CD1a-Reactive T Cells in Peanut Allergy



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COVID IMPACT STATEMENT

The University of Oxford has released guidance to graduate students regarding the effects of the coronavirus pandemic on our studies. We have been advised by the departmental director of graduate studies to detail any disruption of our work.

Most of all, I am thankful that I have not suffered personal loss or health issues during the Covid-19 pandemic. However, lab activity was disrupted by university closures (from March until July 2020), with subsequent restrictions in lab access throughout, in line with social distancing protocols pertaining to University and national guidance.

During initial lockdown procedures, donor blood samples were limited, however I was fortunate to have access to samples which had been frozen previously. Nonetheless, I had planned to further delineate some mechanisms outlined in *Chapter 7* and *Chapter 8*, and the pandemic impacted upon my ability to do so. This would have been achieved through fractionation of peanut lipids and deeper investigation of the interplay between cyclic phosphatidic acid and PPARγ in regulating CD1a expression, and additional functional assays investigating the role of PLD2 in promoting CD1a antigen presentation by dendritic cells. These concepts are discussed in detail during this work.

ABSTRACT

Peanut allergy is the most common cause of food-induced anaphylaxis with 3% prevalence in Western countries, and is associated with atopic dermatitis and null mutations in the gene encoding filaggrin. Langerhans cells (LCs) express high levels of CD1a, which presents lipid antigens to T cells and contributes towards allergic disease, in part through phospholipase generation of neolipid antigens.

It was investigated whether peanuts drive an allergen-reactive CD1a-mediated T cell response. This hypothesis was tested using *ex vivo* T cell ELISpot analyses from peanut-allergic patients and controls, and peanut phospholipase D (PLD) biochemical assays for production of phosphatidic acid (PA) and phosphatidylglycerol (PG) in dendritic cells (DCs). Further investigations were performed to determine whether endogenous human PLD regulates CD1a expression in DCs.

Peanut-derived lipids did not induce a direct CD1a-dependent T cell response, however elevated frequencies of circulating peanut PLD-responsive CD1a-reactive T cells were observed in peanut-allergic individuals, and this was driven by enzymatic activity. Filaggrin inhibited the formation of PA but not PG, and reduced peanut PLD-specific CD1a-reactive T cell responses. Aquaporin 3 (AQP3), which is overexpressed in atopic skin and facilitates PG production by PLD, was also required for these responses. Isoelectric focusing confirmed binding of PA and PG to CD1a, and peanut-allergic patients demonstrated greater CD1a-reactivity to both lipids. Endogenous PLD2 activity was required for CD1a protein and mRNA expression in LCs and DCs, and this correlated with inhibition of PPARγ activity.

In summary, the data show that peanut-derived PLD generates CD1a neoantigens leading to PG- and PA-reactive T cells in peanut-allergic individuals. Filaggrin insufficiency and AQP3 overexpression in atopic patients may dysregulate normal barrier sensing and modulate lipid antigen generation and so predispose to generation of CD1a-reactive T cells. Given its additional effect in facilitating CD1a expression, PLD may exert a dual action in driving CD1a responses.

ABBREVIATIONS

α-GalCer	Alpha-galactosylceramide
AA	Arachidonic acid
AD	Atopic dermatitis
APC	Antigen-presenting cell
AQP	Aquaporin
ATF	Activating transcription factor
ATRA	All-trans-retinoic acid
*β*2-M	*β*2-microglobulin
BG	Birbeck granule
BK	Bradykinin
C2PG	Cyclopentone prostaglandin
CCL	C-C Motif Chemokine Ligand
CCR	CC chemokine receptor
cDC	Conventional dendritic cell
CE	Cornified envelope
C:E	Chloroform: ethanol
CER	Ceramide
CLA	Cutaneous lymphocyte antigen
cPA	Cyclic phosphatidic acid
$cPLA_2$	Cytosolic Phospholipase A_2
CRD	Carbohydrate recognition domain
CREB	cAMP Response Element-Binding Protein
CyD	Cytochalasin D
DAMP	Danger-associated molecular pattern

DC	Dendritic cell
DDM	Didehydroxymycobactin
EFA	Essential fatty acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
ERK	Extracellular signal-regulated kinase
ET	Endothelin
FA	Fatty acid
FcεRI	Fc epsilon receptor I
FFA	Free fatty acid
GM-CSF	Granulocyte-macrophage colony stimulating factor
HDM	House dust mite
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
MoDC	Monocyte-derived dendritic cell
ICAM	Intercellular adhesion molecule
iDC	Immature dendritic cell
IEF	Isoelectric focusing
IFN-γ	Gamma-interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cell
LA	Linoleic acid
LC	Langerhans cell

LPA	Lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class I
MUFA	Monounsaturated fatty acids
MUTZ3-LC	MUTZ3-derived Langerhans cell
NF-κB	Nuclear factor kappa B
NMF	Natural moisturising factor
nsLTP	non-specific lipid-transfer protein
OAS	Oral allergy syndrome
OB	Oil body
OLA	Oleic acid
OVA	Ovalbumin
PA	Phosphatidic acid
PC	Phosphatidylcholine
PCA	Pyrrolidone-5-carboxylic acid
pDC	Plasmacytoid dendritic cell
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
pI	Isoelectric point

PKC	Protein kinase C
PlA	Palmitic acid
PLA_2	Phospholipase A_2
PLA_2G3	secretory phospholipase A_2 group III
PLA_2G4A	cytoplasmic phospholipase A_2 group IVA
PLA_2G4D	cytoplasmic phospholipase A_2 group IVD
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
Po	Peanut oil
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor-gamma
PPRE	Peroxisome proliferator response element
PS	Phosphatidylserine
pTLE	Peanut total lipid extract
PUFA	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation endproducts
SB	Stratum basale
SC	Stratum corneum
SFA	Saturated fatty acid
SG	Stratum granulosum
SM	Sphingomyelin
SMase	Sphingomyelinase
$sPLA_2$	Secretory Phospholipase A_2
SPT	Skin prick test
SS	Stratum spinosum

SUMO	Small Ubiquitin-like Modifier
TAG	Triacylglycerol
TCR	T cell receptor
TEWL	Transepidermal water loss
TF	Transcription factor
TGF	Transforming growth factor
TLE	Total lipid extract
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoietin
UCA	Trans-urocanic acid
UFA	Unsaturated fatty acid

WORD COUNT

ACKNOWLEDGEMENTS 2
COVID IMPACT STATEMENT 3
ABSTRACT 4
ABBREVIATIONS 6
WORD COUNT 10
INTRODUCTION 16
1.1. PEANUT ALLERGY EPIDEMIOLOGY 16
1.1.1. Overall prevalence and trends 16
1.1.2. Explanations for trends in prevalence 17
1.2. PEANUT ALLERGY CLINICAL FEATURES AND DIAGNOSIS 18
1.2.1. Clinical features 18
1.2.2. Diagnosis 19
1.2.3. Current treatments for peanut allergy 20
1.3. PEANUT ALLERGEN SENSITISATION 21
1.4. PEANUT ALLERGENS 23
1.4.1. Overview 23
1.4.2. Lipid allergens 24
1.4.3. Peanut proteins 25
1.5. THE SKIN BARRIER: A FOCUS ON EPIDERMAL LIPIDS AND FILAGGRIN 27
1.5.1. The structure of the epidermis 27
1.5.2. Lipids in the healthy epidermis 27
1.5.3. Dysregulation of skin lipids in atopic dermatitis 29
1.5.4. The role of filaggrin in normal skin homeostasis 30
1.5.5. The role of filaggrin in atopic dermatitis and peanut allergy 32
1.5.6. The interaction between filaggrin and skin lipids 33
1.6. PHOSPHOLIPASE D 33
1.6.1. Overview of PLD 33
1.6.2. Structure and cellular localization of PLD 34
1.6.3. Function of PLD 36
1.6.3.1. Hydrolysis of phosphatidylcholine 36
1.6.3.2. Hydrolysis of sphingomyelin 39
1.6.3.3. Transphosphatidylation of phosphatidylcholine 40
1.6.4. Aquaporin-3: a membrane transporter required for PG production in skin 42
1.6.4.1. AQP structure and function 42
1.6.4.2. The interaction between AQP3 and PLD 44
1.6.4.3. AQP3 and allergic disease 45
1.6.5. The role of PLD in regulating immune system health and disease 46
1.7. CD1A 47
1.7.1. Overview of CD1 molecules 47

1.7.2. CD1a structure, expression and cellular localisation 47
1.7.2.1. CD1a structure 47
1.7.2.2. CD1a cellular expression 48
1.7.2.3. Regulation of CD1a in antigen-presenting cells 49
1.7.2.4. Intracellular trafficking of CD1a 50
1.7.3. CD1a lipid antigen presentation 51
1.7.3.1. Overview of CD1a lipid antigens 51
1.7.3.2. Molecular dynamics of CD1a antigen presentation 51
1.7.4. The relevance of CD1a in allergic disease 54
1.7.5. PA and PG as candidate CD1a-mediated allergens 55

AIMS AND OBJECTIVES 57

MATERIALS AND METHODS 59

1.8. REAGENTS 59

1.9. RECOMBINANT PROTEIN EXPRESSION 61

1.9.1. Expression of recombinant filaggrin 61
1.9.2. Expression of recombinant CD1a 61

1.10. CELL CULTURE 61

1.10.1. Cell culture media 61
1.10.2. Cell lines 62
1.10.3. PBMC isolation from human blood 63
1.10.4. Isolation of human monocytes and T cells 63
1.10.5. Generation of MUTZ3-derived Langerhans cells 64
1.10.6. Generation of monocyte-derived dendritic cells 65

1.11. LIPID EXTRACTION FROM MODCS 65

1.12. MEASUREMENT OF PPAR-GAMMA ACTIVITY 66

1.12.1. Nuclear protein extraction 66
1.12.2. Measurement of PPARγ activity 66

1.13. MEASUREMENT OF MODC PHAGOCYTIC ACTIVITY 67

1.14. DISSOLVING PEANUT LIPIDS, AND MISCIBILITY STUDIES 67

1.15. ISOLATING ANTIGENIC COMPOUNDS FROM RAW PEANUTS 68

1.15.1. Lipid extraction and miscibility studies 68
1.15.2. Protein extraction 68
1.15.3. Isolating oil bodies 69

1.16. ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAYS 70

1.16.1. General technique 70
1.16.2. Variations in technique 73

1.17. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) 74

1.18. QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QPCR) 75

1.18.1. mRNA extraction *75*
1.18.2. cDNA synthesis *75*
1.18.3. qPCR *76*
1.19. FLOW CYTOMETRY 77

1.20. CELL-FREE BIOCHEMICAL EXPERIMENTS 78

1.20.1. Assessment of peanut PLD hydrolysis activity *78*
1.20.2. Assessment of peanut PLD transphosphatidylation Activity *79*
1.20.3. Assessment of additional enzymatic activity of peanut PLD *79*
1.21. CELL-BASED BIOCHEMICAL TESTS 80

1.21.1. Assessment of PA production in MoDCs *80*
1.21.2. Assessment of PG production in MoDCs *80*
1.22. ISOELECTRIC FOCUSING 81

1.22.1. Assessment of CD1a-lipid binding *81*
1.22.2. Assessment of Ara h 1-phosphatidylglycerol binding *82*
1.23. DIFFERENTIAL GENE EXPRESSION ANALYSIS 82

1.24. STATISTICS 83

PHOSPHOLIPASE D GENERATES PRO-INFLAMMATORY CD1A LIGANDS IN PEANUT ALLERGEN-SENSITISED T CELLS 84
1.25. CURRENT KNOWLEDGE 84

1.25.1. The role of phospholipase enzymes in allergic disease *84*
1.25.2. Peanut-derived PLD produces candidate CD1a antigens *85*
1.25.3. Objectives *88*
1.26. RESULTS 88

1.26.1. Peanut PLD inhibits CD1a-reactive T cells derived from healthy individuals *88*
1.26.2. Peanut PLD produces CD1a ligands through enzymatic action to promote an inflammatory phenotype in T cells derived from peanut-allergic patients *91*
1.26.3. Peanut PLD-derived CD1a ligands do not promote a Type 2, Type 17 or Type 22 cytokine response in T cells *95*
1.26.4. Peanut PLD produces the CD1a-binding phospholipids, PG and PA, in MoDCs *98*
1.26.5. Filaggrin inhibits CD1a-dependent inflammation by peanut PLD *103*
1.26.6. Peanut PLD upregulates AQP3 expression in antigen-presenting cells, which is required for CD1a-reactivity in peanut-allergic patients *106*
1.26.7. Peanut PLD possesses multimodal enzymatic activity *109*
1.27. DISCUSSION 110

1.27.1. Characterising the functional response of peanut PLD in healthy T cells *110*
1.27.2. Peanut PLD promotes CD1a-reactive T cell responses in peanut-allergic patients *113*

1.27.3. Peanut PLD promotes a Type I-only response in CD1a-reactive T cells: roles in disease pathogenesis 115
1.27.4. Filaggrin insufficiency in AD may promote generation of PA-reactive T cells 116
1.27.5. Upregulated AQP3 in atopic skin may drive production of PG-reactive T cells 118
1.28. CHAPTER SUMMARY 119

PEANUT ALLERGY IS ASSOCIATED WITH ELEVATED PA- AND PG-REACTIVE T CELLS 120
1.29. CURRENT KNOWLEDGE 120

1.29.1. Peanut proteins and lipids drive allergic responses 120
1.29.2. The potential for peanut lipids to modulate CD1a mechanisms of disease 121
1.29.3. Peanut proteins as potential modulators of CD1a-reactive responses 124
1.29.4. Objectives 125
1.30. RESULTS 126

1.30.1. Peanut oil and total lipid extract from peanuts do not stimulate T cells via CD1a 126
1.30.2. PG inhibits IFN-γ production in CD1a-restricted T cells from healthy controls 131
1.30.1. PG and PA stimulate IFN-γ production in peanut-allergic CD1a-restricted T cells 135
1.30.1. Peanut-derived protein extracts, skin prick tests and oil bodies do not stimulate CD1a-reactive T cell responses 139
1.30.2. Ara h 1 binds PG to elicit CD1a-reactive T cell responses 142
1.31. DISCUSSION 146

1.31.1. Pure peanut lipids do not stimulate CD1a-reactive responses in healthy T cells 146
1.31.2. PG inhibits IFN-γ production in CD1a-restricted T cells from healthy controls 149
1.31.3. PG and PA stimulate CD1a-restricted responses in peanut-allergic patients 151
1.31.4. Peanut protein extracts, skin prick tests and oil bodies do not act via CD1a 153
1.31.5. Ara h 1 enhances CD1a-reactivity, potentially through binding PG 154
1.32. CHAPTER SUMMARY 157

HUMAN PLD ACTIVITY REGULATES CD1A EXPRESSION IN DENDRITIC CELLS 158
1.33. CURRENT KNOWLEDGE 158

1.33.1. Overview of CD1a-regulatory mechanisms 158
1.33.2. Endogenous PLD may modulate CD1a expression 162
1.33.3. Objectives 163
1.34. RESULTS 165

1.34.1. Endogenous PLD activity is required for CD1a expression in MUTZ3-derived LCs 165
1.34.2. PLD2, but not PLD1, inhibition reduces CD1a expression in MoDCs 174
1.34.3. PLD2 inhibits PPARγ activity, allowing production of MoDCs which can induce CD1a-reactive T cell responses 179
1.35. DISCUSSION 184

1.35.1. PLD drives expression of CD1a in LCs 184

1.35.2. PLD2 activity is required for CD1a expression in DCs 189
1.35.3. PLD2 inhibits PPARγ and modulates DC function 191
1.35.4. Chapter summary 194

DISCUSSION AND FUTURE PERSPECTIVES 195

1.36. UNTANGLING THE SOURCE OF CD1A-REACTIVITY IN PEANUT ALLERGY 197

1.37. CHARACTERISING CD1A-REACTIVE T CELLS IN PEANUT-ALLERGIC PATIENTS .. 199

1.38. REGULATION OF CD1A IN DC SUBSETS 203

1.39. USING MOUSE MODELS TO EXPLORE PLD-BASED THERAPEUTICS 205

CONCLUSION 210

BIBLIOGRAPHY 212

INTRODUCTION

1.1. Peanut allergy epidemiology

1.1.1. Overall prevalence and trends

Food allergy prevalence is increasing in Western countries, with 8% of young children affected in the UK and up to 10% in Australia, with an increasing trend during the past 30 years (1-3). In the UK, hospitalisation rates due to food-induced anaphylaxis rose from 1.2 to 2.4 cases per 100,000 patients between 1998 and 2012 (4). Common food allergens which account for the most serious disease burden include peanuts, tree nuts, cow's milk, soy, wheat, sesame, eggs, fish and shellfish (5, 6). The population-based HealthNuts study recruited 5276 children over a 12 year period and reported a peanut allergy prevalence of 3% of infants at the age of 12 months (7). Alarmingly, this prevalence has doubled in children in the UK over the past decade (2).

Peanut allergy is affected by risk criteria including age, sex, ethnicity, genetics, demographics, environmental and concomitant health factors. Multiple studies have evidenced that prevalence is highest in young children and adolescents compared to adults (8-10). Male gender has been listed as a clear risk factor for infants developing food allergy (7, 11). This switches to a female predominance in adults, potentially due to induction of Th2 polarisation and mast cell degranulation by oestrogens during the oestrus cycle (12, 13). Data also suggest a racial disparity, with elevated rates of food allergy in Black and Asian, compared to White, children (14). Moreover, obesity is an important factor, given that a high body mass index positively correlates with serum total IgE in patients with food allergy, with adipokines likely playing a role (15).

Food allergy has been reported to be prevalent in 20-80% of atopic dermatitis (AD) cases (16). Most food-allergic children have a history of AD that usually predates development of the food allergy (6, 17). The HealthNuts study found that 50% of infants with eczema develop food allergy by 1 year of age (18). The Melbourne Atopy Cohort study recruited 620 children at birth and found that at 1 year of age, children with mild and severe eczema had an increased risk of IgE food sensitisation (three- and six-fold respectively) compared to children without eczema (19). Correspondingly, 95% of peanut-allergic individuals suffer from atopic diseases including eczema (20).

1.1.2. Explanations for trends in prevalence

Food allergies, as with many chronic diseases, result from a complex interplay between genetics and environmental exposure during early life. Environmental drivers which may contribute towards the rising trend in food allergy include increasing rates of AD, urbanisation, microbial exposure, antibiotic usage, allergen exposure and changing dietary habits. Population-wide studies have demonstrated a positive correlation between an urban lifestyle and development of food allergies (21). This significant difference remained apparent even after adjusting for covariates such as income, age and ethnicity. Increasing antibiotic usage may also play a role, given that the hygiene hypothesis states that inadequate early exposure to infectious microorganisms stunts immune development, consequently leading to atopic diseases (22, 23). Finally, Western diets are markedly high in fat and low in fibre (24). High fat diets have been shown to promote gut colonization by *Bacteroides* species, which have been implicated in food allergy pathogenesis. Conversely, dietary fibre suppresses food-allergic responses in mice, possibly through metabolism by gut bacteria to short chain fatty acids (SCFAs), which induce an acidic environment and promote food allergen tolerance (25, 26). Other pro-allergic dietary trends include a high ratio of omega-6 to omega-3 fatty acids and reduced intake of antioxidants (27, 28).

Despite extensive prevalence data, food allergy is heterogenous in terms of manifestations and severity, and studies vary in terms of inclusion criteria and methodology. Moreover, there is a poor correlation between self-reported and challenge-confirmed food allergy (29). Nonetheless there are sufficient data documenting the clear increase in peanut allergy, necessitating further understanding of its pathogenesis to inform new treatments.

1.2. Peanut allergy clinical features and diagnosis

1.2.1. Clinical features

Peanut-allergic individuals usually have a personal or family history of atopic disease, with the initial allergic reaction typically developing in infants between 14 and 24 months (20). Clinical manifestations primarily involve the skin, respiratory tract and gastrointestinal tract, as shown in *Table 1* (30, 31). Immediate IgE-mediated reactions occur within a few minutes to hours after ingestion, and patients most commonly present with acute urticaria with or without angioedema. Cutaneous exposure rarely results in a severe reaction, and immediate symptoms after ingestion are often oral itching and lip swelling (32). Whilst many allergic reactions are mild, they can be moderate or severe, ranging from vomiting and abdominal pain to life-threatening anaphylaxis (33). In the United Kingdom, nut allergy causes the highest rate of anaphylactic death in adolescents and young adults (4). Reactions are unpredictable however severity may be modulated by the amount of peanut consumed or concomitant health factors such as asthma status.

Target system	% of patients
Cutaneous Urticaria, erythema, angiodema	89
Respiratory Wheezing, stridor, cough, dyspnea, throat tightness, nasal congestion	42
Gastrointestinal Vomiting, diarrhea, abdominal pain	26
Cardiovascular Hypotension, arrhythmia, cardiac arrest	4

Table 1: **Frequency of target system involvement in first presentation of peanut allergy.** *Modified from (31).*

1.2.2. Diagnosis

An accurate clinical history is the foundation of diagnosing peanut allergy, given that patients who describe two isolated, and compelling, reactions have an 80% probability of being affected (34). Young children may struggle to describe their symptoms and clinicians must monitor closely for mild signs, such as spitting out food, and more severe symptoms, such as hoarseness secondary to airway narrowing (35). Great care must be taken to differentiate primary nut allergy from oral allergy syndrome (OAS), which arises in patients sensitized to pollens who display cross-reactivity with specific fruits, vegetables or nuts (36).

In suspect cases, diagnosis can be confirmed using skin prick tests (SPTs), measurement of allergen-specific IgE, component allergy testing and occasionally oral food challenge (37). SPTs involve placing a drop of peanut extract onto the forearm, and then gently pricking the skin to observe a reaction (38). Combined with a relevant clinical history, a resulting wheal size of $\geq$ 3mm is diagnostic for peanut allergy.

Serum-specific IgE (sIgE) can be a useful diagnostic modality in cases where total peanut sIgE $\geq$ 15 KU/L (positive predictive value of 90%) (39). However, it is less clear-cut at lower concentrations of sIgE, with one study reporting 40% false positive and 22% false negative diagnoses when using nut sIgE (33). During recent years, component allergy testing has been used in food allergy diagnosis and involves measuring sIgE against specific allergenic proteins, often using ImmunoCAP technology (40). sIgE directed against the major peanut allergen Ara h 2 is more sensitive and specific than total peanut IgE, however Ara h 1, 3 and 6 are less predictive (41, 42). The major peanut allergens will later be reviewed in depth (see *Chapter 3.5.3*).

Oral food challenge is a less commonly employed method of definitively diagnosing peanut allergy (43). Challenge doses are tailored based on patient presentation and clinical history, starting with a low dose (e.g. 1/16th of a peanut) and increasing sequentially with 15-30 minute observation intervals (33). This is an effective diagnostic method however it should only be performed in select cases in a clinical setting with access to appropriate medical equipment, given the risk of a severe reaction.

1.2.3. Current treatments for peanut allergy

Until recently, management of peanut allergy consists of two primary strategies: strict peanut avoidance and carriage of self-injectable epinephrine (44). Educating patients and families is important in helping them identify foods containing peanuts and environmental risk of exposure, such as in schools or airplanes. Allergen avoidance has clear effects on daily activities and quality of life, and new treatments are therefore highly sought (45). A peanut-derived biological protein (AR101) was recently identified as a candidate for oral immunotherapy and underwent a Phase III clinical trial (46). 551 peanut-allergic participants were administered either active drug (escalating dose up to 300mg/day) or placebo for a period of 6 months. Individuals were challenged with peanuts at the end of the study, with significantly fewer participants in the active-drug group developing moderate and severe reactions (25% and 5%) compared to the placebo group (59% and 11%). In 2020, Hourihane et al reported another AR101 trial across 227 patients in multiple European hospitals (47). By the end of the 6-month escalating dose period, patients tolerated 1000mg of AR101 protein, with clinically significant improvements in participants aged 8-12 years. Adverse events were reported in all cases, however these were mainly mild or moderate. Authors concluded that AR101 induces rapid desensitisation to peanut protein, and has been authorized by both the US Food and Drug Administration and European Medicines Agency (48, 49). Whilst AR101 offers promise of an option for managing patients, there were

still non-responders in the active-drug group, indicating that AR101 does not provide a definitive cure and at best, provides an increase in tolerated amount of ingestion.

1.3. Peanut allergen sensitisation

Peanut allergy is characterised by an IgE-mediated type 1 hypersensitivity reaction. An allergic reaction occurs when peanut allergens penetrate mucosal barriers and encounter cell-bound IgE. Peanut-specific IgE binds to high-affinity receptors on basophils, mast cells and dendritic cells (DCs). Subsequent cell activation leads to degranulation and release of cytokines and chemokines. This facilitates further inflammatory cell recruitment and amplification of the response (50).

Several studies have investigated the routes of sensitisation to peanut allergens (51). Initially it was proposed that peanut allergy is the result of early consumption of food allergens, as evidenced in some mouse and human studies (52, 53). However, 72-81% of clinical manifestations occur on the first known exposure to peanut, suggesting prior occult sensitization (54). Additional routes of exposure include *in utero*, via breast milk and via environmental exposure (52). An epicutaneous route of sensitisation to peanuts has long been hypothesized (55). Prolonged epicutaneous exposure to peanut protein in mice and subsequent oral challenge leads to potent Th2-type immunity with high levels of IL-4 and serum IgE (56). Tordesillas et al observed that repeated topical exposure of peanut allergens to the skin of mice led to sensitization and anaphylaxis upon re-challenge (57). Moreover, topical application of peanut extract induces cytokine expression in murine skin and cultured human keratinocytes. A human cohort study demonstrated that the use of peanut-containing topical creams was more commonly used in children that subsequently developed peanut allergy compared to control infants (58). These data provide evidence that peanut sensitisation may occur in part via the skin.

It is well known that skin barrier dysfunction contributes to the pathogenesis of AD (59). A major hypothesis is that skin barrier dysfunction in atopic individuals facilitates entry of food allergens, promoting epicutaneous sensitization (60). Perkin et al. demonstrated in three-month-old infants that there was a strong association between AD and food sensitisation, including to peanuts (2016). Another group demonstrated that increased transepidermal water loss (TEWL) at birth and two months of age is predictive of eczema at 1 year of age (61). The same group went on to show that increased TEWL at birth was predictive of food allergy at 2 years of age (62). Disruption of the stratum corneum (SC) by mechanical tape stripping stimulates the production of proinflammatory cytokines including TNF-α and IL-1β and may promote maturation of Langerhans cells (LCs) into immunostimulatory DCs (63, 64). This effect is also seen when lipids are extracted from the skin using organic solvents. Mouse models have demonstrated that disruption of the SC promotes epicutaneous immunisation with peanut proteins, migration of antigen-loaded LCs to local lymph nodes and a dominant Th2 response (65).

A recent large-scale randomised trial investigated the effects of early peanut consumption on subsequent development of peanut allergy (2). 640 infants aged 4-11 months with severe eczema were placed in one of two groups during the first 5 years of life. One group were fed at least 6g of peanut protein per week, whilst the other group avoided all sources of peanut. At 60 months of age, the prevalence of peanut allergy was 13.7% in the avoidance group and 1.9% in the consumption group ($p<0.001$). Overall, this introduces the Dual Allergen Exposure Hypothesis where early epicutaneous exposure to peanut antigens leads to sensitisation, and early oral exposure is more important in promoting tolerance (66).

Peanut protein is detectable in the home environment, spreading quickly and easily around the home via direct contact (67, 68). Environmental allergen concentration may play a role in the pathophysiology and treatment of asthma (69-72). This exemplifies the complex nature of atopic disease and the interplay between genetic, immunological and environmental factors. It would be therefore prudent to further investigate the effects of environmental sensitisation via the skin in the context of peanut allergy.

1.4. Peanut allergens

1.4.1. Overview

The peanut belongs to the legume family of plants, and consists of a shell, seed coat and cotyledon (the main edible part) (73). The cotyledon is composed of 49% fat, 26% protein and 11% fibre (74). Approximately 95% of total peanut lipid content is stored as triacylglycerols (TAGs), with the remaining content being present as free fatty acids (FFAs), diacylglycerols (DAGs) and phospholipids (*Table 2*) (75). Lipids are stored in oil bodies (OBs; oleosomes), intracellular spherical organelles composed of an inner TAG core and an outer monolayer of phospholipids interlinked with structural proteins including oleosins. (76). Protein composition includes seed storage proteins, non-specific lipid-transfer proteins (nsLTPs) and an array of enzymes including phospholipases (77). These proteins serve to store organic compounds, transfer lipids inside the cell and protect against drought stress, respectively.

Effect of Postharvest Treatment on Total Phospholipids[a]

	Phospholipid (% area)					Total phospholipid (mg/100g DWT)
Treatment	PA	PG	PE	PI	PC	
Control	2.2	2.5	13.3	15.7	66.4	500
Immature	4.5	2.3	14	7.6	71.7	700
Heat cured	9.5	1.1	16	15.4	58.1	900
Freeze-damaged	28.3	14.1	15.2	33.5	8.8	250

[a]PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidycholine; DWT, dry weight

Table 2: **Example total phospholipid content in peanuts**. *Modified from (75).*

1.4.2. Lipid allergens

There are no published data describing the allergenic effects of peanut-derived lipids. However, peanut seeds comprise approximately 50% oil, and serve as a potential reservoir for lipid antigens (78). Most peanut fatty acid (FA) content consists of oleic (OLA; $C_{18:1}$), palmitic (PlA; $C_{16:0}$) or linoleic acids (LA; $C_{18:2}$); 95% of which are incorporated within TAGs (79, 80). FFAs and DAGs are detectable, but at a much lower level (0.7% and 0.5%, respectively) (81). FA ratios differ between peanut cultivars; for example, species from the USA contain higher levels of OLA (35.6%) than those from Africa (13.9%) (79, 82). During the past 20 years, high-oleate peanuts (up to 80%) have been selected for in breeding lines given their good frying stability (80). Moreover, oleate content increases with peanut maturity
(83). Given that OLA is a CD1a ligand (discussed later in detail), these observations may provide a novel rationale for recent increases in peanut allergy prevalence in Western countries (84).

In comparison to FAs, phospholipids from the cell membrane account for only 0.5% of peanut oil (Po) lipid content (75). However, important phospholipid constituents include the CD1a ligands phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG), in addition to phosphatidic acid (PA) (85). Importantly, peanut phospholipids contain unsaturated acyl chains, making them particularly suitable CD1a ligand candidates (75). Combined, these findings signal the potential for peanut lipids to act as exogenous allergens.

1.4.3.Peanut proteins

To date, the World Health Organisation has classified 17 peanut proteins as allergens that lead to a specific IgE response. Ara h 1, 2, 3 and 6 (seed storage proteins) are all classified as major allergens in the UK as they lead to an IgE response in most allergic patients (86, 87). Ara h 1 is a 7S vicilin-like globulin with high thermal stability, Ara h 2 is a conglutin with trypsin inhibitor activity, and Ara h 3 is a glycinin (88, 89). Ara h 2 and Ara h 6 share a 59% sequence identity and exhibit some cross-reactivity (90, 91). Such factors contribute towards the high allergenicity of these proteins, for example through evasion of host defence by the gastrointestinal system.

The remaining allergens are considered minor allergens as they less frequently result in fatal anaphylaxis (92). Importantly, roasting has been shown to increase allergenicity in a number of these proteins, likely through structural changes and unmasking of epitopes during cooking (the 'Maillard reaction') (93). A common feature of many food allergens, including nsLTPs, prolamin storage proteins, 2S albumins and some cupins, is their ability to bind lipids (94). Ara h 8 shares 48% sequence identity with the Birch pollen allergen Bet v 1, and is important in patients with birch pollen- associated peanut allergy, often resulting in OAS (95). At its core, Ara h 8 has a large hydrophobic cavity which can strongly bind a range of lipids (96). Lipid binding alters the structure of the protein, increasing proteolytic and thermal stability and allowing safer transit through the gastrointestinal tract (97). Consequently, patients' sera showed greater IgE reactivity when exposed to Ara h 8 that had undergone roasting or formed lipophilic associations. Angelina et al. noted a similar effect with Ara h 1, which bound PG to confer resistance to gastric digestion by human monocyte-derived DCs (MoDCs) and enhanced stability to microsomal degradation (98). Furthermore, peanut lipids facilitated a

proinflammatory environment by increasing the IL-4/IL-10 ratio and stimulating IL-1β production by MoDCs.

Recently, oleosins have been identified as important allergens in several plant allergies (99-101). Oleosins are highly lipophilic proteins with a large hydrophobic domain, which function to stabilise plant OBs and regulate lipid content through enzymatic, including phospholipase A_2 (PLA_2), activity (102). The oleosin Ara h 15 has been identified as a clinically relevant peanut allergen (103). 52 peanut-allergic patients displayed IgE reactivity to at least two different peanut oleosins. Conventional SPTs undergo aqueous extraction during formulation, which eliminates the lipophilic oleosin fraction. Given the number of false negative responses, the authors concluded it was highly probable that patients with a history of peanut allergy and negative diagnostic tests may be oleosin IgE- positive. Literature is currently limited with respect to the exact mechanism by which patients become sensitised to oleosins. However, a murine study demonstrated that the coadministration of 30% corn oil and 3% FA ester significantly increases plasma uptake of the oleosin Gly m Bd 30k, a major soy bean allergen (104). Furthermore, artificial OBs may be used to entrap substances and facilitate faster uptake via the gut and skin (105, 106). This suggests a lipid carrier- mediated mechanism of allergen uptake and raises the question of whether OBs may act as a transfer vehicle for a range of other non-oleosin plant allergens.

Plant nsLTPs function to transfer phospholipids and galactolipids across membranes (107). The nSLTP Ara h 9 is a major allergen in peanut-allergic patients from the Mediterranean region (108). This differs from the UK, where fewer patients show IgE reactivity to Ara h 9 (109). A study in Spanish patients demonstrated 38.1% cosensitisation to peach nsLTP Pru p 3, suggesting Pru p 3 may play a role in sensitising

Mediterranean populations (110). Interestingly, lipid ligand binding enhances the susceptibility of wheat nsLTP to proteolysis and gastric digestion through displacement of a tyrosine residue from the internal hydrophobic cavity (111). In summary, lipids may play an important role in positively and negatively regulating the allergenicity of proteins, depending on ethnicity, environmental exposure and the source of allergen. To further understand the interplay between peanut constituents and the epidermis, it is necessary to discuss the structure and function of the skin barrier.

1.5. The skin barrier: a focus on epidermal lipids and filaggrin

1.5.1. The structure of the epidermis

The skin barrier comprises the outer layer of the epidermis, the SC, and serves as a first line defence against pathogens and allergens (*Figure 1*) (112). Each layer of the epidermis represents a different stage of keratinocyte differentiation, with a tightly controlled calcium gradient regulating terminal differentiation: low concentrations in the stratum basale (SB) and stratum spinosum (SS), high concentrations in the stratum granulosum (SG), and the lowest levels in the SC (113, 114). In the SC, terminally differentiated keratinocytes extrude their nuclei and undergo cell death to become corneocytes, which are sealed together by corneodesmosomes containing the adhesive protein, corneodesmin (115). The resulting cornified envelope (CE) is a complex structure of cross-linked proteins, lipids and corneocytes, commonly denoted “bricks and mortar”. Several SC proteins contribute to barrier defence, including loricrin, involucrin and, notably, filaggrin.

1.5.2. Lipids in the healthy epidermis

Lipids form a major component of the epidermal extracellular matrix and CE, serving to preserve barrier structure and reduce TEWL, offering chemical and mechanical resistance against microbes (115). The epidermal lipid milieu is refined by specific enzymatic activity and increases in calcium concentrations, inducing release of lipids from keratinocyte

lamellar bodies (116-118). Important lipids include ceramides (CERs; derived from cellular phospholipids), FFAs and cholesterol, evidenced in numerous studies. Unique epidermal CER species, including those which comprise very long chain FAs (up to 34 carbon atoms) attached to the sphingosine headgroup, confer the best barrier protection (119). Serine palmitoyl transferase initiates the processing of sphingolipids into CERs, and inhibiting this reaction reduces lamellar body formation (120).

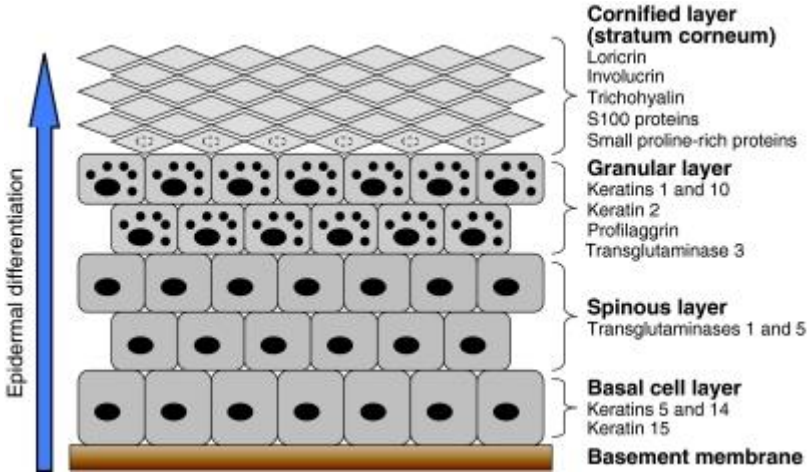


Figure 1. **The skin barrier: cross-section and layers of the epidermis**. *Obtained from (121).*

Cholesterol sulphate protects against corneodesmosome degradation by inhibiting serine proteases (122). Accordingly, 3 β-hydroxysterol-delta converts desmosterol to cholesterol, with mice deficient in this enzyme demonstrating a lack of epidermal cholesterol and lethal dermopathy (123). Similarly, mice deficient in stearoyl-CoA sesaturase 2, which desaturates epidermal FFAs, die shortly after birth (124). There are various homeostatic processes to protect against epidermal insult. Disruption of skin barrier function initiates a cascade in which β-glucocerebrosidase (GCS) and sphingomyelinase (SMase) drive the synthesis and secretion of lamellar bodies (120). In turn, this elevates cholesterol, FA and CER levels, restoring the permeability barrier. Importantly, providing exogenous calcium alters the normal gradient, inhibiting this repair process. Epidermal secretory PLA_2

(particularly $sPLA_2$ group IIF) also regulates this process through production of phospholipids and FFAs; the latter contributing to SC acidification and optimal GCS and SMase activity (125). This further evidences the negative effect of the elevated pH of atopic skin.

Extracutaneous lipids also play a role in lamellar body formation. Dietary essential fatty acids (EFAs) are present in the SC, and whilst their mode of entry is unknown, they may be shuttled by low-density lipoprotein receptors and FA transporters given the expression of these proteins in keratinocytes and the epidermis (human and murine), respectively (126, 127).

Skin surface lipids are known to have antimicrobial effects, with saturated FAs (SFAs) and unsaturated FAs (UFAs) exhibiting potent activity against gram-positive pathogens such as *S. pyogenes* and *S. aureus* (128). Lauric and sapienic acid (hydrolysis products of sebum-derived triglycerides) exert marked germicidal activity, whilst phospholipids and sphingosine moieties have more moderate effects (129). In conclusion, lipids and their associated pathways offer structural and functional protection against external insults to the SC, such as penetration of allergens.

1.5.3. Dysregulation of skin lipids in atopic dermatitis

Patients with AD exhibit an altered epidermal lipid profile, including a reduced average CER chain length and increases in the CER subclasses [AH] and [AS] (130, 131). Topical application of CER[EOS] and CER[NS] was shown to restore organization of skin lipids in a barrier repair model, demonstrating the functional interaction between CERs and the SC lipid matrix (132). Mutations in the gene encoding ABHD5/CGI-58, a protein involved in the formation of acylceramides, results in Chanarin-Dorfman syndrome, which has an AD-like phenotype (133).

Similarly, FFAs demonstrate a shorter chain length in AD (134). Given their cellular toxicity, FFAs are stored in phospholipids and released by phospholipases, however this reaction is stunted in AD, providing a potential explanation for altered FFA content (135). Equally, incomplete degradation of phospholipids may give rise to additional pro-inflammatory lipid antigens, discussed in further detail later. Elevations in monounsaturated FAs (MUFAs) have been observed in human AD skin compared to healthy controls, and increased MUFAs were shown to disrupt the normal organisation of lamellar lipids (136, 137). However, a more recent study concluded that decreased MUFA content in AD drove pathogenic *S. aureus* growth, and whilst results appear contradictory, they suggest a strong role of FFAs in AD pathogenesis (138). The same study observed increased cholesterol-3-sulphate levels in AD skin, which positively correlated with *S. aureus* colonisation. Whilst epidermal lipid dysregulation is both a cause and consequence of skin barrier disruption, proteins are also vital in AD pathogenesis; notably, filaggrin.

1.5.4. The role of filaggrin in normal skin homeostasis

Filaggrin, or filament-aggregating protein, plays a crucial role in maintaining the strength and integrity of the skin (115). Filaggrin is synthesized from the giant precursor profilaggrin, which is extruded from keratohyalin granules in the SG in response to higher calcium levels (*Figure 2*) (139). Conversion from the proprotein occurs during terminal differentiation and involves a complex process of proteolysis and dephosphorylation to produce filaggrin monomers (140).

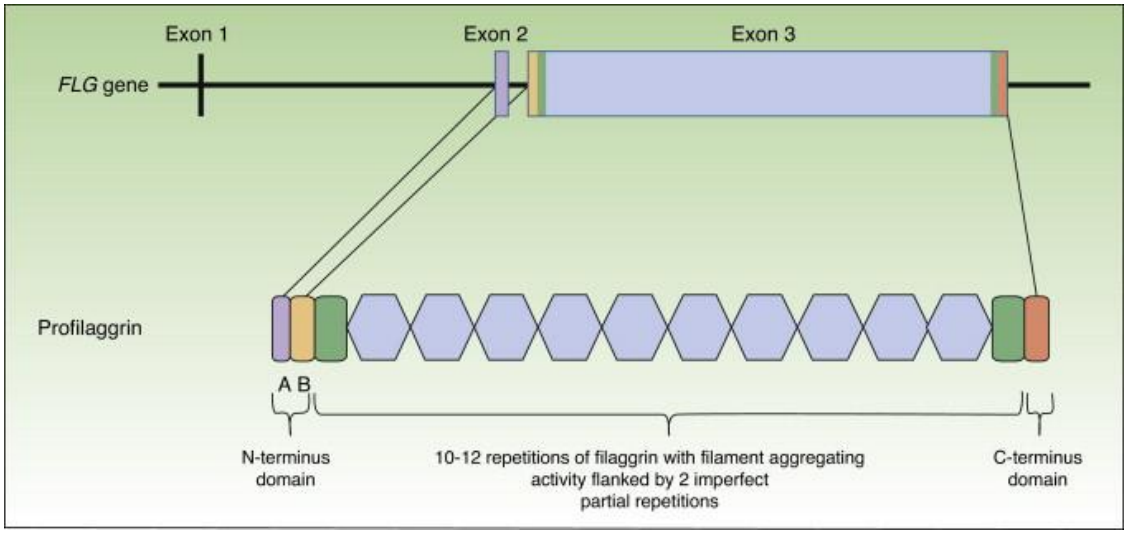


Figure 2. Structure of the *FLG* gene and profilaggrin: The *FLG* gene is situated in the epidermal differentiation complex (chromosome 1) and encodes profilaggrin, which comprises 10-12 repeated filaggrin units. Profilaggrin possesses a C-terminus domain with uncertain function and an N-terminus domain, which confers calcium binding and facilitates nuclear localisation upon cleavage of each filaggrin monomer. *Obtained from (141).*

A major function of filaggrin is the aggregation and compaction of keratin intermediate filaments into macrofibrils, producing an insoluble scaffold for the CE. Interactions between this keratin matrix and structural CE proteins are mediated by transglutaminases (142). In addition to its structural importance, filaggrin plays an active dynamic role in epidermal function. Proteolytic processing of filaggrin releases amino acids which are further converted into pyrrolidone-5-carboxylic acid (PCA) and trans-urocanic acid (UCA), essential in maintaining an acidic pH at the SC surface (4.5–5.3) (143, 144). Acidic conditions are vital in regulating enzymatic and metabolic activity, and for maintaining an antimicrobial environment by inhibiting growth of pathogens such as *S. aureus* (128, 145, 146). Equally, UCA is photoprotective against UV-B radiation (147). Finally, hygroscopic amino acid products are processed into natural moisturising factor (NMF), which prevents TEWL and adheres to non-pathogenic bacteria, inhibiting overgrowth of opportunistic *S. aureus* (148, 149). Thus, filaggrin works through a variety of processes to maintain normal epidermal function.

1.5.5. The role of filaggrin in atopic dermatitis and peanut allergy

Loss-of-function mutations in the filaggrin gene (*FLG*) are associated with a greater risk of having skin barrier dysfunction, AD and allergic sensitisation (150, 151). Disruption of normal filaggrin content leads to the loss of downstream products such as NMF, increasing TEWL and affecting the skin microbiome as previously discussed (152, 153). Reductions in PCA and UCA contributes towards the higher skin surface pH observed in AD, activating serine proteases which degrade corneodesmosomes and compromise CE integrity (154). In addition to structural effects, filaggrin deficiency promotes an excessive Th2 response by increasing the expression of epidermal thymic stromal lymphopoietin (TSLP) (155).

Up to 60% of Europeans with moderate-severe AD demonstrate *FLG* mutations, with R501X and 2282del4 being the most prominent (150, 152). Accordingly, filaggrin expression in umbilical cord blood is associated with development of AD in the first year of life (156). In the HealthNuts cohort, *FLG* null mutations were more common in food-allergic children compared to those without food allergy (7). A peanut-specific study found a strong association between *FLG* loss-of-function mutations and IgE-mediated peanut allergy, with odds ratios (ORs) of 1.9-5.3 in different ethnic cohorts (157). Interestingly this effect was maintained even after correcting for coexisting AD. Using data from the Manchester Asthma and Allergy study, Brough et al. determined that children who carried *FLG* mutations had an increased risk of peanut sensitisation and peanut allergy (158). They found that for every 2.7-fold increase in house dust peanut exposure during infancy, there was a six-fold increase in peanut sensitisation and 3.3-fold increase in development of peanut allergy at school-age. Considering other proteins, a recent genome-wide association study identified a strong association between SNPs in the epidermal adhesion protein integrin α6 (ITGA6) and peanut allergy (159). The same study included a meta-analysis of data from affected Canadian, American, Australian, German and Dutch populations, concluding that loci in or near ITGA6, MMP12/MMP13, ANGPT4, EXOC4 and C11orf30

were significantly associated. This provides evidence that individuals with a disrupted skin barrier are at a greater risk of peanut sensitisation. Investigations into peanut sensitisation should be carried out, using AD as a model for barrier dysfunction.

1.5.6. The interaction between filaggrin and skin lipids

There is limited evidence detailing modulation of skin lipid composition by filaggrin. Human *FLG*– skin constructs contain significantly reduced PCA and UCA filaggrin degradation products, leading to increased $sPLA_2$ group IIA activity to produce FAs and maintain skin acidity (160). This build-up in FFAs results in disordered intercellular lipid lamellae and permeability dysfunction. Filaggrin and lipids demonstrate a two-way functional relationship, given that cholesterol-3-sulphate (which is upregulated in AD) increases RORα expression and production of pro-filaggrin in normal human epidermal keratinocytes (138, 161). Conversely, in a German cohort of 49 patients, CER composition was altered by clinical AD status, rather than *FLG* mutations (162). Nonetheless, filaggrin has a functional role in skin lipid homeostasis, with impairment of these processes impacting on skin integrity, permeability and acidity. This functional role potentially extends to interacting with phospholipase D (PLD), a lipid-modulating enzyme expressed in peanuts.

1.6. Phospholipase D

1.6.1. Overview of PLD

Peanuts and other oil seeds possess a variety of lipases with roles including facilitating germination (163). PLD is a major plant lipid-degrading enzyme, initially identified in cabbage (164, 165). PLD has since been found in numerous organisms, such as plants (166, 167), yeasts (168), bacteria (169) and mammals (170, 171). More recently, PLD has been recognised as important in protecting peanuts against drought stress, a

major predisposing factor for aflatoxin contamination (172). Therefore, functional genomic studies have been implemented to investigate PLD gene mutations and protein function in drought-sensitive lines (173, 174). PLD is classically recognized as an enzyme which hydrolyses PC; a major component of biological membranes (*Figure 3*) (175). During the past decade, additional PLD reactions have been reported in plants, bacteria and humans, with recent data implicating a role in skin immunology (176). Reactions of relevance to peanut allergy will be discussed, including comparative structural biology across PLD isoforms.

1.6.2. Structure and cellular localization of PLD

Multiple PLD isoforms exist, although plants possess a more complex PLD gene family compared to mammals (177). To date, only PLD isoforms from *Streptomyces* sp. and *Salmonella typhimurium* have been resolved by xray crystallography (*Figure 4*) (178, 179). In summary, 18 α helices flank two β sheets, with two flexible loops facilitating docking onto the membrane for the hydrolysis reaction to occur. Commonalities between crystal structures indicate these bacterial enzymes are active as bi-lobed monomers or dimers, and this is likely conserved in plants and mammals.

The two major isoforms in peanuts are PLDα1 and PLDα2, with PLD1 and PLD2 existing in humans. Human PLD1 and PLD2 have distinct subcellular localisations. PLD1 is predominantly located in perinuclear structures including Golgi, early endosomes, late endosomes and multi- vesicular bodies/lysosomes (180). PLD2 is found largely in the plasma membrane and appears to cycle via the early endosome compartment (181). Subcellular localisation is largely determined by 'PX' and 'PH' domains, which function to mediate protein binding to membrane lipids (181, 182).

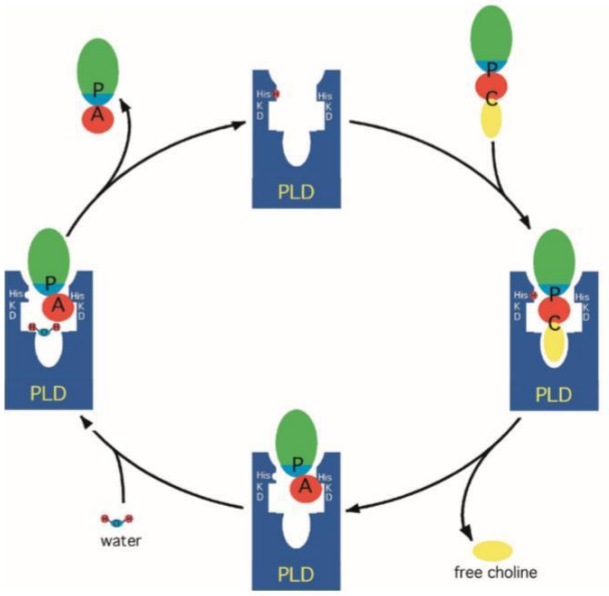


Figure 3. Diagram of phospholipase D (PLD) hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA). The amino acids of the two PLD catalytic domains are represented in white: histidine (His), lysine (K) and aspartic acid (D). PC enters the PLD active pocket and is hydrolysed to PA and choline. *Obtained from (183).*

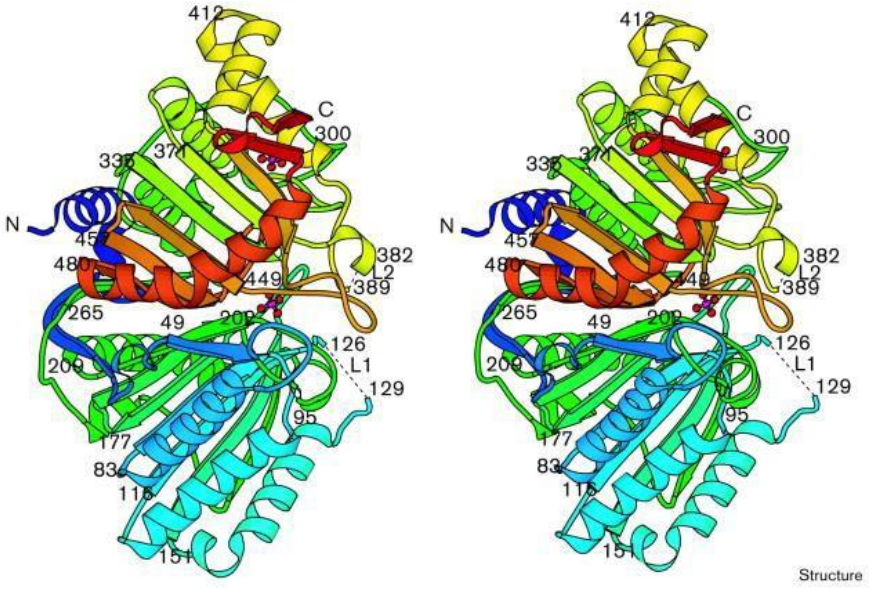


Figure 4. Stereographic illustration of phospholipase D (PLD) from *Streptomyces* sp. strain PMF coloured according to the amino acid sequence. Two flexible loops (labelled L1 and L2) facilitate docking onto the membrane when hydrolysis takes place. *Obtained from (179).*

Human and peanut PLD share approximately 26% protein sequence identity (Uniprot BLAST data, 2019). *Figure 5* is a diagram of the key components in the primary protein sequence of human PLD1 and PLD2 (184). Importantly, both isoforms possess two H*X*K*XXXX*D motifs, embedded within the catalytic site. Such HKD motifs are conserved across all plant, yeast and mammalian PLD isoforms. The conserved histidine, lysine and aspartate residues are required for hydrolysis, possibly through forming a catalytic triad (185). This suggests that, despite taxonomic differences in PLD structure and function, peanut PLD could process PC of both plant and animal origin.

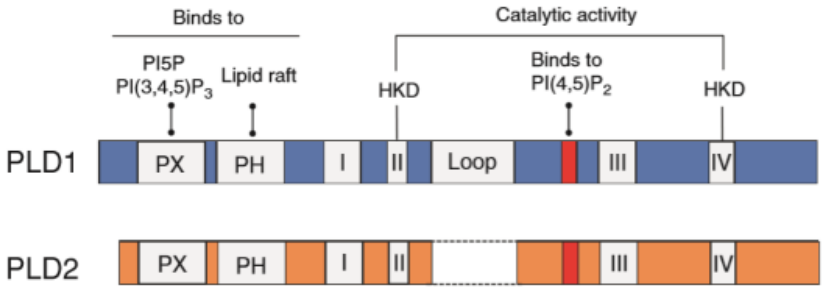


Figure 5. Key components in the primary protein sequence of human PLD1 and PLD2. Left hand side: the PX and PH domains are important for subcellular localisation. Right hand side: two HKD motifs exist in the catalytic site and are required for the hydrolysis reaction. *Obtained from (184).*

1.6.3. Function of PLD

1.6.3.1. Hydrolysis of phosphatidylcholine

PLD was initially identified as an enzyme which hydrolyses the terminal phosphodiester bond of the principal substrate PC to produce the signaling molecule PA and choline (186, 187). This core reaction is of great relevance in both plants and humans. The majority of peanut phospholipid content is PC and is concentrated at the cell membrane (75, 188). Equally, PC accounts for >50% of the phospholipid content in most eukaryotic membranes (189). In peanuts, drought induces PLD to degrade membrane phospholipids, leading to a subsequent accumulation of PA in stress signalling (190). Transgenic plant models have been used to further explore this phenomenon (191). In plants, PA has multifunctional roles in mediation of seed germination, the response to reactive oxygen species,

protecting against cold and drought tolerance, defence against microbes, root growth, cytoskeletal rearrangement and wound response (177). Furthermore, PLD has been described as a pathogenicity factor in *Mycobacteria tuberculosis, Streptomyces* spp., *Actinobacteria* spp., and a number of yeasts (192).

In humans, PLD enzymes play vital roles in cells through maintaining cell membrane integrity and participating in cell signalling. This is achieved through direct protein-protein interactions with a multitude of proteins, and through the actions of PA, which possesses a unique ability to modulate membrane curvature (184). In mammalian cells, PA produced by PLD has a variety of functions. The mammalian target of rapamycin (mTOR) is a protein kinase which regulates cell growth and proliferation via downstream targets. PLD1 produces PA which binds to mTOR and promotes protein synthesis to activate this signalling cascade (193). PA also has important roles in vesicular trafficking, secretion and endocytosis. For example, PLD1-produced PA may be essential for the secretion of insulin from pancreatic beta cells (194). Understanding of the exact mechanism of membrane cargo movement is limited. Potentially, PA activates type I PI(4)P 5-kinases to increase PIP_2 synthesis, which in turn regulates vesicular trafficking (195). Equally, PA may influence vesicle transport via altering membrane structure, evidenced by the important role of PA in tethering proteins, including Raf-1, to membranes (196). Importantly, PA and lysophosphatidic acid (LPA) have a reciprocal relationship in stabilising the cell membrane, with PA inducing spontaneous negative curvature and LPA inducing spontaneous positive curvature (*Figure 6*) (197). PA can be hydrolysed to LPA by PLA_2, and LPA can be converted to PA through the activity of lysophosphatidic acid acyltransferase (LPAAT) enzymes, thus representing a fluid system (198). Furthermore, lysophospholipases can interconvert different lysophospholipids, including processing of LPA to lysophosphatidylcholine (LPC) and vice versa. Given the

importance of the PLD-PA pathway in membrane transport, it is plausible that PLD and PA directly interact with antigen-presenting proteins, whilst indirect effects on membrane dynamics may influence ligand entry.

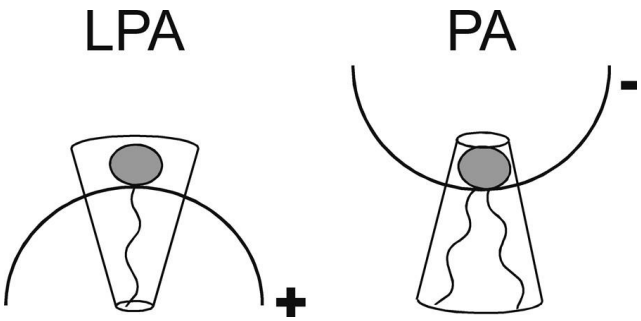


Figure 6. Phosphatidic acid (PA) induces spontaneous negative membrane curvature and lysophosphatidic acid (LPA) induces spontaneous positive curvature. *Obtained from (197).*

Both PA and PLD are involved in cytoskeletal rearrangements and cell differentiation. PLD can interact directly with several proteins, including actin and tubulin. Actin filaments regulate PLD activity in a polymerisation-dependent manner; monomeric G-actin inhibits PLD activity, whereas polymeric F-actin stimulates activity (199). Recently it was shown that tubulin binding to PLD2 inhibits muscarinic receptor-linked PLD2 activation (200). Interestingly, PLD has been shown to promote myogenesis through production of PA which directly binds to actin fibres (201). Effects differ between PLD isoforms, with overexpression of PLD1 promoting actin polymerisation, whereas increased expression of PLD2 has inhibitory effects. Given that filamentous proteins can modulate human PLD activity, and vice versa, this warrants further investigation of other protein candidates that are important in disease.

1.6.3.2. Hydrolysis of sphingomyelin

Another important PLD function is the hydrolysis of sphingomyelin (SM) to CER 1-phosphate and choline (202, 203). SM is detectable in peanuts and implicated in cold tolerance, however the role of PLD in its regulation remains to be explored (204, 205). In mammalian cells, SM accounts for a smaller proportion of the cell membrane than PC, mainly occupying the outer leaflet of the phospholipid bilayer (206). SM metabolism by a variety of SMases plays an important role in the regulation of CER content (207).

CERs are important bioactive molecules with multiple cellular functions including growth, proliferation and apoptosis (208-210). In humans, CERs consist of 6 isoforms, CERS 1-6, with specific tissue predilections (211-213). CERs act via several mechanisms, including binding protease cathepsin D, regulating protein kinase C (PKC) activity and activating the protein phosphatases PP1A and PP2A, which are central in the DNA damage response (214-216). *Streptococcus chromofuscus* PLD was shown to potently inhibit mouse epidermal keratinocyte proliferation and differentiation, given that CER products impede DNA synthesis (217). With respect to immune cell function, CERs regulate inflammation in multiple settings including cancer and inflammatory bowel disease (218). DCs function to present endogenous and exogenous antigens to T cells, mounting an immune response (219). Ceramides bear remarkable similarity to the lipopolysaccharide (LPS) component lipid A and, correspondingly, C_2-CERs inhibit DC macropinocytosis and antigen presentation to T cells (220). Equally, LPS induces CER production in macrophages, suggesting bacteria synergise with the host in order to propagate infection (221). CERs also play a role in the viral response. Exposure of DCs to Measles virus leads to ligation of intercellular adhesion molecule 3 (ICAM-3), inducing SMase activation and increased cellular CER content (222). In murine influenza models, C_8-CER induces DCs to produce the pro-inflammatory cytokine TNF-α and enhances T cell responses (223). Finally, the

glycolipid galactosylceramide (GalCer) is expressed in human DCs and binds the human immunodeficiency virus (HIV)-1 envelope glycoprotein gp120; and therefore may be implicated in viral entry (224). CERs, including GalCer, are important lipid antigens, which are discussed in *Chapter 3.7.3.1* (85, 225). It is therefore clear that CER products of the PLD reaction exert several cellular and immune effects.

CERs both positively and negatively regulate PLD under different conditions. In human fibroblasts, treatment with exogenous short-chain CERs and *S. aureus* SMase led to activation of PLD, and this was likely potentiated by the protein bradykinin (BK) (226). Conversely, Jones et al observed that CERs inhibited BK-induced activation of PLD in human skin fibroblasts (SF 155) (227). Moreover, CERs are potent inhibitors of PLD, as demonstrated in numerous studies (228, 229). The stimulatory and inhibitory activity of CERs on PLD function is dependent on specific factors including cell type and culture conditions, evidencing a tightly regulated axis.

1.6.3.3. Transphosphatidylation of phosphatidylcholine

In the presence of primary alcohols such as ethanol, glycerol or 1-butanol, PLD catalyses a transphosphatidylation reaction (230). Specifically, the phosphatidyl moiety of a phospholipid substrate is transferred to an alcohol to produce the corresponding phosphatidylalcohol. This reaction is well documented in mammals, plants, fungi, bacteria, and is demonstrated in *Figure 7* (231-238). In peanuts, a membrane-specific PLD specifically transphosphatidylates the sphingolipid glycosylinositol phosphoceramide (GIPC) to yield phytoceramide-1-phosphoalcohols (PC1P) (239). Another important product of transphosphatidylation within the skin is PG; this reaction requires recruitment of Aquaporin-3 (AQP3), which is further discussed in *Chapter 3.6.4* (240, 241).

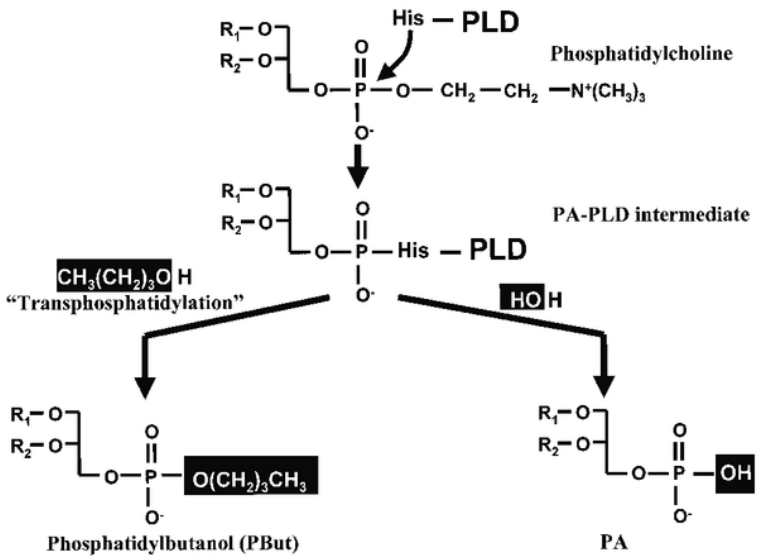


Figure 7. Phospholipase D (PLD) catalyses a two-step transphosphatidylation reaction. In this example, PLD catalyses the conversion of PC to phosphatidylbutanol (PBut), releasing free choline. The first step involves hydrolysis of PC, in which PA is covalently linked to PLD via a histidine residue. During the second step, PA is transferred onto the acceptor alcohol, butanol, to produce PBut (left). In the absence of a primary alcohol, H_2O acts as an acceptor molecule to produce free PA (right). *Obtained from (242).*

In murine and human studies, PG has been unveiled as an important mediator of skin health and disease. During normal epidermal turnover, PG species have distinct effects on regulating keratinocyte differentiation (243). In a murine model, PG species containing saturated or monounsaturated carbon chains stimulated the growth of slowly dividing keratinocytes; conversely, species with polyunsaturated carbon tails, such as soy PG, inhibited overproliferation. In a later study, the same group observed that soy PG reduced ear oedema in a 12-*O*-tetradecanoylphorbol-13-acetate (TPA) mouse psoriasis model, also reducing TNF-α levels and $CD45^+$ cell infiltration into the skin (244). Given the polyunsaturated structure of peanut PG, it is plausible to suggest that it exerts similar effects (245).

Efforts have been made to elucidate the mechanism of action of PG in regulating cell growth and development. PG is a precursor to cardiolipin, a key mitochondrial lipid which

has wide-ranging roles in ATP production and apoptosis (246). PG is also known to bind protein kinase C ßII (PKCßII) which mediates fundamental cellular process including mitosis (247). Moreover, PG is an important component of pulmonary surfactant and inhibits LPS-induced TLR4 activation (248). S100A9 is an antimicrobial peptide which is upregulated in psoriatic skin and animal models of psoriasis (249). In a murine imiquimod psoriasis model, topical PG inhibited S100A9-elicited TLR2/4 activation in primary keratinocytes, reducing production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (250). PG also blocked downstream activation of the transcription factor (TF) nuclear factor-kappa B (NF-κB), and similar results were observed in a macrophage cell line. Consequently, topical PG inhibited psoriatic lesions *in vivo*. Whilst PG is clearly involved in innate mechanisms of skin inflammation, further investigations are warranted to determine its additional effects via the adaptive immune system.

1.6.4. Aquaporin-3: a membrane transporter required for PG production in skin

1.6.4.1. AQP structure and function

AQPs are a group of small, hydrophobic membrane proteins, with at least 13 AQPs identified in humans (251). AQP monomers comprise six membrane-spanning helical domains, connected by two intracellular and three extracellular loops, and two smaller helices (*Figure 8*) (252). Loops 2 and 5 possess conserved asparagine-proline-alanine sequences (NPA motifs), creating a characteristic ‘hourglass’ shape. This complex structure contains both hydrophilic and hydrophobic elements, allowing bidirectional passage of water or glycerol (253). Consequently, AQPs 1, 2, 4, 5 and 8 transport water, whilst AQPs 3, 7, 9 and 10 have additional glycerol-selectivity and are denoted as “aquaglyceroporins”.

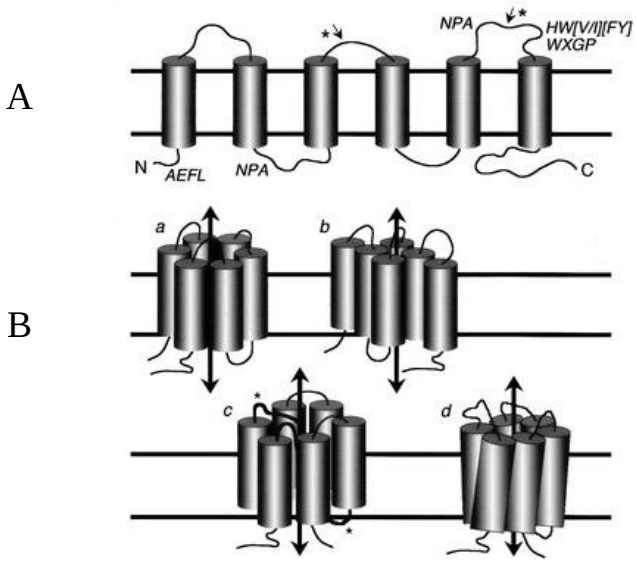


Figure 8. **The structure of Aquaporins**. Schematic showing: A) the 6 membrane-spanning domains, including the N and C-termini, NPA motifs and amino acids specific to aquaglyceroporins B) Schematic showing the different tertiary structures of the domains, with arrows representing the bi-directional passage of water. *Obtained from (252).*

AQP3 is a highly expressed in the epidermis, concentrated mostly in keratinocytes, but also in DCs, LCs, macrophages, eosinophils and neutrophils (254-258). Accordingly, AQP3 plays vital roles in skin homeostasis, transporting glycerol derived from the blood and sebaceous glands (259, 260). AQP3-deficient mice have dehydrated, inelastic skin compared with wild-type, in addition to a reduced SC glycerol content (256, 261). Furthermore, reductions in SC water and glycerol content are strongly correlated, and oral glycerol administration restores skin elasticity in AQP3-null mice (259). This validates glycerol as an important regulator of skin hydration via AQP3 transport, exemplified by the presence of glycerol in topical skin treatments.

AQP3 plays a vital role in wound healing. Following skin tape-stripping, AQP3-deficient mice exhibit delayed barrier repair, and this can be restored by oral administration of glycerol (259, 261). AQP3 deficiency impairs p38 mitogen-activated protein kinase (MAPK) cell signalling in keratinocytes, inhibiting proliferation in response to epidermal growth factor (EGF) (262). AQP3 is also required for proper keratinocyte migration during wound healing, potentially through water influx remodelling cellular shape. AQP3-

dependent modulation of wound healing has been identified in additional cell subsets, including colonocytes and corneal epithelial cells (263, 264).

1.6.4.2. The interaction between AQP3 and PLD

Initial studies demonstrated that human PLD2 colocalises with AQP3 in calveolin-rich microdomains in primary keratinocytes and HaCaT cells (241, 265). These plasma membrane microdomains contain abundant sphingolipids and cholesterol and express the structural protein caveolin-1 (266). Confocal microscopy and co-immunoprecipitation analysis strongly suggested a functional coupling mechanism whereby AQP3 chaperones glycerol onto PLD2 to facilitate production of PG (241). In turn, PG halts keratinocyte proliferation and stimulates early differentiation of keratinocytes in rapidly dividing cells (240). This mechanism was tested by co-transfecting keratinocytes with AQP3 and promoters of differentiation or proliferation (240). AQP3 co-expression decreased the activity of keratin 5, a promoter of proliferation, and increased the activity of keratin 10 and involucrin, both promoters of early differentiation. This emulated the effects of PG, and provision of exogenous PG or glycerol both led to inhibition of DNA synthesis in proliferative cells. More recently, Tsai et al re-expressed AQP3 in keratinocytes derived from AQP3-null mice (267). This induced PLD2-dependent expression of differentiation markers including keratin 10 and loricrin, with no effect on the proliferation marker, keratin 5. Combined, these data provide strong evidence for an AQP3-PLD2-PG signaling module existing exclusively within the skin (*Figure 9*). This evolutionary mechanism may therefore be harnessed by peanut-derived PLD in order to produce PG species.

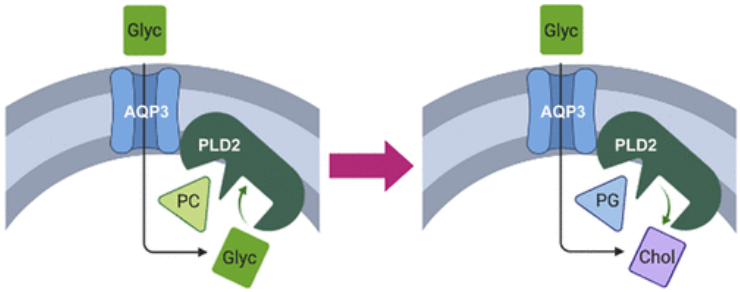


Figure 9. The epidermal Aquaporin-3-phospholipase D2-phosphatidylglycerol (AQP3-PLD2-PG) signaling module. AQP3 facilitates glycerol uptake and transport onto PLD2, which subsequently transphosphatidylates phosphatidylcholine (PC) to PG. *Obtained from (268).*

1.6.4.3. AQP3 and allergic disease

Given the physiological roles of AQP3, studies have been performed to investigate its involvement in AD, which is partly characterised by increased TEWL and aberrant wound healing responses. Using DNA microarray analysis, Olsson et al detected AQP3 expression is upregulated in eczema lesions compared with healthy skin (269). Immunohistochemistry further revealed that AQP3 accumulated in the SB and SS of lesional skin. Increased AQP3 mRNA and protein expression in AD were echoed in later studies in humans and mice, with a positive correlation between AQP3 expression and TEWL (269, 270). Contrastingly, AQP3 was reduced in spongiotic eczema lesions, given that the hallmark intercellular oedema potentially arises from impaired keratinocyte water uptake (271). Despite this very specific phenotype, increased AQP3 in lesional skin provides additional machinery for PG production which can be subsequently presented by LCs to T cells. Interestingly, AQP3 transcription downregulates during healthy mouse keratinocyte differentiation, likely leading to reduced PG production by transphosphatidylation (240). This temporal downregulation appears to be absent in AD lesions, given that elevated AQP3 is detected in the SS which comprises maturing keratinocytes and LCs (268). Upregulated AQP3 in AD lesional skin may therefore lead to increased PG content which can induce immunological effects.

1.6.5. The role of PLD in regulating immune system health and disease

Studies have implicated PLD in regulating the immune response and its role in diseases such as multiple sclerosis, Alzheimer's, vascular disease and cancer (272-275). Enzymatic activity and protein expression of PLD1b and PLD2 were upregulated during the differentiation of MoDCs (276). Interestingly, phagocytic activity of DCs was enhanced by PA, and inhibited by n-butanol, which competitively inhibits PA production. Therefore, PLD produces PA in mature DCs, facilitating protein and lipid antigen capture. A similar phenomenon has also been investigated in sPLA$_2$ group III (PLA$_2$G3), which catalyses the hydrolysis of LPC at the sn-2 position to LPA and a FA (277). PLA$_2$G3 treatment induced functional and phenotypic maturation in differentiating MoDCs, similar to that seen after LPS treatment. This was shown to be dependent on enzymatic activity. Numerous studies have demonstrated the ability of phospholipase enzymes to process lipids and produce neoantigens for subsequent presentation to T cells (278-280). These phenomena offer additional pathways by which PLD and PLA$_2$ modulate DC maturation and subsequent activity.

In the context of allergy, an early study by Oh et al demonstrated the potential importance of PLD in House dust mite (HDM) reactivity (281). They found that treatment of HDM-reactive T cells with the major HDM allergen, Der f 2, led to a doubling of PLD activity. The same group later clarified the mechanism, describing the role of PLD1 in HDM-induced asthma (282). Treatment of human bronchial cells with Der f 2 activated the phospholipase C-γ (PLCγ)/protein kinase C-α (PKC-α)/p38 MAPK pathway, which was shown to be an upstream activator of PLD, whilst also leading to expression and production of IL-13. Authors noted that activating transcription factor-2 (ATF-2) was activated by Der f 2, and this activation was controlled by PLD1. Blocking either the PLCγ/PKC-α/p38 MAPK pathway or ATF-2 inhibited Der f 2-induced IL-13 release,

suggesting PLD1 acts as an important regulator in this disease. More recently, the therapeutic potential of these findings has been explored using intranasal treatment of Der f 2-sensitised mice with AP180, a PLD1-inhibitory peptide (283). AP180 treatment decreased both PLD activity and IL-13 production in bronchial epithelia, and most importantly reduced airway inflammation. Given the parallels drawn between asthma, AD and food allergy, it is possible that PLD has similar roles in regulating the Th2 response against peanut allergens.

1.7. CD1a

1.7.1. Overview of CD1 molecules

The CD1 family comprises a group of heterogeneous transmembrane glycoproteins that present lipid and glycolipid antigens to T cells. CD1 molecules are β2-microglobulin (β2m)- associated and bear structural resemblance to major histocompatibility complex class I (MHC I) (284). However, unlike MHC, the genes encoding CD1 are relatively non-polymorphic (285). Consequently, there are three classes of human CD1 molecules: group 1 (CD1a, b and c), group 2 (CD1d) and group 3 (CD1e) (284). This well-conserved system allows immune surveillance of self and foreign lipids of intra- and extracellular origin, reinforcing CD1 molecules as important regulators of inflammation. Given the high expression of CD1a in human skin, in addition to data which will be discussed, CD1a likely plays a key role in skin lipid-surveillance and dermatological disease (286).

1.7.2. CD1a structure, expression and cellular localisation

1.7.2.1. CD1a structure

Resembling MHC I, all CD1 isoforms comprise a heavy chain, composed of three extracellular antigen-binding helical domains (α1, α2 and α3), a transmembrane domain and an intracellular tail (287). The α3 domain is non-covalently bonded to β2-microglobulin,

resulting in a folded heterodimer. CD1 diverges from MHC I in that the binding pocket is flanked by hydrophobic amino acid residues. Consequently, all CD1 molecules contain an A' pocket and F' pocket, which house non-polar and polar lipid moieties, respectively (287). Quaternary structure differs between isoforms, as shown in *Figure 10*; for example, CD1b possesses additional C' and T' pockets to house larger lipid chains (288). Uniquely, the CD1a A' pocket is buried under an A' roof without connection to other pockets, and its small capacity (~1350 Å) solely accommodates shorter carbon chains (≤24 carbons) (289). The F' pocket is comparatively hydrophilic and exposed to the extracellular space, allowing binding of lipopeptides and interaction between polar lipid components and the T cell receptor (TCR) (290).

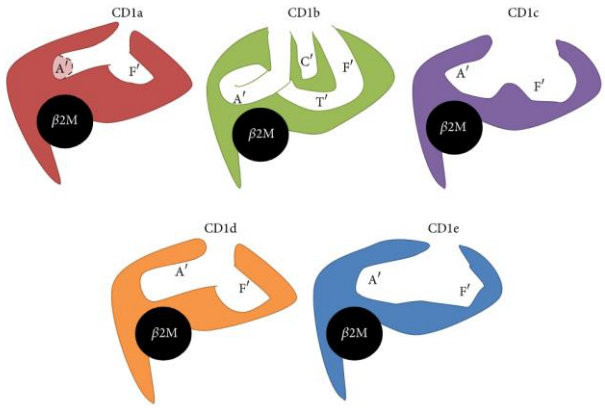


Figure 10. Comparison of CD1 molecule binding grooves. Differential structure of CD1 molecules, folded by *β*-2 microglobulin (*β*2M). *Obtained from* (291).

1.7.2.2. CD1a cellular expression

Group 1 CD1 molecules are primarily detected on professional antigen-presenting cells (APCs) (292). CD1a possesses a specific expression profile and is highly expressed on LCs in the epidermis. LCs are most prominent in the SS, embedded between keratinocytes, and possess elongated dendritic processes which express MHC class II (MHC II) and CD1a (293). Consequently, they are important sentinels of skin barrier breach, presenting exogenous peptide and lipid antigens to T cells. The constitutive high expression of CD1a reflects its importance in ordinary skin lipid surveillance and as a phenotypic marker (294).

In addition to LCs, several other dendritiform cells express CD1a and are increased in frequency during skin inflammation (295). Epidermal $CD1a^{+}CD11b^{+}CD1c^{+}$ myeloid DCs (mDCs) have been identified in atopic skin and can be differentiated from their LC counterparts by a comparatively lower expression of CD1a and lack of Birbeck Granules (BGs), enigmatic organelles specific to LCs (296). CD1a can be induced on both dermal DCs, situated in the upper layers of the dermis, by factors including chemotactic CC chemokine receptor type 7 (CCR7) ligands (297). Recently identified, during acute sterile skin inflammation there is early infiltration of $CD1a^{+}BDCA\text{-}2^{+}CD123^{int}$ DCs, which mimic plasmacytoid DCs (pDCs) and potentially contribute to the wound healing response (298). In addition to DCs, skin-resident type 2 innate lymphoid cells (ILC2s) express CD1a when exposed to TSLP produced during skin inflammation (299). Interestingly, CD1d is also present on ILCs, with the highest expression on ILC3s (300). Finally**,** CD1a is expressed by immature $CD4^{+}/CD8^{+}$ cortical thymocytes and downregulated upon maturation, suggesting a role in thymic selection (301, 302). In conclusion, upregulation of CD1a in immune cell subsets during inflammation highlights a functional role in skin physiopathology. This is of particular relevance when considering epicutaneous sensitisation to peanuts, given that peanut lipid and phospholipase allergens may breach an atopic skin barrier and modulate CD1a antigen presentation.

1.7.2.3. Regulation of CD1a in antigen-presenting cells

Relatively little is known about the regulation of CD1a molecules on APCs. The peroxisome proliferator-activated receptor γ (PPARγ) pathway, acting through the production of all-trans- retinoic acid (ATRA) which binds the retinoic acid receptor (RARα), has been shown to downregulate CD1a expression (303). Some studies have investigated regulation of the *CD1A* gene. The proximal promoter region of *CD1A* has little homology to that of MHC I or II (304). Also, the *CD1A* promoter lacks TATA and therefore has multiple transcription initiation sites (305). One study noted that activation

of the activating transcription factor/cAMP Response Element Binding Protein (ATF/CREB) family decreased *CD1A* transcription, through binding of the *CD1A* promoter in human monocytes (306). Given the upregulation of PLD expression during differentiation of MoDCs, it would be interesting to investigate the roles of PLD1 and PLD2 in regulating CD1a mRNA and protein expression (276). This offers a potential endogenous mechanism of modulating the CD1a-autoreactive response.

1.7.2.4. Intracellular trafficking of CD1a

CD1 molecules display distinct intracellular trafficking patterns directly linked to their amino acid structure (307). CD1b, c and d possess specific cytoplasmic tail tyrosine-based endosomal targeting motifs which predilect their respective endosomal and lysosomal recycling compartments (308). For example, the motif expressed on human CD1b binds to the AP3 adaptor protein complex, leading to accumulation in late endosomes and lysosomes (309). Conversely, guinea pig CD1b3 lacks this motif, leading to trafficking via early endosomes (310). Given that mice lack CD1b, murine CD1d has evolved to interact with AP3 and traffic along the CD1b endosomal route (311). Such phenomena evidence the finely tuned recycling patterns and have given rise to the "traffic hypothesis" of species-specific CD1 trafficking (310).

Amongst CD1 molecules, CD1a lacks a tyrosine-based cytoplasmic sorting motif and uniquely localises to the early endosomal/recycling compartment via a Rab22a- and ADP ribosylation factor 6-dependent process (312, 313). This demonstrates CD1a has evolved to present lipids that localise to these compartments. Using confocal microscopy, Cernadas et al. demonstrated colocalisation of sulfatide and CD1a in early endocytic and recycling compartments in Hela CD1a-transfected cell lines and MoDCs (312). Furthermore, wild type CD1a more efficiently presented sulfatide to CD1a-restricted sulfatide-specific T

cells, compared to CD1a:CD1b tail chimeras which trafficked via the late endosome/lysosomal compartments. This strongly suggests that the intracellular trafficking route is essential for efficient presentation of lipid antigens that pass through these compartments. Equally, CD1a has been shown to spontaneously accumulate in BGs, which have a hypothetical role in CD1a-lipid loading (314). Consequently, our group have developed a strong interest in investigating potential proteins involved in lipid loading. Given the similar recycling pattern of human PLD isoforms (particularly PLD2) and their ability to directly interact with several proteins, PLD may play a role in this mechanism.

1.7.3. CD1a lipid antigen presentation

1.7.3.1. Overview of CD1a lipid antigens

Several CD1a lipid antigens have been identified. Multiple endogenous lipids can be presented by CD1a, including skin oils, FFAs, wax esters, TAGs, squalene and phospholipids (e.g. LPC, sulfatide and SM) (85). Fewer exogenous lipid ligands have been identified, however include the lipopeptide didehydroxymycobactin (DDM) from *Mycobacterium tuberculosis*, PC and PE from cypress pollen, urushiol from poison-ivy and farnesol from balsam of Peru (85, 315-319). To date, crystal structures of CD1a in complex with sulfatide, LPC, SM, OLA, DDM and farnesol have been resolved (84, 315, 320, 321). There are no published structural or functional data describing CD1a presentation of peanut-derived lipids, necessitating this as a new focus of research.

1.7.3.2. Molecular dynamics of CD1a antigen presentation

CD1a can present lipid species with polar head groups in addition to hydrophobic lipids (85, 320). Such profound differences in CD1a antigen polarity and aliphatic structure have led to two opposing models of presentation to the TCR. Some polar lipids are hypothesised to be presented in an ‘MHC-like’ manner, in which the TCR interacts with both CD1a and

the cognate antigen (322). This is exemplified by the crystal structure of CD1a in conjunction with sulfatide, in which the sulphated galactose headgroup is exposed at the F' pocket for TCR recognition (320). A similar mechanism of direct TCR recognition is observed with mycobacterial DDM, in which two peptidic branches protrude further out of the F' pocket (321). Surprisingly, urushiol (derived from the allergen poison ivy) occupies the CD1a binding cleft in a reverse orientation, with the catechol headgroup housed by the A' pocket and the C_{15} acyl chain exposed at the F' portal for potential TCR recognition (319).

A second model of CD1a antigen presentation is 'absence of interference' and has gained most traction amongst CD1 research groups (323). In a landmark study, Birkinshaw et al identified an additional model of TCR activation, observing that LPC and OLA enhanced engagement of the CD1a-autoreactive BK6 TCR, however sulfatide and SM inhibited signalling (84). The BK6 TCR interacted solely with the CD1a A' roof without contacting the lipid ligands nestled underneath. This strongly suggests some ligands act to stabilise the A' roof and modulate TCR interactions indirectly. Shorter-chain lipids without polar headgroups, such as OLA with a C_{18} chain, fully nestled into the binding groove and were permissive to TCR-CD1a docking. Conversely, lipids with strongly polar headgroups, including sulfatide and SM, were non-permissive as they disrupted the TCR-CD1a contact zone. Specifically, the galactosyl sulphate headgroup of sulfatide disrupted Arg76-Glu154 interactions of the A' roof, leading to reorientation of surrounding amino acids and inhibiting binding of the BK6 TCR CDR3β loop. Similar effects were observed with SM, which possesses a long C_{24} aliphatic chain, leading to protrusion of the phosphocholine headgroup. Interestingly, despite its identical headgroup, LPC was identified as permissive given its shorter C_{18} acyl chain and reduced protrusion of phosphorylcholine; the headgroup was laterally displaced from the TCR docking site, and a central CD1a salt bridge conferred

further protection. Moreover, BK6 TCR binding to CD1a-OLA and CD1a-LPC was identical, demonstrating a common docking mode between these permissive ligands. This paradigm was supported by a later study, in which 'headless' apolar oils derived from sebum (primarily squalene and wax esters) promoted T cell activation by CD1a and could displace inhibitory polar membrane lipids (85). Recently, Nicolai et al reported the finding that farnesol, a contact dermatitis allergen, displaces inhibitory polar lipids to become buried deeply within the CD1a binding cleft given its short C_{15} chain (315). Although the terminal hydroxyl group is partially exposed at the F' portal, unsaturated bonds in its main hydrocarbon chain form van der Waals interactions with CD1a amino acids, including Trp14, stabilising the cleft and positioning farnesol away from the TCR to prevent interference. Overall, these dichotomous methods of TCR signalling through cognate interaction or "absence of interference" are governed by TCR clonality and lipid antigen factors, including structure and polarity. Peanut lipid content chiefly comprises FAs and the relatively non-polar PC, suggesting their potential action as exogenous CD1a lipids may occur via the interference model (79). This provides a strong rationale to perform functional T cell assays to inform future CD1a-peanut lipid crystal structures. Examples of CD1a-antigen structures are shown in *Figure 11* (84).

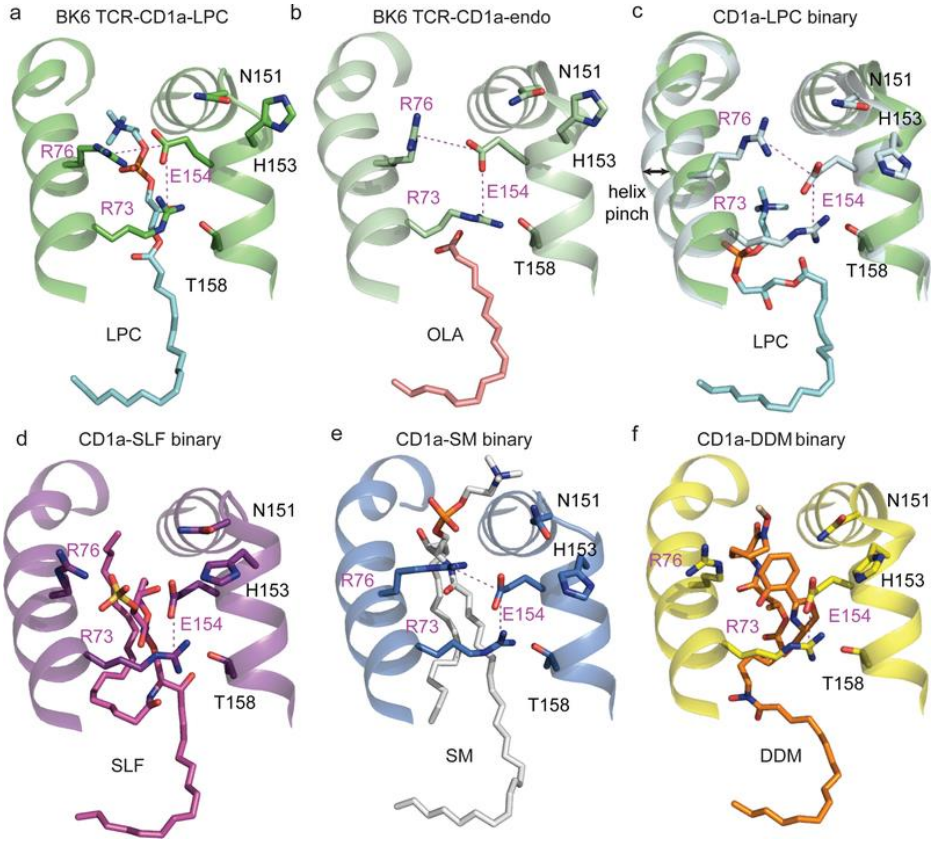


Figure 11. Examples of binary and ternary CD1a-antigen structures. Ternary complexes include: A) BK6 TCR-CD1a-LPC and B) BK6 TCR-CD1a-endo (oleic acid). Binary complexes include C) CD1a-LPC, D) CD1a-sulfatide (SLF), E) CD1a-sphingomyelin (SM) and F) CD1a-dideoxymycobactin (DDM). Key: CD1a represented as ribbons; lipid antigens represented as sticks; CD1a salt bridges represented as dashed lines. *Obtained from (84).*

1.7.4. The relevance of CD1a in allergic disease

Studies have demonstrated that up to 10% of peripheral blood T cells have CD1a-autoreactivity (318). Moreover, CD1a-reactive T cells were found to express cutaneous lymphocyte antigen (CLA), a carbohydrate epitope that identifies T cells that participate in immune responses. This provides strong evidence that CD1a-reactive T cells are a component of the skin-homing T cell subset. As a result, studies have implicated CD1a-autoreactive T cells as important in inflammatory skin disease (279, 280). Our group have produced multiple studies that show CD1a plays a critical role in skin inflammation (e.g. Cheung et al. 2016; Jarrett et al. 2016). Jarrett et al observed that HDM allergen induced a CD1a-specific T cell response in the blood and skin of patients

with AD. Unexpectedly, this effect was induced by sPLA2 within the protein fraction of HDM, suggesting that HDM allergen generates neolipid antigens that can be presented by CD1a. Moreover, filaggrin inhibited PLA_2 activity in biochemical experiments, implying a novel skin barrier sensing mechanism. PLD activity is modulated by proteins including actin and tubulin, and therefore may interact with filaggrin in a similar or novel manner (200). A similar mechanism was replicated by Cheung et al., who showed that cytosolic PLA_2 ($cPLA_2$) in exosomes released by mast cells could induce CD1a neoantigen generation in neighbouring cells (279). Finally, Hardman et al. made the interesting discovery that ILC2s can be induced to express CD1a and can present endogenous antigens, a mechanism dependent on $cPLA_2$ group IVA (PLA_2G4A) expression (299). PLD processes membrane PC to produce PA, which can be easily hydrolysed by endogenous PLA_2 to form the known CD1a ligand LPC (324, 325). Potentially, therefore, both exogenous peanut PLD and endogenous PLD1/PLD2 play a role in CD1a neoantigen generation.

1.7.5.PA and PG as candidate CD1a-mediated allergens

A recent lipidomic study by Wang et al detected an upregulation of PA in facial lesions of atopic infants (326). Using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), authors detected a 3-fold upregulation in PA possessing $C_{16\text{-}22}$ aliphatic chains, which are potentially suitable for CD1a presentation. This raises the hypothesis that endogenous elevations in PA in atopic skin lead to production of circulating PA-reactive T cells. Subsequent generation of PA by exogenous peanut PLD may therefore lead to hyperresponsiveness of PA-reactive T cells, propagating CD1a-mediated inflammation in peanut-allergic patients.

To date, no studies have investigated the presence of PG in eczema lesions, however PG is clearly implicated in regulating skin inflammation and is a known CD1a ligand (85, 244,

250). Importantly, PG has been identified in CD1a eluates by mass spectrometry, demonstrating binding (85). Published and unpublished data have demonstrated PG to be a permissive and inhibitory CD1a ligand depending on TCR clonality, leading to the hypothesis that a component of its immunomodulatory action may be in fact via CD1a, including PG derived from peanuts (85). PG-reactive T cells may therefore play a pro-inflammatory role within our disease model. Finally, filaggrin may modulate the peanut PLD hydrolysis and transphosphatidylation reactions that give rise to these circulating T cells.

AIMS AND OBJECTIVES

To date, no studies have investigated the role of CD1a in peanut (or food) allergy. Also, limited exogenous lipid ligands have been identified. Given the importance of human PLD isoforms in regulating a wide range of cellular processes, including DC maturation and function, and the parallels drawn between exogenous peanut PLD and exogenous HDM PLA_2, this enzyme may offer a novel pathway in CD1a antigen generation.

We therefore wish to test the following hypotheses:

- **Hypothesis 1** – Exogenous peanut PLD may generate neolipid self-antigens for subsequent presentation by CD1a to T cells, and this effect is exacerbated in cases of barrier dysfunction.
- **Hypothesis 2** – Peanut-derived lipids and proteins may act as direct CD1a antigens or modulate CD1a responses, respectively.
- **Hypothesis 3** – Endogenous human PLD1 and PLD2 may have roles in regulating CD1a mRNA and protein expression.

Specific aims are listed below, which will allow testing of these hypotheses:

For hypothesis 1:

- Investigate the capacity of peanut PLD to induce CD1a-specific T cell responses in healthy control, peanut-allergic and patients with AD (as a model of barrier dysfunction).

- Perform biochemical experiments to investigate the source of enzymatic activity that leads to these responses and investigate whether filaggrin modulates these effects.

For Hypothesis 2: Investigate the capacity of peanut lipids to act directly as CD1a ligands and define their nature.

For hypothesis 3: Measure human PLD1 and PLD2 expression in an LC cell line and primary MoDCs, and investigate whether manipulating PLD function has effects on CD1a transcription and expression.

1.8. Reagents

	Purchased Reagents			
Proteins	**Full name**	**Form**	**Source**	**Cat No**
	PLD from peanut (*Arachis hypogea*)	Lyophilized powder (≥60 units/mg protein)	Sigma-Aldrich	P0515-2.5KU
	PLD from *Streptomyces chromofuscus*	Aqueous solution (≥50,000 units/mL)	Sigma-Aldrich	P0065-25KU
	Arachis hypogea (peanut) Soluprick SQ Sol	Soluprick solution (10% glycerol)	ALK-Abello	-
Lipids	**Full name**	**Form**	**Source**	**Cat No**
	Groundnut (peanut) Oil	Pure oil (1g/ml fat [w/v]])	Tesco	-
	L-α-phosphatidylcholine from egg yolk	Lyophilised powder (25mg)	Avanti Polar Lipids	840051P-25MG
	L-α-lysophosphatidylcholine from egg yolk	Lyophilised powder (25mg)	Avanti Polar Lipids	840074P-25MG
	17:0 phosphatidylglycerol	Lyophilised powder (25mg)	Avanti Polar Lipids	830456P-25MG
	18:1 (Δ-9 cis) phosphatidylglycerol	Lyophilised powder (25mg)	Avanti Polar Lipids	840475P-25MG
	18:2 phosphatidylglycerol	Lyophilised powder (25mg)	Avanti Polar Lipids	840485P-25MG
	L-α-phosphatidic acid from soy	Lyophilised powder (25mg)	Avanti Polar Lipids	830071P-25MG
	Sphingomyelin from porcine brain	Lyophilised powder (25mg)	Avanti Polar Lipids	860062P-25MG
Inhibitors	**Full name**	**Form**	**Source**	**Cat No**
	FIPI hydrochloride	Lyophilised powder (5mg)	Sigma-Aldrich	F5807-5MG
	VU0155069	Lyophilised powder (5mg)	Sigma-Aldrich	SML2279-5MG
	ML298	Lyophilised powder (5mg)	Sigma-Aldrich	SML1077-5MG
	DFP00173	Lyophilised powder (5mg)	Sigma-Aldrich	HY-126073-5MG
Other	**Full name**	**Form**	**Source**	**Cat No**
	Redskin (raw) peanuts	Whole peanuts (500g)	Holland & Barrett	-

Table 3. Antigens and inhibitors purchased for experiments

	Purchased Flow Cytometry Antibodies					
Specific antibody	**Antibody**	**Fluorophore**	**Clone**	**Isotype**	**Source**	**Cat No**
	Anti-human CD1a	APC	HI149	Mouse IgG1, κ	Biolegend	300110
	Anti-human CD1c	BV605	L161	Mouse IgG1, κ	Biolegend	331537
	Anti-human CD11c	PE-Cy7	3.9	Mouse IgG1, κ	Biolegend	301607
	Anti-human HLA-DR	FITC	Tü36	Mouse IgG2b, κ	Biolegend	361603
	Anti-human CD11c	PB	3.9	Mouse IgG1, κ	Biolegend	301601
	Anti-human CD14	PerCP	M5E2	Mouse IgG2a, κ	Biolegend	301847
Isotype control	**Antibody**	**Fluorophore**	**Clone**	**Isotype**	**Source**	**Cat No**
	Mouse IgG1, κ	APC	MOPC-21	Mouse IgG1, κ	Biolegend	400119
	Mouse IgG1, κ	BV605	MOPC-21	Mouse IgG1, κ	Biolegend	400161
	Mouse IgG1, κ	PE-Cy7	MOPC-22	Mouse IgG1, κ	Biolegend	400125
	Mouse IgG2b, κ	FITC	MPC-11	Mouse IgG2b, κ	Biolegend	400309
	Mouse IgG1, κ	PB	MOPC-21	Mouse IgG1, κ	Biolegend	400101
	Mouse IgG2a, κ	PerCP	MOPC-173	Mouse IgG2a, κ	Biolegend	400256
Live/Dead	**Antibody**	**Fluorophore**	**Clone**	**Isotype**	**Source**	**Cat No**
	N/A	Fixable Violet	N/A	N/A	Thermo Fisher	L34955
	N/A	Fixable Aqua	N/A	N/A	Thermo Fisher	L34966

Table 4. Purchased flow cytometry antibodies, isotype controls and live/dead stains.

	Purchased blocking antibodies (for ELISpots)				
Blocking Antibody	**Antibody**	**Clone**	**Isotype**	**Source**	**Cat No**
	Ultra-LEAF purified anti-human HLA-DR	L243	Mouse IgG2a, κ	Biolegend	307665
	Ultra-LEAF purified anti-human HLA-A,B,C	W6/32	Mouse IgG2a, κ	Biolegend	311427
	Purified anti-human CD1a	HI149	Mouse IgG1, κ	Biolegend	300101
Isotype	**Antibody**	**Clone**	**Isotype**	**Source**	**Cat No**
	Purified Mouse IgG1, κ	MOPC-21	Mouse IgG1, κ	Biolegend	400101

Table 5. Purchased blocking antibodies and isotype controls, used in functional T cell assays.

1.9. Recombinant protein expression

1.9.1. Expression of recombinant filaggrin

Recombinant filaggrin was kindly provided by Dr Ewa Podobas and Prof Andrzej Dziembowski (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw). Filaggrin synthesis and purification was performed as previously described (280). Briefly, a sequence- and ligation-independent cloning method was used to construct the expression plasmid. The construct contains N-terminal His6-tagged SUMO protein sequence that can be enzymatically cleaved by SUMO protease and was transformed into *Escherichia coli* for subsequent protein expression. Recombinant filaggrin was purified using an ÄKTAxpress chromatography system. Bacterial cell lysates were loaded onto an Agarose column (Quiagen), and proteins were cleaved with SUMO protease, desalted, and eluted. Purified filaggrin was isolated using SDS-polacrylamide gel electrophoresis. Control SUMO protein was used as an experimental control in functional T cell assays.

1.9.2. Expression of recombinant CD1a

CD1a recombinant proteins were obtained from the NIH tetramer facility. CD1a recombinant proteins were also kindly gifted by Dr Demin Li (JR Hospital, Oxford) and purified in-house by Jess Ng and Dr Yi-Ling Chen (WIMM, Oxford).

1.10. Cell culture

1.10.1. Cell culture media

- RPMI (Thermo Fisher)
- RHEPES: RPMI supplemented with HEPES buffer (Thermo Fisher)
- COMPLETE MEDIA: RPMI supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, non-essential amino acids, sodium pyruvate,

HEPES buffer, 2-mercaptoethanol (Thermo Fisher) and 10% fetal calf serum (FCS; Sigma-Aldrich)

- T CELL MEDIUM: RPMI supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, non-essential amino acids, sodium pyruvate, HEPES buffer, 2-mercaptoethanol and 5% heat-inactivated human serum (Thermo Fisher).
- MUTZ3 MEDIA - MEM-α containing 20% FCS, 100-fold dilution of 100x NEAA, 10mM HEPES, 5mM sodium pyruvate, 50µM 2-mercaptoethanol, 100IU/ml sodium-penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 10% 5637-conditioned medium (Thermo Fisher) (327).
- K562 MEDIA - RPMI supplemented with 10% FCS, 100-fold dilution of 100x NEAA, 10mM HEPES, 5mM sodium pyruvate, 50µM 2-mercaptoethanol, 100IU/ml sodium-penicillin, 100µg/ml streptomycin, 2mM L-glutamine and the selection agent G418 (Thermo Fisher).

1.10.2. Cell lines

- K562: K562 cells transfected with either CD1a-expressing vector or empty vector were kindly donated by Professor Branch Moody (Brigham and Women's Hospital, Harvard University). K562 cells were cultured in complete medium supplemented with the selection agent G418.
- MUTZ3 and 5637: MUTZ3 (AC-295) and 5637 (ACC-35) cells were purchased from DSMZ. MUTZ3 were maintained in MUTZ3 media and were cultured in 24 well plates (Costar, Corning) at a concentration of 0.5-1.5 million cells/ml.

1.10.3. PBMC isolation from human blood

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood donated by healthy control (HC), atopic (AD) or peanut-allergic (PN) donors, under local ethics approval (09/H0606/71). PBMC preparation was performed using density gradient centrifugation. Blood samples were diluted 1:1 with RPMI and layered with 15ml Lymphoprep (STEMCELL Technologies) in a 50ml Falcon tube. Samples were centrifuged at 2000 RPM for 20 minutes with deceleration switched off. The PBMC-rich buffy coat layer was extracted and washed in RHepes at 1800 RPM for 10 minutes. Samples were then washed a second time in RHEPES at 1500RPM for 5 minutes, and finally resuspended in complete media containing 10% FCS.

1.10.4. Isolation of human monocytes and T cells

Human primary monocytes were isolated from PBMCs using CD14 MACS magnetic separation beads (Miltenyi Biotec). PBMCs were washed twice by resuspending in 50ml complete media containing 10% FCS and centrifuging at 1500 RPM for 5 minutes. PBMCs were resuspended in a solution containing 80µl MACS buffer (PBS containing 0.5% BSA and 2mM EDTA). 20µl CD3 Microbeads per 10 million cells were added to cell suspensions and incubated at 4^{o}C for 15 minutes, with gentle shaking every 5 minutes. Samples were washed in 5ml of MACS buffers and centrifuged at 1500RPM for 5 minutes, and then resuspended in 1ml of MACS buffer. LS columns were inserted into a MACS magnetic cell separator and pre-loaded with 3ml of MACS buffer, and the columns were not allowed to dry. Cell suspensions were passed though the LS column and washed 3 times with 1ml of MACS buffer, and the $CD14^{-}$ fraction was stored on ice for later processing (maximum 30 minutes). The column was removed from the magnet and 5ml of MACS buffer was added. The plunger was then used to flush the $CD14^{+}$ cells through the column. $CD14^{+}$ cells were washed in MACS buffer at 1500 RPM and resuspended in complete

RPMI medium containing 10% CD1a-selective FCS and maintained at a concentration of 2 million cells/ml in 24 well plates (Costar, Corning). To isolate CD3+ T cells, the same process was performed using CD3 MACS Beads (Miltenyi Biotec) on whole PBMC or $CD14^-$ PBMC. Following the plunging stage, $CD3^+$ cells were washed and resuspended in T cell medium in the presence of 1nM IL-2 (PeproTech).

1.10.5. Generation of MUTZ3-derived Langerhans cells

MUTZ3-derived Langerhans cells (MUTZ3-LCs) were cultured from the human acute myeloid leukemia cell line MUTZ3 as previously described with minor changes (328) . MUTZ3 were maintained in MEM-α (Thermo Fisher) containing 20% FCS, 100-fold dilution of 100x NEAA, 10mM HEPES, 5mM sodium pyruvate, 50µM 2-mercaptoethanol, 100IU/ml sodium-penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 10% conditioned medium obtained by culturing the 5637 human bladder carcinoma cell line (327), and were cultured in 24 well plates (Costar, Corning) at a concentration of 0.5-1.5 million cells/ml. MUTZ3 were differentiated into MUTZ3-derived LCs over 10 days in a TGF-β-dependent manner. MUTZ3 were maintained in MEM-α containing 20% FCS, 10mM HEPES, 5mM sodium pyruvate, 50µM 2-mercaptoethanol, 100IU/ml sodium-penicillin, 100µg/ml streptomycin and 2mM L-glutamine in 12-well plates (Costar, Corning) at a concentration of 0.5 million cells/ml. Recombinant human GM-CSF (100ng/ml; Biolegend, CA, USA), TNF-α (2.5ng/ml; Biolegend, CA, USA) and TGF-β (10ng/ml; Biolegend, CA, USA) were added on days 0, 4 and 7. To examine the effect of PLD inhibition on maturation of MUTZ3-LCs, FIPI hydrochloride (dual PLD1/PLD2 inhibitor; 50nM) or no inhibitor was added to cell cultures on days 0, 4 and 7. Flow cytometry and qPCR phenotypic analysis was performed on days 0, 4, 7 and 10. Light microscopy was performed on days 0 and 10 of differentiation using an EVOS FLoid Imaging System (Thermo Fisher). Fully differentiated MUTZ3-LCs were fixed in a solution

of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer, and Transmission electron microscopy (TEM) was kindly performed by Dr Errin Johnson (Sir William Dunn School of Pathology, Oxford).

1.10.6. Generation of monocyte-derived dendritic cells

Human monocytes were differentiated into monocyte-derived dendritic cells (MoDCs). Human $CD14^+$ monocytes were isolated as previously outlined and cultured in complete RPMI medium containing 10% CD1a-selective FCS (Sigma-Aldrich) and maintained at a concentration of 2 million cells/ml in 24 well plates (Costar; Corning, UK). Human monocyte-derived dendritic cells (MoDCs) were differentiated over 5 days by adding recombinant human IL-4 (50ng/ml; BioLegend, CA, USA) and GM-CSF (100ng/ml; Biolegend, CA, USA) on days 0 and 3. To examine the effect of PLD inhibition on maturation, VUO155069 (PLD1 inhibitor; 50nM; Sigma-Aldrich) or ML298 (PLD2 inhibitor; 10μM; Sigma-Aldrich) or no inhibitor was added on days 0 and 3. Flow cytometry phenotypic analysis was performed on days 0 and 5, for measuring of CD1a expression. MoDCs from select donors were treated with 100μg/ml peanut PLD (Sigma-Aldrich) to determine measure the effect on CD1a expression at 18 hours, as measured by flow cytometry.

1.11. Lipid extraction from MoDCs

Lipids were extracted from fully differentiated MoDCs using a chloroform-free Lipid Extraction Kit (ab211044, Abcam) according to manufacturer's guidelines. The lipid-rich supernatant was carefully collected and transferred to a clean 2ml amber glass vial (Sigma-Aldrich) and dried under a gentle nitrogen stream.

1.12. Measurement of PPAR-gamma activity

1.12.1. Nuclear protein extraction

Nuclear protein extracts were prepared used a Nuclear Extraction Kit (Abcam). During differentiation of control or PLD2-inhibited MoDCs, at 24 hours, $2x10^6$ cells were harvested and washed with PBS once by centrifugation at 1000 RPM for 5 minutes, and the supernatant was discarded. Cells were resuspended in 200µl of pre-extraction buffer and incubated on ice for 10 minutes. Samples were centrifuged at 1200RPM for 10 minutes and cytoplasmic extracts were removed and stored. DTT and PIC solution were diluted in extraction buffer at a 1:1000 ratio. 10µl of extraction buffer were added to nuclear pellets, which were incubated on ice for 15 minutes and vortexed every 3 minutes. Samples were subsequently sonicated for 10 seconds 3 times and centrifuged at 14000 RPM for 10 minutes at 4^oC. Nuclear protein supernatants were extracted, and protein concentration was determined by nanodrop (Thermo Fisher).

1.12.2. Measurement of PPARγ activity

PPARγ activity was measured using the PPARγ Transcription Factor Assay Kit (Abcam, UK). PPARγ activity was detected using an ELISA system which measures binding to a specific dsDNA sequence containing the peroxisome proliferator response element (PPRE). 90µl Complete Transcription Factor Binding Assay Buffer (CTFB) and 10µl nuclear protein extract were added to sample wells in a PPAR 96-Well Strip Plate. 90µl CTFB and 10µl PPARγ positive control were added to positive control wells. Negative controls included blank wells and non-specific binding (NSB) wells, to which only 100µl CTFB was added. All wells were prepared in duplicate. The plate was incubated for 1 hour at RT. PPARγ Primary Antibody was diluted 1:100 in antibody binding buffer (ABB) and 100µl was added to each well, apart from blank wells. The plate was incubated for 1 hour at RT, washed as previously, and residual buffer discarded. Goat anti-rabbit HRP conjugate was

diluted 1:100 in ABB and 100µl was added to each well, apart from blank wells. Samples were incubated for 1 hour at RT. 100µl of developing solution was added to each well and the plate was incubated for 30 minutes at RT. 100µl of stop solution were added per well and absorbance at 450nm was read within 5 minutes, using a Clariostar Plus Microplate Reader (BMG Labtech). PPARγ activity was proportional to absorbance (OD).

1.13. Measurement of MoDC phagocytic Activity

MoDCs were differentiated normally or in the presence of PLD2 inhibitor (10µM ML298; Sigma-Aldrich) and assessed for phagocytic activity using a colorimetric Phagocytosis Assay Kit (ab211156, Abcam). The test used pre-labelled zymosan particles which are phagocytosed by cells and react with a specific substrate which induces a colorimetric signal. Cells were resuspended in complete media containing 10% CD1a-selective FCS at a concentration of $1x10^6$ cells/ml. 100µl of cells ($1x10^5$ cells) were added to wells in a 96-well plate and incubated overnight at 37°C and 5% CO_2. Cytochalasin D was then added to the indicated wells to reach a 2µM concentration. Subsequently, manufacturer instructions were followed and absorbance was measured at 405nm using a ClarioStar Plus Microplate Reader (BMG Labtech).

1.14. Dissolving peanut lipids, and miscibility studies

All lipids were dissolved, tested, and stored in 2ml amber or clear screw-top glass vials (Sigma-Aldrich). Commercial peanut oil (Po; 1000mg/ml fat [w/v]) was obtained from Tesco, UK. In a 2ml amber glass vial (Sigma-Aldrich), 10µl of Po (10mg fat) was added to 990µl of dimethylsulfoxide (DMSO), chloroform, ethanol or chloroform: ethanol (C:E) mixed at a ratio of 2:1 (all obtained from Sigma-Aldrich), to obtain a stock concentration of 10mg/ml Po. Samples were vortexed for 30 seconds, and sonicated for 10 seconds, three times. Immediately, a 100µl aliquot of sample was transferred to a 2ml clear glass vial

(Sigma-Aldrich) and assessed for miscibility using a qualitative grading system ('None' = visibly immiscible, with sample separating from solvent within the vial; 'Partial' = some dissolution, often accompanied by a slightly turbid colour change; 'Complete' = complete dissolution, with a clear appearance).

1.15. Isolating antigenic compounds from raw peanuts

1.15.1. Lipid extraction and miscibility studies

Total lipid extraction (TLE) was performed according to a modified Bligh-dyer method (329). Briefly, crude lipid extraction was initially performed by homogenising a 50g sample of whole raw peanuts (Holland & Barrett, UK) in a BLX520 food blender (Kenwood, UK) for 60 seconds. A 20g sample of ground peanuts was then added to 60ml solution of chloroform/methanol (2:1 ratio) and agitated for 1 hour at RT. This solution was filtered, added to 30ml of saturated sodium chloride (NaCl) solution and centrifuged at 3000RPM for 5 minutes. The upper aqueous phase was discarded, and the lower organic phase was washed with saturated NaCl and centrifuged twice more. The organic phase was collected, and chloroform solvent was evaporated using a SpeedVac (Thermo Fisher). 100µl aliquots of peanut TLE (pTLE) were subsequently transferred to 2ml clear glass vials (Sigma-Aldrich) and mixed with preparations of PBS containing 0.01%, 0.05%, 0.1% or 0.5% of Tween-20 emulsifying agent (Sigma-Aldrich) to reach a final concentration of 10mg/ml pTLE. pTLE/PBST solutions were sonicated for 10 seconds, followed by vortexing for 30 seconds, and this process was repeated four more times. Miscibility was then assessed using a qualitative grading system (see *Chapter 5.7*).

1.15.2. Protein extraction

100g of raw peanuts (Holland & Barrett, UK) and 100ml DW were homogenised in a BLX520 food blender (Kenwood, UK) for 60 seconds. The solution was filtered and 1

volume (100µl) of creamy filtrate was added to 4 volumes (400µl) of methanol in a 5ml amber glass vial (Sigma-Aldrich) and vortexed for 30 seconds. 1 volume (100µl) of chloroform was then added and vortexed for 30 seconds. Samples were centrifuged for 2 minutes at 14000*g*, resulting in two layers (water/methanol at top; chloroform at bottom), with a thin disc of protein precipitates at the interface between the two layers. The upper aqueous layer was gently aspirated and discarded, and the protein precipitates were washed by adding 4 volumes (400µl) of methanol, vortexing for 30 seconds and centrifuging for 2 minutes at 14000*g*. The supernatant was again discarded, leaving protein precipitates floating on top of the organic lower phase. Chloroform was evaporated using a gentle stream of nitrogen gas and protein precipitates were resuspended in 1ml PBS and sonicated for 30 seconds, 3 times. Undissolved precipitates were discarded, and 500µl of aqueous proteinaceous solution was transferred to a 1ml plastic Eppendorf tube. Protein concentration was confirmed using NanoDrop (Thermo Fisher).

1.15.3. Isolating oil bodies

Peanut oil bodies (OB) were isolated using a modification of the protocol performed by Rodelas-Angelia et al (330). 50g of raw peanuts (Holland & Barrett) were soaked in 450ml of pre-chilled buffer containing sodium bicarbonate (0.1M, pH 8.0 pH 8.0) and sodium azide (0.02mM) for 16 hours at 4^{o}C. Peanut seeds were filtered and soaking medium was discarded. Seeds were resuspended in 450ml of pre-chilled buffer containing sodium bicarbonate (0.1M, pH 8.0 pH 8.0) and sodium azide (0.02mM), and homogenised at high power for 90 seconds in a BLX520 food blender (Kenwood). The ground material was filtered using 3 layers of cheesecloth (KitchenCraft), and the yellow “peanut milk” filtrate was transferred to 50ml centrifuge tubes. The filtrate was centrifuged at 10000*g* for 30 minutes at 4^{o}C in an Avanti JXN-26 High-Speed Centrifuge (Beckman Coulter). The crude oil body preparation (COB) was visualised as a floating cream pad and collected using a

sterile spatula. COB was gently mixed with the spatula and weighed using scales. 5g of COB was washed in 20ml of 9M urea (1:4 [w/v]) and centrifuged at 10000*g* for 30 minutes at RT. COB was collected and weighed as previously, and 5g of COB was dissolved in 20ml DW (1:4 [w/v]), before centrifugation at 10000*g* for 30 minutes at 4°C. COB was washed in DW and centrifuged twice further to remove residual urea, to obtain washed oil body preparation (WOB). A stock solution of 20mg/ml WOB in DW was prepared for future use.

1.16. Enzyme-linked immunospot (ELISpot) assays

1.16.1. General technique

CD1a-reactivity was assessed using IFN-γ, IL-22 or IL-13 ELISpot (Mabtech AB). $CD3^+$ T cells were isolated, as previously outlined, and rested for 3 days in T cell medium. ELISpots were performed using a 3-day protocol. For MoDC ELISpots, $CD14^+$ monocytes were isolated and MoDCs were generated as previously outlined and used immediately. Autologous $CD3^+$ T cells were isolated, as previously outlined, and rested for 3 days in T cell medium.

On day 1, ELISpot plates (96-well multiscreen IP-plates, MilliporeSigma) were treated with 50µl/well 35% ethanol for one minute and washed six times with 200µl/well deionised water (DW). Plates were then coated with 100µl/well anti-IFN-γ (15µg/ml), anti-IL-22 (2µg/ml) or anti-IL-13 (2µg/ml) capture antibody (Mabtech AB, Sweden), and incubated overnight at 4°C. Human polyclonal T cells were washed twice and rested in complete media containing 10% FCS. APCs (K562 cells or MoDCs) were cultured in complete media containing 10% FCS and pulsed overnight with antigen or inhibitor at various concentrations and combinations (***Table 6***), as outlined in the relevant results Chapter.

	Chapter I (peanut PLD ELISpots)		
Enzymes	**Name**	**Final Concentration**	**Solvent**
	Peanut PLD	0.1, 1, 10, 50, 100µg/ml	DW
	Streptomyces PLD	0.025, 0.25, 2.5U/ml	DW
Lipids	**Name**	**Final Concentration**	**Solvent**
	PC	100µg/ml	Ethanol
	SM	1, 10, 100µg/ml	Ethanol
Inhibitors	**Name**	**Final Concentration**	**Solvent**
	FIPI (peanut PLD inhibitor)	10µM	DMSO
	DFP00173 (AQP3 inhibitor)	0.4µM	DMSO
Filaggrin	**Name**	**Final Concentration**	**Solvent**
	Filaggrin	1µg/ml	PBS
	SUMO (control for filaggrin)	1µg/ml	PBS

	Chapter II (peanut Lipid ELISpots)		
Lipids	**Name**	**Final Concentration**	**Solvent**
	Peanut oil	1, 10, 100µg/ml	Chloroform: ethanol (2:1)
	Peanut TLE	1, 10, 50µg/ml	PBST (0.01-0.5%)
	17:0 PG	100µg/ml	Ethanol
	18:1 (Δ-cis 9) PG	100µg/ml	Ethanol
	18:2 PG	100µg/ml	Ethanol
	Soy PA	100µg/ml	Ethanol
Peanut extracts	**Name**	**Final Concentration**	**Solvent**
	Peanut protein	1, 5, 10µg/ml	PBS
	Peanut SPT	1, 5, 10µg/ml	Soluprick solvent (10% glycerol)
	OB	10, 50, 100µg/ml	DW

	Chapter II((endogenous PLD2 ELISpots)		
Lipids	**Name**	**Final Concentration**	**Solvent**
	LPC	100µg/ml	Ethanol
Inhibitors	**Name**	**Final Concentration**	**Solvent**
	FIPI (dual PLD1/PLD2 inhibitor)	50nM	DMSO
	VUO155069 (PLD1 inhibitor)	50nM	DMSO
	ML298 (PLD2 inhibitor)	10µM	DMSO

Table 6. Antigens and inhibitors used to treat APCs during day 1 of the ELISpot assay.

On day 2, APCs were washed three times in RHEPES and resuspended in complete media containing 10% FCS. T cells were similarly washed and resuspended. ELISpot plates were washed six times with RPMI and blocked with complete media containing 10% FCS for 1 hour at 37^{o}C. In K562 experiments, K562 cells were preincubated for 1 hour with no antibody or combinations of blocking antibodies (α-CD1a [10μg/ml; clone: HI149, mouse IgG1]; isotype of α-CD1a [10μg/ml; clone: MOPC-21, mouse IgG1]; BioLegend, CA, USA). In MoDC experiments, MoDCs were preincubated for 1 hour with combinations of blocking antibodies (α-HLA ABC [10μg/ml, clone: W6/32, mouse IgG2a, κ], anti-HLA DR [10μg/ml, clone: LN3, mouse IgG2b, κ], α-CD1a [10μg/ml; clone: HI149, mouse IgG1]; isotype of α-CD1a [10μg/ml; clone: MOPC-21, mouse IgG1]). APCs were then cocultured with T cells in ELISpot plates, in complete media supplemented with 10% FCS. In K562 experiments, each well contained 25,000 K562 cells and 50,000 T cells. In MoDC experiments, each well contained 10,000 MoDCs and 30,000 T cells. Control wells contained T cells alone, in the absence of APCs. Positive control T cells were treated with 10ng/ml Phorbol myristate acetate (PMA; Sigma-Aldrich) and 500ng/ml Ionomycin (Sigma-Aldrich). Negative control T cells were cultured in media alone. Cells were then incubated overnight for a minimum of 16 hours at 37^{o}C and 5% CO_2.

On day 3, plates were washed six times in PBS and incubated with 50μl /well of biotin-linked anti-IFN-γ, anti-IL-22 or anti-IL13 antibody (1μg /ml; Mabtech AB) for 2 hours at room temperature (RT). Repeat washing in PBS was performed 6 times and plates were incubated with 50μl /well streptavidin-alkaline phosphatase (1μg /ml, Mabtech AB) for 1 hour at RT. After washing plates 3 times with PBS and twice with DW, spots were developed using 100 μl /well alkaline phosphatase conjugate substrate (BioRad). Substrate was maintained in wells for 20 minutes or until spots could be visualised. Plates were then left to dry overnight.

Spots were counted using an automated ELISpot reader (Autimmun Diagnostika GmbH). The mean number of spots/well was calculated for each condition. T cell negative controls were subtracted from all conditions to eliminate background T cell activity. For cohort studies, results were either expressed as whole spots or fold change with respect to unpulsed APCs. For some K562 experiments, K562-EV and K562-CD1a whole spot data are presented, to illustrate data from each cohort. For other K562 experiment, K562-EV data were subtracted from K562-CD1a data in each condition, demonstrating the frequency of CD1a-reactive T cell responses. All MoDC data were presented as whole spots.

1.16.2. Variations in technique

For select experiment, modifications were applied to the general ELISpot protocol.

- DONORS: Donors differed between experiments, as outlined in the relevant results chapter. Donors for MoDCs and/or polyclonal T cells included: HCs, peanut-allergic individuals and patients with AD. Donors used in each experiment are demonstrated in the relevant results chapter, and this includes individual donor data and mean data across groups.
- PH MODULATION OF PLD ACTIVITY: To assess the effect on pH in modulating peanut PLD activity, prior to antigen pulsing on day 1, complete media containing 10% FCS was adjusted to 4 separate pH conditions (pH 4.5, 5.5, 6.5 or 7.4), using hydrochloric acid (HCl; Sigma-Aldrich) and sodium hydroxide (NaOH; Sigma-Aldrich). At each pH condition, 100ug/ml peanut PLD was preincubated with 100ug/ml PC (L-α-phosphatidylcholine from egg yolk [Avanti Polar Lipids] dissolved in 100% ethanol) for 2 hours at 37^{o}C in 5% CO_2. K562 cells were then resuspended in each reaction mixture, representing the antigen-pulsing stage. This was followed by the ordinary ELISpot protocol.

- COMPARISON OF CONTROL AND PLD2-INHIBITED MODC: To assess whether PLD2 activity during differentiation of MoDCs modulates their capacity to present CD1a antigens, during the antigen pulsing stage, control MoDCss or MoDCs differentiated in the presence of PLD2 inhibitor (50nM ML298; Sigma-Aldrich) were treated with LPC (L-α-lysophosphatidylcholine from egg yolk [Avanti Polar Lipids] dissolved in 100% ethanol). To investigate the effect of PLD2 inhibitor in altering CD1a antigen presentation by already-mature APCs, during antigen pulsing, K562 cells or control MoDCs were pulsed with LPC (L-α-lysophosphatidylcholine from egg yolk [Avanti Polar Lipids] dissolved in 100% ethanol) and PLD2 inhibitor (50nM ML298; Sigma-Aldrich) or no inhibitor. Subsequently, the usual ELISpot method was performed.

1.17. Enzyme-linked immunosorbent assay (ELISA)

IL-4, IL-13 and IL-22 levels in supernatants taken from K562/T cell cocultures, as dictated in the relevant results chapters, were measured by ELISA (IL-4, IL-13 and IL-22 Human ELISA kits; Thermo Fisher). 100µl/well capture antibody, diluted 1:250 in coating buffer, was added to a 96-well plate (Corning Costar) and incubated overnight at 4ºC. Wells were washed 3 times with 250µl/well Wash Buffer, blocked with 200µl of ELISA Diluent and incubated at RT for one hour. Wells were washed once more with Wash Buffer. Samples, negative controls, and standards containing known cytokine levels were added to appropriate wells in duplicate (final volume 200µl in ELISA Diluent) and incubated overnight at 4ºC. After washing 5 times with Wash Buffer, 100µl/well Detection Antibody was added to all wells, and the plate was sealed and incubated at RT for 1 hour. Wells were washed 5 times as previously. 100µl/well streptavidin-HRP, diluted 1:100 in ELISA Diluent, was added to all wells and the plate was sealed and incubated at RT for 30 minutes. After repeat washing 5 times, 100µl/well TMB solution was added to all wells, and the plate

was incubated for 15 minutes at RT. 100µl/well Stop Solution was added to each well, and the plate was incubated for a further 15 minutes at RT. The plate was read at 450nm using a ClarioStar Plus Microplate Reader (BMG Labtech).

1.18. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was used to detect mRNA expression in APCs during differentiation of MUTZ3-LCs and MoDCs, and after treatment of MoDCs with peanut PLD. qPCR was used to detect cytokine mRNA expression in T cells following coculture with antigen-pulsed K562 cells. Further detail is outlined in the relevant results chapters.

1.18.1. mRNA extraction

mRNA extraction from cells was performed using a TurboCapture 96 mRNA kit (72251, Qiagen). Each well contains immobilised oligo-dT which hybridises to sample mRNA. Briefly, lysis solution was prepared, containing 2% v/v β-mercaptoethanol (Sigma-Aldrich) in 2x buffer TCL. $1x10^5$ cells suspended in 50µl media were diluted 1:1 in lysis solution and added to each well in a TurboCapture plate. Turbocapture plates were sealed with AlumaSeal and incubated at RT for 90 minutes. Lysates were discarded and the plate was washed 3 times using 200µL buffer TCW per well. Residual liquid was removed, and cDNA synthesis was performed directly in each well containing mRNA.

1.18.2. cDNA synthesis

cDNA synthesis was performed using a protocol developed in-house. 20µL of cDNA reaction mixture (*Table 7*) was added to each TurboCapture well (Qiagen). The plate was sealed with optical adhesive film and DNA was amplified in a T100 Thermal Cycler (Bio-Rad).

	cDNA Synthesis		
Reaction Mix	**Component**	**Volume/well (µl)**	**Source**
	Nuclease-free water	10	Thermo Fisher
	5x First Strand Buffer	4	Thermo Fisher
	dNTP (10mM)	1	Thermo Fisher
	DTT (0.1M)	2	Thermo Fisher
	Random Hexamers (50µM)	1	Thermo Fisher
	RnaseOUT (100mM)	1	Thermo Fisher
	Reverse Transcriptase (200 U/ÂµL)	1	Thermo Fisher
	Total volume	**20µl**	-

PCR machine setting	**Stage**	**Temperature (^{o}C)**	**Time (min)**
	Annealing	25	10
	Synthesis	37	50
	Heat inactivation	70	15
	Storage	4	∞

Table 7. cDNA reaction mixture components and thermal cycler settings.

1.18.3. qPCR

qPCR was performed using gene-specific Taqman probes (Thermo Fisher) and Taqman gene expression master mix (Thermo Fisher), with details outlined in *Table 8*. 8µl PCR master mix was transferred to each well of a 96- or 384-well optical reaction plate (Thermo Fisher). 2µl of sample cDNA was added to each well and the plate was sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). qPCR was performed using a QuantStudio 7 Real-Time PCR System (Applied Biosystems). ΔCt was calculated by subtracting the Ct value of the gene of interest from that of the endogenous housekeeping gene *GAPDH* (in APCs) or *CD2* (in T cells). Relative gene expression was expressed as $2^{-\Delta Ct}$.

	qPCR		
Master Mix	**Component**	**Volume/well (µl)**	**Source**
	Master Mix (2x)	5	Thermo Fisher
	TaqMan Gene Expression Assay (20x)	0.5	Thermo Fisher
	Nuclease-free water	2.5	Thermo Fisher
	Total Volume	**8**	-

	Target Gene	**Assay ID**	**Source**
TaqMan Gene Expression Assays	*GAPDH*	Hs02786624_g1	Thermo Fisher
	CD2	Hs01040179_g1	Thermo Fisher
	CD1A	Hs00381754_g1	Thermo Fisher
	CD207	Hs00210453_m1	Thermo Fisher
	PLD1	Hs05044256_s1	Thermo Fisher
	PLD2	Hs01093234_m1	Thermo Fisher
	PLA2G4A	Hs00996912_m1	Thermo Fisher
	PLA2G4C	Hs00603557_m1	Thermo Fisher
	TNF	Hs00174128_m1	Thermo Fisher
	IL1B	Hs01555410_m1	Thermo Fisher
	IL6	Hs00174131_m1	Thermo Fisher
	IL12A	Hs01073447_m1	Thermo Fisher
	TGFB1	Hs00998133_m1	Thermo Fisher
	IL10	Hs00961622_m1	Thermo Fisher
	IL4	Hs00174122_m1	Thermo Fisher
	IL5	Hs01548712_g1	Thermo Fisher
	IL13	Hs00174379_m1	Thermo Fisher
	IL17A	Hs00174383_m1	Thermo Fisher
	IL22	Hs05034439_s1	Thermo Fisher

Table 8. qPCR master mix components and TaqMan probes used.

1.19. Flow cytometry

Flow cytometry was used for immunophenotyping of cell populations. Samples were incubated with specific or corresponding isotype monoclonal antibodies for 30 minutes at 4oC. A full list of antibodies, including their fluorophore conjugates and clones, are demonstrated in *Table 9*. Data was acquired using an Attune NXT Flow Cytometer (Thermo Fisher) equipped with Attune NXT Flow Cytometer Software and results were analysed using FlowJo software (OR, USA). Positive cell populations were selected based on gating to fluorescence minus one (FMO) controls. MFI of positive cell populations was determined by calculating the geometric mean of their fluorescence intensity. In some

experiments, the mean frequencies of cell populations or mean MFI of positive cell populations were calculated across multiple donors or experimental repeats.

	MUTZ3-LC Flow Cytometry Phenotyping					
	Antibody	**Fluorophore**	**Dilution**	**Clone**	**Isotype**	**Source**
Specific antibody	Anti-human CD1a	APC	1:50	HI149	Mouse IgG1, κ	Biolegend
	Anti-human CD1c	BV605	1:50	L161	Mouse IgG1, κ	Biolegend
	Anti-human CD11c	PE-Cy7	1:50	3.9	Mouse IgG1, κ	Biolegend
Isotype control	CD1a isotype	APC	1:50	MOPC-21	Mouse IgG1, κ	Biolegend
	CD1c isotype	BV605	1:50	MOPC-21	Mouse IgG1, κ	Biolegend
	CD11c isotype	PE-Cy7	1:50	MOPC-22	Mouse IgG1, κ	Biolegend
Live/Dead	N/A	Fixable Violet	1:1000	N/A	N/A	Thermo Fisher

	MoDC Flow Cytometry Phenotyping					
	Antibody	**Fluorophore**		**Clone**	**Isotype**	**Source**
Specific antibody	Anti-human CD1a	APC	1:50	HI149	Mouse IgG1, κ	Biolegend
	Anti-human HLA-DR	FITC	1:50	Tü36	Mouse IgG2b, κ	Biolegend
	Anti-human CD11c	PB	1:50	3.9	Mouse IgG1, κ	Biolegend
	Anti-human CD14	PerCP	1:50	M5E2	Mouse IgG2a, κ	Biolegend
Isotype control	CD1a isotype	APC	1:50	MOPC-21	Mouse IgG1, κ	Biolegend
	HLA-DR isotype	FITC	1:50	MPC-11	Mouse IgG2b, κ	Biolegend
	CD11c isotype	PB	1:50	MOPC-21	Mouse IgG1, κ	Biolegend
	CD14 isotype	PerCP	1:50	MOPC-173	Mouse IgG2a, κ	Biolegend
Live/Dead	N/A	Fixable Aqua	0.73611111	N/A	N/A	Thermo Fisher

Table 9. Flow cytometry panels used for MUTZ3-LCs and MoDCs immunophenotyping.

1.20. Cell-free biochemical experiments

1.20.1. Assessment of peanut PLD hydrolysis activity

PLD hydrolysis was assessed using an Amplex Red Phospholipase D Assay Kit (A12219, Thermo Fisher). PLD hydrolyses PC to PA. The kit contains choline oxidase, which oxidises choline to betaine and H_2O_2. In the presence of HRP, H_2O_2 reacts with Amplex red reagent to generate fluorescent resorufin (331). Peanut PLD (Sigma-Aldrich) was diluted to 100µg/ml in DW and 100µl was added to wells in a 96-well plate (Corning Costar). A range of PLD inhibitors including 10µM FIPI (dual PLD1/PLD2 inhibitor), 10µM VU0155069

(PLD1 inhibitor) and 10µM ML298 (PLD2 inhibitor) were added to specific wells. 1µg/ml filaggrin was also added to specific reaction wells, to detect inhibition of PLD hydrolysis activity. Subsequently, the protocol was followed according to manufacturer's guidelines and fluorescence was measured at excitation/emission = 540/590nm using a ClarioStar Plus Microplate Reader (BMG Labtech).

1.20.2. Assessment of peanut PLD transphosphatidylation Activity

PLD catalyses the conversion of membrane PC to PG and choline. Transphosphatidylation by PLD was measured indirectly using a fluorometric Phosphatidylglycerol Assay Kit (ab273288, Abcam). The kit utilises enzymes which interact with PG in the sample and produce an intermediate, which in turn reacts with a probe to generate a fluorometric signal. All wells were prepared in duplicate. In specific wells of a 96-well plate (Corning Costar), 100µg/ml PLD and 100µg/ml PC were mixed in 50µl of Assay Buffer, in the presence or absence of PLD inhibitors including 10µM FIPI (dual PLD1/PLD2 inhibitor), 10µM VU0155069 (PLD1 inhibitor) and 10µM ML298 (PLD2 inhibitor). 1µg/ml filaggrin was also added to specific reaction wells, to detect inhibition of PLD transphosphatidylation activity. The plate was incubated at 37^{o}C for 1 hour. Subsequently, the protocol was followed according to manufacturer's guidelines and fluorescence was read at excitation/emission = 535/587nm using a ClarioStar Plus Microplate Reader (GMB Labtech).

1.20.3. Assessment of additional enzymatic activity of peanut PLD

$sPLA_2$, $cPLA_2$ and SMase activities of stock solutions (10mg/ml) of peanut PLD were detected using specific kits, according to manufacturers' instructions. The $sPLA_2$ kit (Cayman Chemicals) uses the 1,2-dithio analogue of diheptanoyl PC. Upon hydrolysis of the thio ester bond at the sn-2 position by PLA_2, free thiols are detected using 5,5' -dithio-

bis-(2-nitrobenzoic acid) (DNTB), producing a colorimetric product (405nm). The $cPLA_2$ kit (Abcam) uses arachidonoyl thio-PC. Hydrolysis of the arachidonoyl thioester bond at the sn-2 position by PLA_2 releases a free thiol which is detected by DTNB, resulting in a colorimetric product (405nm). The SMase kit (Sigma-Aldrich) determines SMase activity using a coupled enzyme assay, resulting in a colorimetric (655nm) product proportional to the SMase activity present.

1.21. Cell-based biochemical tests

HC MoDCs were generated as previously outlined. Cells were treated with peanut PLD (Sigma-Aldrich) for 16 hours in the presence or absence of 1µg/ml filaggrin, and the presence or absence of specific PLD inhibitors, including 10µM FIPI (dual PLD1/PLD2 inhibitor), 10µM VU0155069 (PLD1 inhibitor) and 10µM ML298 (PLD2 inhibitor). Lipids were then extracted from cells, as previously outlined, and measured for PA content (produced through hydrolysis) or PG content (produced through transphosphatidylation).

1.21.1. Assessment of PA production in MoDCs

Phosphatidic acid content was measured using a fluorometric Phosphatidic Acid Assay Kit (Abcam). PA in the sample is converted by specific enzymes to produce an intermediate, which further converts to a fluorescent product. The protocol was followed according to manufacturer's instructions and fluorescence was read at excitation/emission = 535/587nm using a ClarioStar Plus Microplate Reader (BMG Labtech).

1.21.2. Assessment of PG production in MoDCs

MoDC PG content was measured using the same Phosphatidylglycerol Assay Kit (Abcam, UK) as used in cell-free assays, following the manufacturer's protocol.

1.22. Isoelectric focusing

1.22.1. Assessment of CD1a-lipid binding

IEF assessment of CD1a binding to phospholipids was performed as a modification of a previous protocol (332). Recombinant CD1a was acquired from the NIH tetramer facility or kindly provided by Demin Li and purified in-house by Jess Ng and Yi-Ling Chen (see *Chapter 5.2.2*). Sample phospholipids included 17:0 PG, 18:1 (Δ9-Cis) PG, 18:2 PG and soy PA (Avanti Polar Lipids), and the positive control phospholipid was the ganglioside GD3 (Avanti Polar Lipids). Sample lipids were dissolved in DW containing 0.25% tyloxapol and 2% DMSO, to reach a stock concentration of 2.5mM. GD3 was dissolved in the same solvent to reach a stock concentration of 750µM. 10µg CD1a monomers, dissolved in PBS, were co-incubated with candidate phospholipid in a 1:100 molar ratio, or with GD3 positive control in a 1:30 molar ratio, overnight at RT. Negative controls included CD1a monomers incubated alone in PBS ('CD1a endo') and CD1a monomers incubated with tyloxapol/DMSO solvent ('Sample mock' and 'GD3 mock'). Samples, controls and IEF ladder were loaded into a Novex pH 3-7 IEF Protein Gel and electrophoresis was performed in an XCell Surelock Electrophoresis system, which contained specific IEF anode and cathode buffers (Thermo Fisher). For each condition, 10µg of CD1a protein was loaded onto the gel. The electrophoresis program included running samples at 100V for 1 hour, followed by 200V for 1 hour, and finally 500V for 30 minutes. The gel was fixed in 12% trichloroacetic acid (TCA) on a heat plate at 50^{o}C for 45 minutes, and then rinsed 5 times in 100ml DW for 5 minutes. The gel was stained in 50ml SimplyBlue Safestain (Thermo Fisher) for 2 hours, and residual stain was removed by rinsing in 50ml DW. Gels were visualised using an iBright Gel Imaging System (Thermo Fisher).

1.22.2. Assessment of Ara h 1-phosphatidylglycerol binding

Assessment of Ara h 1 binding to PG by IEF followed a similar protocol to assays using CD1a. Recombinant Ara h 1 was acquired from CusaBio. The PG species 18:1 (Δ-9 cis) PG or 18:2 PG were dissolved in DW containing 0.25% tyloxapol and 2% DMSO, to reach a stock concentration of 1.8mM. 10µg recombinant Ara h 1, dissolved in PBS, was co-incubated with 18:1 (Δ-9 cis) PG or 18:2 PG in a 1:100 molar ratio overnight at RT. Negative controls included Ara h 1 incubated alone in PBS ('Ara h 1') and Ara h 1 incubated with tyloxapol/DMSO solvent ('Sample mock'). No positive controls for Ara h 1-lipid interactions were included, as only PG has been identified to interact with Ara h 1, and this was the first instance of this assay being performed (98). The remainder of the assay followed an identical protocol as per CD1a IEF.

1.23. Differential gene expression analysis

Ruiter et al isolated human mDCs from buffy coats of non-identified blood bank donors (333). mDCs were stimulated with peanut protein extract for 24 hours and total RNA was extracted. Gene array analysis was performed using the GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, CA, USA), performed at the Microarray Shared Resource Facility (Icahn School of Medicine at Mount Sinai, USA). Raw gene expression data (GSE159706) were downloaded from GEO Datasets. Gene microarray data analysis was performed using RStudio software (version 1.4.1103). Expression values were $\log_2$-transformed using the *RMA* function from the package *Affy* (version 1.70.0), accessed using *Bioconductor* (version 3.1.4). Differential expression analysis was performed using the package *limma* (version 3.48.3). Log_2 fold change values, and FDR-adjusted p values, comparing stimulated and unstimulated samples were calculated using the *lmFit*, *makeContrasts*, *eBayes*, *topTable* and *decideTests* functions in *limma*. Volcano plots were

generated with the *volcanoplot* function in *limma*, with significance thresholds of 0.6 Log_2FC and padj<0.05.

1.24. Statistics

Statistical analyses were performed using GraphPad Prism (version 9.2.0) and RStudio (version 1.4.1103). Healthy donor, atopic dermatitis and peanut-allergic donors were compared with respect to CD1a-reactivity using two-way ANOVA. In experiments with three or more treatments in one group, such as comparing HC CD1a responses to PLD which has been exposed to different pH conditions, one-way ANOVA was applied. In experiments comparing response between 2 conditions, two-tailed unpaired or paired t-tests were used.

PHOSPHOLIPASE D GENERATES PRO-INFLAMMATORY CD1A LIGANDS IN PEANUT ALLERGEN-SENSITISED T CELLS

1.25. Current knowledge

1.25.1. The role of phospholipase enzymes in allergic disease

Phospholipase enzymes are important drivers of allergy pathogenesis, and are differentiated by their isoform-specific reaction products (334). Isoforms range from Phospholipase A through to D and can be divided into further subgroups. PLA_2s cleave phospholipids at sn-2 acyl bond to produce arachidonic acid (AA) and LPA, and are the most heavily studied isoforms in atopic diseases (335). Two major subsets of PLA_2s exist: $sPLA_2$s which exist in cytoplasmic granules, and $cPLA_2$s in the cytosol. PLA_2s have multiple implications given their widespread production by a range of leukocytes involved in allergic disease, including mast cells, DCs and T cells (336). This is, in part, due to the downstream products of AA and LPA metabolism, which are eicosanoids and platelet activating factor, respectively (337). Such lipid mediators have wide-spanning roles, including inflammatory cell recruitment and modulation of vascular permeability, and are beyond the scope of this thesis. Phospholipases B (PLB) and C (PLC) are less extensively studied in the context of allergy. Fungi express PLB activities and, although poorly understood, this may contribute towards interactions with the host and subsequent disease (338). PLCβ3 regulates Fc-epsilon receptor I (FcεRI)-mediated mast cell activation through recruitment of the protein phosphatase SHP-1, and PLCε is vital in facilitating the production of proinflammatory cytokines by keratinocytes in a murine allergic contact hypersensitivity model (339, 340). Finally, PLCδ1 protects epidermal barrier integrity through regulation of p38 MAPK activity (341).

Importantly, PLA_2 enzymes are key components of the adaptive immune system, modulating T cell and DC function. For example, GIIA $sPLA_2$ has been linked with increased IL-2Ra in T cells, and GV is more greatly expressed in Th2 subsets (342, 343). $sPLA_2$ GIII induce the maturation of human DCs, promoting their ability to migrate and stimulate T cells (277, 344). $sPLA_2$s have been shown to possess additional mechanistic roles in DCs, including binding of mammalian GIB and GIIA $sPLA_2$s to mannose receptors expressed on some DC subsets, including CD205 (345). Remarkably, bee venom PLA_2 ($bvPLA_2$) is structurally identical to mammalian GIII $sPLA_2$ and binds the mannose receptor CD206 in HDM-sensitised mice, reducing eczema lesions (346). Clear reductions in serum IgE and Th1/Th2 cytokines (including IFN-γ, IL-4 and IL-6) accompany the $bvPLA_2$-CD206 interaction, leading to further studies demonstrating its therapeutic potential in atopic dermatitis (AD) (347). This phenomenon of foreign phospholipases modulating the immune system of mammalian hosts has led to their implication in CD1a biology, given that $bvPLA_2$ generates lysophospholipid neoantigens which can be presented to CD1a-reactive T cells (278). Coupled with the fact that HDM-derived PLA_2 induces similar effects, and CD1a-reactive T cells are upregulated in the blood of patients with AD, an exciting field of exogenous phospholipase-driven allergology has arisen over the past decade. We propose to explore this paradigm in the context of exogenous peanut-derived PLD, and how it may contribute towards peanut allergy.

1.25.2. Peanut-derived PLD produces candidate CD1a antigens

PLD is expressed in plants, mammals, and bacteria, and has been extensively reviewed in the Introduction of this thesis, with respect to its functional role in atopic disease. Whilst there are kingdom-specific differences in their function, such as bacterial isoforms favouring a more alkaline environment compared to that of plants, they possess conserved HKD catalytic domains (see *Chapter 3.6.2*) (348-350). Consequently, they serve as an ideal

exogenous phospholipase candidate for harnessing host catabolism of phospholipids. To date, there is no published literature describing the production of PA and PG from host PC by peanut PLD. It is therefore prudent to explore this avenue, given the evidence that these lipid products are implicated in CD1 biology.

PA is a small phospholipid, consisting of a glycerol backbone with FA chains bonded to C_1 and C_2, and a phosphate headgroup situated at C_3 which confers a single negative charge (351, 352). This simple structure evidences its role as an essential building block in the biosynthesis of other phospholipids (353). Consequently, several PA-containing compounds have been identified as CD1a antigens, including PG, PC, PE, PI and phosphatidylserine (PS) (85). Given the central dogma in CD1a biology that hydrophilic head groups can interfere with TCR-CD1a docking, PA poses as an interesting ligand candidate, with a modest charge and identical aliphatic structure to those already identified (354). PA is upregulated in atopic facial lesions of infants, granting credence to our theory that affected individuals possess elevated circulating PA-reactive T cells (326). Moreover, PA, LPA and LPC are interconvertible through the complex action of PLA_2s and lysophospholipases, as described in *Chapter 3.6.3.1* (197, 198). Xray crystallography has been employed to resolve the binary structure of LPC in complex with CD1a and the CD1a-autoreactive BK6 TCR (BK6-CD1a-LPC) (84). Therefore, peanut PLD has the potential to generate CD1a antigens through direct (PA) or indirect (LPC) action.

PG possesses a similar structure to PA, except an additional glycerol molecule is esterified to the phosphate headgroup (355). Under normal physiological conditions, PG is rare in mammalian cell membranes, however it is both a cause and result of the cellular stress response through fine regulation of its biosynthesis (356). This has raised the potential for PG to play a role in CD1-mediated disease processes, which are characterised by fine-

tuning of cellular phospholipids. PG is a known CD1b ligand, having been identified as the immunodominant lipid antigen in circulating CD1b-reactive T cells via cognate αβ TCR recognition (357). In a landmark study, PG was identified as a CD1a ligand, and PG-loaded CD1a proteins abrogated IFN-γ production when incubated with CD1a-reactive BC2 T cell clones (85). The status of PG as a non-permissive CD1a ligand for the BC2 TCR contrasts with its permissiveness for several other TCR clones (unpublished data by collaborators). However, there are a paucity of data describing its role in polyclonal lipid-reactive T cells or in allergic disease. Given that human PLD recruits AQP3 to generate PG within the skin via transphosphatidylation, we hypothesise that peanut-derived PLD hijacks this mechanism to produce PG for subsequent recognition by circulating CD1a-reactive T cells (240).

Peanut allergy is commonly associated with a prior history of AD, and skin barrier defects are a major predisposition towards peanut sensitisation (57, 65, 358). Epicutaneous exposure to peanut PLD in atopic infants may promote generation of PG and PA, modulating the circulating lipid-reactive T cell repertoire. Potentially, filaggrin deficiency alters the biochemical action of PLD during the generation of CD1a neolipids, in a manner similar to HDM-derived PLA_2 (280). Furthermore, AQP3 is elevated in lesional atopic skin, and we aim to modulate AQP3 function and determine the effect on the peanut PLD mechanism (269). Food allergies, including peanut allergy, are characterised by peptide-specific Th2 responses leading to B cell class switching and IgE generation (359). Elevated type I cytokine responses have also been identified in food-allergic children, with food-challenge inducing excessive IFN-γ production by peripheral blood mononuclear cells (PBMCs) (360). Invariant Natural Killer T cells (iNKT cells) are CD1d-reactive and produce a mixture of Th1 and Th2 cytokines (361). We wish to determine whether CD1a-reactive T cells act in a similar manner and contribute towards the complex Th1/Th2 cytokine milieu that facilitates propagation of peanut allergy.

1.25.3. Objectives

To address these questions, the following overarching objectives have been generated:

1. Compare CD1a-reactive T cell responses to peanut PLD between healthy, peanut-allergic and atopic individuals using functional T cell assays.
2. Determine whether peanut PLD leads to the production of PA and PG in human DCs, and whether these lipids can bind to CD1a.
3. Ascertain whether filaggrin and AQP3 play a functional role in promoting or inhibiting the peanut PLD mechanism.

Untangling these objectives will allow us to further understand the interplay between peanut products, skin barrier dysfunction and lipid-reactive T cells in the development of peanut allergy.

1.26. Results

1.26.1. Peanut PLD inhibits CD1a-reactive T cells derived from healthy individuals

CD1a-autoreactive T cells are a normal component of the human T cell repertoire (de Jong et al. 2010). K562 cells are a human myeloid leukaemia cell line which do not express MHC I or II, and transfection with a CD1a-expression vector (provided by Branch Moody, Harvard) allows for monitoring of CD1a ligand presentation that is not influenced by peptide antigen-presentation pathways; thus, modelling K562 cells as APCs (Lozzio and Lozzio, 1977) (de Jong et al., 2010). Furthermore, K562 cells provide a homogenous population which eliminates donor variability in APCs, facilitating a better comparison of T

cell responses across populations. The enzyme-linked immunospot (ELISpot) assay for IFN-γ secretion can be used to sensitively quantify the frequency of CD1a-reactive T cells (Subramaniam et al., 2016). ELISpot for IFN-γ secretion during coculture of human polyclonal T cells with K562 transfected with CD1a (K562-CD1a) or empty vector (K562-EV) therefore provides a sensitive indicator of CD1a antigen presentation. This model can be manipulated by pulsing K562 cells with potential lipid ligands, or lipid-modifying enzymes, prior to coculture.

Peanuts contain abundant enzymes which govern key metabolic pathways such as glycolysis and lipid catabolism (172, 362). PLD plays a key role in modifying the phospholipid milieu within the seed to protect against drought susceptibility and subsequent aflatoxin contamination. We initially sought to determine if this enzyme may alter the phospholipid content of the K562 'DC surrogates' and modify subsequent T cell responses. Initially, K562 cells were pulsed with increasing concentrations of peanut-derived PLD (dissolved in DW) and IFN-γ ELISpot was performed using T cells from 6 healthy control (HC) donors (*Figure 12A*). Pulsing the K562-CD1a cells with the maximum concentration of 100μg/ml PLD led to a significant reduction in IFN-γ production ($p<0.001$), whereas lower concentrations (0.1-50μg/ml) led to a non-significant trend of reduction. There was no significant change in IFN-γ production after pulsing the K562-EV cells. Therefore, 100ug/ml PLD was observed to induce a CD1a-dependent inhibition of IFN-γ production by T cells, and this concentration was selected for all future experiments.

Given that peanut PLD has optimal enzymatic function at pH 5.6, its immunomodulatory effect was investigated under different pH conditions within our ELISpot system (*Figure 12B*) (172). Peanut PLD was preincubated with 100ug/ml PC substrate (dissolved in ethanol) at a pH range of 4.5-7.4 for 2 hours. Reaction mixtures were then neutralised with

HEPES buffering agent and added to the K562 cells prior to coculture. K562 and T cell viabilities were unaffected by the pH of the preincubation. K562-EV spot numbers were subtracted from that of K562-CD1a to give a better visual representation of the CD1a-specific effect. Pulsing with the reaction mixture led to a significant inhibition of IFN-γ production under all pH conditions ($p<0.0001$). Preincubation at a pH of 4.5 led to a more pronounced inhibition than pH 6.5 ($p<0.05$) and 7.4 ($p<0.01$). Similarly, spot counts were significantly lower in the pH 5.5 condition compared to pH 7.4 ($p<0.05$). This indicated that peanut PLD exerted more pronounced effects under acidic conditions, consistent with the aforementioned pH optimum.

Streptomyces spp. are well known for expressing bacterial PLD with high enzyme activity (Uesugi and Hatanaka, 2009). As part of our preliminary experiments, we aimed to determine any peanut PLD-specific effects through comparison with Stre*ptomyces*-derived PLD (*Figure 12C*). One unit of PLD from *Streptomyces chromofuscus* will release 1 μmol of choline from L-α-PC per hour at pH 8.0 and 37 °C (363). *Streptomyces*-derived PLD evidenced a dose-dependent increase in IFN-γ production by HC T cells, with maximal response at a concentration of 2.5 Units/ml (2.22-fold increase; $P<0.01$). This suggests that *Streptomyces* PLD results in a stimulatory CD1a-mediated effect in HC T cells, compared to an inhibition by peanut PLD. A direct comparison between peanut and *Streptomyces* PLD is also shown in the figure, which further demonstrates the unique inhibitory effect of peanut PLD. It was noted that in this particular experiment, the peanut PLD showed a trend towards inhibition compared to unpulsed target cells.

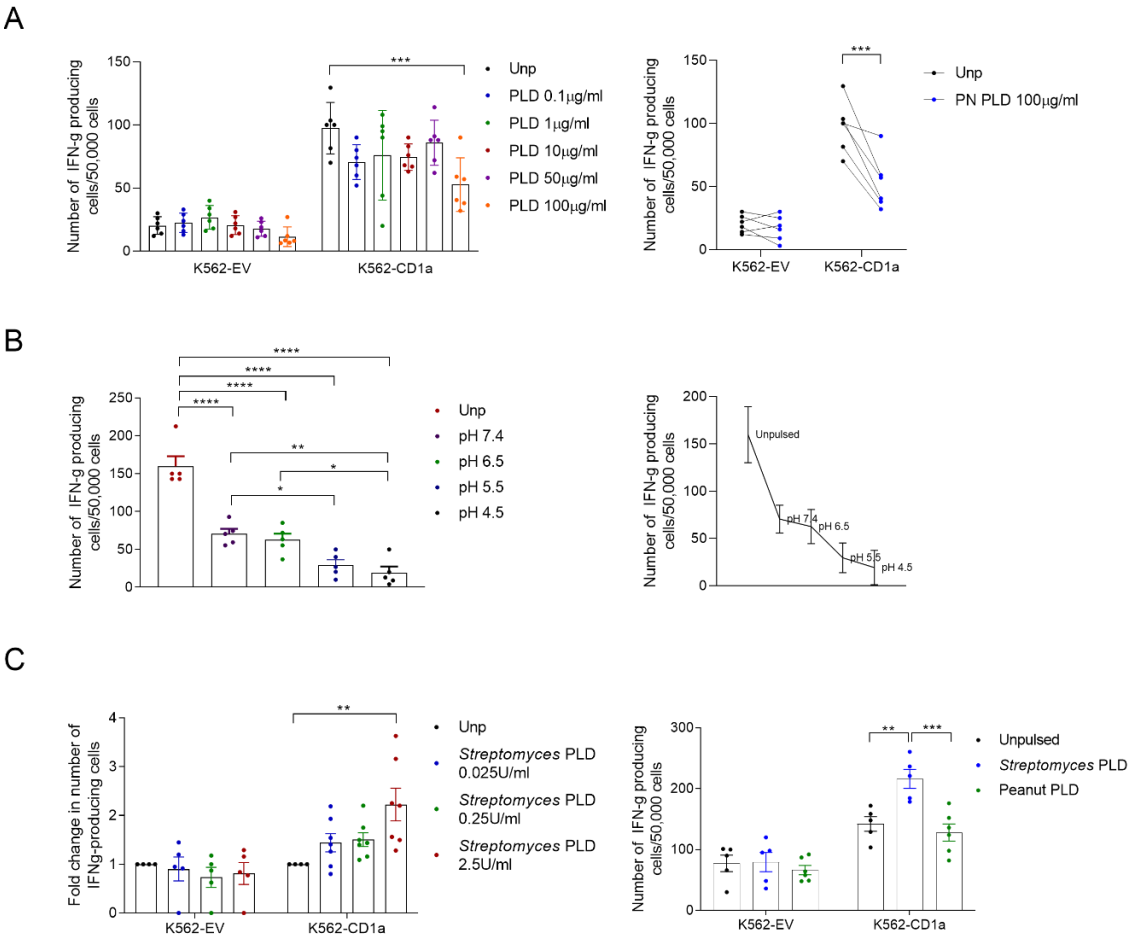


Figure 12. Peanut phospholipase D (PLD) acts via CD1a to inhibit IFN-γ production in healthy T cells. K562 cells transfected with CD1a (K562-CD1a), or transfected with empty vector (K562-EV), were pulsed with PLD and cocultured with blood-derived healthy control T cells (separated by MACS CD3 magnetic cell sorter beads from donor PBMCs) overnight. IFN-γ production was measured by enzyme-linked immunospot (ELISpot) assay. A) Left: K562 were pulsed with different concentrations of peanut-derived PLD; right: summary data showing K562 pulsed with 100μg/ml peanut PLD. B) Peanut PLD was preincubated with phosphpatidylcholine (PC) substrate (dissolved in ethanol) under different pH conditions for 2 hours. Reaction mixtures were then neutralised with HEPES buffering agent and added to the K562 cells prior to coculture. K562-EV spots were subtracted from K562-CD1a spots to aid visualisation and eliminate non-specific background C) Top: K562 cells were pulsed with different concentrations of *Streptomyces*-derived PLD; bottom: K562 cells were pulsed with peanut PLD or *Streptomyces* PLD (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

1.26.2. Peanut PLD produces CD1a ligands through enzymatic action to promote an inflammatory phenotype in T cells derived from peanut-allergic patients

Our group have demonstrated that exogenous and endogenous PLA_2 isoforms can generate CD1a neoantigens, and CD1a-reactive T cells are enriched in atopic patients (Cheung et al., 2016; Jarrett et al., 2016). We aimed to determine if exogenous peanut PLD can produce CD1a antigens in a similar manner. PLD catalyses the conversion of cell membrane PC to PA and PG through hydrolysis and transphosphatidylation, respectively, and these reaction

products were hypothesised to function as CD1a neoantigens which propagate inflammation in peanut-allergic patients (175, 364). Utilising the K562 ELISpot platform, HCs (n=6) were compared with peanut-allergic individuals (n=6) (*Figure 13A*). A striking induction was observed of IFN-γ production by peanut PLD in the peanut-allergic cohort (unpulsed mean = 43.5; PLD mean = 199.75; $p<0.001$), in comparison to an inhibitory effect in HCs (unpulsed mean = 103.8; PLD mean = 52.2; $p<0.01$). Interestingly, CD1a-autoreactivity in T cells, quantified by counting the number of spots in the unpulsed conditions, was elevated in HCs compared to peanut-allergics ($p<0.001$).

Next, it was investigated whether the observed effects of peanut PLD were induced by its enzymatic action, suggesting direct CD1a neoantigen generation, or indirect effects. FIPI is a potent small-molecule inhibitor of PLD, with effects at nanomolar concentrations, and was added to our K562 model (*Figure 13B*) (365). In peanut-allergic (n=6) patients, treatment with 50nm FIPI partially abrogated the IFN-γ-inducing effect of peanut PLD (PLD mean = 153.5 spots; PLD + FIPI mean = 101.7 spots; $p<0.05$). Equally, within the PLD + FIPI condition, IFN-γ production was increased compared to the unpulsed condition (unpulsed mean = 66.5; PLD + FIPI mean = 101.7; $p<0.05$). In HCs (n=6), there was a trend suggesting peanut PLD alone downregulates IFN-γ (in line with previous data), although this was not significant, and this trend was not apparent when K562 cells were cotreated with PLD and FIPI. The lack of significance may be partly due to the large upregulations seen in the peanut-allergic cohort 'masking' the effects. Combined, these results indicate that peanut-allergic patients possess elevated circulating CD1a-reactive T cells which produce IFN-γ in response to K562 lipids generated enzymatically by peanut PLD.

Investigations were performed to determine whether peanut PLD induces similar modulatory effects in MoDCs, providing a more representative model of lipid antigen-presentation. Donor MoDCs express high CD1a and MHCII, and better reflect the complex interplay between the lipid and peptide-presentation pathways (366). Therefore, the ELISpot model was adapted to incorporate MoDCs as APCs, cocultured with autologous T cells. Prior to pulsing, MoDCs were pre-treated with anti-MHC I/II antibodies (α-HLA), to eliminate peptide-specific background, and anti-CD1a antibodies (α-CD1a) or CD1a isotype to detect the presence of CD1a antigen-presentation. *Figure 13C* shows IFN-γ production in cocultures derived from HCs (n=5) and peanut-allergic (n=5) individuals. CD1a-autoreactivity is demonstrated by the significant reduction in spot counts upon addition of anti-CD1a blocking antibody (α-CD1a) to the unpulsed conditions (HCs: $p<0.05$; peanut-allergic: $p<0.05$). Peanut PLD induced IFN-γ production in the peanut-allergic cohort ($p<0.05$), and this was confirmed to be a CD1a-specific effect, given that α-CD1a reduced spot numbers ($p<0.001$). In HCs, PLD inhibited IFN-γ production ($p<0.05$) to a level comparable to α-CD1a treatment. Thus, MoDC coculture data mirrored the findings in K562 experiments.

Finally, experiments were performed to confirm whether peanut PLD induces changes in CD1a expression. HC MoDCs (n=3) were treated with peanut PLD and phenotypic marker expression was measured at 18 hours, mimicking the pulsing stage of the ELISpot (*Figure 13D*). Protein and mRNA expression of CD1a remained high and did not change significantly, allowing confirmation that ELISpot data were not confounded by altered availability of CD1a.

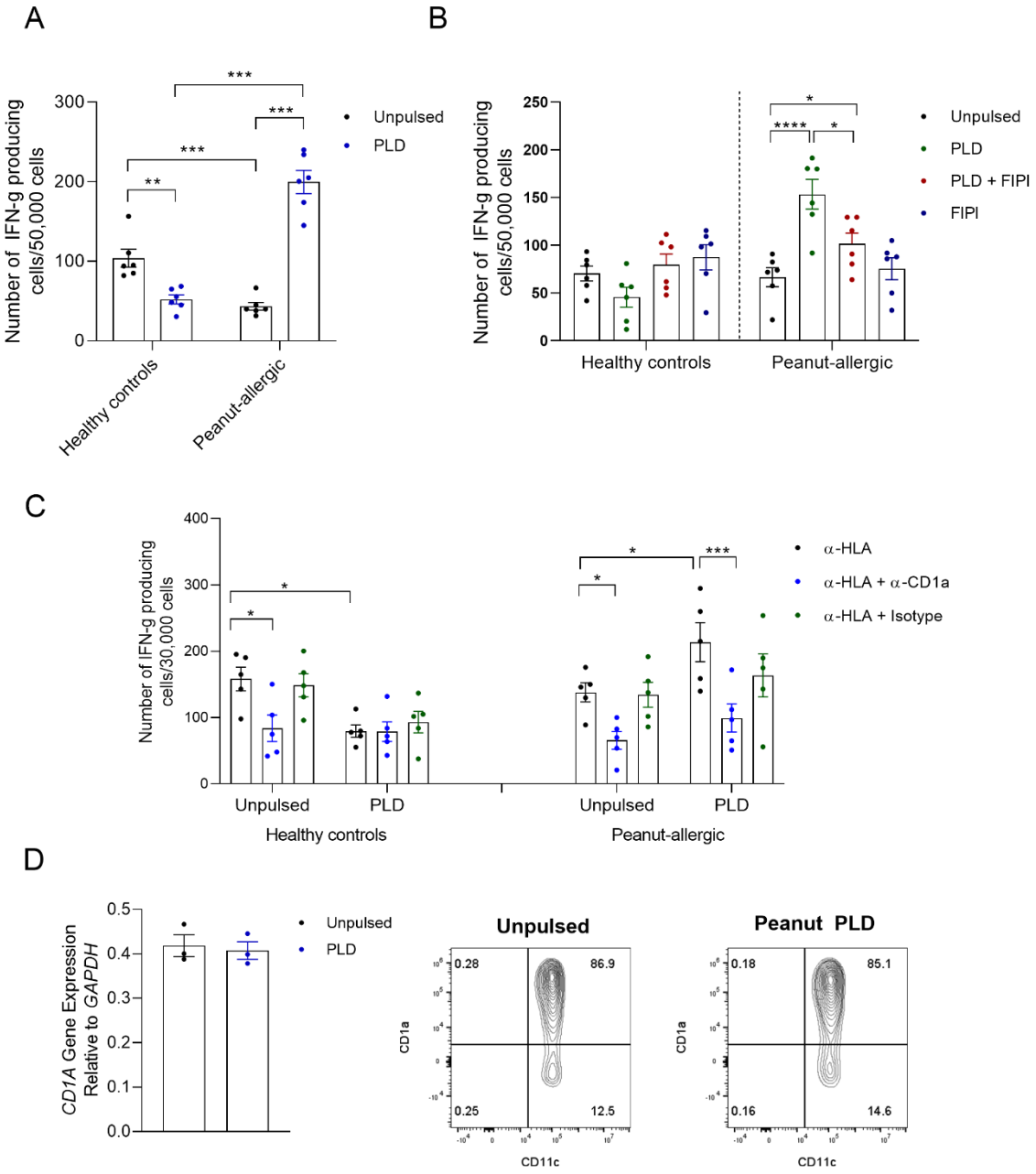


Figure 13. Peanut phospholipase D (PLD) enzymatic activity induces antigen-presenting cells to promote a CD1a-reactive Type I response in peanut-allergic patients. A) K562 cells were treated with peanut PLD and cocultured with CD3+ T cells derived from healthy individuals or peanut-allergic patients, and IFN-γ production was detected by ELISpot. B) K562 cells were pulsed with 100ug/ml peanut PLD +/- 10μM PLD inhibitor (FIPI), cocultured with HC or peanut-allergic T cells and IFN-γ ELISpot was performed. C) Human monocytes were isolated from PBMCs using CD14 MACS separation beads and differentiated into monocyte-derived dendritic cells (MoDCs). MoDCs were pre-incubated with blocking antibody for 1 hour (combinations of anti-HLA Ab, anti-CD1a Ab and IgG1 isotype for CD1a Ab) and treated with peanut PLD. MoDCs were cocultured with T cells and IFN-γ ELISpot was performed. D) MoDCs were harvested after pulsing with PLD. *CD1A* mRNA transcription was analysed by quantitative polymerase chain reaction (qPCR; left) and surface CD1a protein expression was assessed by flow cytometry (right). (*p<0.05; ***p<0.001). (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

1.26.3. Peanut PLD-derived CD1a ligands do not promote a Type 2, Type 17 or Type 22 cytokine response in T cells

Food allergies are characterised by an increased production of cytokines by Th2 cells, including IL-4 which promotes IgE class switching of B cells (367). IL-22 is critical in controlling epithelial homeostasis and is highly upregulated in human and murine AD lesional skin, facilitating production of Type 2 cytokines by keratinocytes (368, 369). IL-17 plays fundamental roles in the pathogenesis of AD and psoriasis, and is downregulated in PBMCs derived from individuals with peanut allergy (370). This inspired experiments to determine whether peanut PLD induces, or inhibits, CD1a-reactive T cells with a Th2, Th17 or Th22 phenotype.

Using a modified system, K562 cells were treated with peanut or *Streptomyces* PLD and, 3 hours after coculture with T cells from HCs (n=6) or peanut-allergic (n=6) individuals, quantitative polymerase chain reaction (qPCR) was performed to measure *IL4*, *IL5*, *IL13*, *IL17A* and *IL22* mRNA expression in cells (*Figure 14A*). To ensure this was representative of cytokine production in T cells, as opposed to K562 cells, values were normalised to expression of *CD2*. *IL4* and *IL13* expression were very low across both cohorts under both pulsing conditions, with Ct values consistently above 37-38. There was a trend suggesting mild induction of *IL4* production in HCs by both PLD isoforms, although this was not significant. *Streptomyces* PLD induced *IL4* production in peanut-allergic individuals ($p<0.05$) however this was unlikely relevant given the low overall expression. Although *IL-13* was significantly higher in peanut-allergic patients compared with HCs ($p<0.0001$), its expression was unaffected by PLD treatment. There was no mRNA expression of *IL5*, *IL17A* or *IL22* in any sample.

Given the lack of conclusive mRNA data, K562 cells were treated with peanut PLD and measured for IL-4, IL-13 and IL-22 cytokine release in K562/T cell coculture supernatants

at 16 hours using enzyme-linked immunosorbent assay (ELISA) (*Figure 14B*). K562-EV data were subtracted from K562-CD1a data to allow easier comparison between groups. IL-4 and IL-13 were detected in peanut-allergic patients, but not HCs (IL4: $p<0.0001$; IL-13: $p<0.01$), and PLD treatment led to no change in cytokine release. Approximate IL-4 and IL-13 protein concentrations in peanut-allergic patients were 11pg/ml and 8pg/ml, respectively. IL-22 was not detected in any sample. Given our sole finding that type-2 cytokines were elevated in peanut-allergic supernatants, we aimed to determine if this involved any CD1a-dependent effects. Experiments were repeated in this cohort (n=4), leading to the observation that CD1a antibody-blocking led to no change in IL-4 or IL-13 release across pulsing conditions (*Figure 15A*). Furthermore, no difference was evident between K562-EV and K562-CD1a coculture supernatants, definitively excluding that previous observations were due to a CD1a-dependent mechanism.

Finally, given the apparent higher sensitivity of the assay, ELISpots were performed using MoDCs and T cells from HCs (n=5) to identify the presence of IL-13- and IL-22-releasing cells (*Figure 15B*). No IL-13-releasing cells were detected in any sample (left panel). PLD treatment led to a marked induction of IL-22 release in one donor (right panel; unpulsed = 15 spots; PLD = 95 spots), however this was not inhibited by CD1a blocking (unpulsed = 9 spots; PLD = 120 spots) and presumed to be a peptide-specific effect. IL-22-releasing cells were not detected in the other donors. In summary, using this system, CD1a-reactive Th-2, Th-17 or Th-22 cells were not detected in HC or peanut-allergic patients. Therefore, given previous observations which highlighted production of type-1 cytokines, only IFN-γ was measured in subsequent functional experiments.

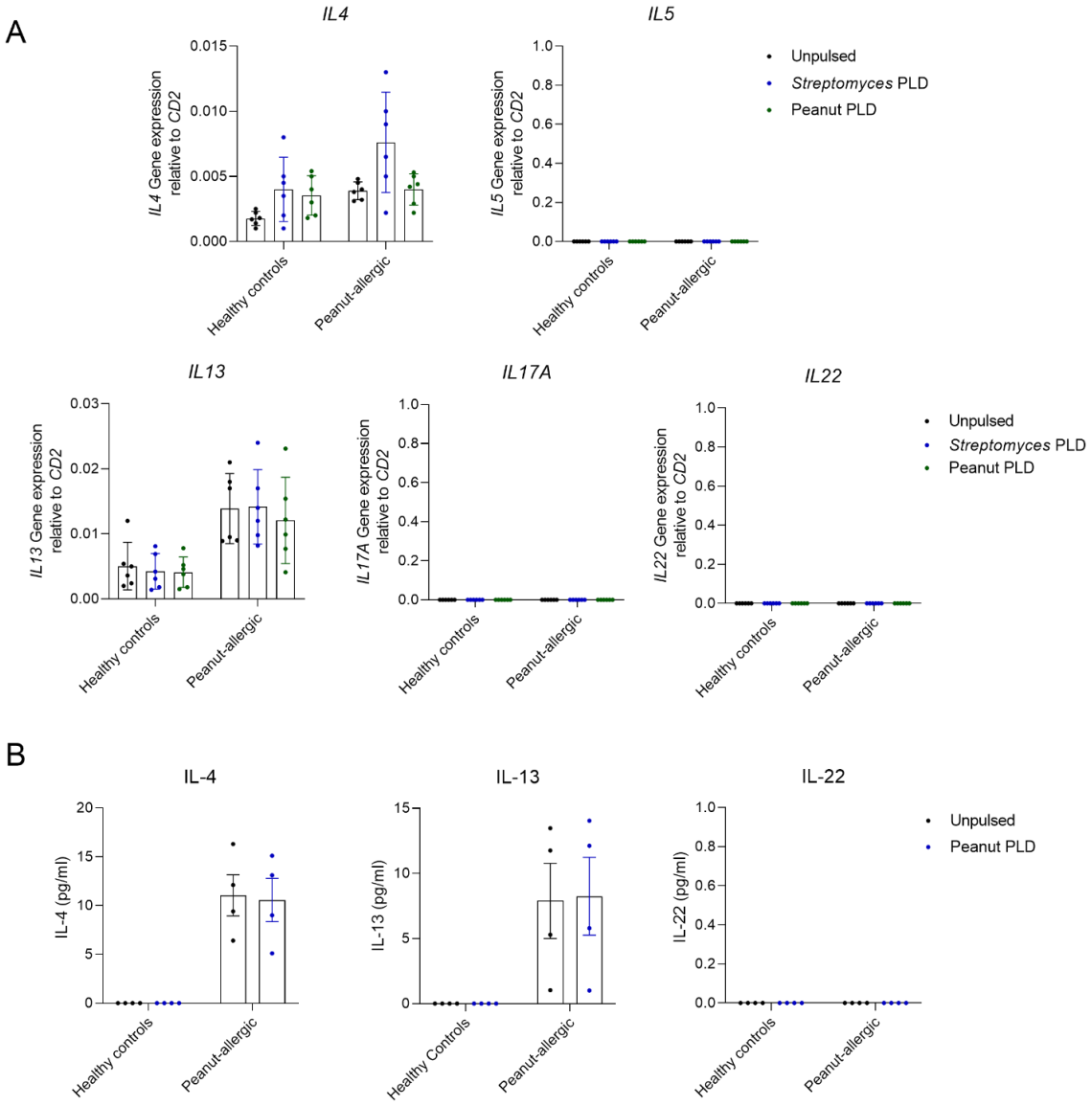


Figure 14. Peanut phospholipase D (PLD)-derived CD1a ligands do not promote a Type 2, Type 17 or Type 22 cytokine response in T cells derived from healthy individuals or peanut-allergic patients. K562 cells were treated with peanut PLD and cocultured with CD3+ T cells derived from healthy individuals and peanut-allergic patients. A) K562 and T cells were harvested after 3 hours of coculture. *IL4*, *IL5* *IL13*, *IL17A* and *IL22* mRNA transcription was analysed using quantitative polymerase chain reaction (qPCR), normalised to T cell expression of *CD2*. B) Supernatants were obtained from K562/T cell cocultures after 16 hours, and IL-4, IL-13 and IL-22 protein levels were quantified using enzyme-linked immunosorbent assay (ELISA).

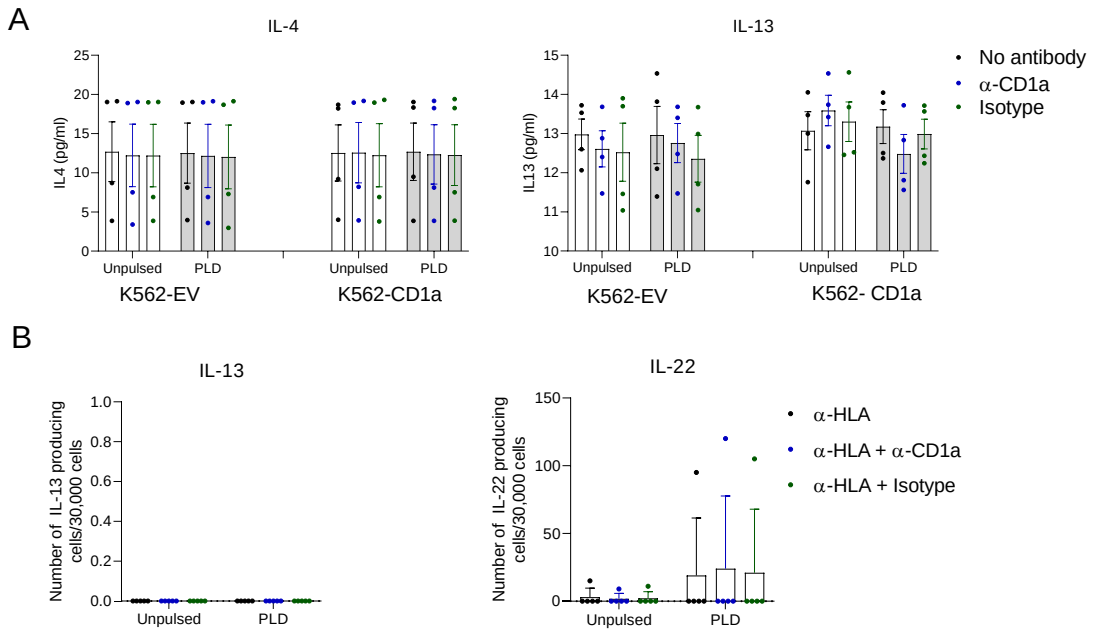


Figure 15. Peanut phospholipase D (PLD)-derived CD1a ligands do not promote a Type 2 or Type 22 cytokine response in T cells derived from healthy individuals or peanut-allergic patients. Antigen-presenting cells (K562 cells or MoDCs) were treated with peanut PLD and cocultured with CD3+ T cells derived from healthy individuals or peanut-allergic patients. A) K562 cells were preincubated with blocking antibody for 1 hour (anti-CD1a Ab or IgG1 isotype for CD1a Ab) prior to antigen pulsing and coculture with peanut-allergic T cells overnight. IL-4 and IL-13 protein levels in coculture supernatants were quantified by ELISA. D) Healthy control MoDCs were generated. MoDCs were pre-incubated with blocking antibody for 1 hour (combinations of anti-HLA Ab, anti-CD1a Ab and IgG1 isotype for CD1a Ab), pulsed with antigen and cocultured with autologous T cells overnight. ELISpot was subsequently performed.

1.26.4. Peanut PLD produces the CD1a-binding phospholipids, PG and PA, in MoDCs

Given the observation that peanut PLD induces CD1a-reactive T cell responses, the underlying mechanisms were investigated. Previously, our lab published the important finding that exogenous PLA_2 derived from HDM generates neolipid antigens in DCs that induce CD1a-reactive T cell responses, contributing towards the pathogenesis of eczema (280). This overarching concept was harnessed to explore the lipid-modulatory behaviour of peanut PLD and resulting production of CD1a ligands.

Initially, cell-free biochemical assays were used to characterise the capacity for peanut PLD to catalyse hydrolysis and transphosphatidylation reactions (*Figure 16A*). All experiments were performed in duplicate and repeated three times, with a final concentration of PLD of

100ug/ml that mirrored the conditions in functional T cell assays. A range of PLD inhibitors were tested for their ability to inhibit the peanut PLD isoform, including 10μM FIPI (dual PLD1/PLD2 inhibitor), 10μM VU0155069 (PLD1 inhibitor) or 10μM ML298 (PLD2 inhibitor) based on published concentrations. PLD hydrolytic activity was 5326 arbitrary units (AU), a measure of fluorescence, and this activity was markedly reduced upon the addition of all inhibitors tested (FIPI = 2348 AU, $p<0.0001$; VU0155069 = 2601 AU, $p<0.0001$; ML298 = 3018 AU, $p<0.001$). Transphosphatidylation activity of PLD was expressed indirectly as a concentration of the reaction product, PG, which was 36.91μM for PLD alone. All inhibitors led to a significant reduction in PLD activity, with the addition of FIPI, VU0155069 and ML298 leading to PG concentrations of 15.86μM ($p<0.01$), 17.07 μM ($p<0.01$) and 21.95 μM ($p<0.05$), respectively. There was no significant difference between the inhibitors in terms of efficacy, and this was apparent in both hydrolysis and transphosphatidylation assays, although concentration titrations may help to define differences. These preliminary experiments therefore provided evidence that peanut PLD catalyses both reactions, and this range of inhibitors could be utilised in subsequent cell-based assays.

To interrogate the model, it was investigated whether peanut PLD generates PG and PA in the DC membrane (*Figure 16B*). MoDCs were generated from 5 HC donors and treated with 100ug/ml PLD for 16 hours, and 10 μM FIPI was included as an inhibitor in select conditions. Cell lipids were subsequently extracted, and assays performed to assess PG or PA production (3 independent experiments for each assay). Basal PG and PA production were 10.3 μM and 11.78 mU/ml, respectively. PLD treatment significantly induced production of PG (26.43 μM, $p<0.05$) and PA (39.29 mU/ml, $p<0.01$). Concurrent FIPI treatment abrogated this effect, reducing concentrations of PG and PA to 12.09 μM ($p<0.05$) and 20.52 mU/ml ($p<0.05$), respectively. There was no significant difference in

phospholipid production between the unpulsed and concurrent PLD/FIPI conditions. In conclusion, peanut PLD enzymatic activity generates PG and PA in MoDCs.

Isoelectric focusing (IEF) is a sensitive method of separating charged molecules based on their isoelectric point (pI), the pH at which the net charge across the molecule is zero (371). Briefly, protein complexes with a negative net charge charged have a lower pI (and vice versa). Protein binding to lipid ligands, which are often negatively charged, thus confers further negative charge to the complex, reducing the pI and visible as migration down the IEF gel in the form of displaced, moved or smeared bands. This phenomenon has been validated as a method of assessing binding of CD1 molecules to phospholipid ligands (332). Using IEF, it was investigated whether CD1a binds to PG and PA, which each confer a negative charge of -1.

Three separate PG species (17:0 PG, 18:1 [Δ9-Cis] PG and 18:2 PG) were initially assessed using the IEF model, as we hypothesised their aliphatic structures were conducive for entry into the CD1a binding groove (*Figure 16C, left panel*). CD1a monomers (dissolved in PBS and kindly provided by Jess Ng and Dr Yi-Ling Chen) were coincubated overnight with a 100x molar excess of each PG species (dissolved in organic solvent; see *3.15.1*) and subjected to IEF. Multiple experimental controls were included. The ganglioside GD3 (dissolved in a different organic solvent mixture; see *see 3.15.1*), strongly binds CD1a and was used as a positive control (30x molar excess). Negative controls included ‘CD1a endo’, ‘GD3 mock’ and ‘Sample Mock’. ‘CD1a endo’ represents CD1a, dissolved in PBS, which has not been coincubated with lipids or organic solvent, and is therefore bound to endogenous expression vector-derived lipids. ‘GD3 mock’ and ‘Sample Mock’ represent CD1a which has been coincubated with the respective organic solvents, to confirm their inherent hydrophobicity has not displaced endogenous lipids. The smeared appearance of

all samples evidences differential lipid binding of individual CD1a proteins, with darker regions (*blue arrows*) representing the most commonly bound ligands. Negative control samples had a pI of ~6.2-6.9, corresponding with the theoretical value of 6.56 for CD1a (372). The GD3 positive control demonstrated a downward shift, with a pI of ~5.3-6, confirming binding of GD3 to CD1a, and displacement of endogenous lipids. Amongst samples, 17:0 PG produced a shift comparable to the positive control (pI ~5.3-5.6), representing PG binding. Remarkably, 18:1 (Δ9-Cis) PG and 18:2 PG induced strong downward shifts (pI ~ 4.7-5.3), confirming complete displacement of endogenous lipids and the generation of CD1a-PG complexes with significantly reduced net charges. Given their strong binding to CD1a, these two PG species were selected for subsequent IEF assays.

Next, soy-derived PA was incorporated into IEF experiments; selected because it is composed of a mixture of PA species with CD1a-amenable FA distributions, ranging from C16:0 to C18:2 (*Figure 16C, right panel*) (373). 18:1 (Δ9-Cis) PG and 18:2 PG were included for comparison, given their strong CD1a binding. A different source of CD1a was used (obtained from the NIH Tetramer Core Facility), with different physicochemical characteristics (pI: ~7; Mw: 50kDa) and more focused bands due to a narrower range of tightly bound endogenously lipids. Consequently, interpretation relied on monitoring for displacement of endogenous lipids by the sample species, as opposed to smeared shifts. 'CD1a Endo' and 'Sample Mock' controls were identical, indicating the sample organic solvent did not lead to displacement of endogenous lipids. Surprisingly, 'GD3 Mock' led to a slight upward shift in pI and was likely due to an interaction between the NIH CD1a and the GD3 solvent, which uniquely contained chloroform and methanol. The GD3 positive control demonstrated clear displacement of endogenous lipids, visible as a much fainter band, demonstrating CD1a binding. 18:1 (Δ9-Cis) PG, 18:2 PG and Soy PA also evidenced displacement and binding. Sample bands were slightly darker and more crimped compared

to the GD3 control, suggesting slightly weaker displacement. In summary, 17:0 PG, 18:1 (Δ9-Cis) PG, 18:2 PG and Soy PA all demonstrated CD1a binding.

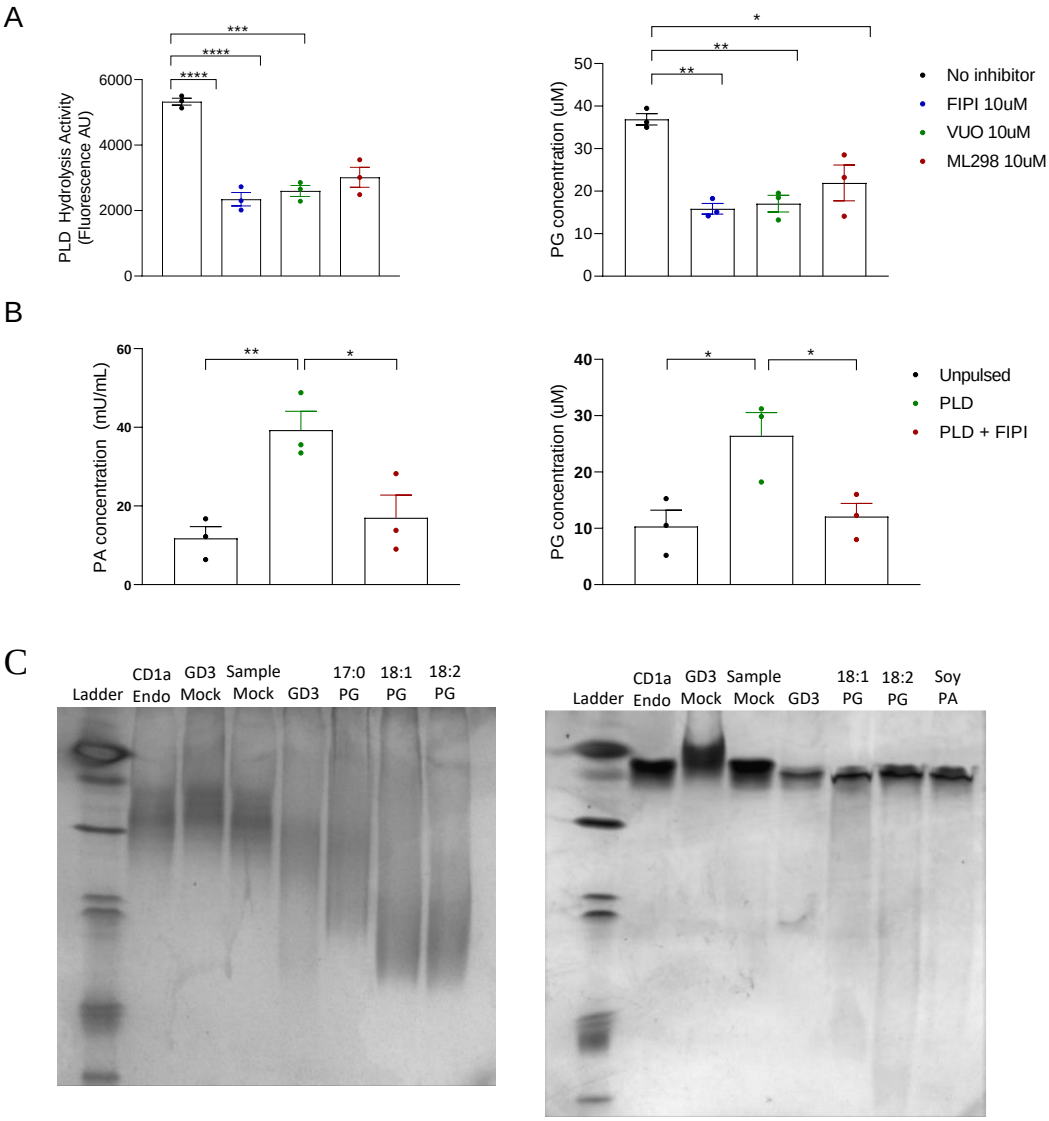


Figure 16. Peanut phospholipase D (PLD) produces the CD1a-binding phospholipids, phosphatidic acid (PA) and phosphatidylglycerol (PG), in MoDCs. A) Peanut PLD was assessed for hydrolysis (production of PA; left) and transphosphatidylation (production of PG; right) activity using cell-free biochemical assays, in the presence/absence of inhibitors: FIPI (10μM; PLD1/PLD2 inhibitor), VU0155069 (10μM; PLD1 inhibitor), ML298 (10μM, PLD2 inhibitor). B) MoDCs were generated from healthy control PBMCs and treated with peanut PLD in the presence/absence of inhibitor. Cell lipids were subsequently extracted, and biochemical assays performed to assess PA or PG production. C) CD1a monomers were coincubated overnight with different PG species (17:0 PG, 18:1 [Δ9-Cis] PG or 18:2 PG, dissolved in organic solvent) and analysed by isoelectric focusing (IEF). GD3, dissolved in organic solvent, was used as a positive control for CD1a binding. Negative controls included CD1a which had been incubated alone ('CD1a endo') and CD1a which had been incubated with organic solvent ('GD3 mock' and 'Sample Mock').

1.26.5. Filaggrin inhibits CD1a-dependent inflammation by peanut PLD

Filaggrin exists in close coordination with epidermal lipids and lipid-modulating enzymes, with cholesterol analogues inducing filaggrin expression and filaggrin deficiency promoting FA generation (*see 4.5.4*) (160, 161). Consequently, filaggrin inhibits the generation of CD1a neolipid antigens by $sPLA_2$ (280). We aimed to determine if filaggrin exerts similar inhibitory effects on peanut PLD and modifies production of PA and PG in APCs.

Initially, the hydrolytic activity of peanut PLD was assessed following coincubation with recombinant monomeric filaggrin at concentrations of 0.1-100 µg/ml (*Figure 17A, left panel*). Resting PLD activity was 5277 AU, and this was significantly inhibited by filaggrin at a concentration of 0.1 µg/ml (2303 AU; $p<0.001$), 1 µg/ml (2283 AU; $p<0.001$) and 10 µg/ml (2359 AU; $p<0.001$). 100 µg/ml filaggrin did not significantly alter PLD hydrolytic activity. Consequently, 1 µg/ml filaggrin was selected for subsequent biochemical experiments, using a similar platform as previously with identical inhibitor conditions. In cell-free assays, the remarkable observation was made that filaggrin inhibits PLD-mediated PA production, by hydrolysis, with an efficacy comparable to the small-molecule inhibitors (*Figure 17B, left panel***;** no inhibitor = 4868 AU; filaggrin inhibition= 2308.7 AU; $p<0.05$). Conversely, filaggrin did not inhibit PG generation by transphosphatidylation (*Figure 17B, right panel*; no inhibitor = 38.3 µM; filaggrin = 42.2 µM; $p = 0.7323$). This effect was then assessed in a cell-based model, using MoDCs (*Figure 17C*). In MoDCs, 1 µg/ml filaggrin produced similar results, inhibiting PLD-mediated production of PA (*Figure 17C, left panel*; PLD treatment = 38.63 mU/ml; PLD and filaggrin cotreatment = 18.64 mU/ml; $p<0.05$), but not PG (*Figure 17C, right panel*; PLD treatment = 42.24 µM; PLD and filaggrin cotreatment = 40.22 µM). This highlighted an extraordinary unimodal effect, whereby filaggrin selectively inhibits PLD-mediated hydrolysis in DCs, reducing downstream PA products.

Next, the effect of filaggrin was explored within the K562 ELISpot system to investigate whether it modulated the CD1a-mediated response to peanut PLD (*Figure 18A*). In peanut-allergic patients (n=6), peanut PLD induced CD1a-mediated production of IFN-γ (unpulsed mean = 71.2 spots; PLD treatment mean = 163.3 spots; $p<0.0001$), whereas combined treatment with peanut PLD and filaggrin abrogated this response (mean = 84.8 spots; $p<0.0001$). In HCs (n=6), the aforementioned inhibitory effect of peanut PLD was observed (unpulsed mean = 103.8 spots; PLD treatment mean = 50.5 spots; $p<0.05$), however filaggrin did not alter this effect (mean = 54 spots; $p > 0.9999$). In both patient cohorts, treatment with filaggrin alone (HC mean = 114.2 spots; peanut-allergic mean = 81.8 spots) or SUMO recombinant control protein (HC mean = 116.8 spots; peanut-allergic mean = 69.5 spots) led to no significant change in IFN-γ production, when compared with the unpulsed condition. This confirmed that filaggrin inhibits peanut PLD-mediated induction of CD1a-mediated T cell responses in peanut-allergic patients.

Filaggrin null mutations are a strong risk factor for atopic eczema (OR: 1.48) (374). Given that many atopic patients are born with filaggrin deficiency, prior to development of peanut allergy, they were investigated for the presence of CD1a-restricted responses to peanut PLD (*Figure 18B*). In the K562 model, PLD treatment induced IFN-γ responses in patients with AD (n=8; unpulsed mean = 89.1 spots; PLD treatment mean = 146.6 spot; $p < 0.05$). No change was observed in the HC cohort (n=8), and there was no inhibition of IFN- γ release, in contrast with previous experiments. Interestingly, in the AD cohort, IFN-γ induction by peanut PLD (~1.7-fold upregulation) was less than that previously noted in the peanut-allergic cohort (~4.6-fold upregulation), however these data are derived from separate experiments and therefore statistics cannot be performed for accurate comparison.

In summary, these data demonstrated that filaggrin inhibits production of PA in APCs by peanut PLD, preventing subsequent induction of CD1a-mediated T cell responses in peanut-allergic patients. Moreover, patients with AD, who often have *FLG* null mutations or have

filaggrin insufficiency (even with wild type *FLG*), exhibit CD1a-reactivity to peanut PLD, although possibly to a lesser extent.

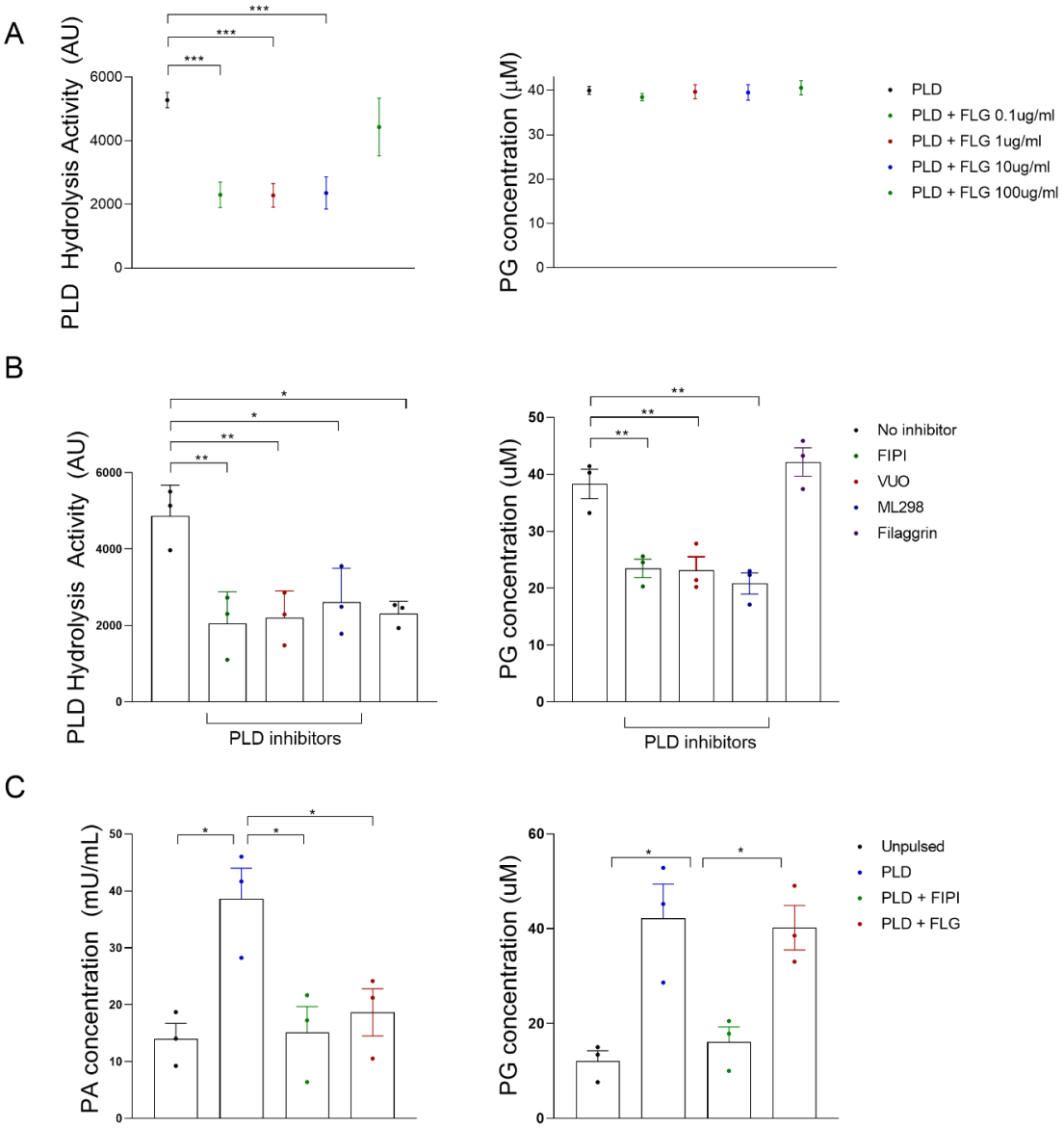


Figure 17. Filaggrin inhibits phosphatidic acid (PA), but not phosphatidylglycerol (PG), formation by peanut phospholipase D (PLD) in monocyte-derived dendritic cells (MoDCs). A) Peanut PLD (100µg/ml) was coincubated with different concentrations of recombinant filaggrin monomers and cell-free biochemical assays were performed to measure hydrolysis and transphosphatidylation activity. B) PLD biochemical assays were repeated using 1µg/ml recombinant filaggrin and a range of PLD inhibitors: FIPI (10µM; PLD1/PLD2 inhibitor), VU0155069 (10µM; PLD1 inhibitor), ML298 (10µM, PLD2 inhibitor) 1 C) MoDCs were generated from healthy control PBMCs treated with peanut PLD +/- recombinant filaggrin or FIPI inhibitor. Cells were harvested and lipids extracted to measure production of PA (left) and PG (right) (*p<0.05; **p<0.01; ***p<0.001).

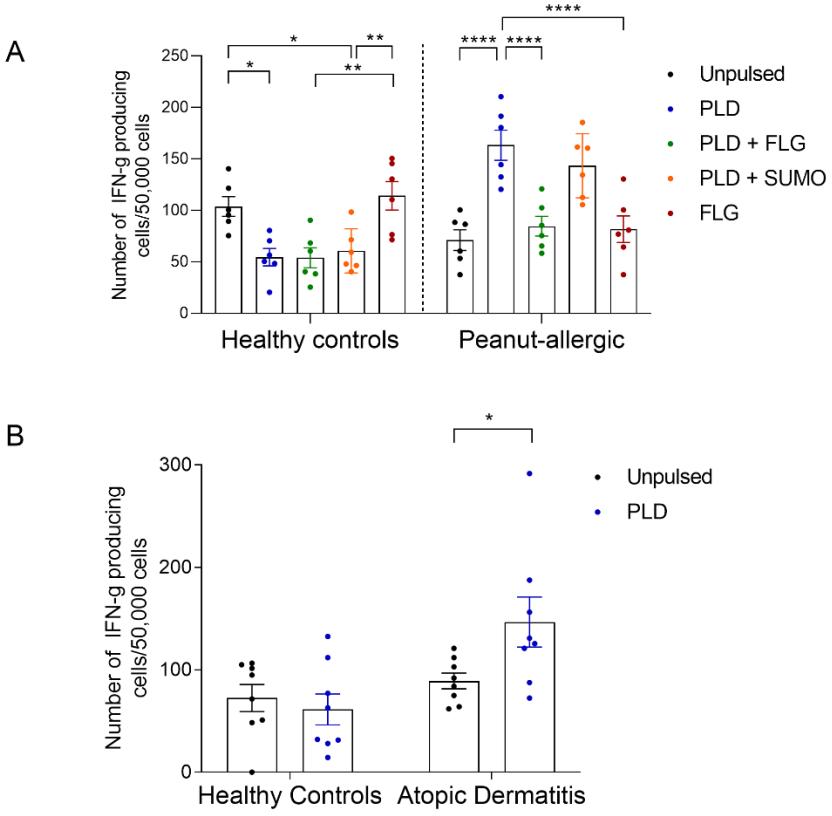


Figure 18. filaggrin inhibits CD1a-reactive T cell responses to peanut phospholipase D (PLD) in peanut-allergic individuals, and peanut PLD induces CD1a-reactive responses in patients with atopic dermatitis (AD). K562 cells were pulsed with A) Peanut PLD +/- recombinant filaggrin monomers +/- recombinant Small Ubiquitin-like Modifier (SUMO) control for filaggrin and cocultured with T cells from healthy controls (HC) or peanut-allergic patients. B) Peanut PLD and cocultured with T cells from HCs or patients with AD. IFN-γ ELISpots were then performed as previously described (*p<0.05; **p<0.01; ****p<0.0001).

1.26.6. Peanut PLD upregulates AQP3 expression in antigen-presenting cells, which is required for CD1a-reactivity in peanut-allergic patients

AQPs comprise a family of transmembrane water and glycerol transporters, which have relevance in a range of neurological, endocrinological and immunological disease processes (375). AQP3 belongs to the aquaglyceroporin class, selectively transporting glycerol substrates onto PLD to produce PG within the skin (240, 251). Thus, it was postulated that peanut PLD recruits AQP3 within our disease model.

Initially, AQP3 expression was measured in APCs used in cocultures (*Figure 19A*). Initially, K562-EV (n=3) or K562-CD1a (n=3) cells were pulsed with 100µg/ml peanut PLD for 12 hours, cells were harvested and analysed by qPCR for *AQP3* mRNA expression

relative to the housekeeping gene *GAPDH*. Strikingly, PLD treatment led to a sharp induction of *AQP3* expression in the K562-CD1a subset (~15-fold; $p<0.0001$) compared with no change in K562-EV cells (*Figure 19A, left panel*). Subsequently, the experiment was repeated in MoDCs generated from healthy donors (n=3), again revealing a significant induction of *AQP3* expression in cells treated with PLD (*Figure 19A, right panel*). These surprising observations demonstrated that AQP3 was not only present in our APCs, but could be also induced further by peanut PLD, suggesting an interesting positive feedback mechanism.

Having made this interesting observation, we sought to determine if this effect was conserved in other DC subsets using other sources of peanut protein (*Figure 19B*). Previously, Ruiter et al isolated mDCs from anonymous blood bank donors, extracted and pooled cellular RNA, performed gene expression profiling by chip array and uploaded raw data to the publicly available GEO DataSets (GEO accession GSE159706) (333). Briefly, differential gene expression analysis was performed using RStudio Software (*limma* package) and volcano plots were generated illustrating gene expression in PLD-treated cells relative to untreated cells. Peanut protein treatment led to ~ 2.5-fold upregulation in *AQP3* expression ($p=0.004$) and a remarkable ~9.3-fold upregulation in *CD1A* expression ($p= 0.000006$). These bioinformatic results supported our prior *in vitro* analysis and the notion of a dynamic signalling unit that exists between CD1a, AQP3 and peanut constituents such as PLD.

Finally, AQP3 activity was inhibited in the ELISpot model (*Figure 19C*). Within the peanut-allergic cohort (n=6), peanut PLD treatment led to the expected induction of IFN-γ release by T cells (unpulsed mean = 94.2 spots; PLD treatment mean = 161.8 spots; $p<0.001$), however this was abrogated by simultaneous addition of AQP3 inhibitor (mean = 103.4 spots; $p<0.05$). AQP3 inhibition alone led to no significant change in IFN-γ secretion (mean = 80.7 spots). Conversely, in HCs (n=6), PLD treatment led to a trend suggesting

inhibition (unpulsed mean = 87.2 spots; PLD treatment mean = 60.3 spots; non-significant) and AQP3 inhibition led to no appreciable change (PLD/inhibitor cotreatment mean = 110.7 spots; inhibitor treatment mean = 94.2 spots). Overall, these results demonstrate that AQP3 functional activity is required for the induction of CD1a-reactive T cells by peanut PLD in this coculture system.

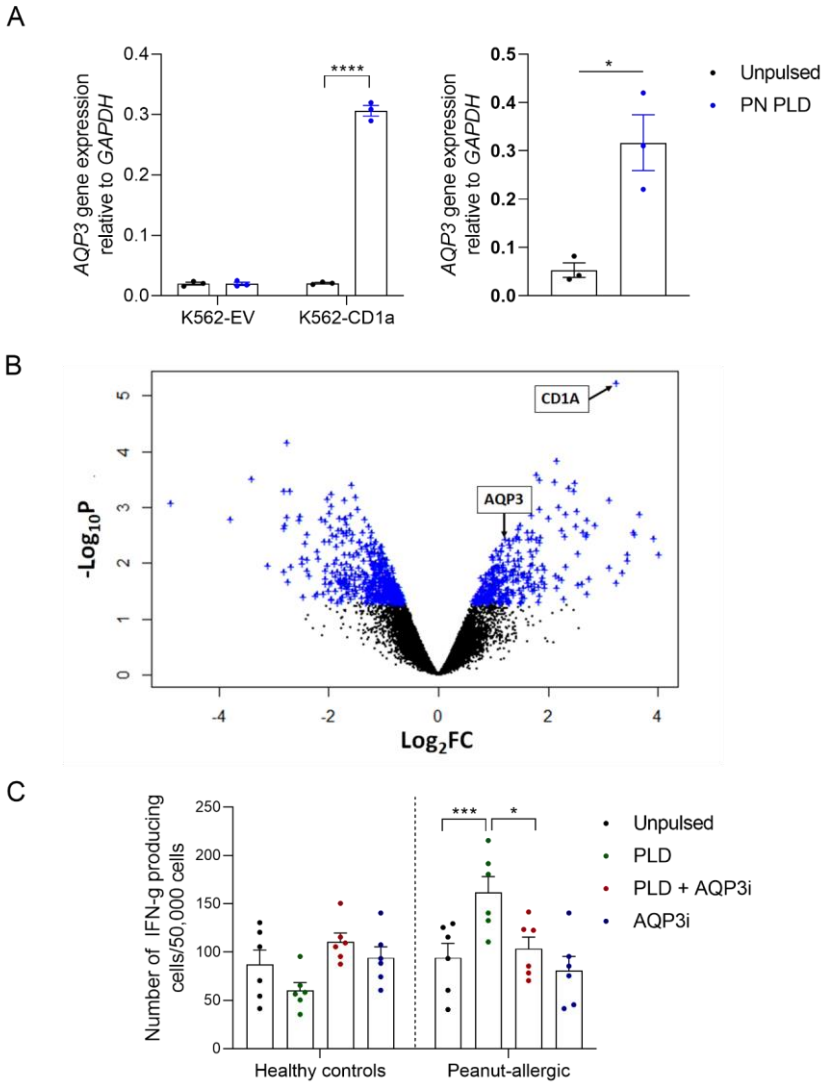


Figure 19. Dendritic cells express Aquaporin-3, which is upregulated by peanut PLD and contributes towards CD1a-reactive T cell responses in peanut-allergic patients. A) K562 cells or monocyte-derived dendritic cells (MoDCs) from healthy controls (HCs) were treated with peanut PLD for 12 hours and *AQP3* mRNA expression (relative to GAPDH) was determined using quantitative polymerase chain reaction (qPCR). B) Previously, human myeloids DCs (mDCs) (n=7) had been treated with peanut protein extract, and RNA was pooled before gene expression profiling by array. (333). GEO accession GSE159706 was obtained, and differential gene expression analysis performed using R software to create volcano plots. Blue crosses represent genes with significant differential expression between treatment conditions (adjusted p value < 0.05; $Log_2FC > 0.6$ or $Log_2FC < -0.6$). C) K562 cells were pulsed with 100ug/ml peanut PLD +/- 0.4μM AQP3 inhibitor (DFP00173), cocultured with HC or peanut-allergic T cells and IFN-γ ELISpot was performed (*p<0.05; ***p<0.001).

1.26.7. Peanut PLD possesses multimodal enzymatic activity

Having investigated the functional consequences of PLD-induced hydrolysis and transphosphatidylation, it was determined if peanut PLD possesses other enzyme activities that may play a role in CD1a antigen presentation. This was achieved using several biochemical assays. *Figure 20A* (*left panel*) demonstrates the $sPLA_2$ activity of peanut PLD. Stock PLD (10mg/ml) had an activity of 0.09 µmol/min/ml and lay within the optimal detection range of the test (0.02-0.2 µmol/min/ml). This was approximately one twentieth of the activity of the PLA_2 positive control. *Figure 20A* (*middle panel*) shows stock peanut PLD has a $cPLA_2$ activity of 9.9 nmol/min/ml, approximately one quarter the activity of the PLA_2 +ve control (39.3 nmol/min/ml). Finally, SMase activity of peanut PLD was detectable at 0.18 milliunits/ml, where one unit will hydrolyse 1 µmole of TNPAL-sphingomyelin per min at pH 7.4 at 37 °C (*Figure 20A, right panel*). This was approximately one sixth the activity of the SMase positive control (1.21 milliunits/ml). Overall, these data demonstrate that peanut PLD possesses additional $sPLA_2$, $cPLA_2$ and SMase activities.

PLD has previously been shown to possess SMase activity, catalysing the hydrolysis of SM to CER 1-phosphate and choline (Flores-Diaz et al., 2016). A recent study demonstrated that SM species with long-chain acyl chains inhibit CD1a , and therefore catabolism of this sphingolipid might dampen inhibitory effects (de Jong et al., 2014). Therefore, this function was investigated, and IFN-γ ELISpots were performed in 3 HC patients using peanut PLD and exogenous SM (*Figure 20B*). Results suggest a dose-dependent SM inhibition of CD1a activity, which was ameliorated with the addition of peanut PLD. Increased numbers of donors are required to investigate if this effect is significant. Furthermore, there was marked ethanol vehicle inhibition in this experiment, possibly confounding CD1a-dependent effects. Despite these factors, peanut PLD can potentially modulate CD1a antigen

presentation through multiple enzymatic pathways.

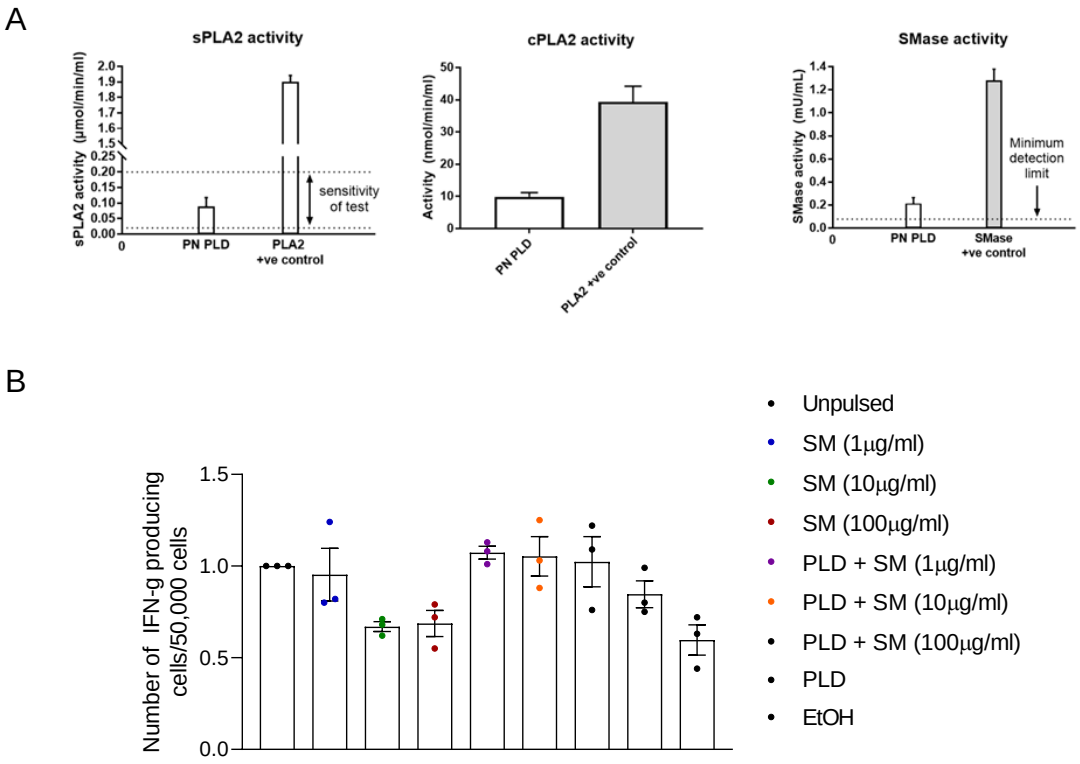


Figure 20. Peanut phospholipase D (PLD) possesses multimodal enzyme activity, however sphingomyelinase (SMase) activity does not modulate a CD1a response. A) Cell-free biochemical assays demonstrating the secretory phosholipase A_2 ($sPLA_2$; (left), cytosolic PLA_2 ($cPLA_2$; middle) and SMase activity (right) of peanut PLD. B) K562 cells were pulsed with 50µg/ml peanut PLD +/- varying concentrations of sphingomyelin (SM). PLD alone, SM alone and ethanol vehicle (EtOH) were used as controls. IFN-γ ELISpots were performed as previously, and fold changes calculated.

1.27. Discussion

1.27.1. Characterising the functional response of peanut PLD in healthy T cells

Phospholipase enzymes are biological regulators which produce key phospholipids which mediate fundamental cellular activity (334). Consequently, phospholipase-driven processes are conserved across animals, bacteria, fungi and plants (338, 376-378). Considering CD1a biology, their endogenous immunomodulatory potential has been identified, given that $cPLA_2$ in mast cells produces CD1a neolipid antigens that contribute towards inflammation in psoriasis (279). However, the discovery that HDM-derived PLA_2 similarly contributes towards eczema evidences the ability of exogenous isoforms to harness host phospholipids

and leukocytes to amount an immune response (280). This phenomenon was explored in the context of peanut allergy, given that peanuts contain abundant PLD (172).

Initial experiments involved determining the interaction between peanut PLD and APCs (using both CD1a-transfected K562 cells and primary MoDCs), and the resulting modulation of CD1a-reactive T cell responses. Surprisingly, peanut PLD induced inhibition of IFN-γ release by HC T cells. Whilst our data suggest this is CD1a-mediated, it is difficult to confirm with certainty using our coculture model for two reasons. Firstly, non-transfected K562 cells induce much lower IFN-γ release by T cells compared with their CD1a-transfected counterparts, and this reduced reactivity may have masked non-specific inhibition. Secondly, it is not possible to definitively confirm CD1a-dependent inhibition using MoDC coculture ELISpot experiments. Naturally, prospective neoantigens exert identical inhibitory effects irrespective of treatment with α-CD1a or Isotype antibody.

Multiple arguments exist to support the notion that PLD induced inhibition independent from CD1a-mediated processes. PLD isoforms are known to modulate the innate immune system through their reaction products. For example, PG inhibits TLR activation by a range of danger-associated molecular patterns (DAMPs), including the antimicrobial peptides S100A9 (TLR2/TLR4) and β-defensin-2 (TLR2) (250). Ordinarily, the TLR co-receptor CD14 presents DAMPs to TLR2 and TLR4, however this mechanism is disrupted when CD14 binds PG (248, 379-381). Interestingly, antibodies directed against the LPS binding site on CD14 also inhibit PG binding, suggesting common sites of interaction (248). Given that MoDCs possess the required innate machinery, including TLRs and detectable CD14, it is plausible that PG inhibited TLR-induced activation of T cells (382, 383). PG has also been shown to inhibit mRNA expression and secretion of TNFα by RAW264.7 cells, which have a monocytic phenotype (250). TNF has been shown to augment levels of IL-2R on

CD4 and CD8 T cells, increasing cytokine production, and therefore PG may have inhibited this process within our experiments (384, 385). In addition to PG, PA exerts wide-ranging cellular effects, including binding of the guanine nucleotide exchange factors (GEFs) DOCK2 and SOS, which activate Rho GTPases, including Rac1 and Ras (386, 387). Potentially, exogenous PLD produces excessive PA, which disturbs the tight regulation of the PA-GEF-Rho GTPase axis that is necessary for efficient T cell activation. Moreover, PA binds to cAMP-PDE4A1, and cAMP-elevating agents have been shown to inhibit peptide antigen-presentation by DCs (388, 389). Finally, insertion of PA into lipid membranes induces a negative curvature, affecting membrane remodelling and vesicle production, with potential resulting effects on DC function (390). Overall, therefore, whilst PLD may have induced CD1a-dependent inhibition in HC T cells, this is potentially confounded by the multitude of mechanisms discussed. *Chapter 7* explores the potential CD1a-mediated effects of the PLD reaction products, PG and PA.

Peanut PLD is reported to function optimally at ~pH 5.5, and this was investigated within our functional coculture assays (348). Remarkably, acidic assay conditions led to a more pronounced inhibition of IFN-γ release by HC T cells, with the strongest inhibition at pH 4.5. Strong evidence suggests that sensitisation to peanuts occurs via dysfunctional atopic skin, and the SC of AD lesions is more alkaline (~pH 6.5) compared to that in healthy epidermis (~pH 5.5) (358, 391). Our results raise the question of whether a healthy, acidic, environment propagates the anti-inflammatory effects of peanut PLD (391). Normal skin acidification is a concerted effort between: the generation of FFAs by $sPLA_2$, the pumping of protons by sodium-hydrogen exchanger 1 (NHE1) protein into the extracellular space surrounding the SG and filaggrin catabolism to produce degradation products (145, 392, 393). Disturbances in these processes lead to an increased risk of AD. Disease is more frequent at anatomical sites with an elevated pH, including the cheeks of infants (394).

Equally, application of alkaline soaps can induce atopic lesions, potentially exacerbated by the removal of protective lipids (395). Consequently, acidification of atopic skin is an emerging therapeutic goal, and may partly explain the beneficial effect of colloidal oatmeal which is present in many topical treatments (396). Similarly, in a mouse HDM-sensitisation model, acidified Cetaphil cream (pH<3) was protective against development of atopic lesions (397). Potentially, alkaline eczematous skin ablates the anti-inflammatory effect of peanut PLD and contributes towards peanut sensitisation. Further pH-based experiments in atopic and peanut-allergic donors are required to test this hypothesis.

In additional preliminary experiments, peanut PLD was determined to exert a unique inhibitory effect in HCs, compared with induction of IFN-γ by *Streptomyces* PLD. This observation may be partly due to the differing pH optima between *Streptomyces* PLD (~7.35) and peanut PLD (~5.5), given the neutral assay conditions (348, 363). Streptomyces infections are rarely reported within the human literature, however the catalytic HKD motif of PLD is strongly conserved across bacterial species (398-400). Our incidental observations suggest a future avenue of investigating the CD1a-mediated effect of PLD expressed by opportunistic microorganisms in AD, such as *Staphylococcus aureus* (401). Conversely, the hygiene hypothesis states that inadequate early exposure to infectious microorganisms stunts immune development, consequently leading to increased prevalence of atopic diseases and food allergies (402). Comparison between healthy, atopic and peanut-allergic cohorts would help us understand whether bacterial PLD exacerbates or protects against allergic sensitisation.

1.27.2. Peanut PLD promotes CD1a-reactive T cell responses in peanut-allergic patients

Following characterisation of the effects of peanut PLD in HC donors, these were compared in peanut-allergic individuals. These data highlight an unforeseen paradigm where peanut

PLD enzymatic activity induces DCs to promote IFN-γ production in peanut-sensitised T cells, whilst exerting a reciprocal inhibitory effect in HC T cells. Importantly, this occurs via a CD1a-mechanism of action. A higher lipid content in human DCs is associated with a stronger immune response, however the K562 cell line eliminates inter-donor variability in phospholipid content (403). Their homogeneity allowed confirmation that peanut-allergic patients possess elevated circulating CD1a-reactive T cells. Patients with AD and psoriasis demonstrate similar elevated CD1a-reactivity to HDM-derived PLA_2 and mast cell $cPLA_2$, respectively (279, 280). This inserts our findings into a growing field of CD1a biology in which exogenous allergens, and intrinsic cellular proteins, provoke excessive lipid-reactive responses through enzymatic action.

PLD inhibition did not completely abrogate CD1a-reactive IFN-γ production in peanut-allergic patients. This suggests peanut PLD possesses additional effects to direct phospholipid synthesis and CD1a-restricted presentation to T cells. Lipids can also act as adjuvants which stimulate TLRs and amplify CD1-restricted responses; interestingly, this can occur concurrently with, or days prior to, CD1-restricted T cell activation (404). PA has been implicated in TLR-4 mediated PLA_2G4A activation, and PLD may therefore indirectly induce PLA_2-derived CD1a neoantigens (405). Studies suggest further upstream roles of PLD in TLR signalling. In a murine model of sepsis, LPS-induced inflammation was ameliorated by blocking PLD and its downstream signaling molecules Sphingosine Kinase 1 (SphK1), extracellular signal-regulated kinase 1/2 (ERK1/2) and NF-κB (406). In pDCs, SphK1 is critical for TLR7/9-induced production of type I IFN (407). ERK is involved in the regulation of TLR2, TL4 and TLR9 in mouse DCs, and NF-κB is activated by all TLRs (408, 409). Finally, PLD is required for subcellular recycling of TLR9 from endosomes to autophagosomes (410). K562 cells express TLR2, TLR3, TLR4, and TLR7, and PLD may

have acted via additional, indirect, mechanisms to contribute towards CD1a-reactive responses. (411).

1.27.3. Peanut PLD promotes a Type I-only response in CD1a-reactive T cells: roles in disease pathogenesis

Peanut-allergic patients possess elevated circulating peptide allergen-specific Th2 cells, compared with Th1 skewing in healthy individuals and children who outgrow peanut allergy (412, 413). Whilst Th17 responses are well established in psoriasis pathogenesis, there are limited data in the context of peanut allergy. IL-17 production was impaired in CD4+ T cells from peanut-allergic children with at least one additional food allergy, implicating Th17 responses in food tolerance (370). Atopic dermatitis is partly characterised by epidermal infiltration of Th22 cells which induce Th2-biased immunity, and IL-22 blockade is a promising therapeutic approach (368, 414). Consequently, it was investigated whether peanut PLD induces CD1a-reactive T cells to produce these cytokines. Our data demonstrate no appreciable production of Type 2, 17 or 22 cytokines in healthy or peanut-allergic donors. Surprisingly, therefore, observed CD1a-reactive responses were confined to IFN-γ production. In future, additional methods of measuring cytokine production should be used, including flow cytometric secretion assays and tetramer-based cell sorting, as further discussed in *Chapter 9.2*.

Importantly, IFN-γ production has been shown to be increased in food-allergic children and is functionally significant (360). IFN-γ upregulates MHC II expression on keratinocytes, which can subsequently present the HDM allergen Der p 1 to CD4+ T cells and induce IL-4 production (415-417). Equally, in primary normal bronchial epithelial cells, IFN-γ markedly increases the expression of the IL-4 receptor (IL-4R) and C-C Motif Chemokine Ligand 17 (CCL17), a powerful chemokine which helps T cells target the skin and is upregulated in AD lesions (418). This is accompanied by increased expression of Lecithin–cholesterol acyltransferase (LCAT), driving production of cholesteryl esters. Given that

cholesteryl esters bind to CD1c, stabilise its conformation and enhance binding of CD1c self-reactive T cells, this may facilitate antigen presentation by $CD1c^+$ LCs in the skin (419). Furthermore, lipid-reactive T cells have been demonstrated to facilitate peptide-specific T cell responses. Through production of IFN-γ and/or IL-4, CD1d-restricted iNKT cells mature DCs through 'licensing'(361). iNKT-licensed DCs express upregulated MHC and costimulatory molecules, including CD80 and CD86, and produce CCL17 to promote recruitment of CCR4+ CD8+ T cells. Licensing is a complex process which may include simultaneous activation of DCs by PAMPs and/or other Th cells (420). Finally, recombinant IFN-γ is known to induce maturation of DCs and provoke similar phenotypic changes (421). Consequently, the delicate cytokine environment within the skin is likely integral in this cross-priming process. Overall, peanut PLD-responsive CD1a-reactive T cells may demonstrate similar adjuvant activity. Production of IFN-γ may induce peptide-specific Th2 responses through a range of mechanisms, for subsequent generation of IgE.

1.27.4. Filaggrin insufficiency in AD may promote generation of PA-reactive T cells

Cell-free biochemical experiments demonstrated that peanut PLD exhibits high activity which can be blocked by a range of small molecule inhibitors. The inhibitors tested were developed for use with endogenous human PLD1 and PLD2 isoforms, and to our knowledge, this is the first documented evidence of their use with peanut PLD (422-424). Their efficacy with peanut PLD may be due to the conserved HKD catalytic domain conserved across all PLD isoforms (see *Chapter 3.6.2*) (350). Cell-based experiments demonstrated that peanut PLD possesses enzymatic activity which leads to generation of PA and PG in MoDCs. Both phospholipid products bound to CD1a, suggesting they mediate CD1a-dependent effects previously observed in peanut-allergic patients. To this end, inhibition of PLD activity abrogates CD1a-reactivity in peanut-allergic T cells, and we propose this occurs through reduced production of PG and PA. Using IEF, a range of CD1a

candidate lipid species were tested, including 17:0 PG, 18:1 (Δ9-Cis) PG, 18:2 PG and Soy PA, which each possess acyl chains of suitable length for nesting in the CD1a groove (287). Importantly, this artificial model is based on the assumption that these lipid species were produced by peanut PLD, and suggestions for further experiments are discussed in *Chapter 9.1*. However, given that PLD can produce a wide range of PA and PG species, and the fact all species bound strongly by IEF, we suggest this assumption is justified (425, 426).

Barrier dysfunction is a key factor in the pathogenesis of eczema and there is an increased incidence of filaggrin null mutations in patients with AD (Agrawal and Woodfolk, 2014, Palmer et al., 2006). Reduced barrier integrity has been shown to increase epicutaneous immunisation by peanut proteins, reflecting increased peanut protein-specific IgE reactivity in patients with AD (Hill et al., 2000; Strid et al., 2004). In a similar manner, barrier dysfunction may promote penetration of PLD-derived lipids into the deeper layers of the epidermis. Subsequent detection by sentinel CD1a+ DCs/LCs and presentation to naïve T cells in peripheral lymph nodes would therefore increase production of circulating lipid-specific CD1a-reactive T cells in patients with AD. It was observed that filaggrin inhibits peanut PLD-induced production of PA in MoDCs. This is concordant with findings reported by Jarret et al, which showed that filaggrin inhibits $sPLA_2$ (Jarrett et al., 2016). Our biochemical data suggest a novel barrier sensing mechanism, whereby filaggrin functionally inhibits hydrolysis by peanut PLD. Consequently, deficient filaggrin in atopic patients may result in loss of this inhibition, increased PLD activity, and subsequent CD1a neoantigen (PA) generation in DCs. This may also explain why PA is upregulated in the lesional facial skin of atopic infants (326). Several laboratories have shown that PA produced by PLD is essential for activation of mTOR (Gomez-Cambronero, 2010). Increased activation levels of mTOR (specifically the subunit, Raptor) are correlated with decreased filaggrin expression in patients with AD, further suggesting an inverse relationship between filaggrin and PLD hydrolytic activity (Naeem et al., 2017). This

theory is supported by functional ELISpot data, presented in *Chapter 6.2.5*, in which T cells derived from patients with AD showed exaggerated CD1a-reactive responses to peanut PLD. Moreover, filaggrin abrogated PLD-specific CD1a-reactive responses in peanut-allergic patients. Taken together, epicutaneous application of peanut PLD to filaggrin-deficient skin may promote generation of PA-specific T cells, contributing towards peanut sensitisation. Equally, PLD may more readily produce neoantigens in atopic patients with filaggrin mutations, stimulating a CD1a response that contributes towards ongoing inflammation.

In addition to PLD activity, peanut PLD possessed detectable $sPLA_2$, $cPLA_2$ and SMase activities. Moreover, PLD displayed $cPLA_2$ and SMase activities that were one-quarter and one-sixth that of their respective positive controls. It was hypothesised that PLD breaks down SM, therefore protecting against CD1a inhibition. However, no significant effect was detected in ELISpot experiments. Conversely, our group has produced data showing that staphylococcal SMase C does possess this effect (unpublished), demonstrating the importance of this mechanism in CD1a signalling. Furthermore, our group have demonstrated that $sPLA_2$ and $cPLA_2$ are important in CD1a neoantigen generation (Cheung et al., 2016; Jarrett et al., 2016). Interestingly, $sPLA_2$ group X has high catalytic activity towards the PLD substrate PC (Atout et al., 2012). This supports the fact that phospholipase species can have multiple enzymatic activities, suggesting that neoantigen generation is a complex process of coordination between different enzymes.

1.27.5. Upregulated AQP3 in atopic skin may drive production of PG-reactive T cells

Given that filaggrin did not modulate the transphosphatidylation activity of peanut PLD, it was hypothesised that upregulation of AQP3 in atopic skin leads to PG-sensitisation (269). Experiments confirmed that peanut PLD treatment of MoDCs led to marked upregulations

in *AQP3* mRNA. Differential gene expression analysis of mDCs treated with crude peanut protein evidenced similar upregulations in *AQP3,* in addition to a vast increase in *CD1A* expression. This posits an interesting concept, whereby exogenous peanut PLD recruits, and induces, endogenous AQP3 to generate PG which can be presented by CD1a. Moreover, it lends much credence to our hypothesis of a dynamic peanut PLD-AQP3-CD1a signaling module. Supporting this theory, AQP3 activity was shown to be required for induction of PLD-responsive CD1a-reactive T cell responses in peanut-allergic patients. Elevated AQP3 in atopic lesional skin may promote this process and gives rise to excessive production of PG-reactive T cells, contributing towards peanut sensitisation. This introduces AQP3 inhibition as a potential therapeutic strategy in peanut-allergic patients, and this topic is discussed in detail in *Chapter 9.4***.**

1.28. Chapter summary

In conclusion, we have highlighted new concepts in CD1a biology that link AD and peanut sensitisation through generation of T cells which are reactive to the products of peanut phospholipase activity. This is directed by dysregulation of epidermal constituents in atopic patients, including reductions in filaggrin content and excessive expression of AQP3. Consequently, this raised the hypothesis that peanut-allergic patients possess elevated PG- and PA-reactive T cells, and this is explored in the next chapter. In *Chapter 9*, future studies are proposed which further investigate the phenotype and function of peanut PLD-responsive T cells, in addition to experiments that probe CD1a-lipid-TCR interactions. Moreover, animal models will be discussed, including their potential to inform peanut PLD-based therapeutics.

Peanut allergy is associated with elevated PA- and PG-reactive T cells

1.29. Current knowledge

1.29.1. Peanut proteins and lipids drive allergic responses

Peanuts are the edible seeds of the legume crop, *Arachis hypogaea*, and are technically a variety of pea (427). The first records of their cultivation were documented by the ancient Inca Empire of Peru, however their modern popularity originated during the 19th century American civil war with the development of hundreds of peanut products (428). Today, peanuts are consumed globally, with thousands of cultivars adapted to meet specific requirements in terms of flavour, aesthetics and suitability for further processing (429). World leaders in peanut production include China (~40%) and India (~15%), with the USA and Nigeria each accounting for 7% of volume (430). Their ubiquitous consumption has given rise to a wide variety of preparatory methods; for example, they are predominantly eaten roasted in Western society, in contrast with a preference towards boiled, raw or fried nuts in both China and India. This dichotomy has driven the major hypothesis that roasting confers allergenicity, providing an explanation for the rise in peanut allergy prevalence in Western countries, as reviewed in *Chapter 3.4.3* (97, 431). Hypoallergenic cultivars have been recently identified using monoclonal antibody-based ELISA technology, which demonstrated reduced levels of the major allergens Ara h 1, 2, 3, 6 and 8 (432). These studies support the theory that variations between peanut constituents, and their modifications during food processing, impact upon symptoms and severity of peanut allergy. Peanuts contain a wealth of proteins and lipids, serving a variety of functions in preserving seed structure and function, in addition to defence against plant pathogens ((73). Lipid co-oxidation of peanut proteins is a major cause of food degradation during storage, affecting both food quality and nutrient availability (433). Equally, proteins regulate the delicate balance of lipids within seeds, including PLD-mediated production of PA, which

protects against drought stress and subsequent aflatoxin contamination (172). Therefore, the bidirectional functional relationship between peanuts and lipids has important implications on public health, including allergic immune responses. This provides a clear rationale that in addition to patient-specific factors, including genotype and environmental factors which predispose to epicutaneous sensitisation, molecular variations between peanuts also contributes towards development of peanut allergy. Considering the marked immunogenicity of affected patients, we wished to determine if constituent proteins and lipids contribute toward CD1a-reactive T cell responses and drive allergic disease.

1.29.2. The potential for peanut lipids to modulate CD1a mechanisms of disease

Peanut seeds and refined Po, from which protein content has been eliminated, contain high lipid content, including FAs, phospholipids and various TAGs (75, 79). Peanut whole lipid extracts have been shown to enhance HDM-induced production of endothelin-1 (ET-1) in keratinocytes; ET-1 is a peptide which promotes pruritus in AD lesions (434). This suggests that peanut lipids facilitate skin barrier damage, thereby promoting epicutaneous sensitisation to other peanut constituents. In broader terms, peanut-derived lipids are known to modulate both innate and adaptive immune responses and will be discussed in the context of DC and CD1a biology, to inform their potential effects on regulating peanut allergy.

FAs are carboxylic acids with long saturated or unsaturated chains (435). Po contains both SFAs and UFAs, with the latter predominating, as in other vegetable oils (79, 436). UFAs can be further subdivided into MUFAs and polyunsaturated FAs (PUFAs) (436). OLA ($C_{18:1}$) is the major peanut MUFA constituent, accounting for 35.6-58.3% of total FA content, whilst the most common PUFA detected is LA ($C_{18:2}$), representing 20.9-43.2% of content (79). Conversely, the SFA PlA ($C_{16:0}$) makes up 9.3-13.0% of overall content.

Outside CD1a biology, dietary FAs have been demonstrated to play wide-spanning roles across immune homeostasis and disease pathogenesis. Functioning as integral structural components of phospholipids and as a major metabolic energy source, dietary FAs possess key immunomodulatory properties including regulating inflammatory processes (437-440). Dietary LA has been demonstrated to promote ERK-mediated IL-10 induction in DCs, with corresponding upregulations in IL-10R expression and downregulations in IL-12 (441). Such changes lead towards skewing from a Th1 to a Th2 phenotype, which is a hallmark of peanut allergy (442). Furthermore, LA and other PUFA species increase epithelial permeability, as evidenced in airway epithelial cells (443). Although not investigated to date, we suggest that similar permeability disruption may occur in keratinocytes upon epicutaneous exposure to LA, promoting deeper access of other peanut allergens during sensitisation. Conversely, OLA has been reported to have anti-inflammatory effects, through FA oxidation-driven induction of $FOXP3^+$ Tregs (444). In a murine study of sepsis, OLA supplementation increased expression of IL-10 and reduced levels of IL-1β and TNF-α in peritoneal lavage fluid (445). Furthermore, application of OLA to the skin of mice has been demonstrated to promote wound healing, partly through decreased expression of cyclooxygenase 2 (COX2) (446). Finally, PlA, and other SFA species, are known pro-inflammatory mediators, acting through multiple processes. In a human study, PlA was shown to function as TLR4 ligand, promoting TLR4 endocytosis by MoDCs and subsequent IL-1β production. (447). Authors further noted that PlA induced MoDC maturation and activation through upregulation of the costimulatory molecules CD86 and CD83. Consequently, SFA-rich diets increase the frequency of pro-inflammatory $CD11c^+CD103^-CD11b^+$ DCs in the gut, whilst reducing tolerogenic $CD11c^+CD103^+CD11b^+$ subpopulations (448). As a major peanut derived SFA, PlA may induce similar effects within the skin.

Clearly, dietary FAs have a complex role in promoting and inhibiting inflammation; given the high content of OLA, LA and PlA in peanut seeds, we seek to determine if these effects are apparent in CD1a-reactive T cells. As reviewed during the introductory chapter, OLA is a known CD1a ligand, evidenced in xray crystallography studies (84). Whilst no structural studies exist to prove their CD1a-binding capacity, LA and PlA possess acyl structures of appropriate length for nesting within the CD1a binding groove (287). Consequently, they offer promising targets for our functional T cell assays.

Po is a good source of other apolar lipids, including squalene (270-1296 mg/kg) and sterols (900-4344mg/kg), 3.8% of which comprises cholesterol (449, 450). As previously discussed, squalene is a sebaceous CD1a autoantigen, offering an interesting exogenous target in peanuts (85). Similarly, cholesteryl esters have been demonstrated to activate the BC2 CD1a-reactive T cell line (85). Po contains 0.15-0.90% sterol esters, and may stimulate similar responses (81). Moreover, cholesterol analogues are upregulated in the skin of patients with AD, and it would be interesting to determine if they possess increased circulating cholesteryl-reactive T cells (138). Given that AD predisposes towards peanut sensitisation, this T cell pool may be responsive to peanut-derived cholesteryl derivatives, contributing towards disease. Finally, Po comprises several other plant-specific cholesterol-like compounds, including β-Sitosterol (47.4-69.0% of total sterols), which may also act via CD1a (450).

Amongst polar compounds, refined Po comprises abundant phospholipids, consisting of PC (38.3–66.4%), PE (13.3–21.9%, PA (2.2-11.8%), PG (up to 2.5%) and multiple sphingolipids (78µmol/kg) (79, 204). PA and PG are generated in plants by PLD, through hydrolysis and transphosphatidylation, respectively (451, 452). Their involvement in governing innate and adaptive immune responses has been extensively reviewed in previous

chapters. PG is known to bind CD1a, and PA is a key constituent of most phospholipids (85, 353). We hypothesise these phospholipid species are responsible for enhanced CD1a-mediated effects of PLD in peanut-allergic patients, as evidenced in *Chapter 6.2.2*. Moreover, PC and PE, with acyl chain structures of 18:2/18:2, derived from cypress pollen have been shown to act as exogenous CD1a ligands (Agea et al., 2005). In summary, peanuts may act as a reservoir of direct CD1a ligands, necessitating investigation of CD1a responses to Po and lipid extracts from seeds.

1.29.3. Peanut proteins as potential modulators of CD1a-reactive responses

Peanuts contain a matrix of proteins with many functions including providing structural integrity, energy storage and modulating lipids to assist in key processes such as seed germination (73). Having previously confirmed the relevance of phospholipase activity, we will review proteins which demonstrate lipophilic activity, in order to inform their impact on CD1a biology and peanut-allergic responses.

To date, 18 major protein peanut allergens have been identified and proven to initiate IgE-mediated responses and induce clinical anaphylaxis in sensitised patients, as reviewed in the Introductory Chapter (453). Ara h 1 is a vicilin-like seed storage protein which cross-reacts with other food allergens and is recognised by more than 90% of peanut-allergic individuals, often leading to severe symptoms (454). Importantly, this protein has been demonstrated to bind to PG, which enhances allergenicity through multiple mechanisms, including reduced uptake by DCs (98). In another study, peanut lipid extracts possessed adjuvant activity which enhanced Ara h 1-induced production of IL-8, IL-6, and TNF-α by in human primary keratinocytes (434). Equally, Ara h 1 inhibited peanut lipid-induced IL-10 release, promoting a proinflammatory phenotype. Ara h 8 exhibits 48% protein sequence homology to the major birch pollen allergen Bet v 1, leading to high IgE cross-reactivity

(95, 455). Consequently, Ara h 8 may be the causative agent of OAS in birch-allergic patients. Moreover, lipid binding confers proteolytic stability to Ara h 8, endorsing allergenicity (97). Therefore, both allergens demonstrate a functional relationship with lipids, including Ara h 1-PG lipid binding, which modulates clinical responses. We wish to investigate whether their lipid interaction capacity additionally modulates CD1a-reactive T cell responses.

Another group of strongly lipophilic peanut proteins comprise the plant-specific oleosins, some of which have been identified as major peanut allergens (456). Oleosins contain both hydrophilic and hydrophobic domains, allowing direct interaction with phospholipids, as previously reviewed (457). Correspondingly, they are an important constituent of plant oleosomes, or OBs, spherical organelles containing a phospholipid monolayer embedded with oleosins. This matrix of lipophilic proteins and phospholipids makes OBs another promising target for CD1a; particularly given the monolayer structure which provides easier access to phospholipid antigens. Moreover, phospholipid content of peanut OBs comprises 61.6% PC and 5.0% PE (458). Both phospholipid species have been identified as CD1a antigens, reinforcing our hypothesis that peanut OBs stimulate lipid-reactive T cell responses. Potentially, oleosins chaperone PC and PE from the phospholipid monolayer of OBs onto CD1a, in a manner analogous to loading of α-galactosylceramide (α-GalCer) onto CD1d by saposins (459).

1.29.4. Objectives

We wish to determine if peanut protein and lipids act as exogenous CD1a ligands, widening understanding of CD1a-reactivity to foreign substances. Additionally, we aim to further

elucidate the lipid-modulatory role of peanut PLD, through assessment of CD1a-reactivity towards its reaction products, PG and PA.

To address these aims, the following objectives have been developed:

1. Investigate the capacity for pure peanut lipids to stimulate CD1a-reactive T cells
2. Compare CD1a-reactive T cell responses to PG and PA between healthy and peanut-allergic individuals. This will model the effects of peanut phospholipids and further our understanding of peanut PLD responses.
3. Investigate the effect of peanut proteins within our CD1a antigen presentation system, including the major protein allergens Ara h 1 and Ara h 8, peanut protein extracts, peanut SPT and oleosins embedded in OBs.

1.30. Results

1.30.1. Peanut oil and total lipid extract from peanuts do not stimulate T cells via CD1a

Refined Po contains abundant FAs (>60% OLA) and phospholipids, consisting of PC (38.3–66.4%), PE (13.3–21.9%), PA (2.2-11.8%) and PG (up to 2.5%) (79). We hypothesised that pure lipid extracts from peanuts may serve as a novel, and yet undiscovered, source of CD1a ligands. To test this hypothesis, Po and peanut total lipid extracts (pTLE) were incorporated into the ELISpot platform. Given their pure lipid nature, Po and pTLE must often be dissolved in non-polar organic solvents and/or aqueous solutions containing detergents (460). Detergents are amphipathic molecules which integrate with lipids and form spherical monolayer micelles, which are miscible in water (461). We screened a panel of commonly-used organic solvents and detergent mixtures for dissolution of Po and TLE, respectively. As described in *Chapter 5.7*, 10mg/ml solutions of Po were prepared in dimethylsulfoxide (DMSO), chloroform, ethanol and chloroform: ethanol (C:E) mixed at a ratio of 2:1 (*Figure 21A, top panel*). Samples were vortexed for 30

seconds and assessed for their miscibility in each solvent using a qualitative grading system ('None' = visibly immiscible, with sample separating from solvent within the tube; 'Partial' = some dissolution, often accompanied by a slightly turbid colour change; 'Complete' = complete dissolution, with a clear appearance). Po was completely immiscible with DMSO at this concentration, and only partial dissolution was achieved in pure ethanol. However, complete miscibility was observed in both chloroform and C:E 2:1. Next, chloroform: methanol lipid extraction (using a modified Bligh and Dyer method; see *Chapter 5.8.1*) was performed on raw peanuts to produce pTLE. 10mg/ml solutions of pTLE were prepared in different formulations of PBST, containing 0.01-0.5% of the emulsifying agent Tween (*Figure 21A, bottom panel*). Samples were sonicated for 10 seconds, followed by vortexing for 30 seconds, and this process was repeated four more times. Miscibility was then scored using a similar system, with identical grading criteria except that 'complete' dissolution represented both clear solutions and homogenous milky emulsions. Whilst pTLE was immiscible in 0.01% PBST, all other concentrations (0.05%, 0.1% and 0.5%) led to complete dissolution. Therefore, these preliminary experiments highlighted that Po dissolves in both chloroform and C:E 2:1, whilst pTLE is miscible in PBST at concentrations of 0.05-0.5%.

Following the identification of appropriate solvents and detergents for dissolution, during further trial experiments, their effects were tested within the K562 system. To maintain the lowest possible concentration of chloroform in assays, given its known cytotoxicity, C:E 2:1 was selected as the model Po solvent. K562-EV and K562-CD1a cells were suspended in RPMI and pulsed with C:E 2:1, to reach final solvent concentrations ranging from 0.1-5% v/v (*Figure 21B*). Cells were incubated overnight 37^{o}C and 5% CO_2 for 12 hours (identical conditions to ELISpots), and cell viability was then quantified. In both K562-EV and K562-CD1a subsets, at lower C:E concentrations of 0.1% and 0.5% v/v, cell death was

≤ 2%. At medium concentrations of 1% and 2% v/v, cell death ranged from 3.5-5%. A high concentration of 5% v/v, C:E 2:1 led to marked cell death which was significantly greater compared with all other concentrations (K562-EV cell death = 26%; $p<0.0001$; K562-CD1a cell death = 36%; $p<0.0001$). In summary, organic vehicle control led to clear dose-dependent cell death. We therefore aimed to minimised this in future ELISpots assays by ensuring <1% v/v organic solvent within cell suspensions.

Tween 20, also named polysorbate 20, is a nonionic surfactant containing a derivative of the sugar alcohol sorbitol and lauric acid (462). Lauric acid is a 12-carbon saturated fat found in many vegetable oils (463). Whilst its chain length is atypical for CD1a ligands, shorter-chain ligands such as urushiol (C_{15}) have been identified. Therefore, we recognised the potential of Tween 20 to stimulate CD1a-mediated responses and confound interpretation of responses to pTLE. PBST solutions containing 0.01-0.5% Tween 20 were therefore incorporated into preliminary K562 ELISpot assays, measuring HC (n=5) T cell IFN-γ release (*Figure 21C*). Within the K562-EV subset, no significant change in cytokine release was observed, regardless of the concentration of Tween 20. Interestingly, pulsing of K562-CD1a cells with 0.5% PBST led to a significant induction of IFN-γ secretion (unpulsed mean spots = 70.4; 0.5% PBST mean spots = 100.6; $p<0.05$). 0.1% PBST did not lead to a significant change, however a trend suggesting induction was observed (mean spots = 86.2). Finally, 0.01% PBST (mean spots = 72) and 0.05% PBST (mean spots = 66) led to no change in IFN- γ release. In summary, Tween-20 stimulated CD1a responses at the higher concentration of 0.5%. Moreover, 0.05% PBST was selected for dissolution of pTLE in future ELISpot experiments, to maximise miscibility and minimise vehicular interference.

A

	Detergent	Ratio or percentage	Miscibility
Peanut oil	DMSO	100%	None
	CHCl3	100%	Complete
	EtOH	100%	Partial
	C:E	2:1	Complete
pTLE	PBST	0.01%	None
	PBST	0.05%	Complete
	PBST	0.10%	Complete
	PBST	0.50%	Complete

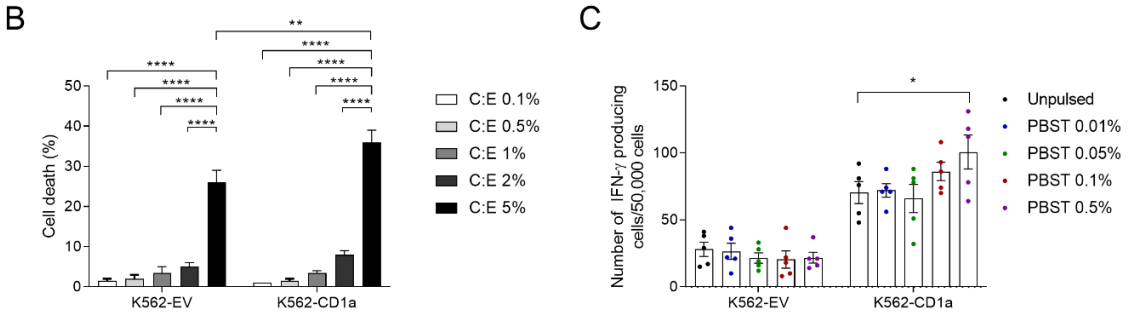


Figure 21. Preliminary peanut oil and total lipid extraction (TLE) dissolution studies in solvents. A) Miscibility of peanut oil when mixed directly with various organic solvents and peanut TLE (pTLE) when mixed with differing concentrations of PBS Tween (PBST) and sonicated ('None' = visibly not dissolved; 'Partial' = colour change in solvent/detergent, with additional undissolved lipid settling at bottom of tube; 'Complete' = clear solution develops after mixing). B) Effect of different concentrations of chloroform: ethanol (C:E) 2:1 on K562 viability, quantified using Trypan Blue and an automated cell counter (Countess II, Thermo Fisher). C) K562 cells were pulsed with pTLE dissolved in PBST, prior to coculture with healthy control T cells overnight. IFN-γ production was measured by ELISpot (*p<0.05).

Following this groundwork, in which appropriate solvents and detergents had been identified, Po and pTLE were investigated for their potential to influence CD1a responses. K562 cells were pulsed with Po dissolved in C:E 2:1 and ELISpots were performed using polyclonal T cells from 4 HC donors. Pulsing K562-CD1a cells with 100µg/ml Po showed a trend suggesting an increase in IFN-γ production, although this was not found to be statistically significant (*Figure 22A, left panel*) (mean fold change = 1.50; SEM = 0.286). *Figure 22A* further demonstrates variability in CD1a response within donors. For example, 1ug/ml Po led to an increase in IFN-γ secretion in C2299 (*middle panel*), however there was a decrease in W006 (*right panel*); this pattern was reversed when pulsing with 10ug/ml Po. This may be partly explained by the wide range of lipids present in refined Po, which may simultaneously act as stimulatory and inhibitory ligands. Furthermore, despite maintaining <1% v/v organic solvent in concordance with our trial assays and previous experiments by

Lepore et al. (464), C:E 2:1 vehicle control led to reductions in IFN-γ secretion, as evident in both donors. Equally, cell death was still evident and varied between donors (0-50%). Therefore, CD1a responses may have been partly affected by vehicle-induced cellular stress mechanisms. To investigate whether CD1a ligand content varies between Po and whole peanuts, pTLE was prepared from raw peanuts, which was subsequently emulsified in PBS-Tween 20 (PBST) (0.05%) for cellular assays. Regardless of the concentration of pTLE pulsed onto K562-CD1a cells, ELISpot assays did not detect any change in IFN-γ secretion by HC (n=4) T cells (*Figure 22B, left panel*). Despite the apparent fold changes within the K562-EV subset, this is likely a function of low spot numbers, as demonstrated in individual donor data for C2345 (*middle panel*) and C2349 (*right panel*). In summary, neither Po nor pTLE clearly stimulated CD1a-reactive T cells in healthy donors.

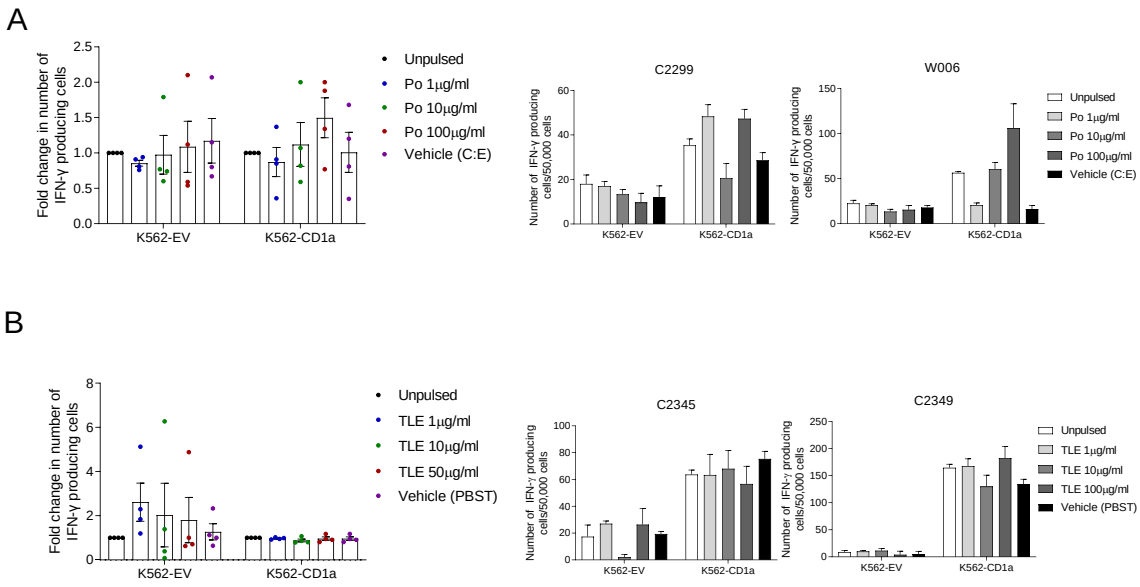


Figure 22. Peanut oil (Po) and peanut total lipid extract (pTLE) do not stimulate CD1a-reactive T cells. K562 cells were pulsed with peanut-derived lipids and their solvents, prior to coculture with healthy control (HC) T cells overnight. IFN-γ production was measured by enzyme-linked immunospot (ELISpot). A) Effect of Po, dissolved in C:E 2:1. Left: fold change in IFN-γ production across 3 HC donors. Middle and right panels: example data from two HC donors (W006 and C2299), showing whole spot counts B) Effect of pTLE, dissolved in PBST 0.05%. Left: fold change in IFN-γ production across 4 HC donors. Right: example data from two donors (C2345 and C2349), showing whole spot counts (*p<0.05).

1.30.2. PG inhibits IFN-γ production in CD1a-restricted T cells from healthy controls

Po and pTLE comprise a wide variety of lipids which vary in terms of molecular structure and polarity (79). We therefore elected to investigate the individual CD1a-modulatory effects of PG and PA, which are abundantly present in peanut seeds and products of the PLD reaction (364, 451). Previous studies suggest that the CD1a binding groove can accommodate lipids with C_{15-24} acyl chains, including a higher affinity towards those which contain one unsaturated bond (287). To model peanut-derived phospholipids which are amenable for CD1a antigen presentation, ELISpot assays were performed incorporating 17:0 PG, 18:1 (Δ9-Cis) PG, 18:2 PG and soy-derived PA; the latter of which contains a mixture of unsaturated and saturated aliphatic chains ranging from 16-18 carbons in length (373).

In preliminary experiments, K562-EV or K562-CD1a cells were treated with each phospholipid species (*Figure 23A*). K562 cells were subsequently cocultured with HC T cells (n=6) overnight and IFN-γ ELISpots were performed. Overall, coculture of T cells with K562-CD1a cells led to significantly higher IFN-γ secretion than coculture with K562-EV cells ($p<0.0001$), indicating the presence of CD1a-reactive T cells in HCs. Within the K562-CD1a subset, compared with the unpulsed condition (mean spots = 94.8), all PG species led to a significant reduction in IFN-γ release (17:0 PG: mean spots = 58.2 [$p<0.05$]; 18:1 Δ9-Cis PG: mean spots = 48 [$p<0.01$]; 18:2 PG: mean spots = 51.5 [$p<0.01$]). In contrast, soy PA led to no appreciable change in IFN-γ secretion (mean spots = 85). For all conditions, treatment of K562-EV cells did not modulate cytokine production (unpulsed mean spots = 16.2; 17:0 PG mean spots = 20.7; 18:1 Δ9-Cis PG mean spots = 22; 18:2 PG mean spots = 15.3; soy PA mean spots = 23.2). These initial experiments therefore suggested that PG suppresses CD1a-mediated IFN-γ cytokine production in HC T cells.

To further clarify CD1a-specificity, CD1a-blocking antibodies were incorporated into subsequent experiments. K562 cells were pre-treated with anti-CD1a antibody (α-CD1a) or CD1a isotype for 1 hour, to confirm a CD1a-specific effect, and then an identical ELISpot model was employed, including treatment and coculture conditions with HC T cells (n=6) (*Figure 23B*). K562-EV data were subtracted from K562-CD1a data to give an easier visualisation. In the unpulsed condition, CD1a-blocking significantly reduced IFN-γ production (mean spots = 67.5) compared with no antibody (mean spots = 149; $p<0.01$) or isotype (mean spots = 132.8; $p<0.05$). There was no significant difference between cells pre-treated with no antibody or isotype. This represented CD1a-autoreactivity of HC T cells and demonstrated that our CD1a-blocking protocol was efficacious. Amongst conditions which had been treated with PG and no antibody, 18:1 (Δ9-Cis) PG (mean spots = 71.9) and 18:2 PG (mean spots = 72.6) led to a significant inhibition of IFN-γ release compared with the unpulsed condition (both $p<0.05$). This inhibitory effect was comparable to CD1a antibody-blocking and isotype treatment in both conditions. When no antibody was used, treatment with 17:0 PG (mean spots = 122) or soy PA (mean spots = 131.5) did not significantly change IFN-γ secretion. Within the soy PA condition, CD1a blocking reduced cytokine production (mean spots = 54.4; $p<0.05$), whereas isotype treatment led to no change (mean spots = 140.1). Incorporation of anti-CD1a antibodies therefore recapitulated the apparent CD1a-inhibitory effects of 18:1 (Δ9-Cis) PG and 18:2 PG, and the absent effects of soy PA, within our system. Conversely, the previous inhibitory effects of 17:0 PG were not repeatable in this instance.

K562-CD1a cells express abundantly high surface CD1a, which can be used to investigate T cell reactivity to lipid antigens (465). This artificial system assumes that the bulk of lipid antigen generation and presentation occurs at the surface of the APC. In fact, lipid antigen presentation by DCs incorporates a complex CD1a recycling system, with CD1a exchange

between the early endosomal pathway and the cell membrane (313). Moreover, in LCs, CD1a-lipid recycling is proposed to incorporate the enigmatic BGs, as outlined in *Chapter 3.7.2* and further explored in *Chapter 8.3.1*. Given the discrepancies observed in the effect of 17:0 PG within the K562 ELISpot system, repeat experiments were performed using HC MoDCs, which would potentially provide a more realistic representation of antigen presentation. As in previous experiments, MoDCs (n=5) were pre-treated with anti-MHC I/II antibodies (α-HLA), to eliminate peptide-specific background, and anti-CD1a antibodies (α-CD1a) or isotype to detect the presence of CD1a antigen-presentation. MoDCs were then subjected to identical phospholipid pulsing conditions and cocultured with autologous T cells overnight, and IFN-γ ELISpot was performed (*Figure 23C*). CD1a-autoreactivity was again confirmed through analysis of the unpulsed condition, which demonstrated a significant reduction in IFN-γ production upon CD1a blocking (α-HLA mean spots = 152; α-HLA and α-CD1a cotreatment mean spots = 78.8; $p<0.01$). α-HLA and isotype antibody cotreatment led to no change (mean spots = 155.1). Remarkably, considering conditions which incorporated PG and monotreatment of α-HLA, only 18:1 (Δ9-Cis) PG led to a downregulation in IFN-γ production (mean spots = 84; $p<0.05$). Again, this was comparable to both α-CD1a (mean spots = 81.6) and isotype (mean spots = 100.2) antibody treatment. On the contrary, when MoDCs were pre-treated with only α-HLA, 17:0 PG (mean spots = 182) and 18:2 PG (mean spots = 168.2) led to no significant change in cytokine secretion. In both conditions, CD1a antibody blocking abrogated IFN-γ secretion as expected (17:0 PG mean spots = 100.8; 18:2 PG mean spots = 80.8). These effects were not observed in cells which had been pre-treated with α-HLA and isotype (17:0 PG mean spots = 187.9; 18:2 mean spots = 170.2). In line with previous observations, soy PA treatment did not modulate CD1a-mediated responses (α-HLA mean spots = 160.8), whilst CD1a antibody-blocking led to reductions in cytokine production (α-HLA and α-CD1a cotreatment mean spots = 74.6; α-HLA and isotype cotreatment mean spots = 150.6).

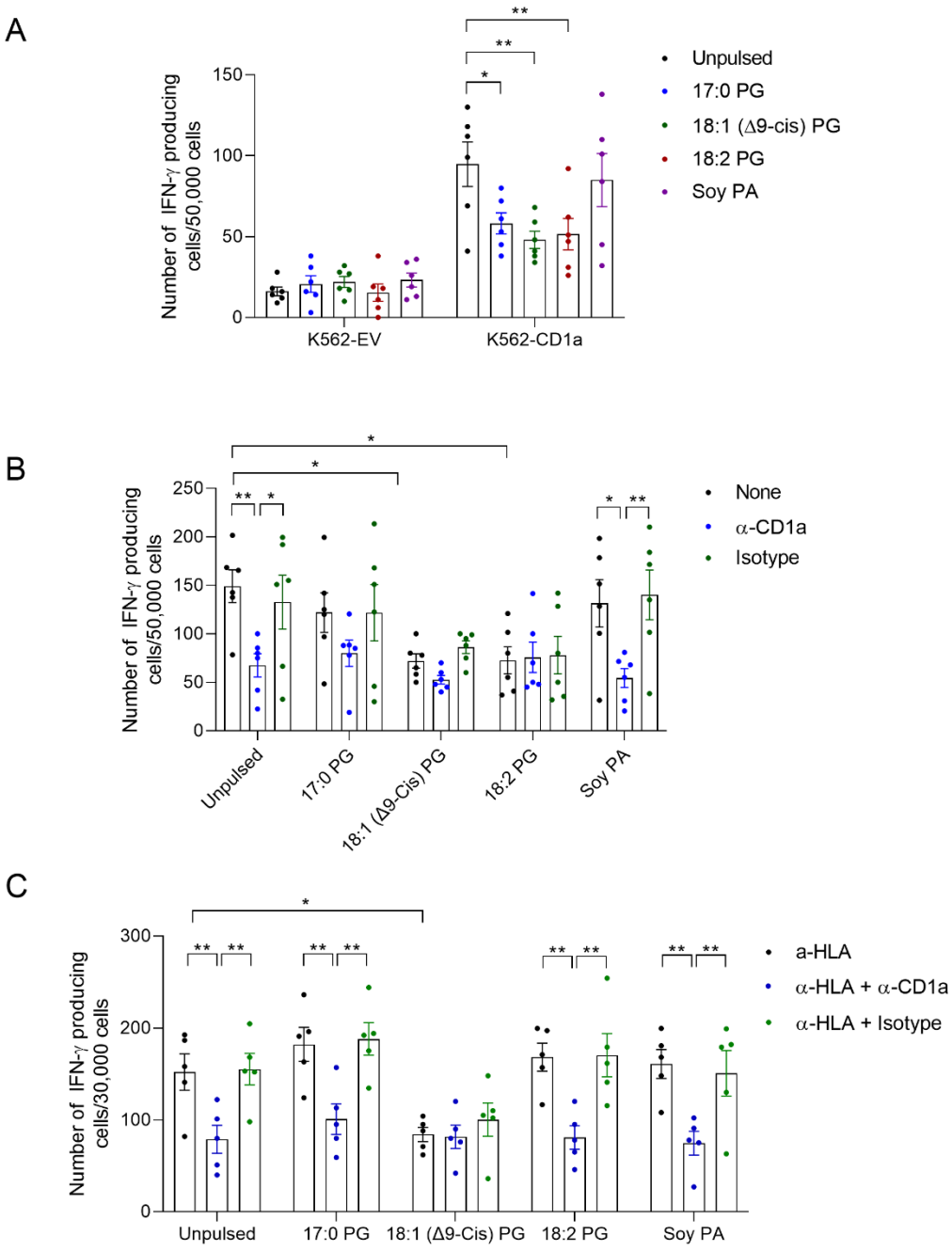


Figure 23. Phosphatidylglycerol (PG) inhibits IFN-γ production in CD1a-restricted T cells from healthy control individuals. A) K562 cells were treated with a range of PG species (17:0 PG, 18:1 (Δ-cis9) PG, 18:2 PG) or soy phosphatidic acid (PA), and cocultured with CD3+ T cells derived from healthy controls (HCs) overnight. IFN-γ production was measured by enzyme-linked immunospot (ELISpot) assay. B) K562 cells were preincubated with blocking antibody for 1 hour (anti-CD1a Ab or IgG1 isotype for CD1a) and ELISpots were repeated in HCs C) Monocyte-derived dendritic cells (MoDCs) were generated from healthy individuals and pre-incubated with blocking antibody for 1 hour (combinations of anti-HLA, anti-CD1a and IgG1 isotype for CD1a). MoDCs were cocultured with autologous T cells and IFN-γ ELISpot was performed. (*p<0.05; **p<0.01).

Overall, these data demonstrate that, in HCs, 18:1 (Δ9-Cis) PG showed repeatable CD1a-mediated inhibition in the K562 and MoDC systems, suggesting a biologically relevant effect. This was therefore selected as the model PG species for subsequent K562 and MoDC ELISpots. Conversely, the inhibitory effects of 17:0 PG and 18:2 PG were uniquely observed in K562, but not primary cell, cocultures. Moreover, the inhibitory effect of 17:0 PG was only observed in one set of K562 experiments. Given the discrepancies between APC platforms, these PG species would be used in future K562 assays only. Finally, soy PA did not modulate CD1a-reactivity in HCs using either APC platform, however it was maintained in all subsequent MoDC and K562 assays to examine differential effects in peanut-allergic patients.

1.30.1. PG and PA stimulate IFN-γ production in peanut-allergic CD1a-restricted T cells

Having characterised the CD1a-mediated effects of individual PG and PA species in HCs, we subsequently investigated their comparative effects in peanut-allergic patients. Our reasons for doing this were two-fold. Firstly, wished to use explore the CD1a-modulatory activity of peanut-derived PG and PA, using our lipid species as a model. Secondly, we previously identified that the enzymatic activity of peanut-derived PLD drives CD1a-mediated inflammation in peanut-allergic patients, as outlined in Chapter I. Having observed that peanut PLD generates PG and PA in MoDCs, we wished to further test our hypothesis that these phospholipid reaction products are responsible for elevated T cell reactivity in patients.

Initially, utilising our K562 ELISpot platform, a range of phospholipid species were used to compare CD1a-reactivity between HCs (n=6) and peanut-allergic (n=6) patients. As expected, compared with the unpulsed condition (*Figure 24A*; mean spots = 105.5), 17:0

PG, 18:1 (Δ9-Cis) PG and 18:2 PG led to a reduction in IFN-γ release by HC T cells (respective mean spots = 44.6 [p<0.01), 49.1 [p<0.01), 55.8 [p<0.05). However, in peanut-allergic patients, treatment of K562 cells with 18:1 (Δ9-Cis) PG and 18:2 PG led to a greater than two-fold induction of IFN-γ production (unpulsed mean spots = 52.5; 18:1 [Δ9-Cis] PG mean spots = 106.9, p<0.05; 18:2 PG mean spots = 124.8, p<0.01). Treatment with 17:0 PG demonstrated a trend suggesting an increase in cytokine production, however this was not significant (mean spots = 86.8). Similar to previous experiments comparing patient groups, CD1a-autoreactivity of peanut-allergic patients was approximately half of that observed in HCs (p<0.05). Next, repeat K562 ELISpots were conducted, using the same donors to compare the effects of soy PA (*Figure 24B*). Whereas PA led to no change in IFNγ production in HCs (unpulsed mean spots = 91.2; soy PA mean spots = 84.7), a marked induction was observed in peanut-allergic individuals (unpulsed mean spots = 67; soy PA mean spots = 147; p<0.01). Whilst not significant, there was an observable trend suggesting reduced CD1a-autoreactivity in peanut-allergic patients compared with HCs. Our K562 assays therefore highlighted the important observations that peanut-allergic patients possess elevated circulating CD1a-reactive T cells which respond to 18:1 (Δ9-Cis) PG, 18:2 PG and soy PA.

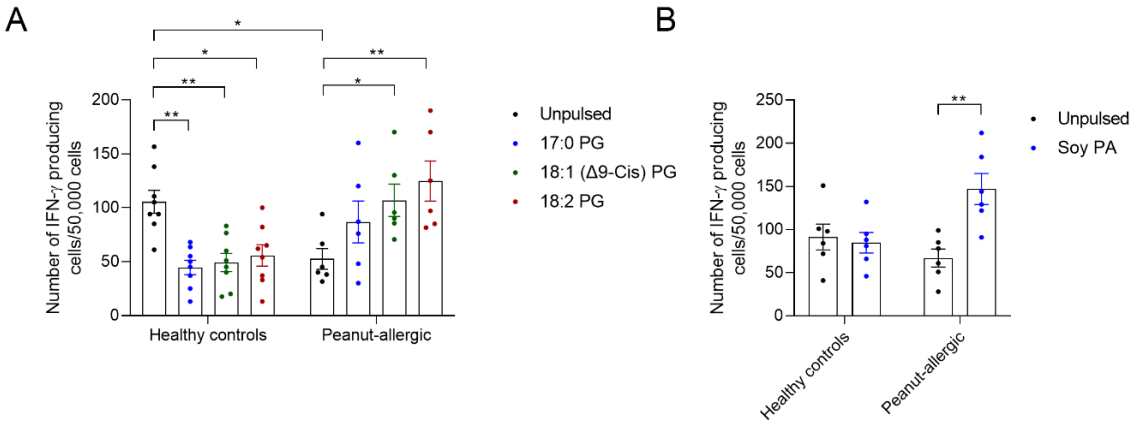


Figure 24. Phosphatidylglycerol (PG) and phosphatidic acid (PA) stimulate IFN-γ production in CD1a-restricted T cells from peanut-allergic patients. K562 cells were treated with phospholipids and cocultured with CD3+ T cells derived from healthy controls or peanut-allergic individuals. IFN-γ production was measured by ELIspot assay. A) Effect of 17:0 PG, 18:1 (Δ-cis9) PG and 18:2 PG. B) Effect of soy PA (*p<0.05; **p<0.01).

Given their important roles in mediating cellular functions, PG and PA were presumed to possess additional non CD1a-mediated effects (244, 353). Previous experiments in HCs suggested these effects differed between K562 cells and MoDCs (*Figure 23*). Consequently, the MoDC ELISpot system was used to compare CD1a responses to 18:1 (Δ9-Cis) PG and soy PA between HC (n=5) and peanut-allergic (n=5) cohorts. Combinations of α-HLA, α-CD1a and IgG1 isotype antibodies were used as previously outlined. *Figure 25A* demonstrates the effects of 18:1 (Δ9-Cis) PG. Amongst unpulsed MoDCs, in both patient groups, pre-treatment with concurrent α-HLA and α-CD1a antibodies reduced IFN-γ production compared with antibody controls (HC: $p<0.001$; peanut-allergic: $p<0.001$). This confirmed that our antibody protocol effectively blocked CD1a-reactivity in both cohorts. In HC MoDCs which had been pre-incubated with α-HLA only, 18:1 (Δ9-Cis) PG treatment led to a significant inhibition (mean spots = 84.1, $p<0.001$) compared with unpulsed cells (mean spots = 176.7). This inhibitory effect was reproduced in cells which had been pre-treated with additional α-CD1a (mean spots = 70.2) or isotype (mean spots = 75.2) antibody. Alternatively, in peanut-allergic patients, 18:1 (Δ9-Cis) PG induced marked IFN-γ secretion, as demonstrated by comparing unpulsed cells (mean spots = 203.2) with PG-treated cells (mean spots = 218.8, $p<0.05$) which had been preincubated with α-HLA alone. Pre-incubation of MoDCs with concurrent α-HLA and α-CD1a antibodies, and subsequent treatment with PG, abrogated this IFN-γ response (mean spots = 88, $p<0.0001$). This differed with the isotype control, which maintained high IFN-γ secretion (mean spots = 210.2). From these data, similarly to that seen in K562 cells, it was concluded that 18:1 (Δ9-Cis) PG induces CD1a-mediated inflammatory responses in peanut-allergic patients, but inhibitory responses in HCs.

Figure 25B demonstrates the effect of soy PA in repeat MoDC experiments, using the same donors. Again, CD1a antibody blocking was effective in both patient groups, as observed

by the reduction in IFN-γ release upon α-CD1a blocking of unpulsed cells (HC: $p<0.05$; peanut-allergic: $p<0.05$). When HC MoDCs had been preincubated with α-HLA alone, soy PA treatment led to no effect on IFN-γ secretion (unpulsed mean spots = 106; soy PA mean spots = 123.6). This starkly contrasted with peanut-allergic patients, in which soy PA induced a marked increase in cytokine production (unpulsed mean spots = 87.4; soy PA mean spots = 167.5; $p<0.01$). CD1a-blocking abrogated this effect (mean spots = 64.8; $p<0.0001$), whereas the effect remained with IgG1 isotype (mean spots = 122.8). Together, these data signalled that soy PA acts via CD1a to induce IFN-γ production by circulating T cells from peanut-allergic patients, but not those from HCs.

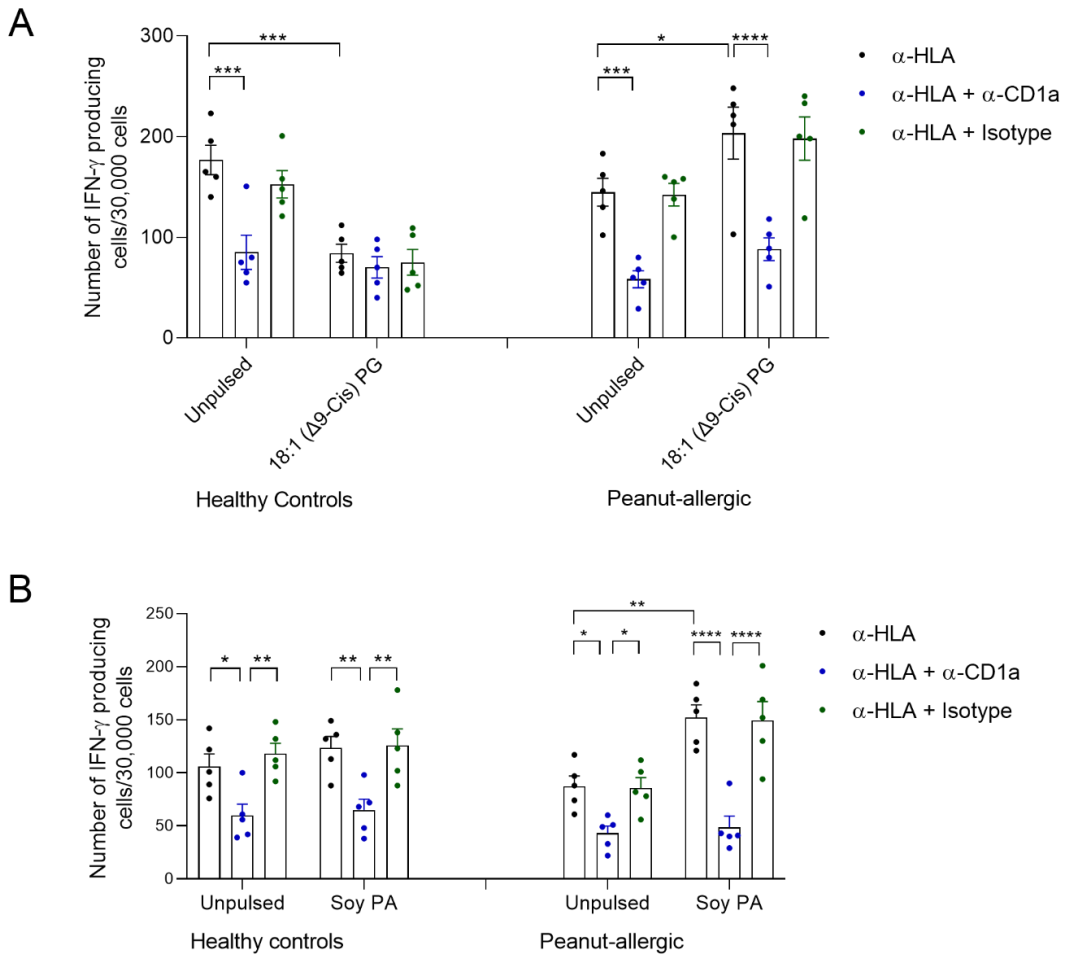


Figure 25. MoDCs present phosphatidylglycerol (PG) and phosphatidic acid (PA) to CD1a-restricted T cells in peanut-allergic patients. Monocyte-derived dendritic cells (MoDCs) were generated from healthy and peanut-allergic individuals and pre-incubated with blocking antibody for 1 hour (combinations of anti-HLA, anti-CD1a and IgG1 isotype for CD1a) prior to treatment with phospholipids. MoDCs were cocultured with autologous T cells and IFN-γ ELISpot was performed. A) Effect of 18:1 (Δ9-Cis) PG. B) Effect of soy PA (*$p<0.05$; **$p<0.01$; ***$p<0.001$); ****$p<0.0001$).

In summary, our experiments demonstrate the presence of increased PG- and PA-reactive T cells in peanut-allergic patients, compared with HCs. Specifically, reactivity to 18:1 (Δ9-Cis) PG, 18:2 PG and soy PA was confirmed. Moreover, MoDCs – primary cells which possess natural CD1a-lipid recycling machinery – efficiently processed and presented 18:1 (Δ9-Cis) PG and soy PA to circulating peanut-allergic T cells.

1.30.1. Peanut-derived protein extracts, skin prick tests and oil bodies do not stimulate CD1a-reactive T cell responses

Our previous experiments had highlighted the role of PG and PLD, both of which are constituents of peanuts seeds, in stimulating peanut-sensitised CD1a-reactive T cells (79, 172). This suggested that peanut proteins and lipids may work in tandem to affect CD1a antigenicity. This notion was further explored through investigating the CD1a-modulatory capacity of novel peanut-derived substances, including those with dual hydrophilic-lipophilic nature.

Prior work, discussed in *Chapter* I, demonstrates the stimulatory role of peanut PLD in CD1a biology and we wished to determine if other protein constituents of raw peanuts might induce similar effects. Briefly, raw peanuts were subjected to chloroform: methanol extraction, and the proteinaceous aqueous phase was obtained (see *Chapter 5.8.2*). K562-EV and K562-CD1a cells were treated with different concentrations of peanut protein solutions, diluted in PBS, and subsequently cocultured with HC (n=4) T cells overnight for IFN-γ ELISpot (*Figure 26A*). Data were presented as fold change with respect to unpulsed cells. No statistical difference in cytokine secretion was detected between conditions for either K562 subset. Whilst pulsing K562-EV cells with 5µg/ml protein solution led to a trend suggesting induction, this was likely a function of lower spot numbers within this

subset. Therefore, peanut protein from raw peanuts did not modulate CD1a responses in HC T cells.

The peanut skin prick test (SPT) is an important component of allergy diagnosis in suspect cases (466). Although there are minor variations in formulation, whole peanut SPTs are prepared from complete seeds and contain high concentrations of most major protein allergens. Due to their medical-grade status and high level of quality control during production to ensure allergenicity, we elected to investigate whether a component of their action is via stimulating CD1a-reactive T cells. Different concentrations of peanut SPT stock solutions were prepared using SPT negative control solution, a solvent containing multiple constituents including 10% glycerol. Stock SPT solutions were then tested for CD1a-reactivity of HC (n=4) T cells using the K562 ELISpot system (*Figure 26B*). No statistical fold change was observed in any condition, however there was an increasing trend suggesting dose-related induction of IFN-γ production within the K562-EV subset (fold changes: 1μg/ml = 1.38; 5μg/ml = 1.89; 10μg/ml = 2.08; vehicle = 1.42). Again, this was potentially a function of lower spot numbers observed for K562-EV cocultures. Therefore, in this model, peanut SPT did not activate HC T cells via CD1a.

Peanut protein extracts and SPT are produced through aqueous extraction, with repeated stringent washing (101). This process eliminates lipophilic substances, and potentially denatures phospholipase enzymes, which may have otherwise acted to influence CD1a signalling. We therefore elected to investigate the CD1a-modulatory capacity of OBs, which contain highly lipophilic proteins, oleosins. Modifying the protocol of Rodelas-Angelia et al, peanut OBs were prepared from raw peanuts using sucrose density gradient washing, followed by repeated urea washing and centrifugation (see *Chapter 5.8.3*) (330). Milky OB preparations were then dissolved in deionised water (DW). Using HC (n=4) T

cell donors, K562 ELISpots were performed, pulsing with 10-100 μg/ml OBs dissolved in DW (*Figure 26C*).

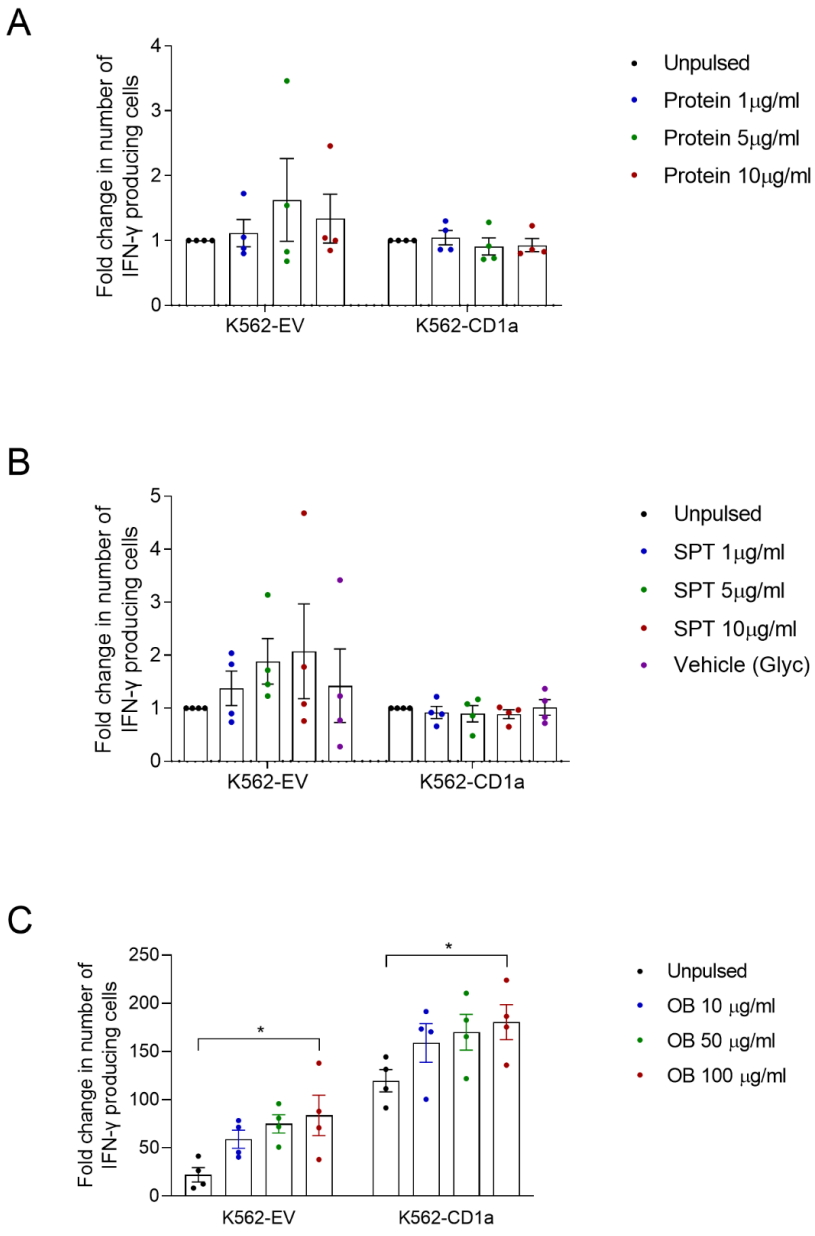


Figure 26. Peanut-derived protein extracts, skin prick test (SPT) and oil bodies (OBs) do not stimulate CD1a-reactive T cell responses in healthy individuals. K562 cells were treated with a range of proteins or lipophilic substances, and cocultured with CD3+ T cells derived from healthy controls. IFN-γ production was measured by ELISpot assay. A) Effect of peanut protein extracts from raw peanuts, obtained from the aqueous phase during chloroform: methanol extraction. Data presented as fold change with respect to unpulsed K562 cells. B) Effect of peanut SPT, compared with vehicle control containing 10% glycerol (Glyc). Data presented as fold change with respect to unpulsed K562 cells. C) Effect of peanut OBs, obtained from raw peanuts through sucrosedensity-gradient washing, urea washing and centrifugation. Data presented as whole spots (*$p<0.05$; **$p<0.01$).

In both cell subsets, there was a trend suggesting a dose-dependent increase in IFN-γ production, although lower pulsing concentrations did not produce statistically significant results. At a higher concentration of 100 µg/ml, in K562-EV cells, a significant increase in IFN-γ production was observed (unpulsed mean spots = 22.3; 100 µg/ml mean spots = 83.8; $p<0.05$). A similar effect was noted in K562-CD1a cells (unpulsed mean spots = 119.8; 100 µg/ml mean spots = 180.5; $p<0.05$). Whilst these data did not exclude CD1a-reactive T cell responses to OBs, there were clear non CD1a-specific mechanisms at play which confounded interpretation, potentially including innate responses to peptide constituents of OBs.

1.30.2. Ara h 1 binds PG to elicit CD1a-reactive T cell responses

Given that enhanced PG-responsiveness was observed in CD1a-reactive T cells of peanut-allergic patients, we wished to determine if interactions between peanut protein and lipid allergens have a facilitatory or curbing effect. Initially, it was investigated whether the lipophilic activities of Ara h 1 and Ara h 8 modulate CD1a responses within our K562 system. K562 cells were pulsed with serial concentrations (1-100 µg/ml) of Ara h 1 (*Figure 27A*) or Ara h 8 (*Figure 27B*), cocultured with T cells from HCs (n=3) overnight, and IFN-γ ELISpots were performed. Neither allergen altered responses within the empty vector subset. At a concentration of 100 µg/ml, Ara h 1 stimulated CD1a-reactivity in T cells (unpulsed mean spots = 54.7; Ara h 1 mean spots = 90.3; $p< 0.01$). There was no significant increase in IFN-γ production at lower concentrations of Ara h 1, however 10 µg/ml led to a trend suggesting induction (mean spots =74.7). Conversely, Ara h 8 did not alter IFN-γ production in CD1a-reactive T cells. Preliminary results therefore suggested Ara h 1 exhibits CD1a-modulatory activity, and this allergen was used henceforth.

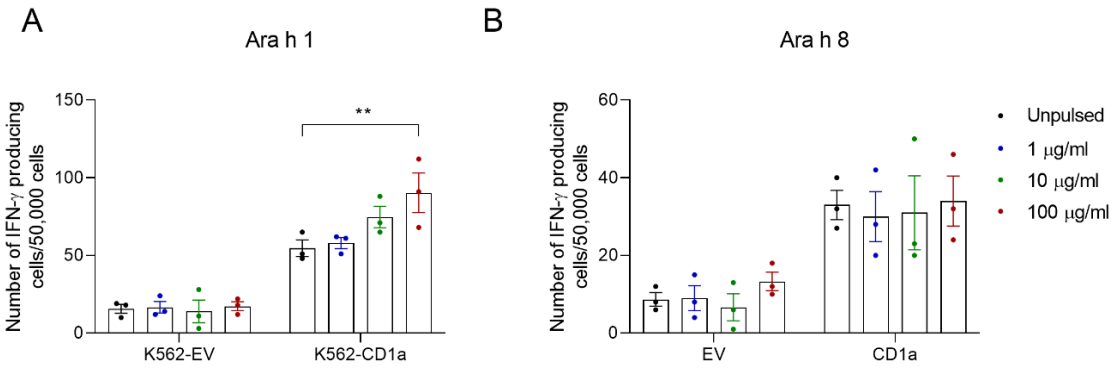


Figure 27. The major peanut allergen Ara h 1 induces CD1a-reactive T cell responses. K562 cells were treated with A) Ara h 1 or B) Ara h 8, and cocultured with CD3+ T cells derived from healthy controls overnight. IFN-γ production was measured by ELISpot assay (**p<0.01).

Next, Ara h 1 was investigated for its capacity to induce differential CD1a responses in peanut-allergic patients. K562 cells were pulsed with 100 µg/ml Ara h 1 and cocultured with HC (n=4) or peanut-allergic (n=4) T cells (*Figure 28A*). The left panel demonstrates whole spot data, with empty vector data subtracted to remove non CD1a-specific background. In peanut-allergic individuals, Ara h 1 induced a marked induction of IFN-γ production (unpulsed mean spots = 74.3; Ara h 1 mean spots = 156.5). There was no equivalent significant increase in HCs, although a trend suggested a milder induction (unpulsed mean spots = 82.5; Ara h 1 mean spots = 127.3). These findings suggested responses were more pronounced in peanut-allergic patients, and so we standardised data to eliminate inter-donor variability in CD1a-autoreactivity, allowing for more accurate comparison. For each patient cohort, we calculated the fold change in IFN-γ secretion induced by Ara h 1 treatment with respect to unpulsed cells (*Figure 28A, right panel*). Interestingly, in HCs, Ara h 1 treatment revealed a significant 1.56-fold increase in cytokine production compared with the unpulsed condition ($p<0.001$). Remarkably, this effect was exacerbated in peanut-allergic patients, which demonstrated a 2.1-fold increase ($p<0.0001$). Moreover, this fold increase was significantly higher in peanut-allergic patients compared with HCs ($p<0.001$). In summary, Ara h 1 induces T cell responses via a CD1a-dependent mechanism, and this effect is exacerbated in peanut-allergic patients.

Following the important findings that both PG and Ara h 1 stimulate CD1a-reactivity in peanut-allergic patients, it was hypothesised that a component of this mechanism was related to Ara h 1-PG binding. Previously, Ara h 1 had been shown to interact with PG vesicles derived from egg L-α-PG, which contains a heterogeneous distribution of fatty acids, with acyl structures ranging from C_{16-22} and containing up to five unsaturated double bonds (98, 467). Consequently, potentially Ara h 1 bound with differential affinity to various PG species within the mixture. Given that 18:1 (Δ-9 cis) PG and 18:2 PG induced CD1a responses in peanut-allergic individuals, it was investigated whether Ara h 1 can bind to these individual species, assessed using IEF (*Figure 28B*). Recombinant Ara h 1 (dissolved in PBS) was coincubated overnight with 100X Molar excess of 18:1 (Δ-9 cis) PG or 18:2 PG (dissolved in organic solvent; see *Chapter 5.15.2*), and subsequently subjected to IEF. Two negative controls were used in this assay: 'Ara h 1' and 'Sample mock' 'Ara h 1' represents Ara h 1, dissolved alone in PBS, which was incubated alone. 'Sample mock' represents Ara h 1 coincubated with organic solvent overnight, to determine if such lipophilic detergents strip endogenously bound lipids from Ara h 1 and alter its isoelectric point (pI). No positive controls for Ara h 1-lipid binding were incorporated into the assay, as no previous studies have reported use of this technique. All samples demonstrate a 'ladder' appearance, representing multiple permutations of Ara h 1 with different associate pI values. Both negative controls appeared identical, revealing pI values of ~6-7. Both experimental samples demonstrated downward displacement of bands. Remarkably, coincubation of Ara h 1 with 18:1 (Δ-9 cis) PG induced the formation of two new bands with pI values less than 3.5 (*blue arrow, left*). This was accompanied by visible displacement of the original band, especially within its upper region (*red arrow, left*). Equally, coincubation with 18:2 PG led to the production of a single new band with a pI value less than 3.5 (*blue arrow, right*). Upper band displacement was visible to a lesser

extent (*red arrow*, *right*), corresponding with the fact that only a single band formed. Therefore, our data demonstrate that Ara h 1 binds to 18:1 (Δ-9 cis) PG and 18:2 PG. To our knowledge, this is the first instance which has confirmed binding with these specific PG species.

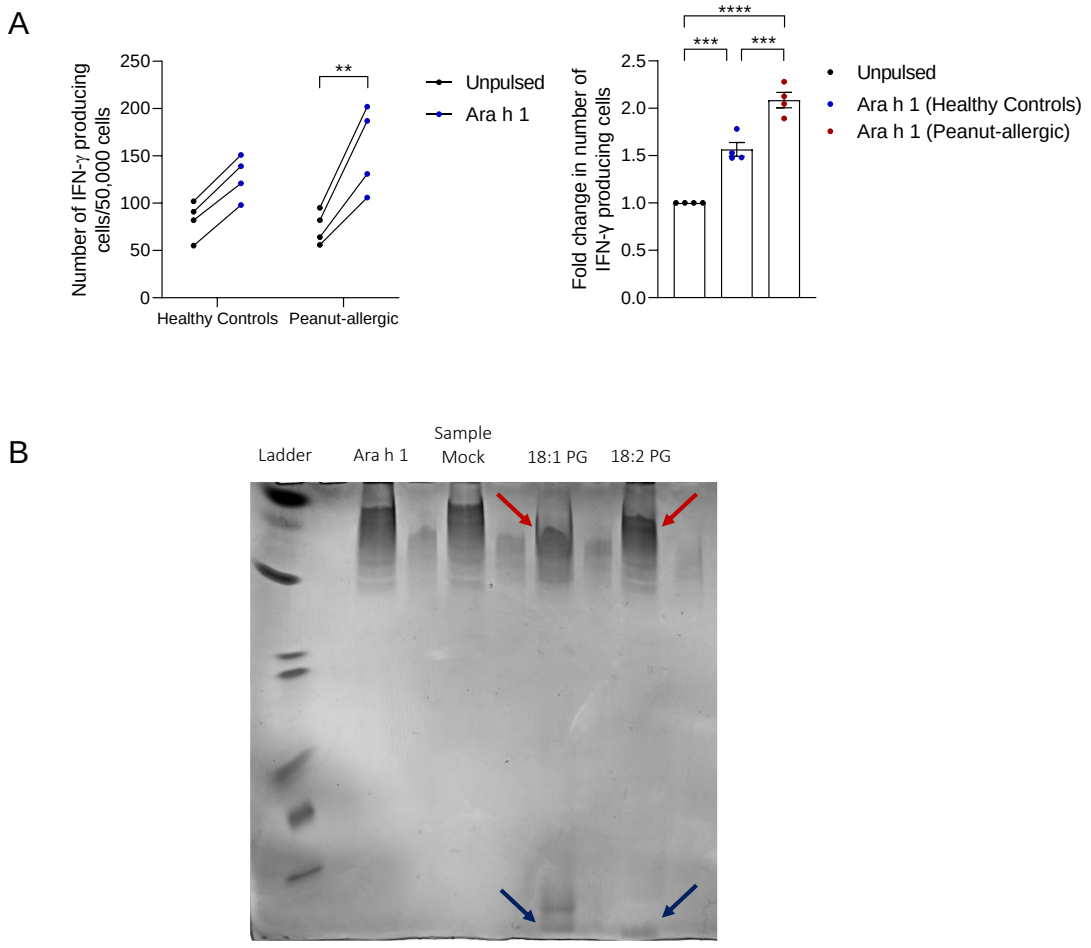


Figure 28. The major peanut allergen Ara h 1 elicits CD1a-reactive T cell responses, exacerbated in peanut-allergic patients, and binds phosphatidylglycerol (PG). A) K562 cells were treated with Ara h 1 and cocultured with T cells derived from healthy control or peanut-allergic patients, and IFN-γ ELISpots were performed. Data are expressed in terms of IFN-γ-producing spots (left) and fold change with respect to untreated K562s (right). B) Recombinant Ara h 1 was coincubated overnight with different PG species (18:1 [Δ9-Cis] PG or 18:2 PG, dissolved in organic solvent) and analysed by isoelectric focusing (IEF). Negative controls included Ara h 1 which had been incubated alone and Ara h 1 which had been incubated with organic solvent ('Sample Mock'). Red arrows indicate displaced bands. Blue arrows indicate newly-formed bands (**p<0.01; ***p<0.001); ****p<0.0001).

In summary, Ara h 1, an allergen known to demonstrate lipophilic interactions, induces T cell responses via a CD1a-dependent mechanism. This effect is exacerbated in peanut-allergic individuals, demonstrating another peanut protein constituent, in addition to PLD, which may contribute towards lipid-reactivity. Notably, Ara h 1 can bind specific PG species which we have previously shown to stimulate CD1a-reactive T cell responses in peanut-allergic patients.

1.31. Discussion

1.31.1. Pure peanut lipids do not stimulate CD1a-reactive responses in healthy T cells

Peanuts are an abundant source of lipids and proteins, including phospholipase enzymes, offering a wealth of targets that might modulate CD1a antigen presentation (75, 468). We first investigated whether Po lipids could be directly presented by CD1a to polyclonal T cells. The ELISpot experiments in HCs demonstrated no clear evidence of CD1a-mediated responses, as only a trend was observed which suggested IFN-γ production at a pulsing concentration of 100 μg/ml Po. This was surprising, given the strong candidacy of OLA ($C_{18:1}$), LA ($C_{18:2}$) and PlA ($C_{16:0}$) as CD1a ligands (287). It must be noted that the K562 system is an artificial model which eliminates MHC signalling. Potentially, CD1a-reactive T cells require co-stimulation with cytokines derived from peanut protein-specific T cells (via MHC) and lipid allergens (via CD1a) in order to mount an effective cytokine response. Food lipids are known to act as adjuvants for the peptide-specific response. Alcocer et al. demonstrated that Brazil nut lipids stimulate CD1d-restricted iNKT cells to license a Th2 response and are required for sensitisation to the major Brazil nut allergen Ber e 1 (469). In another study, sphingolipid isolated from cow's milk induced iNKT cell proliferation and production of the Th2 cytokines IL-4, IL-5 and IL-13, whilst the iNKT cell agonist α-galactosylceramide stimulated IFN-γ production. Moreover, iNKT cells from children with milk allergy produced more IL-4 and IL-13 in response to this sphingolipid than those from nonallergic children (470). Specific sphingolipid recognition by iNKT cells may therefore

facilitate the induction of a cow's milk-protein specific Th2 response and subsequent sensitisation. This concept of exogenous food proteins and lipids working together to modulate host defence is a relatively new but interesting field, and future experiments are discussed in *Chapter 9.1*.

Confounding factors exist which may have additionally impacted upon our findings, including heterogeneous lipid content of Po and inter-donor variability in CD1a-reactivity. The lack of a significant CD1a-reactive response to Po may be a function of peanut-specific factors, such as heterogeneous FA and phospholipid content. Peanuts contains a wide FA distribution, with $C_{16\text{-}24:0}$ acyl chains (471). The distributions are dependent on the specific peanut cultivar, with the largest variations observed in OLA, LA and PlA, the three major peanut FAs (472). Genotypic differences have been observed between countries, including variances between cultivars grown in Argentina, USA, Bolivia, Africa and Poland (82, 450, 473, 474). Within countries, there are further regional variations in FA content, according to specific downstream processing and use (475). Furthermore, certain peanut lines are bred to incorporate high-OLA content, and reduce LA levels, in order to enhance shelf life (80). Consequently, peanuts are characterised based on their OLA/LA (O/L) ratios, with a higher level being preferable (476). Such variations may have major implications on the immune response to the FA content of Po. For our experiments, Po was bought commercially, and no information is available regarding the country of origin. Given that OLA is a known stimulatory CD1a ligand, it is possible that low O/L cultivars were pressed to produce this oil, reducing antigen availability (84). Equally, it is entirely possible that our Po contained a mixture of different breeding lines, confounding interpretation through the previously discussed non-specific anti- and pro-inflammatory effects of OLA, LA and PlA (441, 446, 447). In future, batches of Po should be assessed by high performance liquid

chromatography-mass spectrometry (HPLC-MS) to confirm FA content and allow more accurate measurement of responses.

Po contains a range of phospholipids, including PC, PE, PG, PA and SM (75, 204, 477). Exogenous cypress pollen PC and PE have been identified as stimulatory CD1a antigens, whereas long-chain SM ($C_{24:1}$) is an established inhibitory ligand (85, 317, 465). Previously, PG has been shown to inhibit CD1a-reactive T cell responses in HCs; this is supported by our own data demonstrating inhibitory responses in HCs to PLD, a PG-producing enzyme (85). Given that peanut phospholipid content is 38.3-66.4% PC, 13.3-21.9% PE and up to 2.5% PG, and the sphingolipid content is 78µmol/kg, Po is likely a mixture of stimulatory and inhibitory CD1a phospholipid ligands (79, 204). Again, phospholipid content is dependent on breeding line and maturation status, with elevated PC, PE and PA observed in both high-OLA cultivars and immature seeds (75, 478). Consequently, whilst Po may indeed exert no CD1a-reactivity, interpretation may in fact be confounded by heterogeneous CD1a responses or reduced phospholipid content within our batches of oil.

Inter-donor variability led to differing CD1a-reactive T cell responses observed at different pulsing concentrations of Po. These ELISpot experiments used polyclonal T cells, introducing two factors that promote inter-donor variability. Firstly, the proportion of CD1a-reactive T cells within the PBMC pool will vary between individuals, affecting overall CD1a reactivity to lipids. Secondly, CD1a-reactive T cells from different individuals may recognise different lipid components of peanut oil, depending on prior sensitisation. This could introduce an overall inhibitory effect to peanut SM, and a stimulatory effect in patients sensitised to peanut PC, PE, PG or PA. To explore further, specific phospholipid components could be analysed and extracted from peanuts using HPLC-MS and subsequently pulsed onto target cells (479). Identification of specific

phospholipid-reactive T cells from patients would allow generation of peanut phospholipid-specific T cell clones. Furthermore, chloroform-containing organic solvents are required to dissolve and pulse peanut oil in ELISpots, due to the high concentration of non-polar triglycerides (480). Fractionation of polar lipids would allow the use of chloroform-free organic solvents, that may exhibit less vehicle interference. These concepts are further explored in *Chapter 9.1*.

Given the lack of clear CD1a-reactive responses observed in Po, pTLE was prepared from raw peanuts and tested within the ELISpot system. ELISpots demonstrated no evidence of IFN-γ secretion by T cells, regardless of pulsing concentration. Even at the individual donor level, no trends were observed, in contrast with peanut oil, and may be due to a combination of factors. Chloroform: methanol extraction carries the risk of losing highly polar lipids (and potential CD1a ligands), which move into the aqueous phase and are discarded (481). Also, Po is produced through methods including hot-pressing, where temperatures exceed 105^{o}C (482). Cooking is known to increase peanut protein allergenicity (483). In a different manner, the cooking process might influence lipid antigen content or lipase activity. For example, cooking can degrade lipids and produce lipid peroxides (484). Lipid peroxidation is a process by which oxidants such as free radicals attack lipids containing carbon-carbon double bonds, to produce lipid peroxyl radicals (485). Potentially, specific lipid peroxides in roasted nuts or peanut oil have increased CD1a specificity compared to lipids from unroasted nuts. Future studies should employ fractionation of roasted and unroasted peanut lipids, followed by pulsing within our K562 ELISpot system, to test this hypothesis.

1.31.2. PG inhibits IFN-γ production in CD1a-restricted T cells from healthy controls

Having identified no clear evidence of CD1a-reactivity against heterogeneous peanut lipids, we examined responses to pure phospholipid preparations. A range of PG (17:0 PG, 18:1 Δ-

cis 9 PG or 18:2 PG) and PA species were investigated, given their relative abundance in peanut seeds (79). Whereas PA led to no effect, PG inhibited CD1a-reactive T cell responses in HCs, and this was apparent across all PG species in initial K562 experiments (Figure 10A). Whilst this may be attributed to PG acting as a non-permissive CD1a ligand, PG also acts via a range of non-CD1a mediated mechanisms, as extensively discussed in Chapter I, including inhibition of TLR2 and TLR4 responses (248, 250). Interestingly, increased consumption of peanuts correlates with reduced serum C-reactive protein (CRP) and IL-6 in non-sensitised patients (486). Potentially, peanut-derived PG inhibits TLR2, contributing towards reductions in IL-6 (487). Furthermore, in a murine model of inflammation, injection of PG-rich nanoliposomes reduced serum CRP, and we propose peanut PG acts in a similar manner (488).

In subsequent K562 ELISpot experiments, 17:0 PG led to no significant inhibition of T cell responses, whereas 18:1 Δ-cis 9 PG and 18:2 PG maintained robust reduction of IFN-γ release (*Figure 23B*). Potentially, 17:0 PG exhibits weaker binding to CD1a compared with other species, as evidenced by a reduced shift in pI in the previous chapter (*Figure 16C*). This may be a function of its shorter and saturated acyl structure, however its capacity to bind CD1a remains to be explored through structural studies such as xray crystallography. Equally, whilst unsaturated PG species are known to exhibit anti-proliferative and anti-inflammatory effects via TLR2 and TLR 4 inhibition, saturated PG species do not exert these effects (243, 244). Consequently, 17:0 PG may demonstrate reduced inhibition of both CD1a-mediated and innate T cell responses.

In MoDC experiments, 18:1 Δ-cis 9 PG inhibited CD1a-reactive responses in HC T cells, whereas this effect was not observed for 17:0 PG or 18:2 PG. The notable discrepancy between the effects of PG species on MoDCs and K562 cells may originate from differing

CD1a structure and lipid-loading. K562 cells are an MHC-deficient myeloid cell line which are amenable to transfection of antigen-presenting molecules, as demonstrated in numerous studies (489, 490). Consequently, CD1a-transfected K562 cells are frequently used to investigate T cell reactivity to CD1a antigens (280, 465, 491). Specifically, K562 cells are transfected with pcDNA3.1 or pcDNA3 vector containing cDNA encoding CD1a, which is delivered to the cell membrane and is highly expressed (318). Thus, in K562-CD1a cells, we suspect most lipid binding occurs directly at the cell surface, through an artificial mechanism. Conversely, differentiation of MoDCs is driven by treatment with IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF), both of which contribute towards 'natural' CD1a expression, resulting in lipid loading in early endosomal compartments (492-494). Contrasting lipid loading mechanisms may give rise to the differing responses to PG observed between K562 cells and MoDCs. Moreover, CD1a structure varies with expression technique, as demonstrated by the differing pI values of CD1a generated in-house and that obtained from NIH (*Figure 16C*). We suggest this alters the conformation of the lipid-binding groove, leading to an altered affinity for specific PG species. Notably, it has previously been suggested that CD1a targets phospholipids with a single unsaturated double bond at the cis-9 position, potentially explaining the robust effects of 18:1 Δ-cis 9 PG (495). Therefore, this species was selected for subsequent MoDC experiments to model the effects of peanut PG.

1.31.3. PG and PA stimulate CD1a-restricted responses in peanut-allergic patients

During previous experiments, peanut PLD induced production of PA and PG in MoDCs, and both species bound CD1a (*Figures 16B and C*). Importantly, peanut PLD induced elevated CD1a-reactive T cell responses in peanut-allergic patients. Moreover, filaggrin inhibited, and AQP3 promoted, this effect (*Figures 18A and 19C*). This allowed us to hypothesise that filaggrin deficiency and AQP3 overexpression in atopic skin promote sensitisation to PA and PG, produced by peanut PLD (150, 269). Therefore, by

incorporating PA and PG into our ELISpot experiments, we could investigate the capacity of both exogenous peanut-derived phospholipids and endogenous PLD-generated lipids to modulate CD1a responses in individuals with peanut allergy.

These data reveal that peanut-allergic patients possess elevated PG- and PA-reactive CD1a-reactive T cells, in contrast with an inhibitory response observed in HCs to PG. This allowed us to draw two fundamental insights. Firstly, PG- and PA- induced highly similar responses to peanut PLD, lending strong support to our peanut PLD mechanism of sensitisation. Whilst previous studies have confirmed the presence of upregulated PLA_2-responsive CD1a-reactive T cells in atopic and psoriatic patients, we have presented the first study investigating the effect of phospholipase reaction products (279, 280). Secondly, PG and PA are major phospholipid constituents of peanuts, and our data may have highlighted the first example of exogenous food-derived phospholipids which drive primary CD1a responses in allergic disease. To date, studies in this area are limited to the adjuvant activity of food lipids, which act via CD1d to potentiate peptide-specific responses, as previously discussed (469, 470). Whilst these PG and PA species serve as a useful model, future studies should employ fractionation and isolation of phospholipids from peanuts using HPLC-MS, as further discussed in *Chapter 9.1*. Subsequent ELISpot experiments in healthy, AD and peanut-allergic individuals could then be used to assess production of Type 2 cytokines, including IL-4 and IL-13, which are hallmark for peanut allergy (412). This would provide robust support to our claims that peanut lipids induce CD1a-reactive T cells which license peptide-specific responses, as outlined in *Chapter 6.3.3* (361). In addition to stimulating CD1a-mediated responses, peanut-derived phospholipids may also promote inflammation through other mechanisms. PA is known to induce production of pro-inflammatory IL-6, TNF-α and IL-1β in Raw 264.7 monocytes, and this is mediated by the Akt pathway (496). Moreover, PA promotes B cell proliferation, and we propose this

facilitates IgE production following peanut peptide-specific responses (497). Equally, *Staphylococcus aureus*-derived PG promotes hydrolysis of human skin CERs by ceramidases, contributing towards barrier dysfunction and epicutaneous sensitisation (498). Potentially, peanut PG exerts a similar effect.

1.31.4. Peanut protein extracts, skin prick tests and oil bodies do not act via CD1a

Given the paucity of CD1a-reactive responses to peanut lipids, peanut proteins were incorporated into K562 ELISpot assays. We had hypothesised that peanut protein enzymatic activity would modulate responses to K562 cell lipids. Interestingly, peanut protein extracts led to no change in IFN-γ production in CD1a-reactive T cells from HCs. This lack of effect was surprising, given the marked inhibitory effect of peanut PLD which was previously observed in HC T cells (*Figure12A*). We concluded that the extraction procedure, which incorporates chloroform and methanol to remove lipid fractions, potentially denatured enzymes within the aqueous phase. Chloroform is a commonly-used protein denaturing agent, often utilised to separate protein from DNA and RNA (499). As an alcohol, methanol disrupts protein tertiary structures through protonation of amino acid residues (500). We considered this may have disturbed the HKD catalytic domain of PLD, in addition to other allergenic peanut proteins. Future experiments should incorporate specific extraction techniques for target enzymes, which can then be used in antigen-presentation assays. PLD has been successfully purified from peanuts using a sequence of hexane delipidation, acetone precipitation, Sepharose fractionation, elution and testing of fractions for PLD activity (501). Furthermore, this protocol could be modified to identify and extract other phospholipase species, including PLA_2 and PLC, for subsequent functional analysis.

Mirroring peanut protein extracts, peanut SPT did not induce CD1a-reactive T cell responses. In order to generate SPT, peanuts are subjected to aqueous extraction, eliminating hydrophobic proteins which may otherwise contribute towards CD1a-reactivity

(502). Oleosins are strongly lipophilic proteins which induce IgE responses in peanut, hazelnut and sesame allergy (101, 103, 503). Their strong association with lipids within OBs affords oleosins with poor aqueous solubility; consequently, they are difficult to extract and are underrepresented in diagnostic SPT extracts (103). Therefore, peanut OBs were extracted and incorporated into the K562 ELISpot system, to infer effects of their constituent oleosins and phospholipids. Interestingly, following pulsing with 100µg/ml OB solution, both EV- and CD1a-transfected K562 cells induced significant IFN-γ responses in HC T cells. Whilst this did not eliminate the possibility of CD1a-reactive T cell responses, this demonstrated clear non-specific effects. Reviewed in detail in *Chapters 4.4.3* and *7.1.3*, OBs contain a TAG core, enveloped by a monophospholipid layer which is embedded with proteins including oleosins (457). TAGs are major cellular energy stores and have been shown to upregulate CD40 and CD80 on THP1 monocytes in a dose-dependent manner (504). We suggest that, upon endocytosis by K562 cells, peanut OBs release TAGs and contribute towards upregulation of costimulatory molecules which promote T cell responses. Equally, the strongly hydrophobic nature of oleosins may prevent their release from OBs during ELISpot pulsing (457). Schwager et al recombinantly expressed water-soluble Ara h 15, a peanut oleosin, in *Escherichia coli* (103). This was achieved by modifying the hydrophobic core domain of Ara h 15 through insertion of a His-tag linked to a Glycin-Alanin-Glycin-spacer. Utilising such techniques would allow us to assess the effect of pure oleosins in modulating CD1a responses, given their potential ability to assist in CD1a lipid loading.

1.31.5. Ara h 1 enhances CD1a-reactivity, potentially through binding PG

Following previous experiments, we hypothesised the lack of CD1a-reactive T cell responses to peanut protein extracts was due to the elimination of lipophilic species during extraction. Therefore, recombinant Ara h 1 and Ara h 8 were incorporated into the ELISpot

system, given their functional lipid-interaction capacity (97, 98). Whereas no effect was observed with Ara h 8, remarkably Ara h 1 induced CD1a-reactive T cell responses, which was exacerbated in peanut-allergic individuals. This is the first example of a major peanut protein allergen modulating CD1a-reactivity. Previously, Angelina et al demonstrated a dose-dependent aggregation of PG vesicles in response to Ara h 1, thereby evidencing Ara h 1 interacts with PG (98). Importantly, this was shown to exert a functional effect, given that PG vesicles protected Ara h 1 from gastric digestion, preserving IgE binding sites. Furthermore, MoDCs produced significantly more IL-1β in the presence of both Ara h 1 and peanut-derived lipids. Clearly, therefore, PG and peanut lipids endorse allergenicity of Ara h 1, and we hypothesised that Ara h 1 exerts a reciprocal effect, by promoting presentation of PG to CD1a-reactive T cells in peanut-allergic patients. However, in that study, PG was derived from egg yolk which comprises a heterogeneous mixture of $C_{16\text{-}22}$ species with multiple saturation states (98, 505). Therefore, we sought to confirm specific binding of Ara h 1 to 18:1 (Δ9-Cis) PG and 18:2 PG, which were responsible for our observed effect in peanut-allergic individuals.

In IEF binding assays, there were extraordinary reductions in the pI of Ara h 1 in response to coincubation with our PG species. 18:1 (Δ9-Cis) PG led to a particularly marked effect, generating two new bands with pI values that were less than 3.5. 18:2 PG induced a similar outcome, with the addition of a single band. These bands represent Ara h 1 with significantly reduced molecular charge, evidencing newly-formed lipid-bound complexes (506). This allowed us to draw key insights. A reduction in molecular charge of this magnitude likely represents alterations in the tertiary structure of Ara h 1, through alterations in amino acid protonation and electrostatic interactions (507). Potentially, this unveils and stabilises IgE binding sites as reported previously (98). The high affinity Fc receptor for IgE, FcεRI, is largely expressed on the surface of mast cells and basophils,

mediating their activation in allergic disease (508). However, in atopic patients, FcεRI is also detected on the surface of LCs and DCs, facilitating rapid uptake and processing of peptide allergens, and subsequent presentation to $CD4^+$ T cells via MHC II (509-512). We suggest IgE-mediated cross-presentation plays an equivalent role in propagating CD1a-reactive responses to peanut allergens. Upon binding peanut PG, specific IgE binding sites on Ara h 1 may be unveiled, facilitating DC uptake of both protein and lipid allergens. This would provide a further explanation for increased Ara h 1-responsiveness of peanut-sensitised CD1a-reactive T cells, and potential experiments are discussed in *Chapter 9.1*.

In addition to promoting PG uptake, Ara h 1 may directly deliver PG to CD1a for subsequent presentation to lipid-specific T cells. Given their poor solubility in aqueous cytosol, protein loading with lipids is often mediated by lipid transfer proteins (LTPs) (513). Understanding of CD1-lipid loading is relatively limited and largely confined to CD1b and CD1d. Saposins are a group of small, non-enzymatic proteins which facilitate endosomal lipid loading of CD1 molecules (513). Consequently, Saposin B (SapB) and Saposin C (SapC) are required for loading of lipids onto CD1d and CD1b, respectively (459, 514). Given its dual hydrophilic and hydrophobic capacity, we hypothesise that Ara h 1 facilitates PG-loading of CD1a in a similar manner (515). To test this, we initially recommend further characterisation of Ara h 1-PG interactions through methods such as vehicle co-sedimentation, varying concentrations of each component to identify the protein-lipid binding affinity (516). Subsequently, confocal microscopy could be employed to investigate co-localisation of fluorescently tagged Ara h 1, PG and CD1a in MoDCs. Moreover, interaction with early endosome markers, including early endosome antigen 1 (EEA1) and transferrin, would assist in localising the sites of lipid loading (517, 518).

1.32. Chapter summary

In summary, we have demonstrated that peanut-allergic patients possess elevated PG- and PA-responsive CD1a-reactive T cells. This bears relevance to allergic sensitisation to food-derived lipids, given the abundance of PG and PA in peanut seeds. Further investigation should involve exploring lipid-reactivity to phospholipid species isolated directly from peanuts. Moreover, our findings lend further support to the peanut PLD mechanism of lipid sensitisation in atopic patients, introduced in the previous chapter. Importantly, we noted that Ara h 1 also induces CD1a-reactive T cell responses in peanut-allergic patients, and this may be related to its inherent ability to interact with PG. This provokes intriguing questions on how protein-lipid interplay modulates lipid reactivity, and future experiments are outlined in *Chapter 9*.

HUMAN PLD ACTIVITY REGULATES CD1A EXPRESSION IN DENDRITIC CELLS

1.33. Current knowledge

During previous chapters, new mechanisms were outlined by which peanut-derived PLD drives CD1a-reactivity in peanut-allergic patients. This evidenced the strong immunomodulatory potential of PLD, leading us to consider the effects of the human endogenous isoforms, PLD1 and PLD2, in modulating CD1a biology. Both isoforms have been extensively reviewed in the Introductory chapter (*Chapter 3.6*), with wide-ranging effects on cellular function and protein expression via production of important metabolic intermediates including PA and PG (272, 387, 519, 520). Consequently, PLD is a key enzyme in maintaining homeostasis of lipids and their protein targets. We aim to understand whether endogenous PLD regulates CD1a expression at the cellular level, providing additional roles which extend beyond its ability to produce CD1a ligands. To demonstrate this rationale, current knowledge of processes which shape CD1a expression will be reviewed, including potential functions for human PLD1 and PLD2.

1.33.1. Overview of CD1a-regulatory mechanisms

Despite its discovery three decades ago, transcriptional regulation of CD1a remains poorly understood (521). DCs are heterogeneous and possess distinct CD1a expression profiles, which vary from low in mDCs to high in LCs (522). Given that mDCs reside in blood and LCs exist in skin, local microenvironment of tissues plays a role in modulating genetic expression of *CD1A* (523). Extracellular regulatory factors include cytokines, immunoglobulins (Igs) and lipids (306, 522, 524). Several cytokines promote CD1a expression. Granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 are well-known for their ability to induce expression of group I CD1 molecules on MoDCs

(525, 526). Cotreatment of monocytes with IL-3 and IL-4 induces similar effects, in addition to other proinflammatory cytokines IL-1β, IL-6 and TNF-α (527, 528). Conversely, induction is suppressed by anti-inflammatory IL-10 (528). Clearly, cytokine milieu, which is predilected by tissue localisation and disease-state, plays an important role in controlling CD1a levels.

Igs have been shown to play equivalent functions. In a study by Smed-Sörensen et al, exposure of MoDCs to media enriched with IgG eliminated group I CD1 expression and upregulated CD1d expression, and this was mediated by FcγRIIa (522). The opposite effect was observed with IgG-depleted media, demonstrating the inverse relationship between regulation of group I CD1 molecules and CD1d. Moreover, IgG interactions modulated the ability of MoDCs to present antigens to CD1-restricted T cells. This provided further insight into the discrepancy between $CD1a^{low}$ mDCs and $CD1a^{High}$ LCs, given that IgG is much more highly concentrated in blood compared with skin under nonpathological conditions.

Serum lipids have also been demonstrated to regulate CD1a expression on DCs. Previously, human AB serum and fetal calf serum (FCS) were shown to induced a dose-dependent inhibitory effect on the production of $CD1a^{+}$ MoDCs (524). This effect was mediated by lipoproteins, suggesting inhibition was a result of lipid constituents of serum. In another study, fractionation of human serum revealed that constituent polar lipids LPA and cardiolipin potently inhibit group I CD1 molecule expression on MoDCs (529). PPARγ is a ligand-activated nuclear TF which is highly expressed in adipose tissue and predominantly regulates fatty acid storage (530). Endogenous PPARγ agonists include fatty acids, and fatty-acid derived eicosanoids and prostaglandins (531). As with other PPAR isoforms, activated PPARγ forms heterodimers with the retinoid X receptor to trigger transcription of

target genes (532). Lipid modulation of PPARγ activity is perhaps the most well-defined axis in regulating CD1a expression (524, 533). In a landmark study, PPARγ was shown to immediately upregulate in MoDCs, leading to downregulations in *CD1A* and upregulations in *CD1D* transcription (533). This was confirmed to induce functional effects, with increased CD1d expression inducing proliferation of iNKT cells. These effects were recapitulated in another study, in which PPARγ expression was restricted to $CD1a^-$ cells, corresponding with elevated transcription of its target genes apolipoprotein E (ApoE) and fatty-acid-binding protein-4 (FABP4) (524). Importantly, Szatmari et al identified that PPARγ induces the expression of retinol and enzymes, including retinol dehydrogenase 10, which induce its conversion to ATRA. Through binding of RARα, ATRA promoted expression of CD1d, and inhibited expression of CD1a, in human DCs. Therefore, lipids are integral in modulating CD1 expression profiles, through their induction of, and production by, PPARγ. Outside the effects of PPARγ, there is limited understanding of regulation of *CD1A* by TFs. The ATF/CREB family of TFs are activated by phosphorylation and regulate diverse cellular processes including mRNA expression (534). In an early study, Hiromatsu et al identified a cAMP response element (CRE) 965bp upstream of the *CD1A* translation start site (535). This proximal promoter region was later shown to bind CREB-1 and ATF-2, resulting in reduced *CD1A* transcription (306). Recently, Mahiddine et al provided further insight into this mechanism, observing that ATF-2 activity is modulated by the relative abundance of short and long isoforms of CD99 (536). CD99 long form (CD99LF) expression in immature DCs (iDCs) induced phosphorylation of ATF-2, which subsequently downregulated *CD1A* transcription. Reduced CD1a-reactive T cell responses may therefore contribute towards cAMP-induced suppression of TNF-α, IL-17 and IFN-γ production (537, 538).

Finally, antigen-specific factors modulate CD1 expression in DCs, which are often correlated with disease states. Treatment of healthy individuals with tetanus toxoid vaccine triggered GM-CSF production and resulted in upregulated CD1a, -b and -c on monocytes (539). Conversely, *Leishmania donovani*- infected DCs downregulate their transcriptional and surface expression of group I CD1 molecules, reducing their ability to stimulate *Mycobacterium tuberculosis*-restricted T cell responses, although the mechanism was unclear (540, 541). *M. tuberculosis* expresses multiple CD1 ligands, including DDM, fatty acids, mycolic acid, mannosyl phosphomycoketide and the sulfoglycolipid Ac2SGL, and therefore possesses a complex relationship with the transcriptional regulation of CD1 molecules (542-545). Two studies demonstrate that mycobacterial components promote CD1 expression. Infection of $CD1^-$ myeloid precursors with live *M. tuberculosis* induced group I CD1 expression via activation of TLR-2 by mycobacterial cell wall lipids (546). Similarly, Videira et al noted that BCG administration upregulates *CD1A*, *CD1B* and *CD1C* transcription in urothelium biopsies from bladder cancer patients (547). Furthermore, dermal granulomas from leprosy patients exhibited upregulated expression of CD1a, b and c, and this reflected an attempt by the host immune system to amount increased CD1-mediated defence against pathogen (548). However, a contrasting body of evidence exists to suggest that mycobacteria suppress CD1 expression in order to evade immune responses (549). Infection of iDCs with *M. tuberculosis* eliminated CD1 expression within 24 hours (550). GM-CSF and IL-4 treatment induced lower frequencies of $CD1a^+$ MoDCs in patients with pulmonary tuberculosis (551). This aberrant CD1 expression is partly explained by phosphorylation of p38 MAPK, which is initiated by mycobacteria (552). In other studies, differentiation of monocytes to MoDCs in the presence of BCG induced similar suppression of CD1a levels, and monocytes infected with *M. smegmatis* developed directly into $CD1a^-$ $CD83^+$ mDCs (553-555). Contrasting results between studies may be explained by the fact that BCG induces production of both GM-CSF and IL-10 by mononuclear cells, which

exert opposing effects on CD1 expression (556). Equally, *M. tuberculosis* activates *IL-10* gene transcription through p38 MAPK and PI3K/AKT pathways, triggering IL-10 release by monocytes and subsequent CD1 suppression (557, 558). In addition to transcription, mycobacterial lipids may interfere with the complex cycle of CD1a recycling and loading, outlined in the Introduction. These processes include several molecular targets, including apolipoproteins and saposins involved in lipid loading, which may be disturbed by mycobacterial constituents, interfering with normal CD1 trafficking to the cell membrane (559, 560). Finally, microRNAs (miRNAs) are non-coding RNAs which regulate gene expression (561). Seven miRNAs (33a, 33b, 224, 421, 495, 590-3p, and 590-5p) have been identified which may control transcriptional activity of *CD1A* by targeting its 3'UTR region of *CD1A* (562). Pathogens, including mycobacteria, contain abundant noncoding RNA and may therefore utilise this mechanism to further control CD1a expression (563).

1.33.2. Endogenous PLD may modulate CD1a expression

Given the identification of a growing number of mechanisms which modulate CD1a expression, we wish to explore the role of endogenous PLD isoforms. Phospholipases have been implicated in MoDC maturation, and we aim to further characterise these effects in the context of PLD, PPARγ and CD1a (277). In addition to agonists, several PPARγ antagonists have been identified (564-566). Cyclic PA (cPA) is a natural analogue of the CD1a ligand, LPA, which forms a 5-carbon ring between the sn-2 hydroxyl group and sn-3 phosphate group (567). cPA is produced by intermolecular transphosphatidylation of LPC by PLD, giving rise to the unusual ring structure. Whilst cPA and LPA share some receptors, this structural difference gives rise to opposing cellular effects; for example, LPA promotes cell proliferation whilst cPA demonstrates potent antimitogenic activity (568, 569). Importantly, PLD2-generated cPA has been shown to bind PPARγ with high affinity and inhibit its activation through induction of conformational changes and preventing

binding of exogenous agonists (570-572). This corresponded with downregulations in transcription of PPARγ target genes *FABP4* and *CD36*, and downstream lipid accumulation was noted in RAW264.7 monocytes and primary mouse macrophages. This further differentiates cPA from LPA, which is a PPARγ agonist (573). Furthermore, these interactions have been shown to regulate pathology, given that cPA-mediated inhibition of PPARγ suppressed proliferation of the colonic cancer cell lines HT-29 and DLD-1 (571, 572). Given that PLD inhibits PPARγ via production of cPA, and reduced PPARγ activity promotes CD1a expression on DCs, we aim to determine if endogenous PLD activity is required for CD1a expression (574). This would provide an additional pro-inflammatory role of PLD in stimulating CD1a-reactive T cells. Furthermore, PLD1 and PLD2 exert several effects on DC function outlined in the introductory chapter (*Chapter, 4.6).* including promotion of antigen uptake through phagocytosis (276). Therefore, it will also be investigated whether phenotypic alterations in DCs, induced by inhibition of their PLD activity, alter their capacity to present CD1a antigens to T cells.

1.33.3. Objectives

We seek to harness the novel functions of endogenous PLD, to further inform current understanding of mechanisms regulating CD1a expression. To address this aim, the following objectives have been developed:

1. Investigate the role of endogenous PLD activity in promoting CD1a expression in MUTZ3-LCs, an inducible LC line.
2. Determine the comparative role of PLD activity in driving CD1a expression in primary MoDCs..
3. Characterise the effects of PLD inhibition in MoDCs, including PPARγ activity, antigen uptake and presentation to CD1a-restricted T cells.

1.34. Results

1.34.1. Endogenous PLD activity is required for CD1a expression in MUTZ3-derived LCs

Endogenous PLD is known to upregulate during differentiation of monocytes into MoDCs (276). Given previous findings that exogenous PLD induces CD1a responses (see *Chapter 6*), we wished to determine if endogenous human PLD activity affects CD1a mRNA and protein expression in LC. MUTZ3 cells are a $CD34^+$ acute myelomonocytic leukemia cell line, which bear similarity to DC precursors (575). MUTZ3 cells can be differentiated into an LC phenotype ($CD1a^+CD207^+$) over 10 days using a cytokine cocktail of GM-CSF, TGF-β and TNF-α added on D0, D4 and D7 (328). The MUTZ3-LC model was used to test our hypothesis, as it offers renewable source of cells with an LC/DC phenotype.

Initially, it was investigated whether MUTZ3-LCs are a suitable phenotype. Light microscopy images were obtained demonstrating the transition of small, round, heterogeneously dispersed, undifferentiated MUTZ3 cells (*Figure 29A, left panel*), to larger MUTZ3-LCs (*Figure 29A, right panel*), which aggregate under activation and possess characteristic elongated dendritic processes. Transmission electron microscopy (TEM) was performed on differentiated MUTZ3-LCs, with dendrites clearly visible at lower power (*Figure 29B, left panel*; *blue arrows*). At higher magnification, we observed the presence of BGs, confirming an LC-specific phenotype (*Figure 29B, right panel*; *red arrows*). Although BGs are classically recognised as having a 'tennis racket' shape, this is dependent on acquiring a specific section during image acquisition. Due to oblique sectioning, our images demonstrate the 'handle' structure of BGs. Therefore, microscopy images suggested this cell line possesses an LC phenotype, and MUTZ3-LCs were used as surrogates for primary cells in subsequent experiments.

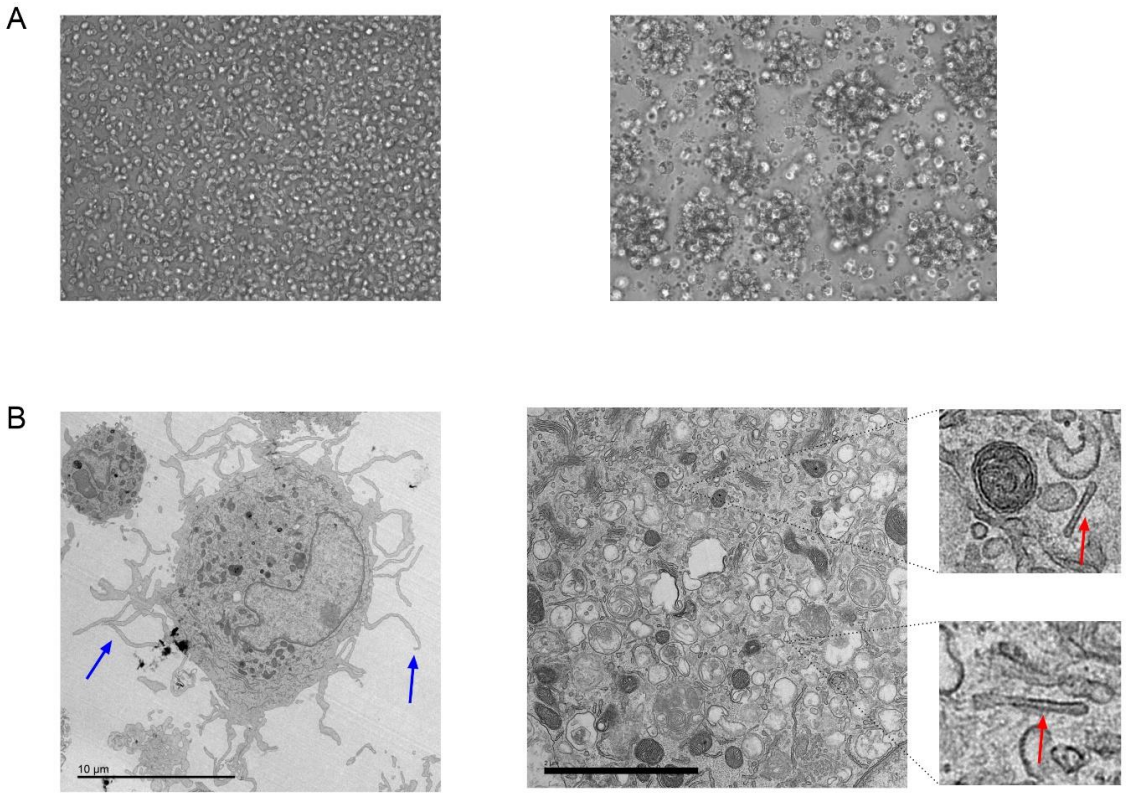


Figure 29. MUTZ3-derived Langerhans cells (MUTZ3-LCs) possess an LC-like ultrastructure. MUTZ3-LCs were cultured over 10 days with TNF-α, GM-CSF and TGF-β, as previously described. A) Light microscopy images showing undifferentiated MUTZ3 cells (left panel; D0) and MUTZ3-LCs (right panel; D10). B) Transmission electron microscopy (TEM) were obtained of MUTZ3-LCs. Dendritic processes are visible lower magnification (left panel; blue arrows). Characteristic LC Birbeck Granules are visible at higher magnification (right panel; red arrows).

Next, investigations were performed to determine the role of endogenous PLD in regulating CD1a expression during differentiation of MUTZ3-LCs. Cells were treated with no inhibitor or dual PLD1/PLD2 inhibitor (50nM FIPI hydrochloride) on day 0, day 4 and day 7, prior to the addition of cytokines. Three independent experiments were performed. *Figure 30A* illustrates flow cytometry data taken from one experiment, demonstrating frequencies of $CD1a^+$ cells on day 0 (prior to any cytokine or inhibitor treatment), day 7 and day 10 of differentiation. Standard gating techniques were used, including initial selection of cells with suitable FSC and SCC characteristics, and elimination of doublets and dead cells (576). Gating to fluorescence minus one (FMO) and isotype controls were different between days, given the morphological changes in cells as they transition towards an LC phenotype. On day 0, before treatment with PLD2 inhibitor, CD1a-expressing cells were

low or absent (*Figure 30A, left and middle columns*; control group = 1.78%; PLD-inhibited group = 2.53%). The control group demonstrated sequential increases in CD1a-expressing cells on day 7 (18.9%) and day 10 (89.3%) of differentiation, the latter corresponding with expected MUTZ3-LC expression levels (*Figure 30A, left column*) (575). Remarkably, PLD inhibition abrogated this effect, with 5.30% and 34.3% of cells expressing CD1a on day 7 and 10, respectively (*Figure 30A, middle column*). Histogram plots show the frequency of CD1a-expressing cells, normalised to the mode, in both populations (*Figure 30A, right column*; dotted line = control cells; solid line = PLD-inhibited cells). These data demonstrate clear reductions in numbers of $CD1a^+$ cells on day 7 and day 10, compared with control cells, suggesting this is induced by inhibition of PLD.

To confirm the observed effect of PLD inhibition was repeatable, data was aggregated from three HC donors. Within the control cohort, the mean frequency of CD1a-expressing cells increased from 1.09% on day 0 to 12.5% on day 7 ($p<0.05$) and 84.8% on day 10 ($p<0.0001$) (*Figure 30B*). The increase between day 7 and 10 was also significant ($p<0.001$). Strikingly, PLD inhibition induced a 52.1% reduction in the frequency of $CD1a^+$ cells at day 10 (32.7%), compared with the control group ($p<0.0001$). Moreover, in FIPI-treated cells, there was no significant change in the frequency of $CD1a^+$ cells between day 0 and day 7 (3.7%). Increases were observed between day 0 and day 10 ($p<0.01$), and day 7 and day 10 ($p<0.01$), however increments were less than that of control cells. Next, it was investigated whether FIPI treatment modulates absolute CD1a expression in cells. Across three donors, CD1a mean fluorescence intensity (MFI) was obtained by calculating the average geometric mean fluorescence in $CD1a^+$ cells on day 10 (*Figure 30C*). CD1a MFI was downregulated by 88.5% in MUTZ3-LCs which had been differentiated in the presence of PLD inhibitor, compared with control cells (control cells = 49023 AU; PLD-inhibited cells = 5669 AU; $p<0.01$). Moreover, cell viability was >85% and was unaffected by FIPI

treatment. Therefore, PLD activity is required for production of MUTZ3-LCs which express CD1a, and also for optimal expression of CD1a in individual cells.

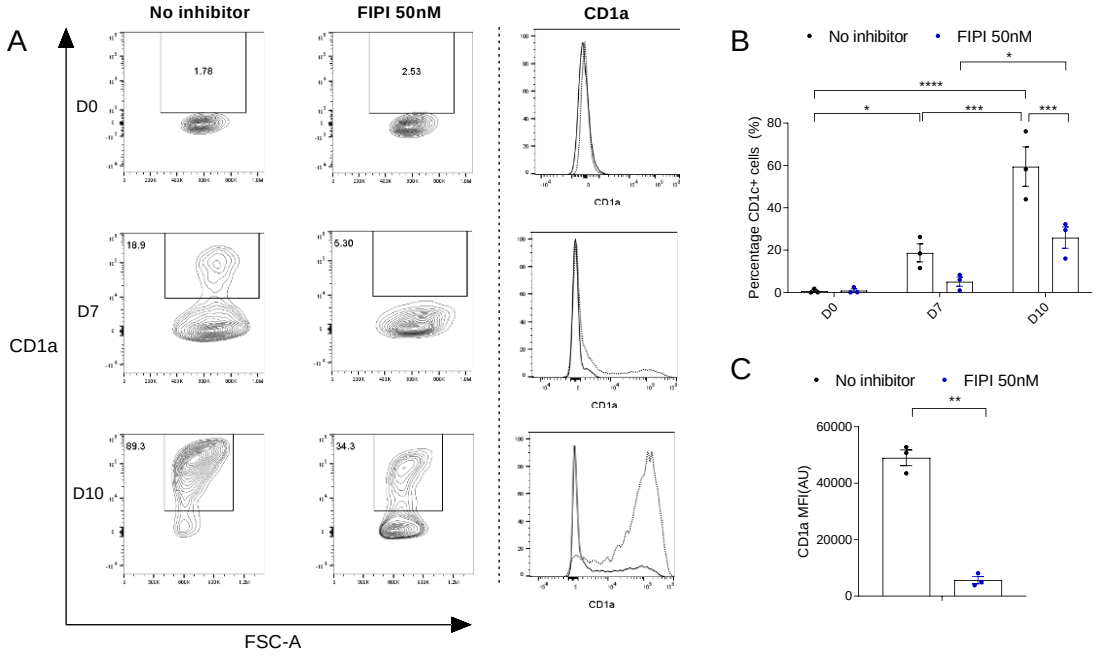


Figure 30. Phospholipase D (PLD) inhibition reduces CD1a protein expression in MUTZ3-derived Langerhans cells (MUTZ3-LCs). MUTZ3-LCs were cultured over 10 days with TNF-α, GM-CSF and TGF-β, as previously described. On D0, D4 and D7, cells were treated with dual PLD1/PLD2 inhibitor (FIPI; 50nM) or no inhibitor. Flow cytometric data is shown for D0, D7 and D10. A) Frequency of CD1a-expressing cells. Left and middle columns show contour plots and right column shows histograms comparing untreated and PLD-inhibited cells (dotted line = control cells; solid line = PLD-inhibited cells). B) Mean frequency of $CD1a^+$ cells in both treatment groups on day 10, across 3 experimental repeats. C) CD1a MFI of $CD1a^+$ cells in both treatment groups on day 10, across 3 experimental repeats.

Given the clear relationship between cellular PLD activity and CD1a expression, we elected to determine if this was an upstream effect on normal DC differentiation or a downstream effect specific for CD1a. Within the same experiments, cells were analysed by flow cytometry for CD11c and CD1c expression, to reflect the development of a DC phenotype which expresses other group I CD1 molecules (*Figure 31A*). CD11c was expressed approximately half of cells on day 0 (control cells = 47.6%; PLD-inhibited cells = 56.6%) on day 0, as expected in this cell line, increasing in both cohorts on day 7 (control cells = 92%; PLD-inhibited cells = 79.6%) and day 10 (control cells = 95%; PLD-inhibited cells = 98.3%) (Figure 20A, left and middle columns) (577). On day 0, CD1c was expressed on

0.48% of control cells, increasing to 26.22% and 76.12% on days 7 and 10 of differentiation, respectively (*Figure 31A, left and middle columns*). This effect was abrogated when cells were differentiated in the presence of FIPI, leading to $CD1c^+$ cell frequencies of 0.97% and 29.52% on days 7 and 10, respectively. Histogram plots further illustrate the frequency of $CD1c^+$ cells, normalised to the mode, in each treatment cohort (*Figure 31A, right column***;** dotted line = control cells; solid line = PLD-inhibited cells). Across treatment conditions in three experiments, mean frequencies of $CD1c^+$ cells were calculated at day 10 (*Figure 31B*). In control MUTZ3-LC, compared with day 0, increased numbers of CD1c-expressing cells were observed at day 7 (18% increase; $p<0.05$) and day 10 (59% increase; $p<0.0001$). Differentiation in the presence of FIPI significantly reduced $CD1c^+$ cells at day 10, compared with control cells (33.5% decrease; $p<0.001$). Within the $CD1c^+$ cell fraction which had been treated with FIPI, CD1c MFI was reduced at day 10 (46% decrease; $p<0.05$), demonstrating that inhibition of PLD reduces CD1c expression at a cellular level (*Figure 31C*). Our data demonstrate that endogenous PLD activity is required for CD1c protein expression in this cell line, however it is not required for CD11c expression. Given that PLD inhibition suppressed both CD1a and CD1c protein expression, this suggests that PLD plays a role in regulating the expression of group I CD1 family members.

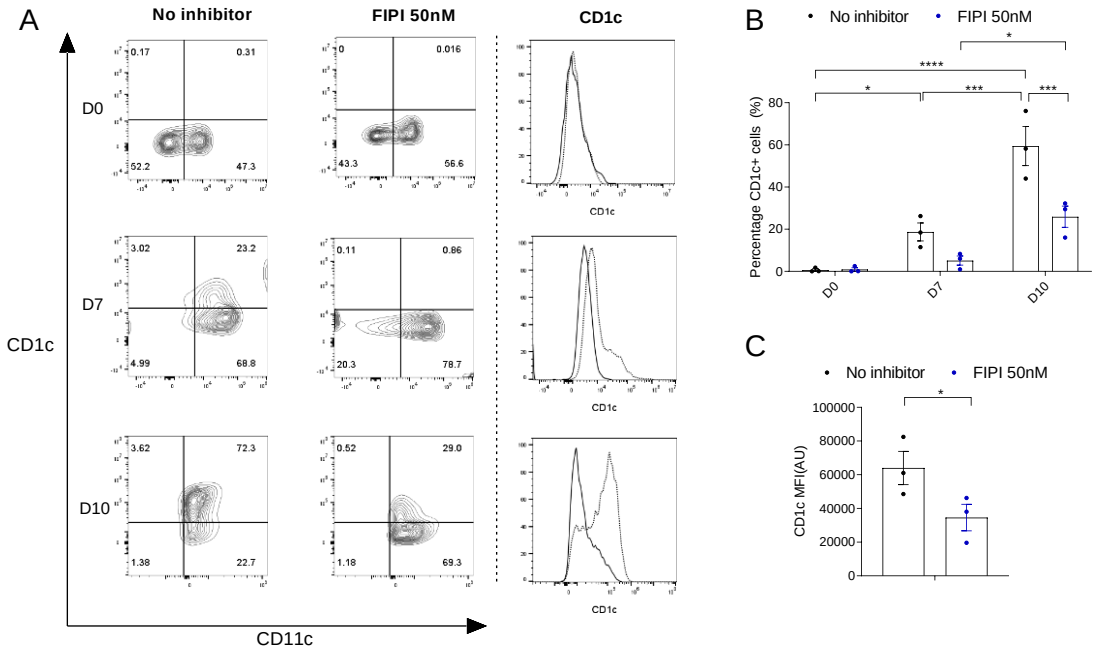


Figure 31. Phospholipase D (PLD) inhibition reduces CD1c protein expression in MUTZ3-derived Langerhans cells (MUTZ3-LCs). MUTZ3-LCs were cultured over 10 days with TNF-α, GM-CSF and TGF-β. On D0, D4 and D7, cells were treated with dual PLD1/PLD2 inhibitor (FIPI; 50nM) or no inhibitor. Flow cytometric data is shown for D0, D7 and D10. A) Frequency of CD1c- and CD11c-expressing cells. Left and middle columns show contour plots of CD1c and CD11c expression. Histograms in right column display the frequency of CD1c-expressing cells, comparing untreated and PLD-inhibited cells (dotted line = untreated cells; solid line = PLD-inhibited cells). B) Mean frequency of $CD1c^+$ cells in both treatment groups on day 10, across 3 experimental repeats. C) CD1c MFI of $CD1c^+$ cells in both treatment groups on day 10, across 3 experimental repeats (*p<0.05; ***p<0.001; ****p<0.0001).

Next, specific PLD isoforms were examined for their role in regulating mRNA expression in MUTZ3-LCs. Cells were separated into various PLD inhibitor culture conditions (no inhibitor; PLD1 inhibitor = VU0155069 50nM; PLD2 inhibitor = ML298 10μM; dual PLD1/PLD2 inhibitor = FIPI 50nM), with inhibitors added on day 0, day 4 and day 7, prior to addition of cytokines. Briefly, RNA extraction was performed on cells on day 0 (prior to addition of inhibitors or cytokines), day 4, day 7 and day 10 of differentiation. cDNA synthesis and qPCR were subsequently performed, and mRNA transcription was normalised to expression of the housekeeping gene GAPDH. *Figure 32A* shows mRNA transcription of genes encoding phospholipase isoforms during differentiation. *PLD1* and *PLD2* expression increased between day 0 and day 7 (*Figure 32A, top two panels*; *PLD1* = 10-fold increase; $p<0.05$; *PLD2* = 13-fold increase; $p<0.01$), and between day 7 and day 10

(*PLD1* = 3-fold increase; $p<0.0001$; *PLD2* = 2-fold increase; $p<0.05$). This confirms that *PLD1* and *PLD2* transcription increase throughout differentiation in control cells. Notably, on day 10, *PLD1* transcription was reduced by ~95% when either PLD1 or PLD2 activity were inhibited ($p<0.0001$), whereas *PLD2* transcription was not inhibited by any blocker. *PLD1*, but not *PLD2*, is known to be transcriptionally activated by both PLD1 and PLD2 activity (578). Therefore, this confirms the expected mRNA expression profile, suggesting this is a functional model of PLD inhibition in MUTZ3-LCs. Furthermore, inhibition of PLD1 enhanced transcription of *PLD2* by 57% ($p<0.01$) in fully differentiated cells, suggesting a feedback mechanism**.** Amongst other phospholipase isoforms, *PLA2G4C* transcription increased between day 0 and day 10 (*Figure 32A, bottom left panel*; 30-fold increase; $p<0.0001$). Furthermore, PLD2 and dual PLD inhibition of cells reduced *PLA2G4C* expression on day 10 (PLD2-inhibited cells = 2-fold decrease; $p<0.05$; dual PLD-inhibited cells = 2.6-fold decrease; $p<0.01$). *PLA2G4A* showed no clear pattern of expression during differentiation in control cells, and PLD inhibition led to no clear effect on *PLA2G4A* transcription (*Figure 32A, bottom right panel*). Consequently, *PLA2G4C*, but not *PLA2G4A*, expression appears to be modulated by PLD2 activity.

Figure 32B demonstrates *CD1A* and *CD207* (langerin) expression, which were both highly expressed in fully differentiated cells (Ct values <20). In line with protein data, *CD1A* expression increased between day 0 and day 7 (800-fold increase; $p<0.01$), and between day 7 and day 10 (2.3-fold; $p<0.0001$), during differentiation of control MUTZ3-LCs (*Figure 32B, left panel*). Unexpectedly, in fully differentiated cells (day 10), *CD1A* transcription was unaffected by PLD1 inhibition, whereas PLD2 and dual inhibition reduced transcription by 43.3% ($p<0.01$) and 59.9% ($p<0.0001$), respectively, compared with control cells. *CD207* expression demonstrated marked upregulation between day 0 and day 10 (4400-fold increase; $p<0.0001$), however transcription was not modulated by any PLD

inhibitor (*Figure 32B, right panel*). Our data demonstrate that *CD1A* and *CD207* expression increase throughout differentiation of MUTZ3-LCs, and PLD activity is required for *CD1A* transcription.

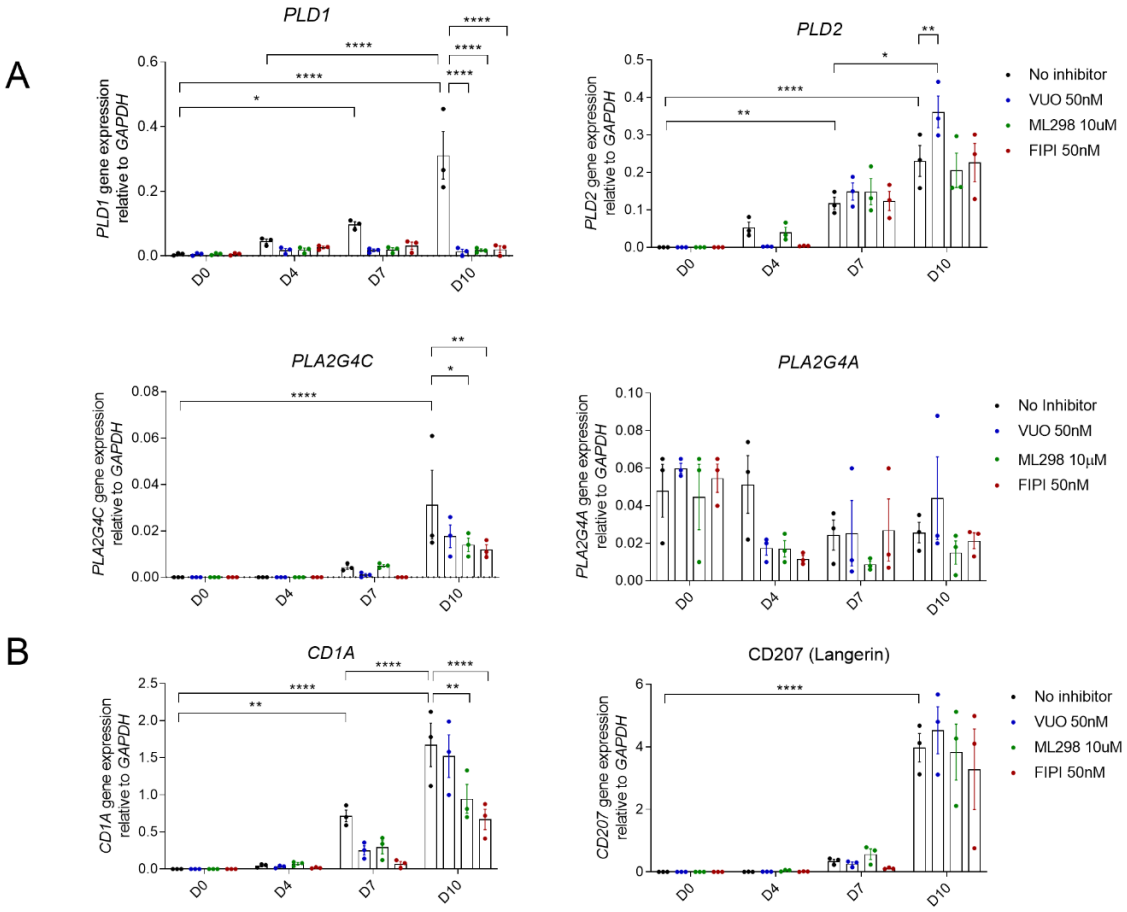


Figure 32. Phospholipase D (PLD) inhibition reduces *CD1A* mRNA expression in MUTZ3-derived Langerhans cells (MUTZ3-LCs). MUTZ3-LCs were cultured over 10 days with TNF-α, GM-CSF and TGF-β. On D0, D4 and D7, cells were treated with PLD1 inhibitor (VU0155069; 50nM), PLD2 inhibitor (ML298; 10µM), dual PLD1/PLD2 inhibitor (FIPI; 50nM) or no inhibitor. Cells were isolated and quantitative polymerase chain reaction (qPCR) was performed on D0, D7 and D10 of differentiation, normalised to expression of *GAPDH*. Data are shown from 3 independent experiments. A) mRNA expression of *PLD1*, *PLD2*, *PLA2G4C* and *PLA2G4A*. B) mRNA expression of *CD1A* and *CD207* (*p<0.05; **p<0.01; ****p<0.0001).

To determine the effect of PLD inhibition on cytokine production by differentiating MUTZ3-LC, qPCR was performed for a range of pro- and anti-inflammatory cytokines which reflect LC function (*Figure 33*). In control cells, transcription of pro-inflammatory cytokines *TNF* and *IL1B* increased throughout differentiation, culminating in high levels of expression (Ct values <20), compared with day 0 (*Figure 33A, top panels*; *TNF*: 150-fold

increase; p<0.0001; *IL1B*: 16-fold increase; p<0.0001). PLD1 inhibition led to no significant change in either cytokine. PLD2 and dual inhibition significantly reduced transcription of *TNF* by 77.3% (p<0.0001) and 80% (p<0.001), respectively. Similar effects were noted with *IL1B*, given that PLD2 inhibition reduced mRNA transcription by 62%, and dual inhibition reduced mRNA transcription by 59% (p<0.001). Other pro-inflammatory cytokines *IL6* and *IL12A* were not expressed under any condition *(Figure 33A, bottom panels*). Therefore, PLD2 activity is required for the transcription of *TNF* and *IL1B* in fully differentiated MUTZ3-LCs. Amongst anti-inflammatory cytokines tested, *TGFB* was highly expressed throughout differentiation (Ct values <20), with no overriding pattern (*Figure 33B, left panel*). This coincided with an uneven response to PLD inhibition, which was apparent on day 4 (PLD1 inhibition: 58% reduction; p<0.05; PLD2 inhibition: 51% reduction; p<0.05) and day 7 (PLD2 inhibition: 49% reduction; p<0.05; dual inhibition: 48% reduction; p<0.05), compared with control cells. In fully differentiated cells, *TGFB* transcription was not modulated by any PLD inhibitor. Moreover, *IL10* was not expressed in any sample (*Figure 33B, right panel*).

Overall, these data demonstrate that PLD activity is required for protein and mRNA expression of CD1a in an LC-like line. The qPCR data suggests this is driven by PLD2, given that PLD1 inhibition did not modulate transcription of *CD1A*. Furthermore, PLD2 activity is required for the production of proinflammatory TNF-α and IL-1β by MUTZ3-LCs. Factoring in the effect on CD1c, this suggests that PLD2 is an upstream regulator of DC maturation. These insights drove us to explore the role of PLD in regulating the phenotype and function of primary DCs.

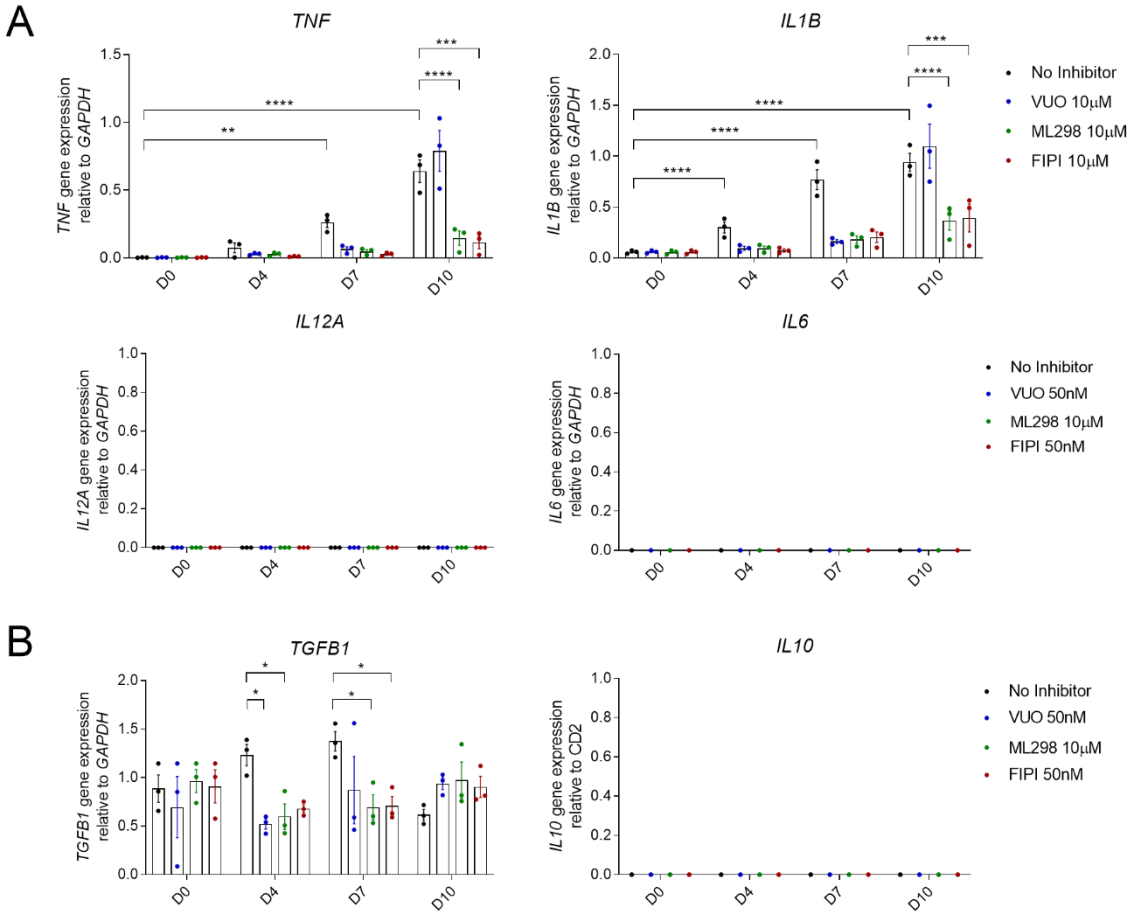


Figure 33. Phospholipase D (PLD) inhibition reduces pro-inflammatory *TNF* and *IL1B* cytokine mRNA expression in MUTZ3-derived Langerhans cells (MUTZ3-LCs). MUTZ3-LCs were cultured over 10 days with TNF-α, GM-CSF and TGF-β. On D0, D4 and D7, cells were treated with PLD1 inhibitor (VU0155069; 50nM), PLD2 inhibitor (ML298; 10µM), dual PLD1/PLD2 inhibitor (FIPI; 50nM) or no inhibitor. Cells were isolated and quantitative polymerase chain reaction (qPCR) was performed on D0, D7 and D10 of differentiation, normalised to expression of *GAPDH*. Data are shown from 3 independent experiments. A) mRNA expression of proinflammatory cytokines *TNF*, *IL1B*, *IL12A* and *IL6*. B) mRNA expression of anti-inflammatory cytokines *TGFB1* and *IL10* (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

1.34.2. PLD2, but not PLD1, inhibition reduces CD1a expression in MoDCs

Our data had identified that PLD2 activity facilitates mRNA and protein expression of CD1a during differentiation of a dendritiform cell line. Next, it was investigated whether PLD2 plays a similar role in the differentiation of primary cells. HC MoDCs (n=5) were generated over 5 days by treating donor monocytes with GM-CSF and IL-4 on day 0 and day 3 of differentiation. Cells were either untreated or treated with PLD1 (VU0155869 50nM**)** or PLD2 inhibitor (ML298 10µM) on day 0 and day 3 of differentiation. *Figure 34A*

shows representative flow cytometry data from two donors, W016 and W019, highlighting the frequencies of $CD11c^+$ and $CD14^+$ cell populations on day 0 (pre-treatment) and day 5 of differentiation. Whilst on day 0, most cells were $CD14^+CD11c^-$ monocytes (W016 = 91.2%; W019 = 95.1%), the predominant cells on day 5 were $CD14^-CD11c^+$ MoDCs, and this was apparent in control (W016 = 1.07%; W019 = 0.56%), PLD1-inhibited (W016 = 99.3%; W019 = 99.5%) and PLD2-inhibited cells (W016 = 97.1%; W019 = 99.4%). Histograms comparing control $CD11c^+$ cell populations between day 0 and day 5 demonstrate the generation of MoDCs (*Figure 34B***;** dotted line = day 0; solid line = day 5). From these data, we concluded that our culture conditions were conducive to generation of $CD11c^+$ MoDCs, and importantly this was not altered by PLD inhibition.

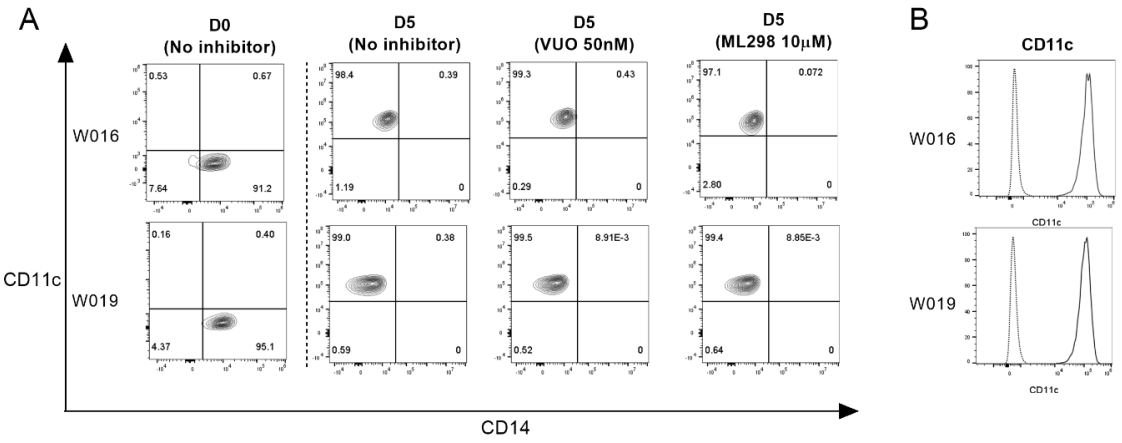


Figure 34. PLD2 inhibition does not alter CD11c protein expression during differentiation of monocyte-derived dendritic cells (MoDCs). Healthy control MoDCs were cultured over 5 days with GM-CSF and IL-4, as previously described. On D0 and D3, no inhibitor, PLD1 inhibitor (VU0155069; 50nM) or PLD2 inhibitor (ML298; 10μM) was added to cells. Phenotypic analysis was performed on D0 (prior to addition of any inhibitor) and D5, using flow cytometry. Gating to FMO/isotype controls was different between days, as cells become larger and undergo morphological changes as they transition towards a DC phenotype (A) CD11c and CD14 surface expression in donors W016 and W019, throughout differentiation and across treatment conditions. B) Histograms showing the frequency of CD11c-expressing control cells from donors W016 and W019, comparing between D0 and D5 (dotted line = D0; solid line = D5).

Next, experiments were performed to investigate the specific effect of PLD inhibition on CD1a expression in MoDCs. *Figure 35A* shows representative flow cytometry data from two donors, W016 and W019, highlighting the frequencies of $CD1a^+$ and $HLA\text{-}DR^+$ cell populations on day 0 (pre-treatment) and day 5 of differentiation. In both donors, there was

an observable upregulation of CD1a-expressing cells between day 0 (W016 = 0.015%; W019 = 0.13%) and day 5 (W016 = 84.8%; W019 = 57.17%), as expected with this differentiation protocol (579). Whilst PLD1 inhibition did not alter the generation of cells expressing CD1a on day 5 (W016 = 89.1%; W019 = 59.816%), PLD2 inhibition abrogated their production (W016 = 12.9%; W019 = 3.51%). This is further demonstrated in modal histogram plots which compare $CD1a^+$ cell frequencies between untreated and PLD2-inhibited cells on day 5 in both donors (*Figure 35B*; dotted line = control cells; solid line = PLD2-inhibited cells). Mean data were compiled across all five donors, demonstrating upregulation in the frequency of control $CD1a^+$ MoDCs during differentiation *(Figure 35C*; day 0 mean = 0.13%; day 5 mean = 55.7%; $p<0.0001$). Compared with control cells on day 5, differentiation in the presence of PLD1 inhibitor did not significantly alter the frequency of CD1a-expressing cells (mean = 57.74%), however PLD2 inhibition induced a significant reduction (mean = 25.0%; $p<0.01$). Throughout differentiation, cell viability was >80%, and was unaffected by inhibitor treatment. Having confirmed that PLD2, but not PLD1, inhibition reduces the number of fully differentiated MoDCs which express CD1a, we sought to determine its effect on cellular expression of CD1a. Across donors, CD1a MFI of fully differentiated $CD1a^+$ MoDCs was compared between untreated and PLD2-inhibited cells (*Figure 35D*). The data show that PLD2 inhibition led to a 58% reduction in CD1a MFI (control cells = 60215 AU; PLD2-inhibited cells = 25612 AU). In conclusion, PLD2 activity is required for the generation of $CD1a^+$ MoDCs and promotes CD1a expression in individual MoDCs.

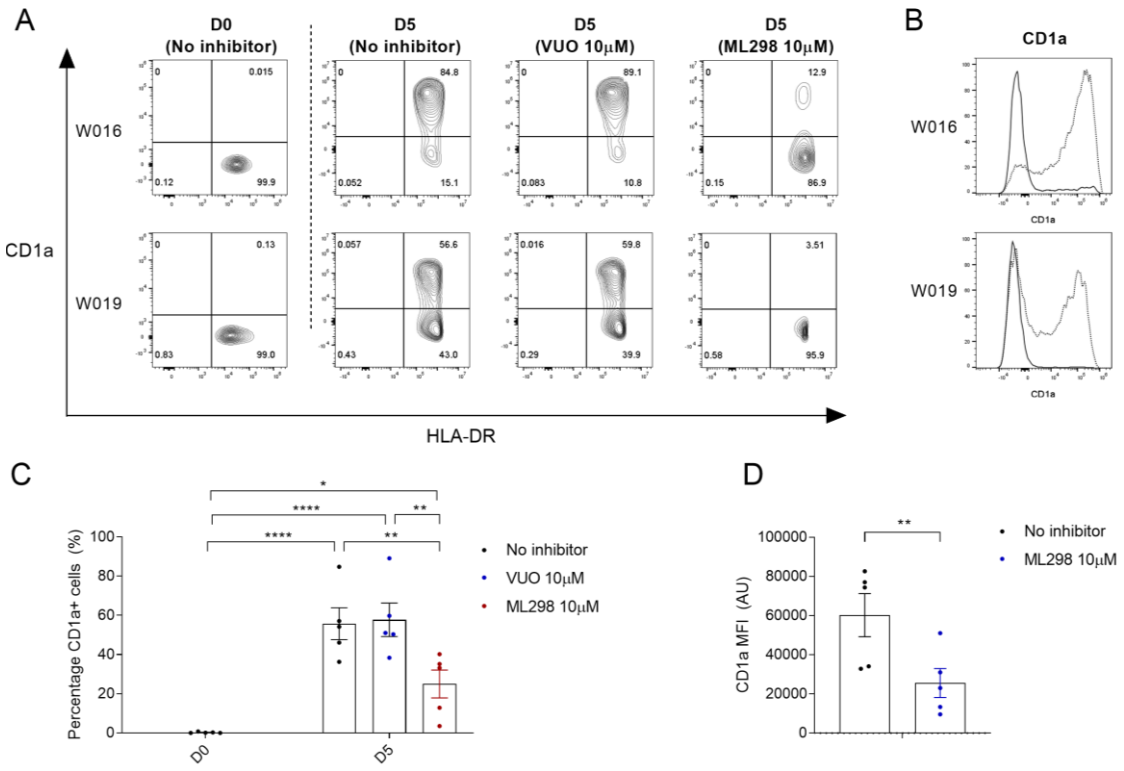


Figure 35. PLD2 inhibition markedly reduces CD1a protein expression during differentiation of MoDC. HC MoDC (n=5) were cultured over 5 days with GM-CSF and IL-4, as previously described. On D0 and D3, no inhibitor, PLD1 inhibitor (VU0155069; 50nM) or PLD2 inhibitor (ML298; 10μM) was added to cells. Phenotypic analysis was performed on D0 (prior to addition of any inhibitor) and D5, using flow cytometry. Gating to FMO/isotype controls was different between days, as cells become larger and undergo morphological changes as they transition towards a DC phenotype (A) CD1a and HLA-DR surface expression in donors W016 and W019, throughout differentiation and across treatment conditions. B) Histograms showing the frequency of CD1a-expressing cells from donors W016 and W019 at day 5, comparing untreated (dotted line) and PLD2-inhibited cells (solid line). C) Mean frequency of CD1a+ cells across donors during differentiation. D) CD1a MFI in CD1a+ MoDC on D5, comparing untreated and PLD2-inhibited cells (*p<0.05; **p<0.01; ****p<0.0001).

To investigate whether PLD1 and PLD2 regulate mRNA expression in HC MoDCs (n=3), qPCR was performed on cells on day 0 (pre-treatment) and day 5, using similar protocols as previously outlined. In control cells, *CD1A* transcription was absent at day 0 of differentiation, and demonstrated marked upregulation at day 5 (*Figure 36A, top left panel*; p<0.0001). In fully differentiated MoDCs, *CD1A* expression was upregulated by inhibition of PLD1 (1.4-fold upregulation; p<0.001) and downregulated by inhibition of PLD2 (23-fold downregulation; p<0.0001). This was concordant with results previously observed in MUTZ3-LCs, further reinforcing the concept that PLD2 activity is required for CD1a expression in DCs. Interestingly, inhibition of either PLD isoform was accompanied by an

upregulation in *PLD2* transcription (PLD1-inhibition = 1.75-fold increase; $p<0.05$; PLD2-inhibition = 1.6-fold increase; $p<0.05$), whereas *PLD1* and *PLA2G4A* transcription were unaffected (*Figure 36A, top right and bottom panels*). This suggests a novel feedback mechanism whereby PLD2 transcription is upregulated in response to loss of PLD activity. Amongst cytokines measured, *TNF*, *IL1B* and *TGFB1* were highly expressed at day 0 (*Figure 36B, top panels and bottom left panel*). *IL1B* and *TGFB1* showed trends suggesting upregulation in differentiated control MoDC, whereas *TNF* showed a trend suggesting downregulation. Neither PLD inhibitor modulated expression of these cytokines. Finally, *IL12A* and *IL6* were poorly expressed, demonstrated by the wide variability between donors (*Figure 36B, bottom middle and right panels*).

Taken together, the data show that PLD2, but not PLD1, activity during differentiation of both MUTZ3-LCs and MoDCs is required for mRNA and protein expression of CD1a. In MoDCs, a protective mechanism was observed, in which further PLD2 is recruited to counteract reductions in activity. Whilst PLD2 did not modulate mRNA expression of pro- and anti-inflammatory cytokines in primary cells, we hypothesised that PLD2 may regulate other processes in DCs, including lipid antigen uptake and presentation via CD1a.

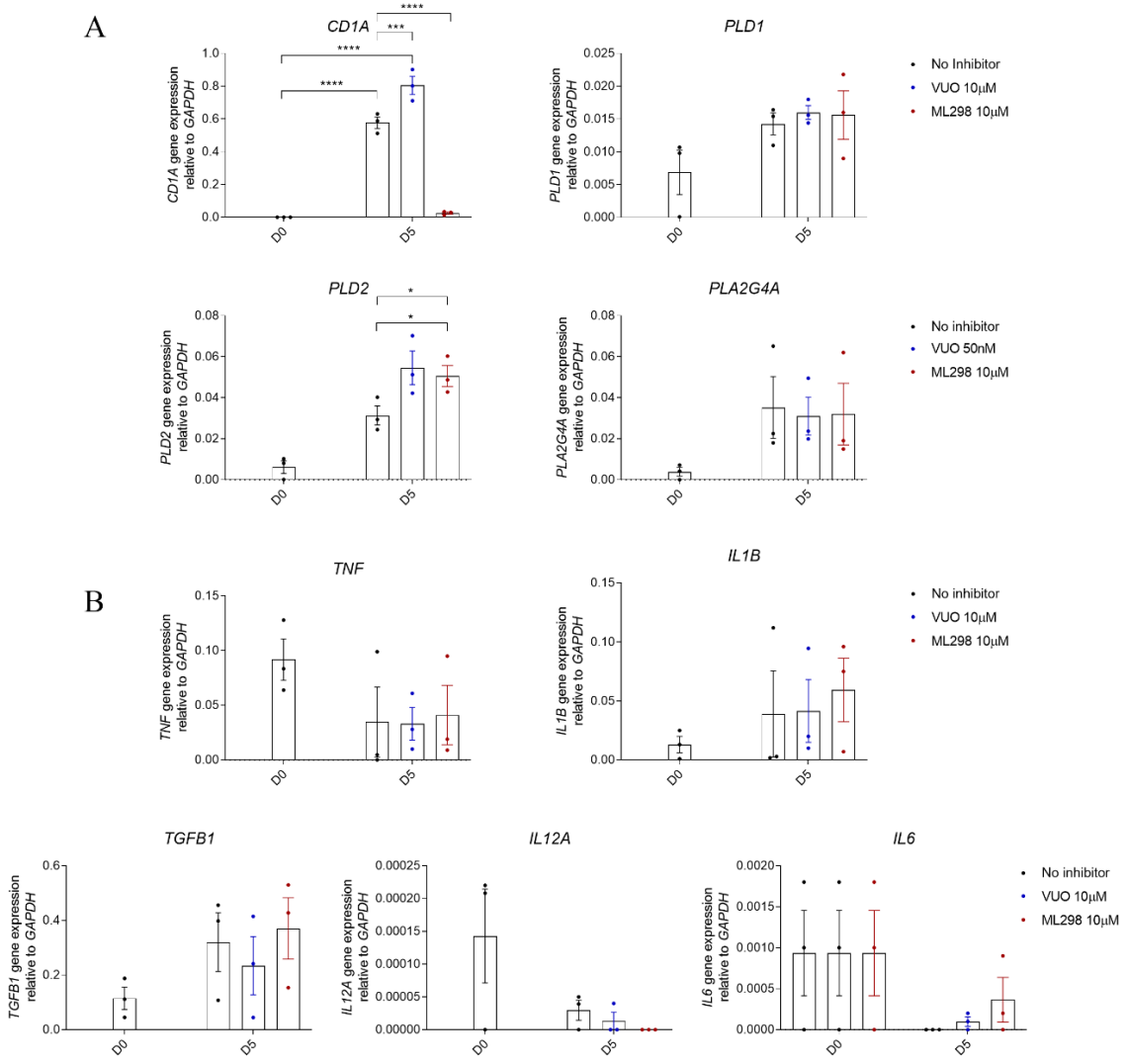


Figure 36. PLD2 inhibition markedly reduces CD1A and pro-inflammatory cytokine mRNA expression in MoDCs. HC MoDC (n=3) were cultured over 5 days with GM-CSF and IL-4, as previously described. On D0 and D3, no inhibitor, PLD1 inhibitor (VU0155069; 50nM) or PLD2 inhibitor (ML298; 10µM) was added to cells. Cells were isolated and quantitative polymerase chain reaction (qPCR) was performed on day 0 and day 5 of differentiation, normalised to expression of *GAPDH*. A) mRNA expression of *CD1A*, *PLD1*, *PLD2* and *PLA2G4A* . B) mRNA expression of *TNF*, *IL1B*, *TGFB*, *IL12A* and *IL6*. (*p<0.05; ***p<0.001; ****p<0.0001).

1.34.3. PLD2 inhibits PPARγ activity, allowing production of MoDCs which can induce CD1a-reactive T cell responses

PLD2 produces the PPARγ-antagonist cPA, and PPARγ activity downregulates expression of group I CD1 molecules on DCs (533, 570). Experiments were planned to determine whether PLD2 inhibition increases PPARγ activity during MoDC differentiation, contributing towards downregulations in CD1a and CD1c. Briefly, HC MoDCs (n=3) were

differentiated in the presence of PLD2 inhibitor (ML298; 10μM) or no inhibitor. At 24 hours, nuclear protein extracts were prepared and assessed, using ELISA, for binding of PPARγ to a specific dsDNA sequence containing the peroxisome proliferator response element (PPRE; see *Chapter 5.5*). In this assay, PPARγ activity is proportional to light absorbance at 450nM. Compared with control cells (OD = 0.06), the PPARγ content was increased in the positive control (OD=0.39; $p<0.01$), confirming functionality of the test (*Figure 37A*). Strikingly, PLD2-inhibition also led to increased PPARγ activity, compared with control (OD = 0.21; $p<0.05$). This supported our hypothesis that PPARγ activation acts as the intermediary between PLD2 inhibition and group CD1 downregulation in MoDCs.

PPARγ activity has been demonstrated to promote phagocytic programs in macrophages and endocytosis in DCs (533, 574). Despite the observed inverse relationship between PLD2 and PPARγ activity, PLD activity has also been shown to promote phagocytosis (520). Therefore, it was investigated whether inhibition of PLD2 promotes phagocytosis in MoDCs, via PPARγ activation, or inhibits phagocytosis, via loss of inherent PLD-dependent signal transduction. As performed previously, HC MoDCs (n=3) were generated in the presence or absence of PLD2 inhibitor. Fully differentiated MoDCs were then compared with respect to their ability to uptake pre-labelled zymosan, a particle frequently used in phagocytosis assays (*Figure 37B*). Therefore, light absorbance at 450nM was proportional to zymosan uptake. The phagocytosis inhibitor cytochalasin D (CyD) was used to confirm uptake was by phagocytosis. In both control and PLD2-inhibited cells, CyD abrogated phagocytosis (Control cells [no inhibitor OD = 0.68; CyD OD = 0.102; $p<0.0001$]; PLD2-inhibited cells [no inhibitor OD = 0.368; CyD OD = 0.104; $p<0.05$]). Moreover, PLD2-inhibited cells possessed reduced phagocytic function compared with control cells ($p<0.05$). These results confirmed that, despite upregulations in PPARγ activity, PLD2 inhibition during differentiation impairs phagocytosis of antigen by MoDCs.

Having characterised phenotypic and functional modulation of MoDCs by PLD2 inhibition during differentiation, we sought to explore the resulting effect on antigen-presentation capacity. Monocytes were obtained from two HC donors, C2345 and C2346, and differentiated into MoDCs in the presence or absence of PLD2 inhibitor. Control and PLD2-inhibited MoDCs were subsequently compared in terms of their ability to present the CD1a ligand LPC to autologous T cells, using the IFN-γ ELISpot platform (*Figure 37C*). In C2345, preincubation of control MoDCs with α-HLA and subsequent pulsing with LPC induced IFN-γ production by T cells (*Figure 37C, left panel*; unpulsed = 118 spots; LPC = 188 spots). IFN-γ induction by LPC appeared to be blocked upon addition of α-CD1a antibody (92.5 spots), but not isotype antibody (171.5 spots). Similar results were observed in control MoDCs from C2346, in which α-HLA blocking and treatment with LPC induced IFN-γ secretion (*Figure 37C, right panel*; unpulsed = 87.5 spots; LPC = 128.5 spots). Again, this was inhibited by α-CD1a antibody pre-treatment (spots = 67), but not by isotype (spots = 165.5). In both donors, MoDCs which had been differentiated in the presence of PLD2 inhibitor induced markedly less IFN-γ release, and this was not modulated by antibody pre-treatment or pulsing with LPC (C2345 = 43.5-64.5 spots; C2346 = 19-37 spots). The data therefore suggest that PLD2 inhibition during differentiation of MoDCs stunts their ability to induce activation of CD1a-reactive T cells, and we hypothesise this is due to downregulation of CD1a. However, further donors are required to calculate means and draw accurate conclusions.

Next, we aimed to understand the role of PLD2 in regulating CD1a antigen presentation in normal MoDCs. Fully differentiated control MoDCs (C2345 and C2346) were pulsed with combinations of LPC and PLD2 inhibitor in the ELISpot model, to test the hypothesis that PLD2 inhibition would compromise LPC uptake and presentation to CD1a-reactive T cells. Moreover, it was theorised that PLD2 inhibition would downregulate CD1a at the time of

pulsing, further ameliorating IFN-γ responses. In both donors, pre-treatment of MoDCs with α-HLA and subsequent treatment with LPC induced IFN-γ production by polyclonal T cells (*Figure 37D***;** C2345 [unpulsed = 98 spots; LPC = 137 spots]; C2346 [unpulsed = 105.5 spots; LPC = 168 spots]). Surprisingly, cotreatment with PLD2 inhibitor and LPC did not inhibit this response (C2345 = 122 spots; C2346 = 179 spots). Equally, treatment with PLD2 inhibitor alone induced responses which were similar to unpulsed cells (C2345 = 86.5 spots; C2346 = 106.5 spots). α-CD1a-blocking abrogated IFN-γ production under all pulsing conditions, to similar levels (C2345 = 49-72.5 spots; C2346 = 46-65.5 spots), whereas isotype did not inhibit cytokine release. Overall, these data suggested CD1a-reactivity to LPC, however this was not modulated by PLD2 inhibitor. Further investigations in a larger number of donors are required to confirm this.

Finally, experiments were performed to determine whether PLD2 modulates LPC antigen presentation by K562 cells. K562 cells were pulsed with LPC +/- PLD2 inhibitor, cocultured with polyclonal T cells (n=3) and IFN-γ secretion was measured by ELISpot. Treatment of K562 cells with LPC or PLD2 inhibitor did not modulate cytokine responses (*Figure 37E*; unpulsed = 27 spots; LPC = 35.3 spots; LPC + PLD2 inhibitor = 26.3 spots; PLD2 inhibitor alone = 25.7 spots). In K562-CD1a cells, compared with the unpulsed condition (mean = 93 spots), treatment with LPC led to an induction of IFN-γ release (mean = 161.3 spots; $p<0.05$). Cotreatment with LPC and PLD2 inhibitor did not modulate cytokine secretion (mean spots = 182), and PLD2 inhibitor alone induced similar responses to the unpulsed condition (mean spots = 70). Similar to results observed in MoDCs, we concluded that PLD2 inhibition does not alter presentation of LPC to CD1a-reactive T cells by K562.

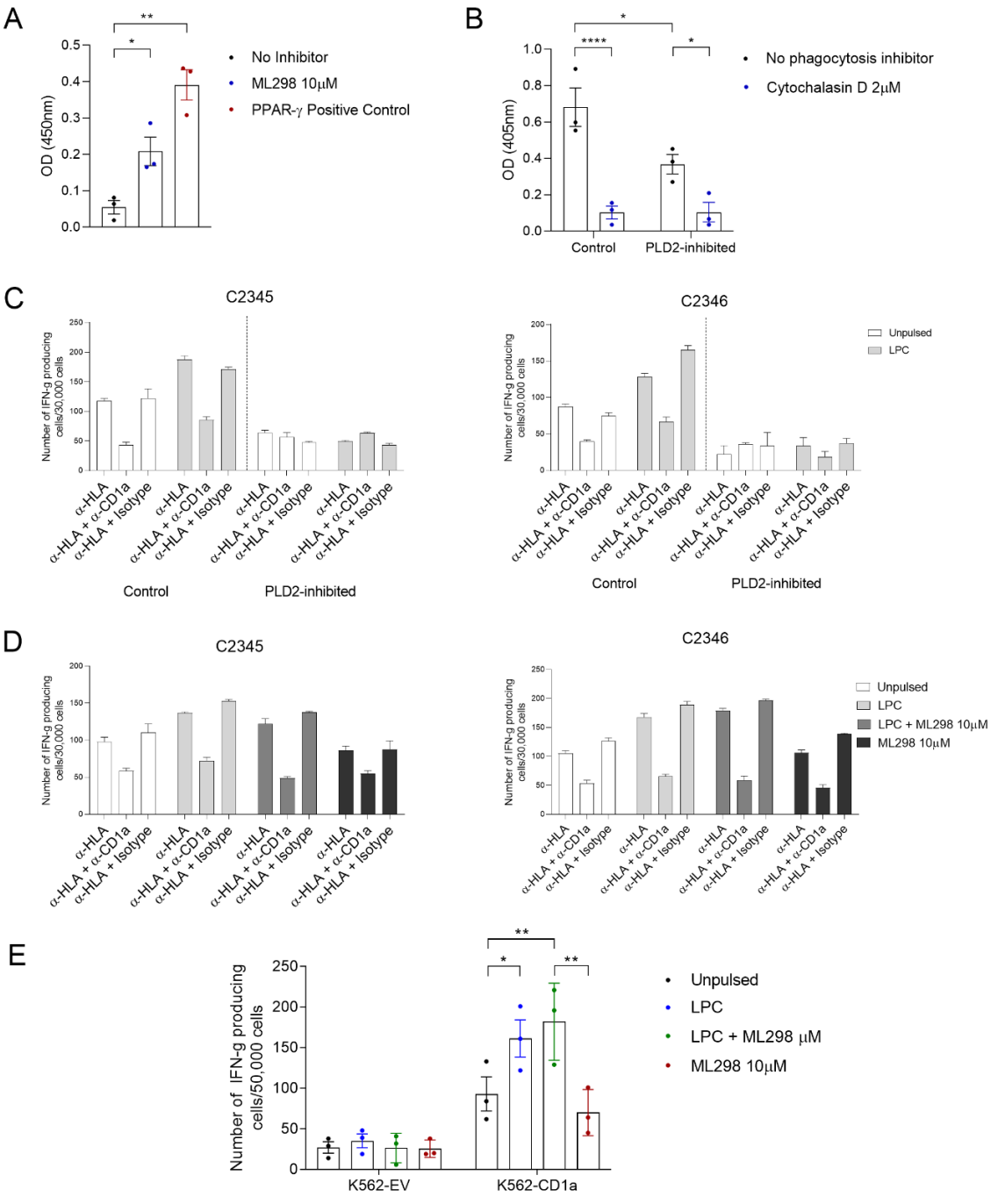


Figure 37. PLD2 inhibits PPARγ, allowing production of monocyte-derived dendritic cells (MoDCs) which can uptake antigens and induce CD1a-reactive T cell responses. Healthy control MoDCs were differentiated in the presence of PLD2 inhibitor (ML298; 10μM) or no inhibitor A) At 24 hours, nuclear protein extracts were prepared and assessed for PPARγ activity by measuring binding to the peroxisome proliferator response element (PPRE), using ELISA. PPARγ activity was proportional to light absorbance (OD) at 450nm. B) On D5, MoDC were treated with pre-labelled zymosan +/- phagocytosis inhibitor (2μM cytochalasin). Zymosan uptake via phagocytosis was proportional to light absorbance (OD) at 405nM. C) Control and PLD2-inhibited MoDCs were pre-incubated with blocking antibodies, treated with LPC and cocultured with autologous T cells. IFN-γ ELISpot data shown for 2 donors: C2345 (left) and C2346 (right) D) Control MoDCs were pre-incubated with blocking antibodies, treated with LPC and cocultured with autologous T cells. IFN-γ ELISpot data shown for 2 donors: C2345 (left) and C2346 (right) E) K562 cells were treated with LPC and cocultured with HC T cells (n=3). IFN-γ ELISpot data shown (*p<0.05; **p<0.01; ****p<0.0001).

In summary, our data allowed us to draw key insights. Firstly, inhibition of PLD2 activity during differentiation of MoDCs promotes PPARγ activity, and we suggest this underpins the observed downregulations in CD1a expression. Secondly, PLD2 activity promotes differentiation of MoDCs which can effectively uptake antigen, and loss of this activity leads to ameliorated presentation of LPC via CD1a. Finally, PLD2 treatment of K562 cells and fully differentiated MoDCs does not modulate their ability to present LPC to CD1a-reactive T cells.

1.35. Discussion

1.35.1. PLD drives expression of CD1a in LCs

Under normal homeostasis, skin DCs can be divided into three major subsets: epidermal LCs, dermal type 1 conventional DCs (cDC1s) and dermal type 2 conventional DCs (cDC2s) (580). Additional subtypes arise in inflamed skin, including pDCs and MoDCs (581). CD1 regulation is therefore a complex process across multiple cell types (further discussed in *Chapter 9.3*), and we initially elected to focus on LCs. Given their strategic location within the epidermis, allowing sampling of antigens that penetrate the SC, LCs offer a first line of defence against pathogens. LCs can be distinguished by their high expression of CD1a and langerin (CD207) (582). This raises important questions regarding their ability to capture lipid antigens and initiate CD1a-reactive T cell responses. Whilst the precise role of LCs in allergic sensitisation is under debate, human atopic skin possesses elevated numbers of activated LCs, which express CD80 and CD86 (583, 584). This was corroborated in mice with vitamin D3-induced AD, in which LC expressed elevated maturation markers CD40, CD86 and CCR7 (585). Furthermore, in OVA-sensitised mice, LCs uptake epicutaneous antigen and induce $CD62L^{+}$ Tregs, suggesting an additional function in skin-induced tolerance (586). Importantly, CD1a and langerin expression is upregulated in LC from eczema lesions, suggesting these proteins may synergise in

processing of lipid antigens (587). Therefore, LCs present a vehicle for induction of CD1a-reactive responses, which may benefit or hinder epicutaneous sensitisation to peanut antigens. However, there is a paucity of data describing mechanisms which underpin their CD1a expression and give rise to such responses.

To investigate transcriptional and protein regulation of CD1a in LCs, the MUTZ3 cell line was differentiated into an LC phenotype over 10 days in the presence of TNF-α, GM-CSF and TGF-β (588). This offered a renewable source of LCs, which expressed CD1a, Langerin and BGs. Equally, this eliminated the issues associated with inter-donor variability and acquiring sufficient primary cells from patient skin (589). Initially, imaging was performed to confirm their phenotype was conducive for further experiments. Light microscopy confirmed their growth from small, dispersed CD34+ MUTZ3 precursors into larger, aggregated cells which possess elongated dendritic processes. Notably, LC-specific BGs were visible on TEM, however their 'tennis racket' shape could not be appreciated due to oblique sectioning, revealing only the 'handle' (590). Nonetheless, their presence suggested MUTZ3-LC were a suitable surrogate for primary LCs in subsequent experiments.

Flow cytometry data showed that dual PLD1/PLD2 inhibition led to a >50% reduction surface CD1a protein expression on MUTZ3-LCs, whilst maintaining normal levels of the DC/LC marker CD11c. This suggests that PLD activity is required for normal CD1a expression in MUTZ3-LCs. Moreover, PLD inhibition led to a 46% reduction in CD1c expression. This would suggest that PLD exerts an upstream action in regulating CD1 molecules. To our knowledge, this is the first example of cellular phospholipases modulating phenotype or function of LCs. Endogenous PLD isoforms are known to mediate many cellular processes, including gene regulation, through both direct protein-protein

interactions and the production of PA (184). For example, PA activates mTOR, a major regulator of cell growth and proliferation (Foster and Xu, 2003). Equally, PLD-induced PA has been shown to activate the Ras-ERK/PI3K/NFκβ pathway, which controls DNA transcription, cytokine production and cell survival (282, 591, 592). Therefore, it is possible that PLD activity is required for normal LC maturation, of which CD1a expression is a component. This is reflected in keratinocytes, in which sustained activation of PLD is associated with normal differentiation; moreover, PLD may mediate late differentiation through production of diacylglycerol and activation of specific PKC isoforms (593). Finally, glycosylphosphatidylinositol-specific phospholipase D1 (GPLD1) binds MHCI and data suggest it enters the MHCI processing pathway, which incorporates early endosomes (594, 595). Given the similar structure of MHCI and CD1 molecules, PLD may play a similar role in loading lipid cargo onto CD1a, promoting normal trafficking and expression at the cell surface (287).

Next, investigations were performed to determine the role of individual PLD isoforms in regulating *CD1A* transcription. qPCR data showed that PLD2 inhibition downregulated *CD1A* by >40%, with dual PLD1/PLD2 inhibition leading to a greater reduction of 59.9%. Unexpectedly, PLD1 inhibition alone had no such effect, suggesting PLD2 plays a more significant role in *CD1A* transcription in MUTZ3-LCs. The PI3K/AkT/mTOR pathway is essential in regulating DC development, and mTORC1 activity is critical for the maintenance of LC numbers in murine skin (596). PLD-generated PA is a key activator of mTOR, and potentially this function is commanded by PLD2 (597). We demonstrated that *PLD1* and *PLD2* transcription upregulates during the differentiation of MUTZ3-LCs, in line with a previous observations by Kang et al (276). Notably, *PLD1* transcription was reduced in response to PLD1 or PLD2 inhibition. Endogenous PLD1/PLD2 are known to promote PLD1 but not PLD2 expression via production of PA, which activates the Ras-ERK/PI3K-

NFκβ pathway; NFκβ subsequently binds the *PLD1* promoter region (578). Therefore, inhibition of either isoform will stunt PLD1 expression. Equally, PLD1 inhibition enhanced PLD2 transcription. Potentially, upon inhibition of PLD1, PLD2 'takes over' the production of PA to mediate cellular processes. This may explain why PLD1 inhibition alone led to no effect on *CD1A* transcription, but dual PLD1/PLD2 inhibition exacerbated downregulation. Whilst *PLA2G4A* was poorly expressed in this cell line, *PLA2G4C* was upregulated in differentiated MUTZ3-LC, and this effect was inhibited by PLD2 and dual PLD1/PLD2 inhibition. Recently, Cheung et al identified that IFN-α-induced mast cells transfer cPLA$_2$ group IVD (PLA$_2$G4D) inside exosomes to neighbouring LCs, leading to generation of CD1a neolipids (279). Our data suggest LCs contain inherent cPLA$_2$ group IVC (PLA$_2$GIVC) activity, allowing innate production of LPA which can be presented to CD1a-reactive T cells. Moreover, this is potentiated by PLD2 activity, representing a novel interplay between phospholipase isoforms in promoting CD1a antigen generation. Finally, inhibition of PLD2, but not PLD1, reduced expression of *IL-1β* and *TNF-α* in differentiated MUTZ3-LCs. This supports the notion that PLD regulates upstream DC maturation and function. Cotreatment of monocytes with IL-1β and TNF-α induces CD1a expression on MoDCs, reinforcing a specific role for PLD2 in driving production of these cytokines in LCs and promoting CD1a expression (528). We noted that *TGFB* expression remained high throughout differentiation, regardless of PLD inhibitor treatment. TGF-β is a critical factor in development of CD207$^+$LCs, given that TGF-β-deficient mice lack epidermal LCs (598). Kaplan et al demonstrated this process involves autocrine production of cytokine (599). Equally, LC development is abrogated in mice which lack ld2, a signaling molecule downstream of TGF-βR (600). Furthermore, TGF-β supplementation specifically induces langerin expression in human CD1c$^+$ DCs and murine MoDCs (601, 602). Consistently high *TGFB* transcription in our MUTZ3-LC may have driven the high *CD207* (langerin) transcription observed, overriding the anti-maturation effects of PLD inhibition.

Our data suggested PLD activity is required for CD1a and CD1c expression in LCs. This has provoked further questions regarding additional effects impacting LC ultrastructure and lipid antigen loading. The function of BGs remains poorly understood, however an early study demonstrated that they are langerin^{+} subdomains of the endosomal recycling compartment (603). Langerin is expressed predominantly on LCs, but also on dermal interstitial CD103^{+} DCs and lymph node resident CD8^{+} DCs (604, 605). As a C-type lectin, langerin binds carbohydrate moieties present in viral glycoproteins and fungal cell walls, with examples including HIV and *Candida* (606, 607). This suggests langerin facilitates pathogen, and antigen, uptake via receptor-mediated endocytosis, analogous to the action of DC-SIGN (608). In immature LCs, langerin traffics from the plasma membrane to EEA1^{+} early/sorting endosomes, and BGs form as a consequence of langerin accumulation (603). The langerin carbohydrate recognition domain (CRD) is crucial for this interaction, and contributes towards membrane zipping during BG formation, giving rise to their unique shape (609). Consequently, BGs reduce dramatically in number and size upon LC maturation, due to a corresponding reduction in langerin recycling. CD1a possesses remarkable overlap with langerin, given its early endocytic recycling pathway and binding affinity for glycosylated ligands (307). Equally, treatment of CD1a^{High} DCs and LCs with anti-langerin antibodies abrogated their capacity to present *M. leprae* to CD1a-restricted T cells (610). Moreover, CD1a itself is a glycoprotein and may interact directly with the langerin CRD; this is supported by unpublished co-immunoprecipitation data from our lab. It is therefore possible that langerin plays a role in the capture of extracellular ligands, which are transferred onto CD1a during intracellular recycling. To investigate this hypothesis, we suggest performing confocal microscopy to determine if CD1a and langerin colocalise in EEA1^{+} early endosomes. If this is ascertained, we suggest CD1a may also play a role in BG formation. Subsequent TEM studies in PLD2-inhibited MUTZ3-LCs, which express low CD1a, would identify potential corresponding reductions in BGs.

Furthermore, we suggest transfecting MUTZ3 cells with gene constructs for tail-deleted CD1a, which lacks an early endosome-homing tyrosine motif, to determine if this interferes with BG formation in differentiated MUTZ3-LCs (313).

It is important to recognise there are limitations of drawing inferences on DC CD1 regulation using data acquired using a cell line. In human skin equivalents, MUTZ3-LCs migrate from the epidermis to dermis in response to allergen exposure, and therefore possess some characteristics similar to primary LCs (588). Consequently, MUTZ3 have been successfully used in experiments for investigation of skin sensitisation and development of cancer vaccines (611, 612). However, MUTZ3-derived DCs (MUTZ3-DCs) are transcriptionally different to primary dermal DCs and LCs, more closely resembling tonsillar mDCs (613). Equally, MUTZ3-DCs are limited in their ability to respond to influenza vaccine components, compared with MoDCs (614). Whilst no comparative data exist for MUTZ3-LCs, we needed to determine whether the observed role of PLD2 in regulating CD1a expression was applicable to primary cells, or if this was a MUTZ3-specific phenomenon.

1.35.2. PLD2 activity is required for CD1a expression in DCs

MoDCs serve as a useful inducible model, which reduces the requirement for isolating primary DCs from skin (579). Therefore, we investigated the effects of PLD1 and PLD2 in regulating CD1a mRNA and protein expression in MoDCs. Inhibition of PLD2, but not PLD1, activity during MoDC differentiation led to marked downregulation of CD1a protein expression, as determined by flow cytometry. This was accompanied by the expected $CD11c^{+}CD14^{-}$HLA-DR^{+} DC phenotype. Therefore, normal PLD2 function is required for CD1a expression on MoDCs, in contrast to PLD1 which demonstrated no effect when inhibited. This difference may be explained by differences in subcellular localisation

between isoforms. PLD1 is found largely in perinuclear structures (180), whilst PLD2 localises mainly to the plasma membrane (615). Both isoforms possess 'PH' domains, which drive entry of PLD1 into lipid rafts during internalisation and are required for localisation of PLD2 to the plasma membrane. Interestingly, deletion of the 'PH' domain in PLD2 led to exclusive accumulation in early endosomes, the major compartment for CD1a trafficking (314). This suggests that PLD2 exclusively recycles via this compartment, bringing it into regular close contact with CD1a. Conversely, PLD1 non-selectively traffics via early endosomes, late endosomes and lysosomes, suggesting less contact with CD1a. This introduces the concept that, in addition to neoantigen generation and CD1a regulation, PLD2 may play a role in protein-protein interactions with CD1a. Saposins have been shown to facilitate the loading of lipids onto CD1d for the subsequent presentation to iNKT cells through activation of hydrolases and lipid editing (616). However, saposins possess no enzymatic action and therefore the mechanism of hydrolase activation remains unclear. In contrast, PLD is a hydrolase by definition and we hypothesise that PLD2 may act via directly modifying lipids to facilitate loading onto CD1a in early endosomes. Our group has an ongoing interest in this area, with additional work investigating the role of bacterial acyloxyacyl hydrolase (AOAH) in CD1a-lipid loading (data unpublished).

Assessed using qPCR, PLD2 inhibited *CD1A* transcription in MoDCs in a similar manner to MUTZ3-LCs. This strengthens our argument that PLD2 specifically drives transcription of *CD1A*. Notably, PLD1 inhibition induced both *PLD2* and *CD1A* expression. These data suggest that PLD1 inhibition leads to increased *PLD2* transcription, which further promotes transcription of *CD1A*. We propose that the interplay between *PLD1*/*PLD2* and *PLD2*/*CD1A* occurs through similar mechanisms as previously discussed. Notably, *PLA2G4A* expression was upregulated in differentiated MoDCs but was not modulated by PLD inhibition. PLA_2G4A is classically associated with mast cells, catalysing production of LPA from membrane phospholipids, and is upregulated in atopic disease (335, 617, 618).

To our knowledge, we present the first longitudinal data describing its expression during differentiation of MoDCs. Recently, Hardman et al showed that ILC2s express PLA_2G4A and can be induced to express CD1a with TSLP (299). Moreover, treatment of ILC2s with recombinant PLA_2G4A induced CD1a-reactive T cell responses. Similar investigations should be performed to determine the role of MoDC-derived PLA_2G4A in generating CD1a antigens. *TNF*, *IL1B*, *IL12A*, *IL6* and *TGFB* mRNA expression data were equivocal, with no significant changes noted during differentiation. This was likely because they were resting cells, and repeat experiments should be performed in which cytokine responses are compared after treatment with maturation factors such as LPS, which activates cells via TLR-4 (619). This would allow clearer assessment of the role of PLD2 in promoting differentiation of functionally competent MoDCs.

1.35.3. PLD2 inhibits PPARγ and modulates DC function

Following the discovery that PLD2 activity drives CD1a protein and mRNA expression in MoDCs, assays were performed to interrogate the mechanisms of transcriptional regulation. Our data suggest that PLD2 activity possesses an inverse relationship with PPARγ expression during early differentiation of MoDCs. PPARγ expression is associated with downregulations in group I CD1 molecules, as outlined in *Chapter 8.1.1* (533). This implies that PLD2 promotes CD1a expression via inhibition of PPARγ, and we present the first data which demonstrate this signaling axis (outlined in *Figure* 38). PLD2 is known to inhibit PPARγ through production of the specific antagonist cPA, and further experiments should be implemented to investigate this interaction (570). Initially, this would involve characterisation of PPARγ target gene expression in control and PLD2-inhibited MoDCs, including *FABP4*, liver X receptor alpha (*LXRα*) and Angiopoietin-like 4 (*ANGPTL4*) (620-622). This would confirm increased functional activity of PPARγ in response to PLD2 inhibition. Western blot of nuclear protein extracts would allow further confirmation of the presence of upregulated phosphorylated (active) PPARγ in PLD2-inhibited cells.

Subsequently, TLE could be performed on nuclear protein extracts and assessed for cPA content using targeted liquid chromatography techniques (623). Confirmation of reduced nuclear cPA content and elevated PPARγ activity in PLD2-inhibited MoDCs would support our proposed mechanism. PLD2 inhibition, and resulting PPARγ activation, represents a viable therapeutic strategy in inflammatory diseases including allergy, and is further discussed in *Chapter 9.4*.

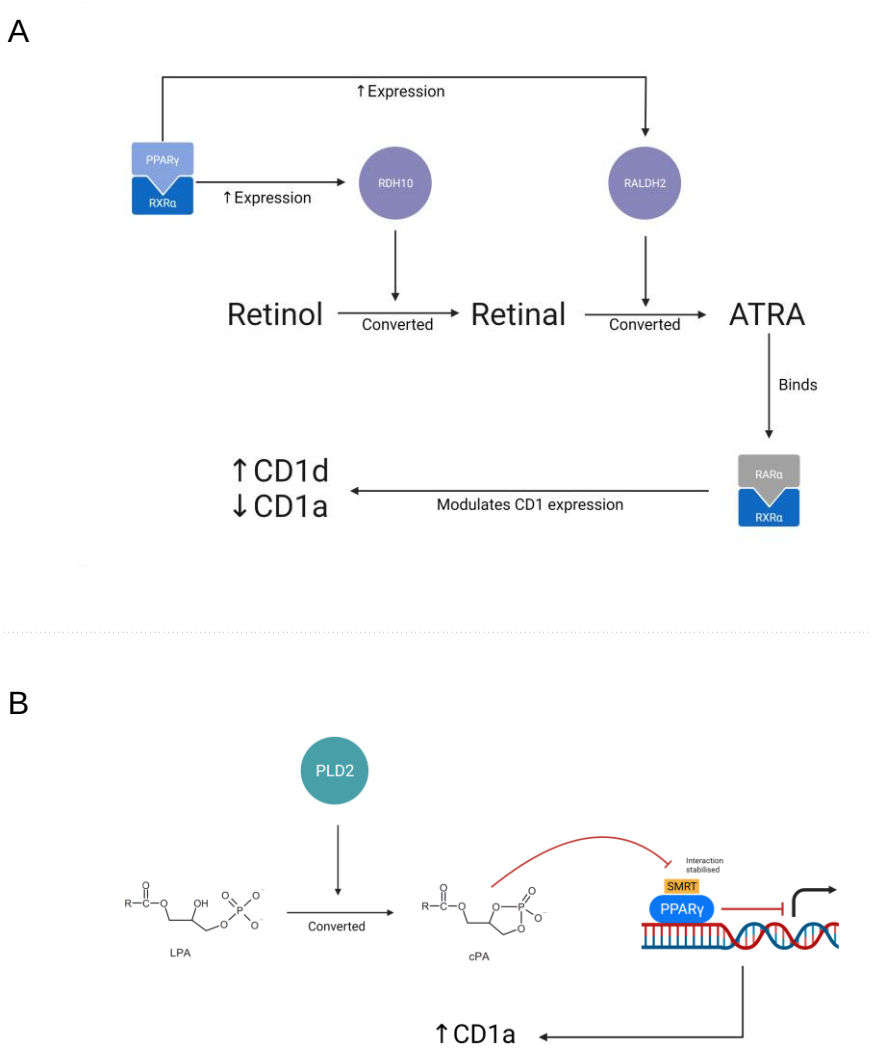


Figure 38. Phospholipase D2 (PLD2) promotes CD1a expression through inhibition of PPARγ. A) Ordinarily, PPARγ inhibits expression of CD1a in dendritic cells (DCs). PPARγ forms a heterodimer with Retinoid X receptor alpha (RXRα), inducing expression of retinol dehydrogenase 10 (RDH10) and retinaldehyde dehydrogenase 2 (RALDH2). These enzymes sequentially process retinol to all-trans retinoic acid (ATRA), which is an RARα agonist, leading to subsequent downregulation in CD1a expression. *Modified from (624).* B) PLD2 converts lysophosphatidic acid (LPA) to cyclic phosphatidic acid (cPA), which is a potent inhibitor of PPARγ, acting through stabilising its interaction with the corepressor SMRT. *Modified from (570).* This suggests PLD2 inhibits PPARγ, promoting CD1a expression. *Made using Biorender.*

Next, assays were performed to measure the antigen uptake capacity of MoDCs which had been differentiated in the presence of PLD2 inhibitor. Notably, PLD2 activity was required during differentiation for efficient phagocytosis, in line with previous observations (520). Although PPARγ promotes phagocytic mechanisms, and PLD2 inhibition increases PPARγ, we concluded that PLD2 activity plays a more dominant role, thereby explaining the reduction in antigen uptake (533, 574). PLD2 localises at the plasma membrane of macrophages and is required for phagosome formation and early internalisation, mediated by PA (625). In RAW264.7 monocytes, PLD2 is activated by the small GTPase Arf6 in order to promote phagocytosis (626). Furthermore, phagocytosis of *M. tuberculosis* is associated with increased PLD activity in monocyte derived macrophages (MDM) (627). Remarkably, treatment of MDM with exogenous PLD derived from *S. chromofuscus* promoted phagocytosis. Reflecting upon the first results chapter (*Chapter 6*), potentially peanut PLD exerts a similar effect in DCs and promotes peanut antigen uptake. In addition to phagocytosis, PLD2 is required internalisation of several signaling receptors through endocytosis, including EGF receptor (EGFR), angiotensin II receptor (AT1R) and multiple G-coupled protein receptors (GPCRs) (615, 628, 629). Studies suggest these effects are due to PLD-induced activation of small GTPases which potentiate clathrin-mediated endocytosis, and through modulation of membrane curvature by PA (630, 631). The small Rho GTPases Rac1, Cdc42 and RhoA promote antigen uptake by DCs, and we suggest measuring their activity in MoDCs which have been differentiated in the presence of PLD2 inhibitor, compared with controls (632). This will help determine if such hydrolases mediate PLD2-driven phagocytosis and endocytosis. Finally, flow cytometric assays should be performed in both MoDC cohorts, to determine if PLD2 inhibition reduces uptake of FITC-labelled LPC and peanut PLD. This would help determine if endogenous PLD2 potentiates uptake of LPC and peanut PLD to provoke CD1a-reactive T cell responses.

Finally, functional T cell assays were performed to measure the effect of PLD2 inhibition in modulating presentation of CD1a antigens. In two donors, trends suggested that PLD2 inhibition during differentiation of MoDC markedly stunted their ability to present LPC to CD1a-reactive T cells. We propose this was a direct result of CD1a downregulation on APCs and suggest expanding the cohort to provide firm conclusions. Fully differentiated MoDCs (from the same donors) were also with PLD2 inhibitor and assessed for their capacity to present LPC. Whilst the data suggest that endogenous PLD2 does not modulate CD1a antigen-presentation in already-mature DCs, further donors are required to fully elucidate. In K562 experiments, LPC induced CD1a-reactive T cell responses in healthy donors, however this was not modulated by concurrent PLD2 inhibition. It must be noted that K562-CD1a are transfectant cells, which are not subject to natural cellular regulation, and therefore PLD2 is not expected to drive CD1a expression (491). Future experiments should incorporate a wider range of allergenic substances that modulate CD1a-reactivity, including peanut PLD, HDM and candida (280).

1.35.4. Chapter summary

In summary, we have identified that endogenous PLD2 activity is required for expression of group I CD1 molecules in an LC line and primary human DC. In part, this appears to involve inhibition of the nuclear TF PPARγ. This new and novel mechanism appears to modulate DC function, through facilitation of antigen uptake and enabling of CD1a ligand presentation. In the next chapter (*Chapter 9*), these concepts will be further discussed, with a focus on differential CD1 transcriptional regulation between different DC subsets and the potential to develop PLD2-specific inhibitors in managing allergic disease.

DISCUSSION AND FUTURE PERSPECTIVES

The work of the thesis has highlighted that PLD plays a key role in the peanut-allergic response to CD1a-presented lipids. Peanut-derived PLD generates PA and PG from MoDC membrane phospholipids, which are recognised by CD1a-reactive T cells in peanut-allergic patients. In these patients, elevated PLD-, PA- and PG- responsive T cells were demonstrated, lending strong support to this concept. Importantly, this system is closely linked with barrier dysfunction, given that atopic skin contains reduced filaggrin content and elevated AQP3 expression. Both factors facilitate excessive production of PA and PG, respectively, leading to enhanced generation of lipid-reactive T cells. This provides a novel explanation for epicutaneous sensitisation to peanuts in atopic patients, directly implicating a role for CD1a. Equally, it is suggested that peanut-derived PA and PG may play a direct role in peanut lipid sensitisation. Moreover, peanut protein constituents provoke CD1a-reactive responses. It was observed that Ara h 1 induces CD1a-reactivity in peanut-allergic patients, and this may be a function of its ability to bind PG and modulate consequent responses. This provides the first evidence of a peanut major protein allergen acting through CD1a systems of biology, inciting the notion that peanut sensitisation is a product of plant protein and lipid interactions. Finally, it was demonstrated that human PLD2 activity is required for CD1a expression in LC and DC, and this appears to be related to inhibition of PPARγ, which ordinarily inhibits transcription of group I CD1 molecules. Endogenous PLD2 appears to accommodate CD1a antigen presentation, and potentially peanut PLD hijacks this system, manifesting as peanut allergen sensitisation and subsequent inflammatory responses. *Figure* 39 summarises these insights. Further investigations are demanded to unravel these insights, as detailed within each results chapter. Here, I recap on important future experiments, and present additional recommendations which are poignant in both peanut allergy and CD1a biology as a whole.

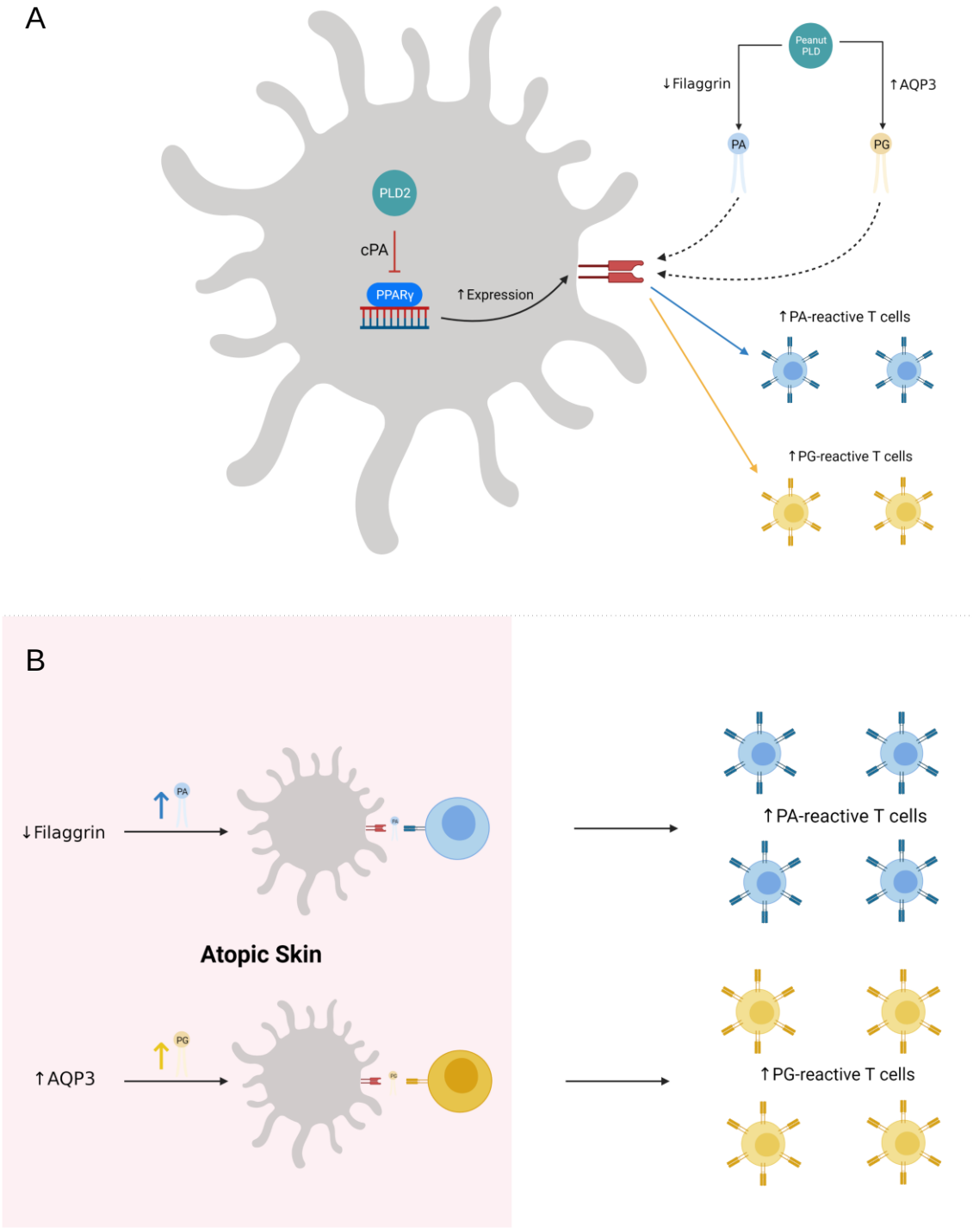


Figure 39. Phospholipase D (PLD) exerts dual action in driving CD1a expression and promoting generation of CD1a ligands in peanut allergy. A) Dual action of PLD. Left: endogenous PLD2 catalyses the production of cyclic phosphatidic acid (cPA), as previously outlined. cPA inhibits PPARγ activity in dendritic cells (DCs), promoting CD1a expression. Right: exogenous peanut PLD generates phosphatidic acid (PA) and phosphatidylglycerol (PG) in DCs, exacerbated by filaggrin insufficiency and aquaporin-3 (AQP3) overexpression in atopic skin. This leads to generation of PA- and PG-reactive T cells, contributing towards peanut sensitisation. B) Detailed schematic demonstrating phospholipid generation in atopic skin, and subsequent generation of lipid-reactive T cells. *Made using Biorender.*

1.36. Untangling the source of CD1a-reactivity in peanut allergy

In peanut-allergic patients, elevated CD1a-reactivity to PA and PG were observed, and suggest this was a potential result of two mechanisms. Firstly, exogenous peanut PLD generates these species in MoDCs; secondly, this is a direct result of peanut lipid exposure through a defective skin barrier. Whilst the biochemical and ELISpot experiments, which incorporated filaggrin treatment and AQP3 inhibition, supported the former mechanism, further experiments are required to elucidate. Definitive confirmation would require pulsing CD1a-transfected cells with peanut PLD, elution of CD1a-lipid complexes and identification of PA and PG species using mass spectroscopic techniques (85). Specific CD1a-PA or CD1a-PG complexes could then be tested for their capacity to activate T cells from healthy, AD and peanut-allergic donors. To further investigate the latter mechanism, peanut lipid purification techniques must be employed. No significant CD1a-reactive T cell responses were observed against peanut oil or TLE from raw peanuts. However, these preparations comprise heterogeneous mixtures of lipids, and roasted peanuts may need to be utilised to harness the CD1a antigen potential of lipid peroxides (485). Therefore, HPLC-MS fractionation of peanut oil, and whole roasted and unroasted peanuts, would allow determination of individual exogenous lipid species and their peroxide derivates, including PA, PG, SM, OLA, LA and PlA (75, 79). Lipid fractions could then be pulsed onto plate-bound CD1a, eliminating the requirement for APC, and subsequently exposed to polyclonal T cells from healthy, AD and peanut-allergic individuals (491). Through measurement of a range of cytokines including type 2, IFNγ and IL-22, this would shed light on whether peanut lipids give rise to a component of the PA- and PG-reactive responses observed in peanut-allergic patients. Generation of phospholipid-reactive T cell clones would allow for TCR sequencing and subsequent resolution of CD1a-TCR-lipid ternary structures using xray crystallography (84). Identification of primary peanut-derived CD1a ligands would be

of great importance in allergology and CD1 biology, in a similar vein to PC and PS derived from cypress pollen (317).

CD1a-dependent effects of peanut protein extracts, SPT or oleosin-containing OBs, were not observed and it was deemed this was related to the extraction techniques, with individual experimental plans outlined in *Chapter 7.3.4*. Purified protein allergens were therefore used in the assays, and surprisingly it was observed that Ara h 1 induces CD1a-reactive T cell responses, in addition to binding PG species. Ara h 1 binding to PG and peanut lipids endorses allergenicity through multiple mechanisms including unveiling IgE epitopes (98). It is hypothesised that Ara h 1-PG complexes are more readily processed by DCs through IgE-mediated cross-presentation, facilitating PG uptake and presentation to CD1a-reactive T cells in peanut-allergic patients (*Chapter 7.3.5*). To explore this hypothesis, Ara h 1 and Ara h 1-PG complexes could be compared with respect to their binding to Ara h 1-specific IgE, using ImmunoCAP technology (633). If differences are noted, Ara h 1 and Ara h 1-PG complexes could be coincubated with peanut allergic sera and pulsed onto MoDCs for subsequent ELISpots. Incorporation of anti-CD1a and specific anti-IgE antibodies would help dissect the functional role of IgE-mediated cross presentation in lipid-reactivity to peanut allergens. Equally, food lipids are known to act as adjuvants and license peptide-specific Th2 responses in allergy (469). It is suggested that peanut lipids, including PG, promote T cell recognition of Ara h 1, inducing a cascade of events which results in the production of Ara h 1-specific IgE. MoDCs should be treated with Ara h 1 in the presence/absence of peanut-derived lipids, including PG. Concurrent MHC- and CD1a-blocking antibodies would allow determination of whether CD1a-presented peanut lipids potentiate peptide-specific responses to Ara h 1. Moreover, these experiments can be extended to other major allergens, including Ara h 2, 3 and 6. Confirmation of these effects would identify a bidirectional relationship between Ara h 1

and lipids in endorsing allergenicity, and the mechanics of Ara h 1-PG interactions could be further explored, as detailed in *Chapter 7.3.5*.

1.37. Characterising CD1a-reactive T cells in peanut-allergic patients

CD1a-reactive T cells in peanut-allergic patients should be further characterised across several criteria. Firstly, the method of identifying CD1a-reactivity was restricted to measurement of IFNγ in circulating T cells. Previously, our group demonstrated that the skin of patients with AD is enriched with HDM-responsive CD1a-reactive T cells (isolated from skin suction blister fluid) (280). Similarly, in patients with psoriasis, skin-derived T cells are more responsive to PLA_2G4D compared with HCs, and this occurs via a CD1a mechanism of action (279). These studies incorporated only circulating lipid-reactive T cells, and so suggest future experiments investigating the role of PLD in modulating antigen presentation to skin-resident T cells. Functional assays could incorporate flow cytometric secretion assays to explore type 2 cytokine production.

Secondly, the data demonstrate a marked difference in polyclonal T cell responses between patient groups, suggesting the benefit of investigating monoclonal responses. 95% of human T cells express a TCR comprising an alpha (α) and beta (β) chain, encoded by *TRA* and *TRB*, respectively (634). Although gamma-delta (γδ) CD1a-reactive T cells have been described, structural studies which identify CD1a antigen specific TCRs are restricted to the αβ repertoire so far, for example in the context of LPC (84). Similarly, iNKT cells, which are CD1d-reactive and extensively studied, are of the αβ T cell lineage (635). Recent advances in sequencing technology have allowed identification of peanut protein-specific TCRβ sequences in CD4+ memory T cells from peanut-allergic patients (636). Antigen-specific complementarity-determining region 3 β (CDR3β) repertoires from peanut-allergic patients expressed >7000 unique amino acid sequences and were markedly more

convergent than that from HCs. This is in concordance with an older study which demonstrated selective convergence of TCR V beta 11 (TCRVβ11) clonality in peanut-allergic patients following treatment with crude peanut protein (637). Potentially, lipid-specific TCRs are convergent in peanut-allergic patients, giving rise to this cohort's exclusive production of IFN-γ in response to PLD, PG and PA. Notably, CD1a-autoreactivity was higher in the healthy cohort, whereas PLD, PG or PA stimulation led to greater responses in the patient group. This is unlikely attributed to a batch effect, given that ELISpot plates each incorporated one HC and one peanut-allergic donor. Potentially, peanut-sensitised TCRs recognise a narrower range of PLD-associated lipids. It would be interesting to generate PLD-, PG- and PA- specific T cell clones from HC and peanut-allergic patients and perform TCR sequencing to test this hypothesis. Ultimately, ternary crystal structures would help characterise the binding mechanics of PLD-derived lipids within the CD1a groove, and subsequent interactions with the TCR.

Thirdly, unconventional TCRs may also play a role in the CD1a response to peanut PLD products. γδ T cells are rare and enigmatic, and their epithelial-resident status makes them an interesting target in skin biology (638). Their TCRs consist of a γ and δ chain, encoded by *TRG* and *TRD* respectively, and recognise a chemically diverse range of ligands, including CD1-presented lipids (639). TCR Vδ gene segment usage predicts ligand recognition, and divides γδ T cells into Vδ1, Vδ2 and Vδ3 subpopulations (640). In CD1 biology, mainly it is the CD1c- and CD1d-restricted T cells which have been shown to express γδ TCRs, and generally belong to the Vδ1 subpopulation (641-645). Remarkably, Vδ1 T cells can recognise several non-classical MHC-like molecules, even without loaded antigen (639). This draws an interesting parallel with the 'absence of interference' model of TCR recognition of CD1a, as outlined in the Introduction (*Chapter 3.7.3.2*) (323). This is supported by recent unpublished data by Rossjohn et al, demonstrating γδ T cell TCR

binding to CD1a. Induction of peanut allergy in mice resulted in marked decreases in intestinal-resident γδ T cells, and blockade of the γδ TCR induced Th2 cytokine production, suggesting they play a role in promoting oral tolerance (646). Potentially, peanut-allergic patients express lower levels of lipid-specific γδ T cells in the skin, leading to the observed inflammatory responses to peanut PLD. Thus, comparison of αβ and γδ TCR clonality between patient groups seems prudent.

Fourthly, it is necessary to characterise the origin of lipid-reactive T cells in peanut-allergic patients. I present two possible scenarios. Given that peanut products are detectable in the environment, it is suggested that epicutaneous exposure to peanut-derived PLD, PG and PA promotes LC processing of lipids (647). This process is facilitated by filaggrin deficiency and AQP3 overexpression in atopic skin and may contribute directly towards peanut sensitisation through generation of PG- and PA-reactive T cells (150, 269). Conversely, these peanut products may promote inflammation in patients with pre-existing lipid-reactive T cells. Wang et al demonstrated that PA is upregulated in facial eczema lesions of infants (326). Patients with eczema have increased risk of asthma (OR 2.68-11.53) and food sensitisation (1.72-12.64), given their status as atopic diseases (648). Whilst PG has not been measured in AD skin, Carraro et al. detected elevated PG in the urine of children with early-onset asthma compared to those with transient wheezing (649). The authors concluded this was a result of elevated PG content in pulmonary surfactant of asthmatic patients, leading to increased urinary elimination. Potentially, members of this cohort had concurrent AD, with increased PG production in lesional skin (269). Therefore, atopic patients may already possess elevated PG- and PA-reactive T cells, which are activated by peanut PLD, PG and PA and facilitate peptide-specific responses in peanut-sensitised patients. To explore further, it is necessary to gather more information on the nature of T cell populations. Whilst ELISpot is a sensitive method of quantifying CD1a-reactive T cells, it

only provides information on effector production of cytokines (491). CD1a tetramers could be used to accurately compare the frequency of PG- and PA-reactive T cells between healthy, AD and peanut-allergic patients. For example, evidence of tetramer binding in peanut-allergic patients, but absence of binding in HCs and patients with AD, would suggest a primary sensitisation pathway. Tetramer-binding cells could be phenotyped, to determine the presence of naïve ($CD45RO^{-}CLA^{-}$), memory ($CD45RO^{+}CLA^{-}$) and skin-homing memory ($CD45RO^{+}CLA^{+}$) T cell populations (318). This would further allow us to answer the fundamental question of whether peanut-allergic patients possess naïve CD1a-reactive T cells with inherent PG- and PA-binding capacity, or whether sensitisation occurs peripherally.

Finally, further studies should dissect the transcriptional mechanisms of cytokine production by CD1a-reactive T cells. T-bet is a TF expressed in myeloid and lymphoid lineages which recruits chromatin-modifying enzymes that regulates IFN-γ expression and promote differentiation of Th1 cells (650). CD1d-restricted iNKT cells are categorised according to surface markers, cytokine production and TF expression (291). Consequently, the NKT1 subtype produce IFN-γ and express high amounts of T-bet (651). Considering other non-conventional T cells, T-bet deficiency markedly abrogates levels of IFN-γ-producing γδ T cells (652). To date, understanding of transcriptional regulation of CD1a-restricted T cells is limited. Our data demonstrated marked induction of IFN-γ in peanut-allergic individuals, and it would be interesting to compare T-bet expression and activity between patient cohorts using western blot analyses on cells (653). This may help phenotype CD1a-reactive T cells based on their TF expression and subsequent propensity to produce Type I or II cytokines. Furthermore, in CD1a-reactive T cells, the T-bet-specific loci of *IFN-γ* could be assessed using ATAC-seq (654). This would help elucidate whether

IFN-γ induction in peanut lipid-reactive T cells is a function of chromatin rearrangement, thereby highlighting new understanding of the downstream effects of CD1a-TCR docking.

1.38. Regulation of CD1a in DC subsets

It was observed that PLD2 activity promotes group I CD1 expression in LCs and DCs, and the data suggest this was a function of PPARγ inhibition. It is possible this is through production of the PLD2 antagonist cPA and so specific experiments are proposed in *Chapter 8.3.3* (570). Whilst the data support a strong role for PLD2 in promoting CD1a expression in LCs and DCs, there is considerable divergence between the development of certain subtypes. LC express DC-like characteristics, including a dendritic phenotype and the ability to migrate to lymph nodes and present antigen to T cells (655). However, current research suggests they are not clearly of a DC lineage, and may in fact originate from tissue-resident macrophage progenitors which are seeded into foetal tissues during development (656). After birth, LCs fully differentiate in response to microenvironmental factors such as tissue-specific TFs and production of TGF-β by keratinocytes, gaining expression of molecules including CD1a and langerin (599, 657, 658). These 'long-term' LCs have self-renewing capability and represent the bulk epidermal LCs under the steady state. This differs ontogenically from classical DCs, which arise from DC-committed bone marrow progenitors (659). Therefore, our findings in MUTZ3-LC may represent additional upstream effects in diversification of macrophage progenitors, driven by PLD. Macrophages can be subdivided into two subsets: $CD64^{+}CD80^{+}$ M1 macrophages are proinflammatory and activated by IFN-γ or LPS, and $CD11b^{+}CD209^{+}$ M2 macrophages which promote wound healing and are activated by type 2 cytokines (660). PLD2 interacts with TLR4 to promote macrophage polarisation towards an M2 phenotype, and MUTZ3 express TLR4 (661, 662). Given that approximately 40% of M2 macrophages are $CD1a^{+}CD1b^{+}$, compared with 10% of M1 macrophages, this suggests PLD2 may induce

group I CD1 expression through generation of LC with partial M2 characteristics (663). Equally, during skin inflammation, blood Gri-1^{High} monocytes quickly infiltrate the skin and differentiate into 'short-term' LCs, providing additional reserves for host defence (582). This LC subset shares a common progenitor with MoDCs, suggesting their CD1a is acquired in a manner like conventional DCs. In summary, regulatory mechanisms of CD1a expression in LCs are intricate and may be dependent on the specific LC subset. To represent 'short-term' LCs, we suggest generating monocyte-derived LCs (MoLCs) with GM-CSF, IL-4 and TGF-β, and incorporating PLD inhibitors during this process (664). To represent 'long-term' LCs, extracting primary LCs from healthy epidermis and culturing in the presence of PLD inhibitor may be required (665). Phenotyping experiments would help dissect differences in PLD-driven CD1 regulation between primary LCs of differing ontogeny.

To represent primary DCs, these studies utilised MoDC, which presented a valuable model for early investigations in PLD2 regulation of CD1a expression. However, the skin contains a heterogeneous mixture of DC subtypes (666). With the exception of epidermal LC, cDC are derived from common myeloid progenitors (CMPs) which develop into macrophage dendritic cell progenitors (MDPs) (659). MDPs either give rise to monocytes or common dendritic cell progenitors (CDPs), the latter of which are upstream of CD11c^{-}CD123^{+} pDCs, CD11c^{Low}CD141^{+} cDC1s and CD11c^{High}CD1c^{+} cDC2s (667, 668). cDC1s and cDC2s are present in skin under steady state conditions, whereas MoDCs and pDCs are recruited during skin inflammation (669, 670). Notably, there is further divergence within these progenitor pathways, representing a complex network of differentially produced skin DC present under different conditions. cDC1s, cDC2s and MoDCs express CD1a, and whilst pDCs do not typically express CD1a, CD1a^{+}BDCA-2+CD123^{int} DCs were recently identified during acute sterile skin inflammation (298, 671). Single cell RNA sequencing to

identify cDCs and pDCs from healthy and atopic skin and comparing their differential expression of PLD1, PLD2 and CD1a would be of interest. Moreover, cDCs and pDCs could be isolated, and MoDCs generated, from both patient cohorts and cultured in the presence of PLD inhibitors, to determine differential effects on CD1a expression. This would allow investigation of whether PLD2-driven CD1a expression plays an important role in DCs derived from CDPs, in addition to that already observed in MoDCs.

1.39. Using mouse models to explore PLD-based therapeutics

Within these experiments, potential therapeutic indications for, peanut PLD-, AQP3- and endogenous PLD2-inhibition have been identified. Direct inhibition of peanut PLD in atopic skin represents a viable therapeutic strategy in preventing peanut lipid sensitisation. To our knowledge, no peanut PLD-specific inhibitors have been developed, and therefore a panel of endogenous human PLD inhibitors were tested to identify their efficacy. FIPI hydrochloride was tested for peanut PLD functional assays, which also demonstrates activity against human PLD1 and PLD2. Whilst FIPI exerted potent peanut PLD-inhibitory effects, this redundancy with human isoforms may confound data in future mouse studies. Whilst all PLD isoforms share the same HKD catalytic domain (see *Chapter 3.6.1*), human and plant isoforms share only 26% primary protein sequence identity (Uniprot BLAST data, 2019). This presents an opportunity for development of specific small molecule inhibitors for peanut PLD. Peanut PLD crystal structure determination would aid in raising a panel of candidate molecules which can be tested using high-throughput screening methods (672). Epicutaneous peanut sensitisation could then be performed in CD1a-transgenic mice in the presence or absence of peanut PLD inhibitors, to determine the resulting effect on development of lipid-reactive T cells (57). This would require much groundwork and collaboration, however the tools, including CD1a-transgenic mouse models, are available (319).

Aquaporins are another set of compelling drug targets across a range of conditions including skin disease, cancer and neurological disease (673). Consequently, a wide range of small molecule inhibitors have emerged from experimental and computational screens. In a mouse model, AQP3-mediated hydrogen peroxide transport facilitated activation of NF-κB signalling in keratinocytes and development of psoriasis (674). Furthermore, exposure to a tumour initiator did not lead to development of skin tumours in AQP3-null murine epidermis (675). AQP3 inhibition may therefore protect against skin tumour growth and inflammatory skin disease. The data in this thesis has raised the hypothesis that topical AQP3 inhibitors may reduce CD1a-mediated inflammation. Robust CD1a-transgenic mouse models are required to test this theory, in which AQP3-null or control mice are sensitised to peanuts and compared in terms of their CD1a-restricted responses to peanut PLD. If AQP3-deficiency nullifies the generation of PG-reactive T cells, then AQP3 inhibitors represent a viable topical strategy in combatting peanut lipid sensitisation in atopic patients.

Finally, endogenous PLD2 inhibition presents a novel pharmacological approach, through induction of PPARγ activity and inhibition of CD1a expression on DCs. There is ample evidence that manipulation of CD1a expression modifies inflammatory responses. *M. tuberculosis* induces CD1 downregulation to evade CD1-dependent immunity and HIV interferes with intracellular trafficking of CD1a (562, 676). Several pharmacological agents have been identified that possess CD1-modulatory properties. The anti-asthmatic drug suplatast tosilate significantly downregulates CD1a expression on MoDCs, potentially contributing towards its effect in preventing Th2 responses (677). In asthmatic bronchial mucosa, the frequency of $CD1a^+$ DCs is elevated, and inhaled beclomethasone diproniate reduces their number to that seen in healthy epithelium (678). Topical tacrolimus is an immunomodulatory drug and second-line treatment of moderate to severe atopic eczema

(679). Tacrolimus treatment reduces CD1a MFI in MoDCs, and significantly inhibits LPS-induced CD1a expression (680). Differentiation of MoDCs in the presence of the corticosteroid dexamethasone promotes ERK activity, skewing them towards an IL-10-producing phenotype and ablating CD1a expression (681). Outside allergic disease, trimethylpsoralen (TMP) bath PUVA therapy markedly reduced $CD1a^+$ LC frequencies in psoriatic skin (682). Similarly, topical imiquimod application to squamous cell carcinoma in patients reduces the frequency of CD1a-expressing epidermal DCs by 75% (683). Gold sodium thiomalate is a commonly used rheumatoid arthritis therapy, and inhibits CD1a expression in mature MoDCs (684). A range of other drugs downregulate CD1 expression, including antitumoural, monoclonal antibody and anti-hypertensive therapies (685-687). This sets a precedent for CD1 modulation in combatting disease, suggesting endogenous PLD2 inhibitors can be used in this manner.

PPARγ induction in DCs, in response to PLD2 inhibitors, provides further support for their use. Thiazolidinediones (TZDs) are a family of heterocyclic compounds which specifically activate PPARγ, and are successfully used to treat diabetes, through their antihyperglycemic, hypoglycemic and hypolipidemic effects (688). Within the skin, TZD PPARγ agonists stimulate normal mouse keratinocyte differentiation and accelerate barrier recovery following injury (689). Moreover, TZD treatment reduces the cutaneous inflammatory responses in an oxazolone model of allergic contact dermatitis. Similarly, TZDs inhibit keratinocyte proliferation in response to tape-stripping (690). Consequently, PPARγ agonists represent a major therapeutic target for allergic dermatitis and hyperproliferative disorders such as psoriasis (691). PPARγ activity is similarly protective against development of food allergy. Gu et al demonstrated that OVA-sensitised mice possess reduced intestinal PPARγ activity compared with control mice, and this was associated with intestinal barrier destruction (692). In another study, female OVA-sensitised

mice were more vulnerable to food allergy than their male counterparts, and this was partly associated with reduced gut PPARγ levels (693). Interestingly, CD1a-expressing monocytes mediate inflammation in inflammatory intestinal disease, and we posit their CD1a expression is partly a function of reduced PPARγ activity (694). Equally, $CD1d^{+}CD11c^{+}$ cells regulate normal intestinal microbiota, through presentation of lipids to iNKTs, and control the intestinal IgA repertoire (695). PPARγ induces CD1d expression in DCs and may therefore preserve intestinal immune homeostasis (624). Consequently, data suggest that PPARγ preserves both skin and intestinal barrier function, implicating a role for PLD2 inhibition in preventing peanut epicutaneous sensitisation and inhibiting inflammation upon oral challenge. Importantly, whilst TZDs are effective PPARγ agonists, they can lead to potent side effects including oedema, weight gain and congestive heart failure (696). This calls for additional therapeutic promoters of PPARγ, and we PLD2 inhibitors may fit this criterion.

In addition to its CD1-centric effects, pharmacological inhibition of PLD2 may influence other aspects of the allergic response. Our data demonstrates that PLD2 inhibition promotes PPARγ activity in DCs. Chinetti et al demonstrated that PPARγ activation induced caspase-linked apoptosis of human macrophages (697). Persistent pro-inflammatory activity of macrophages is associated with the pathogenesis of chronic AD, and therefore PLD2 inhibition may counteract this (698). COX-2-derived cyclopentone prostaglandins (C2PGs) are PPARγ agonists which potently oppose inflammation in mononuclear cells (699). Moreover, C2PGs including 15d-PGJ_2 inhibit oxidative burst and cytokine production in macrophages (700, 701). This is reflected in animal models of inflammatory bowel disease, in which PPARγ agonists hinder development of colitis (702, 703). The Th1 cytokine IFN-γ skews macrophages towards an M1 phenotype, contributing towards skin inflammation (704). M1 release high levels of the chemoattractant HMGB1, which binds to RAGE, in

turn activating ERK1/2- and NFκB p65-dependent production of proinflammatory cytokines (705, 706). PPARγ activation has been suggested to interfere with this process and ameliorate murine AD symptoms (706). Conversely, Th2 cytokines induce M2, which express high levels of IL-10 and promote skin repair in humans and mice (707, 708). M2 are important in alleviating allergic dermatitis, and PPARγ primes their production from monocytes (709). Therefore, PPARγ is a central regulator of the intricate M1-M2 network of signalling cascades. Potentially, PLD2 inhibition presents a novel drug target which promotes PPARγ activity, leading to induction of M2 and reduction in AD symptoms. Disparate data exist, given that PLD2 has been shown to bind TLR4 and promote M1 induction via IL4-JAK3 signaling (661). Equally, M2 produced matrix metalloproteinase 12 (MMP12) in a murine model of allergic contact dermatitis, exacerbating symptoms (710). This evidences the complex immunological nature of allergic skin disease. While providing *in vitro* data supports its efficacy, treatment of AD-phenotype mice with PLD2 inhibitor and characterisation of leukocyte populations (including CD1a expression on epidermal DC) would help unfurl the relative importance of each pathway discussed.

Whilst PLD2 inhibition represents a viable strategy, there are potential risks associated with inhibiting a key metabolic enzyme. PLD2-knockout mice generated longer PA species, and this correlated with cerebellar architectural defects and olfactory dysfunction (711). Moreover, PLD2 was required for proliferation of murine cytotoxic $CD8^+$ T cells, and *PLD-2* ablation significantly stunted anti-tumour responses (712). Whilst we are proposing topical, and not systemic administration, of PLD2 inhibitors, this necessitates careful monitoring for extra-dermatological effects during their use in mouse models. Adverse effects would nonetheless provide additional support for inhibiting peanut derived PLD, which does not regulate mammalian homeostasis.

CONCLUSION

This thesis provides the first evidence that CD1a-reactive T cells contribute towards peanut allergy. The data show this was facilitated by PLD, a ubiquitous enzyme which had never previously been implicated in CD1 lipid-reactivity. Exogenous peanut-derived PLD generated PA and PG in DCs; a reaction which was inhibited by filaggrin and exacerbated by AQP3 activity. Peanut-allergic patients demonstrated elevated circulating PLD-, PA and PG-responsive CD1a-reactive T cells. This introduces a new paradigm that filaggrin insufficiency and AQP3 overexpression in patients with AD accelerates epicutaneous sensitisation to peanuts. Moreover, the major peanut allergen Ara h 1 induced CD1a-reactivity, and this may be related to its ability to bind PG and modulate subsequent responses. Peanut-derived phospholipids may have also contributed towards the generation of lipid-reactive T cells, and peanut lipid fractionation studies are indicated to test this hypothesis. Endogenous human PLD2 was found to drive CD1a expression in both LCs and DCs, and this was potentially through inhibition of the TF PPARδ. This has provoked questions regarding potential differences in CD1 transcriptional regulation that exist between different DC subsets. Identification of PLD-specific upregulation of CD1a expression in atopic patients would provide new insights into factors which predispose to epicutaneous sensitisation, including to peanut antigens. These findings establish a dual effect of self and foreign PLD isoforms in enhancing CD1a expression and promoting generation of permissive ligands which enhance allergic disease. Future studies are indicated which interrogate the nature of PA- and PG-reactive T cells, using TCR sequencing and xray crystallography studies to define the mechanics of CD1a-ligand

binding and TCR recognition. Further phenotyping of lipid-reactive T cells, including their full spectrum of cytokine production, will clarify additional roles in peanut allergy pathogenesis. Equally, elucidating the mechanisms underlying their production of IFN-γ will draw new insights across CD1a biology. Animal studies are required to develop novel therapeutics, including PLD and AQP3 inhibitors which may modulate CD1a expression and inhibit development of PA and PG-reactive T cells, with consequent functions in preventing sensitisation and ameliorating inflammation.

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