesised that the lipids will activate NKT cells to increase Th2 cytokine secretion, thus enhancing allergic sensitisation to an allergen.

The results can then be compared between allergic and non-allergic donors to look at differences between NKT cell lipid-activation profiles. Non-allergic participants will be recruited using existing ethics by posters placed around the University of Nottingham. Participants will not be eligible if they work in the same department that is running the study.

STATISTICS

Methods

Miss G Hopkins will perform all statistical analyses on UoN computers to compare results of participants with different demographics, using non-identifiable data (Study

codes used instead of participant names). All statistical analysis will be carried out using Prism or SPSS. Dr Lucy Fairclough, the chief investigator, will supervise and ensure all statistical analyses are correct.

Sample size and justification

The aim is to recruit 15 allergic subjects. 15 healthy participants will be recruited through existing ethics. This sample size is based upon previous studies of this nature. The actual sample size will be determined by how many subjects the research team has in their database with the relevant allergies, and how many of these will be willing to participate, as well as how many can be recruited within the time-frame.

Primary Outcome Assessment

Primary outcomes:

The measurement of NKT cell Th1 and Th2 cytokine responses to allergen-associated lipid stimuli.

The participant's NKT cells will be phenotyped by flow cytometry to distinguish differences in NKT cells to healthy controls.

The NKT cells will also be measured for their proliferation in response to allergen-associated lipid stimuli, in comparison to healthy controls.

All outcomes are laboratory based and will be measured by flow cytometry analysis.

Assessment of safety

As this study in in vitro, and all primary end points are laboratory experiment outcomes, there are no safety requirements.

Procedures for missing, unused and spurious data. N/a.

Definition of populations analysed

Full Analysis set: All participants, who donated blood twice, as required, and for whom at least one post-baseline assessment of the primary endpoint is available.

Per protocol set: All participants in the Full Analysis set who are deemed to have no major protocol violations that could interfere with the objectives of the study.

Participants who do not give blood twice will be excluded from the analysis, as this means all desired cell types cannot be analysed to reach the outcomes.

ADVERSE EVENTS

No major adverse events are envisaged with the study of this nature. There is the chance of mild bruising and feeling faint when their blood is being collected. Participants will be advised to eat and drink before the procedure to minimise feeling faint.

ETHICAL AND REGULATORY ASPECTS

ETHICS COMMITTEE AND REGULATORY APPROVALS

The study will not be initiated before the protocol, informed consent forms and participant and favourable opinion from the Research Ethics Committee (REC), the respective National Health Service (NHS) or other healthcare provider's Research & Development (R&D) department, and the Health Research Authority (HRA) if required. Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant information sheets have been reviewed and received approval / favourable opinion from the REC and R&D departments. A protocol amendment intended to eliminate an apparent immediate hazard to participants may be implemented immediately providing that the REC are notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The trial will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice, and the UK Department of Health Policy Framework for Health and Social Care, 2017.

INFORMED CONSENT AND PARTICIPANT INFORMATION

The process for obtaining participant informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator or their nominee and the participant shall both sign and date the Informed Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Trial Master File. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the trial.

The decision regarding participation in the study is entirely voluntary. The investigator or their nominee shall emphasize to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No trial-specific interventions will be done before informed consent has been obtained.

The investigator will inform the participant of any relevant information that becomes available during the course of the study, and will discuss with them, whether they wish to continue with the study. If applicable they will be asked to sign revised consent forms.

If the Informed Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

RECORDS

Case Report Forms

Each participant will be assigned a trial identity code number by the research team for use on CRFs, other study documents and the electronic database. As the study is only recruiting 5 participants, the participant study number along with their type of allergy will be sufficient identifiers.

CRFs will be treated as confidential documents and held securely in accordance with regulations. The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Study Number, to permit identification of all participants enrolled in the study, in accordance with regulatory requirements and for follow-up as required

CRFs shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Study Delegation Log.'

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the CRF.

Sample Labelling

Each participant will be assigned a study identity code number for use on the samples, consent forms and other study documents and the electronic database. The documents and database will also use note their type of allergy.

Source documents

Source documents shall be filed at the investigator's site in a locked office and may include but are not limited to, consent forms, laboratory results and records. A CRF may also completely serve as its own source data. Only research members as listed on the Delegation Log shall have access to study documentation other than the regulatory requirements listed below.

Direct access to source data / documents

The CRF and all source documents, including progress notes and copies of laboratory results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities (e.g. DH, Human Tissue Authority).

DATA PROTECTION

All investigators will endeavour to protect the rights of the trial's participants to privacy and informed consent, and will adhere to the Data Protection Act, 2018. The CRF will only collect the minimum required information for the purposes of the trial. CRFs will be held securely, in a locked room, or locked cupboard or cabinet. Access to the information will be limited to the research staff and investigators and relevant regulatory authorities (see above). Computer held data including the trial database will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method).

Only the research team will have access to subject medical records. The UoN research team will only receive a spreadsheet with participant study numbers and some personal data such as age, sex, ethnicity, any current medication the participant is taking, and what allergies they have.

Electronic data will be backed up every 24 hours to both local and remote media in encrypted format.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for trial participants and trial staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but trial participants may have recourse through the NHS complaints procedures.

The University of Nottingham as research Sponsor indemnifies its staff with both public liability insurance and clinical trials insurance in of claims made by research subjects.

TRIAL CONDUCT

Study conduct may be subject to systems audit of the Study Master File for inclusion of essential documents; permissions to conduct the study; Study Delegation Log; CVs of staff and training received; local document control procedures; consent procedures and recruitment logs; adherence to procedures defined in the protocol (e.g. inclusion / exclusion criteria, correct randomisation, timeliness of visits); adverse event recording and reporting; accountability of trial materials and equipment calibration logs.

TRIAL DATA

Monitoring of study data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Chief investigator (Academic Supervisor) shall carry out monitoring of study data as an ongoing activity.

Entries on CRFs will be verified by inspection against the source data. A sample of CRFs (10% or as per the study risk assessment) will be checked on a regular basis for verification of all entries made. In addition the subsequent capture of the data on the trial database will be checked. Where corrections are required these will carry a full audit trail and justification.

Study data and evidence of monitoring and systems audits will be made available for inspection by REC as required.

RECORD RETENTION AND ARCHIVING

In compliance with the ICH/GCP guidelines, regulations and in accordance with the University of Nottingham Research Code of Conduct and Research Ethics, the Chief or local Principal Investigator will maintain all records and documents regarding the conduct of the study. These will be retained for at least 7 years or for longer if required. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.

The study Master File and study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all trial databases and associated meta-data encryption codes.

DISCONTINUATION OF THE TRIAL BY THE SPONSOR

The Sponsor reserves the right to discontinue this trial at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons. The Sponsor shall take advice from the Trial Steering Committee and Data Monitoring Committee as appropriate in making this decision.

STATEMENT OF CONFIDENTIALITY

Individual participant medical information obtained as a result of this study are considered confidential and disclosure to third parties is prohibited with the exceptions noted above.

Participant confidentiality will be further ensured by utilising identification code numbers to correspond to treatment data in the computer files.

Such medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.

If information is disclosed during the study that could pose a risk of harm to the participant or others, the researcher will discuss this with the CI and where appropriate report accordingly.

Data generated as a result of this trial will be available for inspection on request by the participating physicians, the University of Nottingham representatives, the REC, local R&D Departments and the regulatory authorities.

PUBLICATION AND DISSEMINATION POLICY

A full study report will be submitted for publication in a peer reviewed medical journal as well as form part of a PhD thesis. Participants will not be identified in any publications. Participants will have the option on the consent form to be notified of any resulting publication, should they wish to access the publication.

The research team are employed by the University of Nottingham and have appropriate rights to publish the data

Authors must acknowledge that the study was performed with the support of Nottingham University Hospitals NHS Trust and funded by Unilever and the BBSRC.

USER AND PUBLIC INVOLVEMENT

No involvement from the public will be sought for this study.

STUDY FINANCES

Funding source

This study is funded by Unilever and The BBSRC.

Participant stipends and payments

An inconvenience allowance of £25 will be given to all participants after their second blood draw only. The allowance covers their participation in the study, as well as any travel costs incurred from attending the QMC or Cripps Health Centre.

REFERENCES

Fairclough, L., RA Urbanowicz, RA., J Corne, J., and JR Lamb, J.R. Killer T cells in COPD. Clinical Sciences 2008; 114: 533-541

Mirotti L, Florsheim E, Rundqvist L, Larsson G, Spinozzi F, Leite-De-Moraes M, et al. Lipids are required for the development of Brazil nut allergy: The role of mouse and human iNKT cells. Allergy: European Journal of Allergy and Clinical Immunology. 2013;68(1):74-83.

Bii. Patient Information Sheet

Participant Information Sheet

(Final Version 5.0: 11-03-2022)

IRAS Project ID: 272958

Title of Study: The Role of NKT Cells and Lipid Stimuli in the Development of Allergic Sensitisation

Name of Chief Investigator: Dr Lucy Fairclough

Name of Clinical Investigator: Dr Simon Royal, Dr Alexandra Croom

Name of Local Researchers: Dr David Onion, Miss Georgina Hopkins, Miss Stephanie Pearson

You are being invited to take part in this research, as part of a PhD project. Before you decide to do so, it is important you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with any friends or relatives, if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part. If you decide to take part you can keep this information sheet and then you will be asked to sign a consent form, which you may also keep.

What is the purpose of the study?

Allergies are on the rise, now affecting around 30% of adults and 40% of children. Allergies propose a major health concern, with extreme reactions leading to anaphylactic shock and even death. The mechanisms underlying the development of an allergy are still not fully understood, making allergy management difficult. This research will investigate the role of lipids (including fats and oils) in the development of allergies by stimulating natural killer t-cells (NKT cells) of the blood with lipids. NKT cells are a rare type of cell found in the blood, which can interact with lipids to regulate inflammatory immune responses. This work will help understand if lipids are influencing the development of allergies, which can then provide potential tools for developing future techniques to study allergy.

Why have I been invited?

You are being invited to take part because you have a food allergy of interest to our study, and you have responded to our recruitment email or invitation within the clinic. We are inviting 5 participants like you to take part. Your blood is needed to compare to blood taken from people with no allergies, so we can determine any differences in NKT cells and their sensitivity to lipids.

Do I Have to Take Part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect your legal rights. If you choose to decline the study invitation, or withdraw from the study, this will not affect any care you currently/will receive.

What will happen to me if I take part?

If you agree to take part in the study, the clinical investigator will ask you to attend Cripps Health Centre, or the clinical immunology and allergy clinic within the Queen's Medical Centre (QMC). Once you have given the investigator your informed written consent and asked any questions you may have, you will be asked to complete a short questionnaire to confirm your eligibility in the study. This will include questions regarding your age, sex, ethnicity, current medications/treatments, and list of substances you are allergic to. Your answers will be anonymised so the researchers will not be able to identify you. The local NHS research team will also view your medical records to access these details. Only the local NHS research team and your usual healthcare team will have access to your medical records.

Once you have completed the questionnaire, you will then have 50 mL of blood taken from your arm by a trained professional. The procedure will use a needle and syringe to collect the blood sample, usually lasting no more than 5 minutes. Before giving blood, there is no need to stop any medication you are taking. It is recommended that you should avoid donating more than 500 mL of blood over a 6-month period and allow at least 3 months before giving blood in other studies. As this study involves taking two blood samples of 50 mL 8 days apart, you will be asked to return to the clinic 8 days after giving your first blood sample. Here you will have your second (and final) blood sample taken by a trained healthcare professional, and you will then not be required to participate in any further activities. The blood samples will be taken to the immunology lab within the University of Nottingham for processing. The cells extracted will be stimulated with lipids to study its effect on allergic sensitisation and compared with cells taken from participants with no allergies. The research project will end once all participant bloods have been analysed (approximately 1.5 years).

Expenses and payments

Participants will be paid an inconvenience allowance (£25) to participate in the study and help cover any travel costs to the QMC.

What are the Possible Disadvantages of Taking Part?

There may be some initial discomfort when inserting the needle for blood taking, as well as a feeling of faintness. There may also be some slight bruising around the needle insertion area, but this should clear up within a few days. If you are at all worried, please do not hesitate to contact us.

What are the possible benefits of taking part?

We cannot promise the study will help you directly, but the information we get from this study may help aid future research into the mechanisms of allergic sensitisation.

What happens when the research study stops?

All research data will be kept on a secure internal server. Data will be analysed and will be used for PhD projects and/or scientific publications.

What if There is a Problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers' contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting the Cripps Health Centre complaints by telephone: 0115 846 8888. Or, you can contact Nottingham University Hospitals Trust PALS (Patient Advice and Liaison Service) by telephone: 0800 183 0204, by email: pals@nuh.nhs.uk, or you can find further contact information at:

https://www.nuh.nhs.uk/share-your-feedback

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against the University of Nottingham but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will My Taking Part in this Study be Kept Confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence. If you join the study, we will use information collected from you and your medical records during the course of the research. This information will be kept strictly confidential, stored in a secure and locked office, and on a password protected database at the University of Nottingham. Under UK Data Protection laws the University is the Data Controller (legally responsible for the data security) and the Chief Investigator of this study (named above) is the Data Custodian (manages access to the data). This means we are responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited as we need to manage your information in specific ways to comply with certain laws and for the research to be reliable and accurate. To safeguard your rights we will use the minimum personally – identifiable information possible.

You can find out more about how we use your information and to read our privacy notice at:

https://www.nottingham.ac.uk/utilities/privacy.aspx

The data collected for the study will be looked at and stored by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people from regulatory organisations to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty

Your contact information will be kept by the NHS organisation for up to 12 months after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies (unless you advise us that you do not wish to be contacted). This information will be kept separately from the research data collected and only those who need to will have access to it. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team given permission by the data custodian will have access to your personal data.

In accordance with the University of Nottingham's, the Government's and our funders' policies we may share our research data with researchers in other Universities and organisations, including those in other countries, for research in health and social care. Sharing research data is important to allow peer scrutiny, re-use (and therefore avoiding duplication of research) and to understand the bigger picture in particular areas of research. Data sharing in this way is usually anonymised (so that you could not be identified) but if we need to share identifiable information we will seek your consent for this and ensure it is secure. You will be made aware then if the data is to be shared with countries whose data protection laws differ to those of the UK and how we will protect your confidentiality.

What will happen if I don't want to carry on with the study?

If you decide to take part, you are still free to withdraw at any time and without giving a reason. This would not affect your legal rights. If you withdraw we will no longer collect any information about you or from you but we will keep the information about you that we have already obtained as we are not allowed to tamper with study records and this information may have already been used in some analyses and may still be used in the final study analyses. To safeguard your rights, we will use the minimum personally-identifiable information possible. If you withdraw, you can also request any samples of yours to be destroyed, preventing use of them in further experiments.

Involvement of the General Practitioner/Family doctor (GP)

Your GP will NOT be notified of your participation in this study.

What will happen to the samples I give?

Blood samples will be handled in accordance with the local laboratory regulations. They will be labelled with a code and stored in a dedicated secure laboratory fridge. Access is limited to the members of this research team, until the experiments have been completed. Also, a database of all samples collected and used will be maintained in line with good laboratory practice and legal regulations such as the Human Tissue Act 2004. All blood samples and experimental data will be given a code, so no one can be identified. Plasma samples will be isolated from the blood and stored within the lab's secure -80 °C freezer for up to 5 years after the end of the study, until they are analysed. After use, the samples and any extracted cells will be disposed of in line with university guidelines and the Human Tissue Act 2004.

What Will Happen to the Results of the Research Study?

The results of this study will be published, but you will not be identifiable. The results may be used to conduct further investigations into lipids and NKT cells in allergic sensitisation. The study results can be shared with you if you choose to agree with this option on the consent form.

Who is Organising and Funding the Research?

The research is organised by the University of Nottingham. It is funded by a PhD studentship provided by Unilever and The Biotechnology and Biological Sciences Research Council (BBSRC).

Who Has Reviewed the Study?

The study has been reviewed and approved by the NHS HRA Research Ethics Committee.

Contact for Further Information

Dr Lucy Fairclough – Chief Investigator

lucy.fairclough@nottingham.ac.uk

0115 8230729

Georgina Hopkins – Local Researcher

georgina.hopkins@nottingham.ac.uk

Thank you for reading this information sheet.

Biii. Participant Consent Form

CONSENT FORM

(Final Version 4.0: 11-03-2022)

Title of Study: The Role of NKT Cells and Lipid Stimuli in the Development of Allergic Sensitisation.

IRAS Project ID: 272958

Name of Researchers: Dr Lucy Fairclough, Dr Simon Royal, Dr Alexandra Croom, Dr David Onion, Miss Stephanie Pearson, and Miss Georgina Hopkins

Name of Participant:

Study Volunteer Number:

1. I confirm that I have read and understand the information sheet version number 5.0 dated 11/03/2022 for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis.

3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential.

4. I understand and agree that two 50 mL blood samples will be taken 8 days apart for analysis of the role of NKT cells and lipids in allergic sensitisation.

5. I have not been a subject in any other research study in the last three months which involved: taking a drug; having an invasive procedure (e.g. blood sample >50ml) or exposure to ionising radiation.

6. I agree to take part in the above study.

7. (Optional) I would like to be contacted if the results of the study are published.

_____	_____	_____
Name of Participant	Date	Signature

_____	_____	_____
Name of Person taking consent	Date	Signature

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

Biv. Participant Questionnaire

Questionnaire

(Final Version 5.0: 11-03-2022)

Title of Study: The Role of NKT Cells and Lipid Stimuli in the Development of Allergic Sensitisation.

IRAS Project ID: 272958

Name of Researchers: Dr Lucy Fairclough, Dr Simon Royal, Dr Alexandra Croom, Dr David Onion, Miss Stephanie Pearson, and Miss Georgina Hopkins

Study Volunteer Number:

Please answer these questions to the best of your knowledge. Your answers will be anonymised with a study volunteer number, so the researchers will not be able to identify you.

Sex ……..

Date of Birth ……..

Ethnic Group (Please tick one option that best describes your ethnic group/background)

	White		**Asian/Asian British**
☐	English/Welsh/Scottish/Northern Irish/British	☐	Indian
☐	Irish	☐	Pakistani
☐	Gypsy or Irish Traveller	☐	Bangladeshi

PLEASE TURN OVER

☐ Any other White background, please describe

……

☐ Chinese

☐ Any other Asian background, please describe.

……

Mixed/Multiple ethnic groups

☐ White and Black Caribbean

☐ White and Black African

☐ White and Asian

☐ Any other Mixed/Multiple ethnic background, please describe

……..

Black/African/Caribbean/Black British

☐ African

☐ Caribbean

☐ Any other Black/African/Caribbean background, please describe

…….

Other ethnic group

☐ Arab

☐ Any other ethnic group, please describe

……

Do you have a diagnosed IgE-mediated food allergy? (please circle)
Yes/No

If Yes, please state which type of food allergies (e.g. peanut, soy)

……

Do you suffer from any other allergies? (please circle)
Yes/No

If Yes, please state which type of allergies you have and, if known, what the trigger(s) is/are. (e.g. hay fever triggered by grass pollen, asthma triggered by mould, or eczema triggered by soap, etc.)

……

……

How were you diagnosed? (e.g. GP, skin-prick test, self-diagnosed)

……
……

Do you currently smoke? (please circle)
Yes/No

Are you currently on any medication or undergoing any treatment? (Including over the counter medicine e.g. aspirin). (please circle)
Yes/No

If Yes, please state what:

……

……

Have you had a cold in the last 2 weeks? (please circle)
Yes/No

Thank you for taking the time to complete this questionnaire