IDENTIFICATION OF LIPID SPECIFIC NKT RECEPTORS IN THE CONTEXT OF BRAZIL NUT ALLERGY

Rui Wang

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

September 2019

Abstract

The mechanisms that lead to the development of allergy are still poorly described. In particular what makes an ordinary protein to be recognised as allergen is not yet defined. In order to study the intrinsic allergenicity of proteins, our group has used nut proteins as a model system. The 2S storage albumin from Brazil nut (Ber e 1) in particular is regarded as a major allergen and historically was the first allergen to be transgenically transferred from one plant to another. Whilst Ber e 1 is able to sensitise allergic individuals, the purified protein is not highly immunogenic in animals. Within this project the ongoing hypothesis is that natural lipids present in the nut are required for Ber e 1 to act as an allergen.

For this, in the study presented in this PhD dissertation, the main focuses were: (i) to develop *in vitro* techniques to use stable human cell lines to generate transiently transfected cells for the assessment of natural lipids to act as ligands in the activation of non-conventional T cells; (ii) to identify lipid-binding NKT cells from primary human cultures using a single cell system and NGS with the aim of isolating and characterizing lipid-binding TCR activation; (iii) to express lipid-specific TCRs in surrogate stable human cell lines in order to facilitate a construction of a cell screening lipid library.

During this work it was shown that the recombinant TRAV10 protein receptor expressed in Jurkat Lucia cells increased over 72 hours and that CD69 is a better marker for measuring activation in primary NKT cells. Using FACS sorting, primary lipid specific NKT cells from six volunteers (4 allergic and 2 healthy) were successfully isolated from PBMCs and the TCRs sequenced. Over 200 TCR sequences were analysed and nut lipid specific sequences identified. Three pairs of nut lipid-specific-TCR sequences were then cloned into an in house designed acceptor bidirectional plasmid engineered to contain specific type IIS restriction enzymes. Surrogate T cells (Jurkat 76) were subsequently transiently transfected with the nut specific TCRs and co-cultured with human APC presenting lipids. In

this system all three transiently transfected T cell lines showed higher IL-2 expression in response to nut lipids than to α-GalCer. The transfected cell lines were used to screen Brazil nut lipid fractions and showed specificity towards phosphatidylethanolamine (PE), phosphatidylserine (PS) and their derivative phospholipids.

In the final and exploratory chapter and in order to facilitate the search for new lipid binding-TCRs, a rapid high-throughput methodology for sequencing long-length TCR transcripts using targeted capture and next generation Nanopore sequencing (MinION) was described. Human PBMCs from a healthy volunteer were isolated and co-cultured with APCs in the presence of six different lipids before RNA isolation. A total of 40,862 transcripts were obtained in the first MinION run, which uniquely aligned to TCR constant regions. Initial analyses involving only TCR alpha chains have shown that 3 were specific to α-GalCer, 13 to Sulfatide, 4 to Lactosylceramides, 27 to Brazil nut lipid, 29 to Lipopolysaccharides (LPS) and 3 to Lyso-phosphatidylethanolamine (LPE). The full bioinformatic processing of the remaining data is ongoing.

The results shown here therefore further suggest that particular classes of nut lipids are involved in the activation of NKT cells from nut allergic patients and that α/β and γ/δ TCR receptors are involved in the mechanisms of activation. The results from this study will help to characterise the intrinsic factors linked to Ber e 1 allergenicity and ultimately will help to define what makes a common protein, within a food matrix context, to function as an allergen to a group of susceptible individuals. The surrogate systems presented here are important first steps in the establishment of human cell-specific lipid responsive libraries for the study of natural lipid substances.

Acknowledgement

I would like to express my great appreciation to Associate Professor Marcos Alcocer for the tremendous and continuous guidance of my PhD study. He is the man leading me into the fascinating scientific fields of immunology. I cannot imagine a better supervisor in my PhD research. It is an honour to have my doctoral training under his patience and advice.

I would also like to express my gratitude to my co-supervisor Dr. Lucy Fairclough. I am very grateful to Dr. Ashfaq Ghumra. I am extremely thankful to him for sharing his expertise selflessly. He always helped and inspired me during my PhD study.

A special thanks to Dr. David Onion, Nicola Croxall, Prof. Sean May, Dr. Marcos Castellanos Uribe, Dr. Iqbal Khan, Nadine Holmes, Ronja Pscheid, Ling Yu Lea Kan and everyone from the School of Bioscience. Without assistance and support from all of you, I would not be able to complete my PhD study.

Finally, a sincere gratitude from deep inside to my family. To my dearest parents, thanks for providing me the motivation, the encouragement throughout my work and life. To my grandparents and my family members, thanks for your unfailing support to me. To my beloved boyfriend Dr. Xiaodong Peng, thanks for your unselfish accompany and support from the beginning.

Publications

Wang R, Pscheid R, Ghumra A, Kan LY, Cochrane S, Fairclough L, Alcocer M (2019) 'Towards a surrogate system to express human lipid binding TCRs', *Biotechnology Letters*, 41(10), pp. 1095-1104. doi: 10.1007/s10529-019-02713-2.

Wang R, Ghumra A, Cochrane S, Fairclough L, Broughton Rm Michaelson L, Beaudoin F, Alcocer M (2019) 'Defining lipids and TCRs involved in the intrinsic allergenicity of nut proteins' (Submitted to Allergy)

Wang NX, Yang W, Yang H, Yang CP, **Wang R,** White S, Salter A, Alcocer M (2019) 'Identification and characterization of serine proteinase as specific allergens in yellow mealworm (Tenebrio Molitor) larva' (In preparation)

Wang NX, Yang W, Yang H, Yang CP, **Wang R,** White S, Salter A, Alcocer M (2019) 'Allergen immunoassay of common edible insects: a systematic review and meta-analysis' (In preparation)

Communications in congresses

Wang R, Ghumra A, Cochrane S, Fairclough L, Alcocer M. Identification Of Nut Lipid Specific NKT Receptors And Their Role On The Intrinsic Allergenicity Of Nut Proteins. *The European Academy of Allergy and Clinical Immunology (EAACI), Lisbon Portugal, June 2019*. Oral Presentation.

Abbreviations

APC: Antigen-presenting cell

bGH: Bovine Growth Hormone

BSA: Bovine serum albumin

CO_2: Carbon dioxide

CDRs: Complementary-determining regions

CD3-FITC: Cluster of differentiation 3-Fluorescein isothiocyanate

CD25-ECD: Cluster of differentiation 25-R Phycoerythrin-Texas Red-X

CD56-PE: Cluster of differentiation 56-R Phycoerythrin

CD69-PC5: Cluster of differentiation 69-Phycoerhthrin-Cy5 Conjugate

CRAC: Ca2+ release-activated Ca2+ channel

CRISPR: Clustered regularly interspaced short palindromic repeats

DAG: Diacylglycerol

DC: Dendritic cell

DGKA: Diacylglycerol kinase-α

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EB: Elution Buffer

ELISA: Enzyme-linked immune sorbent assay

ESI-MS/MS: Electrospray ionization tandem mass spectrometry

EXT: External

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

FoxP3:	Forkhead box P3
GALT:	Gut-associated lymphoid tissue
GATA-3:	Trans-acting T cell-specific transcription factor 3
GI:	Gastrointestinal
GL:	Glycolipid
GM-CSF:	Granulocyte-macrophage colony stimulating factor
HDL:	High-density lipoproteins
HEPES:	Hydroxyethyl piperazineethanesulfonic acid
HGP:	Human Genome Project
HRP:	Horseradish peroxidase
IDL:	Intermediate low-density lipoproteins
IFN:	Interferon
Ig:	Immunoglobulin
INT:	Internal
IKK:	IκB Kinase
IL:	Interleukin
iNKT:	invariant Natural Killer T
IRF:	Interferon regulatory factor
LC:	Langerhans Cell
LDL:	Low-density lipoproteins
LPE:	Lyso-phosphatidylethanolamine
LPI:	Lyso-phosphatidylinnositol
LPS:	Lipopolysaccharides
MAIT:	Mucosal associated invariant T
MEM:	Minimum essential medium

MHC:	Major histocompatibility complex
MLNs:	Mesenteric lymph nodes
M cells:	Microfold cells
NGS:	Next Generation sequencing
NF-AT:	Nuclear factor of activated T cells
NF-κB:	Nuclear factor kappa B
NKT:	Natural killer T
NL:	Neutral lipid
NMR:	Nuclear Magnetic Resonance
ONT:	Oxford Nanopore Technology
PA:	Phosphatidic acid
PBMC:	Peripheral Blood Mononuclear Cell
PBS:	Phosphate buffered saline
PC:	Phosphatidylcholine
PCA:	Principal Component Analysis
PCR:	Polymerase chain reaction
PE:	Phosphatidylethanolamine
Pen-Strep:	Penicillin-Streptomycin
PKC:	Protein kinase C
PL:	Phospholipid
PP:	Peyer's Patches
PS:	Phosphatidylserine
PMA:	Phorbol myristate acetate
RAP:	Rapid Adapter
RAST:	Radioallergosorbent test

RIN: RNA Integrity Number

RPMI: Roswell Park Memorial Institute

RT: Reverse transcription

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

SBS: Sequencing by synthesis

SEAP: Secreted embryonic alkaline phosphatase

SFA8: Sunflower albumin 8

SMRT: Single Molecule Real Time

SPE: Solid-phase extraction

SREBP: Sterol Regulatory Element Binding Protein

T-bet: T-box transcription factor TBX21

TRAV: T cell receptor α variable region

TRBV: T cell receptor β variable region

TCR: T-cell receptor

TGF-β: Transforming growth factor beta

TLC: Thin Layer Chromatography

TLE: Total lipid extract

TMB: Tetramethylbenzidine

TNF-α: Tumour necrosis factor-α

TRDV: T cell receptor variable region

Treg: Regulatory T

TRGV: T cell receptor variable region

Th: T helper

T-CD4+: Thymocyte-related CD4+ T cell

VLDL: Very low-density lipoproteins

α-GalCer: α-galactosylceramide

Contents

Chapter 1 General introduction

1.1 Tolerance to food antigens

In a healthy individual, the body has immune tolerance to most food antigens. To maintain immune tolerance, the immune system is not only able to distinguish between self and non-self, but also harmless non-self and threatening non-self. The gastrointestinal (GI) tract is an important barrier for the immune system to identify and maintain tolerance. It is the largest contact between the body and the external environment, with an intestinal mucosal surface area of more than 300 square meters. The GI tract is in contact with a large number of foreign antigens, including more than 30 kilograms of food protein ingested each year, and more than 1,000 kinds bacteria in trillions (Commins, 2015). The GI tract mainly consists of the gut-associated lymphoid tissue (GALT) and lymphoid organs, which is responsible for general immune surveillance in the gut. Epithelial Microfold cells (M cells) and Peyer's Patches (PP) in the intestinal lumen facilitate uptake through phagocytosis and transcytosis, and may be required in the development of immune tolerance (Suzuki *et al.*, 2008; Jung, Hugot and Barreau, 2010). Mesenteric lymph nodes (MLNs) mainly contribute to mucosal immunity, and is essential for recognition of pathogens, commensal bacteria and protein antigens (Brandtzaeg *et al.*, 2008; Pabst and Mowat, 2012).

Tolerance occurs when there is sustained hyporesponsiveness in the immune system following exposure to microorganisms and foreign antigens. This is achieved by the

complex interactions of regulatory cells to prevent unnecessary damage to the host. Studies have shown that a low dose of antigen exposure cause higher production of antigen-specific regulatory cells such as regulatory T (Treg) cells, while a high dose of antigens result in T cell anergy or deletion (Friedman and Weiner, 1994). CD103+ Dendritic cells (DCs), a subset of DCs present in the lymph nodes and lamina propria, play a major role in immune tolerance by producing suppressive immunoregulatory factors such as transforming growth factor beta (TGF-β and retinoic acid to promote forkhead box P3 (FoxP3) positive Treg cell proliferation (Coombes *et al.*, 2007). CX3CR1+ macrophages sample and transfer antigens from the lumen to CD103+ macrophages through a gap junction, and facilitate antigen presentation and Interleukin (IL)-10 secretion to maintain immune tolerance (Hadis *et al.*, 2011; Mazzini *et al.*, 2014; Chinthrajah *et al.*, 2016; Yu, Freeland and Nadeau, 2016).

1.2 Food allergy

Allergy is defined as a chronic disorder in which antigens cause a skewed immune response, and are associated with many diseases such as asthma and allergic rhinitis (Yazdanbakhsh, Kremsner and Van Ree, 2002; Dreborg, 2015). Allergens are substances that induce an immune reaction in certain people, but are normally harmless for most individuals. They are derived from a plethora of sources such as dust, chemicals, microbes, food, and pollen (Kay *et al.*, 2008; Dave *et al.*, 2011).

Around the world, it is estimated that roughly 10-40 % of people are suffering from at least one form of allergy (Blaiss, M.S., Canonica, G. W., Holgate, S.T, Lockey, R.F. and Pawankar, 2013). The number of cases related to allergy is on the rise, with the estimation that 1 in 2 people in the EU will be suffering from allergy disorders by the year 2025 (EAACI, 2016). This will affect the productivity and impoverish the wellbeing of people.

The clinical symptoms of allergy can vary from mild irritation to life-threatening anaphylactic shock. So far, no definitive therapies are available for allergies. On the diagnostic front, many tests such as skin prick test, radioallergosorbent test (RAST), Enzyme-linked immune sorbent assay (ELISA), protein microarrays are often used for quantifying IgE antibodies (Salazar *et al.*, 2017). It is generally accepted that the most effective palliative method to treat allergy is avoidance of contact with the offending material.

Food allergy is defined as an adverse immune response to the ingestion of food proteins. Ingested food is digested into small protein fragments in the gastrointestinal tract and then absorbed by the lumen epithelial cells. It is attractive that some food proteins intake may cause an allergic immune response, leading to food allergy. Some of the clinical signs of IgE-mediated food allergy include urticaria (hives), diarrhoea, chest tightness, gastrointestinal pain, and angioedema of skin or mouth (Waserman and Watson, 2011). The severity and reaction time are

dependent among individuals, and symptoms usually occur within two hours of exposure to allergen (Burks *et al.*, 2012).

The joint and synergistic effects of multiple cells limit the allergy response caused by bacteria and food proteins, limit microorganisms in the intestine, and identify pathogens (Chinthrajah *et al.*, 2016). When the body fails to maintain this proper balance, it will lead to the loss of tolerance and lead to an immune response to harmless food antigens, such as IgE-mediated food allergy. Hypersensitivity is divided into 4 types, and most food allergies are type I IgE-mediated hypersensitivity reactions. Sensitization during an allergic response is a process that induces IgE production upon the first contact with an antigen, which primes the body to react to these substances in the following encounters. Firstly, IgE is produced and binds to FcεRI receptors on mast cells and basophils. The antigen specifically binds to the IgE- FcεRI complex, activating mast cells and basophils (Galli, Tsai and Piliponsky, 2008; Wang *et al.*, 2010). The effector stage occurs during subsequent exposures of the same antigen, and has two phases: early phase and late phase. In early phase, the antigen triggers the release from a series of bioactive substances from these activated cells, such as histamine, leukotrienes and kinins. These bioactive substances cause telangiectasia, increased vessel wall permeability, smooth muscle contraction and increased adenoid secretion. Degranulation of mast cells and basophils can result in the classic symptoms of Type 1 hypersensitivity such as anaphylactic shock, asthma and abdominal pain (Mak,

T.W., Saunders, M.E., Jett, 2014; Satitsuksanoa *et al.*, 2018; Anvari *et al.*, 2019). The late phase is commonly taking place in asthmatic patients, and normally arises 2-6 hours after antigen exposure (Galli, Tsai and Piliponsky, 2008). Eosinophils along with macrophages and other activated immune cells migrate into the inflammation site, secreting chemokines and cytokines to cause further tissue damage, and can cause swelling and smooth muscle contraction (Mak, T.W., Saunders, M.E., Jett, 2014).

Natural Killer T (NKT) cells are a subset of unconventional T cells that could participate in the development of food allergy. Once activated, NKT cells may drive the acquired immune response to an antigen through T helper (Th) 2 cell responses and lead to Immunoglobulin E (IgE) production, without clonal expansion. This will activate various immune cells and thus NKT cells play a central role in bridging innate and adaptive immune responses (Nishimura *et al.*, 2000; Tsuda *et al.*, 2001; Mirotti *et al.*, 2013). Jyonouchi *et al.* reported that children with milk allergies had NKT cells, and had a greater Th2 response to milk sphingomyelin compared to healthy controls (Jyonouchi *et al.*, 2011). Furthermore, most NKT cells recognize lipid antigens presented by CD1, a major histocompatibility complex (MHC) class I-like antigen-presenting molecule expressed in antigen presenting cells (APCs). Recent work has shown that a natural lipid fraction from Brazil nut provided an essential adjuvant activity for sensitisation to the Brazil nut allergen Ber e 1, and that NKT cells play a critical role in the development of Brazil nut-allergic response

(Mirotti *et al.*, 2013). It is well-established that T and B cells are significantly involved during an allergic response (Rocklin *et al.*, 1980). The underlying mechanisms behind the sensitisation phase of allergy are still poorly described. Particularly, what makes an allergen is not yet defined.

Allergy	Prevalence in young children, %	Prognosis
Cow's milk	0.3 - 3.5 (<0.5 in adults)	> 80% outgrown by 16 y
Hen's egg	0.5 - 8.0 (<0.5 in adults)	> 80% outgrown by 16 y
Wheat	<1	Majority outgrow – 65% by 12 y
Fish	<0.2 (children) and <0.5 (adults)	Usually allergic for life
Shellfish	<0.5 (children) and <2.5 (adults)	Usually allergic for life
Peanut	0.06 - 5.90	20% outgrown
Tree nut	0.2 - 1.4	10% outgrown
Plant food	0.1 - 4.3	

Table 1 Summary of prevalence of allergy to individual food allergens (Gray et al., 2014).

Currently an increased prevalence of allergy has been observed, with young children and infants in particular more susceptible to develop allergy, especially from food (Walker and Wing, 2010). Many sources are implicated in food allergies such as milk, eggs, wheat, fish, shellfish, peanuts, tree nuts and plant food (Rona *et al.*, 2007). These allergies affect individuals of all ages and are usually permanent. An

illustrative breakdown of prevalence by food products is also summarised in Table 1. There is a strong impression that there has been an increase in the rate of referrals for food allergy, with the most obvious rise in peanut and tree-nut allergies in the UK (Stiefel *et al.*, 2017). Together they are now the commonest food cause of anaphylaxis affecting approximately 1.5 % of the general population in the UK (Grundy *et al.*, 2002). Several families of proteins are implicated in nut allergy; their clinical relevance and the proteins involved are depicted in Table 2.

Tree nut	Protein family	Component	Protein type	Homologue / Clinical relevance
Hazelnut	Pan-allergens	Cor a 1	PR-10	Homolog of Bet v 1: PFS
		Cor a 2	Profilin	Homolog of Bet v 2: PFS
		Cor a 8	LTP	Systemic reactions in children from Mediterranean areas
	Storage proteins	Cor a 9	11S globulin	Systemic reactions
		Cor a 11	7S globulin	Systemic reactions
		Cor a 14	2S albumin	Systemic reactions
Cashew	Storage proteins	Ana o 1	7S globulin	
		Ana o 2	11S globulin	
		Ana o 3	2S albumin	Systemic reactions
Pistachio	Storage proteins	Pis v 1	2S albumin	Homolog of Ana o 3; systemic reactions
		Pis v 2	11S globulin	Homolog of Ana o 2; systemic reactions
		Pis v 3	7S globulin	
		Pis v 5	11S globulin	Homolog of Ana o 1
Walnut	Storage proteins	Jug r 1	2S albumin	Systemic reactions
		Jug r 2	7S globulin	Systemic reactions
		Jug r 4	11S globulin	Systemic reactions
	Pan-allergens	Jug r 3	LTP	Systemic reactions in Mediterranean individuals
		Jug r 5	Profilin	
Pecan	Storage proteins	Car i 1	2S albumin	Homolog of Jug r 1
		Car i 2	7S globulin	Systemic reactions
		Car i 4	11S globulin	Homolog of Jug r 4
Almond	Storage proteins	Pru du 6	11S globulin	
	Pan-allergens	Pru du 3	LTP	
		Pru du 4	Profilin	
Pine nut	Storage proteins	Pin p 1	2S albumin	Systemic reactions
Brazil nut	Storage proteins	Ber e 1	2S albumin	Systemic reactions
		Ber e 2	11S globulin	

** Systemic reactions: e.g., asthma, food-induced pulmonary haemosiderosis, dermatitis herpetiformis, shock, uterine contractions, etc.*

Table 2 Individual tree nut components (Blanc et al., 2015; Weinberger and Sicherer, 2018), their homologues and clinical relevance of allergy.

Despite the relative seasonal consumption in Europe, Brazil nut (*Bertholletia*

excelsa) has been identified as a common tree nut allergy (Weinberger and Sicherer, 2018). Due mainly to its extremely high content of sulphur amino acid, Ber e 1, the major protein allergen from Brazil nut, has attracted much scientific and press attention in many fronts. As described by Alcocer *et al.* (Alcocer, Rundqvist and Larsson, 2012), Ber e 1 has been used as a model protein in early processing studies of plant storage proteins; in early plant transgenic work; in plant vacuolar targeting studies; as a target in early nutritional supplementation experiments; as one of the first food allergens to be transferred from one plant to other. The first case of systemic allergic reaction caused by food allergen transferred in semen was also reported (Bansal *et al.*, 2007).

As mentioned previously the mechanisms that lead to the development of food allergy (sensitization) are still elusive; however once established, the cascade of events leading to elicitation of symptoms are well documented (Alcocer, Rundqvist and Larsson, 2012). In recent years and using Ber e 1 as model protein system, our group and others have searched for materials other than protein that might be co-presented during the sensitization phase. Cumulative evidence has shown that large numbers of protein allergens possess a lipid binding site or lipid transfer functions (Mirotti *et al.*, 2013; Jappe *et al.*, 2019). It has been described that the major allergen 2S albumin from Brazil nut Ber e 1 for instance, when injected in mice did not elicit a strong antibody production, suggesting that components other than the proteins might influence the intrinsic sensitization potential of this protein. Indeed, lipids

from Brazil nuts were shown to be required for the sensitization of mice and for the induction of functional IgE (Dearman, Alcocer and Kimber, 2007; Alcocer, Rundqvist and Larsson, 2012; Mirotti *et al.*, 2013).

1.3 Lipids

Lipids are major components of the human body that participate in physiological functions and cell structure formation. One important characteristic of the group is that they are hydrophobic substances that dissolve well in organic solvents, and are therefore transported through carrier proteins and membrane channels within the body. Lipids, whose levels are maintained by the liver and adipose tissue, depending on their classes and fatty acid composition result in a wide range of molecules with differing solubility, charge, shape and size that facilitates their function in a variety of cellular processes including signalling, defence, apoptosis and senescence, which could be beneficial or harmful (Burdge and Calder, 2015).

Dietary lipids in particular are macronutrients required for the regulation of metabolism, which are primarily obtained from food. They are also important to facilitate the absorption of fat-soluble vitamins. Fatty acids are classified into essential and non-essential, where essential fatty acids are unable to be synthesized by the body and need to be obtained from diet (Singh, 2005). Once digested and absorbed in the intestine, lipids enter the bloodstream and are carried by

chylomicrons that consist of phospholipids and apoproteins on the periphery with a cholesterol-rich interior. These are found in the bloodstream and liver in order to transport cholesterol to adipose tissues or to the liver. There are five major classes of lipoproteins: Chylomicron, very low-density lipoproteins (VLDL), high-density lipoproteins (HDL), intermediate low-density lipoproteins (IDL) and low-density lipoproteins (LDL), each having varied lipid composition, apoproteins, size and density (Feingold and Grunfeld, 2000). Dietary lipids, depending on the type of lipid and intake amount, have been shown to affect the immune response and inflammatory state. Endres *et al.* (Endres *et al.*, 1993) has shown that n-3 polyunsaturated fats commonly present in oily fish possess anti-inflammatory properties in patients where cytokines such as interleukin 1 (IL-1) and tumour necrosis factor as well as T lymphocyte production are suppressed (Pablo Martinez and Álvarez De Cienfuegos, 2000). Studies have shown that populations with a high n-3 unsaturated fatty acid diet tend to have lower incidences of autoimmune and inflammatory diseases (Patterson *et al.*, 2012).

From a molecular perspective, the main regulator of intracellular lipid processing is the Sterol Regulatory Element Binding Protein (SREBP), important mediators of inflammatory signals brought on by immune activation (Leavy, 2011). Within the immune system, lipid antigens are internalized by the antigen presenting cell (APC) and presented on the surface of the CD1 molecules. CD1 are MHC class I like molecules that are expressed on the surface of APCs, and are capable of presenting

lipid antigens on the surface instead of protein fragments (de Jong, 2015). There are four human isoforms of CD1 molecules (CD1a, CD1b, CD1c, and CD1d) expressed on APCs and they are known to undergo endosomal trafficking, which are internalised into endosomal vesicles and recycled (Jayawardena-Wolf *et al.*, 2001). Lipid antigens presented via CD1 are recognized by T cells that produce a response to the changes in extracellular environment (Dowds *et al.*, 2014).

Lipid antigens are substances that can be considered harmful to the body under certain circumstances, and might directly trigger activation of the immune system. They are separated into two categories, namely self-lipids and foreign lipids, depending on their origin and capability of self-synthesis (Barral and Brenner, 2007). Lipids can trigger a T helper (Th) 1 or Th2 response in the immune system, where Th1 response is a pro-inflammatory reaction against parasitic infections and autoimmune conditions, and Th2 are related to cell atopy, which promotes an anti-inflammatory response and allergy (Okada *et al.*, 2010).

It is now accepted that lipid binding is a common characteristic observed for members of several protein families that include allergens. Binding or colocalization of immunostimulatory or immunomodulatory lipids could significantly contribute to the allergenicity of proteins. For this, several roles have been ascribed to lipids such as facilitating the passage of an allergen through the intestinal epithelial barrier, altering their degradation within the gastrointestinal tract, or both. However, the role

of lipids as adjuvants bound to the protein allergens as stipulated in our initial hypothesis has only been described in few cases. As shown in Table 3 lipids as ligands are described in Bet v 1-like proteins, non-specific lipid transfer proteins, Globulin, 2S albumins and lipocalins. These proteins contain hydrophobic binding sites for lipids, and it is proposed that their immunomodulation lead to enhanced Th2 response actions (Jappe *et al.*, 2019).

Protein family	Source	Allergen	Allergological relevance	Mode of lipid/ligand interaction	Resulting effects
Bet v 1 like	Birch (*Betula verrucosa*)	Bet v 1	Major allergen, associated with mild allergic reactions	Binds ligands via hydrophobic pocket	Binds and permeabilizes membranes
	Peanut (*Arachis hypogaea*)	Ara h 8	Minor allergen, associated with mild allergic reactions, marker for pollen-associated food allergy	Binds lipids via hydrophobic pocket	Delayed enzymatic digestion, increased thermal stability, enhanced uptake in intestinal mucosa
Non-specific lipid transfer protein	Peach (*Prunus persica*)	Pru p 3	Pan-allergen, associated with severe allergic reactions (Mediterranean area)	Binds fatty acids in inner hydrophobic cavity	Induction of conformational changes that lead to increased IgE-binding
	Peanut (*Arachis hypogaea*)	Ara h 9	Pan-allergen, associated with severe allergic reactions (Mediterranean area)	Binds lipids, phospholipids in inner hydrophobic cavity	Unknown
	Grape (*Vitas vinifera*)	Vit v1	Pan-allergen, associated with severe allergic reactions (Mediterranean area)	binds phosphatidylcholine	Delayed enzymatic digestion
Globulin	Peanut (*Arachis hypogaea*)	Ara h 1	Major allergen, associated with severe allergic reactions	Interaction with phosphatidylglycerol vesicles	Delayed enzymatic digestion
	Mustard (*Sinapis alba*)	Sin a 2	Major allergen, associated with severe allergic reactions	Interaction with phosphatidylglycerol vesicles and mustard lipids	Protection against enzymatic digestion & microsomal degradation, activation of human DCs
2S Albumin	Brazil nut (*Bertholletia excelsa*)	Ber e 1	Major allergen, potentially associated with severe allergic reactions	Lipid-binding hydrophobic cavity is assumed	Co-administration with brazil nut lipids induced IgE and IgG1-response in mice and IL-4 in murine and human CD1d-restricted iNKT cells
	Peanut (*Arachis hypogaea*)	Ara h 2	Major allergen, associated with severe allergic reactions (marker allergen)	None	Might inhibit tryptic degradation of co-administered peanut allergens
Oleosins	Peanut (*Arachis hypogaea*)	Ara h 10 Ara h 11 Ara h 14 Ara h 15	Potential major allergens, associated with severe allergic reactions (potential marker allergens)	Bind phospholipids and lipids via hydrophobic domain creating an oil body	Potentially enhanced uptake of oil bodies via lipid-carrier-mediated transport mechanism
	Sesame (*Sesamum indicum*) Hazelnut (*Corylus avellana*)	Ses i 4 Ses i 5 Cor a 12 Cor a 13	Potential major allergens, associated with severe allergic reactions (potential marker allergens	Bind phospholipids and lipids via hydrophobic domain creating an oil body	
Lipocalin	Cow's milk (*Bos domesticus*)	Bos d 5	Major allergen	Carries hydrophobic molecules, phosphatidylcholine	Insertion into bilayers, protection against enzymatic digestion
	Dog (*Canis familiaris*)	Can f 6	Unknown	Binds LPS	Enhancement of LPS/TLR4-signaling
Secreto-globulin	Cat (*Felis domesticus*)	Fel d 1	Major allergen	Potentially binds TLR-ligands	Enhancement of TLR2 and TLR4 signaling
Group 2 mite allergen	House dust mite (*Dermatophagoides pteronyssinus*)	Der p 2	Major allergen, more often recognized by asthmatics	Binds LPS due to structural similarity with MD-2	Enhancement of LPS/TLR4-signaling resulting in airway Th2 inflammation
	House dust mite (*Dermatophagoides farinae*)	Der f 2	Major allergen, more often recognized by asthmatics	Binds LPS due to structural similarity with MD-2	
Group 5/7 mite allergen	House dust mite (*Dermatophago-ides pteronyssinus*)	Der p5	Minor allergen, more often recognized by asthmatics	Hydrophobic cavities that might bind apolar ligands	Potential stimulation of TLR2
		Der p 7	Minor allergen, more often recognized by asthmatics	Hydrophobic cavities that might bind apolar ligands	
Group 13 mite allergen	House dust mite (*Dermatophago-ides pteronyssinus*)	Der p 13	Minor allergen	Selective binding of fatty acids in inner cavity	Induction of airway epithelial cell activation through TLR2-MyD88-NF-κB and MAPK-dependent mechanisms
Group 14 mite allergen	House dust mite (*Dermatophago-ides pteronyssinus*)	Der p 14	Minor allergen	Potential transporter of lipids	Unknown
Group 21 mite allergen	House dust mite (*Dermatophago-ides pteronyssinus*)	Der p 21	Minor allergen	Potentially binds lipids from house dust mite	Activation of airway epithelial cells through TLR2 signalling

Table 3 Allergens and their interaction with lipids (Jappe et al., 2019).

1.4 NKT cells

Natural killer T (NKT) cells are a unique lymphocyte subpopulation characterized by co-expression of surface markers from conventional T cells (CD3) and NK cells (CD56). They were first identified and named by Hercend *et al.* in 1984 (Hercend *et al.*, 1984). Their most characteristic function is the ability to be rapidly activated and produce Th1 and Th2 cytokines in response to T-cell receptor engagement bound to hydrophobic molecules. Stimulation of NKT cells also results in the activation of other cells, including dendritic cells, NK cells, macrophages, B cells and conventional T cells. NKT cells are a focus of current immunology research as they bridge innate and adaptive immune systems. Because of their potent immuno-modulatory properties, NKT cells are considered to influence a wide range of diverse disease conditions such as autoimmune diseases, allergy and tumor rejection, as well as infections (Wu and Kaer, 2011). Significant progress has been made in the mechanisms of origin, selection, differentiation and maturation of NKT cells. However, some viewpoints are still controversial, and need to be further investigated. From the practical point of view, several potential therapeutic applications of functional NKT cells have been suggested including cancer treatment (Fujii *et al.*, 2013).

As shown in Table 4, heterogeneity is a characteristic of NKT cell populations (Bendelac, Savage and Teyton, 2007). Two main populations of NKT cells are

described: type I and type II cells (Godfrey *et al.*, 2004). Type I NKT cells, also known as invariant natural killer T (iNKT), can be CD1d restricted and express invariant TCRs which can recognize the glycolipid α-Galactosyl Ceramide (α-GalCer). α-GalCer isolated from the sea sponge *Agelas mauritianus* is a well described lipid activator of iNKT cells. Type II NKT cells form most of the remaining NKT cells expressing variant TCRs and can recognise besides α-GalCer a range of other hydrophobic molecules (Pellicci *et al.*, 2011). Most NKT cells recognize lipid antigens presented by CD1, an MHC class I-like antigen-presenting molecule expressed in APCs. CD1 consists of a binding pocket adapted for glycolipid antigens. NKT-like cell populations are mucosal associated invariant T (MAIT) cells, thymocyte-related CD4+ T cells (T-CD4+), CD8+ T cells and γδ T cells. Most of these NKT-like cell populations share the same properties with iNKT cells.

Features	αβ T	NKT cells		NKT-like cells			
		Type I	Type II	MA1T	T-CD4+	CD8+	γδ T
TCR repertoire	Diverse	Vα14Jα18 Vβ8.2, Vβ7, Vβ2	Diverse, some have Vα3.2Jα9, Vα8Jα9, Vβ8	Vα19Jα33 Vβ8, Vβ6	Diverse	Diverse	Vγ1.1Vδ6.3
MHC-restriction	MHC I or MHC II	CD1d	CD1d	MR-1	MHC II	MHC I	Not determined
Selecting cells	TEC	Double positive	Double positive	B cells or DCs?	Double positive	Double positive	Not determined
TCR ligands	Peptide antigens	iGb3, α-GalCer	Sulfatide, Lyso-sulfatide	Hydrophilic antigens or lipids	Not determined	Not determined	Non-peptidic antigens
Positive selection	Yes	Yes	Yes	Yes	Yes	Yes	No
CD4／CD8 expression	CD4+ or CD8+	CD4+ or double negative	CD4+ or double negative	Primarily double negative	CD4+	CD8+	No
Activated Phenotype	After antigen exposure	Yes	Yes	Yes	Yes	Yes	Yes
SLAM-SAP dependent	No	Yes	Yes	No	Yes	Yes	Yes
PLZF expression	No	Yes	Yes	Yes	Yes	Yes	Yes
α-GalCer reactivity	No	Yes	No	No	No	No	No

MR-1, major histocompatibility molecule related-1; MAIT, mucosal-associated invariant T; TEC, thymic epithelial cells; SLAM, signaling lymphocytic activation molecule; SAP, SLAM-associated molecule; iGb3, isoglobotrihexosylceramide; PLZF, promyelocytic zinc finger.

Table 4 Differences and similarities between conventional T cells, NKT cells and NKT-like cells (Das, Sant'Angelo and Nichols, 2010).

Type I NKT cells, iNKT cells are more abundant and well described presenting NK1.1 (NKR-PIC) or CD161 (NKR-PIA) molecules. Mouse type I NKT cells express a specific α chain (Vα14-Jα18) in combination with a restricted number of β chains (Vβ8.2, Vβ7, or Vβ2) (Matsuda *et al.*, 2001). Human type I NKT cells express a TCR that contains Vα24-Jα18 paired with Vβ11 (Porcelli and Modlin, 1999). Depending on the CD4 and CD8 expression, iNKT cells can be isolated as

CD4+ and CD8+ as well as double negative (CD4-CD8-) iNKT cells (Bendelac, Savage and Teyton, 2007; Matsuda *et al.*, 2008).

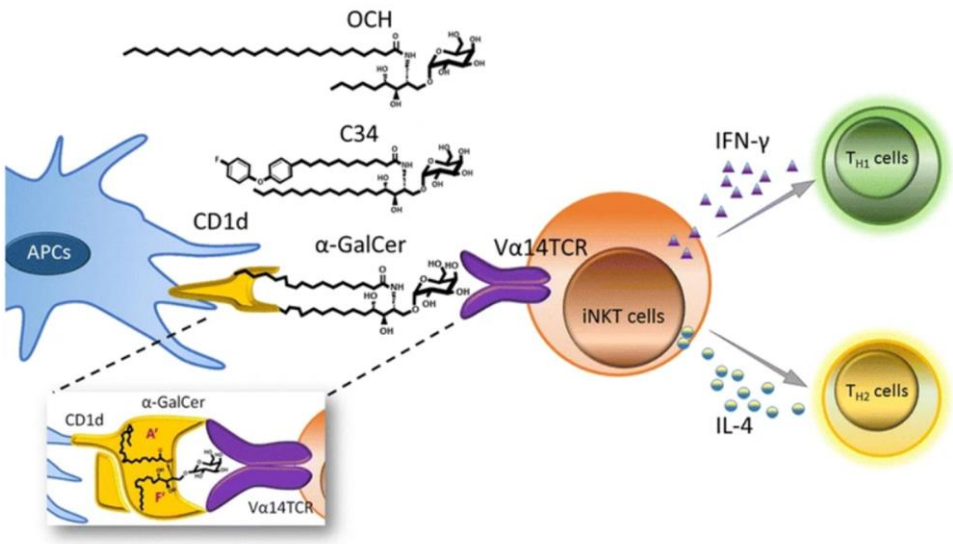


Figure 1 Activation of iNKT cells by α-GalCer and its analogs (Hung, Huang and Yu, 2017).

iNKT cells are hence a type of specialized T cell subset that is involved in immune regulation triggered by hydrophobic molecules. As shown in Figure 1, following activation by antigens such as α-GalCer, iNKT cells rapidly produce a series of important cytokines including (Th1) IFN-γ and (Th2) IL-4 (Hung, Huang and Yu, 2017). At the same time, iNKT cells have a significant part in the stimulation and regulation of other immune cells, like dendritic cells, natural killer cells, T and B cells (Figure 2). iNKT cells can be activated by lipid-coated APCs and up-regulated CD40L. iNKT cytokine released and CD40/CD40L interactions lead to DC stimulation and maturation, which leads to antigen-specific T cell responses. IFN γ and IL-4, secreted by iNKT cells, can stimulate NK cells and B cells to release IFN γ,

antibodies and lead to memory responses. IL-2 produced by iNKT cells can lead to Treg cells proliferation. This process modulates the body's immune response in order to enhance the effects of immunity, anti-tumorigenesis, suppression of tissue rejection from transplantation and other immune responses (Bendelac, Savage and Teyton, 2007; Matsuda *et al.*, 2008).

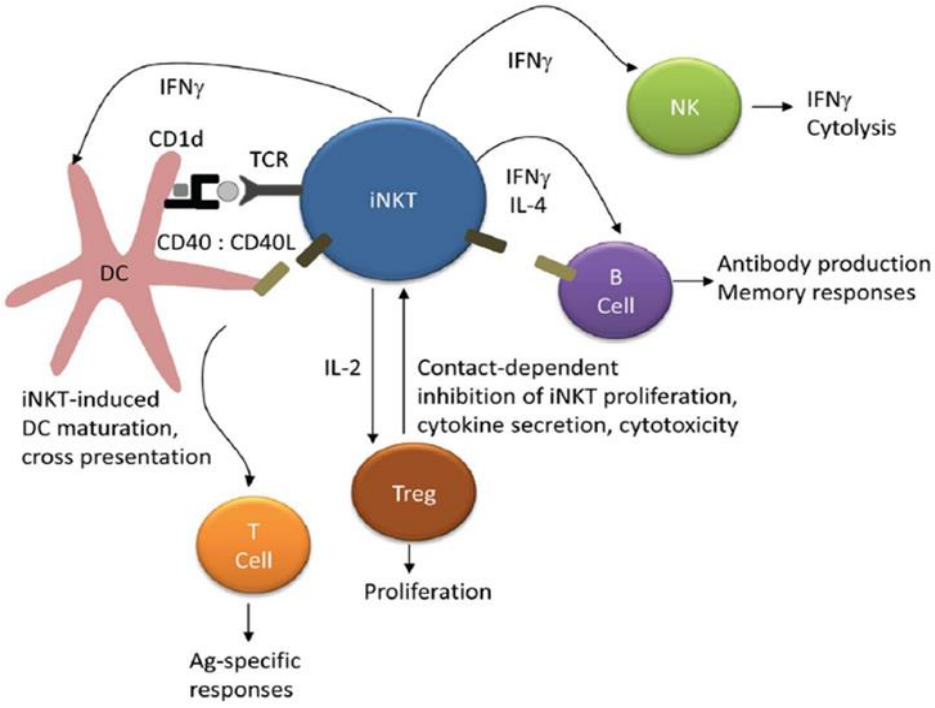


Figure 2 iNKT regulation of NK, T cell and B cell activation (Juno, Keynan and Fowke, 2012).

In contrast to both conventional T lymphocytes and natural killer cells, NKT cells recognize hydrophobic antigens rather than peptides (Girardi and Zajonc, 2012). Moreover, by promoting the secretion of Th1 or Th2 cytokines, they play key regulatory roles. As previously demonstrated by our group, they seem to be the main group of cells involved in the recognition of lipid nuts during the allergic response (Mirotti *et al.*, 2013).

1.5 T cell receptor

The features of human T cell receptor (TCR) complex structure were first described by Haskins *et al.* (Haskins *et al.*, 1983). Thereafter, a great number of studies has been dedicated to this field. Details of TCR engaged T cell activation has been described in Cantrell *et al.* (Cantrell, 1996).

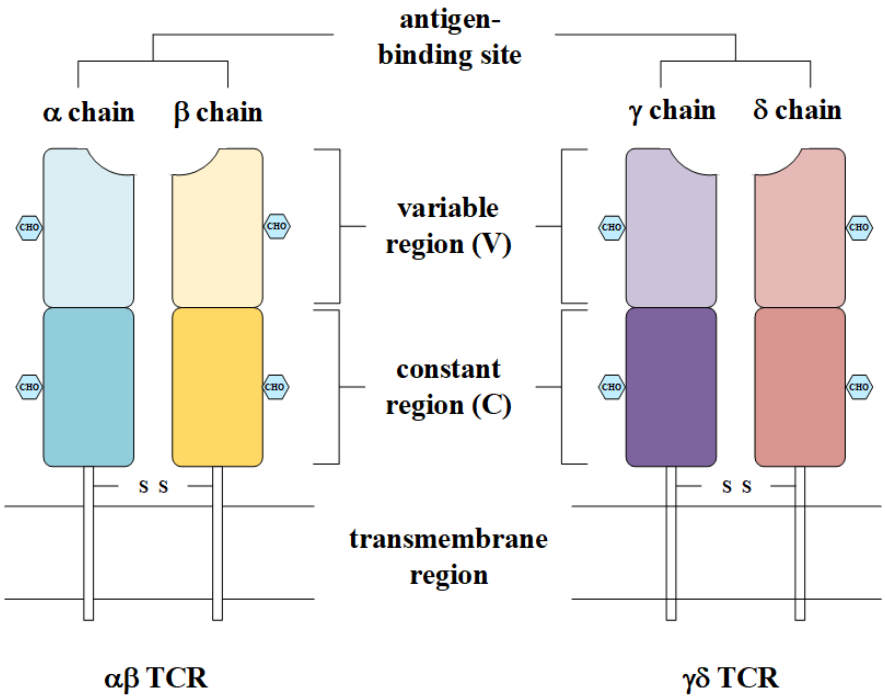


Figure 3 The structure of alpha beta (αβ) and gamma delta (γδ) T cell receptors (TCR).

The T cell receptor on the NKT cell recognises the hydrophobic molecules presented by the CD1 receptor on the APC, this recognition then triggers an intracellular cascade, causing the release of cytokines. TCRs are highly diverse heterodimers (Figure 3), consisting of a combination of α and β chains (αβ TCR) and, γ and δ chains (γδ TCR). Each chain is divided into a variable (V) region, important for

antigen recognition, and a constant (C) domain. The α- and γ-chain V domains are encoded by V (TRAV and TRGV respectively) and joining (J; TRAJ and TRGJ) gene segments, whereas β- and δ-chain V domains are encoded by V (TRBV and TRDV), diversity (D; TRBD and TRDD), and J (TRBJ and TRDJ) genes. The V domain comprises three complementary-determining regions (CDRs) in each chain of the TCR. The CDR1, CDR2 and CDR3 loops are located inside the V domains. The CDR3 loop causes the highest diversity (Sullivan and Kronenberg, 2005).

As shown in Figure 4, the V region of TCR α- and γ-chain is encoded by a number of V and J genes, while TCR β and δ chains are encoded by V, D and J genes. During T cell development, the loci that encode T-cell receptor α-, β-, γ- and δ-chains are rearranged. For both loci, V and J gene segments, and an additional D gene segment for the β- and δ-chains, are recombined to form the final rearranged αβ or γδ TCR DNA sequence. This process also involves the deletion and insertion of nucleotides at the V-D, D-J and V-J domains. Following transcription, the sequences between the recombined V(D)J regions and the gene encoding the constant (C) region are removed by splicing. The CDR3 is encoded by the V (D) J junction, whereas the CDR1 and CDR2 loops are encoded within the germline V gene. During V(D)J recombination, one random allele of each gene segment is recombined with the others to form a functional variable region (Rosati *et al.*, 2017). The surrogate cell lines (Jurkat Lucia, Jurkat Dual and Jurkat 76) which were used in this study are human TCR αβ/γδ null cell lines.

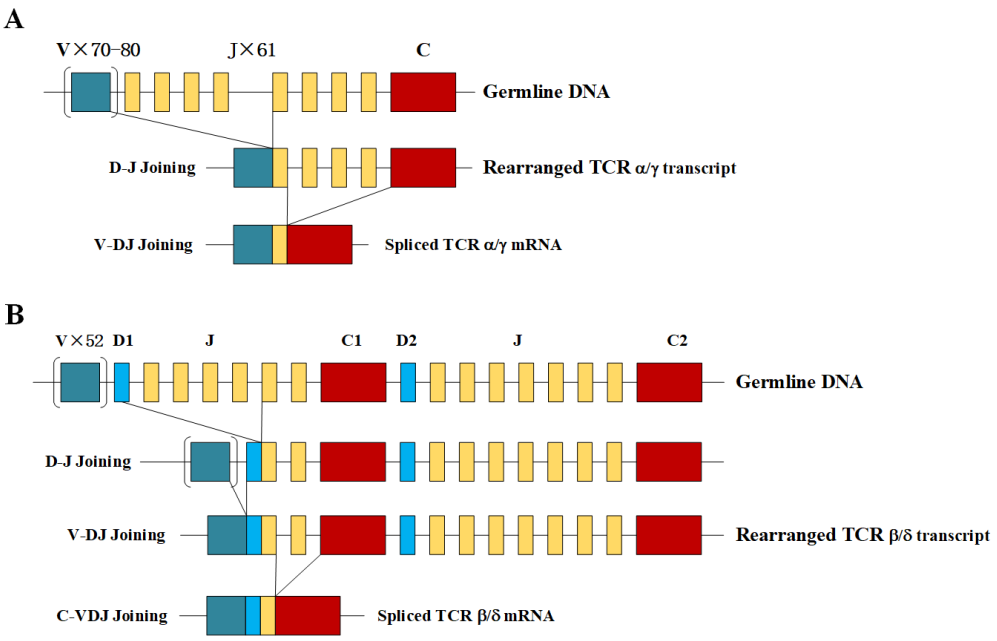


Figure 4 TCR V(D)J regions recombination. A) Alpha and Gamma chains of TCRs. B) Beta and Delta chains of TCRs.

1.6 Dendritic cells

Dendritic cells (DC) were first described in the lymph nodes of mice by the American scholar Steinman in 1973 (Steinman and Cohn, 1973). DCs are known for their dendritic protrusions on the cell membrane during maturation. As its main function is antigen presentation, it is the most powerful antigen presenting cell (APC) in the immune system, binding and presenting antigens to stimulate T cells *in vivo*.

Besides heart and brain, DCs are widely distributed in small numbers in various tissues and organs of the human body. This includes the spleen, lymph nodes, thymus and other lymphoid organs, and non-lymphoid organs such as skin, blood, liver *etc.* (Scandella *et al.*, 2002). The proportion of DCs in human peripheral blood accounts for less than 1% of mononuclear cells (Kassianos *et al.*, 2010).

DCs are mainly derived from bone marrow and lymphoid tissues. Depending on their developmental stage, DCs can be divided into two types: immature and mature DCs. Different developmental stages determine functional properties and cell phenotype of DCs (Shurin *et al.*, 2005). Under normal conditions, most dendritic cells in the body exist in an immature state. Immature DCs overexpress chemokine receptors, complement receptors, macrophage colony-stimulated receptors, and have low or no expression of co-stimulatory molecules such as CD80, CD86 and adhesion molecules (Cravens and Lipsky, 2002; Pletinckx *et al.*, 2011). In addition, immature DCs are less able to stimulate the activation of T lymphocytes, but are effective in antigen uptake and processing. DCs can also migrate into lymph nodes and the spleen via the circulatory and lymphatic systems by altering expression of chemokine receptors on their surface. After reaching maturity by binding to the antigen, antigens are presented to T lymphocytes, enhancing the activity and proliferative ability of the T lymphocytes (Castell-Rodríguez *et al.*, 2017). MHC-II molecules are highly expressed in mature DCs, which bind to the antigenic peptide, thereby providing the first signals for T cell activation; through B7-1/CD80, B7-2/CD86 and CD40, etc. (Fujii *et al.*, 2004). Stimulating molecules and adhesion molecules such as ICAM-1 provide the second signal for the activation of T cells, where mature DCs secrete various cytokines (IL-12, IL-18, IL-15, etc.) (Calmeiro *et al.*, 2018). CD83 and CD25 surface molecules mark the maturation of DCs. It has been shown that mature DCs can effectively activate T cells, thereby initiating an immune response, while the antigen uptake and processing ability is weakened. DCs

use the above-mentioned characteristics for antigen presentation, immunomodulation and immunostimulation. DCs are the only APC that activates unsensitized naive T cells (Münz, 2010).

APCs play an immunoregulatory role in the body, as they can ingest, process antigens, and present the processed antigen to T and B lymphocytes (Rosenberg, 2005). APCs can be divided into two sub-groups; professional APCs and non-professional APCs. Professional APCs express MHC-II molecules, synergistically stimulate signaling molecules, undergo extracellular antigen uptake and processing, activate $CD4^+$ T cells, and induce an adaptive immune response. Cells belonging to this group include DCs, mononuclear-macrophages, endothelial cells, B cells, and Langerhans cells (LC). Non-professional APCs are cells with specialized functions that do not express MHC-II molecules or co-stimulatory signaling molecules, but can be induced to express these molecules under specific conditions, especially during a sustained inflammatory response. These cells consist of mainly endothelial cells, epithelial cells and activated T cells.

Lipid antigens are presented by a distinct family of MHC Class I-like proteins, named CD1. Humans express four CD1 proteins, which are CD1a, CD1b, CD1c and CD1d. CD1a-d are trafficked to the cell surface, where they may display lipid antigens to T-cell receptors. In this study, two APC cell lines, Thp-1 and Mutz-3 were used in experiments, the former derived from mouse and latter from human

DCs. Thp-1 cells express CD1a, CD1b and CD1c receptors on their surface, which can bind with and present antigens to activate T cells (Kasinrerk W, Baumruker T, Majdic O, 1993). Human Mutz-3 cells express CD1a, CD1c and CD1d receptors on their surface (Masterson *et al.*, 2002; Larsson, Lindstedt and Borrebaeck, 2006; Yuan, Song and Jiang, 2012).

DCs initiate an immune response in two aspects. On the one hand, DCs present an antigen to the TCR, which provides a signal to initiate activation. On the other hand, DCs with immunomodulatory effects can induce and maintain autologous foreign antigens, leading to immune tolerance. DC cells are unique in antigen presentation, immune activation and tolerance, which play important roles in health and disease (Mbongue *et al.*, 2014).

1.7 Signalling cascade

Signal transduction pathways are a series of downstream intracellular processes triggered as a result of cell activation through specific ligands, and are crucial for gene expression. Activation of T cells requires co-stimulation, mainly from CD28 and TCR. Although there is still debate to whether the activation can cause multiple signal transductions at the same time, studies have found that the co-stimulation of TCR and CD28 enhances the scale of the immune response (Acuto and Michel, 2003; Cheng, Montecalvo and Kane, 2011).

T cell receptor (TCR) mediated signalling is directly associated with pro-inflammatory responses, where calcium ions (Ca^{2+}) and protein kinase C (PKC) are upregulated, and mTOR activity is suppressed (Bronstein-Sitton, 2006). In T cells, nuclear factor of activated T cells (NF-AT) and nuclear factor kappa B (NF-κB) are two of the main types of activation signalling cascade, and this is determined based on the mediator (Figure 5). Other sensitizers such as Ionomycin, Phorbol myristate acetate (PMA) and Concanavalin A can act as calcium ion carriers for stimulation, bypassing TCRs. Therefore, Calcium signalling regulates various major intracellular activities such as differentiation and cell growth, and causes activation of transcription factors. Within the cell nucleus, gene expression of selected proteins is dependent on mediators that target specific genetic sequences for transcription (Rahman and MacNee, 1998).

NF-AT is a transcription factor that is not only involved in immune regulation but also has a role in controlling angiogenesis, circadian rhythms and tumour metastasis. The calcineurin-NF-AT signalling pathway is calcium dependent. Following the entry of calcium ions through the Ca^{2+} release-activated Ca^{2+} channel (CRAC) into the cell, calmodulin upregulates the phosphatase calcineurin, which activates the NF-AT protein, leading to the transcription of the NF-AT gene positioned upstream of the interleukin 2 (IL-2) promoter (Dolmetsch *et al.*, 1997; Müller and Rao, 2007). The cascade leads to gene transcription, where the NF-AT transcription factor is able to bind to the IL-2 promoter, causing the release of IL-2.

Inflammation is required to reduce or eliminate harmful agents from the body. Therefore, when the body is in a chronic inflammatory state due to illness such as atherosclerosis, it triggers the NF-κB pathway to release cytokines. NF-κB is a transcription factor that is regulated by cell marker CD28, which is the main component of the pro-inflammatory cascade (Fisher *et al.*, 2006). This signalling pathway is primarily driven by diacylglycerol (DAG) and its messenger protein kinase C (PKC). During high levels of intracellular calcium, calcineurin activates kinase IκB Kinase (IKK), which then phosphorylates a protein IκB. IκB is a protein associated with the NF-κB protein, which eventually induces cytokine production (Lawrence, 2009). Although Ca^{2+} signalling is required for NF-κB, the origin and mechanisms of its involvement in this pathway and its role in transcriptional regulation are still poorly defined (Liu *et al.*, 2016).

Transcription within the coding region causes the production and release of cytokines such as tumour necrosis factor-α (TNF-α). At high calcium levels, IKK releases p50/p65 and p50/c-Rel, which are transcription factors within the NF-κB gene, allowing transcription of pro-inflammatory and proliferative genes (Oeckinghaus and Ghosh, 2009).

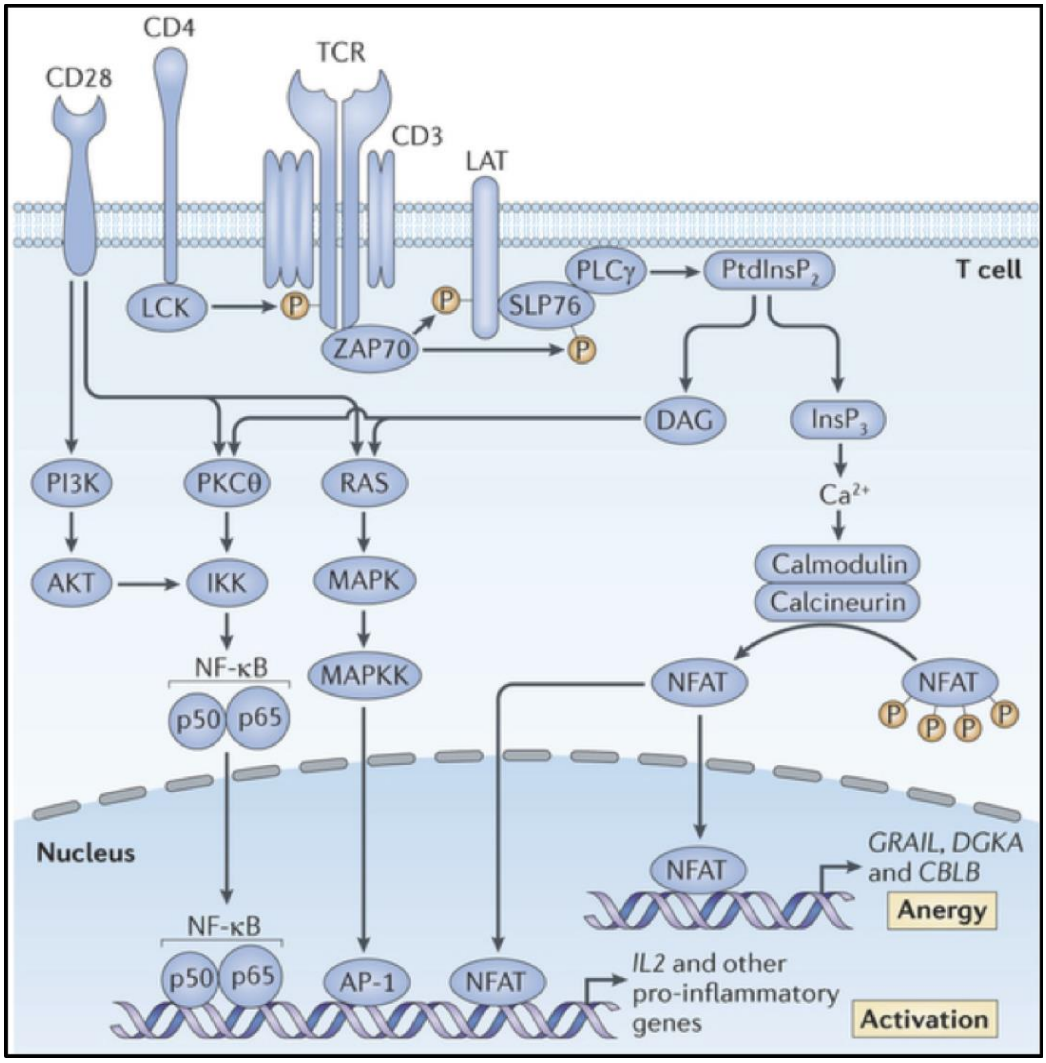


Figure 5 TCR signaling pathways (Pollizzi and Powell, 2014). The activation of Ca^{2+}/calmodulin-dependent signaling pathways and protein kinase C (PKC) activates the nuclear factor of activated T cells (NF-AT) and nuclear factor kappa B (NF-κB) proteins, leading to the transcription of the NF-AT and NF-κB genes positioned upstream of the interleukin 2 (IL-2) promoter, and causing the release of IL-2 and cell activation. When NFAT is activated in the absence of AP-1 activation, the expression of genes such as diacylglycerol kinase-α (DGKA), which relates to anergy in lymphocytes, will inhibit full T cell activation.

Ionomycin, PMA and Concanavalin A can act as calcium ion carriers to induce the production of IL-2 and activate the expression of both NF-AT and NF-κB reporter genes. PMA can be used to activate human T cells via the activation of PKC (Paulsen *et al.*, 2009). Ionomycin, a type of calcium ionophore, can stimulate human T cells by activating the Ca^{2+}/calmodulin-dependent signalling pathways (Chatila *et*

al., 1989). Concanavalin A, a known TCR/CD3 complex ligand, stimulates T cells primarily via the component of the TCR/CD3 complex (Miao *et al.*, 2005) (Figure 6).

Lymphocytes produce some cytokines which contribute to stimulation of their differentiation. Generally, cytokines are produced by the activation of T-cell transcription factors. The expression of two T-cell transcription factors, T-box transcription factor TBX21 (T-bet) and trans-acting T cell-specific transcription factor 3 (GATA-3), is considered to be important in allergy study. T-bet is an important Th1 cell transcription factor. It plays a decisive role in the differentiation of Th1 cells (Szabo *et al.*, 2000). GATA-3 plays an important role in the differentiation of Th2 cells. It is a specific transcription factor for Th2 cells, which can promote the differentiation of Th2 cells from the differentiated or differentiating Th cells (Pai *et al.*, 2008).

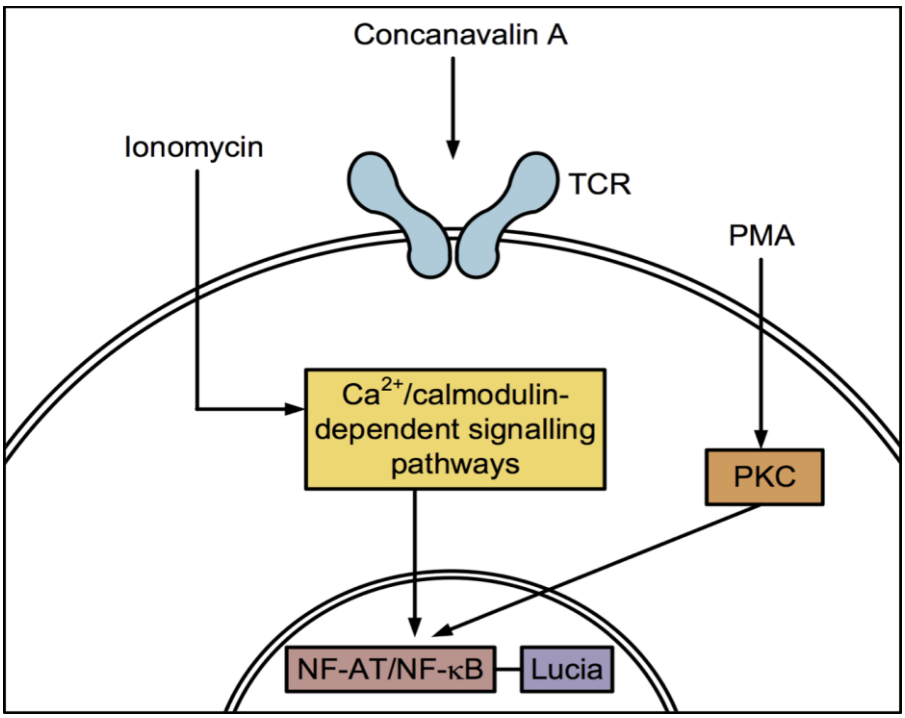


Figure 6 Multiple regulations occur in the NF-AT/NF-kB pathways by T cell stimulators.

1.8 Luminescence

A reporter gene assay is a highly sensitive and reproducible technique that involves the detection of successful transcription at the targeted region. It mainly uses a biomarker that is activated upon a chemical reaction, producing an observable and quantifiable result. Although immunological techniques are relatively accurate, a reporter gene assay is a rapid method of producing results as it could measure the outcome directly from its source upon stimulation. Luciferase is one of the enzymes usually derived from fireflies that catalyse an oxidative reaction of the substrate, luciferin, to produce oxyluciferin (Nakatsu *et al.*, 2006; Lomakina, Modestova and Ugarova, 2015). This reaction emits light at wavelengths of 470 nm to 700 nm, which can be read by a luminometer. Measuring luciferase activity can be performed

in vivo for xenografts in tissue samples and *in vitro* for luciferase-transduced cells.

In the Lucia™ Luciferase assay (QUANTI-Luc™), a synthetic promoter fused to a completely novel secreted luciferase expressed reporter gene from marine planktons and crustaceans expressing luminescence has been cloned (InvivoGen, 2012a). Lucia luciferase has been engineered for its superior bioluminescence signal. Lucia luciferase can develop a strong signal than the ordinary luciferase, where even a minute amount of luminescence can be detected and measured. The principle of a luciferase reporter assay is shown in Figure 7 (InvivoGen, 2012b).

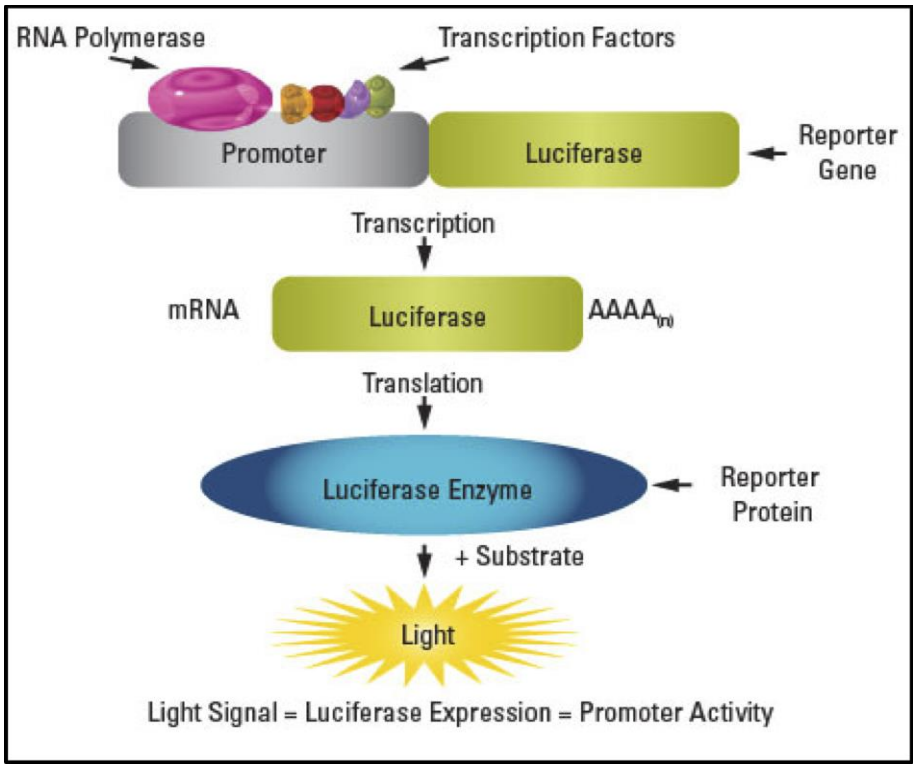


Figure 7 The principle of a luciferase reporter assay (ThermoFisher). This reporter assay was used to detect the activation of surrogate cells.

In a stable transfection, the luciferase reporter gene is incorporated into the cell's chosen DNA sequence. Following the activation of associated responsive elements

and promoters, it will cause gene expression and the production of luminescence which can be measured at 450 nm using a plate reading spectrometer. Therefore, it is particularly effective in measuring cell activation and effectiveness in transcription or knockout genes, and provides information on cell signalling pathways.

1.9 Next Generation sequencing (NGS)

When addressing the sequencing industry development, it is critical to recognize the three major breakthroughs in sequencing technology. The First-generation Sanger sequencing technology is based on Sanger dideoxy chain-termination method to carry out DNA sequencing. The first sequencer became commercialized in 1986, shortly followed by the Human Genome Project (HGP) as one of the major milestones. The assembly of the Human Genome took scientists around the globe almost a decade to complete. Since Sanger sequencing is low-throughput, expensive and time consuming, ambitious projects such as the HGP would not be reproducible in large-scale sequencing projects involving genomes of other organisms (Heather and Chain, 2016).

Twenty years following the release of First-generation sequencing, a revolution took place in sequencing technology. Starting from 2005, the release of Roche's 454, Illumina's Solexa and Life Technologies' Solid became the Second-generation high-

throughput sequencing platform. Second generation high-throughput sequencing involves sequencing by synthesis (SBS) technique, where light emitted from fluorescent-tagged nucleotides on PCR clusters is captured and translated into sequencing information. In comparison with Sanger sequencing, Second-generation sequencing provides higher throughput, lower costs, and is immediately available for researchers. However, errors in short reads from Second-generation high-throughput sequencing technology are increasingly problematic in scientific and medicinal genomic project research (Buermans and den Dunnen, 2014).

In 2011, PacBio launched PacBio RS single molecule real-time sequencer to mark the beginning of Third-generation Single Molecule Real Time (SMRT) technology. PacBio RS single molecule real-time sequencing is conducted through Nanopore technology. Signal detection is achieved through combining fluorescent-tagged phosphonucleotides with modern light capture technology during synthesis, converting signals to sequencing information. Its main advantage is producing long reads of 10 kb in length or more, which is comparatively longer than Illumina (Jain *et al.*, 2018).

In 2014, Oxford Nanopore Technology (ONT) launched single molecule real time sequencers MinION. Nanopore technology is based on capturing changes in electric currents from passing single polymers through nanopores and converts to nucleotide information. This technique produces longer reads compared to PacBio (Deamer,

Akeson and Branton, 2016).

Nanopore sequencing technology, also known as Fourth-generation sequencing, has recently been widely accepted and its use extended to many areas of science. This technique relies on a large number of single-molecule DNA or RNA being driven through pores by guide proteins in real-time. The flow of different nucleotides causes changes in electrical current, which are detected and read as a sequence. As polymerase chain reaction (PCR) is not required in this sequencing technique, so problems with DNA polymerase denaturation are eliminated. DNA molecules are theoretically able to pass through the Nanopore continuously if the strands are constantly separated, and currently Nanopore sequencing could reach reads up to 150 kb (Magi *et al.*, 2018). This technology has undergone three major advances; single DNA nucleotide flow through, precise detection of single nucleotides from enzyme on Nanopore, and precise control of single nucleotide sequencing.

The MinION Nanopore sequencer from ONT is a Nanopore sequencing platform that has now been widely accepted (Figure 8). The MinION Nanopore sequencer's core consists of 2048 Nano holes (pores) organized into 512 channels, all of which are controlled by specialized electronic flow cell. Each nucleotide produces a different frequency and amplitude in current signalling, and these changes are captured during the movement of the single strand DNA or RNA through the Nanopore (Lu, Giordano and Ning, 2016). The principle behind Nanopore

sequencing is the administration of an ionic current while the polymer strand travels through a nanometer-sized hole (nanopore), and the sequence is determined by fluctuations in current measured from passing DNA or RNA nucleotides. Unlike other sequencing techniques, Oxford Nanopore's technology does not require DNA being cleaved into fragments or PCR amplification. With a portable and affordable handheld device, it enables long and quick reads of up to 2Mb of nucleotides. Also, this model could directly read RNA and cDNA, allowing transcriptomes to be sequenced in full length. Oxford Nanopore's technology platform exceeds other sequencing platforms in the following ways; long read length enables sequencing of microorganism genomes, real-time detection for immediate analysis, and portability for convenience (Jain *et al.*, 2018). At present, there is no alternative for a portable sequencer device available on the market.

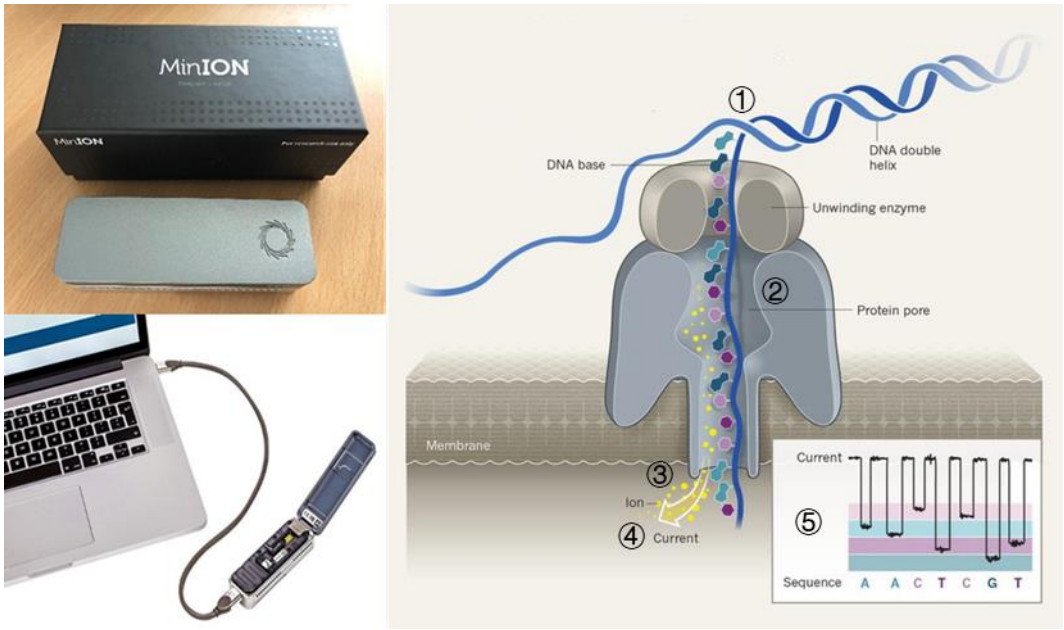


Figure 8 Principles of Nanopore sequencing (Eisenstein, 2017). 1. DNA is split into single strands using Helicase. 2. DNA single stranded nucleotides flow through Nanopore protein, where an adaptor protein captures nucleotide to be read. 3. Each DNA nucleotide causes a distinct change in ionic current as it passes through the Nanopore protein. 4. Adaptor protein detects 5 different changes in ionic current. 5. Sequence information is obtained using graphical models and algorithms according to the changes in current (Eisenstein, 2017).

Chapter 2 Aims and objectives

Lipids and non-peptide ligands can bind to MHC-like CD1 molecules to activate unconventional T cells. The glycolipid α-GalCer has been shown to stimulate NKT cells after it has been presented via CD1 by APCs. NKT cells, a sub-class of unconventional T-cells, can bind to hydrophobic antigens. Once activated, the NKT cells secrete cytokines such IL-2.

As briefly outlined above our previous work strongly suggested that natural hydrophobic ligands co-presented via CD1 to non-conventional T cells play a critical role in the development of the allergic response. Therefore, the aim of this work is to utilize state-of-art *in vitro* techniques to generate transiently transfected, stable human cell lines to assess the ability of natural antigens from a variety of sources to act as ligands in the activation of non-conventional T cells. The proposed high throughput system should be stable, reproducible, robust and sensitive to act as a replacement for animal experiments. The specificity of such activation will be established with a view to identifying lipids for further consideration as potential drivers of allergic sensitisation. Such techniques will also enable other materials of interest, e.g. selected surfactants, to be assessed with regards to immune stimulation and potentially provide a screening assay to enable selection of commercially interesting materials with a reduced risk of immunomodulation.

In order to achieve these, aims and objectives were:

1. To develop *in vitro* techniques to use stable human cell lines to generate

transiently transfected cells for the assessment of natural lipids to act as ligands in the activation of non-conventional T cells;

2. To identify lipid-binding NKT cells from primary human cultures using a single cell system and NGS with the aim of isolating and characterizing lipid-binding TCR activation

3. To express lipid-specific TCRs in surrogate stable human cell lines in order to facilitate construction of a cell screening lipid library.

Chapter 3 Developing a reporter based assay for lipid-binding TCRs

3.1 Introduction

In previous studies our group has shown that Brazil nut lipids were required during the sensitization phase with Ber e 1 (Dearman, Alcocer and Kimber, 2007). The requirement for natural lipids from nuts as a critical component for the intrinsic allergenicity of Ber e 1 was further demonstrated when human T-cell lines derived from nut allergic patients were shown to produce IL-4 to Ber/lipid C in a CD1d dose dependent manner. Jα18 and CD1 knock out experiments further implicated natural killer T (NKT) cells in the response (Mirotti *et al.*, 2013). Altogether, these results suggest that NKT cells play a critical role in the Brazil nut sensitization phase of susceptible individuals.

In order to set an *in vitro* system to characterise the dialogue between lipids and human NKT, a reliable cell line and stable system able to respond to a plethora of hydrophobic compounds and TCRs must be in place. Unfortunately, human NKT cell lines are not available. Therefore, in this chapter, the main aim is to develop a surrogate reporter system. Here a mammalian expression vector was constructed containing, synthesised α and β chains of the TCR (pMJA219: TRAV10/TRBV25) which bind to a well described lipid (α-GalCer) and transfected a range of host mammalian cell lines (Jurkat Lucia and Jurkat Dual) to observe activation upon lipid binding. Both Jurkat Lucia and Jurkat Dual cell lines are TCR negative.

In this chapter the commercial availability of some reporter cell lines such as Jurkat Lucia (InvivoGen) were explored. These cell lines contain a stable integration of an inducible Luciferase reporter construct driven by DNA specific sequences. The Jurkat-Lucia™ NF-AT encodes Luciferase that is highly secreted and driven by an ISG54 minimal promoter fused to six copies of the NF-AT consensus transcriptional response element (InvivoGen). Another cell line described here is the Jurkat Dual IRF (interferon regulatory factor) and NF-κB cell system (InvivoGen). The Dual system contains IRF activation sequences fused to the secreted embryonic alkaline phosphatase (SEAP) and NF-κB activation sequences fused to the Luciferase (InvivoGen). Levels of Lucia luciferase are easily measurable in the cell culture supernatant when using QUANTI-Luc™, a specific and sensitive Lucia luciferase detection reagent.

3.2 Materials and methods

3.2.1 Blood

Blood was obtained from one healthy volunteer and obtained through the Biobank under the ethical medical licence BS25062015.

3.2.2 Cells and Reagents

Jurkat Lucia cells (human NF-AT-Luc reporter T cells) and Jurkat Dual cells (human

IRF-SEAP and NF-κb-Luc reporter T cells) were bought from InvivoGen. Thp-1 (human monocyte/macrophage cell line) was purchased from ATCC. Mutz-3 cell line (human DC cell line) and 5637 cell line were obtained from DSMZ. Roswell Park Memorial Institute (RPMI) 1640 media, α-Minimum Essential Media (MEM), sodium pyruvate, L-Gluthamine and fetal bovine serum (FBS) were purchased from Gibco. Zeocin and blasticidin were purchased from Thermo Fisher Scientific. Fugene® HD Transfection Reagent was bought from Promega. Sodium pyruvate and L-Glutamine were purchased from Gibco. Murine granulocyte-macrophage colony stimulating factor (GM-CSF) was bought from Sigma and human GM-CSF was purchased from PEPROTECH. PMA, Ionomycin, Concanavalin A, Penicillin-Streptomycin (Pen-Strep), hydroxyethyl piperazineethanesulfonic acid (HEPES), α-Mercaptoethanol and Histopaque were purchased from Sigma. QUANTI-Luc™ was bought from InvivoGen. The eBioscience™ Human IL-2 ELISA Ready-Set-Go!™ Kit (2nd generation) was bought from Fisher Scientific. Avidin-horseradish peroxidase (HRP) is used for indirect enzymatic labelling, in conjunction with biotinylated antibodies. Tetramethylbenzidine (TMB) acts as soluble substrates to yield a colorimetric reaction. Mouse anti-human Cluster of differentiation 3-Fluorescein isothiocyanate (CD3-FITC), mouse anti-human CD56-R Phycoerythrin (CD56-PE), mouse anti-human CD69-Phycoerhthrin-Cy5 Conjugate (CD69-PC5) and mouse anti-human Vα24-PE antibody were from Beckman Coulter.

3.2.3 Plasmids

The pMJA219 (TRAV10/TRBV25) plasmid (Figure 9) was designed in our lab. A synthetic expression DNA plasmid (pMJA219, GenBank MH782476) containing TCR receptors has been synthesised by Geneart (Invitrogen) based on the backbone of the mammalian expression plasmid pcDNA3.1(+) (Invitrogen). pMJA219 (TRAV10/TRBV25）is driven by the bidirectional Cytomegalovirus promoter from pBI-CMV1 (Clontech) and flanked by the rabbit β-globin and bGH (bovine Growth Hormone) polyadenylation sequences at the 3' end of TRBV25 and TRAV10 sequences respectively. The complete TCR expression plasmid contains the human α-GalCer responsive TRBV25 (clone BM2a.t, GenBank: DQ341454.1) and TRAV10 (clone J3N.5T, GenBank: DQ341448.1) cDNA complete sequences without introns (Brigl *et al.*, 2006). pMJA219 (TRAV10/TRBV25） carries selectable markers for Ampicillin for Escherichia coli and G418 (Geneticin) for mammalian cells.

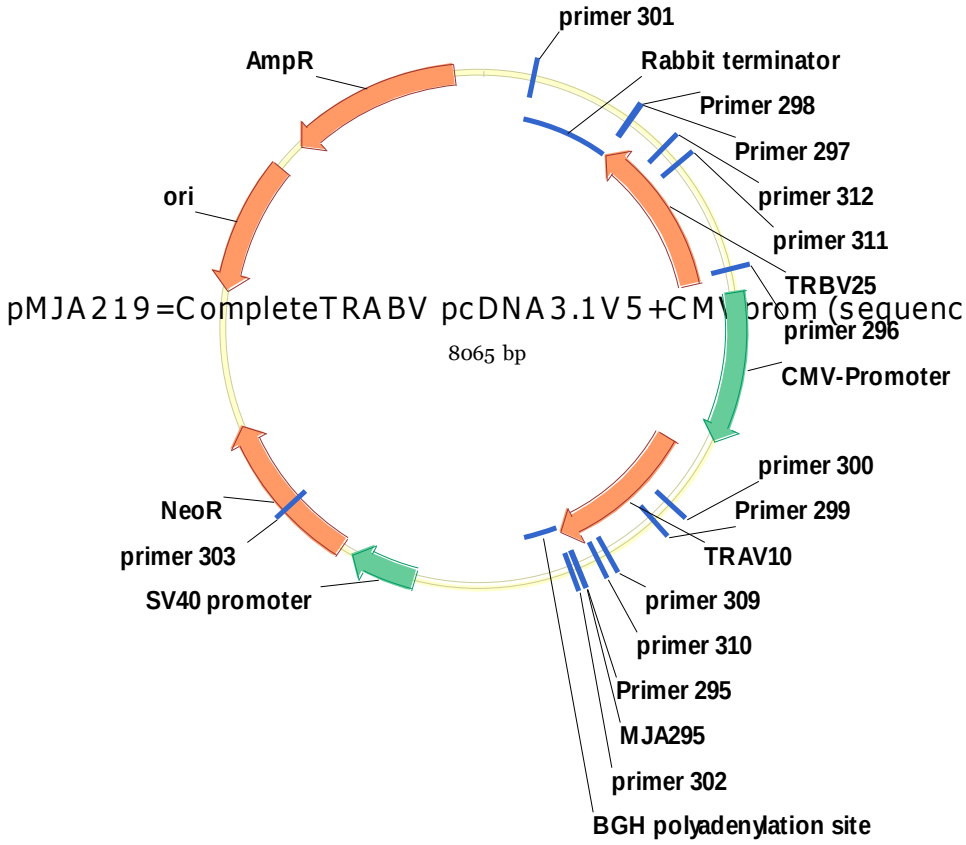


Figure 9 Plasmid map of pMJA219 contains TCR TRAV10/TRBV25 genes (GenBank MH782476).

3.2.4 Cell culture

Jurkat Lucia cells and Jurkat Dual cells were cultured according to the manufacturer's instructions (InvivoGen). Jurkat Lucia cells were cultured in RPMI 1640 10 % FBS and 100 µg/ml Zeocin. The media was changed every three days, by switching between the media with Zeocin and without. Jurkat Dual cells were grown in RPMI 1640 10 % FBS, 100 µg/ml Zeocin, 10 µg/ml Blasticidin and 1 % Pen-Strep. The media was changed every three days, by switching between the media with antibiotics (Zeocin, Blasticitin and Pen-Strep) and without. Mutz-3, a human DC line, was cultured according to the DSMZ instructions. The conditioning

media for Mutz-3 cell growth was provided by the 5637 cell line. The 5637 cell line (cultured in media also described by DSMZ) was produced by allowing the cells to reach a confluency of approximately 80 % - 90 %, and then replenish the cells with fresh media. After 24-hour incubation, the media was collected, filtered, and stored at -20°C. This conditioned media was used to grow Mutz-3 cells. Mutz-3 cells were cultured in α-MEM 20 % FBS, 20 % conditioned media and 5 ng/ml human GM-CSF.

3.2.5 Activation by T-cell stimulators

In this Chapter, all experiments were set up in triplicate wells and repeated twice. 96-well round-bottomed plates were used to activate TCRs by incubating the cells (Jurkat Lucia and Jurkat Dual) with T-cell stimulators (Ionomycin, PMA and Concanavalin A). Cells (4×10^5) were added in each well in 150 µl of specific cell medium. For each well, according to the final concentration of stimulators, a certain volume of Ionomycin, PMA and Concanavalin A was mixed with RPMI media to a total volume of 50 µl, and the mixture added into the correct well to a total final volume of 200 µl. The cells were incubated with Ionomycin, PMA and Concanavalin A in a 96-well round-bottomed plate for 24 h in 37°C and 5 % CO_2. After 24 hours, 120 µl supernatant of each well were collected for measuring T-cell activation by Luminescense (secreted luciferase) and IL-2 by ELISA.

For measuring Luminescence, an Optiplate 96-well plate was used. 20 µl sample and 50 µl QUANTI-Luc solution were pipetted into wells, mixed and immediately measured on a luminometer (Turner Biosystems). After measuring, the data was analysed on excel. Standard deviation was used to calculate statistical significance between the means which are presented as error bars and T test used to determine P-value. $P<0.05$ is considered statistically significant, $p<0.01$ is considered highly statistically significant and $p<0.001$ is considered extremely statistically significant.

IL-2 was measured essentially as described by the manufacturer of the human Ready-Set-Go! Kit in a 384-well ELISA plate. The ELISA plate was coated with 30 µl 1x coating antibody and incubated overnight at 2-8°C. The plate was washed 3 times with PBS containing 0.05 % Tween-20 the next day (wash buffer). Afterwards, the plate was blocked with 60 µl 1x blocking buffer for 1 hour at room temperature. The plate was aspirated and washed again with wash buffer. The standards were made from the supplied lyophilized standard in a dilution series. 30 µl samples and standard samples were pipetted and incubated at room temperature for 2 hours. Then, 30 µl 1x detection antibody was added into the wells and incubated for 1 hour at room temperature, the plate was washed again using wash buffer. After the plate was washed again, 30 µl 1x Avidin-HRP was pipetted into the wells and incubated for 30 min at room temperature. The plate was washed again and 60 µl TMB was added into each well at room temperature for 15 minutes, then 30 µl 2 M H_2SO_4 (stop solution) was added to each well. The absorbance was measured at 450 nm

using the plate reading spectrometer. After measuring, the data were loaded to excel for analysis. Standard curve was used as the comparison with the absorbance results. Standard deviation was used to calculate statistical significance between the means which are presented as error bars. P values indicate significance level as assessed by T test. $P<0.05$ is considered statistically significant, $p<0.01$ is considered highly statistically significant and $p<0.001$ is considered extremely statistically significant.

3.2.6 Transfection and co-culture

96-well round-bottomed plates were used to monitor the time course expression of TCRs in T cells. A number of cells (4×10^5) were added in each well in 100 µl of specific cell medium. Fugene reagent was warmed at room temperature before using. The volume of RPMI media used varies depending on the volume of pMJA219 (TRAV10/TRBV25) plasmid, which was combined with Fugene reagent totalling 50 µl. First, RPMI media was added in each tube, followed by the addition of 3 µl Fugene, and the mixture was incubated for 5 min. Afterwards, 1 µg plasmid was pipetted into the RPMI-Fugene mixture and incubated for 30 mins. After the incubation, the transfection was added to each well in the 96-well round-bottomed plate and incubated at 37°C, 5 % CO_2.

The next day, 4×10^5 APCs (Thp-1 or Mutz-3) in 30 µl of cell culture media were added in each well. Then, 20 µl of 10 µg/ml lipid (α-GalCer) were pipetted into each

well with APCs and incubated for 4 hours at 37°C. The concentration of lipids used in co-culture experiments was optimized by Dr Xiaowei Wang. The total volume of APC and lipid was fixed to 50 µl. After 4 hours' incubation, 50 µl of cells and lipids were pipetted into 96-well round-bottomed plate with the transfected cells. Finally, each well containing the lipid loaded APCs and transfected human T cells should have 200 µl suspension in total. The 96-well round-bottomed plate was incubated for 24 hours at 37°C, 5 % CO_2.

After 24 hours, 120 µl supernatant were collected from each well and Luminescense and IL-2 measured as described above.

3.2.7 Isolation of primary NKT cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood from one healthy volunteer on Histopaque. 10-20 ml of blood were diluted 2:1 in RPMI 1640. A volume of 5 ml histopaque per 15 ml blood/RPMI 1640 mixture were added and centrifuged for 22 min at 800 g. The PBMC layer formed between the serum and Histopaque interface was collected using a Pasteur pipette, washed in RMPI 1640, and centrifuged for 10 min at 300 g. PBMCs were re-suspended in RPMI 1640 10 % FBS and 0.1 % Dimethyl sulfoxide (DMSO) and total number was calculated using a hemocytometer.

3.2.8 Flow cytometric analysis

Cells in each well were harvested into each fluorescence-activated cell sorting (FACS) tubes, 2 ml of PBA (500 ml phosphate buffered saline (PBS) containing 30 % bovine serum albumin (BSA) and 20 % Sodium Azide) was added and centrifuged for 5 min at 300 g. Supernatants were discarded and 5 µl antibody (mouse anti-human Vα24-PE, mouse anti-human CD3-FITC, mouse anti-human CD56-PE or mouse anti-human CD69-PC5) was added according to the manufacturer's instructions and incubated at 4°C for 30 min in the dark. Afterwards, 2 ml of PBA was added to each tube and the cells centrifuged for 5 min at 300 g. Supernatants were discarded, and the pellet re-suspended in 0.5 ml 2 % formaldehyde. Analysis that did not require subsequent expansion of cells was carried out by fixing the cells in a final step using formaldehyde, whereas in cases where cells were sorted for re-culturing, all antibodies staining was carried out with live cells and the final fixation step was omitted. Analysis was carried out on the FC500 at the Flow Cytometry Facility (University of Nottingham). After measurement by FACS machine, the resulting data were processed on Kaluza software for plots and statistics.

3.3 Results and discussion

3.3.1 Develop a reporter based assay for human T cells (Jurkat) expressing lipid-binding T cell receptors (TCRs) (NF-AT and NF-κB)

As described in the introduction of this chapter, the availability of some commercially available reporter cell lines such as Jurkat Lucia (Invivogen) was explored. The Jurkat-Lucia™ NF-AT encodes Luciferase that is driven by an ISG54 minimal promoter fused to six copies of the NF-AT consensus transcriptional response element. Jurkat Dual IRF&NF-κB cell system (Invivogen) contains IRF and NF-κB inducible reporter constructs.

In an initial exploratory experiment both cell lines showed activation (luciferase secretion) when induced by Ionomycin, PMA and Concanavalin A. *In vivo* human T cells are known to release IL-2 and other cytokines when induced by these chemicals (Cameron *et al.*, 2015). As shown in Figure 10, a mixture of PMA and Ionomycin seems to work better than PMA and Concanavalin A for activating Jurkat Lucia cells. PMA and Ionomycin have previously been shown to have a synergistic effect (Chatila *et al.*, 1989).

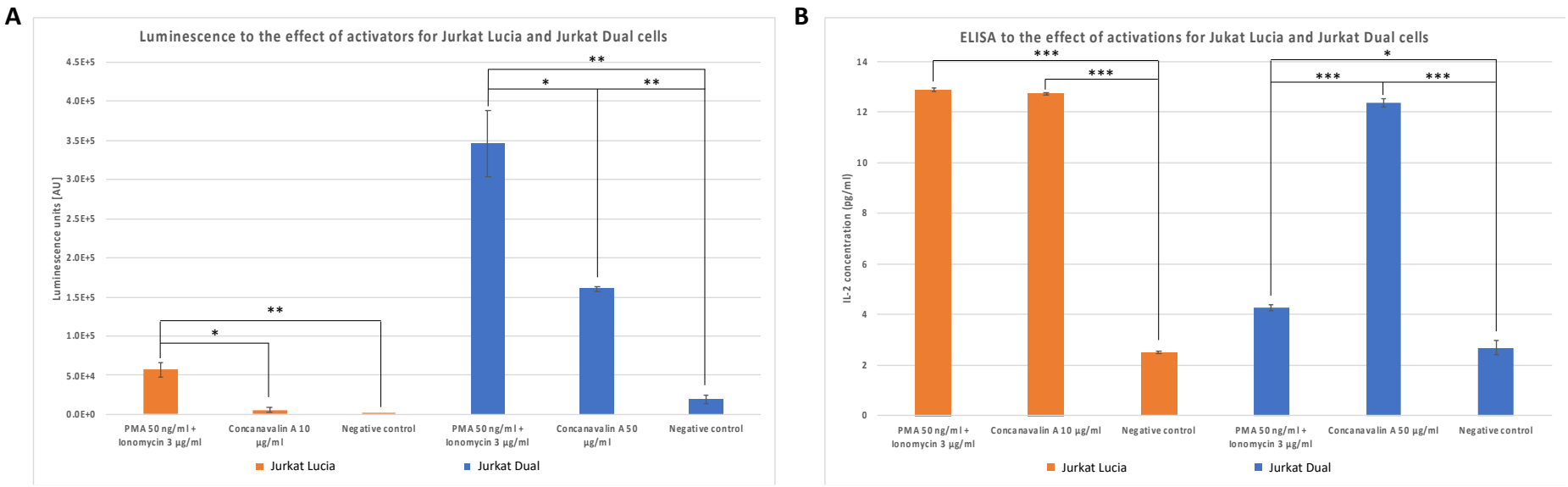


Figure 10 Characterization of the activation of Jurkat Lucia and Jurkat Dual host cell lines in the presence or absence of known T-cell stimulators. A) expression of the luciferase gene, B) production of IL-2. Results are averaged from triplicate wells. (*=p <0.05, **=p <0.01, ***=p <0.001)

Jurkat Dual cells show a significantly ($P<0.05$) higher luminescence when stimulated with PMA and Ionomycin compared to Concanavalin A (Figure 10 A). Contrastingly, ELISA results showed that the Concanavalin A has three-fold higher significant ($P<0.001$) increase in absorbance when compared to PMA and Ionomycin (Figure 10 B). This suggests that different pathways are activated during this process with PMA and Ionomycin activating the luciferase gene in Jurkat Dual cells, whilst Concanavalin A targets the release of the IL-2. Figure 10 A also shows that the luminescence reading of Jurkat Dual cells is significantly ($P<0.01$) higher than Jurkat NF-AT Lucia cells after stimulation of all activators.

3.3.2 Use of different T-cell lines to express recombinant lipid-binding TCRs and optimize the mammalian expression vector containing pMJA219 (TRAV10/TRBV25)

In order to demonstrate whether a new function could be assigned to these reporter cells, the Jurkat Lucia and Jurkat Dual cells were transfected with a TCR expression construct (pMJA219: TRAV10/TRBV25). These TCR genes were previously described as specific for α-GalCer (Brigl *et al.*, 2006). In order to be expressed in equimolar quantities, a CMV commercial bidirectional promoter was introduced in the designed pMJA219 (TRAV10/TRBV25) both genes flanking. The transfected cell lines were then co-cultured with Mutz-3 that were pre-loaded with α-GalCer. As shown in Figure 11, transfected Jurkat Lucia exhibited the highest luminescence

in the presence of lipid-presented APCs. Without lipid the luminescence decreased by around 50%. The reporter Jurkat Lucia NF-AT cells with the recombinant TCR genes showed a significantly ($p<0.05$) higher luminescence compared to the non-transfected cells co-cultured with α-GalCer.

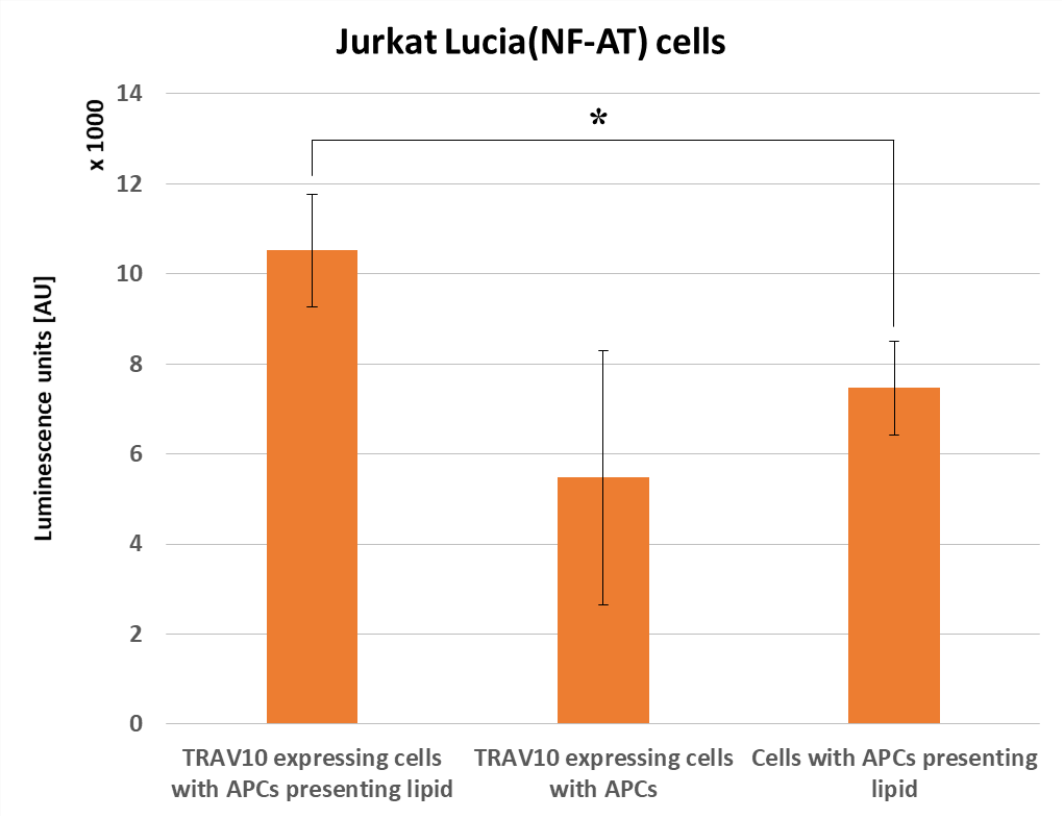


Figure 11 Bar chart showing luminescence assay of transfected Jurkat NF-AT Lucia cells co-cultured with APCs (Mutz-3) loaded with lipid. Results are averaged from triplicate wells. (*=p <0.05)

Jurkat Dual cells (IRF&NF-κB) were also responsive when transfected with TCR TRAV10/TRBV25 expression plasmid (pMJA219) and co-cultured with the human Mutz-3 cells (APCs) presenting α-GalCer (lipid) (Figure 12). Transfected Jurkat Dual cells also had a significantly higher ($P<0.01$) luminescence reading in the presence of lipid-loaded APCs compared to non-transfected cells that are co-

cultured without lipid. By comparing Figure 11 and Figure 12, the luminescence reading of Jurkat Dual cells was higher than Jurkat Lucia cells after stimulation.

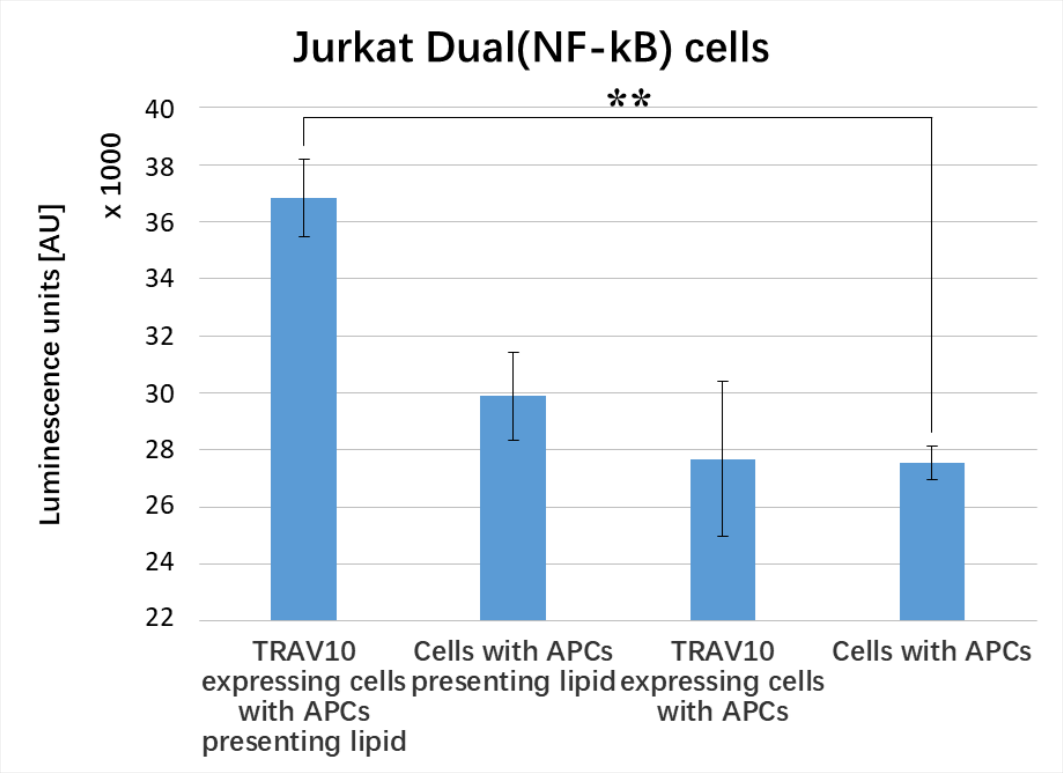


Figure 12 Bar chart showing luminescence assay of transfected Jurkat Dual cells co-cultured with APCs-loaded with lipid. Results are averaged from triplicate wells. (**=p <0.01)

Taken together these initial results have demonstrated that co-culturing T cells with APCs presenting lipid antigens on the CD1 molecule can lead to activation of the luciferase reporter gene in the transfected Jurkat Lucia and Jurkat Dual T cell lines. Further it showed that a functional lipid responding TCR plasmid can be engineered. Compared with the inducing T cell stimulators such as PMA, Ionomycin and Concanavalin A, the luminescence units obtained from co-culture experiments were much smaller. This might be due to T cell endosomal trafficking, where TCRs are internalized and recycled continuously, particularly in unstimulated cells (San José

and Alarcón, 1999; Cesari, 2009). Therefore, during co-culture there may be down-regulation in TCRs, leading to the inability to bind to CD1 on APCs, causing low luminescence units. This suggests that optimisation of co-culture parameters may be required to improve the internal signal of the reporter construct in Jurkat Lucia and Jurkat Dual cells.

Significantly non-transfected Jurkat Lucia and Jurkat Dual cells that were co-cultured with lipid-presented APCs exhibited higher luminescence compared to those without lipid, suggesting that lipids may be involved in activation of Jurkat cells through Ca^{2+} signalling or other mechanisms.

It is important to register that for co-cultures of both Jurkat Lucia and Jurkat Dual cells with lipid-presented APCs, there were many inaccuracies in terms of measuring luminescence units. The experiments were repeated multiple times, but resulted in inconsistent luminescence readings. This may be due to background noise, Jurkat passage number and/or some clustering effect.

3.3.3 Use different APCs to present lipids for expressing recombinant lipid-binding TCRs

The loading of the lipid to CD1 on APC has been described by others (Le Nours *et al.*, 2016). In order to validate the use of the DC cell lines in the development of the lipid responding system, some experiments were carried out with primary human

PBMC cells and FACS isolated from one healthy volunteer. It is important to emphasise that the design and execution of the FACS experiments were carried out mainly by Dr Ashfaq Ghumra a postdoctoral researcher during my training period.

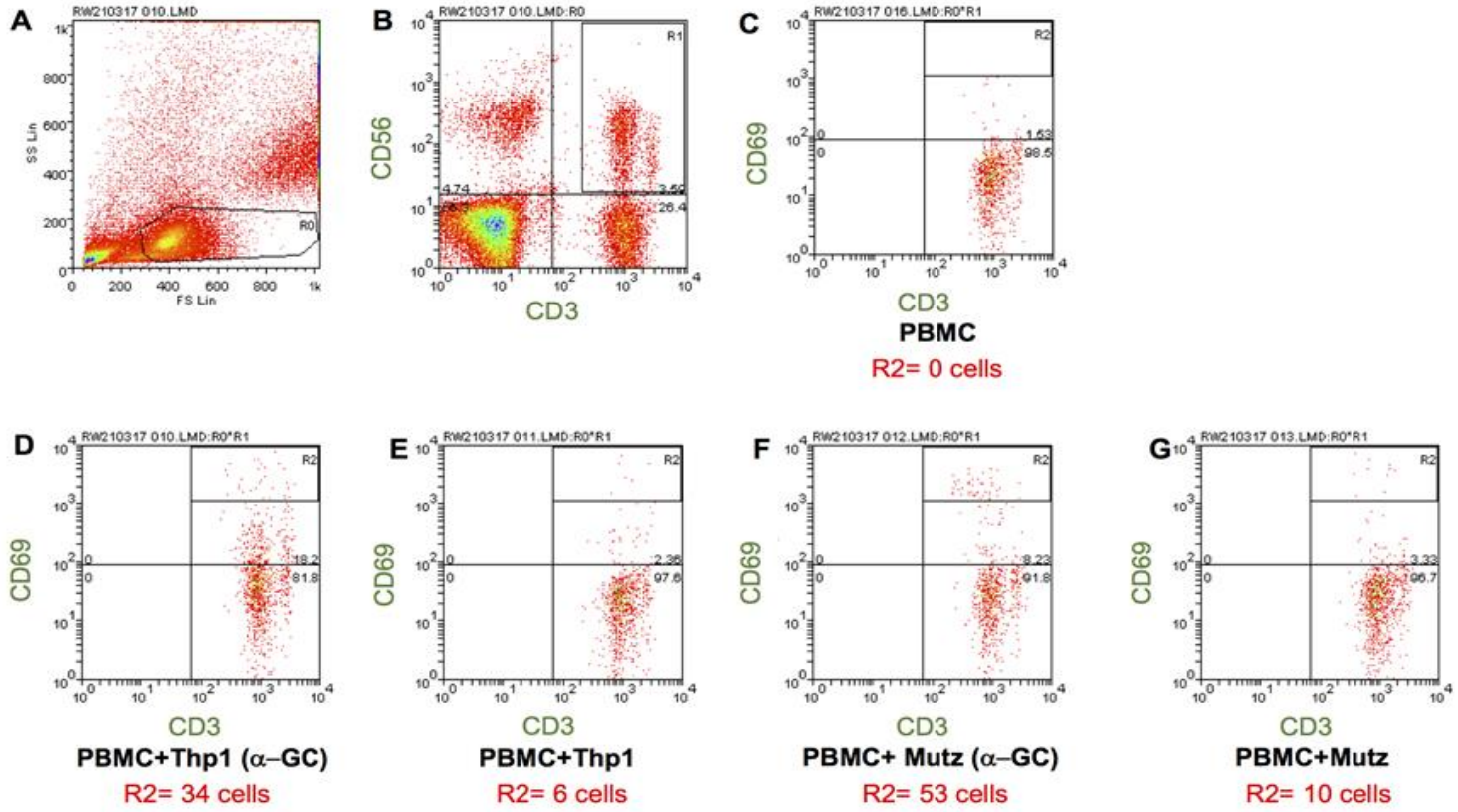


Figure 13 Gating strategy on PBMCs to enrich CD3+CD56+CD69+^{high} NKT cells and percent activated cells following presentation of α–GalCer to NKT cells by Thp1 or Mutz-3 antigen presenting cells. A) Dot plot showing PBMCs, with the lymphocyte gate (R0). B) CD3+CD56+ lymphocyte gate (R1). C) CD3+CD56+CD69+^{high} lymphocytes gate (R2). D) to G) CD3+CD56+CD69+^{high} lymphocytes (R2) after co-culture conditions shown. Results are representative of three repeat experiments. Cell numbers show mean of three repeats.

According to Figure 13 A, lymphocyte-gated events (R0) were collected. The CD3+CD56+ cells purified from all lymphocytes are shown in Figure 13 B (R1). Activated primary NKT cells have markers CD3+CD56+CD69+, therefore cells were further isolated from the R1 gate in different conditions. In Figure 13 C, a gate was established (R2) as a standard to determine the activated cells at different conditions. Figure 13 D and E indicate a gate based on PBMC control was applied to primary NKT cells co-cultured with Thp-1 loaded with or without α-GalCer. There were 34 activated cells within the gate with α-GalCer (Figure 13 D), and 6 activated cells without α-GalCer (Figure 13 E). Figure 13 F and G show the activated population of primary NKT cells co-cultured with Mutz-3 loaded with or without α-GalCer. There are 53 activated cells from the co-culture with α-GalCer (Figure 13 F), and 10 activated cells from co-culture without α-GalCer (Figure 13 G).

Whether the APC cell line is Thp-1 or Mutz-3, the overall results indicated that a higher number of activated cells for co-cultures incubated with α-GalCer is observed, compared with those without α-GalCer. Comparing the primary NKT cells co-cultured with α-GalCer-loaded Thp-1 or Mutz-3, it is observed that co-cultures with the latter has a much higher number of activated primary NKT cells, demonstrating an increased ability for activation. The human DC-Mutz-3 therefore was used in all subsequent work described hereafter.

There is a clear indication that co-cultures without α-GalCer still activate primary NKT cells (shown in gate R2 in Figure 13 E and G). This may be due to the ability of CD69 to be upregulated by autoreactive stimulation. NKT cells are innate-like T cells that recognize specific microbial antigens and also display auto-reactivity to self-antigens, and do not require TCR stimulation (Wang *et al.*, 2008). Basal activation of NKT cells may allow priming of these cells for fast activation upon a signal (Wang *et al.*, 2008). However, the mechanisms behind NKT cell auto-reactivity activation are not yet fully understood. In the current laboratory conditions, the number of activated primary NKT cells were low even in the presence of lipids. There is a possibility that the lipid concentration was too low for activation, or perhaps they were not properly presented by the APCs. Other studies using unconventional T cells also have shown a low number of activated cells, and this appears to be a normal phenomenon *in vitro* (Sánchez-Rodríguez *et al.*, 2011; Sbihi *et al.*, 2017). In an *in vivo* system, it is recognised that the human body is regulated through homeostasis, therefore a massive number of activated T cells can have detrimental effects, as they lead to a cytokine storm (Coico and Sunshine, 2015).

3.3.4 Monitoring the time course expression of TCRs in T cells

In order to optimise the transfection of the recombinant lipid specific TCRs, a time course experiment was set up with the Jurkat cell lines. In this study, Jurkat Lucia

cells were transfected with a α/β TCR expression construct pMJA219 (TRAV10/TRBV25) in a transient system. FACS, a rapid and robust method, was used as a tool to monitor transfection efficiency at the single-cell level. The marker mouse anti-human Vα24-PE, which is specific for TRAV10 TCRs, was used to measure the percentage of transiently transfected Jurkat Lucia cells.

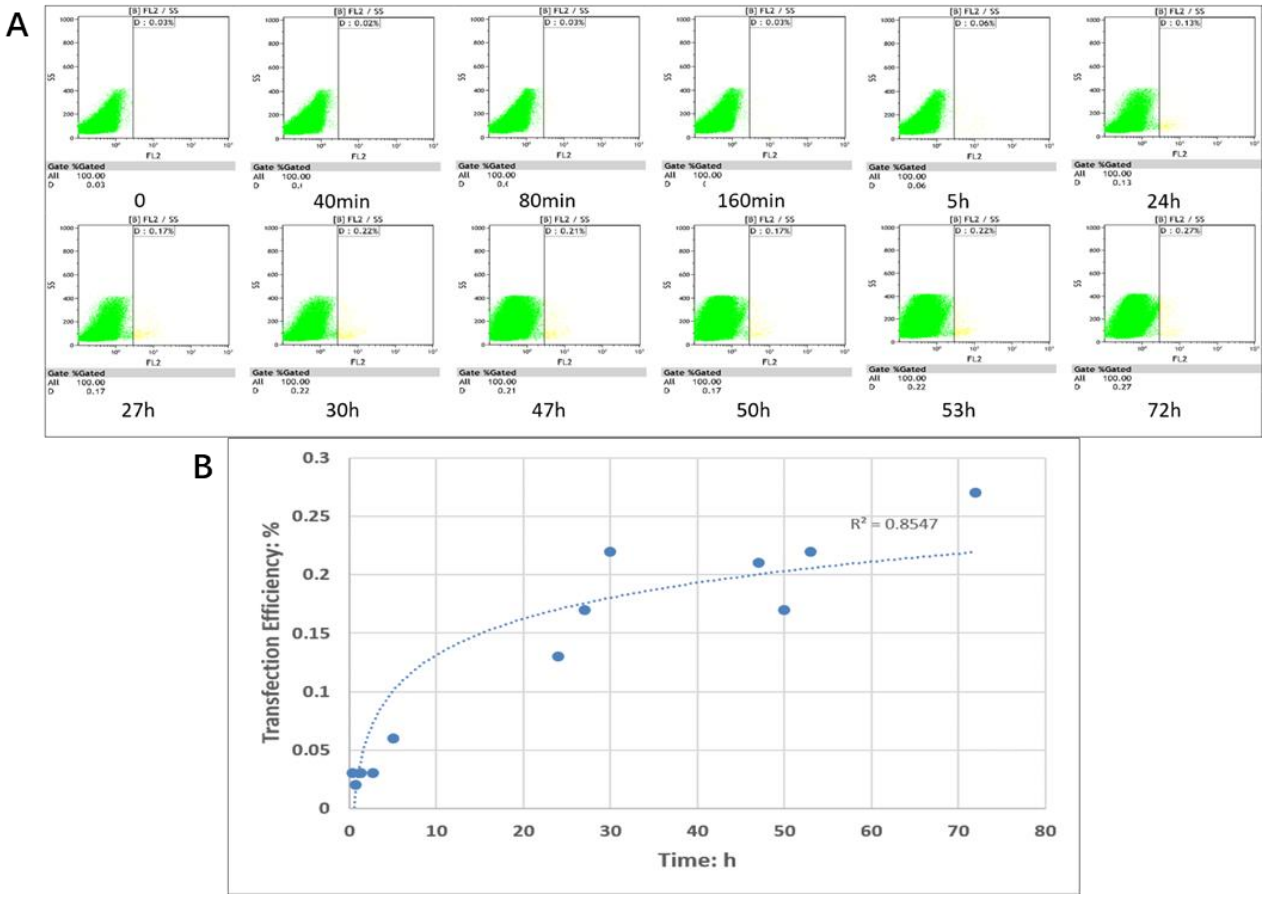


Figure 14 The time course expression of TCRs in human T cells. A) shows the different time course of Jurkat NF-AT Lucia transfected with pMJA219 (TRAV10/TRBV25) TCR genes expressing plasmid. B) shows the summary of the time course expression of TCRs in human T cells. Results show mean of repeats from triplicate experiments.

As shown in Figure 14, the percentage of labelled TCR (pMJA219: TRAV10/TRBV25) Jurkat Lucia cells expressing human TRAV10 protein receptors increased after transfection (time 0). From time 0 until 24 hours transfection, the percentage of anti-Vα24PE labelled cells increased significantly. In normal T cell biology, it is expected that TCRs are internalized and recycled continuously, a phenomena described as T cell endosomal trafficking (San José and Alarcón, 1999; Cesari, 2009). Despite the rapid turn-over reported for these receptors (Liu *et al.*, 2000), in these experimental conditions the level of expression reached a maximum and did not decay up to 72 h.

Obviously, the transfection efficiency was low (around 0.2 %). Homann *et al.* (Homann *et al.*, 2017) showed that a higher transfection efficiency and protein expression can be obtained with a smaller plasmid. Large plasmids require more transfection reagent to complex the DNA and are more difficult to transport across the plasma membrane (Homann *et al.*, 2017). Transfection efficiency can also be affected by multiple experimental parameters such as the choice of the transfection method, health and viability of the cell line, quality and quantity of the nucleic acid used (Kim and Eberwine, 2010). In order to improve the transfection effectively, there are several implemental steps that could be used such as the host cell number and the methods of transfection (lipotransfection, virus mediated or electroporation). Moreover, the condition of Jurkat Lucia cells whether they are clustered or separated may affect the transfection. As will be described in Chapter

4, a German visiting researcher successfully developed and tested a lentivirus expression system with pMJA219 (TRAV10/TRBV25) in our lab.

3.4 Conclusions

The objective of this initial work was to develop a reporter based assay for stable surrogate T cells (Jurkat) expressing lipid-binding TCRs. In this chapter, a mammalian expression vector containing the α and β chains of the TCR was constructed which binds α-GalCer (pMJA219: TRAV10/TRBV25) and transfected host mammalian cell lines to observe activation upon lipid binding. The host cell lines tested were mainly the commercial human Jurkat NF-AT Lucia and Jurkat IRF&NF-κB Dual T-cell. ELISA was used to detect IL2 and a secreted luciferase reporter used to detect T cell activation by Luminescence. The main conclusions were as follow:

- Initial characterization of host cell lines using known T-cell stimulators showed that 50 ng/ml PMA and 3 µg/ml Ionomycin were the most effective at activating Jurkat Lucia (NF-AT) cells whereas 50 µg of Concanavalin A was the most suitable stimulator for Jurkat Dual (IRF&NF-κB) cells.

- Preliminary results showed that APCs presenting lipids showed a higher luminescence when pMJA219 (TRAV10/TRBV25) is expressed in Jurkat Lucia or Jurkat Dual cells.

- The selection of Mutz-3 rather than Thp-1 as APC for lipid loading was confirmed by FACS data using primary NKT cells from one healthy volunteer co-cultured with α-GalCer-loaded APCs.

- Initial results showed that the percentage of labelled TCR Jurkat Lucia cells expressing human TRAV10/TRBV25 protein receptors (pMJA219) after transient transfection increased and was stable for up to 72 hours.

Chapter 4 Towards a surrogate system to express human lipid binding TCRs

Biotechnol Lett (2019) 41:1095–1104
https://doi.org/10.1007/s10529-019-02713-2

ORIGINAL RESEARCH PAPER

Towards a surrogate system to express human lipid binding TCRs

Rui Wang · Ronja Pscheid · Ashfaq Ghumra · Ling Yu Lea Kan · Stella Cochrane · Lucy Fairclough · Marcos J. C. Alcocer

4.1 Introduction

What makes a protein an allergen with respect to the ability to sensitise and, in particular a food allergen, is not well understood. Previously Kean *et al.* (Kean *et al.*, 2006) reported that the 2S albumin from sunflower, and not the major nut allergen Ber e 1 was able to polarize dendritic and T helper (Th) cell responses in mice with production of IL-12 p40 and TNF-α (Kean *et al.*, 2006). Subsequently, it was shown that Brazil nut lipids were required during the sensitization phase with Ber e 1 (Dearman, Alcocer and Kimber, 2007). Later, in kinetic experiments it was also shown that Ber e 1 can indeed accommodate one lipid molecule (stoichiometry 1:1) (Rundqvist *et al.*, 2012; Mirotti *et al.*, 2013). The requirement for natural lipids from nuts as a critical component for the intrinsic allergenicity of Ber e 1 was further demonstrated when human T-cell lines derived from nut allergic patients were shown to produce IL-4 to Ber/lipid C in a CD1d dose dependent manner. Jα18 and CD1 knock out experiments further implicated NKT cells in the response (Mirotti *et al.*, 2013). Altogether, these results illustrate the essential role of the natural lipid fraction in nut protein sensitization and strongly suggest that NKT cells play a critical role in the development of Brazil nut-allergic response.

NKT cells are unique lymphocyte subpopulations characterized by co-expression of surface markers from conventional NK and T cells. NKT cells make up 0.01-2 % of human peripheral blood mononuclear cells and have been shown to be important in

all aspects of immunity such as development, regulation of autoimmune, allergic, infectious and neoplastic responses (Taniguchi, Seino and Nakayama, 2003; O'Konek, Terabe and Berzofsky, 2012; Godfrey *et al.*, 2015). In response to T-cell receptor (TCR) engagement NKT cells rapidly produce cytokines involved in the activation of dendritic cells (DCs), NK cells, macrophages, B cells and conventional T cells amongst others. NKT cells, via TCR engagement, can recognize lipid antigens that are presented by the nonclassical MHC I-like CD1 receptors expressed on the surface of antigen presenting cells (APCs), which is significantly different from T cells (de Jong, 2015). Lipid antigens presented via CD1 generally respond to changes in extracellular environment (Dowds *et al.*, 2014). Within the context of allergies, NKT cells were shown to participate in Th2 responses through a CD1d-dependent mechanism (Mirotti *et al.*, 2013).

One of the major bottlenecks in studies of the interaction of natural lipids with the cells of the human immune system is the lack of human lipid responsive cell lines. In the present study, human TCRs were engineered into a stable murine hybridoma cell line employing well described synthetic TCR sequences and utilizing the widely used marine sponge glycolipid α-galactosylceramide (α-GalCer) as an activator (Bai *et al.*, 2012). Further, murine and human stable DC cell lines has been extended to commercial surrogate human T cells (Jurkat). The transient expression TCRs in several Jurkat backgrounds via plasmid or via lentivirus transfection was also analyzed. The surrogate cell lines (Jurkat Lucia and Jurkat 76) which were used in

this chapter are human TCR null cell lines. Thus, the methodology, results and discussion presented herein will help towards a cell system able to express human responsive lipid binding TCR sequences. These are important first steps in the establishment of cell specific lipid responsive libraries for the study of natural lipid substances.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Antibodies and IL-2 ELISA

Mouse anti-human Vα24-PE antibody was from Beckman Coulter. eBioscience Human IL-2 ELISA Ready-Set-Go Kit was bought from Fisher Scientific.

4.2.1.2 Cell lines

JAWSII a mouse dendritic cell line was purchased from the American Tissue culture collection (ATCC) (#CRL-11904). DN32.D3 a mouse double negative iNKT hybridoma cell line was a gift from Professor Albert Bendelac, Howard Hughes Medical Institute, University of Chicago, USA. Jurkat Lucia cells (human T cell containing NFAT Luciferase reporter gene) were purchased from InvivoGen. Mutz-3 cells (human DC cell line) and ACC 35 cells (human urinary bladder carcinoma)

were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany (# 295 and 5637 respectively). Mutz-3 has been reported to express CD1a, CD1b, CD1c and CD1d (Masterson *et al.*, 2002). 293T cells, a human embryonic Kidney cell, were purchased from the American Tissue culture collection (ATCC) (#CRL-3216). Jurkat 76 is a human TCR αβ/γδ null cell line kindly donated by M. Heemskerk (Heemskerk *et al.*, 2003) that has been reported as a stable recipient for new TCR sequences (Guo *et al.*, 2016).

4.2.1.3 Chemicals

RPMI 1640 media, heat inactivated FBS, opti-MEM, DMEM and α-MEM were purchased from Gibco, UK. Zeocin and blasticidin were purchased from Thermo Fisher Scientific, UK. α-Galactosyl ceramide-KRN7000L was obtained from Tebu-bio Ltd, UK. Murine GM-CSF was purchased from PeproTech, UK. L-α-Lyso phosphatidylethanolamine, HEPES, Beta-Mercaptoethanol, Histopaque, Puromycin, Polyethylenimine, TNF-α, Ionomycin and concanavalin A were purchased from Sigma UK. Sulphatide and glucocerebroside were from Matreya LLC, USA. L-α-Lysophosphatidyl inositol sodium salt, phosphatidic acid and β-sitosterol were from Fluka, UK. Phorbol 12-myristate was from Invivogen. 3-sn-Phosphatidyl coline was from soya bean from BDH, UK. Fugene 6 Transfection Reagent was bought from Promega, UK. Sodium pyruvate and L-Glutamine were

purchased from Gibco. Polybrene was purchased from Millipore Co EMD. Luciferase substrate QUANTI-Luc was bought from InvivoGen. BioSource™ Multiplex Kits for the Luminex Platform-Th1/Th2 were bought from Invitrogen, UK.

4.2.2 Methods

4.2.2.1 Lentivirus expression

The lentivirus expression was carried out using the 3 plasmid second generation system essentially as described by Roth *et al.* (Roth *et al.*, 2017). The psPAX2 (packaging) and pMD2.G (VSV-G expressing envelope) were gifts from Didier Trono (Addgene plasmid 12260 and 12259 respectively). pSin-EF2-Nanog-Pur (target vector) was a gift from James Thomson (Addgene plasmid 16578). Two target vectors were assembled. pMJA285 (Genbank MH782473) contained the bidirectional CMV promoter driving TRAV10 (GenBank DQ341448.1) and TRBV25 (Genbank DQ341454.1) sequences in opposite orientation as described for the pMJA219, XbaI and SpeI fragment were inserted into pSin-EF2-Nanog-Pur. pMJA289 (GenBank MH782475) was assembled by PCR overlapping primers and contained the same TRAV10/TRBV25 constructs in a dicistronic orientation driven by EF2 promoter and the α/β TCR sequences separated by the GSG-2A self-cleavage peptide sequence GSGATNFSLLKQAGDVEDNPGP (Liu *et al.*, 2017).

For the transduction, the target cells (Jurkat Lucia or Jurkat 76) were counted. For each transduction, 10^5 cells were used. The cell suspension was centrifuged (150 g, 5 min) in separated tubes and the supernatant was discarded. Cell pellets were resuspended in 2 ml virus supernatant. Polybrene was added at a final concentration of 8 µg/ml. The suspension was transferred into a 6-well plate. The plate was sealed with parafilm centrifuged for 90 min at 250 g (spin transduction). Afterwards, the cells were carefully resuspended and incubated for another 6 h at 37°C 5 % CO_2. Subsequently, the cells were centrifuged and resuspended in fresh Jurkat media. The supernatant was carefully discarded. 48 h post transfection, the cells were split. Puromycin antibiotic was added to one well at a final concentration of 0.5 µg/ml. One week later, the cells were tested in a co-culture. Furthermore, the cells were stained with mouse anti-human Vα24-PE antibody and analysed in flow cytometry as described below.

4.2.2.2 Co-culture

In this Chapter, all experiments were set up in triplicate wells and repeated twice. DC-lipid load: human DC Mutz-3 were cultured at 37°C, 5 % CO_2 in media containing α-MEM with FBS (20 %), conditioned media (5637 cell line supernatant at 20 %) and human GM-CSF (5 ng/ml) according to cell culture instructions (DSMZ). Mouse DC JAWSII were cultured in the same conditions but in α-MEM with FBS (20 %), supplemented with L-glutamine (4 mM), Na-pyruvate (1 mM)

and murine GM-CSF (5 ng/ml). For lipid loading Mutz-3 or JAWII cells were counted, centrifuged and resuspended in fresh DC media (RPMI 1640, 10 % FBS, 1 % Pen-Strep and 5 % DMSO) to a final density of $2.5x10^4$ cells/30 µl and added onto each well of a 96 well round-bottomed plate. Lipid or α-GalCer was added to Mutz-3 or JAWSII cells to a total 50 µl/well reaction mixture and incubated for 24 h at 37°C, 5 % CO_2.

T cell transfection: Jurkat Lucia cells were cultured in Jurkat media (RPMI 1640, 10 % FBS and 100 µg/ml Zeocin) accordingly to the manufacturer's instructions (InvivoGen). The media was changed every three days, by switching between media with and without Zeocin. Jurkat 76 cells were cultured in Jurkat 76 media (RPMI 1640, 10 % FBS, 1 % L-Glutamine, 1 % sodium pyruvate, 1 % Pen-Strep). DN32.D3 cells were cultured in media containing RPMI 1640, 10 % FBS, 1 % Pen-Strep and 2mM L-Glutamine. For transfection, the cells were counted, centrifuged and resuspended in 100 µl/well fresh media to a final density of $2x10^5$ cells/well and dispensed onto a 96 well round-bottomed plate. The transfections were performed by the procedure described in the Fugene 6 Transfection Reagent protocol (Promega,UK). Briefly, a total of 50 µl of transfection reaction was prepared in RPMI media: 3 µl Fugene 6 reagent was added followed by 1 µg plasmid DNA and incubated for 30 min at room temperature. Finally, all transfection reactions were added drop-wise to each well containing cells and incubated for 24 h at 37°C, 5 % CO_2.

Co-transfection and readout: After 24 h the lipid loaded Mutz-3 cells were mixed and added (50 µl/well) to transfected cells onto a 200 µl final reaction containing $2.4x10^4$ DC+ $2x10^5$ T cells) and incubated overnight at 37°C, 5 % CO_2. PMA (50 ng/ml) and ionomycin (3 µg/ml) were used as positive controls to stimulate T cells. After 24 h incubation, the supernatant was harvested, and the secreted luciferase activity measured by QUANTI-Luc assay containing coelenterazine substrate. For this, 20 µl of each sample was transferred onto Optiplate 96 plate, 50 µl/well QUANTI-Luc added and immediately measured by a luminometer (Turner Biosystems) and analysed in Microsoft Excel software. The remaining supernatant was stored at -20ºC or used in an IL-2 Sandwich ELISA determination (384 well ELISA plate) using HRP and TMB as substrate following the Ready-Set-Go! Kit according to the manufacturer's instructions (Fisher Scientific). The results were read at 450 nm using Tecan Infinite M200 PRO plate reader, data collected by Magellan software and transferred to a Microsoft Excel software spreadsheet for analysis.

4.2.2.3 Flow cytometric analysis

Firstly, cells in each well were harvested into each FACS tube, 2 ml of PBA (500 ml PBS containing 30 % BSA and 20 % Sodium Azide) was added and centrifuged for 5 min at 300 g. Supernatants were discarded, and the cell pellet was re-suspended. Antibody (5 µl mouse anti-human Vα24-PE antibody) was added according to the

manufacturer's instructions and incubated at 4°C for 30 min in the dark. Afterwards, 2 ml of PBA was added to each tube and centrifuged. Supernatants were discarded, and the pellet re-suspended in 0.5 ml PBA. Analysis was carried out on the Flow Beckman Coulter flow cytometer FC500 (Beckman Coulter life Sciences, USA), at the Flow Cytometry Facility (University of Nottingham). After quantification by FC500, the data was analysed using Kaluza software (Beckman Coulter).

4.3 Results and discussion

4.3.1 Lipid responsive TCR, murine system

The murine hybridoma cell line DN32.D3, originally described by A. Bendelac (Lantz and Bendelac, 1994), has been widely used by many groups working with lipid binding and NKT cell activation. The TCR of DN32.D3 cell line consists of Vα14 Jα18 paired with Vβ8, Vβ7 or Vβ2 and the cell line expresses the NK1.1 marker, an identifier of NK cells. The characteristics of DN32.D3 cell line have been described in great detail elsewhere (Kim *et al.*, 2006, 2010; Jordan-Williams, Poston and Taparowsky, 2013). This double negative hybridoma murine cell line when presented in co-culture to the murine DC cell line JawsII showed a sigmoidal IL-2 dose response curve with increasing α-GalCer concentration (Figure 15 A; this experiment has been carried out by Dr Xiaowei Wang). This response is blocked by murine anti-human CD1d antibodies, hence CD1d specific, and is not further

amplified by murine anti-CD3/CD28 beads. Furthermore, the response seemed to be IL-2 specific as only the IL-2 cytokine was detected, as assessed by a limited Luminex panel containing only 6 cytokines (IL-2, 4, 5, 10, 12 and IFN-γ) was used.

As expected, the dose response curve of DN32.D3 against α-GalCer in co-culture is species-dependent. DN32.D3 showed a higher sensitivity to lower levels of α-GalCer when the glycolipid is presented by the murine JAWSII than when presented by the human Mutz-3 DC cell lines (Figure 15 A). Regarding lipid specificity, DN32.D3 has been shown to be quantitatively specific to α-GalCer in the murine JAWSII system (Ghumra and Alcocer, 2017) with a high background response for the other lipids tested (Figure 15 B). When Mutz-3 human DC system was used in co-culture with DN32.D3, it showed lower sensitivity and only presented α-GalCer and LPE not to the other lipids (Figure 15 B).

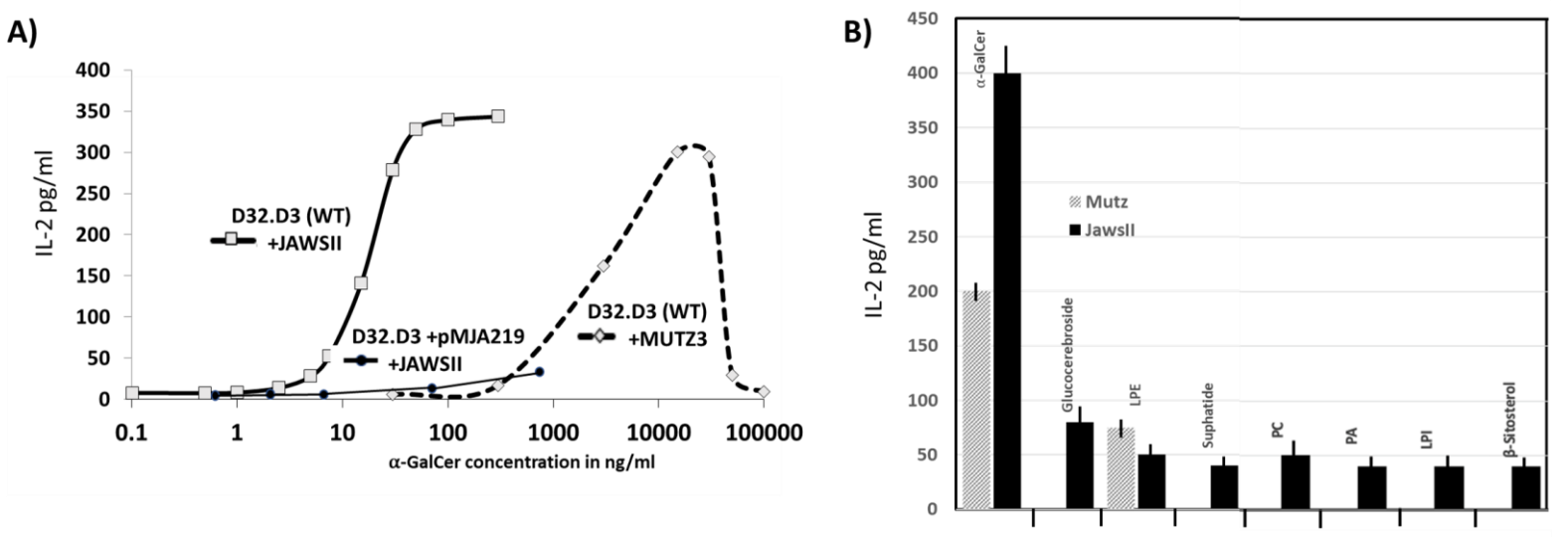


Figure 15 α-GalCer dose response of DN32.D3 co-cultured with JAWSII or Mutz-3 cell lines. A) IL-2 α-GalCer dose response of DN32.D3 wild type and transfected DN32.D3 with the plasmid pMJA219 (TRAV10/TRBV25) and co-cultured with JAWSII or Mutz-3 as indicated after 72 h 37°C. B) IL-2-lipid specificity response for DN32.D3 co-cultured with Mutz-3 or JAWSII, lipids at 1 µg/ml for JAWSII and 10 µg/ml for Mutz-3. LPE: lyso-phosphatidyl ethanolamine, PC: phosphatidyl choline, PA: phosphatidic acid, LPI: lyso-phosphatidylinnositol. All co-cultures were performed in triplicates at density of c.a $5x10^5$ cells/well of DN32.D3 and $5x10^4$ cells/well of APC.

In an attempt to engineer new functions and humanise DN32.D3, a plasmid (pMJA219) containing the α-GalCer responsive human TRAV10 and TRBV25 sequences was constructed (Figure 16). The α/β TCR sequences used in pMJA219 (TRAV10/TRBV25) design have been previously described as α-GalCer specific using lipid loaded tetramers (Brigl *et al.*, 2006). In order to achieve TCR α/β equimolar expression a mammalian pMJA219 (TRAV10/TRBV25) plasmid was designed that contained the bidirectional CMV promoter driving the TCR sequences. After transfection and selection for 3 weeks on Gentamicin, FACS analysis using TRAV10 specific human antibodies (V24α sequence) confirmed that 60-80 % of pMJA219 (TRAV10/TRBV25) DN32.D3 transfected cells displayed the specific human TRAV10 and none on the controls. The levels of transcription obtained with the bidirectional promoter when assessed by Real Time-PCR suggest that the 2 genes are highly expressed. Altogether these results confirmed that the bidirectional promoter is functional and that the murine cell line DN32.D3 can express a human α chain. However, when the pMJA219 (TRAV10/TRBV25) transfected DN32.D3 cells were co-cultured with the murine DC JawsII they did not show any improvement in their activation as measured by IL-2 to detect α-GalCer as shown in Figure 15 A or to respond to PMA/ionomycin. These transfected cells were also unresponsive when co-cultured with the human Mutz-3 system. Whether these results were a product of competition with the murine CD3 as suggested by some authors (Ahmadi *et al.*, 2011) or by mis-pairing with the endogenous murine α/β TCR during ER folding (Sommermeyer *et al.*, 2006; Knies *et al.*, 2016) remains to

be clearly demonstrated. What is clear from this exercise is that the over expression of the human α/β TCR chains clearly disrupted the function of the endogenous murine chains (Figure 15 A).

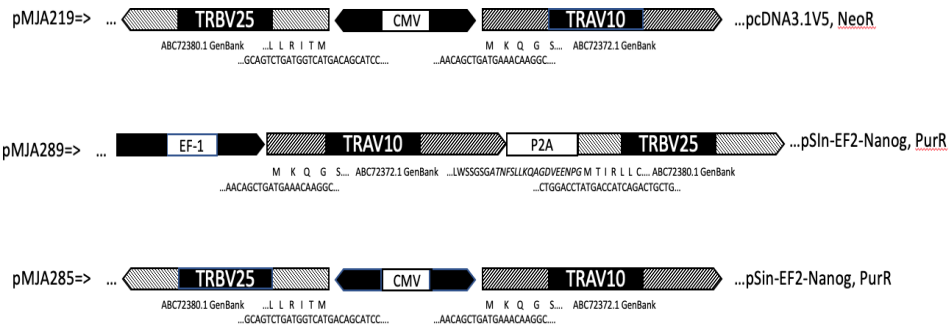


Figure 16 Diagrammatic TCR clones designed and constructed for this work. pMJA219 (TRAV10/TRBV25) is a mammalian NeoR plasmid (GenBank MH782476) driven by a bi-directional CMV promoter (pBI-CMV1, Clontech). pMJA285 (GenBank MH782473) is a similar construct but cloned into the lentivirus pSin-EF2-Nanog, PurR background. pMJA289 (GenBank MH782475) is a polycistronic construct into which the P2A cleavage sequence has been engineered. The AA proteolytic cleavage sequence of the P2A is indicated in italic. pMJA288 is pSin-EF2-Nanog lentivirus vector used in the cloning but without any insert.

Attempts to disrupt the wild type TCR murine chains from DN32.D3 before the human TCR transfection were made by Clustered regularly interspaced short palindromic repeats (CRISPR) by visiting American students, but the resulting clones did not show any murine α/β expression. None of the DN32.D3 knockout cells produced significant amounts of IL-2, or responded to PMA / ionomycin, or were affected by the presence of the human TRAV10 / TRBV25 expression construct. It seems that due to its hybridoma nature and after a large number of passages, DN32.D3 has suffered a great number of recombinations plus cumulative mutations that have resulted in an almost unusable cell line as far as expression of

new TCRs is concerned.

4.3.2 Jurkat Lucia cells

A human stable cell line Jurkat Lucia was used instead of the murine DN32.D3. This cell line contains an inducible secreted coelenterazine luciferase reporter construct regulated by human NFAT binding sites. No data is currently available on TCR or indeed CD3 expression of this particular cell line. pMJA219 (TRAV10/TRBV25) was transfected into Jurkat Lucia and TRAV10 expression was measured by FACS in a time course experiment (Figure 17 A). Poor transfection (a maximum of only 0.3 %) was observed but further analyses were nevertheless carried out. The TRAV10/TRBV25 transfected Jurkat Lucia showed higher levels of luminescence compared to non-transfected cells when co-cultured with the human dendritic cell Mutz-3 loaded with α-GalCer (Figure 17 B). The co-culture experiments were done mainly by an undergraduate student. Whether the APC, in this case Mutz-3, had time and the right conditions to achieve the optimal maturity was tested by incubation in three different maturation/conditioning media (M1, M2 and M3) as shown in Figure 17 B. As reported these media contained different amounts of TNF-α, IL-4, hGM-CFS and different incubation times (Masterson *et al.*, 2002; Ning, Morgan and Pamphilon, 2011). From these findings M1 gave the highest signal and therefore was used throughout the remainder of the study.

These results demonstrated that the synthetic human α/β sequences present in the plasmid pMJA219 (TRAV10/TRBV25) were functional in Jurkat Lucia cells, which responded to α-GalCer when presented by APCs and corroborated previous work by Brigl, van den Elzen *et al.* (Brigl *et al.*, 2006). They also suggested that the commercial human Jurkat Lucia cells could be used for a transient expression of human TCRs in a lipid screening program. The low transfection rate and stability of these cells is questionable and warrants the need to further this line of investigation. Whether, as reported (Guo *et al.*, 2016), an over expression of CD3 could bypass the Jurkat's known TCR expression bottle neck and improve the functionality of the cell line, remains to be shown.

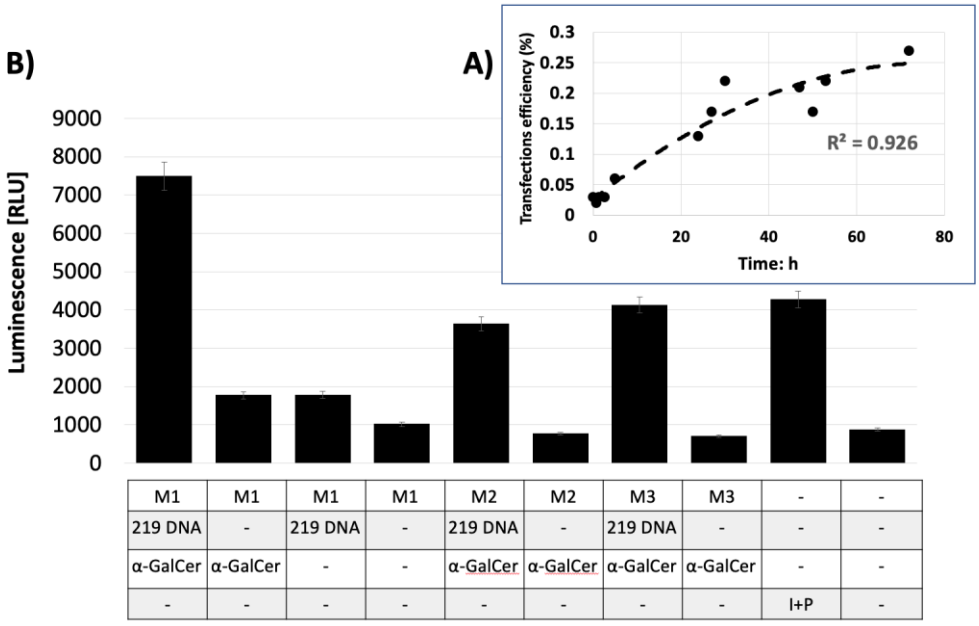


Figure 17 Luminescence of co-cultured human DC Mutz-3 + Jurkat Lucia T cells. A) Time course experiment showing the stability of the transfected TCR genes encoded by pMJA219 (TRAV10/TRBV25) in Jurkat Lucia T cells as measured by V24α expression by FACS. B) Luminescence readings of triplicate wells after 24 h of co-incubation of Mutz-3 and Jurkat Lucia cells at a density of $2x10^5$ each/well at 37°C, 5 % CO_2. M1-3 refers to the maturation treatment that Mutz-3 has undergone. M1=lipid+24h in RPMI 1640, 10 % FBS, 1 % Pen-Strep and 5 % DMSO. M2=Lipid+24h in α-MEM 60 %, 20 % FBS, 100 ng/ml hGM-CSF, 10 ng/ml IL-4, 75 ng/ml TNF-α (Ning, Morgan and Pamphilon, 2011). M3=lipid+24h in α-MEM 60 %, 20 % FBS, 50 ng/ml hGM-CSF, 20 ng/ml IL-4, 12 ng/ml TNF-α (Masterson et al., 2002). I+P=Ionomycin+PMA.

4.3.3 Lentivirus system

One alternative for plasmid driven TCR expression in T lymphocytes is the use of a lentivirus expression system as previously reported (Zhou and Buchholz, 2013). In this 3 plasmid system the packaging and envelope genes are mixed with the target or transfer plasmid (3:1:3) and the virus packed in receptor cells. For this, the target plasmid pMJA285 was designed and constructed to contain the α/β TRAV10/TRBV25 sequences and similarly to pMJA219 (TRAV10/TRBV25), was driven by the CMV bidirectional promoter (Figure 16). In another strategy the target

plasmid pMJA289 (Figure 16) was produced containing the α/β genes in a dicistronic configuration separated by the GSG-2A self-cleavage sequence, as described by Liu *et al.* (Liu *et al.*, 2017). The pMJA 285 and pMJA 289 synthetic plasmid experiments were carried out mainly by Ronja Pscheid in our facilities. When these viruses containing α/β TCR sequences were transfected into the Jurkat Lucia background, the dicistronic construct showed a higher transfection rate than the CMV construct after 72h, as monitored by FACS using anti-human Vα24 antibody (Figure 18 A). The human TCR expression achieved with the protease cleavage construct containing the P2A sequence (pMJA289) was higher than the CMV bidirectional construct. These results are in agreement with Thomas *et al.* (Thomas, Stauss and Morris, 2010), who stated that P2A improves the equimolar expression of both genes as well as leads to higher levels of cell-surface TCRs.

Interestingly, a significantly higher transfection efficiency with the same virus constructs was obtained with the cell line Jurkat 76 when compared with Jurkat Lucia ($p<0.05$) (Figure 18 A), suggesting a more efficient assembling of the TCR/CD3 complex in this cell line. Jurkat 76 is a human TCR αβ/γδ null cell kindly donated by M. Heemskerk (Heemskerk *et al.*, 2003) that has been reported as good and stable recipient for new TCR sequences (Guo *et al.*, 2016). Thus, the results presented here corroborated the findings from both groups and suggest that indeed the mispairing of the endogenous α/β TCR during ER folding in the presence of the new human TCR sequences impaired the functionality of the lipid receptors.

Despite the high transfection efficiency, not all the expressed TCRs seemed to be functional. IL-2 and luciferase release after 24 h co-culture with Mutz-3 cells loaded with α-GalCer were not much increased when compared to the transient plasmid expression of pMJA219 (TRAV10/TRBV25), although the expression of the new human αβ sequences in the Jurkat 76 background produced reliable titration curves (Figure 18 B) and reduced the time for obtaining stable lipid responsive transfected Jurkat cells. The co-culture experiment was done mainly by an undergraduate student. These results ratified the results in Figure 18 B and show a higher response to α-GalCer of Jurkat 76 than Jurkat Lucia when transduced with plasmid pMJA289.

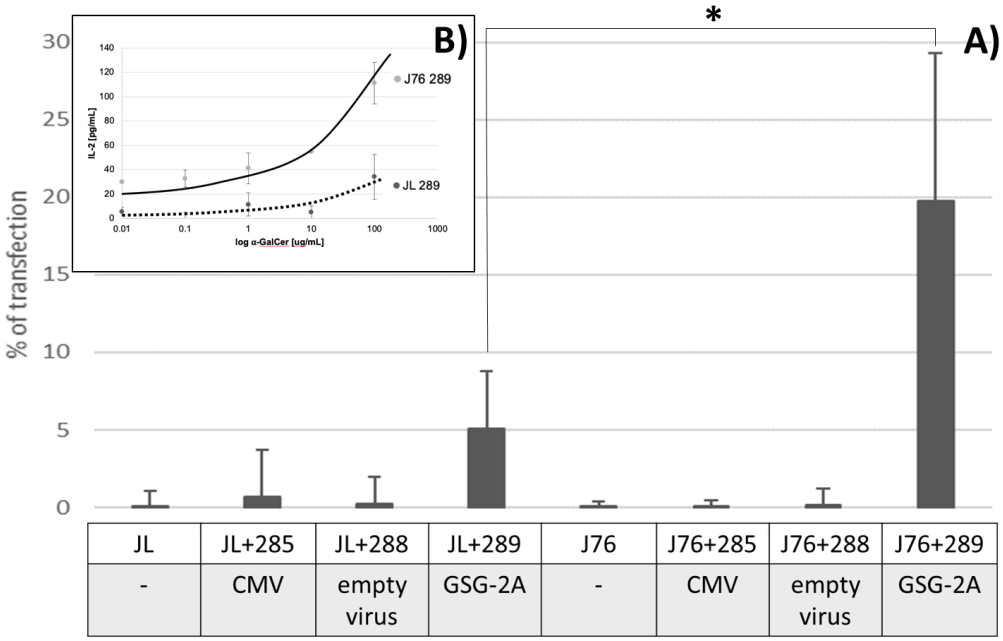


Figure 18 Human Vα24 expression of lentivirus transfected Jurkat cell lines. A) Both cell lines, Jurkat Lucia (JL) and Jurkat 76 (JL76) were transfected with lentivirus, containing the bidirectional CMV (pMJA285) or EF2 promoter + GSG-2A sequences (pMJA289) or none, maintained in normal T cell media without selection for 72 h and subsequently stained with anti-human-Vα24 for FACS analyses. % transfection expressed as the fraction of positive labelled/total cells. B) Titration curve, both cell lines, JL and JL76 transduced with pMJA289, were cocultured with Mutz-3 incubated with different concentrations of α-GalCer. The supernatant was collected after 24 h and IL-2 was measured in triplicate wells. (*=p<0.05)

Furthermore, the different Jurkat Lucia and Jurkat 76 cell lines were co-cultured with Mutz-3 + α-GalCer and only Mutz-3 (Figure 19 C Jurkat Lucia, Figure 19 D Jurkat 76 background). Jurkat Lucia and Jurkat 76 transfected with pMJA 285 showed a notable activation by α-GalCer. The co-cultured experiments were done mainly by an undergraduate student. The expression of the human TCR by lentivirus was further monitored by luminescence and IL-2 expression in the presence of α-GalCer. In a time course experiment and as shown in Figure 19 A and B the assay results from the co-culture one to four weeks post-transduction have significantly changed. The difference between the cell lines with transduced TCR (Jurkat Lucia

pMJA 285 and Jurkat Lucia pMJA 289) was notable higher compared to Jurkat Lucia cells transfected with pMJA 288. These experiments suggest that there is only a limited time frame in which the transduced T-cells can be used. It has been described that after one week, the cells can undergo apoptosis and may become exhausted and lose functions with repetitive re-stimulations (Zhong *et al.*, 2010).

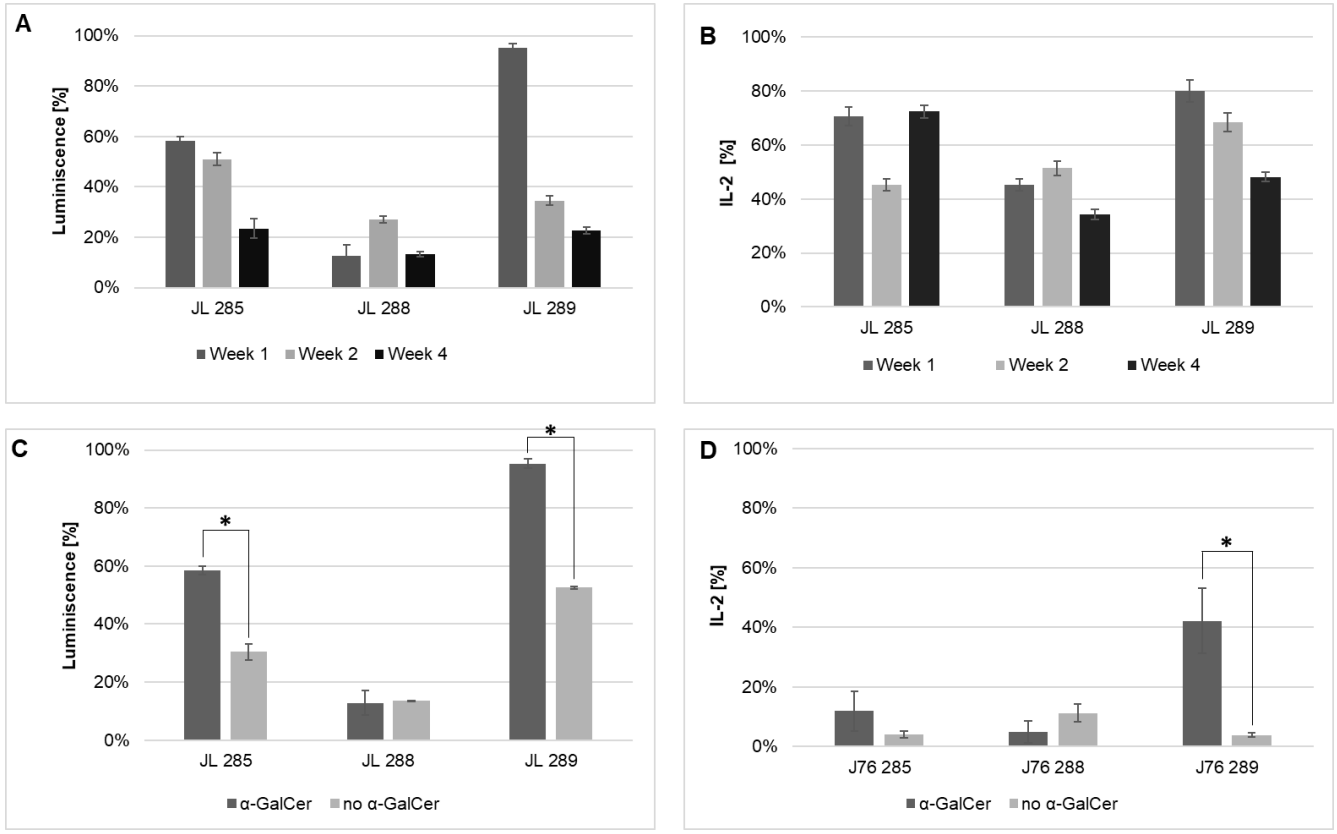


Figure 19 Lentiviral expression of luciferase and IL-2 after co-culture. The different Jurkat cell lines (JL:Jurkat Lucia and J76: Jurkat 76 TCR null) were transduced with pMJA285, pMJA288 (no TCR) and pMJA289. Co-culture time experiments were set Mutz-3 + α-GalCer for 4 weeks. A) JL time course luminescence reading. B) Ibidem but IL-2 measurements. C) JL 24 h co-culture with Mutz-3 +/- α-GalCer. D) ibid using J76 cell line and IL-2 expression. Results are averaged from triplicate wells. (*=p<0.05)

4.4 Conclusions

In this chapter, human TCRs were engineered into a stable murine hybridoma cell line and utilized α-GalCer as an activator. Further, murine and human stable DC cell lines has been extended to commercial surrogate human T cells (Jurkat). The transient expression TCRs in several Jurkat backgrounds via plasmid or via lentivirus transfection was also analyzed. Thus, the study of this chapter will help towards a surrogate cell system able to express human responsive lipid binding TCR sequences. This is important the first step in the establishment of cell specific lipid responsive libraries for the study of natural lipid substances.

The main conclusions were as follows:

- Widely used murine hybridoma cell line DN32.D3 is unusable as far as expression of new TCR sequences are concerned.

- Jurkat Lucia cells, containing as a reporter the secreted coelenterazine luciferase regulated by human NF-AT, are functional and potentially could be used for a transient expression of human TCRs, in a lipid screening program.

- Higher transfection efficiencies were obtained with the lentivirus polycistronic constructs containing the P2A sequence in a TCR αβ/γδ null cell (Jurkat 76).

Chapter 5 Defining lipids and TCRs involved in the intrinsic allergenicity of nut proteins

Article to be submitted to “Allergy” September 2019.

5.1 Introduction

Tree nuts are amongst the most common food allergens, and Brazil nut allergy in particular, is now well documented (Nordlee *et al.*, 2002; Alcocer, Rundqvist and Larsson, 2012). The high sulphur content seed storage 2S albumin Ber e 1 was unquestionably identified as the major allergen in Brazil nuts (Pastorello *et al.*, 1998; Alcocer, Rundqvist and Larsson, 2012). In the 1990s early plant transgenic work explored the high methionine content of Ber e 1 for nutritional supplementation studies. This early transgene work attracted scientific and press attention as this protein turned out to be the first allergen to be transferred from one plant to another (Ewan, 1996; Nordlee *et al.*, 2002). Hence biochemical, immunological and clinical evidence for the allergenicity of Ber e 1 is available (Alcocer, Rundqvist and Larsson, 2012).

Although a great number of proteins are encountered in our normal diets, only few protein families are commonly implicated as food allergens (Radauer and Breiteneder, 2007). Further, what makes an allergen allergenic, and in particular a food allergen, has not yet been defined. Some proteins from the 2S albumin family such as Ara h 2 from peanuts and Ber e 1 from Brazil nut are recognised as major allergens and present high proteolytic, thermal, and chemical stability being therefore resistant to gastro-intestinal conditions (Alcocer *et al.*, 2002; Tengel *et al.*, 2005). However, others 2S albumin proteins with the same characteristics from

highly consumed foods, such as soybean and sunflower seeds, are rarely associated with allergies (Murtagh *et al.*, 2003; Lin *et al.*, 2004). Thus, these biochemical/biophysical characteristics alone do not define the factors responsible for the intrinsic allergenicity of food proteins or are responsible for the initial sensitisation alone.

Previously, using the 2S albumin model system, previous studies have showed that sunflower albumin 8 (SFA8) from sunflower and not Ber e 1 was able to polarise murine dendritic and T helper (Th) cell responses *in vitro* with production of IL-12 p40 and TNF-α; analysis of transcription factors showed increased T-bet with respect to both proteins, but some GATA-3 with respect to Ber e 1 (Kean *et al.*, 2006). T-bet is an important Th1 cell transcription factor. It plays a decisive role in the differentiation of Th1 cells (Szabo *et al.*, 2000). GATA-3 plays an important role in the differentiation of Th2 cells. It is a specific transcription factor for Th2 cells, which can promote the differentiation of Th2 cells from the differentiated or differentiating Th cells (Pai *et al.*, 2008). In *in vivo* experiments it was shown that Brazil nut lipids were required for sensitisation with Ber e 1 and one particular complex lipid fraction (lipid C) was able to induce specific Ber e 1 anaphylactic antibodies in naïve animals (Dearman, Alcocer and Kimber, 2007). In kinetic experiments it has been shown that Ber e 1 can accommodate one lipid molecule (stoichiometry 1:1) with K_d of 5.6±0.1 μM, a result confirmed by Nuclear Magnetic Resonance (NMR) exchange experiments employing 15N-labelled Ber e 1

(Rundqvist *et al.*, 2012; Mirotti *et al.*, 2013). The requirement for natural lipids for the intrinsic allergenicity of Ber e 1 was further confirmed *in vivo* and *in vitro* by monitoring the interaction of Dendritic cells (DCs) and Natural Killer T cells (NKT). NKT cells make up 0.01-2 % of human peripheral blood mononuclear cells (PBMCs) and are important in all aspects of immunity such as development, regulation and in therapy for autoimmune, infectious and neoplastic diseases (O'Konek, Terabe and Berzofsky, 2012) and small molecule recognition in general (Taniguchi, Seino and Nakayama, 2003; Godfrey *et al.*, 2015). In this context it has been shown that CD1 and Jα18 iNKT-deficient mice sensitised to Ber e 1 and lipid C produced lower levels of anaphylactic antibodies when compared to wild type mice. Further, murine NKT cells produced IL-4, not IFN-α, when stimulated with Ber e 1 and lipid C (Mirotti *et al.*, 2013). Experiments with human cells have shown that T cell lines derived from nut-allergic patients produced IL-4 to Ber e 1 with lipid C in a dose-dependent manner when primed with $CD1d^{+}C1R$ transfectants, suggesting a specific role for NKT cells (Mirotti *et al.*, 2013).

These results so far highlight the essential role of natural plant lipid in the allergenicity of Ber e 1 and suggest that non-conventional T cells such as NKT cells are involved as important regulators in the initial sensitisation phase of Brazil nut allergy.

In order to understand these interactions, the specific TCRs from NKT cells

involved in lipid recognition need to be characterised. In the work presented here protocols for isolation of primary human NKT cells were improved and optimised, sequenced and expressed lipid binding TCRs and used these TCRs to screen a Brazil nut lipid library. The results from this study will help to characterise the intrinsic factors linked to Ber e 1 allergenicity and will, ultimately, help to define what makes a common protein within a food matrix context, allergenic to a particular group of susceptible individuals.

Thus, in this study, aiming at isolating large families of primary NKT cells from 4 allergic and 2 healthy human volunteers, NKT cells (CD3+, CD56+) were targeted by FACS. By challenging the NKT cells with lipids, the active cells were individually sorted and the α/β and γ/δ TCR sequences were amplified. The lipid-activated specific populations of TCRs, were then identified, sequenced and cloned into expression constructs to be used in the *in-vitro* system as shown in Figure 20.

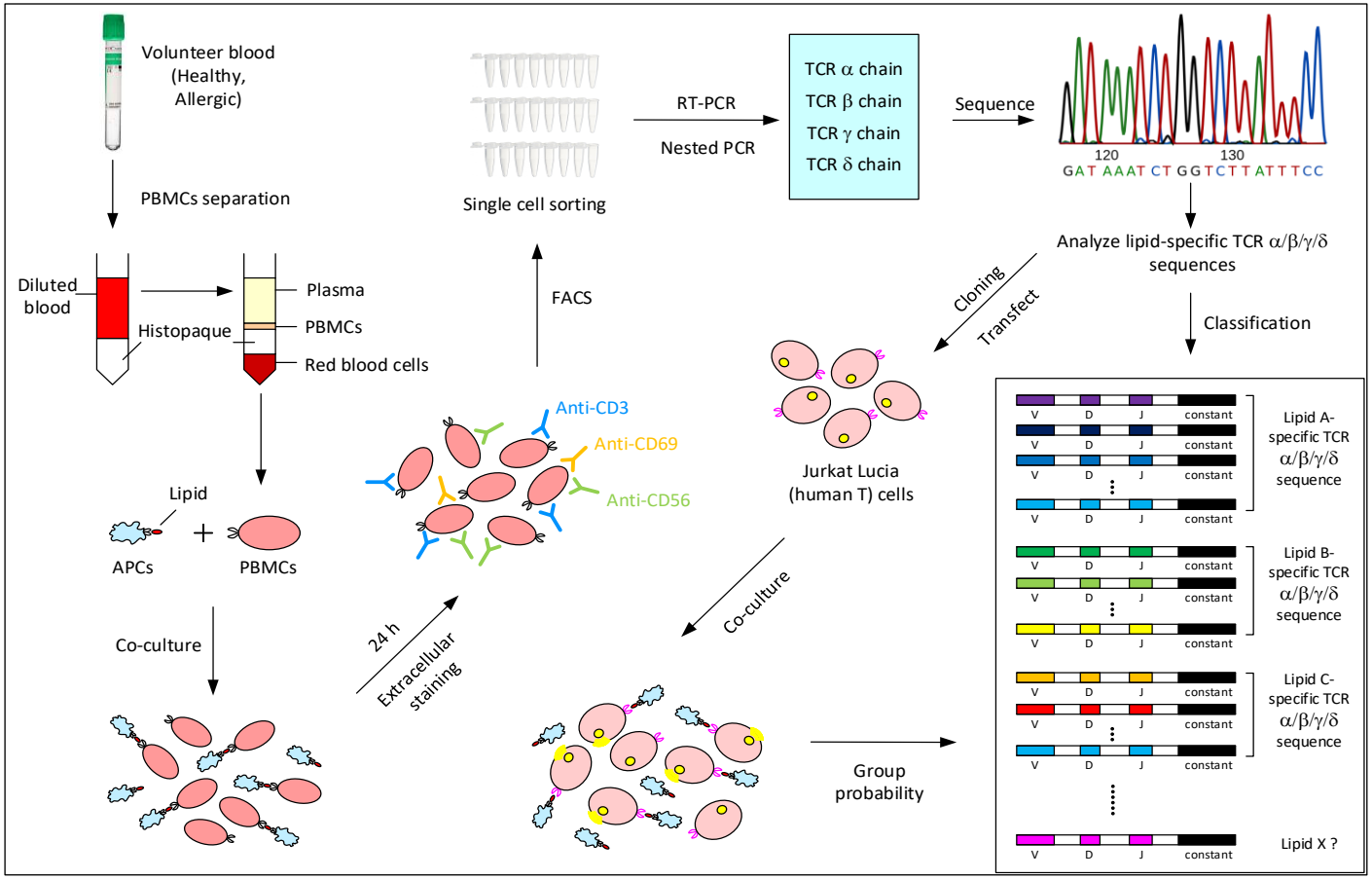


Figure 20 Methods of cloning, expression and functional characterization of paired α/β and γ/δ TCR chains from single primary NKT cell analysis

5.2 Materials and methods

5.2.1 Blood and ethical approval

Ethical medical approval to work with NKT cells from the blood of 4 allergic and 2 healthy volunteers screened by the Nottingham Health Science Biobank at the University of Nottingham is in place (BS25062015 SoL Immunol).

5.2.2 Cells

Jurkat Lucia cells (human T cells containing NFAT Luciferase reporter gene) were purchased from InvivoGen. Jurkat 76 (human T cell TCR null) was kindly donated by Dr M.H.M. Heemskerk, Leiden University Medical Centre, The Netherlands. Jurkat 76 cells were cultured in RPMI 1640 10 % FBS and 1 % Pen-Strep. The media was changed every three days, by switching between the media with Pen-Strep and without. Mutz-3 cells (human DC cell line) and ACC 35 cells (human urinary bladder carcinoma) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany (# 295 and #5637 respectively).

5.2.3 Chemicals

RPMI 1640 media, heat-inactivated FBS and α-MEM were purchased from Gibco,

UK. Zeocin and blasticidin were purchased from Thermo Fisher Scientific, UK. α-GalCer was obtained from Tebu-bio Ltd, UK. Histopaque, ionomycin and concanavalin A were purchased from Sigma UK. Fugene 6 Transfection Reagent was bought from Promega, UK. Sodium pyruvate and L-Glutamine were purchased from Gibco. Luciferase substrate QUANTI-Luc was bought from InvivoGen. SuperScript™ VILO™ Master Mix was bought from Invitrogen. Triton X-100 was bought from Sigma. GoTaq® Green Master Mix was bought from Promega. QIAGEN® Plasmid Midi kit was bought from Qiagen.

5.2.4 Antibodies

Mouse anti-human CD3-FITC, mouse anti-human CD56-PE, mouse anti-human CD69-PC5 and mouse anti-human CD25-R Phycoerythrin-Texas Red-X (CD25-ECD) were bought from Beckman Coulter. α-GalCer Loaded Recombinant CD1d Tetramer and Negative Control Recombinant CD1d Tetramer were purchased from ProImmune, UK. eBioscience Human IL-2 ELISA Ready-Set-Go Kit was bought from Fisher Scientific.

5.2.5 Isolation of primary NKT cells

PBMCs were isolated from blood from healthy and allergic volunteers on Histopaque. 10-20 ml of blood was diluted 2:1 in RPMI 1640. A volume of 5 ml Histopaque per 15 ml blood/RPMI 1640 mixture were added and centrifuged for 22

min at 800 g. The PBMC layer formed between the plasma and Histopaque interface was collected using a Pasteur pipette, washed in RMPI 1640, and centrifuged for 10 min at 300 g. PBMCs were re-suspended in RPMI 1640 10 % FBS and 0.1 % DMSO and total number was calculated using a haemocytometer.

5.2.6 Co-culture

In this Chapter, all experiments were set up in triplicate wells and repeated twice. For cell line work: 96-well round-bottomed plates were used to measure the activation of reporter constructs using CD1 expressing APCs in co-culture experiments. A number of 4×10^5 cells were added in each well in 100 µl of specific cell medium. Before using Fugene reagent, it was warmed at room temperature. The volume of RPMI media used varies depending on the volume of plasmid, which was combined with Fugene reagent totalling 50 µl. First, RPMI media was added in each tube, followed by the addition of 3 µl Fugene, and the mixture was incubated for 5 min. Afterwards, 1µg plasmid was pipetted into the RPMI-Fugene mixture and incubated for 30 mins. After the incubation, the transfected cells were added to each well in the 96-well round-bottomed plate and incubated for 24 h at 37°C, 5 % CO_2. On the other plate, 4×10^5 Mutz-3 with 30 µl of cell culture media were added to each well. Then, 20 µl of 10 µg/ml lipid were pipetted into each well with Mutz-3 cells and incubated for 4 hours at 37°C, 5 % CO_2. The total volume of APC and lipid incubation was 50 µl. After 4 hours incubation, 50 µl of APCs and lipids were

pipetted into 96-well round-bottomed plate with the transfected cells. Each well containing the lipid-loaded APCs and transfected human T cells had 200 µl suspension in total. The 96-well round-bottomed plate was incubated for 24 hours at 37°C, 5% CO_2. After 24 hours, 120 µl of supernatant from each well was collected for measuring T-cell activation by Luminescence (Quanti-Luc) and IL-2 ELISA essentially as described in Wang *et al.* (Wang *et al.*, 2019). Data were analysed using Microsoft Excel software. Error bars indicate standard deviation. P values indicate significance level as assessed by T test.

For FACS: 12-well plates were used to measure the activation of primary NKT cells using CD1d expressing APCs in co-culture experiments. 1×10^6 Mutz-3 cells were added to each well in 500 µl of cell medium (RPMI 1640 10 % FBS and 0.1 % DMSO). A concentration of 5 µg/ml lipids (α-GalCer or Brazil nut) was added and incubated for 1 hour at 37°C, 5% CO_2. Afterwards, 1×10^6 PBMCs with 500 µl of cell media were added to each well. Finally, each well containing the lipid-loaded APCs and PBMCs were cultured in a 1 ml suspension. The 12-well plate was incubated for 24 hours at 37°C, 5% CO_2.

5.2.7 Flow cytometric analysis

Firstly, cells in each well were harvested into each FACS tube, 2 ml of PBA (500 ml PBS containing 30 % BSA and 20% sodium azide) was added and centrifuged for 5

min at 300 g. Supernatants were discarded and antibodies (mouse anti-human CD3-FITC, mouse anti-human CD56-PE and mouse anti-human CD69-PC5) were added according to the manufacturer's instructions and incubated at 4°C for 30 min in the dark. Afterwards, 2 ml of PBA was added to each tube and centrifuged at 300g for 5min. Supernatants were discarded, pellet re-suspended in 0.5 ml PBA. Analysis was carried out on the FC500, MoFlo cell sorter or Astrios cell sorter at the Flow Cytometry Facility (University of Nottingham). After quantification by FACS machine, the data was analysed using the dedicated Kaluza Analysis software from Beckman Coulter. The resulting files, reports documenting statistics were obtained and loaded into Kaluza and the same plots and statistics were generated.

5.2.8 RT-PCR, Nested PCR and Sequencing

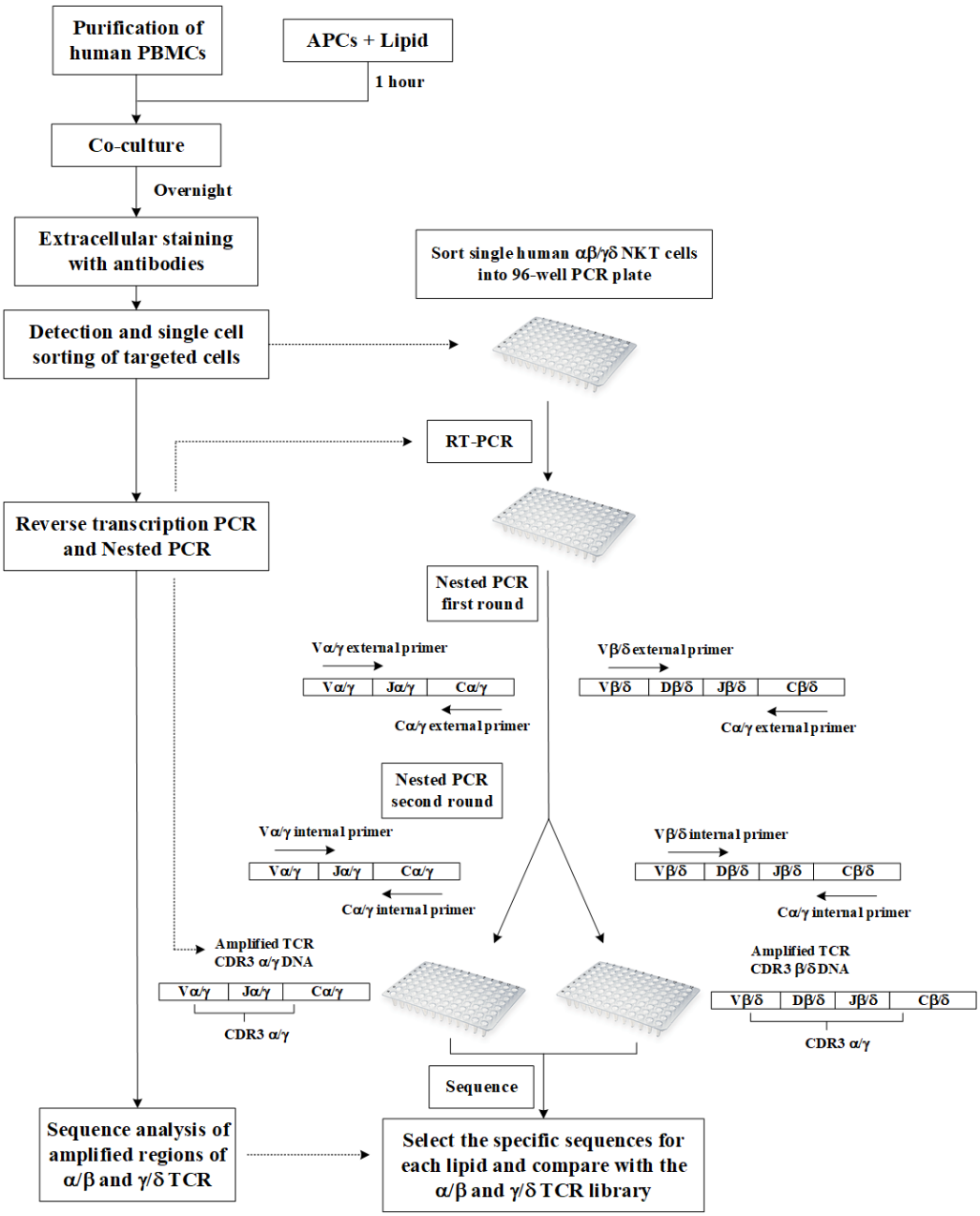


Figure 21 Overview of the multiplex PCR protocol to amplify and sequence paired TCR CDR3 α/β and CDR3 γ/δ. Nested PCR primers targeting TCR genes are shown in Appendix 1.

As shown in Figure 21, after sorting single human α/β or γ/δ T cells into a 96-well PCR plate, the reverse transcription of the α-, β-, γ- and δ-TCR chains was carried out directly to obtain single-cell cDNA without any RNA extraction step. Essentially

0.4 µl VILO, 0.22 µl 1 % Triton and 1.4 µl PCR water were added into each well for the reverse transcription PCR. The cDNA synthesis program was run as follows: 10 min at 25˚C, 90 min at 42˚C, 5 min at 85˚C and hold at 4˚C. Then, a first round of Nested PCR was performed using external primer mixture of 40 TRAV, 27 TRBV, 9 TRGV and 8 TRDV sense and single TRAC, TRBC, TRGC and TRDC antisense primers following reverse transcription PCR. A volume of 12.5 µl Go-Taq, 0.5 µl TRAV-External (EXT)/ TRBV-EXT/ TRGV-EXT/ TRDV-EXT, 0.5 µl TRAC-EXT/ TRBC-EXT/ TRGC-EXT/ TRDC-EXT and 6.5 µl PCR water were added into each well and mixed with 2 µl of reverse transcribed product. The first round Nested PCR program was run as follows: 5 min at 95˚C, 20 s at 95˚C, 20 s at 52˚C, 45 s at 72˚C, repeat 34 times from step 2 to 4, 7 min at 72˚C and hold at 4˚C. Then first-round PCR products were subjected to four separate second-round PCRs using a corresponding internal primers mix (40 TRAV, 27 TRBV, 9 TRGV and 8 TRDV sense primers and single TRAC, TRBC, TRGC and TRDC antisense primers). A volume of 12.5 µl Go-Taq, 0.5 µl TRAV-Internal (INT)/ TRBV-INT/ TRGV-INT/ TRDV-INT, 0.5 µl TRAC-INT/ TRBC-INT/ TRGC-INT/ TRDC-INT, 9 µl PCR water and 2.5 µl of the first round Nested PCR product were added into each well. The second round Nested PCR program was run in similar conditions as the first one. All the primers used for Nested PCR are shown in Appendix 1. The PCR products were run on a 2 % agarose gel to check for the success rate of the PCR amplification, and each positive reaction was purified using the manufacturer's PCR Purification Kit protocol. The DNA fragments were then sequenced using standard

Sanger methods (BioSource, Nottingham) and the results analysed by Vector NTI software (Invitrogen).

Primers targeting α-chain TCR (40 TRAV), β-chain TCR (27 TRBV), γ-chain TCR (9 TRGV) and δ-chain TCR (8 TRDV) genes were as designed by Dash *et al.* (Dash, Wang and Thomas, 2015) and Guo *et al.* (Guo *et al.*, 2016). The primers were designed for all TRAV, TRBV, TRGV and TRDV regions along with antisense primers for their respective constant regions. Two sets of primers (external and internal) were designed in order to perform a nested PCR.

5.2.9 TCR acceptor expression plasmids

Synthetic acceptor expression DNA plasmids pMJA251 and 252 (AddGene: plasmid #128540, plasmid #128541) containing TCR receptors have been synthesised (Geneart) based on the backbone of the mammalian expression plasmid pcDNA3.1(+)/Zeo(+)(Invitrogen) that uses Zeocin as the mammalian selectable marker. pMJA251 and 252 are driven by the bidirectional Cytomegalovirus promoter from pBI-CMV1 (Clontech) and flanked by the rabbit β-globin and bGH (bovine Growth Hormone) polyadenylation sequences at the 5' and 3' end of the constructs sequences respectively. The acceptor plasmids pMJA251 and 252 were engineered to contain two type IIS restriction enzyme Esp31 (BsmB1) sites replacing the VD sequences, so these plasmids only contained the γ/δ and α/β

constant regions. Briefly for new assemblies the primers MJA431 / MJA432 and MJA434 / MJA435 were used to amplify cDNA containing α and β sequences for pMJA297; the primers MJA440 / MJA440 and MJA437 / MJA438 were used to amplify cDNA containing γ and δ sequences for pMJA290; the primers MJA446 / MJA447 and MJA443 / MJA444 were used to amplify cDNA containing γ and δ sequences respectively for pMJA295 in a Go-Taq PCR reaction (95°C-20 s / 57°C-20 s / 72°C-15 s) for 35 cycles. All the primers used for PCR are shown in Appendix 3. After PCR purification (Qiagen purification kit), a 20 µl Golden Gate digestion-ligation reaction containing 200 ng of γ(α) and 200ng of δ(β), 100ng of pMJA251(252), 1 mM ATP, 10 IU of Esp31 (BsmB1) and 10 IU of T4 ligase was set, run (37°C / 5 min 23°C / 5 min) for 12 cycles and kept at 4°C. The resulting reactions was directly transformed into Escherichia coli, selected in ampicillin plates. QIAGEN midi prep was used to carry out the DNA extraction.

5.2.10 Nut lipid isolation and fractionation

Brazil nut total lipid extracts (TLEs) were obtained from our collaborator (Frederik Beaudoin, Rothamstead Experimental Station)) using the Folch method (Folch, Lees and Sloane Stanley, 1957) with the following modifications. Brazil nut tissue was ground in liquid nitrogen using a mortar and pestle and 1 g of powered tissue was mixed with 20 ml chloroform/methanol (2:1; v/v). This suspension was incubated on a tube rotator for 10 min at room temperature and 5 ml of water was

added to induce phase separation. The sample was centrifuged at 1000 g for 5 min and the lower (organic) phase recovered. The upper phase was extracted a second time by adding 15 ml chloroform, vortexing for 30 s and processing as described above. After centrifugation the lower phase was added to the first extract and the sample washed with 10 ml of 1 % potassium chloride in water and vortexing for 30 s. Phase separation was accelerated by centrifugation at 1000 g for 1 min and the lower phase was dried under nitrogen. TLE aliquots were dissolved in chloroform at a concentration of 10 mg/ml and stored at -20˚C.

Fractionation: TLEs were fractionated into neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions by solid-phase extraction (SPE) using LiChrolut Si (40-63 μm) columns (Merck, Germany) and following the manufacturer's instructions. Essentially, after conditioning the columns with chloroform NL, GL, and PL fractions were eluted sequentially using chloroform/acetic acid (99:1; v/v), acetone/methanol 9:1 (v/v), and methanol, respectively. After SPE separation, the eluted fractions were dried under nitrogen and dissolved in chloroform.

The lipid classes were analysed by Thin Layer Chromatography (TLC). Plates for analysis of the PL fraction were impregnated with boric acid as described by Leray *et al.* (Leray *et al.*, 1987). The solvent for NLs separation was hexane / diethyl ether / acetic acid 70:30:1 (v/v), whilst chloroform / ethanol / water / triethylamine 30:35:7:35 (v/v) was used for analysis of GL and PL fractions. TLC plates were

sprayed with primuline (10 mg/l in acetone / water 80:20 v/v) and the bands were visualised under UV light. Individual bands were scraped from the plates and the lipids eluted by washing the silica powder twice with 2 mL of chloroform and pooling both fractions. Chloroform was evaporated under nitrogen and the samples stored at -20˚C. These samples were used for screening assays using lipid specific TCRs and for electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis as described below.

5.2.11 Lipid analysis – MS-Orbitrap (carried out at Rothamstead Experimental Station by Dr Frederik Beaudoin)

Lipid molecular species composition was analysed by high resolution / accurate mass (HR/AM) lipidomics using a Vanquish - Q Exactive Plus UPLC-MS/MS system (Thermo Fisher Scientific). Work flow consisted of using total lipid, lipid recovered from SPE and lipid soaked off silica from TLC and made up to 70 µl. 20 µl was injected into a UPLC/MS (Thermo Vanquish system). Separation was reformed on Thermo Scientific Accucore C18 (2.1 x 150 mm, 2.6 mm) at 35°C with an autosampler tray temperature of 10°C and flow rate at 400 µl min-1 Mobile phase: A=10 mM ammonium formate in 50% Acetonitrile + 0.1% formic acid, B=2 mM ammonium formate in Acetonitrile/propan-2-ol/water 10/88/2 + 0.02% formic acid. Elution gradient was run for 28 minutes from 35% B at start to 100% at 24 mins. Thermo Q Exactive HESI II conditions, sweep plate in use probe position in C.

Conditions were adjusted for separate positive and negative runs, running samples in a single polarity resulted in more identifications. LC/MS at 140K resolution and data independent HCD MS2 experiments (35K resolution) were performed in positive and negative ion modes. Full Scan @ 140,000 resolution m/z 150-1200 Top 15 MS/MS @ 35,000 resolution. The stepped collision energy was 25, 30, 40 replacing 25 with 30 negative ion mode. Sheath gas set to 60, Aux gas 20, sweep gas 1 spray voltage 3.2 KV in positive ion mode with small adjustments in negative ion mode, capillary temperature 320 and aux gas heater set to 370°C. Lipidsearch 4.2 (Thermo Fisher Scientific) experimental workflow was used for lipid characterization potential lipid species were identified separately from positive or negative ion adducts. The data for each replicate were aligned within a chromatographic time window by combining the positive and negative ion annotations and merging these into a single lipid annotation.

5.3 Results and discussion

5.3.1 NKT activation markers

Mutz-3 is a stable human DC cell line that has been reported to express CD1a, CD1b, CD1c and CD1d (Masterson *et al.*, 2002). Whether the isolated NKT primary cells could be activated by lipids presented by the human DC cell line Mutz-3 was ascertained by monitoring NKT activation markers. Several activation markers for

NKT activation have been described in the literature, some for early (CD69 and CD54) and some for late responses (CD25 and CD107a) (Chirathaworn *et al.*, 2002; Bajnok *et al.*, 2017; King and Plun-Favreau, 2017). Initial time course experiments with Mutz-3 as APCs co-cultured with α-GalCer and nut lipid showed that the late activation marker CD25 did not increase significantly after 96h and was, therefore, not an ideal activation marker to be used for sorting activated cells (Figure 22). From the earlier markers, CD54 was up-regulated upon activation but not as much as CD69 and did not show much basal activation as observed with CD69. These preliminary results suggested that the isolation of Mutz-3 presentation of lipid and detection of activated NKT primary cells via CD69 was a feasible enterprise.

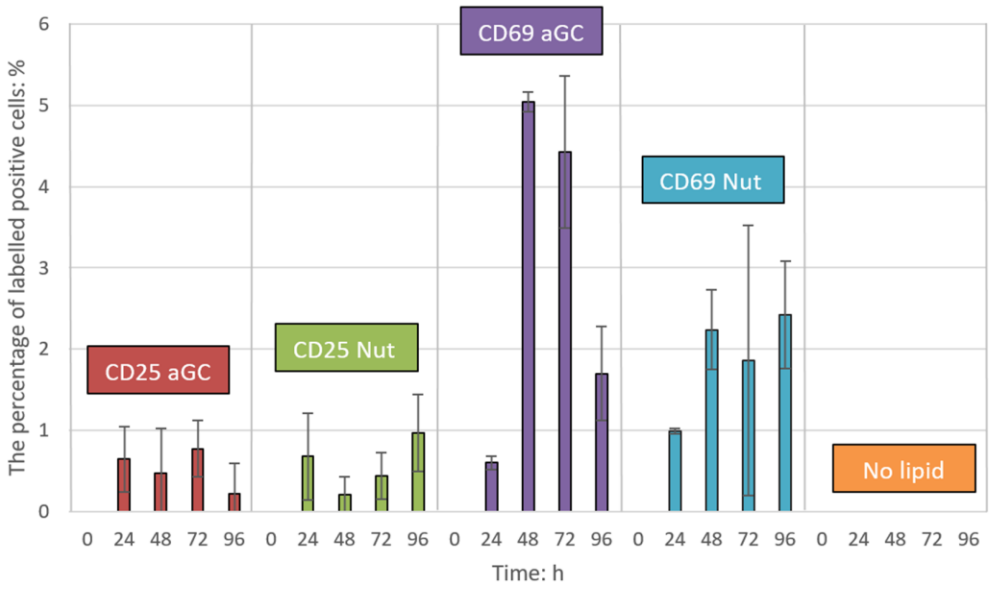


Figure 22 Time course of CD3+CD56+ primary lymphocytes co-cultured with lipid loaded APCs. The CD3+CD56+ activated T cells purified from all lymphocytes were expressed as a percentage of the total of CD3+ cells. CD25 and CD69 activation in NKT primary cells was based on PBMCs as the negative control. Activated T cells were expressed as a percentage of the total of CD3+ cells after 24h, 48h, 72h and 96h co-cultured with Nut lipid loaded APCs. These measurements are the average of triplicate experiments.

5.3.2 TCR sequencing of NKT cells from allergic patients

Based on these preliminary results and on the single cell sequencing methodology described by Dash *et al.* (Dash *et al.*, 2011) and Guo *et al.* (Guo *et al.*, 2016), an optimised protocol as depicted in Figure 20 has been developed and tested. As described in the methodology herein, the main alterations of the protocol described by others were the lipid presentation using human Mutz-3 cells and the emphasis on isolating activated NKT CD3+CD56+CD69+^{high} cells. As shown in Figure 23C by concentrating on CD69+^{high} cells, the overall number of cells to be sequenced was significantly reduced and became manageable.

In order to test the methodology, further PBMCs from six volunteers (4 allergic and 2 healthy) were isolated and co-cultured with a human DC cell line (Mutz-3) as APCs loaded with α-GalCer or the active Brazil nut lipid fraction (lipid C). After 24h the cells were labelled and activated lymphocytes CD3+CD56+CD69+^{high} were sorted into single cells by FACS as shown in the schematic in Figure 23. The total number of CD3+CD56+CD69+^{high} cells varied greatly between the subjects and no statistically significant differences between allergic and healthy individuals were observed. The 6 patients showed consistently higher numbers of CD69+^{high} with both α-GalCer and Brazil nut extract than without lipid, suggesting therefore that NKT cell activation was taking place. Also, statistical analysis on data from 6 volunteers (4 allergic and 2 healthy volunteers) showed that a greater number of

NKT responders to α-GalCer than nut lipid was observed ($p<0.05$).

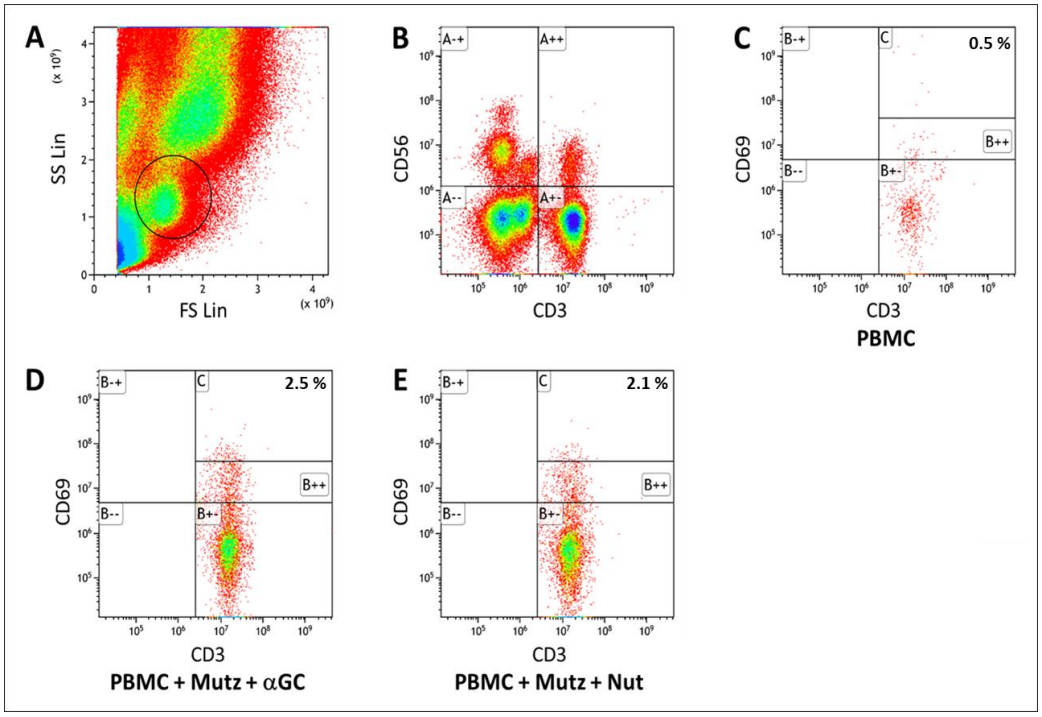


Figure 23 Activation of non-isolated NKT cells co-cultured with lipids loaded APCs. A) The FACS dot plot shows total cells and the lymphocyte gate. B) The FACS plot shows total lymphocytes from A) and staining for CD3 and CD56. C), D) and E) are of CD3+CD56+ cells from B) that showed CD69 activation. Dot plots based on only PBMC cells (C) was applied to other conditions (D and E). These plots are representatives of several measurements obtained from 6 volunteers (4 allergic and 2 healthy volunteers). The percentages of the gated cells showed no statistically significant differences between allergic and healthy individuals.

The α/β and γ/δ TCR sequences from single activated cells were amplified using a specific nested PCR protocol (Dash *et al.*, 2011) and the comprehensive primer sets reported by Thomas & Suzuki's Group (Guo *et al.*, 2016; Kitaura *et al.*, 2016). From this screening around 103 pairs of TCR DNA sequences were obtained; the sequences were analyzed and classified against human TCR sequence libraries using the IMGT/V-QUEST website (Lefranc *et al.*, 2003). A table containing the described sequences has been filed in Appendix 2.

5.3.3 Cloning and characterisation of lipid specific TCRs

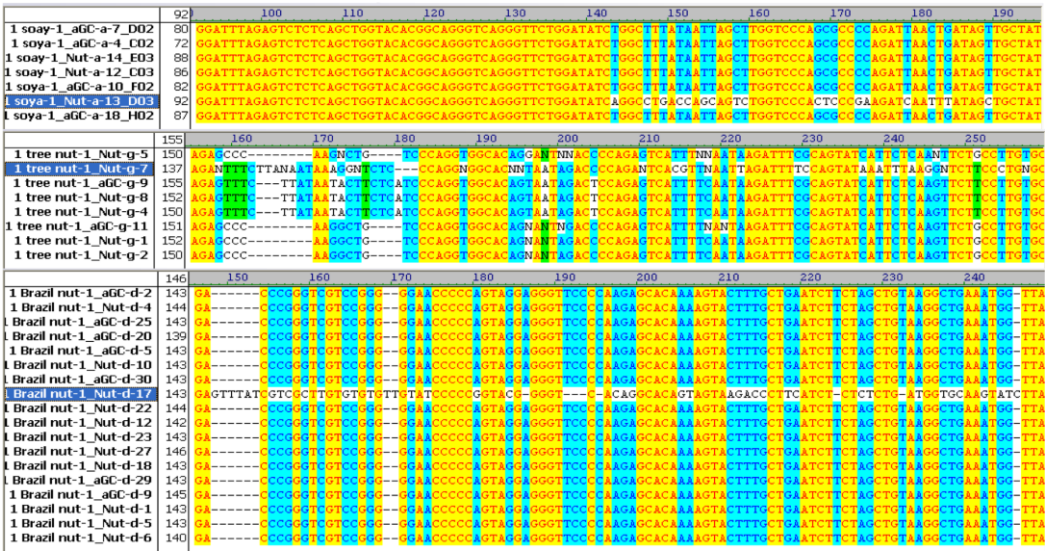


Figure 24 Examples of the alpha, gamma and delta TCR Brazil Nut lipid C-specific sequences. The unique sequences are soya-1 Nut-a-13, tree nut-1 Nut-g-7 and Brazil nut-1 Nut-d-17. Soya-1 Nut-a-13 represents sequences from volunteers who are allergic to soya, and the alpha chain from number 13 cell treated with Brazil nut lipid C.

The DNA alignment of the TCR sequences showed a great number of sequence similarities, suggesting either that the lipid activation protocol is partially selective or there is a potential bias introduced by the multiplex PCR protocol. Attempts to address this problem are ongoing by direct sequencing of RNAs using next generation technology. Notwithstanding, if for each patient the TCR sequences that reacted with α-GalCer were excluded and therefore regarded as background (Figure 24), it was possible to identify three pairs of nut lipid specific TCR sequences, 1 α/β and 2 γ/δ TCR (Figure 25). In order to characterise their specificity, the 3 nut lipid TCR sequences were cloned into the acceptor bidirectional plasmid pMJA251 (for γ/δ TCRs) and 252 (for α/β TCR), engineered to contain two specific type IIS restriction enzyme Esp31 sites. The full constructs were then re-named as pMJA290,

pMJA295 and pMJA297 and filed into Genbank as MK764035, MK764035, MK764035 respectively (Figure 25). pMJA290 is a γ/δ TCR classified by IMGT as γ TRGV4*01F and TRGJ1*02F or TRGJ2*01F, and δ TRDV2*03F, TRDJ1*01F and TRDD3*01F; pMJA295 is a γ/δ TCR classified as γ TRGV8*01F and TRGJ1*02F, and δ TRDV1*01F, TRDJ1*01F and TRDD2*01F or TRDD3*01F; pMJA297 is α/β TCR classified as α TRAV1-2*01F and TRAJ12*01F, and β TRBV6-4*01F or TRBV6-4*02F, TRBJ2-3*01F and TRBD1*01F. The lipid responsive γ/δ phenotype rather than the more abundant α/β was an unexpected result. The different usage of variants of V genes, normally not associated with α-GalCer was expected though, as the nut lipid used (lipid C) is a large mixture of lipid families. Brazil nut lipid C is composed of complex lipids, mainly triglycerides, sterylglycosides, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidic acid as described by our group (Mirotti *et al.*, 2013).

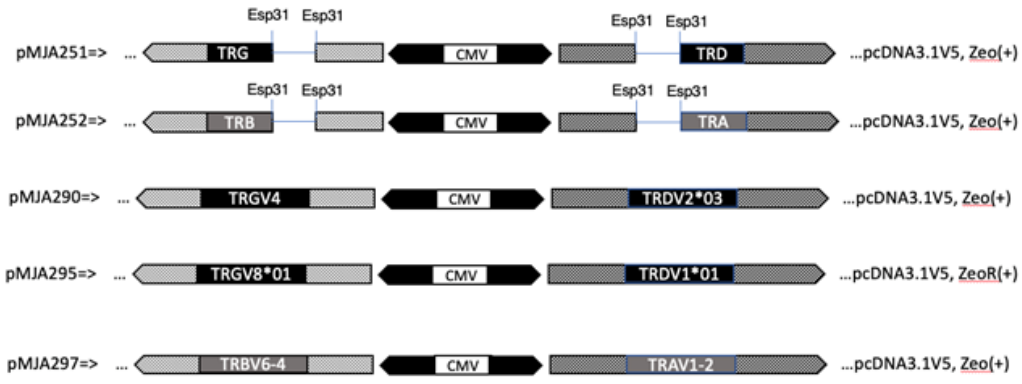


Figure 25 Diagram of plasmid constructs containing the nut specific TCR clones. pMJA251 and 252 = acceptor plasmids. pMJA 290 and 295 are γ/δ and 297 α/β TCRs. The full TCR construct gene sequences were filed at Genbank as MK764035, MK764035, MK764035.

Surrogate T cells (Jurkat 76) were subsequently transiently transfected with the bidirectional CMV expression clones and co-cultured with human Mutz-3 presenting α-GalCer/nut lipids, essentially as previously described (Wang *et al.*, 2019). pMJA219 (TRAV10/TRBV25) containing a synthetic TCR sequence specific to α-GalCer was used as control. In this system all three T cell lines transiently transfected with pMJA290, pMJA295 and pMJA297 TCR plasmids showed higher IL-2 expression in response to nut lipids than to α-GalCer (p-values: pMJA290=0.1872, pMJA295=0.0033, pMJA297=0.0064); only pMJA219 (TRAV10/TRBV25) recognized α-GalCer (Figure 26). In a more sensitive T cell luciferase assay, also described in Wang *et al.* (Wang *et al.*, 2019), the 3 plasmids showed the same degree of specificity towards Mutz-3 presenting nut lipids rather than α-GalCer in co-culture (p-values: pMJA290=0.0924, pMJA295=0.0555, pMJA297=0.0288). These results strongly suggested that the 3 isolated pairs of TCR sequences and expressed plasmids contained nut-specific TCR sequences.

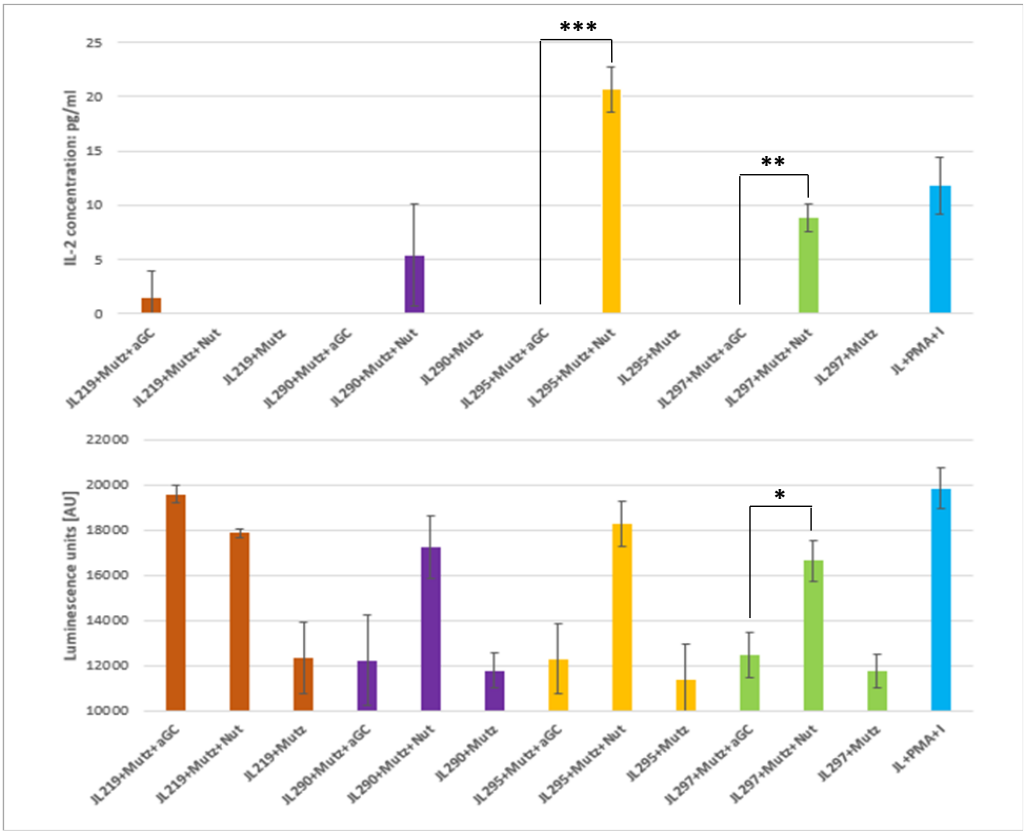


Figure 26 Transient transfection of Jurkat Lucia cells with nut-specific TCRs expression constructs and activated with human APCs coated with lipids. IL-2 (upper) and Luminescence (lower) of Jurkat Lucia cells transfected with nut-specific TCR expression construct and incubated with human APCs coated with αGelCer, nut lipids or without lipid. Results are averaged from triplicate wells. (*=p <0.05, **=p <0.01, ***=p <0.001)

5.3.4 Screening of nut natural lipid fraction C using lipid specific TCRs

The nut lipid fraction C, mainly recognised by the 3 TCR sequences, was investigated by fractionation of nut lipids into three major classes: neutrolipids (NL), glycolipids (GL) and polar lipids (PL). These lipid classes were then further fractionated by Thin Layer Chromatography as described in the methods section done by collaborators. Since neutrolipids did not show any activity in preliminary animal experiments (Dearman, Alcocer and Kimber, 2007) NLs fractions were not used for TCR assays. 15 fractions were isolated from the PL and GL classes (Figure

27) and preparative solutions were individually loaded onto Mutz-3 cells to be presented in co-culture to Jurkat 76 cells transiently transfected with pMJA290, pMJA295 and pMJA297 plasmids. As shown in Figure 27, expressed TCRs from pMJA295 and pMJA297 when loaded with lipids from fractions PL5, PL7 and PL8 from Brazil nuts do preferentially activate the surrogate T cell as shown by release of IL-2. These lipid fractions did not respond to the other TCR sequences or in the absence of the exogenous TCRs.

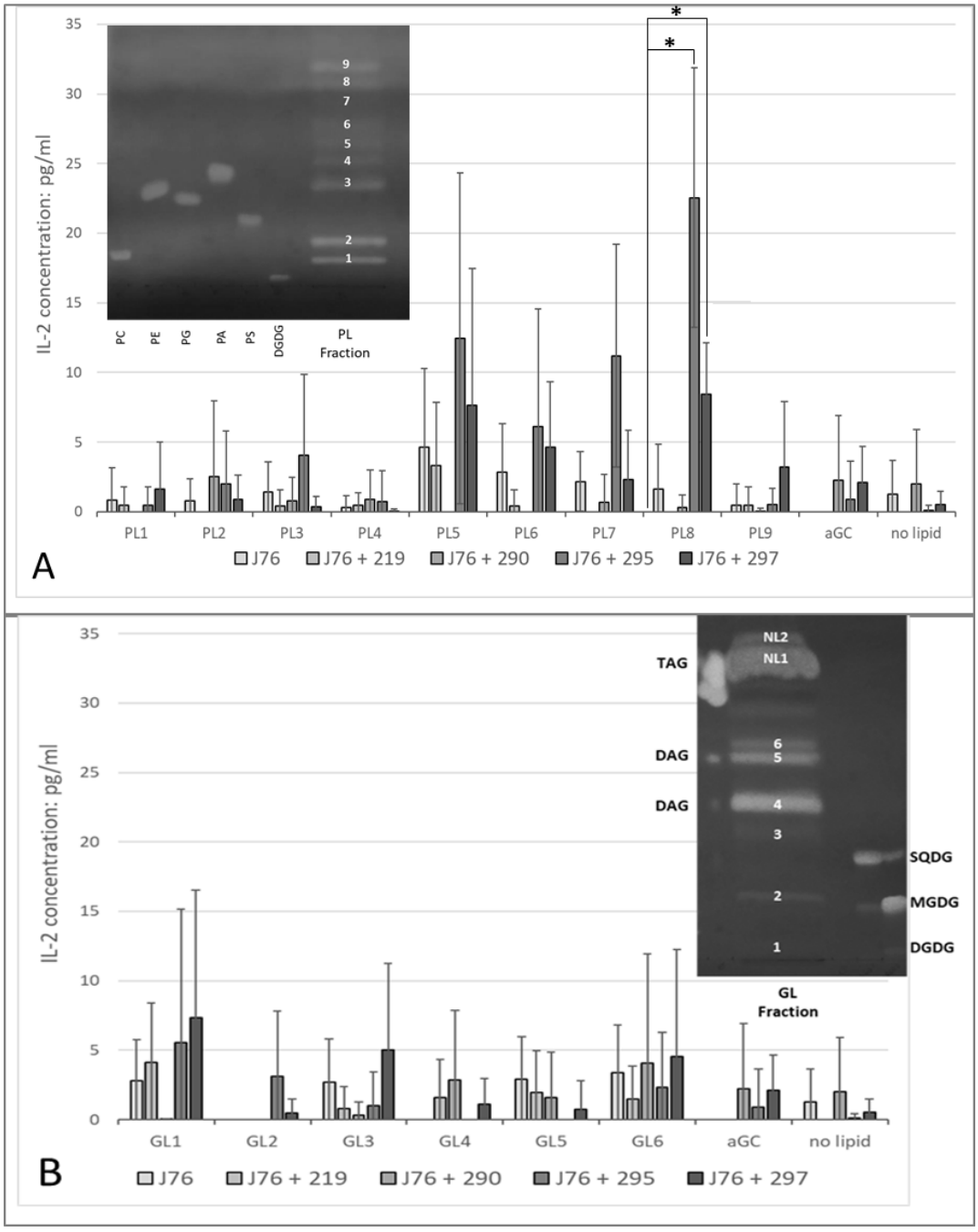


Figure 27 Fractionation of Brazil nut Polar lipids (PL) and Glycolipids (GL) by Thin Layer Chromatography (TLC) and in vitro TCR specific activation. IL-2 release from triplicate co-culture experiments containing Mutz-3 and pMJA290, pMJA295 and pMJA297 transiently transfected Jurkat 76 cells with lipid PL and GL fractions. The inserted photographs showed the fractionated of PL and GL classes by TLC and some known molecular markers as comparison. (*=p<0.05). PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PG: Phosphatidylglycerol, PA: Phosphatidic acid, PS: Phosphatidylserine, DGDG: Digalactosyldiacylglycerol, TAG: Triacylglycerol, DAG: Diacylglycerol, SQDG: Sulfoquinovosyldiacylglycerol, MGDG: Monogalactosyldiacylglycerol.

Fractions PL5, PL7 and PL8 are very minor components of Brazil nut polar lipids

(Figure 27A; insert). The identity of PL1-4 and some of the GL fractions could be tentatively assigned by comparison with molecular markers on TLC (Appendix 4). The identity of the remaining fractions was investigated by ESI-MS/MS using an Orbitrap instrument as described in the method. From Figure 27, it can be inferred that pMJA295 and 297 showed a significant preference for polar lipids in fractions PL8>PL5>PL7, ESI-MS/MS analyses revealed that these fractions (PL5, 7 and 8) contain several classes of lipids but are enriched in a small number of phospholipid molecular species which were either not detected or present at very low levels in other, non-reactive PL and GL fractions (Figure 28). Preliminary and tentative compound identification using accurate mass of compounds and mass fractionation spectra suggested that the most active fractions PL8 and PL5 are enriched in phosphatidylethanol (Pet) and phosphatidylethanolamine (PE) molecular species respectively whereas PL7 also contains phosphatidylmethanol (PMet) and phosphatidylserine (PS) species. It is surprising to find PE and PS species migrating in less polar fractions on TLC (closer to the migration front). This suggests that they may have been complexed with more polar lipids not yet characterised. It was also unexpected to find Pet and PMet species as, to our knowledge, these have never been reported before in plants. These molecular species detected could be PE degradation products formed during the extraction, purification and separation procedures, however it would require phospholipase D activity and this is highly unlikely under consideration.

PE has also been reported by other groups. Using a panel of synthetic PEs, iNKT cells were shown to be activated by PE that contained at least one unsaturated acyl chain (Rauch *et al.*, 2003). More recently PE has been shown to activate NKT cells and induce an antiviral immune response in mice (Singh, Tripathi and Cardell, 2018).

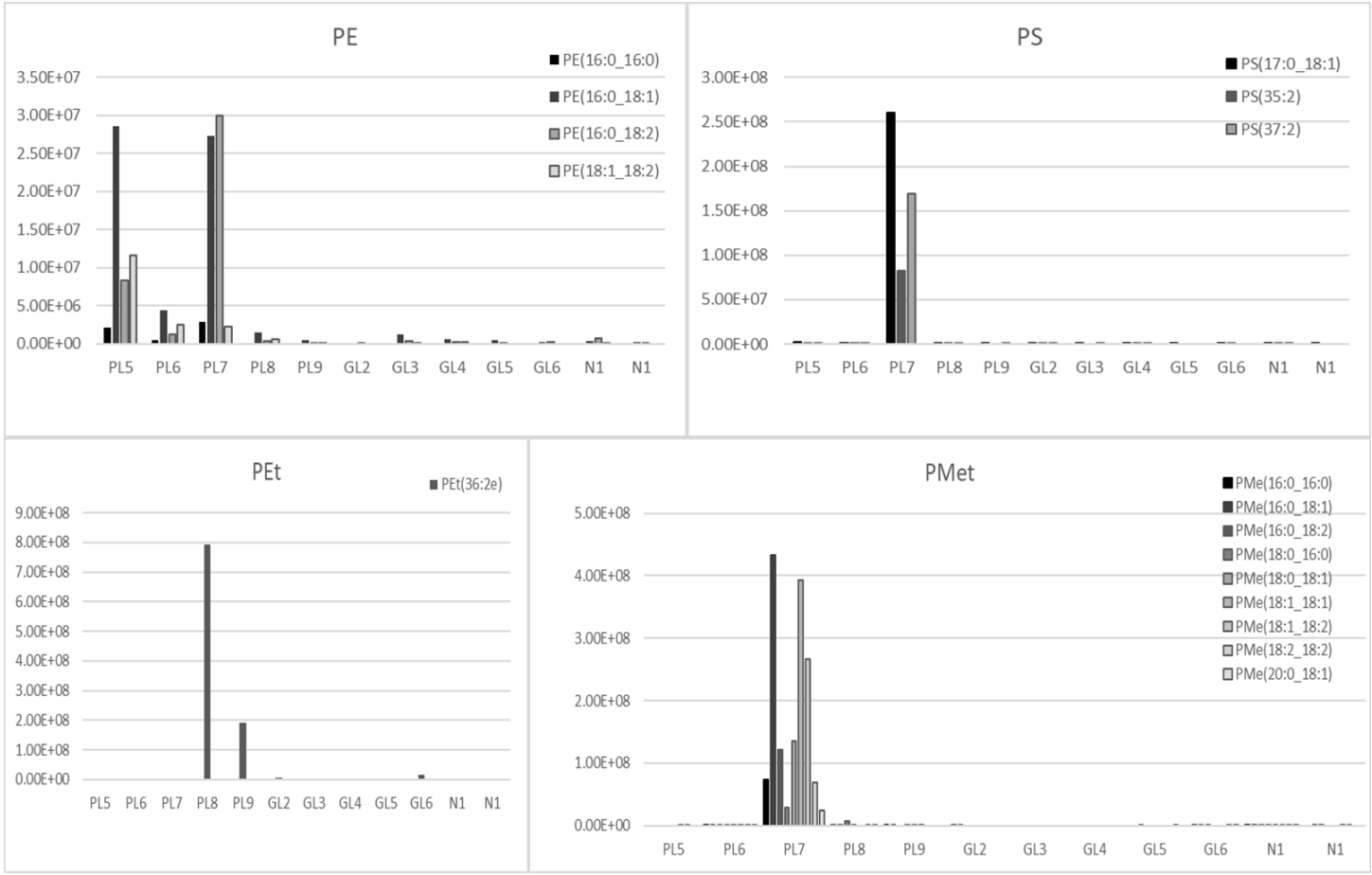


Figure 28 Lipid molecular species found enriched in fractions PL5, PL7 and PL8. PE: Phosphatidylethanolamine, PS: Phosphatidylserine, Pet: Phosphatidylethanol and PMet: Phosphatidylmethanol.

The results shown here suggest that particular classes of Brazil nut lipids are involved in the activation of NKT cells of nut allergic patients and that not only α/β but γ/δ TCRs such as pMJA295 and pMJA297 might be involved in the activation. Previous studies have shown that Ber e 1 can accommodate one lipid molecule (stoichiometry 1:1) with K_d of 5.6±0.1 μM, confirmed by NMR exchange experiments (Rundqvist *et al.*, 2012; Mirotti *et al.*, 2013). Whether the lipids described here are bound to Ber e 1 remains to be demonstrated. The results further support our initial hypothesis that natural plant lipids play an essential role in the intrinsic allergenicity of Ber e 1, important in the initial sensitisation phase. These approached offer a tested protocol for isolation of other lipid active TCR sequences.

Recently the potential role of natural lipids in the intrinsic allergenicity of proteins has also attracted the attention of many other groups. The putative active lipids described here, the campothecin cross-linked lipid described for Pru p 3 (Cubells-Baeza *et al.*, 2017) and the collective lipid ligands described in many other systems and reviewed elsewhere (Jappe *et al.*, 2019) are now a key part of the discussion on intrinsic allergenicity.

In a wider context, these initial findings further corroborate the general idea that an active talk is occurring between our adaptive immune system and innate defense components of the plant kingdom. The putative active lipids described recently as extra danger signals for plant proteins and the large number of plant pathogenesis-

related proteins recognized as major allergens are together further evidence of this talk.

Although exciting, the results presented here are just preliminary and do not unequivocally demonstrate that the nut PE and its derivatives alone are the differentiating factor between a protein (Ber e 1) which is able to sensitise and one which is inactive. For this the lipid needs to be fully characterised, synthesised and tested in *in vivo* sensitisation experiments (mainly Principal Component Analysis (PCA)) with adequate controls. The time and cost of these experiments are unfortunately beyond the scope of this work.

5.4 Conclusions

Of all activation markers tested, it was shown that CD69 is the relative optimal marker for the study as the early marker for activated NKT cells compared with CD25. CD69 is induced by T-cells right after the TCR down-regulation whereas CD25 expression takes comparatively more time. Some researchers observed that an increase in the expression of CD69 happens after 4 hours of T cell activation (Longo *et al.*, 2008) and it takes a longer time for the induction of CD25 expression. CD25 is the receptor for IL-2 and therefore essential if cell activation is to be successful via signalling pathways. Kenji *et al.* found the expression of CD25 expression on human PBMCs was enhanced after 72 hours of cell culture (Takizawa

et al., 2005). In terms of their functions, CD25 helps to understand the behaviour of NKT cells whereas CD69 gives strength to the activation process.

Primary lipid specific NKT cells were successfully isolated from PBMCs from six volunteers (4 allergic and 2 healthy). Two lipid classes (α-GalCer and Nut lipids) were presented by APCs. There was a clear indication that co-cultures without lipids still had activated primary NKT cells. This may be due to the ability of CD69 to upregulate by autoreactive stimulation. The CD3+ CD56+ CD69+^{high} stimulated NKT cells were individually cell sorted, amplified and sequenced. Over 200 TCR sequences were analysed and lipid specific sequences identified. As shown in Figure 24, most of sequences were similar, however, it was obvious that lipid specific sequences were present by comparing with others. Most sequences were not specific for any lipids, which means most of the TCR of activated NKT cells which are stimulated by different lipid-coated APCs are the same. It is interesting that the size of almost all the sequences was bigger than the size from the library. It might because the isolated sequences have some intron regions inside.

In the work presented here protocols for isolation of human primary NKT cells were improved and optimised, lipid binding TCRs were sequences and expressed and these TCRs were used to screen a Brazil nut lipid library. Although not yet final, the results shown here suggest that specific phospholipids (PL8) from nuts are involved in the activation of NKT cells of Brazil nut allergic patients and that γ/δ and α/β

TCR receptors such as the ones expressed by pMJA295 and pMJA297 are involved in the activation. The results from this study will help to characterise the intrinsic factors linked to Ber e 1 allergenicity and will ultimately, help to define what makes a common protein within a food matrix context, to function as an allergen to a particular group of susceptible individuals.

The main conclusions were as follow:

- CD69 is the relative optimal marker for the study as the early marker for activated NKT cells compared with CD25.

- Over 200 TCR sequences were analysed from PBMCs from six volunteers (4 allergic and 2 healthy). Three pairs of Brazil nut lipid C-specific TCR sequences were obtained by analysing all the sequences.

- Specific phospholipids (PL8) from Brazil nut are involved in the activation of NKT cells of Brazil nut allergic patients and that γ/δ and α/β TCR receptors such as the ones expressed by pMJA295 and pMJA297 are involved in the activation.

Chapter 6 Nanopore sequencing

6.1 Introduction

In this final research chapter, the aim was to generate, develop and optimize an experimental workflow to accurately sequence the targeted long-length TCR mRNA transcripts which were responsive to lipids using state-of-the-art NGS technology. RNA extracted from PBMCs co-cultured with a selection of lipids were used to generate lipid specific TCR sequencing libraries. The RNA mixtures were bulk sequenced on Oxford Nanopore MinION and analyzed by modern bioinformatic tools to assess their total TCR repertoire. As this is a very exploratory part of the work, several dead ends, mistakes and details on the optimization of the methodology are described. Due to time constraints, only the analysis of alpha TCR gene libraries from one nut allergic patient is described. Further analysis of the other classes from this and other patients will be carried out by the group and will be the subject of a future joint publication on the technique.

6.2 Materials and methods

6.2.1 Materials

6.2.1.1 Cells

Blood was obtained from a nut allergic volunteer and obtained through the Biobank under the ethical medical licence BS25062015. The Mutz-3 (human DC cell line)

was purchased from DSMZ. Directly conjugated monoclonal antibodies, namely mouse anti-human CD3-FITC, mouse anti-human CD56-PE and mouse anti-human CD69-PC5 were bought from Beckman Coulter.

6.2.1.2 Materials

SuperScript™ VILO™ Master Mix was bought from Invitrogen. Triton X-100 was bought from Sigma. RNeasy® Mini Kit was bought from Qiagen. MinION Nanopore sequence device, Flow cell (FLO-MIN106 R9) and SQK-PCS108/109 kit were bought from Oxford Nanopore Technologies. NEBNext Poly(A) mRNA Magnetic Isolation Beads were bought from New England BioLabs. Lactosylceramides 1500 was bought from MATREYA LLC Lipids and Biochemicals. Lipopolysaccharides (LPS) and Lyso-phosphatidylethanolamine (LPE) were bought from Sigma Aldrich.

6.2.2 Methods

6.2.2.1 Cells and culture conditions

The Mutz-3 cell line was cultured as described in 3.2.4. Primary human NKT cells were isolated as described in 3.2.7.

6.2.2.2 Co-culture

In this Chapter, all experiments were set up in triplicate wells and repeated twice. The activation of primary NKT cells used CD1d expressing APCs in co-culture experiments in 12-well plates. 1×10^6 Mutz-3 cells were added in each well in 500 µl of cell medium (RPMI 1640 10 % FBS and 0.1 % DMSO). A concentration of 5 µg/ml lipids were added into APCs and incubated for 1 hour. Afterwards, 1×10^6 PBMCs with 500 µl of cell media was added in each well. Each well containing the lipid loaded APCs and PBMCs had a final volume of 1 ml. The 12-well plate was incubated for 24 hours at 37°C, 5 % CO_2.

6.2.2.3 Flow cytometric analysis

Cells from each well were harvested into separate FACS tubes, 2 ml of PBA (500 ml PBS containing 30 % BSA and 20% Sodium Azide) was added and centrifuged for 5 min at 300 g. Supernatants were discarded and 5 µl antibodies (mouse anti-human CD3-FITC, mouse anti-human CD56-PE and mouse anti-human CD69-PC5) were added according to the manufacturer's instructions and incubated at 4°C for 30 min in the dark. Afterwards, 2 ml of PBA was added to each tube. Supernatants were discarded, and the pellet re-suspended in 0.5 ml PBA. Analysis and single-cell sorting were carried out in the 96-well PCR plate on the Astrios cell sorter at the Flow Cytometry Facility (University of Nottingham). 20,000 cells were sorted out

into each tube contained 350 µl RLT buffer, or into each tube containing 4 µl VILO, 2.2 µl 1% Triton and 14 µl PCR water.

6.2.2.4 General Nucleotide manipulations (Nanopore SQK-PCS108/109 kits)

Total RNA was extracted by centrifuging 20,000 cells in each FACS tube for 5 min at 300 g in a total volume of 350 µl RLT buffer. RNA extraction was carried out essentially as described by the manufacturer's instructions using RNeasy® Mini Kit (QIAGEN). Poly-A mRNA magnetic isolation was carried out according to the manufacturer's instructions (NEBNext Poly(A) mRNA Magnetic Isolation Module). The qualitative and quantitative quality controls for RNA were carried out using the Agilent 2100 Bioanalyzer system. Reverse transcription and strand switch synthesis was carried out as described in the Sequence-specific cDNA-PCR sequencing SQK-PCS108/109 Nanopore protocol. Essentially 50 ng RNA or 1 ng poly-A RNA containing VNP primer or TCR constant region primers (Appendix 5) were incubated at 65 ºC for 5 min and then snap cooled. For the optimized protocol they were incubated at 90 ºC for 5 min, then snap cooled twice. The strand-switching buffer (SQK-PCS108: 4 µl Superscript IV buffer, 1 µl RNaseOUT, 1 µl 100 mM Dithiothreitol (DTT) and 2 µl SSP primer; SQK-PCS109: 4 µl 5× Reverse transcription (RT) buffer, 1 µl RNaseOUT, 1 µl Nuclease-free water and 2 µl SSP primer) was added into the annealed RNA and incubated at 42 ºC for 2 min. 200 U of Reverse Transcriptase (SQK-PCS108: SuperScript IV Reverse Transcriptase;

SQK-PCS109: Maxima H Minus Reverse Transcriptase) were then added and the mixture incubated at 50 ºC for 10 min, 42 ºC for 10 min, 80 ºC for 10 min and kept at 4 ºC for the SQK-PCS108 or 42 ºC for 90 min, 80 ºC for 5 min and kept at 4 ºC following SQK-PCS109.

6.2.2.5 Amplification /bar coding and attachment of adaptor

A volume of 25 µl LongAmp Taq Master Mix, 1.5 µl cPRM/Bar coding LWB primer, 18.5 µl Nuclease-free water and 5 µl Reverse-transcribed RNA sample were added into a tube for selection of full-length transcripts. The PCR program was run as follows: 30 s at 95˚C, 15 s at 95˚C, 15 s at 62˚C, 50 s at 65˚C, repeat 34 times from step 2 to 4, 6 min at 65˚C and hold at 4˚C. Then, 1 µl NEB Exonuclease 1 (20 units) was added into each PCR tube to clean the AMPure XP beads and incubated at 37 ºC for 15 min followed by 80 ºC for 15 min. All PCR reactions from one nut allergic volunteer and different barcoding were added into a 1.5 ml Eppendorf DNA LoBind tube. AMPure XP beads were added into the reactions and incubated for 5 min at room temperature. The tube was kept on the magnet, the supernatant removed and beads were washed twice with freshly 70 % ethanol without disturbing the pellet. The tube was then removed from the magnetic rack, the pellet resuspended in Elution Buffer (EB), incubated for 10 min at room temperature and kept on the magnet until the eluate was clear and colourless. The amplified DNA was removed into a clean Eppendorf DNA LoBind tube for analysis of the size, quantity and

quality as described above. 1 µl of Rapid Adapter (RAP) was added into the amplified cDNA library (100 ng) and incubated for 5 min at room temperature. The prepared library was then loaded into the MinION flow cell. Priming, loading and running of the flow cell process were carried out essentially as described following manufacturer's instructions (MinION Nanopore sequence device).

6.2.2.6 Bioinformatic tools

All the (DNA) bases were called using the MinKNOW software available at the Oxford Nanopore Technologies web site. The DNA sequences were compiled in MATLAB and separated in classes by barcodes using the Flexbar version 3.0.3 platform (Roehr, Dieterich and Reinert, 2017) and Skewer (Jiang *et al.*, 2014). The texts were visualised and organised using grep tool bar. The bioinformatic support was kindly given by Andrew Ward from ADAC, University of Nottingham. The specific V, D, J genes and their classification were obtained by sequence searches at the IMGT/V-QUEST website. The Veen diagrams were obtained on line at http://bioinformatics.psb.ugent.be/webtools/Venn/.

6.3 Results and discussion

6.3.1 Procedure using purified activated NKT Cells

Human PBMCs from a nut allergic volunteer were isolated and co-cultured with a

stable human DC cell line (Mutz-3 cells) as APCs in the absence or presence of α-GalCer. As shown in Figure 29, co-cultures with lipid (α-GalCer) loaded Mutz-3 cells had a higher number of activated primary NKT cells, demonstrating an increased ability for activation. The CD3+CD56+CD69+^{high} cells which responded to α-GalCer (Figure 29D) were then FACS sorted (20,000 cells/tube) and collected into the lysing reagent and subjected to RNA extraction using RNeasy Mini Kit.

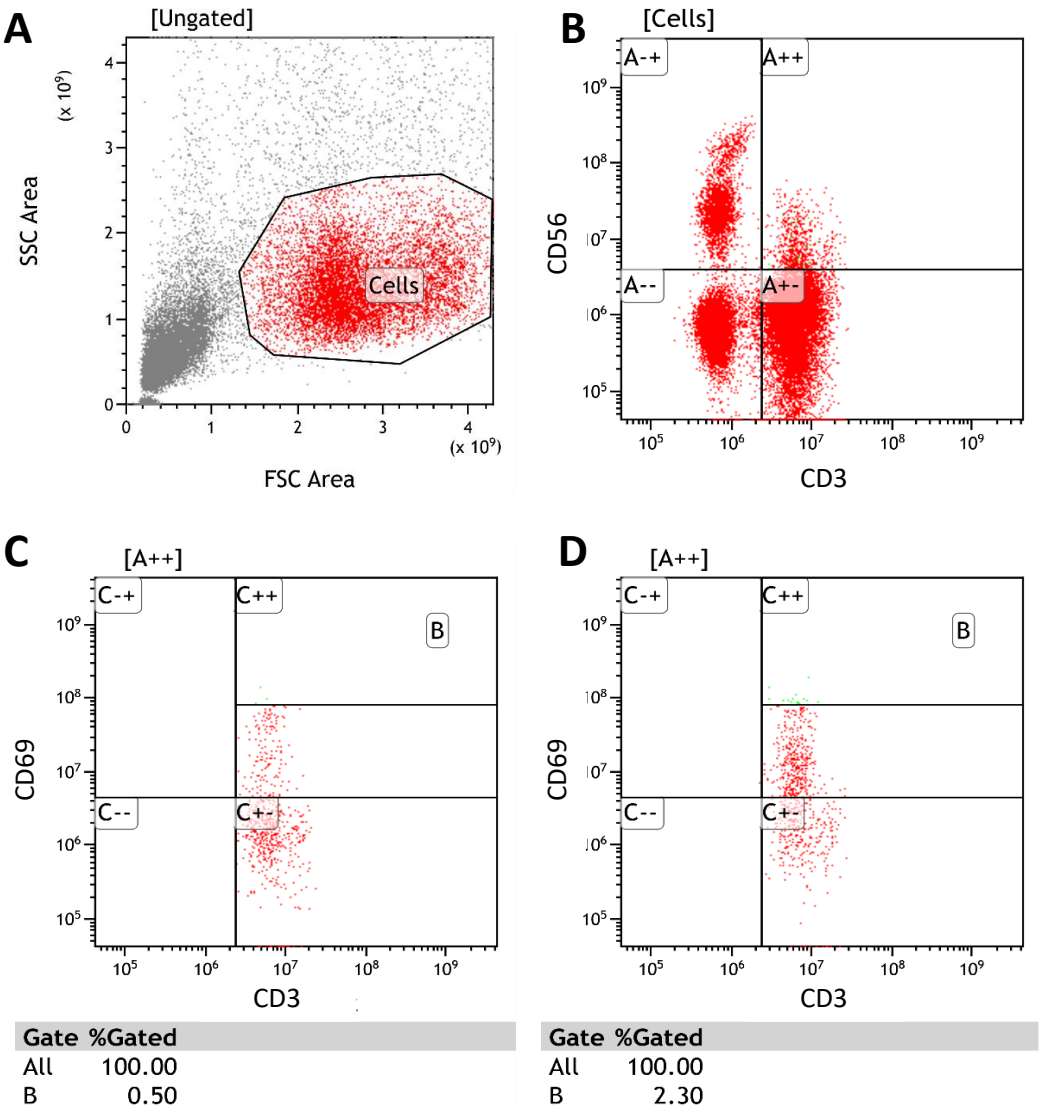


Figure 29 Activation of non-isolated NKT cells co-cultured with lipid loaded Mutz-3 APCs. A) The FACS dot plot shows total cells and the lymphocyte gate. B) The FACS plot shows total lymphocytes from A) and staining for CD3 and CD56. C) and D) are of CD3+CD56+ cells from B) that showed CD69 activation. Dot plots based on only PBMC cells (C) and when applied with α-GalCer (D). These plots are representatives of several measurements obtained from a nut allergy volunteer.

The RNA Integrity Number (RIN) values were utilized to test the quality of the RNA samples (Schroeder *et al.*, 2006). As shown in Figure 30, the RIN number of total RNA extracted from PBMCs co-cultured with α-GalCer-loaded Mutz-3 cells was 1.2, and the poly-A isolated RNA extracted from PBMCs co-cultured with α-GalCer-loaded Mutz-3 cells was too low to be measured. At this point the resulting

procedure was too long (>30h). The following steps were taken: after the PBMCs separation, the NKT cells were co-cultured with lipid-coated APCs for 24h incubation, then extracellular staining (approximately 1.5h) and FACS cell sorting (approximately 2h), and the RNA extracted (approximately 1h) performed. The long and complex experimental procedure may be the main reason that led the RNA degradation, causing the low RIN number.

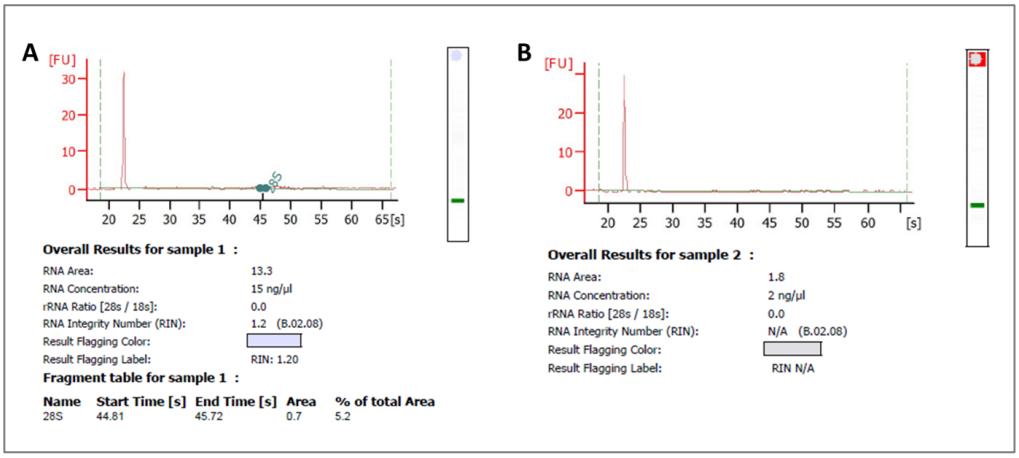


Figure 30 The RNA qualitative and quantitative results from Agilent 2100 Bioanalyzer system. Peaks produced from electropherograms (graph) that depict the size distribution of RNA fragments, with the corresponding gel-like image of RNA fragments (box), and metrics of RNA concentration and integrity (RIN number). Two representative samples with A) the total RNA extracted from PBMCs co-cultured with α-GalCer-loaded Mutz-3 cells, and B) the poly-A isolated RNA extracted from PBMCs co-cultured with α-GalCer-loaded Mutz-3 cells.

Several attempts to amplify the low RIN number RNA using the PCS108 procedure described by Nanopore have failed. In this attempt four methods were used to perform the RNA reverse transcription and strand switching process using the total RNA and poly-A RNA, as described in Figure 31A. In our study, the TCR sequences needed to be separated by different chains (α, β, γ and δ). Hence, 8 constant primers were designed at the 3' end of the TCR constant region, 2 for each constant chain

(Appendix 5).

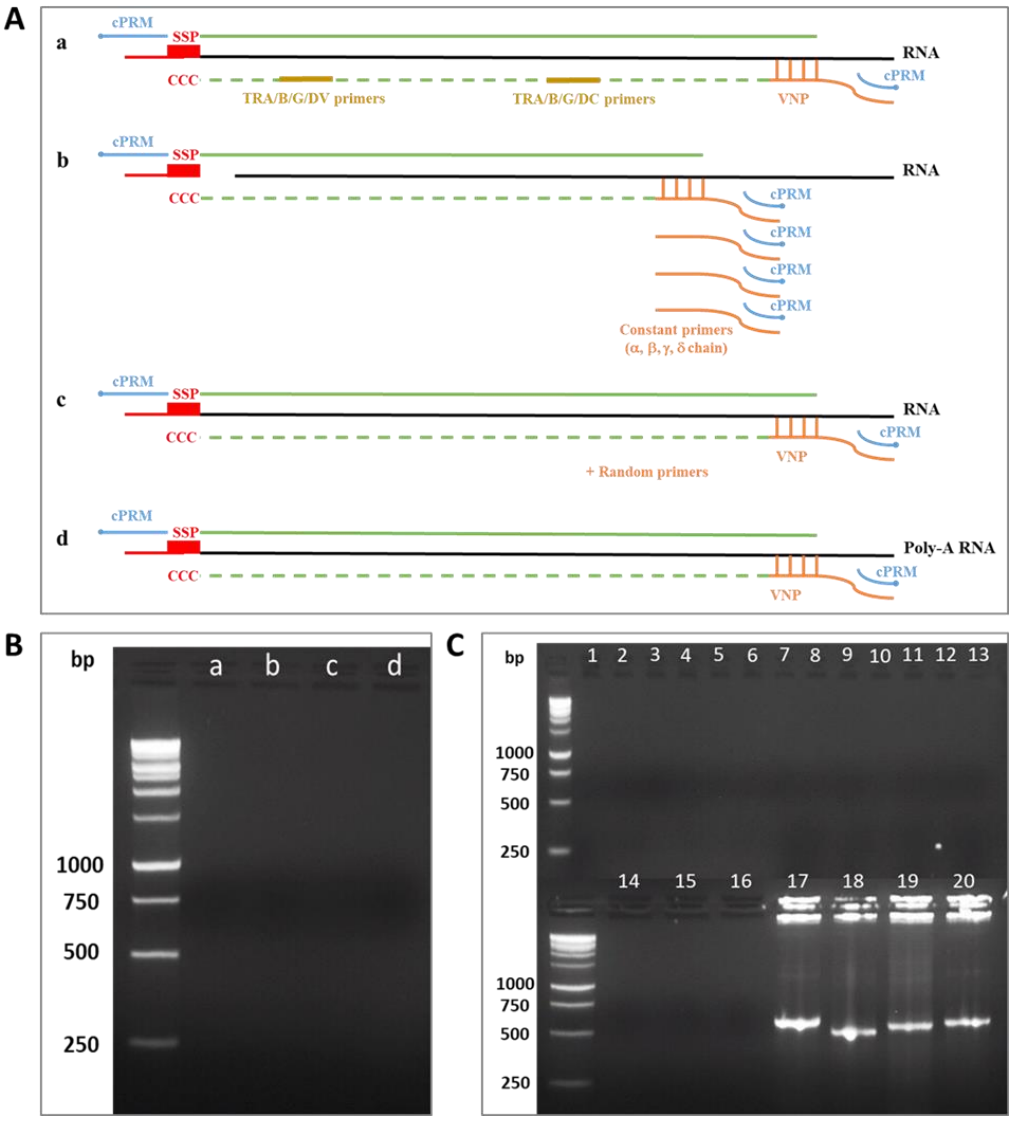


Figure 31 A) RNA reverse transcription, strand switching methods. B) PCR products obtained by using Reverse transcription (RT)-RNA from the four methods with cPRM primers. C) PCR products obtained by using the RT-RNA from four methods with TRA/B/G/DV primers and TRA/B/G/DC primers (1-16). PCR products obtained using TCR plasmids as positive control (17-20). B) and C) Analysis of PCR products on 2% agarose gel. Electrophoresis was at 90 V. Gels were stained with ethidium bromide, and DNA was visualized under UV. 1kb DNA ladder marker.

As shown in Figure 31B all the amplifications described in Figure 31A failed. All the results at this stage strongly suggested that the extracted RNA was degraded. As

described previously, the long and complex experimental process may have led to the RNA degradation. Further, as discussed by others, FACS cell sorting is a stressful process that may reduce cell viability and subsequently the quality of the isolated RNA (Loontiens *et al.*, 2019).

6.3.2 FACS to sort activated NKT cells, VILO to do RT-PCR and nested PCR to sequence

The same FACS cell sorting experiment was used successfully during the single cell isolation in Chapter 5. Whether the second strand cDNA syntheses were the problem has been addressed by using the VILO system as previously described. The activated lipid-specific primary NKT cells (1,000 cells, 2,000 cells, 5,000 cells and 10,000 cells) were isolated by FACS and sorted directly into VILO reaction tubes. Then, each reaction was used to carry out the cDNA synthesis program as described in Chapter 5. The Nested PCR was carried out for the α chain by using TRA/B/G/DV primers and TRA/B/G/DC primers (Appendix 1). As shown in Figure 32, the PCR bands after the nested PCR program using VILO were around 450 bp, similar to our previous results and the bands on the gel were more clearly visible as the number of cells increased.

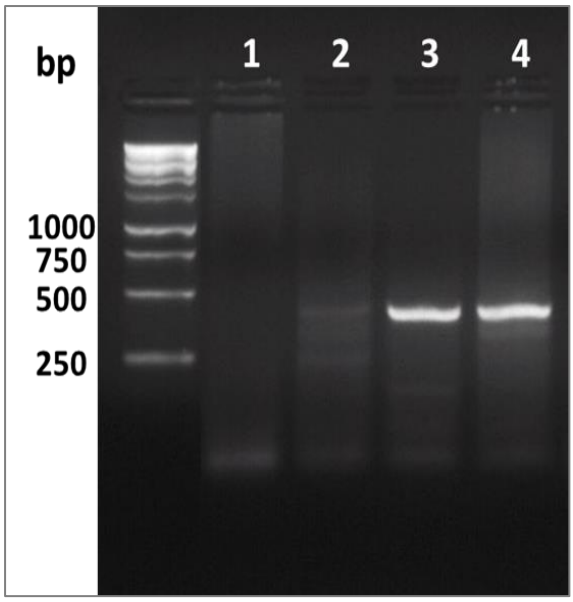


Figure 32 Analysis of PCR products on 2% agarose gel. Samples 1-4 were the Nested PCR products for the α chain from 1,000 cells, 2,000 cells, 5,000 cells and 10,000 cells respectively. Electrophoresis was at 90 V. Gels were stained with ethidium bromide, and DNA was visualized under UV. 1kb DNA ladder marker.

The same procedure was then be used for amplification of the larger fragment using TRA/B/G/DV primers with the constant primers (two primers for each chain) individually and together. As shown in Figure 33, in addition to the primer dimers, there was no visible band on the gel, which means the full length of TCRs was not obtained.

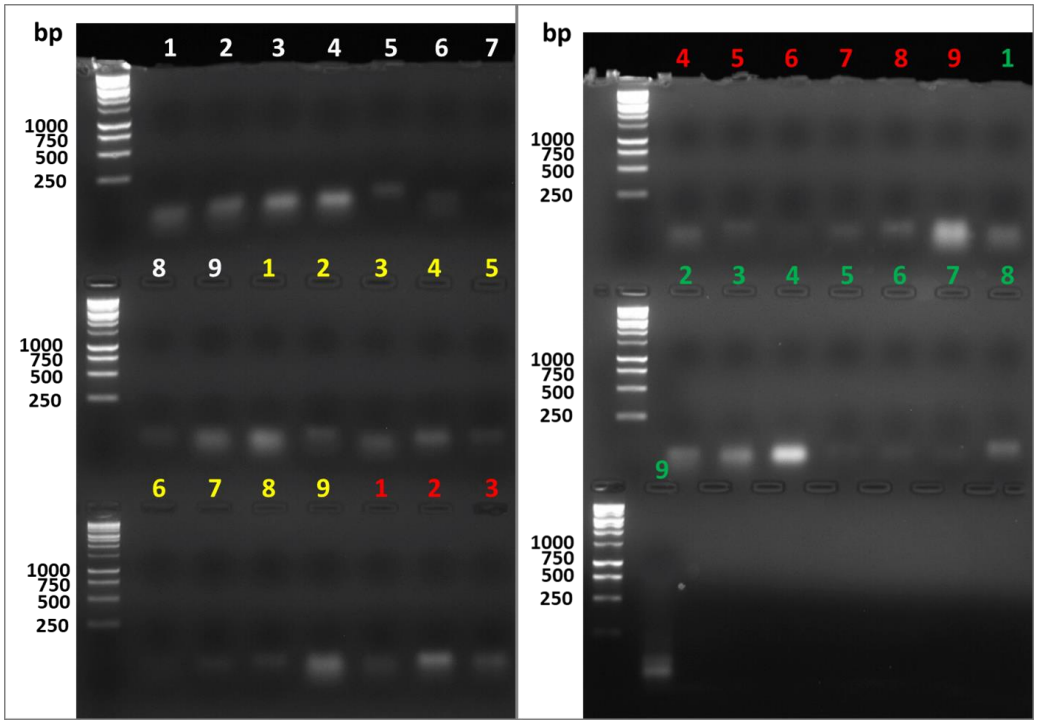


Figure 33 Analysis of PCR products on 2% agarose gel. PCR products obtained by using TRA/B/G/DV primers with constant primers (two primers for each chain) individually and together. Samples 1-9 in white color, in yellow color, in red color and in green color were the PCR products from 1,000 cells, 2,000 cells, 5,000 cells and 10,000 cells respectively. Electrophoresis was at 90 V. Gels were stained with ethidium bromide, and DNA was visualized under UV. 1kb DNA ladder marker.

These results demonstrated that it was not the reverse transcription procedure that was impaired in longer PCR amplifications. Thus, further work on improving the RNA quality was required.

6.3.3 RNA extraction directly after co-culture experiments, without FACS sorting

So far it has been demonstrated that the RNA integrity number was very low after FACS cell sorting system. As described by others, cell lysis should take place immediately after PBMC isolation in order to get the high RNA integrity (Baine, Mallya and Batra, 2013). In addition, the long co-culture incubation time might lead to RNA degradation as well. Thus, checking different co-culture incubation times

was necessary. Therefore, cells were directly collected after co-culture incubation and their RNA extracted. Four different incubation time courses (0h, 2h, 5h and overnight) were set up to check if the co-culture incubation time might affect the RNA integrity. As shown in Figure 34, the RNA RIN number of all treatments were 10, 9.9, 10 and 10 respectively, which strongly suggested that the FACS cell sorting process had a negative effect on the RNA integrity.

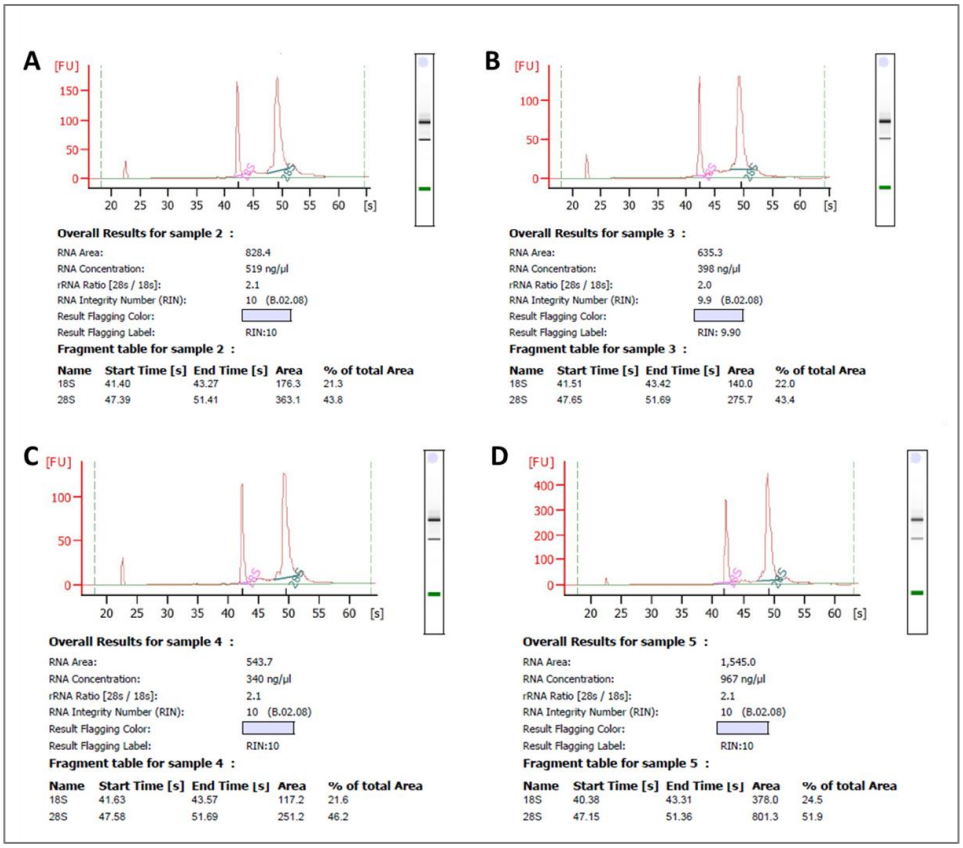


Figure 34 The RNA qualitative and quantitative results from Agilent 2100 Bioanalyzer system. Peaks produced from electropherograms (left) that depict the size distribution of RNA fragments, and the corresponding gel-like image of RNA fragments (right), and metrics of RNA concentration and integrity (RIN number) are shown. Four representative samples with the total RNA extracted from PBMCs co-cultured with α-GalCer-loaded Mutz-3 cells for A) 0h, B) 2h, C) 5h and D) overnight.

Meanwhile the protocols from Oxford Nanopore Technologies (ONT) were used to improve the sequence-specific cDNA-PCR sequencing of SQK-PCS108 and release

the SQK-PCS109. This new kit and protocol brought a few important alterations on the type of reverse-transcriptase and the supply of primers. Using the new protocol and procedure, and as shown in Figure 35 A, samples 1-4 showed a smear on the gel when the VNP primer was used, but there were just primer dimers on the gel for sample 5 when constant primers were used. In order to show whether specific TCR bands were present on the smears, the PCR products from samples 1-4 were re-amplified using TRAV and TRAC specific primers. As shown in Figure 35 B, faint bands of around 450 bp could be seen. This suggests the smear might contain TCR sequences.

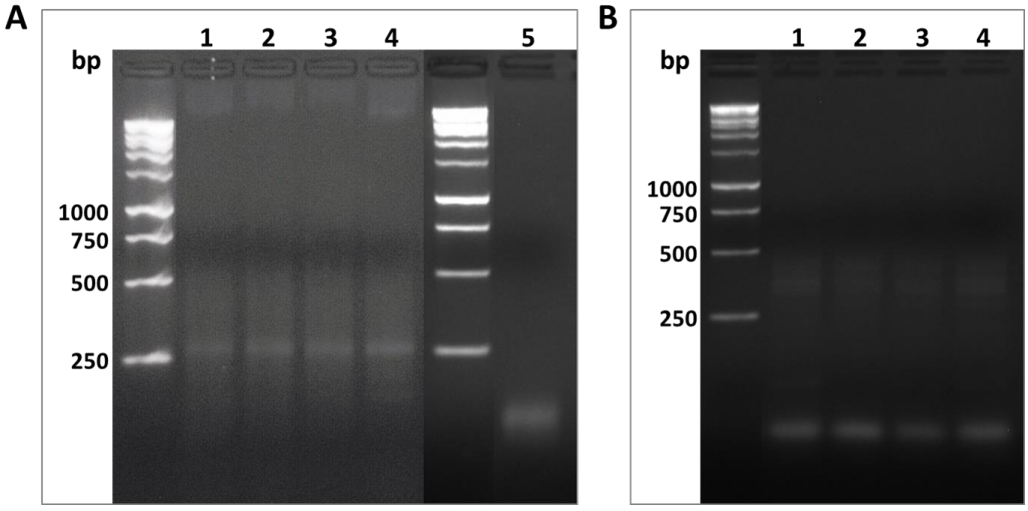


Figure 35 Analysis of PCR products on 2% agarose gel. A) Samples 1-4 were the PCR products obtained by using VNP primer to do the reverse transcription and using cPRM to do the PCR from 0h, 2h, 5h and overnight co-culture incubations respectively. Sample 5 was the PCR product obtained by using constant primers to do the reverse transcription and using a cPRM primer to do PCR from overnight co-culture incubation. B) Sample 1-4 were the PCR products obtained by using the product from A) samples 1-4 with TRAV primer and TRAC primer. Electrophoresis was at 90 V. Gels were stained with ethidium bromide, and DNA was visualized under UV. 1kb DNA ladder marker.

TRA/BV primers and constant primers were then used to check if the full length of

TCRs could be obtained. As shown in Figure 36, there were gel bands when the VNP primer was used to do the RT and there was nothing when the constant primers were used.

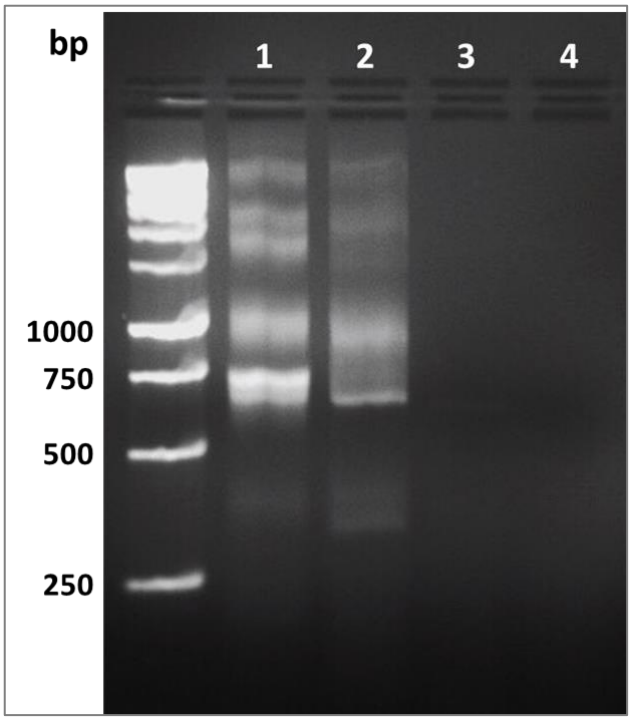


Figure 36 Analysis of PCR products on 2% agarose gel. Samples 1 and 2 were the PCR products obtained by using the RT-RNA (when using VNP primer to do the reverse transcription) with TRA/BV primers and constant primers for α/β chain to do PCR respectively. Samples 3 and 4 were the PCR products obtained by using the RT-RNA (when using constant primers to do the reverse transcription) with TRA/BV primers and constant primers for α/β chain to do PCR respectively. Electrophoresis was at 90 V. Gels were stained with ethidium bromide, and DNA was visualized under UV. 1kb DNA ladder marker.

Up to this point, the experimental protocol had been optimized using the VNP primer from Nanopore essentially as described in their protocol. In this study, four chains of TCRs needed to be separated, so TCR constant region primers were required instead of the VNP primer in order to label the different TCR chains. The VNP primer hybridizes to any poly A-tailed RNA and is tailed with a primer site for subsequent amplification (The Connectivity Database, 2019). Therefore, it was

speculated whether the constant primers' region was hidden, and not available due to secondary RNA structures. In order to test this concept, the heating temperature was increased to 90 ºC twice to break the RNA secondary structure. As shown in Figure 37A-1, after increasing the RNA heating temperature a smear was obtained. When re-amplified with TRAV primers and constant primers for α chain, the larger PCR product of around 700 bp was then visible as expected (Figure 37B). As recommended by Nanopore, AMPure XP beads purification is necessary before Nanopore sequencing. Therefore, as shown in Figure 37 B, the expected bands were brighter after the bead purification. The results so far demonstrated that a large >700bp PCR product is possible if FACS is not used for sorting, and if the specific constant region 3' end primer was used instead VPN and a thermal treatment to break the RNA secondary structure is carried out.

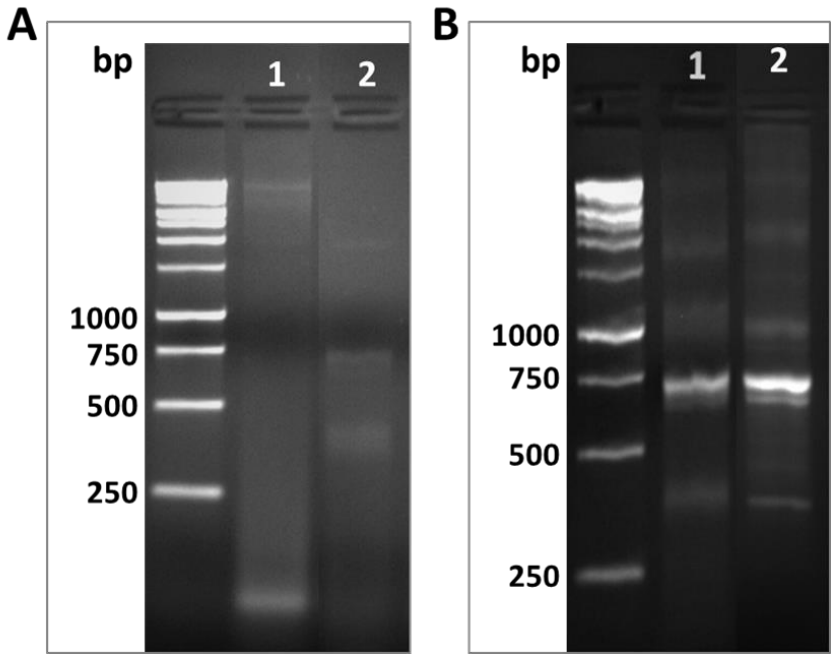


Figure 37 Analysis of PCR products on 2% agarose gel. A) Sample 1 was the PCR product obtained by using the RT-RNA (when using constant primers to do the reverse transcription) with cPRM primers to do the PCR. Sample 2 was the PCR product obtained by using sample 1 with TRAV primers and constant primer for the α chain to do the PCR. B) Sample 1 was the PCR product obtained by using the RT-RNA (when using constant primers to do the reverse transcription) with TRAV primers and constant primer for the α chain to do the PCR. Sample 2 was the PCR product obtained from sample 1 after AMPure XP beads cleaning. Electrophoresis was at 90 V. Gels were stained with ethidium bromide, and DNA was visualized under UV. 1kb DNA ladder marker.

6.3.4 Culture of nut allergic PBMCs with lipids, followed by RNA extraction

Using the optimised conditions, a library of TCR transcripts from a nut allergic volunteer was prepared using six different types of lipids. They were: α-GalCer, Sulfatide, Lactosylceramides, Brazil nut lipid, Lipopolysaccharides (LPS) and Lyso-phosphatidylethanolamine (LPE). α-GalCer and Sulfatides are normally described in NKT activation research (Sullivan and Kronenberg, 2005; Zhang *et al.*, 2011). Lactosylceramide is a known self-antigen (Brennan *et al.*, 2011). The Brazil nut lipid was lipid C described by our group (Mirotti *et al.*, 2013). LPS is a known

bacterial ligand from Escherichia coli and LPE is an allergen source from egg yolk (Bufe and Holst, 2004; van Hoogevest and Wendel, 2014).

After PMBCs extraction, cells were co-cultured and incubated with the different lipids and their RNA extracted. As shown in Figure 38, the RNA RIN number of all treatments was 10, which strongly suggested that the RNA integrity was good to proceed.

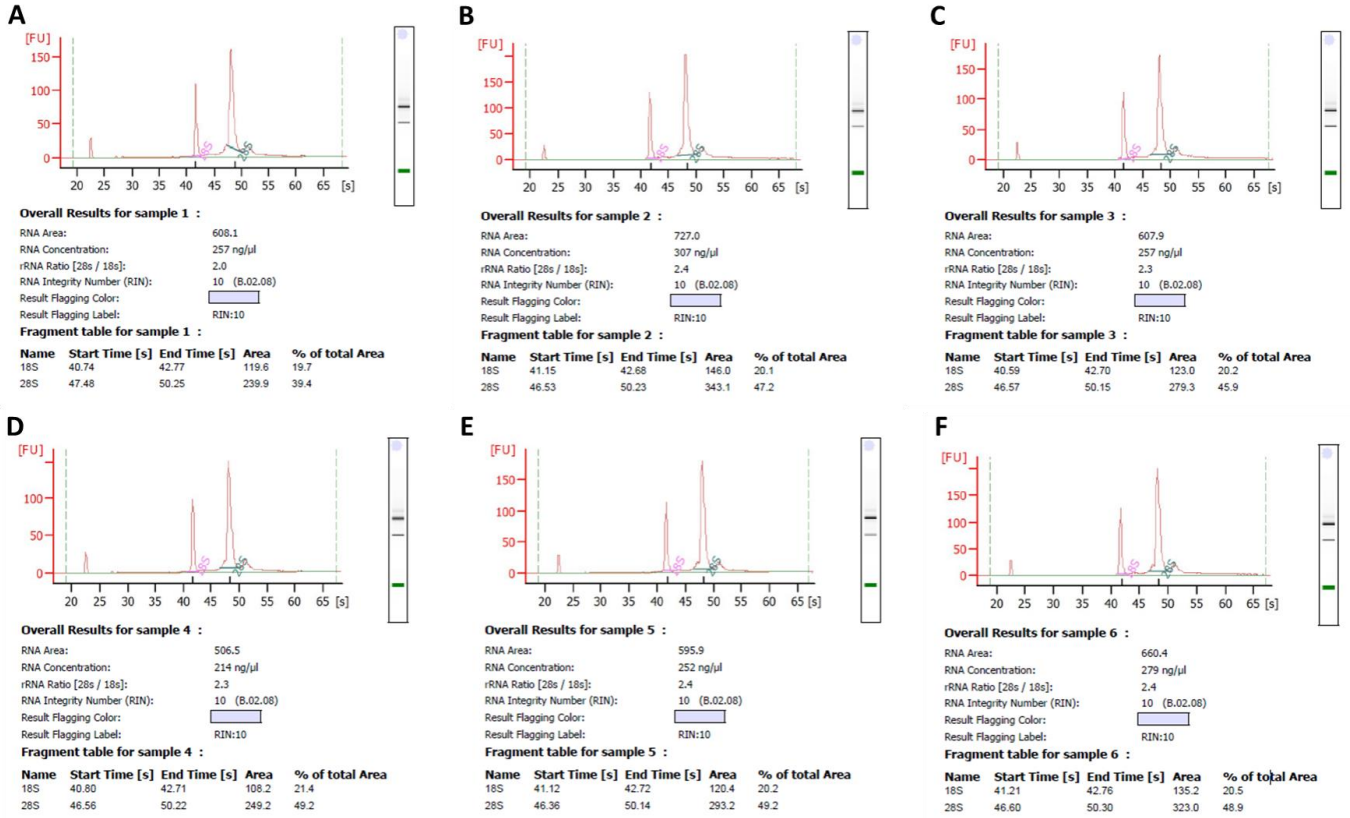


Figure 38 The RNA qualitative and quantitative results from Agilent 2100 Bioanalyzer system. Peaks produced from electropherograms (left) that depict the size distribution of RNA fragments, the corresponding gel-like image of RNA fragments (right), and metrics of RNA concentration and integrity (RIN number). The extracted RNA qualitative and quantitative results from one volunteer. Six representative samples with the total RNA extracted from PBMCs co-cultured with Mutz-3 cells loaded with A) α-GalCer, B) Sulphatide, C) Glycosphingolipids, D) Brazil Nut Lipid, E) Lipopolysaccharides (LPS) and F) Lyso-phosphatidylethanolamine (LPE).

The barcoding kits were as follows: LWB01 for α-GalCer, LWB02 for Sulfatide, LWB03 for Lactosylceramides, LWB04 for Brazil nut lipid C, LWB05 for LPS and LWB06 for LPE. LWB07, 08 and 09 were used as negative controls to label TCR sequences from PBMCs with Mutz-3 (no lipid), PBMCs only and Mutz-3 cells only.

The MinION run took 4.5h and the base calling software took 28h to be accomplished on an iMAC computer with a 1 Terabyte HD (Figure 39). All the sequences were called using the MinKNOW software, compiled in MATLAB and separated into classes by barcodes. The bioinformatic support was kindly given by Andrew Ward from ADAC, University of Nottingham. The sequences were analysed and classified against human TCR sequence libraries using the IMGT/V-QUEST website.

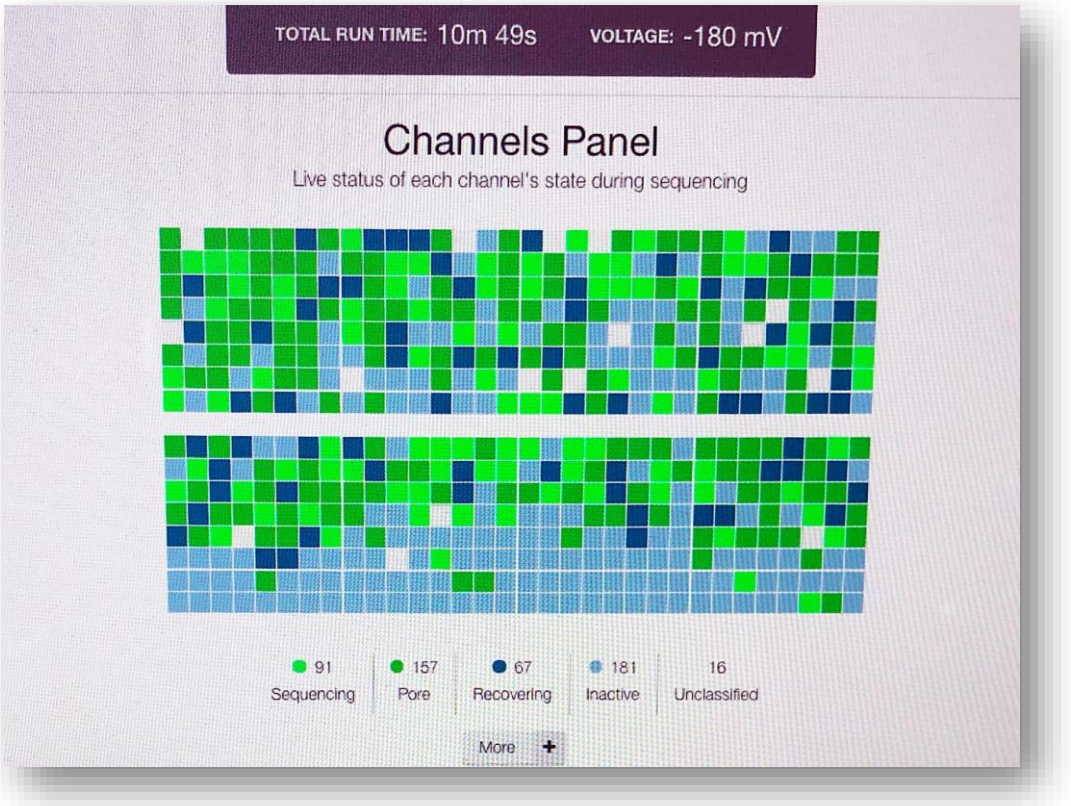


Figure 39 Real-time sequencing analysis on MinION Nanopore sequencer. Sequencing and Pore represents the number of active pores at any point in time. A good library is indicated by more channels in a state of Sequencing compared with Pore. Recovering represents channels that become available for sequencing again. Inactive represents channels that are no longer available for sequencing. Unclassified indicates channels that are not assigned one of the classifications.

As shown in Figure 40, following Nanopore sequencing, 728,566 transcripts were produced using the optimized method. 17.9% of the total Nanopore reads contained sequence lengths over 500 bp. A total of 1,999 sequences with LWB01 barcode, 3,036 with LWB02 barcode, 3,916 with LWB03 barcode, 11,230 with LWB04 barcode, 3,584 with LWB05 barcode, 183 with LWB06 barcode, 7,519 with LWB07 barcode, 7,763 with LWB08 barcode and 1,632 with LWB09 barcode were recovered.

20 TCR α chain sequences with >500bp containing LWB01 were identified, 43 with LWB02, 31 with LWB03, 253 with LWB04, 220 with LWB05 classification, 45 with LWB06, 29 with LWB07, 322 with LWB08 and 84 with LWB09. As the procedure delineated in this protocol should amplify T cell sequences, the large number of TCR α chains for PBMC only was expected. Interestingly the Mutz-3 cells alone also generated some sequences. In a quick search of Mutz-3 genomic sequences (Quentmeier *et al.*, 2019) these results have been confirmed.

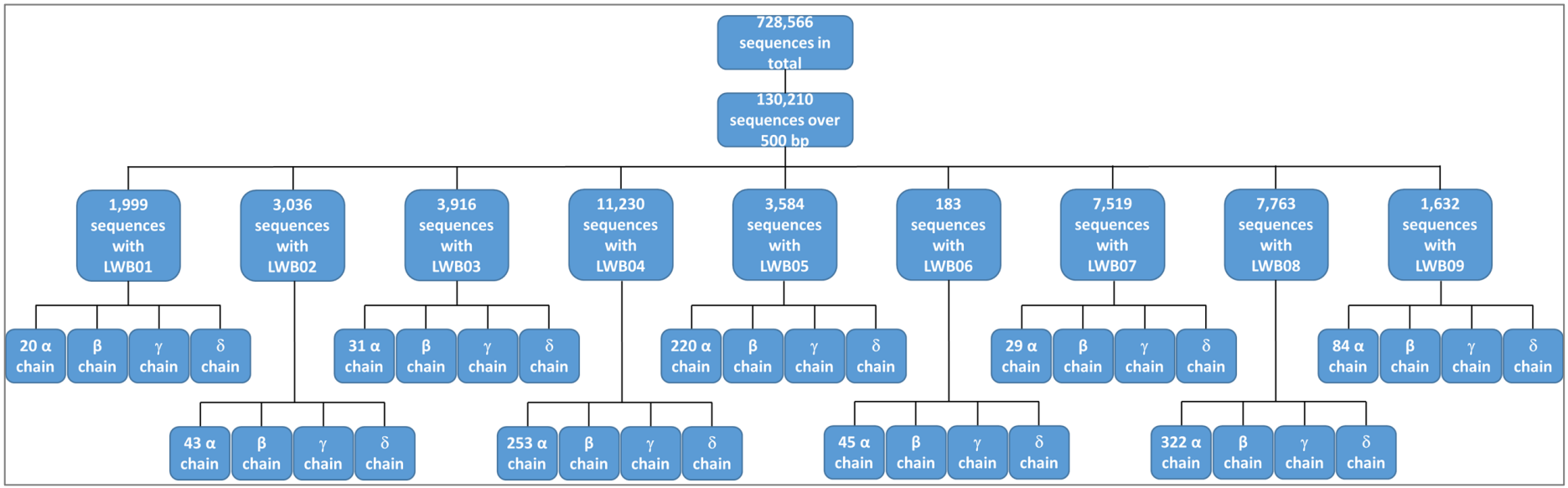
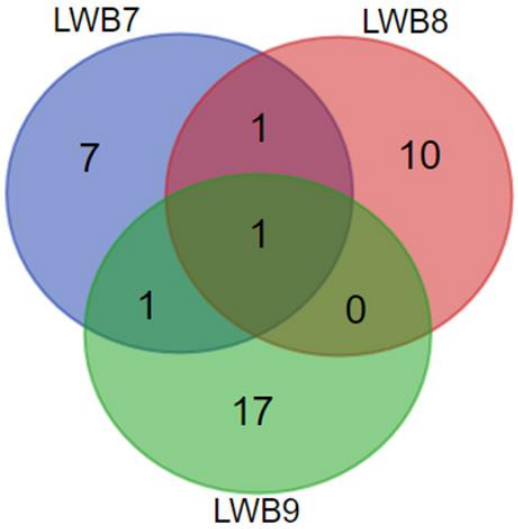


Figure 40 The classification and number of all the TCR alpha (α) transcriptions derived from Nanopore sequencing of PBMC and / or Mutz-3 antigen presenting cells following incubation with the lipids shown. Due to time constraints, only the alpha chain sequences were analyzed. LWB01 for α-GalCer, LWB02 for Sulfatide, LWB03 for Lactosylceramides, LWB04 for Brazil nut lipid C, LWB05 for Lipopolysaccharides (LPS), LWB06 for Lyso-phosphatidylethanolamine (LPE), LWB07 for PBMCs with Mutz-3 without lipid, LWB08 for PBMCs alone without lipid and LWB09 for Mutz-3 cells alone without lipid.

The assignment of the different gene families was carried out using the tools available at the IMGT/V-QUEST. There was little assignment of non-reference V and J genes. PBMCs co-cultured with Mutz-3 (APCs, no lipid), PBMCs alone without lipid and Mutz-3 (APCs) alone without lipid were added to serve as negative controls. As shown in Figure 41, except for non-reference sequences, there are 7 specific TCR α chain sequences obtained from PBMCs co-cultured with Mutz-3 (APCs, no lipid), 10 specific sequences from PBMCs alone without lipid and 17 specific sequences from Mutz-3 (APCs) alone without lipid. In addition to the non-reference sequences, there was just one shared TCRα sequence from LWB07 and LWB08 classification, which was from PBMCs. There was no TCRα sequence shared between PBMCs and Mutz-3 APC cells.



Names	total	elements
LWB7 LWB8 LWB9	1	no results
LWB7 LWB8	1	TRAV14/DV4*03TRAJ37*01
LWB7 LWB9	1	TRAV14/DV4*01
LWB7	7	TRAV19*01TRAJ3*01 TRAV14/DV4*03TRAJ18*01 TRAV14/DV4*01TRAJ8*01 TRAV14/DV4*02TRAJ45*01 TRAV14/DV4*01TRAJ22*01 TRAV14/DV4*04 TRAV3*01TRAJ43*01
LWB8	10	TRAV14/DV4*03TRAJ44*01 TRAV14/DV4*04TRAJ13*02 TRAV14/DV4*01TRAJ14*01 TRAV14/DV4*03TRAJ12*01 TRAV14/DV4*01TRAJ15*02 TRAV14/DV4*04TRAJ44*01 TRAV14/DV4*01TRAJ20*01 TRAV14/DV4*02TRAJ34*01 TRAV14/DV4*01TRAJ13*02 TRAV14/DV4*03TRAJ34*01
LWB9	17	TRAV14/DV4*02TRAJ21*01 TRAV14/DV4*03TRAJ41*01 TRAV14/DV4*01TRAJ49*01 TRAV14/DV4*01TRAJ10*01 TRAV14/DV4*01TRAJ31*01 TRAV14/DV4*01TRAJ12*01 TRAV14/DV4*01TRAJ11*01 TRAV14/DV4*02TRAJ43*01 TRAV14/DV4*03TRAJ13*01 TRAV14/DV4*01TRAJ21*01 TRAV14/DV4*03TRAJ50*01 TRAV16*01TRAJ15*01 TRAV19*01TRAJ16*01 TRAV14/DV4*01TRAJ53*01 TRAV14/DV4*01TRAJ26*01 TRAV14/DV4*01TRAJ30*01 TRAV14/DV4*04TRAJ26*01

Figure 41 The Venn diagram and the TCRα family for negative controls in the absence of lipid (LWB07: PBMCs + Mutz-3, LWB08: PBMCs alone without lipid, LWB09: Mutz-3 alone without lipid).

By removing the same sequences contained in the negative control groups, the bioinformatics alignment and statistical analysis of α chain sequences were performed for the TCR sequences and results are shown in Figure 42. There were 3 α-GalCer-specific TCR α chain sequences, 13 Sulfatide-specific TCR α chain sequences, 4 Lactosylceramides-specific TCR α chain sequences, 27 Brazil nut lipid C-specific TCR α chain sequences, 29 LPS-specific TCR α chain sequences and 3 LPE-specific TCR α chain sequences.

Names	total	elements
LWB1 LWB2 LWB3 LWB4 LWB5 LWB6	1	no results
LWB2 LWB3 LWB4 LWB5 LWB6	1	TRAV14/DV4*01
LWB1 LWB4 LWB5 LWB6	1	TRAV14/DV4*03
LWB2 LWB3 LWB4 LWB5	1	TRAV14/DV4*01TRAJ34*01
LWB1 LWB4 LWB5	1	TRAV14/DV4*04
LWB3 LWB4 LWB5	2	TRAV19*01TRAJ34*01 TRAV14/DV4*04TRAJ4*01
LWB1 LWB2	1	TRAV14/DV4*01TRAJ6*01
LWB1 LWB3	1	TRAV14/DV4*01TRAJ23*01
LWB1 LWB5	2	TRAV14/DV4*04TRAJ13*01 TRAV14/DV4*01TRAJ36*01
LWB1 LWB6	1	TRAV14/DV4*02TRAJ26*01
LWB2 LWB3	1	TRAV14/DV4*03TRAJ48*01
LWB2 LWB5	1	TRAV14/DV4*01TRAJ3*01
LWB3 LWB4	1	TRAV14/DV4*03TRAJ23*01
LWB4 LWB5	8	TRAV14/DV4*01TRAJ58*01 TRAV14/DV4*04TRAJ27*01 TRAV14/DV4*03TRAJ20*01 TRAV14/DV4*03TRAJ49*01 TRAV14/DV4*03TRAJ30*01 TRAV14/DV4*02 TRAV14/DV4*01TRAJ43*01 TRAV14/DV4*03TRAJ54*01
LWB1	3	TRAV14/DV4*02TRAJ42*01 TRAV14/DV4*03TRAJ51*01 TRAV14/DV4*04TRAJ33*01
LWB2	13	TRAV16*01TRAJ22*01 TRAV14/DV4*03TRAJ11*01 TRAV14/DV4*04TRAJ40*01 TRAV14/DV4*01TRAJ4*01 TRAV14/DV4*01TRAJ57*01 TRAV14/DV4*03 TRAJ20*01 TRAV14/DV4*02TRAJ14*01 TRAV14/DV4*03TRAJ21*01 TRAV14/DV4*03TRAJ3*01 TRAV14/DV4*02TRAJ44*01 TRAV16*01TRAJ20*01 TRAV14/DV4*01TRAJ40*01 TRAV14/DV4*03TRAJ26*01
LWB3	4	TRAV14/DV4*03TRAJ14*01 TRAV14/DV4*01TRAJ41*01 TRAV14/DV4*02TRAJ37*01 TRAV14/DV4*03TRAJ39*01
LWB4	27	TRAV14/DV4*04TRAJ51*01 TRAV14/DV4*04TRAJ53*01 TRAV14/DV4*03TRAJ27*01 TRAV29/DV5*01 TRAV16*01TRAJ23*01 TRAV29/DV5*01TRAJ52*01 TRAV14/DV4*04TRAJ42*01 TRAV14/DV4*03TRAJ5*01 TRAV14/DV4*01TRAJ25*01 TRAV14/DV4*01TRAJ48*01 TRAV16*01TRAJ27*01 TRAV14/DV4*03TRAJ53*01 TRAV14/DV4*02TRAJ10*01 TRAV14/DV4*03TRAJ4*01 TRAV14/DV4*01TRDJ1*01 TRAV8-1*02TRAJ10*01 TRAV14/DV4*03TRAJ16*01 TRAV14/DV4*01TRAJ35*01 TRAV14/DV4*03TRAJ47*01 TRAV14/DV4*04TRAJ1*01 TRAV14/DV4*02TRAJ53*01 TRAV14/DV4*04TRAJ38*01 F TRAV14/DV4*03TRAJ57*01 TRAV14/DV4*02TRAJ52*01 TRAV14/DV4*04TRAJ29*01 TRAV14/DV4*03TRAJ22*01 TRAV14/DV4*02TRAJ18*01
LWB5	29	TRAV14/DV4*01TRAJ9*01 TRAV14/DV4*01TRAJ18*01 TRAV14/DV4*02TRAJ41*01 TRAV14/DV4*01TRAJ59*01 TRAV14/DV4*03TRAJ11*01 F TRAV14/DV4*01TRAJ13*01 TRAV14/DV4*01TRAJ54*01 TRAV14/DV4*01TRAJ27*01 TRAV14/DV4*01TRAJ44*01 TRAV14/DV4*02TRAJ23*02 TRAV14/DV4*04TRAJ45*01 TRAV16*01TRAJ7*01 TRAV14/DV4*01TRAJ50*01 TRAV14/DV4*04TRAJ35*01 TRAV14/DV4*01TRAJ5*01 TRAV14/DV4*03TRAJ25*01 TRAV14/DV4*03TRAJ28*01 TRAV14/DV4*03TRAJ43*01 TRAV14/DV4*02TRAJ50*01 TRAV14/DV4*03TRAJ38*01 TRAV14/DV4*03TRAJ40*01 TRAV14/DV4*04TRAJ22*01 TRAV14/DV4*01TRAJ29*01 TRAV16*01TRAJ34*01 TRAV14/DV4*04TRAJ9*01 TRAV14/DV4*03TRAJ52*01 TRAV16*01TRAJ52*01 TRAV14/DV4*01 F TRAV14/DV4*03TRAJ31*01
LWB6	3	TRAV14/DV4*01TRAJ45*01 TRAV14/DV4*01TRAJ38*01 TRAV14/DV4*01TRAJ37*01

Figure 42 The TCRα family analysis for six lipids.

The high number of non-reference sequences might due to a limitation of the high error-rate (5-15%) of base-called Nanopore sequencing data (Rang, Kloosterman and de Ridder, 2018; Singh *et al.*, 2018). This is likely due to the challenges of the

signal processing of the ionic current measurements (Schreiber *et al.*, 2013). In this study, not only many non-reference sequences, but also single-base mutations were detected. In our optimized protocol, a mixture of 40 primers at 5' end of the PCR reaction was used. Interestingly however is that almost all TCR α chain sequences are from TRAV14 families. This suggests that only TRAV14 primers were amplified during the PCR reaction. This certainly is a bias in the methodology used here and might be due to a large number of PCR cycles required to generate sufficient material for Nanopore sequencing, which can distort the distribution of barcodes and sequences (Kebschull and Zador, 2015). Whether this is an allergic specific amplification cannot be discarded at this point and the analysis of other patients might shed some light on this.

These results are only preliminary and the first steps on this comprehensive and reasonably cheap technology. They clearly demonstrate that an amplified long-length TCR sequence library can be obtained using the Nanopore sequencing platform. These initial results are encouraging steps towards generating a full-length lipid-library of TCR transcripts.

6.4 Conclusions

In this Chapter, a rapid high-throughput method was described to sequence full-length TCR transcripts using targeted capture and Nanopore sequencing. The human

PBMCs from an allergic volunteer were isolated and co-cultured with a stable human DC cell line (Mutz-3 cells) as APCs in the absence or presence of six different lipids.

The main conclusions were as follow:

- Following nanopore sequencing, a total of 40,862 transcriptions were obtained, which uniquely aligned to TCR constant regions. There were 3 α-GalCer-specific TCR α chain sequences, 13 Sulfatide-specific TCR α chain sequences, 4 Lactosylceramides-specific TCR α chain sequences, 27 Brazil nut lipid-specific TCR α chain sequences, 29 LPS-specific TCR α chain sequences and 3 LPE-specific TCR α chain sequences.

- This novel method can be applied to high-throughput workflows to obtain targeted full-length TCR mRNA sequences from a large number of cells. The power of this method was demonstrated by combining transcriptome profiling with full-length TCR sequence characterization from thousands of human NKT cells. Using the Nanopore sequencing method, complete TCR sequences should be obtained with a reasonably cheap technology.

Chapter 7 Final discussion

The mechanisms that lead to the development of allergy are still poorly described. What makes an allergen and, in particular a food allergen, has not yet been defined. The storage 2S albumin Ber e 1 is the major allergen in Brazil nut and the first allergen to be transgenically transferred from one plant to another (Nordlee *et al.*, 2002). Previous work has demonstrated that lipids are essential for the allergenicity of proteins, mainly food proteins. In the case of Brazil nut, lipids were required for the induction of antibody response (IgE) to Ber e 1 in mice and possibly in humans. The purified Ber e 1 protein is not immunogenic in animals and plant lipids are essential for the allergenicity (Dearman, Alcocer and Kimber, 2007). Working with nut protein models experimentally, it was shown that natural plant lipids have an essential role in the allergenicity of the 2S albumin Ber e 1, but suggested that non-conventional T cells such as NKT cells are involved as important regulators in the initial allergic sensitisation phase to Brazil nut proteins (Mirotti *et al.*, 2013). This orchestrated action of APC, lipid, NKT (or/and Langerhans Cell (LC) and others) and T cells is activated via an MHC-like molecules (CD1)-lipid-TCR interaction and should result in large cytokine release.

Therefore, the underlying aim of this work was to utilise state-of-the-art *in vitro* techniques to undertake transient transfections using existing stable human cell lines to assess the ability of natural antigens from a variety of sources to act as ligands in the activation of non-conventional T cells. The proposed high throughput system should be stable, reproducible, robust and sensitive enough to act as a replacement

for animal experiments. For this, *in vitro* techniques were used to generate transiently transfected, stable human cell lines, a working protocol to isolate and characterise novel lipid binding TCRs from primary human NKT cells was optimised, a new NGS system was attempted and the expression and validation of the transfection of the isolated TCR into surrogate stable human cell lines was carried out.

The first part of this study aimed to optimize a sensitive *in vitro* TCR-lipid reporter cell system based on commercial cell lines. Initial characterization of human cell lines using known T-cell stimulators found that PMA and Ionomycin were the most suitable activators for Jurkat Lucia (NF-AT) cells, and Concanavalin A was the most effective in Jurkat Dual (IRF&NF-κB) cells. Both Jurkat Lucia and Jurkat Dual cells were able to produce higher signals if transfected with the α-GalCer specific TRAV10/TRBV25 expression plasmid (pMJA219) and when cultured with lipid-loaded APC (CD1). This suggests that the NF-AT and NF-κB signalling were induced on the lipid-TCR activation pathway. It was then concluded that Jurkat cells could be successfully used to emulate the function of NKT cells responding to external lipid stimuli through TCR activation. Other important information taken from the FACS data was that under our experimental co-culture conditions, Mutz-3 cells were a much more efficient lipid APC than Thp-1 cells. The percentage of transfected labelled TCR Jurkat Lucia cells expressing human TRAV10/TRBV25 protein receptors (pMJA219) has increased up to 72 hours.

As our initial hypothesis gravitated around lipid-NKT interaction and to our knowledge no human stable NKT cell line is available, some of the initial work as described in the Chapter 3 was carried out using the DN32.D3 murine cell line. This cell line, responsive only to α-GalCer has been widely used by many groups around the world. In our hands however it has shown to be problematic as discussed in Chapter 4. To overcome this limitation, the expression of human TCR α/β sequences was successfully attempted and achieved by two independent systems, one driven by a bidirectional promoter on a plasmid and another by a traditional lentivirus system. Both systems employed stable DC cell lines as lipid presenting cells, and a stable commercial T cell line as a surrogate system. A significant improvement in efficiency was achieved using TCR αβ/γδ null cell line (Jurkat 76) when using the lentivirus polycistronic constructs system containing the P2A sequence. The results suggested that the mis-pairing of the endogenous α/β TCR during ER folding in the presence of the new human TCR sequences could be impairing the functionality of the TCR lipid receptors. The details of the surrogate systems presented here and all the intermediate steps for its optimisation are now published (Wang *et al.*, 2019).

In order to isolate activated NKT cells from the general population of primary lymphocytes, particular activation markers needed to be characterised. In our experimental conditions the expression of CD69 was strongly induced by lipid stimulation. These results corroborate the work of other groups that observed an increase in the expression of CD69 at the early stage after activation (Longo *et al.*,

2008). Other slower (>72h) activation markers such as CD25 (Takizawa *et al.*, 2005) have been tried without much success. Thus, primary NKT cells co-cultured with α-GalCer-loaded APCs were shown by FACS to have a higher number of CD69+^{high} activated cells than when co-cultured with nut lipid-loaded APCs. The glycolipid α-GalCer (also called KRN7000) was originally identified in a screen for antitumor activity of compounds derived from marine sponges (Kobayashi *et al.*, 1995) and strongly binds to the NKT cell TCRs (Sidobre *et al.*, 2004). The nut lipid fraction C used in our experiments was derived from Brazil Nut and consists of a complex mixture of lipid classes. Previous studies have shown that the Brazil nut lipid fraction C was particularly active in animal sensitisation experiments (Mirotti *et al.*, 2013). As fraction C is a complex mixture of lipids, there is a possibility that the active nut lipid is only a small fraction of the total lipid, hence the reduced activity compared to α-GalCer.

In the work presented protocols for the isolation of single primary human NKT cells by FACS sorting were improved and optimised and the sequenced TCRs involved CD3+CD56+CD69+^{high} activated cells, purified from allergic and healthy volunteers. In this system active CD69+ cells were co-cultured with lipid loaded APC (Mutz-3). Therefore, primary lipid specific NKT cells were successfully isolated from PBMCs from six volunteers (4 allergic and 2 healthy). In this work, two lipids (α-GalCer and Brazil nut lipid C) were loaded to the APC and presented to the primary cells. CD3+CD56+CD69+^{high} stimulated NKT cells were

individually cell sorted, amplified and sequenced. In these experiments, there was a clear indication that co-cultures without lipids produced activated primary NKT cells. This may be due to the ability of CD69 to be upregulated by autoreactive stimulation (Wang *et al.*, 2008). With this in mind CD69+^{high} positive cells were the focus in this study. Over 200 TCR sequences were characterised and lipid specific sequences identified. Either due to the bias induced by the PCR amplification or the overall experimental conditions most of the TCR DNA sequences had similar sequences. Three pairs of nut lipid C-specific TCR sequences (two γ/δ and one α/β) however were identified and were assembled and tested into the *in vitro* cell system. Surrogate T cells (Jurkat 76) were transiently transfected with the nut specific TCR receptors and co-cultured with human APC presenting lipids. In this system all three T cell lines transiently transfected have shown higher IL-2 expression in response to nut lipids than to α-GalCer.

In subsequent experiments, the three nut specific transfected cell lines were used to screen fractions of Brazil nut lipids that have been produced by our collaborators at the Rothamstead experimental station and these recombinant cell lines showed specificity towards particular lipid fractions (Chapter 5). When analysed by ESI-MS/MS these fractions were composed of several classes of lipids but particularly enriched by a small number of phospholipid species. Preliminary and tentative compound identification suggests that the most active fractions PL8 and PL5 are enriched in phosphatidylethanol and phosphaditylethanolamines molecular species

respectively whereas PL7 also contains phosphatidylmethanol and phosphatidylserine species. It was surprising to find PE and PS species migrating in the less polar fractions on TLC (closer to the migration front). This suggests that they might have been complexed with more polar lipids not yet characterised. It was also unexpected to find Pet and PMet species as, to our knowledge, these have never been reported before in plants. These molecular species detected could be PE degradation products formed during the extraction, purification and separation procedures however it would require phospholipase D activity and this is highly unlikely under consideration. Although exciting, the results presented here are just preliminary and do not unequivocally demonstrate that the nut lipids identified in this study are the only the differentiating factor between a protein (Ber e 1) able to sensitise and an inactive one which results in tolerance. However, taken together these results further support our initial hypothesis that natural plant lipids might play an essential role in the intrinsic allergenicity of the nut major allergen Ber e 1.

The potential role of natural lipids or other food matrix components in the intrinsic allergenicity of proteins has also attracted the attention of other groups. Large numbers of protein allergens have been shown to possess lipid binding sites or lipid transfer functions and the collective lipid ligands described in many other systems, reviewed elsewhere (Cubells-Baeza *et al.*, 2017), are now essential components of the discussion on intrinsic allergenicity of proteins. The presence of the hydrophobic site and the observation that other components co-presented with proteins can

influence the initial stages of allergic sensitisation are of growing interest. Recent lipid-ligand binding data of Pru p 3 showed that ligand binding increased IgE binding and basophil activation presumably by exposure of IgE-binding epitopes otherwise buried inside the compact structure (Cubells-Baeza *et al.*, 2017). In the presence of Pru p 3, the lipid-ligand increased allergic sensitization by direct interaction with immune cells (activation of APCs, CD1 presentation and NKT cells). The lipid-ligand carried by Pru p 3 was presented by CD1 receptors expressed in APCs, and then recognized by NKT cells. The lipid-ligand seems to act as adjuvant modulating the immune system to a Th2 response towards IgE production (Tordesillas *et al.*, 2017). This further verified the hypothesis that allergic sensitization is enhanced by the recognition of lipid-ligands carried by some allergens. It has also been reported that the cat allergen Fel d 1 cannot directly activate the immune response, but requires binding to LPS (Herre *et al.*, 2013). Another group found that lipid binding to Par j 1 and Par j 2 from plant pollens might indirectly contribute to the allergenic potential of the molecules (González-Rioja *et al.*, 2009). These studies all suggest that lipids are involved in the intrinsic allergenicity of protein allergens.

In the current study, Ber e 1 lipid needs to be fully characterised, synthesised and few species of animal experiments run (mainly Principal Component Analysis (PCA)) with adequate controls. The time and cost of these experiments were unfortunately beyond the scope of this thesis.

The single-cell sequencing methodology used in Chapter 5 has multiple steps, and is difficult, very time consuming, expensive, limited in scope and prone to PCR biased amplifications. New high-throughput Next Generation Sequencing platforms such as Illumina are currently commercially available for sequencing of TCRs. Illumina technique provides great sensitivity and accuracy for TCR sequencing, and can be used to identify TCR diversity without quantitative restrictions. This powerful platform however can only generate short reads of a cDNA template, limiting the reconstruction of highly diverse sequences such as TCR (Singh *et al.*, 2018). To overcome these limitations and obtain long-read sequences, the nanopore methodology was explored and using a MinION device optimised a short sequencing methodology of TCR mRNA transcripts.

For this and only as an exploratory attempt a protocol was optimised as described in Chapter 6. Total RNA was extracted from PBMCs from human volunteers after co-culture with a selection of lipids. After several modifications in the first run, 728,566 transcripts were successfully produced, 17.9% of total reads containing sequences over 500 bp. As an average TCR sequence is 800bp this is a significant improvement over the other systems. By the time this thesis was written, the alpha chain sequences from one patient were analysed. Within the group containing >500bp, 3 α-GalCer-specific, 13 Sulfatide-specific, 4 Lactosylceramides-specific, 27 Brazil nut lipid-specific, 29 LPS-specific and 3 LPE-specific TCR α chain sequences were detected. The results demonstrate that

MinION Nanopore sequencing technique is a powerful method which can be used to sequence a large numbers TCR sequences. The analysis of and bioinformatic processing of the TCRs for the remaining patients is ongoing in the lab and will certainly be submitted for publication soon.

Nanopore sequencing is a rapid novel technology delivering long reads in real time on a portable instrument at low cost. According to the literature a major limitation of MinION sequencing is its lower read accuracy when compared with short-read technologies (Rang, Kloosterman and de Ridder, 2018). In this study, it has indeed shown to be the case. Many non-reference sequences were generated, and many single-base mutations were present when compared with TCR references at the IMGT database. In the one patient example depicted in this thesis, almost all TCR α chain sequences were from the same V gene family. This suggested that either the patient has a large bias or, most probably, that the PCR cycles required to generate sufficient material for Nanopore sequencing did not work properly. The analysis of other patients using separate four different chains might help to solve this bias. One of the largest drawbacks however for the NGS nanopore approach is the lack of information on the pairing of the TCRs. Pairing of a TCR α/β, γ/δ is essential for antigen recognition (Govers *et al.*, 2010). The production of shuffling β libraries for a few selected α chains as obtained for antibodies might be an option. Other more complex combinatorial arrangements have been proposed by several companies. iRepertoire Inc for instance has developed a sensitive method that cognates pairing

of human TCR α and β V-regions using the short read Illumina platform.

In summary, the results shown in this study further corroborate our previous studies and suggest that natural plant lipids and NKT cells are involved in the intrinsic allergenicity of nut proteins. By defining a putative natural hydrophobic ligand from Brazil nut, the work is one step closer to characterising what makes a common protein an allergen. The initial steps for production and testing of extensive lipid specific cell libraries have been delineated. An initial exploratory NGS system to sequence new TCR sequences has been attempted. It is hoped that the techniques described here will enable other materials of interest to be assessed with regards to their immune stimulation of allergic responses and potentially provide a screening assay to enable selection of commercially interesting materials with a reduced risk of immunomodulation.

Appendix 1

TRA gene(s) targeted by primer	External primer sequence (EXT)	Internal primer sequence (INT)
TRAV1	5′ AACTGCACGTACCAGACATC 3′	5′ GCACCCACATTTCTKTCTTAC 3′
TRAV2	5′ GATGTGCACCAAGACTCC 3′	5′ CACTCTGTGTCCAATGCTTAC 3′
TRAV3	5′ AAGATCAGGTCAACGTTGC 3′	5′ ATGCACCTATTCAGTCTCTGG 3′
TRAV4	5′ CTCCATGGACTCATATGAAGG 3′	5′ ATTATATCACGTGGTACCAACAG 3′
TRAV5	5′ CTTTTCCTGAGTGTCCGAG 3′	5′ TACACAGACAGCTCCTCCAC 3′
TRAV6	5′ CACCCTGACCTGCAACTATAC 3′	5′ TGGTACCGACAAGATCCAG 3′
TRAV7	5′ AGCTGCACGTACTCTGTCAG 3′	5′ ACAATTTGCAGTGGTACAGG 3′
TRAV8-1	5′ CTCACTGGAGTTGGGATG 3′	5′ GTCAACACCTTCAGCTTCTC 3′
TRAV8-2, 8-4	5′ GCCACCCTGGTTAAAGG 3′	5′ AGAGTGAAACCTCCTTCCAC 3′
TRAV8-3	5′ CACTGTCTCTGAAGGAGCC 3′	5′ TTTGAGGCTGAATTTAAGAGG 3′
TRAV8-6	5′ GAGCTGAGGTGCAACTACTC 3′	5′ AACCAAGGACTCCAGCTTC 3′
TRAV8-7	5′ CTAACAGAGGCCACCCAG 3′	5′ ATCAGAGGTTTTGAGGCTG 3′
TRAV9-1, 9-2	5′ TGGTATGTCCAATATCCTGG 3′	5′ GAAACCACTTCTTTCCACTTG 3′
TRAV10	5′ CAAGTGGAGCAGAGTCCTC 3′	5′ GAAAGAACTGCACTCTTCAATG 3′
TRAV12- 1, 12-2, 12-3	5′ CARTGTTCCAGAGGGAGC 3′	5′ AAGATGGAAGGTTTACAGCAC 3′
TRAV13- 1	5′ CATCCTTCAACCCTGAGTG 3′	5′ TCAGACAGTGCCTCAAACTAC 3′
TRAV13- 2	5′ CAGCGCCTCAGACTACTTC 3′	5′ CAGTGAAACATCTCTCTCTGC 3′
TRAV14	5′ AAGATAACTCAAACCCAACCAG 3′	5′ AGGCTGTGACTCTGGACTG 3′
TRAV16	5′ AGTGGAGCTGAAGTGCAAC 3′	5′ GTCCAGTACTCCAGACAACG 3′

TRAV17	5′ GGAGAAGAGGATCCTCAGG 3′	5′ CCACCATGAACTGCAGTTAC 3′
TRAV18	5′ TCCAGTATCTAAACAAAGAGCC 3′	5′ TGACAGTTCCTTCCACCTG 3′
TRAV19	5′ AGGTAACTCAAGCGCAGAC 3′	5′ TGTGACCTTGGACTGTGTG 3′
TRAV20	5′ CACAGTCAGCGGTTTAAGAG 3′	5′ TCTGGTATAGGCAAGATCCTG 3′
TRAV21	5′ TTCCTGCAGCTCTGAGTG 3′	5′ AACTTGGTTCTCAACTGCAG 3′
TRAV22	5′ GTCCTCCAGACCTGATTCTC 3′	5′ CTGACTCTGTGAACAATTTGC 3′
TRAV23	5′ TGCTTATGAGAACACTGCG 3′	5′ TGCATTATTGATAGCCATACG 3′
TRAV24	5′ CTCAGTCACTGCATGTTCAG 3′	5′ TGCCTTACACTGGTACAGATG 3′
TRAV25	5′ GGACTTCACCACGTACTGC 3′	5′ TATAAGCAAAGGCCTGGTG 3′
TRAV26- 1	5′ GCAAACCTGCCTTGTAATC 3′	5′ CGACAGATTCACTCCCAG 3′
TRAV26- 2	5′ AGCCAAATTCAATGGAGAG 3′	5′ TTCACTTGCCTTGTAACCAC 3′
TRAV27	5′ TCAGTTTCTAAGCATCCAAGAG 3′	5′ CTCACTGTGTACTGCAACTCC 3′
TRAV29	5′ GCAAGTTAAGCAAAATTCACC 3′	5′ CTGCTGAAGGTCCTACATTC 3′
TRAV30	5′ CAACAACCAGTGCAGAGTC 3′	5′ AGAAGCATGGTGAAGCAC 3′
TRAV34	5′ AGAACTGGAGCAGAGTCCTC 3′	5′ ATCTCACCATAAACTGCACG 3′
TRAV35	5′ GGTCAACAGCTGAATCAGAG 3′	5′ ACCTGGCTATGGTACAAGC 3′
TRAV36	5′ GAAGACAAGGTGGTACAAAGC 3′	5′ ATCTCTGGTTGTCCACGAG 3′
TRAV38- 1, 38-2	5′ GCACATATGACACCAGTGAG 3′	5′ CAGCAGGCAGATGATTCTC 3′
TRAV39	5′ CTGTTCCTGAGCATGCAG 3′	5′ TCAACCACTTCAGACAGACTG 3′
TRAV40	5′ GCATCTGTGACTATGAACTGC 3′	5′ GGAGGCGGAAATATTAAAGAC 3′
TRAV41	5′ AATGAAGTGGAGCAGAGTCC 3′	5′ TTGTTTATGCTGAGCTCAGG 3′
TRAC	5′ GACCAGCTTGACATCACAG 3′	5′ TGTTGCTCTTGAAGTCCATAG 3′

Table 1 Primers targeting α-chain TCR (TRAV) genes for Nested PCR (Dash, Wang and Thomas, 2015).

TRB gene(s) targeted by primer	External primer sequence (EXT)	Internal primer sequence (INT)
TRBV2	5′ TCGATGATCAATTCTCAGTTG 3′	5′ TTCACTCTGAAGATCCGGTC 3′
TRBV3-1	5′ CAAAATACCTGGTCACACAG 3′	5′ AATCTTCACATCAATTCCCTG 3′
TRBV4-1, 4-2, 4-3	5′ TCGCTTCTCACCTGAATG 3′	5′ CCTGCAGCCAGAAGACTC 3′
TRBV5-1, 5-3, 5-4	5′ GATTCTCAGGKCKCCAGTTC 3′	5′ CTTGGAGCTGGRSGACTC 3′
TRBV5-5, 5-6, 5-7, 5-8	5′ GTACCAACAGGYCCTGGGT 3′	5′ TCTGAGCTGAATGTGAACG 3′
TRBV6-1, 6-2, 6-3, 6-5, 6-6, 6-7, 6-8, 6-9	5′ ACTCAGACCCCAAAATTCC 3′	5′ GTGTRCCCAGGATATGAACC 3′
TRBV6-4	5′ ACTGGCAAAGGAGAAGTCC 3′	5′ TGGTTATAGTGTCTCCAGAGC 3′
TRBV7-1, 7-2, 7-3	5′ TRTGATCCAATTTCAGGTCA 3′	5′ TCYACTCTGAMGWTCCAGCG 3′
TRBV7-4, 7-6, 7-7, 7-8, 7-9	5′ GSWTCTYTGCAGARAGGCC 3′	5′ TGRMGATYCAGCGCACA 3′
TRBV9	5′ GATCACAGCAACTGGACAG 3′	5′ GTACCAACAGAGCCTGGAC 3′
TRBV10-1,		

10-2, 10-3	5′ TGTWCTGGTATCGACAAGACC 3′	5′ TCCYCCTCACTCTGGAGTC 3′
TRBV11-1, 11-2, 11-3	5′ CGATTTTCTGCAGAGACGC 3′	5′ GACTCCACTCTCAAGATCCA 3′
TRBV12-3, 12-4, 12-5	5′ ARGTGACAGARATGGGACAA 3′	5′ CYACTCTGARGATCCAGCC 3′
TRBV13	5′ AGCGATAAAGGAAGCATCC 3′	5′ CATTCTGAACTGAACATGAGC 3′
TRBV14	5′ CCAACAATCGATTCTTAGCTG 3′	5′ ATTCTACTCTGAAGGTGCAGC 3′
TRBV15	5′ AGTGACCCTGAGTTGTTCTC 3′	5′ ATAACTTCCAATCCAGGAGG 3′
TRBV16	5′ GTCTTTGATGAAACAGGTATGC 3′	5′ GAAAGATTTTCAGCTAAGTGCC 3′
TRBV17	5′ CAGACCCCCAGACACAAG 3′	5′ TGTTCACTGGTACCGACAG 3′
TRBV18	5′ CATAGATGAGTCAGGAATGCC 3′	5′ CGATTTTCTGCTGAATTTCC 3′
TRBV19	5′ AGTTGTGAACAGAATTTGAACC 3′	5′ TTCCTCTCACTGTGACATCG 3′
TRBV20-1	5′ AAGTTTCTCATCAACCATGC 3′	5′ACTCTGACAGTGACCAGTGC 3′
TRBV23-1	5′ GCGATTCTCATCTCAATGC 3′	5′ GCAATCCTGTCCTCAGAAC 3′
TRBV24-1	5′ CCTACGGTTGATCTATTACTCC 3′	5′ GATGGATACAGTGTCTCTCGA 3′
TRBV25-1	5′ ACTACACCTCATCCACTATTCC 3′	5′ CAGAGAAGGGAGATCTTTCC 3′
TRBV27, 28	5′ TGGTATCGACAAGACCCAG 3′	5′ TTCYCCCTGATYCTGGAGTC 3′
TRBV29-1	5′ TTCTGGTACCGTCAGCAAC 3′	5′ TCTGACTGTGAGCAACATGAG 3′
TRBV30	5′ TCCAGCTGCTCTTCTACTCC 3′	5′ AGAATCTCTCAGCCTCCAGAC 3′
TRBC	5′ TAGAACTGGACTTGACAGCG 3′	5′ TTCTGATGGCTCAAACACAG 3′

Table 2 Primers targeting β-chain TCR (TRBV) genes for Nested PCR (Dash, Wang and Thomas, 2015).

TRGV gene(s) targeted by primer	External primer sequence	Internal primer sequence
TRGV3.5	5′TCTTCCAACTTGGAAGGG3’	5′GGTCATCTGCTGAAATCAC3′
TRGV7	5′TCTTCCAACTTGCAAGGG3’	5′GGTCATCTGCTGTAATCACTTG3′
TRGVA	5′GGGTCATCCTGTTTCCAG3′	5′TACCTAAGGACCTGTGTAGAGG3′
TRGVB	5′TGGCCTCCCAAAGTACTG3′	5′TCCTCTTTCTATGTCCCAGG3′
TRGV8	5′CCAACTTGGAAGGGAGAAC3′	5′AAAATGCCGTCTACACCC3′
TRGV9	5′CCAGGTCACCTAGAGCAAC3′	5′TGTCCATTTCATATGACGG3′
TRGV10	5′TTATCAAAAGTGGAGCAGTTC3′	5′CAGCTATCCATTTCCACGG3′
TRGV11	5′GAACAACCTGAAATATCTATTTCC3′	5′CATATCTTGGAAGGCATCC3′
TRGV1.2.4.6	5′GGGTCATCTGCTGAAATCAC3′	5′CCAGGAGGGGAAGGC3′
TRGC	5′GGTGTTCCCCTCCTGG3′	5′CCCAGAATCGTGTTGCT3′

Table 3 Primers targeting γ-chain TCR (TRGV) genes for Nested PCR (Guo et al., 2016).

TRDV gene(s) targeted by primer	External primer sequence	Internal primer sequence
TRDV1	5′GCCCAGAAGGTTACTCAAG3′	5′AGCAAAGAGATGATTTTCCTTA3′
TRDV2	5′ATTGAGTTGGTGCCTGAAC3′	5′TATATCAACTGGTACAGGAAGACC3′
TRDV3	5′TGTGACAAAGTAACCCAGAGTTC3′	5′GGTACTGCTCTGCACTTACGAC3′
TRDV4/ TRAV14	5′CAAACCCAACCAGGAATG3′	5′AGGAAAAGGAGGCTGTGAC3′
TRDV5/ TRAV29	5′GCAAGTTAAGCAAAATTCACC3′	5′CTGCTGAAGGTCCTACATTC3′
TRDV6/ TRAV23	5′TTGATAGTCCAGAAAGGAGG3′	5′CGTTTGACTACTTTCCATGG3′
TRDV7/ TRAV36	5′GACAAGGTGGTACAAAGCC3′	5′ATCTCTGGTTGTCCACGAG3′
TRDV8/ TRAV38-2	5′CAGTCACTCAGTCTCAACCAG3′	5′TCTGGTACAAGCAGCCTC3′
TRDC	5′CTTCATATTTACCAAGCTTGACAG3′	5′GATGACAATAGCAGGATCAAAC3′

Table 4 Primers targeting δ-chain TCR (TRDV) genes for Nested PCR (Guo et al., 2016).

Appendix 2

PatientNumber/Lipid α/β or γ/δ chain	Vα/γ gene	J α/γ gene	Vβ/δ gene	J β/δ gene	D β/δ gene
1/ α-GalCer α/β chain	TRAV8-6*01 F	TRAJ32*02 F	TRBV6-2*01 F, or TRBV6-3*01 F	TRBJ2-2*01 F	TRBD1*01 F
	TRAV4*01 F	TRAJ16*01 F	TRBV20-1*01 F, or TRBV20-1*02 F or TRBV20-1*03 (F) or TRBV20-1*04 (F) or TRBV20-1*05 (F)	TRBJ2-7*01 F	TRBD2*01 F
	TRAV1-2*01 F	TRAJ33*01 F	TRBV6-1*01 F	TRBJ1-1*01 F	TRBD2*01 F
	TRAV1-2*01 F	TRAJ33*01 F	TRBV6-1*01 F	Homsap TRBJ2-7*01 F	Homsap TRBD1*01 F
1/ Nut α/β chain	Homsap TRAV1-2*01 F	Homsap TRAJ20*01 F	Homsap TRBV6-4*01 F, or Homsap TRBV6-4*02 F	Homsap TRBJ2-3*01 F	Homsap TRBD2*02 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	Homsap TRBV20-1*01 F, or Homsap TRBV20-1*02 F or Homsap TRBV20-1*03 (F) or Homsap TRBV20-1*04 (F) or Homsap TRBV20-1*05 (F)	Homsap TRBJ1-1*01 F	Homsap TRBD1*01 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	Homsap TRBV20-1*01 F, or Homsap TRBV20-1*02 F or Homsap TRBV20-1*03 (F) or Homsap TRBV20-1*04 (F) or Homsap TRBV20-1*05 (F)	Homsap TRBJ2-1*01 F	Homsap TRBD1*01 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	No results		
1/ Nut γ/δ chain	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F

2/ α-GalCer α/β chain	Homsap TRAV12-2*02 (F)	Homsap TRAJ31*01 F	Homsap TRBV28*01 F	Homsap TRBJ1-4*01 F	Homsap TRBD1*01 F
2/ α-GalCer γ/δ chain	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F, or Homsap TRGV4*02 F	Homsap TRGJ2*01 F	Homsap TRDV2*01 F, or Homsap TRDV2*02 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV8*01 F	Homsap TRGJ1*02 F, or Homsap TRGJP1*01 F or Homsap TRGJP2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
2/ Nut γ/δ chain	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F, or Homsap TRGV4*02 F	Homsap TRGJ1*01 F, or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
3/ α-GalCer α/β chain	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	Homsap TRBV6-4*01 F, or Homsap TRBV6-4*02 F	Homsap TRBJ2-3*01 F	Homsap TRBD1*01 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	Homsap TRBV6-1*01 F	Homsap TRBJ2-6*01 F	Homsap TRBD1*01 F
	Homsap TRAV22*01 F	Homsap TRAJ11*01 F	Homsap TRBV19*01 F	Homsap TRBJ2-3*01 F	No results

	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	No results		
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	Homsap TRBV20-1*01 F, or Homsap TRBV20-1*02 F or Homsap TRBV20-1*03 (F) or Homsap TRBV20-1*04 (F) or Homsap TRBV20-1*05 (F)	Homsap TRBJ2-1*01 F	Homsap TRBD2*02 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	No results		
3/ α-GalCer γ/δ chain	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV2*03 F	Homsap TRDJ3*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV2*03 F	Homsap TRDJ3*01 F	Homsap TRDD3*01 F
	No results		No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
3/ Nut α/β chain	Homsap TRAV10*01 F	Homsap TRAJ18*01 F	Homsap TRBV25-1*01 F	Homsap TRBJ1-1*01 F	Homsap TRBD1*01 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	No results		
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	No results		
	Homsap TRAV1-2*01 F	Homsap TRAJ12*01 F	Homsap TRBV6-4*01 F, or Homsap TRBV6-4*02 F	Homsap TRBJ2-3*01 F	Homsap TRBD1*01 F
	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	No results		
3/ Nut γ/δ chain	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F

	Homsap TRGV3*01 F	Homsap TRGJ1*01 F, or Homsap TRGJ2*01 F	No results		
4/ α-GalCer α/β chain	Homsap TRAV17*01 F	Homsap TRAJ58*01 ORF	Homsap TRBV28*01 F	Homsap TRBJ2-1*01 F	Homsap TRBD1*01 F
4/ α-GalCer γ/δ chain	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		
	No results		No results		
	No results		No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		No results		
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
4/ Nut α/β chain	No results		Homsap TRBV28*01 F	Homsap TRBJ1-5*01 F	Homsap TRBD1*01 F
	Homsap TRAV13-1*01 F	Homsap TRAJ45*01 F	Homsap TRBV5-3*01 ORF, or Homsap TRBV5-4*01 F or Homsap TRBV5-4*02 (F) or Homsap TRBV5-4*03 (F) or Homsap TRBV5-4*04 (F)	Homsap TRBJ1-1*01 F	Homsap TRBD1*01 F
	No results		Homsap TRBV30*01 F, or Homsap TRBV30*05 (F)	Homsap TRBJ2-3*01 F	Homsap TRBD2*01 F

4/ Nut γ/δ chain	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		No results		
	No results		Homsap TRDV2*03 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		No results		
5/ α-GalCer α/β chain	Homsap TRAV1-2*01 F	Homsap TRAJ33*01 F	Homsap TRBV20-1*01 F, or Homsap TRBV20-1*02 F or Homsap TRBV20-1*03 (F) or Homsap TRBV20-1*04 (F) or Homsap TRBV20-1*05 (F)	Homsap TRBJ2-7*01 F	Homsap TRBD1*01 F
	Homsap TRAV3*01 F	Homsap TRAJ28*01 F	Homsap TRBV7-2*01 F, or Homsap TRBV7-2*04 (F)	Homsap TRBJ2-3*01 F	Homsap TRBD2*01 F
	Homsap TRAV1-2*01 F	Homsap TRAJ12*01 F	Homsap TRBV7-9*01 F	Homsap TRBJ1-5*01 F	Homsap TRBD2*02 F

5/ α-GalCer γ/δ chain	Homsap TRGV4*01 F, or Homsap TRGV4*02 F	Homsap TRGJ1*02 F, or Homsap TRGJ2*01 F	No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV9*01 F, or Homsap TRGV9*02 F	Homsap TRGJ1*01 F, or Homsap TRGJ1*02 F or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD1*01 F and Homsap TRDD2*01 F and Homsap TRDD3*01 F
	Homsap TRGV2*02 (F)	Homsap TRGJP*01 F	No results		
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		
	Homsap TRGV4*01 F, or Homsap TRGV4*02 F	Homsap TRGJ1*02 F, or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F
	Homsap TRGV4*02 F	Homsap TRGJ1*02 F, or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F
5/ Nut α/β chain	Homsap TRAV1-2*01 F	Homsap TRAJ12*01 F	Homsap TRBV6-1*01 F	Homsap TRBJ1-4*01 F	No results
	Homsap TRAV14/DV4*02 F	Homsap TRAJ29*01 F	Homsap TRBV20-1*01 F, or Homsap TRBV20-1*02 F or Homsap TRBV20-1*03 (F) or Homsap TRBV20-1*04 (F) or Homsap TRBV20-1*05 (F)	Homsap TRBJ2-7*01 F, or Homsap TRBJ2-7*02 ORF	No results
5/ Nut γ/δ chain	Homsap TRGV4*02 F	Homsap TRGJ1*02 F, or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F

	Homsap TRGV4*02 F	Homsap TRGJ1*02 F, or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		
	Homsap TRGV4*02 F	Homsap TRGJ1*02 F, or Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F
	Homsap TRGV8*01 F	Homsap TRGJ1*02 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD2*01 F and Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV2*02 (F)	Homsap TRGJP2*01 F	No results		
6/ α-GalCer γ/δ chain	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
6/ Nut α/β chain	No results		Homsap TRBV6-4*01 F, or Homsap TRBV6-4*02 F	Homsap TRBJ2-3*01 F	Homsap TRBD2*01 F
6/ Nut γ/δ chain	No results		No results		
	No results		No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*02 F	Homsap TRGJP1*01 F	No results		
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F
	No results		Homsap TRDV1*01 F	Homsap TRDJ1*01 F	Homsap TRDD3*01 F

	Homsap TRGV4*01 F	Homsap TRGJ2*01 F	No results		

Table 5 α-GalCer/Nut lipid-specific TCR analysis.

Appendix 3

Primer name	Sequence (5’ – 3’)
MJA431	TTAATTCGTCTCAGCTGCCATGTGGGGAGTTTTCCTTCTTTATGTTTC
MJA432	TTAATTCGTCTCTTGCTCCGAAGATCAATTTATAGCTGCTATCCAC
MJA434	TTAATTCGTCTCGCTGTCATGAGAATCAGGCTCCTGTGCTGT
MJA435	TTAATTCGTCTCTGGGCCAAAATACTGCGTATCCAC
MJA437	TTAATTCGTCTCAAGCTGATGCAGAGGATCTCCTCCCTCATCC
MJA438	TTAATTCGTCTCTTTTCCAAAGATGAGTTTATCGTCGCTT
MJA440	TTAATTCGTCTCGCATGCAGTGGGCTCTAGCGGTG
MJA441	TTAATTCGTCTCACTGCCAAAGAGTTTCTTATATACTTCTCATCCC
MJA443	TTAATTCGTCTCAAGCTGATGCTGTTCTCCAGCCTGCTGT
MJA444	TTAATTCGTCTCTTTTCCAAAGATGAGTTTATCGGTGTACGTGAGG
MJA446	TTAATTCGTCTCGCATGCTGTTGGCTCTAGCTCTGCTTCTAGCTT
MJA447	TTAATTCGTCTCACTGCCAAAGAGTTTCTTATAATAAAGGCTCTC

Table 6 Primer sequences for amplifying lipid-specific TCRs for pMJA290, pMJA295 and pMJA297.

Appendix 4

TLC fraction	Co-migrating Marker (TLC)
PL1	PC
PL2	PI
PL3	PE
PL4	PA
PL5	-
PL6	-
PL7	-
PL8	-
PL9	-
GL1	DGDG
GL2	MGDG
GL3	-
GL4	1, 2DAG
GL5	1, 3DAG
GL6	TAG

Table 7 Identify of the lipid fractions separated by Thin Layer Chromatography (TLC). PL: Polar lipid, GL: Glycolipid, PC: Phosphatidylcholine, PI: Phosphatidylinositol, PE: Phosphatidylethanolamine, PA: Phosphatidic acid, DGDG: Digalactosyldiacylglycerol, MGDG: Monogalactosyldiacylglycerol, DAG: Diacylglycerol, TAG: Triacylglycerol.

Appendix 5

Primer name	Sequence
MJA464	ACTTGCCTGTCGCTCTATCTTCATAGCCGCAGCGTCATGAGCAGAT
MJA465	ACTTGCCTGTCGCTCTATCTTCATAACCCGGCCACTTTCAGGAGGA
MJA466	ACTTGCCTGTCGCTCTATCTTCATACCATGGCCATCAGCACGAGGG
MJA467	ACTTGCCTGTCGCTCTATCTTCATAGGGCACTGACCAGCACGGC
MJA468	ACTTGCCTGTCGCTCTATCTTCATATTGGCAGTCAAGAGAAAATTGACGG
MJA469	ACTTGCCTGTCGCTCTATCTTCATAACAGCATTCGTAGCCCAAGCACT
MJA470	ACTTGCCTGTCGCTCTATCTTCATATGTGACCCAAGAAAGGTTGGGGTT
MJA471	ACTTGCCTGTCGCTCTATCTTCATAATGCCCAACTTTGTGACCCAAGAA

Table 8 TCR constant region primers (MJA464 and 465 are two TCRα constant primers, MJA466 and 467 are two TCRβ constant primers, MJA468 and 469 are two TCRδ constant primers and MJA470 and 471 are two TCRγ constant primers)

References

Acuto, O. and Michel, F. (2003) 'CD28-mediated co-stimulation: a quantitative support for TCR signalling', *Nature Reviews Immunology*, 3(12), pp. 939–951. doi: 10.1038/nri1248.

Ahmadi, M. *et al.* (2011) 'CD3 limits the efficacy of TCR gene therapy in vivo', *Blood*, 118, pp. 3528–3537. doi: 10.1182/blood-2011-04-346338.

Alcocer, M. J. C. *et al.* (2002) 'The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2 S plant albumins', *Journal of Molecular Biology*, 324(1), pp. 165–175. doi: 10.1016/S0022-2836(02)01061-6.

Alcocer, M., Rundqvist, L. and Larsson, G. (2012) 'Ber e 1 protein: The versatile major allergen from Brazil nut seeds', *Biotechnology Letters*, 34(4), pp. 597–610. doi: 10.1007/s10529-011-0831-1.

Anvari, S. *et al.* (2019) 'IgE-Mediated Food Allergy', *Clinical Reviews in Allergy and Immunology*. Humana Press Inc., 57(2), pp. 244–260. doi: 10.1007/s12016-018-8710-3.

Bai, L. *et al.* (2012) 'Distinct APCs Explain the Cytokine Bias of α-Galactosylceramide Variants In Vivo', *The Journal of Immunology*, 188(7), pp. 3053–3061. doi: 10.4049/jimmunol.1102414.

Baine, M. J., Mallya, K. and Batra, S. K. (2013) 'Quantitative real-time PCR expression analysis of peripheral blood mononuclear cells in pancreatic cancer patients', *Methods in Molecular Biology*, 980, pp. 157–173. doi: 10.1007/978-1-62703-287-2_8.

Bajnok, A. *et al.* (2017) 'The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia', *Mediators of Inflammation*, 2017, pp. 1–7. doi: 10.1155/2017/8045161.

Bansal, A. S. *et al.* (2007) 'Dangerous liaison: Sexually transmitted allergic reaction to Brazil nuts',

Journal of Investigational Allergology and Clinical Immunology, 17, pp. 189–191.

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

Bendelac, A., Savage, P. B. and Teyton, L. (2007) 'The biology of NKT cells.', *Annual Review of Immunology*, 25, pp. 297–336. doi: 10.1146/annurev.immunol.25.022106.141711.

Blaiss, M.S., Canonica, G. W., Holgate, S.T, Lockey, R.F. and Pawankar, R. (2013) *White Book on Allergy (Update: 2013)*. Edited by R. Pawanker et al. World Allergy Organisation. Available at: http://www.worldallergy.org/UserFiles/file/WhiteBook2-2013-v8.pdf.

Blanc, F. *et al.* (2015) 'Further studies on the biological activity of hazelnut allergens', *Clinical and Translational Allergy*, 5, p. 26. doi: 10.1186/s13601-015-0066-7.

Brandtzaeg, P. *et al.* (2008) 'Terminology: Nomenclature of mucosa-associated lymphoid tissue', *Mucosal Immunology*, 1, pp. 31–37. doi: 10.1038/mi.2007.9.

Brennan, P. J. *et al.* (2011) 'Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals', *Nature Immunology*, 12(12), pp. 1202–1211. doi: 10.1038/ni.2143.

Brigl, M. *et al.* (2006) 'Conserved and Heterogeneous Lipid Antigen Specificities of CD1d-Restricted NKT Cell Receptors', *The Journal of Immunology*. American Association of Immunologists, 176(6), pp. 3625–3634. doi: 10.4049/jimmunol.176.6.3625.

Bronstein-Sitton, N. (2006) 'T Cell Signaling and Activation: No Simple Matter', *Pathways*, 2(2), pp. 8–12. Available at: www.alomone.com.

Buermans, H. P. J. and den Dunnen, J. T. (2014) 'Next generation sequencing technology: Advances and applications', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 10(1842), pp. 1932–1941. doi: 10.1016/j.bbadis.2014.06.015.

Bufe, A. and Holst, O. (2004) 'LPS-binding protein as a target molecule in allergy and asthma', *Journal of Allergy and Clinical Immunology*, 114(3), pp. 583–585. doi: 10.1016/j.jaci.2004.06.034.

Burdge, G. C. and Calder, P. C. (2015) 'Introduction to fatty acids and lipids', *World Review of Nutrition and Dietetics*. Edited by K. B. Calder, Philip C, Waitzburg D.L. Karger, pp. 1–16. doi: 10.1159/000365423.

Burks, A. W. *et al.* (2012) 'ICON: Food allergy', *Journal of Allergy and Clinical Immunology*, 129, pp. 906–920. doi: 10.1016/j.jaci.2012.02.001.

Calmeiro, J. *et al.* (2018) 'Highlighting the Role of DC-NK Cell Interplay in Immunobiology and Immunotherapy', in *Dendritic Cells*, pp. 25–45. doi: 10.5772/intechopen.78804.

Cameron, G. *et al.* (2015) 'Antigen Specificity of Type I NKT Cells Is Governed by TCR β-Chain Diversity', *The Journal of Immunology*, 195(10), pp. 4604–4614. doi: 10.4049/jimmunol.1501222.

Cantrell, D. A. (1996) 'T cell antigen receptor signal transduction pathways', *Annual Review of Immunology*, 14, pp. 259–74. doi: 10.1016/S0955-0674(97)80064-6.

Castell-Rodríguez, A. *et al.* (2017) 'Dendritic Cells: Location, Function, and Clinical Implications', in *Biology of Myelomonocytic Cells*, pp. 21–50. doi: 10.5772/intechopen.68352.

Cesari, F. (2009) 'Immune synapses: TCR-CD3 recycling to the synapse', *Nature Reviews Immunology*. Nature Publishing Group, 9(12), pp. 820–821. doi: 10.1038/nri2680.

Chatila, T. *et al.* (1989) 'Mechanisms of T cell activation by the calcium ionophore ionomycin.', *Journal of immunology (Baltimore, Md.: 1950)*, 143(4), pp. 1283–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2545785 (Accessed: 22 June 2017).

Cheng, J., Montecalvo, A. and Kane, L. P. (2011) 'Regulation of NF-κB induction by TCR/CD28', *Immunologic Research*, 50(2–3), pp. 113–117. doi: 10.1007/s12026-011-8216-z.

Chinthrajah, R. S. *et al.* (2016) 'Molecular and cellular mechanisms of food allergy and food tolerance', *Journal of Allergy and Clinical Immunology*, 137(4), pp. 984–997. doi: 10.1016/j.jaci.2016.02.004.

Chirathaworn, C. *et al.* (2002) 'Stimulation Through Intercellular Adhesion Molecule-1 Provides a Second Signal for T Cell Activation', *The Journal of Immunology*, 168(11), pp. 5530–5537. doi: 10.4049/jimmunol.168.11.5530.

Coico, R. and Sunshine, G. (2015) *Immunology : a short course*.

Commins, S. P. (2015) 'Mechanisms of Oral Tolerance', *Pediatric Clinics of North America*. W.B. Saunders, 62(6), pp. 1523–1529. doi: 10.1016/j.pcl.2015.07.013.

Coombes, J. L. *et al.* (2007) 'A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β -and retinoic acid-dependent mechanism', *Journal of Experimental Medicine*, 204, pp. 1757–1764. doi: 10.1084/jem.20070590.

Cravens, P. D. and Lipsky, P. E. (2002) 'Dendritic cells, chemokine receptors and autoimmune inflammatory diseases', *Immunology and Cell Biology*, 80(5), pp. 497–505. doi: 10.1046/j.1440-1711.2002.01118.x.

Cubells-Baeza, N. *et al.* (2017) 'Identification of the ligand of Pru p 3, a peach LTP', *Plant Molecular Biology*, 94, pp. 33–44. doi: 10.1007/s11103-017-0590-z.

Das, R., Sant'Angelo, D. B. and Nichols, K. E. (2010) 'Transcriptional control of invariant NKT cell development', *Immunological Reviews*, 238, pp. 195–215. doi: 10.1111/j.1600-065X.2010.00962.x.

Dash, P. *et al.* (2011) 'Paired analysis of TCRα and TCRβ chains at the single-cell level in mice', *Journal of Clinical Investigation*, 121(1), pp. 288–295. doi: 10.1172/JCI44752.

Dash, P., Wang, G. C. and Thomas, P. G. (2015) 'Single-Cell Analysis of T-Cell Receptor αβ Repertoire', in *Immunosenescence*, pp. 181–197. doi: 10.1007/978-1-4939-2963-4.

Dave, N. D. *et al.* (2011) 'Stress and Allergic Diseases', *Immunology and Allergy Clinics of North America*. NIH Public Access, pp. 55–68. doi: 10.1016/j.iac.2010.09.009.

Deamer, D., Akeson, M. and Branton, D. (2016) 'Three decades of nanopore sequencing', *Nature Biotechnology*, 34, pp. 518–524. doi: 10.1038/nbt.3423.

Dearman, R. J., Alcocer, M. J. C. and Kimber, I. (2007) 'Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1', *Clinical and Experimental Allergy*, 37(4), pp. 582–591. doi: 10.1111/j.1365-2222.2007.02689.x.

Dolmetsch, R. E. *et al.* (1997) 'Differential activation of transcription factors induced by Ca2+ response amplitude and duration', *Nature*, 386(6627), pp. 855–858. doi: 10.1038/386855a0.

Dowds, C. M. *et al.* (2014) 'Lipid antigens in immunity', *Biological Chemistry*, pp. 61–81. doi: 10.1515/hsz-2013-0220.

Dreborg, S. (2015) 'Debates in allergy medicine: Food intolerance does not exist', *World Allergy Organization Journal*. BioMed Central, p. 37. doi: 10.1186/s40413-015-0088-6.

EAACI (2016) *The European Academy of Allergy and Clinical Immunology (EAACI) Tackling the Allergy Crisis in Europe- Concerted Policy Action Needed*. Available at: http://www.eaaci.org/images/media/EAACI_Manifesto_brochure_Interactive.pdf.

Eisenstein, M. (2017) 'An ace in the hole for DNA sequencing', *Nature*, 550, pp. 285–288. doi: 10.1038/550285a.

Endres, S. *et al.* (1993) 'Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation.', *Journal of leukocyte biology*, 54(6), pp. 599–603. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8245713 (Accessed: 23 June 2017).

Ewan, P. W. (1996) 'Clinical study of peanut and nut allergy in 62 consecutive patients: new features and associations.', *BMJ (Clinical research ed.)*. BMJ Publishing Group, 312(7038), pp. 1074–8. doi:

10.1136/bmj.312.7038.1074.

Feingold, K. R. and Grunfeld, C. (2000) *Introduction to Lipids and Lipoproteins*, *Endotext*. MDText.com, Inc. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26247089 (Accessed: 23 June 2017).

Fisher, W. G. *et al.* (2006) 'NFAT and NFκB activation in T lymphocytes: A model of differential activation of gene expression', *Annals of Biomedical Engineering*, 34(11), pp. 1712–1728. doi: 10.1007/s10439-006-9179-4.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) 'A simple method for the isolation and purification of total lipides from animal tissues.', *The Journal of biological chemistry*, 226(1), pp. 497–509.

Friedman, A. and Weiner, H. L. (1994) 'Induction of anergy or active suppression following oral tolerance is determined by antigen dosage', *Proceedings of the National Academy of Sciences of the United States of America*, 14, pp. 6688–6692. doi: 10.1073/pnas.91.14.6688.

Fujii, S. I. *et al.* (2004) 'The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation', *Journal of Experimental Medicine*, 199(12), p. 1607. doi: 10.1084/jem.20040317.

Fujii, S. ichiro *et al.* (2013) 'NKT cells as an ideal anti-tumor immunotherapeutic', *Frontiers in Immunology*. Frontiers, 4, p. 409. doi: 10.3389/fimmu.2013.00409.

Galli, S. J., Tsai, M. and Piliponsky, A. M. (2008) 'The development of allergic inflammation', *Nature*, 454, pp. 445–454. doi: 10.1038/nature07204.

Ghumra, A. and Alcocer, M. (2017) 'The use of a semi-automated system to measure mouse natural killer T (NKT) cell activation by lipid-loaded dendritic cells', in *Methods in Molecular Biology*, pp. 249–262. doi: 10.1007/978-1-4939-6925-8_19.

Girardi, E. and Zajonc, D. M. (2012) ‘Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells’, *Immunol Rev*, 250(1), pp. 167–179. doi: 10.1111/j.1600-065X.2012.01166.x.

Godfrey, D. I. *et al.* (2004) ‘NKT cells: what’s in a name?’, *Nature reviews. Immunology*, 4(3), pp. 231–7. doi: 10.1038/nri1309.

Godfrey, D. I. *et al.* (2015) ‘The burgeoning family of unconventional T cells’, *Nature Immunology*, 16(11), pp. 1114–1123. doi: 10.1038/ni.3298.

González-Rioja, R. *et al.* (2009) ‘Par j 1 and Par j 2, the two major allergens in Parietaria judaica, bind preferentially to monoacylated negative lipids’, *FEBS Journal*, 276(6), pp. 1762–1775. doi: 10.1111/j.1742-4658.2009.06911.x.

Govers, C. *et al.* (2010) ‘T cell receptor gene therapy: strategies for optimizing transgenic TCR pairing’, *Trends in Molecular Medicine*, 16(2), pp. 77–87. doi: 10.1016/j.molmed.2009.12.004.

Gray, C. L. *et al.* (2014) ‘Epidemiology of IgE-mediated food allergy’, *South African Medical Journal*. Health & Medical Publishing Group, 105(1), pp. 68–69. doi: 10.7196/SAMJ.9103.

Grundy, J. *et al.* (2002) ‘Rising prevalence of allergy to peanut in children: Data from 2 sequential cohorts’, *Journal of Allergy and Clinical Immunology*, 110(5), pp. 784–9. doi: 10.1067/mai.2002.128802.

Guo, X. J. *et al.* (2016) ‘Rapid cloning, expression, and functional characterization of paired αβ and γδ T-cell receptor chains from single-cell analysis’, *Molecular Therapy - Methods & Clinical Development*. Official journal of the American Society of Gene & Cell Therapy, 3, p. 15054. doi: 10.1038/mtm.2015.54.

Hadis, U. *et al.* (2011) ‘Intestinal Tolerance Requires Gut Homing and Expansion of FoxP3+ Regulatory T Cells in the Lamina Propria’, *Immunity*, 34, pp. 237–246. doi: 10.1016/j.immuni.2011.01.016.

Haskins, K. *et al.* (1983) 'The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody.', *The Journal of experimental medicine*, 157(4), pp. 1149–69. doi: 10.1084/JEM.157.4.1149.

Heather, J. M. and Chain, B. (2016) 'The sequence of sequencers: The history of sequencing DNA', *Genomics*, 107(1), pp. 1–8. doi: 10.1016/j.ygeno.2015.11.003.

Heemskerk, M. H. M. *et al.* (2003) 'Redirection of antileukemic reactivity of peripheral T lymphocytes using genetransfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region', *Blood*, 102, pp. 3530–3540. doi: 10.1182/blood-2003-05-1524.

Hercend, T. *et al.* (1984) 'Identification of a 140-kDa activation antigen as a target structure for a series of human cloned natural killer cell lines', *European Journal of Immunology*, 14(9), pp. 844–852. doi: 10.1002/eji.1830140914.

Herre, J. *et al.* (2013) 'Allergens as Immunomodulatory Proteins: The Cat Dander Protein Fel d 1 Enhances TLR Activation by Lipid Ligands', *The Journal of Immunology*, 191(4), pp. 1529–35. doi: 10.4049/jimmunol.1300284.

Homann, S. *et al.* (2017) 'A novel rapid and reproducible flow cytometric method for optimization of transfection efficiency in cells', *PLoS ONE*, 12(9), pp. 1–22. doi: 10.1371/journal.pone.0182941.

van Hoogevest, P. and Wendel, A. (2014) 'The use of natural and synthetic phospholipids as pharmaceutical excipients', *European Journal of Lipid Science and Technology*. Wiley-VCH Verlag, 116(9), pp. 1088–1107. doi: 10.1002/ejlt.201400219.

Hung, J. T., Huang, J. R. and Yu, A. L. (2017) 'Tailored design of NKT-stimulatory glycolipids for polarization of immune responses John T Kung', *Journal of Biomedical Science*. Journal of Biomedical Science, pp. 1–10. doi: 10.1186/s12929-017-0325-0.

InvivoGen (2012a) *Introducing Lucia : a new secreted luciferase*, *Review InvivoGen*.

InvivoGen (2012b) *Introducing Lucia: a new secreted luciferase*. Available at: https://www.invivogen.com/sites/default/files/invivogen/old/docs/reviews/review-lucia-luciferase-invivogen.pdf (Accessed: 25 September 2019).

InvivoGen (no date a) *Jurkat-Dual Cells*. Available at: https://www.invivogen.com/sites/default/files/invivogen/products/files/jurkat_dual_tds.pdf (Accessed: 26 September 2019).

InvivoGen (no date b) *Jurkat-Lucia ™ NFAT Cells*. Available at: https://www.invivogen.com/sites/default/files/invivogen/old/PDF/Jurkat_Lucia_NFAT_TDS.pdf (Accessed: 26 September 2019).

Jain, M. *et al.* (2018) 'Nanopore sequencing and assembly of a human genome with ultra-long reads', *Nature Biotechnology*, 36, pp. 338–345. doi: 10.1038/nbt.4060.

Jappe, U. *et al.* (2019) 'Lipophilic allergens, different modes of allergen-lipid interaction and their impact on asthma and allergy', *Frontiers in Immunology*, 10, p. 122. doi: 10.3389/fimmu.2019.00122.

Jayawardena-Wolf, J. *et al.* (2001) 'CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain', *Immunity*, 15(6), pp. 897–908. doi: 10.1016/S1074-7613(01)00240-0.

Jiang, H. *et al.* (2014) 'Skewer: A fast and accurate adapter trimmer for next-generation sequencing paired-end reads', *BMC Bioinformatics*, 15(182), pp. 1–12. doi: 10.1186/1471-2105-15-182.

de Jong, A. (2015) 'Activation of human T cells by CD1 and self-lipids', *Immunological Reviews*, 267(1), pp. 16–29. doi: 10.1111/imr.12322.

Jordan-Williams, K. L., Poston, S. and Taparowsky, E. J. (2013) 'BATF regulates the development and function of IL-17 producing iNKT cells', *BMC Immunology*, 14, p. 16. doi: 10.1186/1471-2172-14-16.

Jung, C., Hugot, J.-P. and Barreau, F. (2010) 'Peyer's Patches: The Immune Sensors of the Intestine', *International Journal of Inflammation*, 2010, p. 823710. doi: 10.4061/2010/823710.

Juno, J. A., Keynan, Y. and Fowke, K. R. (2012) 'Invariant NKT Cells: Regulation and Function during Viral Infection', *PLoS Pathogens*, 8(8), pp. 1–10. doi: 10.1371/journal.ppat.1002838.

Jyonouchi, S. *et al.* (2011) 'Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin', *Journal of Allergy and Clinical Immunology*, 1, pp. 102–109. doi: 10.1016/j.jaci.2011.02.026.

Kasinrerk W, Baumruker T, Majdic O, K. W. and S. H. (1993) 'CD1 molecule expression on human monocytes induced by granulocyte- macrophage colony-stimulating factor', *Journal of Immunology*, 150(2), pp. 579–584.

Kassianos, A. J. *et al.* (2010) 'Isolation of Human Blood DC Subtypes', *Dendritic Cell Protocols*, 595, pp. 45–54. doi: 10.1007/978-1-60761-421-0_3.

Kay, A. B. *et al.* (2008) *Allergy and Allergic Diseases. Volume 1: The Scientific Basis of Allergy and Volume 2: Allergic Diseases: Etiology, Diagnosis and Treatment*. 2nd edn. Blackwell.

Kean, D. E. *et al.* (2006) 'Differential Polarization of Immune Responses by Plant 2S Seed Albumins, Ber e 1, and SFA8', *The Journal of Immunology*, 177(3), pp. 1561–1566. doi: 10.4049/jimmunol.177.3.1561.

Kebschull, J. M. and Zador, A. M. (2015) 'Sources of PCR-induced distortions in high-throughput sequencing data sets', *Nucleic Acids Research*, 43(21), p. 143. doi: 10.1093/nar/gkv717.

Kim, H. S. *et al.* (2010) 'T Cell Ig Domain and Mucin Domain 1 Engagement on Invariant NKT Cells in the Presence of TCR Stimulation Enhances IL-4 Production but Inhibits IFN- Production', *The Journal of Immunology*, 184, pp. 4095–4106. doi: 10.4049/jimmunol.0901991.

Kim, P. J. *et al.* (2006) 'GATA-3 Regulates the Development and Function of Invariant NKT Cells',

The Journal of Immunology, 177, pp. 6650–6659. doi: 10.4049/jimmunol.177.10.6650.

Kim, T. K. and Eberwine, J. H. (2010) 'Mammalian cell transfection: The present and the future', *Analytical and Bioanalytical Chemistry*, 397(8), pp. 3173–3178. doi: 10.1007/s00216-010-3821-6.

King, L. and Plun-Favreau, H. (2017) 'Mitophagy', in *Parkinson's Disease: Molecular Mechanisms Underlying Pathology*. Academic Press, pp. 139–177. doi: 10.1016/B978-0-12-803783-6.00005-5.

Kitaura, K. *et al.* (2016) 'A new high-throughput sequencing method for determining diversity and similarity of T cell receptor (TCR) α and β repertoires and identifying potential new invariant TCR α chains', *BMC Immunology*, 17(1), p. 38. doi: 10.1186/s12865-016-0177-5.

Knies, D. *et al.* (2016) 'An optimized single chain TCR scaffold relying on the assembly with the native CD3-complex prevents residual mispairing with endogenous TCRs in human T-cells', *Oncotarget*, 7, pp. 21199–21221. doi: 10.18632/oncotarget.8385.

Kobayashi, E. *et al.* (1995) 'KRN7000, a novel immunomodulator, and its antitumor activities.', *Oncology research*, 7, pp. 529–34.

Lantz, O. and Bendelac, A. (1994) 'An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans', *The Journal of Experimental Medicine*, 180, p. 1097. doi: 10.1084/jem.180.3.1097.

Larsson, K., Lindstedt, M. and Borrebaeck, C. A. K. (2006) 'Functional and transcriptional profiling of MUTZ-3, a myeloid cell line acting as a model for dendritic cells', *Immunology*. Wiley-Blackwell, 117(2), pp. 156–166. doi: 10.1111/j.1365-2567.2005.02274.x.

Lawrence, T. (2009) 'The nuclear factor NF-κB pathway in inflammation.', *Cold Spring Harbor perspectives in biology*, pp. 1–10. doi: 10.1101/cshperspect.a001651.

Leavy, O. (2011) 'Innate immunity: Linking lipids and inflammasomes', *Nature Reviews Immunology*, 11(6), pp. 368–369. doi: 10.1038/nri2997.

Lefranc, M. P. *et al.* (2003) 'IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains', *Developmental and Comparative Immunology*, 27(1), pp. 55–77. doi: 10.1016/S0145-305X(02)00039-3.

Leray, C. *et al.* (1987) 'Thin-layer chromatography of human platelet phospholipids with fatty acid analysis', *Journal of Chromatography B: Biomedical Sciences and Applications*, 420, pp. 411–416. doi: 10.1016/0378-4347(87)80198-6.

Lin, J. *et al.* (2004) 'The expression and processing of two recombinant 2S albumins from soybean (Glycine max) in the yeast Pichia pastoris', *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1698(2), pp. 203–212. doi: 10.1016/j.bbapap.2003.12.001.

Liu, H. *et al.* (2000) 'On the dynamics of TCR:CD3 complex cell surface expression and downmodulation', *Immunity*, 13(5), pp. 665–675. doi: 10.1016/S1074-7613(00)00066-2.

Liu, X. *et al.* (2016) 'T cell receptor-induced nuclear factor κb (NF-κB) signaling and transcriptional activation are regulated by STIM1- and Orai1-mediated calcium entry', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 291(16), pp. 8440–8452. doi: 10.1074/jbc.M115.713008.

Liu, Z. *et al.* (2017) 'Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector', *Scientific Reports*, 7, p. 2193. doi: 10.1038/s41598-017-02460-2.

Lomakina, G. Y., Modestova, Y. A. and Ugarova, N. N. (2015) 'Bioluminescence assay for cell viability', *Biochemistry (Moscow)*, pp. 701–713. doi: 10.1134/S0006297915060061.

Longo, N. *et al.* (2008) 'Diagnosis of clavulanic acid allergy using basophil activation and leukotriene release by basophils', *Journal of Investigational Allergology and Clinical Immunology*, 18(6), pp. 473–5.

Loontiens, S. *et al.* (2019) 'Purification of high-quality RNA from a small number of fluorescence activated cell sorted zebrafish cells for RNA sequencing purposes', *BMC Genomics*, 20(228), pp. 1–

16. doi: 10.1186/s12864-019-5608-2.

Lu, H., Giordano, F. and Ning, Z. (2016) 'Oxford Nanopore MinION Sequencing and Genome Assembly', *Genomics, Proteomics and Bioinformatics*, 14(5), pp. 265–279. doi: 10.1016/j.gpb.2016.05.004.

Magi, A. *et al.* (2018) 'Nanopore sequencing data analysis: state of the art, applications and challenges', *Briefings in Bioinformatics*, 19(6), pp. 1256–1272. doi: 10.1093/bib/bbx062.

Mak, T.W., Saunders, M.E., Jett, B. D. (2014) 'Chapter 18 - Immune Hypersensitivity', in *Primer to the Immune Response (Second Edition)*, pp. 487–516. Available at: https://booksite.elsevier.com/9780123852458/ (Accessed: 10 March 2020).

Masterson, A. J. *et al.* (2002) 'MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors', *Blood*. American Society of Hematology, 100(2), pp. 701–703. doi: 10.1182/blood.V100.2.701.

Matsuda, J. L. *et al.* (2001) 'Natural killer T cells reactive to a single glycolipid exhibit a highly diverse T cell receptor β repertoire and small clone size', *Proceedings of the National Academy of Sciences of the United States of America*, 98(22), pp. 12636–41. doi: 10.1073/pnas.221445298.

Matsuda, J. L. *et al.* (2008) 'CD1d-restricted iNKT cells, the "Swiss-Army knife" of the immune system', *Current Opinion in Immunology*, pp. 358–368. doi: 10.1016/j.coi.2008.03.018.

Mazzini, E. *et al.* (2014) 'Oral Tolerance Can Be Established via Gap Junction Transfer of Fed Antigens from CX3CR1+ Macrophages to CD103+ Dendritic Cells', *Immunity*, 40, pp. 248–261. doi: 10.1016/j.immuni.2013.12.012.

Mbongue, J. *et al.* (2014) 'The role of dendritic cells in tissue-specific autoimmunity', *Journal of Immunology Research*, 2014, pp. 1–17. doi: 10.1155/2014/857143.

Miao, B. *et al.* (2005) 'T-cell receptor (TCR)/CD3 is involved in sulfated polymannuroguluronate

(SPMG)-induced T lymphocyte activation', *International Immunopharmacology*, 5(7–8), pp. 1171–1182. doi: 10.1016/j.intimp.2005.02.011.

Mirotti, L. *et al.* (2013) 'Lipids are required for the development of Brazil nut allergy: The role of mouse and human iNKT cells', *Allergy: European Journal of Allergy and Clinical Immunology*, 68(1), pp. 74–83. doi: 10.1111/all.12057.

Müller, M. R. and Rao, A. (2007) 'Linking calcineurin activity to leukemogenesis', *Nature Medicine*, pp. 669–671. doi: 10.1038/nm0607-669.

Münz, C. (2010) 'Natural killer cells and autoimmunity', in *Natural Killer Cells*. BioMed Central, pp. 461–467. doi: 10.1016/B978-0-12-370454-2.00034-X.

Murtagh, G. J. *et al.* (2003) 'In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8', *Clinical and Experimental Allergy*, 33(8), pp. 1147–1152. doi: 10.1046/j.1365-2222.2003.01736.x.

Nakatsu, T. *et al.* (2006) 'Structural basis for the spectral difference in luciferase bioluminescence', *Nature*, 440(7082), pp. 372–376. doi: 10.1038/nature04542.

Ning, J., Morgan, D. and Pamphilon, D. (2011) 'A rapid culture technique produces functional dendritic-like cells from human acute myeloid leukemia cell lines', *Journal of Biomedicine and Biotechnology*, 2011, pp. 1–9. doi: 10.1155/2011/172965.

Nishimura, T. *et al.* (2000) 'The interface between innate and acquired immunity: Glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes', *International Immunology*, 12(7), pp. 987–994. doi: 10.1093/intimm/12.7.987.

Nordlee, J. A. *et al.* (2002) 'Identification of a Brazil-Nut Allergen in Transgenic Soybeans', *New England Journal of Medicine*, 334(11), pp. 688–92. doi: 10.1056/nejm199603143341103.

Le Nours, J. *et al.* (2016) 'Atypical natural killer T-cell receptor recognition of CD1d-lipid antigens.', *Nature communications*, 7, p. 10570. doi: 10.1038/ncomms10570.

O'Konek, J. J., Terabe, M. and Berzofsky, J. A. (2012) 'The role of NKT cells in the immune regulation of neoplastic disease', in *Innate Immune Regulation and Cancer Immunotherapy*, pp. 7–21. doi: 10.1007/9781441999146_2.

Oeckinghaus, A. and Ghosh, S. (2009) 'The NF-κB family of transcription factors and its regulation.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 1(4), pp. 1–14. doi: 10.1101/cshperspect.a000034.

Okada, H. *et al.* (2010) 'The "hygiene hypothesis" for autoimmune and allergic diseases: An update', *Clinical and Experimental Immunology*, 160(1), pp. 1–9. doi: 10.1111/j.1365-2249.2010.04139.x.

Pablo Martinez, M. A. and Álvarez De Cienfuegos, G. (2000) 'Modulatory effects of dietary lipids on immune system functions', *Immunology and Cell Biology*, 78(1), pp. 31–39. doi: 10.1046/j.1440-1711.2000.00875.x.

Pabst, O. and Mowat, A. M. (2012) 'Oral tolerance to food protein', *Mucosal Immunology*, 5, pp. 232–239. doi: 10.1038/mi.2012.4.

Pai, S.-Y. *et al.* (2008) 'Distinct Structural Requirements of GATA-3 for the Regulation of Thymocyte and Th2 Cell Differentiation', *The Journal of Immunology*, 180(2), pp. 1050–1059. doi: 10.4049/jimmunol.180.2.1050.

Pastorello, E. A. *et al.* (1998) 'Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy.', *The Journal of allergy and clinical immunology*, 102(6), pp. 1021–1027.

Patterson, E. *et al.* (2012) 'Health implications of high dietary omega-6 polyunsaturated fatty acids', *Journal of Nutrition and Metabolism*, 2012, pp. 1–16.

Paulsen, M. *et al.* (2009) 'FasL cross-linking inhibits activation of human peripheral T cells', *International Immunology*, 21(5), pp. 587–598. doi: 10.1093/intimm/dxp028.

Pellicci, D. G. *et al.* (2011) 'Recognition of β-linked self glycolipids mediated by natural killer T cell antigen receptors', *Nat Immunol*, 12(9), pp. 827–33. doi: 10.1038/ni.2076.

Pletinckx, K. *et al.* (2011) 'Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells', *Frontiers in Immunology*, 2, pp. 1–16. doi: 10.3389/fimmu.2011.00039.

Pollizzi, K. N. and Powell, J. D. (2014) 'Integrating canonical and metabolic signalling programmes in the regulation of T cell responses', *Nature Reviews Immunology*. Nature Publishing Group, pp. 435–446. doi: 10.1038/nri3701.

Porcelli, S. A. and Modlin, R. L. (1999) 'THE CD1 SYSTEM: Antigen-Presenting Molecules for T Cell Recognition of Lipids and Glycolipids', *Annual Review of Immunology*, 17, pp. 297–329. doi: 10.1146/annurev.immunol.17.1.297.

Quentmeier, H. *et al.* (2019) 'The LL-100 panel: 100 cell lines for blood cancer studies', *Scientific Reports*. Nature Publishing Group, 9(1), pp. 1–14. doi: 10.1038/s41598-019-44491-x.

Radauer, C. and Breiteneder, H. (2007) 'Evolutionary biology of plant food allergens', *Journal of Allergy and Clinical Immunology*, 120(3), pp. 518–525. doi: 10.1016/j.jaci.2007.07.024.

Rahman, I. and MacNee, W. (1998) 'Role of transcription factors in inflammatory lung diseases', *Thorax*, 53, pp. 601–612. doi: 10.1136/thx.53.7.601.

Rang, F. J., Kloosterman, W. P. and de Ridder, J. (2018) 'From squiggle to basepair: Computational approaches for improving nanopore sequencing read accuracy', *Genome Biology*, 19(90), pp. 1–11. doi: 10.1186/s13059-018-1462-9.

Rauch, J. *et al.* (2003) 'Structural Features of the Acyl Chain Determine Self-phospholipid Antigen

Recognition by a CD1d-restricted Invariant NKT (iNKT) Cell', *Journal of Biological Chemistry*, 278(48), pp. 47508–15. doi: 10.1074/jbc.M308089200.

Rocklin, R. E. *et al.* (1980) 'Generation of Antigen-Specific Suppressor Cells during Allergy Desensitization', *New England Journal of Medicine*. Massachusetts Medical Society, 302(22), pp. 1213–1219. doi: 10.1056/NEJM198005293022201.

Roehr, J. T., Dieterich, C. and Reinert, K. (2017) 'Flexbar 3.0 - SIMD and multicore parallelization', *Bioinformatics*, 33(18), pp. 2941–2942. doi: 10.1093/bioinformatics/btx330.

Rona, R. J. *et al.* (2007) 'The prevalence of food allergy: A meta-analysis', *Journal of Allergy and Clinical Immunology*, 120(3), pp. 638–646. doi: 10.1016/j.jaci.2007.05.026.

Rosati, E. *et al.* (2017) 'Overview of methodologies for T-cell receptor repertoire analysis', *BMC Biotechnology*, 61, pp. 1–16. doi: 10.1186/s12896-017-0379-9.

Rosenberg, S. A. (2005) 'Cancer immunotherapy comes of age.', *Nature clinical practice. Oncology*. NIH Public Access, 2(3), p. 115. doi: 10.1038/ncponc0101.

Roth, H. *et al.* (2017) 'Flavivirus infection uncouples translation suppression from cellular stress responses', *mBio*, 8(1), pp. 1–18. doi: 10.1128/mBio.02150-16.

Rundqvist, L. *et al.* (2012) 'Solution Structure, Copper Binding and Backbone Dynamics of Recombinant Ber e 1-The Major Allergen from Brazil Nut', *PLoS ONE*, 7(10), pp. 1–11. doi: 10.1371/journal.pone.0046435.

Salazar, A. *et al.* (2017) 'Allergen-Based Diagnostic: Novel and Old Methodologies with New Approaches', in *Allergen*, pp. 77–99. doi: 10.5772/intechopen.69276.

San José, E. and Alarcón, B. (1999) 'Receptor engagement transiently diverts the T cell receptor heterodimer from a constitutive degradation pathway', *Journal of Biological Chemistry*, 274(47), pp. 33740–33746. doi: 10.1074/jbc.274.47.33740.

Sánchez-Rodríguez, E. N. *et al.* (2011) 'Persistence of decidual NK cells and KIR genotypes in healthy pregnant and preeclamptic women: a case-control study in the third trimester of gestation', *Reproductive Biology and Endocrinology*, 9(1), p. 8. doi: 10.1186/1477-7827-9-8.

Satitsuksanoa, P. *et al.* (2018) 'Regulatory Immune Mechanisms in Tolerance to Food Allergy', *Frontiers in immunology*, 9, pp. 1–9. doi: 10.3389/fimmu.2018.02939.

Sbihi, Z. *et al.* (2017) 'iNKT and memory B-cell alterations in HHV-8 multicentric Castleman disease', *Blood*, 129(7), pp. 855–865. doi: 10.1182/blood-2016-06-719716.

Scandella, E. *et al.* (2002) 'Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells', *Blood*, 100(4), pp. 1354–61. doi: 10.1007/s11205-014-0803-7.

Schreiber, J. *et al.* (2013) 'Error rates for nanopore discrimination among cytosine, methylcytosine, and hydroxymethylcytosine along individual DNA strands', *Proceedings of the National Academy of Sciences*, 110(47), pp. 18910–5. doi: 10.1073/pnas.1310615110.

Schroeder, A. *et al.* (2006) 'The RIN: An RNA integrity number for assigning integrity values to RNA measurements', *BMC Molecular Biology*, 7(3), pp. 1–14. doi: 10.1186/1471-2199-7-3.

Shurin, G. V. *et al.* (2005) 'Small Rho GTPases Regulate Antigen Presentation in Dendritic Cells', *The Journal of Immunology*, 174(6), pp. 3394–400. doi: 10.4049/jimmunol.174.6.3394.

Sidobre, S. *et al.* (2004) 'The T cell antigen receptor expressed by Vα14i NKT cells has a unique mode of glycosphingolipid antigen recognition.', *Proceedings of the National Academy of Sciences of the United States of America*, 101(33), pp. 12254–12259. doi: 10.1073/pnas.0404632101.

Singh, A. K., Tripathi, P. and Cardell, S. L. (2018) 'Type II NKT Cells: An Elusive Population With Immunoregulatory Properties', *Frontiers in Immunology*, 9, pp. 1–8. doi: 10.3389/fimmu.2018.01969.

Singh, M. (2005) ‘Essential fatty acids, DHA and human brain.’, *Indian journal of pediatrics*, 72(3), pp. 239–42.

Singh, M. *et al.* (2018) ‘High-throughput targeted long-read single cell sequencing reveals the clonal and transcriptional landscape of lymphocytes’, *bioRxiv*, 10, p. 3120. doi: 10.1101/424945.

Sommermeyer, D. *et al.* (2006) ‘Designer T cells by T cell receptor replacement’, *European Journal of Immunology*, 36, pp. 3052–3059. doi: 10.1002/eji.200636539.

Steinman, R. M. and Cohn, Z. A. (1973) ‘Identification of a novel cell type in peripheral lymphoid organs of mice: I. Morphology, quantitation, tissue distribution’, *Journal of Experimental Medicine*. The Rockefeller University Press, 137(5), pp. 1142–1162. doi: 10.1084/jem.137.5.1142.

Stiefel, G. *et al.* (2017) ‘BSACI guideline for the diagnosis and management of peanut and tree nut allergy’, *Clinical and Experimental Allergy*, 47, pp. 719–739. doi: 10.1111/cea.12957.

Sullivan, B. A. and Kronenberg, M. (2005) ‘Activation or anergy: NKT cells are stunned by α-galactosylceramide’, *Journal of Clinical Investigation*, 115(9), pp. 2328–9. doi: 10.1172/JCI26297.

Suzuki, H. *et al.* (2008) ‘Ovalbumin-Protein σ1 M-Cell Targeting Facilitates Oral Tolerance With Reduction of Antigen-Specific CD4+ T Cells’, *Gastroenterology*, 135, pp. 917–925. doi: 10.1053/j.gastro.2008.05.037.

Szabo, S. J. *et al.* (2000) ‘A novel transcription factor, T-bet, directs Th1 lineage commitment’, *Cell*, 100, pp. 655–669. doi: 10.1016/S0092-8674(00)80702-3.

Takizawa, K. *et al.* (2005) ‘Enhanced expression of CD69 and CD25 antigen on human peripheral blood mononuclear cells by prolactin.’, *Endocrine journal*, 52(5), pp. 635–41. doi: 10.1507/endocrj.52.635.

Taniguchi, M., Seino, K. I. and Nakayama, T. (2003) ‘The NKT cell system: Bridging innate and acquired immunity’, *Nature Immunology*, 4(12), pp. 1164–1165. doi: 10.1038/ni1203-1164.

Tengel, T. *et al.* (2005) 'Complete assignment and secondary structure of the Brazil nut allergen Ber e 1', *Journal of Biomolecular NMR*, 32(4), p. 336. doi: 10.1007/s10858-005-8875-y.

The Connectivity Database (2019) *cDNA sequencing with Oxford Nanopore*.

ThermoFisher (no date) *Luciferase Reporters Luciferase Reporters*. Available at: https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/luciferase-reporters.html# (Accessed: 23 June 2017).

Thomas, S., Stauss, H. J. and Morris, E. C. (2010) 'Molecular immunology lessons from therapeutic T-cell receptor gene transfer', *Immunology*, 129, pp. 170–177. doi: 10.1111/j.1365-2567.2009.03227.x.

Tordesillas, L. *et al.* (2017) 'Mechanisms underlying induction of allergic sensitization by Pru p 3', *Clinical and Experimental Allergy*, 47(11), pp. 1398–1408. doi: 10.1111/cea.12962.

Tsuda, H. *et al.* (2001) 'Characterization of NKT cells in human peripheral blood and decidual lymphocytes', *American Journal of Reproductive Immunology*. Blackwell Munksgaard, 45(5), pp. 295–302. doi: 10.1111/j.8755-8920.2001.450505.x.

Walker, S. and Wing, A. (2010) 'Allergies in children', *The journal of family health care*. Pulsus Group, 20(1), pp. 24–26.

Wang, M. *et al.* (2010) 'Peanut-induced intestinal allergy is mediated through a mast cell-IgE-FceRI-IL-13 pathway', *Journal of Allergy and Clinical Immunology*, 126, pp. 306–316. doi: 10.1016/j.jaci.2010.05.017.

Wang, R. *et al.* (2019) 'Towards a surrogate system to express human lipid binding TCRs', *Biotechnology Letters*. Springer Netherlands, 41(10), pp. 1095–1104. doi: 10.1007/s10529-019-02713-2.

Wang, X. *et al.* (2008) 'Natural killer T-cell autoreactivity leads to a specialized activation state', *Blood*. American Society of Hematology, 112(10), pp. 4128–4138. doi: 10.1182/blood-2008-05-157529.

Waserman, S. and Watson, W. (2011) 'Food allergy.', *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology*, 7 Suppl 1(S1), p. S7. doi: 10.1186/1710-1492-7-S1-S7.

Weinberger, T. and Sicherer, S. (2018) 'Current perspectives on tree nut allergy: A review', *Journal of Asthma and Allergy*, 11, pp. 41–51. doi: 10.2147/JAA.S141636.

Wu, L. and Kaer, L. Van (2011) 'Natural killer T cells in health and disease', *Frontiers in Bioscience - Scholar*, 3, pp. 236–251.

Yazdanbakhsh, M., Kremsner, P. G. and Van Ree, R. (2002) 'Immunology: Allergy, parasites, and the hygiene hypothesis', *Science*, 296(5567), pp. 490–494. doi: 10.1126/science.296.5567.490.

Yu, W., Freeland, D. M. H. and Nadeau, K. C. (2016) 'Food allergy: Immune mechanisms, diagnosis and immunotherapy', *Nature Reviews Immunology*, 16, pp. 751–765. doi: 10.1038/nri.2016.111.

Yuan, C., Song, G. and Jiang, G. (2012) 'The characterization and role of leukemia cell-derived dendritic cells in immunotherapy for leukemic diseases', *Intractable and Rare Diseases Research*. International Advancement Center for Medicine & Health Research Co., 1(2), pp. 53–65. doi: 10.5582/irdr.2012.v1.2.53.

Zhang, G. *et al.* (2011) 'Sulfatide-activated type II NKT cells prevent allergic airway inflammation by inhibiting type I NKT cell function in a mouse model of asthma', *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 301(6), pp. 975–984. doi: 10.1152/ajplung.00114.2011.

Zhong, S. *et al.* (2010) 'Retroviral transduction of T-cell receptors in mouse T-cells', *Journal of Visualized Experiments*, 44(44), p. 2307. doi: 10.3791/2307.

Zhou, Q. and Buchholz, C. J. (2013) ‘Cell type specific gene delivery by lentiviral vectors: New options in immunotherapy’, *OncoImmunology*, 2, p. 22566. doi: 10.4161/onci.22566.