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The role of CD1a-restricted T cells and phospholipase in allergic disease

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By

Sumithra Subramaniam

St Cross College

Supervisor: Dr Graham Ogg

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Abstract

The role of CD1a-restricted T cells and phospholipase in allergic disease

Sumithra Subramaniam St Cross College

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The skin is an important barrier against a range of different environmental challenges. The skin associated immune system is able to detect breaks in the barrier to initiate a protective immune response. Langerhans cells express a high density of CD1a, which presents lipid antigens to T-cells. However little is known about CD1a-restricted lipid antigens and the role of CD1a-restricted T-cells in inflammatory skin disease.

In order to investigate the role of T-cells that react to CD1a presenting lipid in skin disease, wasp and bee venom were studied as an antigen source. Venoms are able to cause allergic hypersensitivity reactions, associated with skin T-cell infiltration, a venom protein-specific T-cell response in allergic individuals and are independent of filaggrin. Using primary antigen presenting cells and target cells lacking surface MHC expression (K562 cells) transfected with CD1a, allowed investigation of polyclonal T-cells responses from unrelated donors.

Bee and wasp venoms were shown to induce CD1a-restricted T-cell responses in both peripheral blood and skin. Surprisingly this activity was not contained within the lipid fraction of the venoms, but instead was mediated through the generation of a lipid ligand by venom phospholipase. Furthermore, wasp venom delivery results in the production of phospholipase products in the skin of humans. A significantly increased frequency of IFNγ-, GM-CSF- and IL-13-producing venom specific CD1a-restricted T-cells was observed in allergic individuals compared to healthy controls. During subcutaneous immunotherapy, frequencies of CD1a-reactive T cells were initially induced, peaking by weeks 5, but then reduced despite escalation of antigen dose. CD1a-reactive T cells were further investigated to characterise their physiological role by generating T cell lines which produced a range of different cytokines, including IL-22 on stimulation with phospholipase and other phospholipase containing allergens.

In summary, we identified a novel pathway of skin inflammation where lipids generated by allergen-derived phospholipase can be recognised by CD1a-restricted T cells which produce type 1 and type 2 cytokines and associate with allergic reactivity. These findings have implications for novel therapeutic strategies for allergic disease.

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Abbreviations

αGalCer	α-Galactosylceramide
AA	Arachidonic acid
ACD	Allergic contact dermatitis
AD	Atopic dermatitis
AHR	Airway hypersensitivity reaction
APC	Antigen presenting cell or Allophycocyanin
β2M	Beta-2-microglobulin
BSA	Bovine serum albumin
BV	Bee venom
CCD	Cross reactive carbohydrate determinants
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDR	Complementarity-determining (hypervariable) region
CHS	Contact hypersensitivity
CLA	Cutaneous lymphocyte antigen
CLIP	Class II-associated invariant chain peptide
cPLA2	Cytosolic phospholipase A2
CTLA	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DDM	Dideoxymycobactin
DETC	Dendritic epidermal T cells
DLN	Draining lymph node
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot

EPB	Epidermal permeability barrier
ER	Endoplasmic reticulum
FA	Fatty acid
FACS	Fluorescence-activated cell sorter (FACS®)
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GWAS	Genome wide association studies
HLA	Human leukocyte antigen
ICS	Intra-cellular cytokine secretion assay
IFN-γ	Gamma-Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T-cells
LC	Langerhans cell
LPL	Lysophospholipid
MACS	Magnetic cell separation (Miltenyi Biotec)
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
MTP	Microsomal triglyceride transfer protein
NOD	Non-obese diabetic
NK	Natural Killer
NKT	Natural Killer T cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PLA1	Phospholipase A1

PLA2	Phospholipase A2
PMA	Phorbol myristoyl acetate
R0	RPMI, glutamine, penicillin and streptomycin
R10	RPMI, 10% FCS, glutamine, penicillin and streptomycin
R10*	RPMI, 10% Human AB serum, glutamine, penicillin and streptomycin
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute- 1640 (media formulation)
RT-PCR	Reverse transriptase polymerase chain reaction
SEM	Standard error of the mean
SIT	Allergen-specific immunotherapy
SLIT	Sublingual immunotherapy
SNP	Single nucleotide polymorphism
sPLA2	Secretory phospholipase A2
SPT	Skin prick test
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TCR	T cell receptor
TEWL	Transepidermal water loss
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like Receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UTR	Untranslated region
UVR	UV radiation
WV	Wasp venom

1 Introduction

1.1 CD1

T cell receptor (TCR) interaction with antigen presented by antigen-presenting molecules is key to mounting an effective immune response. Classical T cells recognise peptide antigens, processed and presented on the cell surface by major histocompatibility complex (MHC) Class I or II molecules, through interactions with αβ or γδ TCR. There are also unconventional populations of T cells, including those that recognise lipids, glycolipids and lipopeptides presented by cluster of differentiation (CD) 1 molecules. The five human CD1 family genes cluster on chromosome 1, encoding glycoproteins that form an antigen presentation system consisting of CD1a, CD1b, CD1c, CD1d and CD1e. CD1 isoforms are classified into three groups based on sequence homology, genomic organisation and cellular distribution (1). Group 1 consists of CD1a, CD1b and CD1c, Group 2 consists of CD1d and Group 3 of CD1e (2). Group 1 and 2 CD1 molecules are expressed on the cell surface, while CD1e is expressed intracellularly in compartments of the endosomal pathway and thought to play a role in lipid processing and loading onto CD1 as they do not reach the cell surface but accumulate in the trans-golgi network in immature dendritic cells (DC) (3).

Structurally, CD1 molecules resemble MHC Class I, composed of a heavy chain non-covalently associated to β2 microglobulin (β2M) (2, 4). The heavy chain has a small cytoplasmic tail, a transmembrane region, and an extracellular antigen-binding domain. The extracellular domain is composed of 3 alpha helices; the α3 helix proximal to the membrane interacts with β2M, supporting the distal helices and shows the most conservation between CD1 isoforms, while the α1 and α2 helices distal to the membrane sit on a 6-strand β-pleated sheet floor interacting with the lipid antigen (2).

Unlike MHC Class I however, CD1 molecules show relatively little polymorphism and differ in the antigen binding groove, which consists of hydrophobic channels and pockets into which lipid acyl chains bind (5). Differences in size, position and hydrophobicity of these pockets determine the range of lipids that can interact and stimulate T cells (5). Together with the fact that the different CD1 isoforms have differential cellular expression, this suggests that CD1-restricted T cells have distinct roles in immune responses.

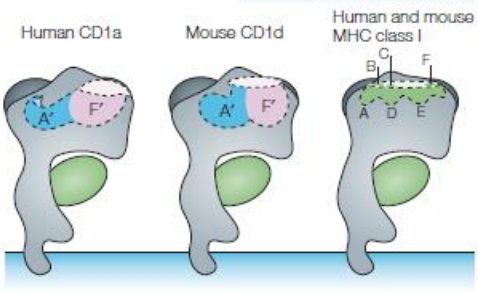


Figure 1-1: Comparison of human and mouse CD1 and MHC molecules (5).

1.1.1 Evolution of CD1

CD1 genes have been identified in a number of mammals now, including primates, cows, horses, pigs and rodents, although not all 5 isoforms are present in all mammals (6). Group I molecules are present in most mammals, except rodents where chromosomal rearrangement resulted in deletion of these genes and so mice only express CD1d. CD1 molecules are highly conserved throughout evolution and the different CD1 isoforms show greater homology to the same isoforms in other species than to different isoforms in the same species, suggesting early diversification of the isoforms, and diverse antigen binding and function (7). The discovery of CD1 in chickens has furthered our understanding of the evolution of these genes. While human CD1 is found on chromosome 1, unlinked to the MHC Class I locus on

chromosome 6, the chicken CD1 gene is located within 50kb of the MHC Class I gene, suggesting that the two were once linked during evolution (6). Indeed the human CD1 gene is associated with an MHC paralogy group (region of chromosome containing some duplicated genes from MHC locus that have diverged after the duplication event leading to a new function) on chromosome 1 (8). Sequence and structural homology of CD1 and MHC Class I molecules suggests that CD1 is the result of MHC Class I gene duplication and distribution to different MHC paralogous regions, although the order of these events is still unclear (8). CD1 genes have now also been identified in reptiles, with similar links to the MHC locus and paralogous regions, although the different isotypes may have developed in independent diversification events (9). This shows a higher degree of evolutionary conservation than previously believed and indicates an important role for these molecules in the immune response.

CD1 genes are relatively non-polymorphic in comparison to MHC. Analysis of the different isoforms revealed two allelic variants at exon 2 and 3, encoding α1 and α2 loops of the antigen-binding groove, in CD1a, CD1d and CD1e but none in CD1b and CD1c (10). CD1a showed changes in amino acids at positions 13 and 51 resulting in replacement of isoleucine and tryptophan (allele 1) with a threonine and cysteine (allele 2) respectively (10). Allele 1 is present at a frequency of 0.13 compared to 0.87 of allele 2 (10). The functional significance of CD1a polymorphism however is unclear. While CD1a polymorphisms was not associated with breast, lung and colorectal cancers (11), they did seem to contribute to susceptibility to multiple sclerosis and Guillain-Barre syndrome (12, 13), although the functional effects are still unknown.

Individuals with low or undetectable CD1a expression have been identified, who were unable to upregulate CD1a at the mRNA and protein level on monocyte-derived dendritic cells (mDC) *in vitro* or present mycobacterial lipid antigens to T cells (14).

This was found to be associated to two single nucleotide polymorphisms (SNP) located at the 5' untranslated region (UTR) of CD1a, which is linked with gene expression. Low CD1a expression has been associated with a more immature DC phenotype, with diminished cytokine secretion and increased phagocytic potential (15, 16). The clinical significance of CD1a deficiency in disease is still unclear, although association with susceptibility to tuberculosis infection has been demonstrated (17).

1.1.2 CD1 assembly and antigen presentation

Lipid antigens require processing and loading onto CD1 molecules by antigen presenting cells (APC) for presentation to T cells. CD1 polypeptides translocate into the endoplasmic reticulum (ER) where they associate with protein folding chaperones, such as calnexin and calreticulin, and then recruit Erp57, which is involved in tertiary structure formation (8). As the heavy chains are folded, ER lipids bind into the groove, acting as chaperone lipids, similar to class II invariant chain peptide (CLIP) with MHC Class II, providing structural stability and preventing binding of non-antigenic lipids until entry into the secretory or endocytic pathway where antigenic lipids may be encountered (5). The heavy chain then rapidly associates non-covalently to β2M via the α3 helix, although the strength of this interaction differs between isoforms. Mice deficient in β2M showed impaired CD1d-restricted T cell development and activation (18) and cells lacking β2M showed sequestration of CD1b in the ER (19) or rapid degradation of the heavy chains (20), highlighting the importance of β2M in formation of CD1 molecules and regulation of their movement from the ER.

Following glycosylation in the Golgi, CD1 molecules exit the ER and traffic to the cell surface. They are then rapidly re-internalised into the endosomal pathway where they encounter antigenic lipids (1). The cytoplasmic tail of the different isoforms contains tyrosine or leucine based sequences that associate with specific adaptor proteins,

directing them into different parts of the endosomal compartments. The adaptor proteins are multimeric complexes, consisting of two large subunits (α,γ,δ or ε interacting with β unit), a medium μ-subunit and a small α-subunit (21). The adaptor proteins interact with the cytoplasmic tail through the binding of the tyrosine residues to the μ-subunit or leucine residue to or β or μ-subunits (21). There are three well characterised adaptor proteins. AP1 is found at the trans-golgi complex and AP3 at the trans-golgi complex and in endosomes, directing CD1 molecules to the endosomal and lysosomal compartments. AP2 is found at the cell surface and promotes the internalisation of CD1b, CD1c and CD1d in clathrin-coated vesicles, from where they are delivered to different endosomal compartments (21).

CD1a has the shortest cytoplasmic tail of the isoforms and does not contain motifs that could bind adaptor proteins. As such it has a dense distribution on the cell surface and only traffics to the early endosomal compartments, which has been demonstrated by imaging studies (21). This is further confirmed by the kinetics of CD1a loading and trafficking, and the neutral pH requirements of lipid antigens loaded onto CD1a (22). CD1a internalisation and trafficking back to the surface is faster than translocation of other molecules from the cell surface to late endosomal compartments (21). Blocking vacuolar ATPases prevents generation of a pH gradient through the endosomal compartments, but does not affect CD1a activation of T cells, therefore a low pH is not required for generation and loading of many CD1a lipid ligands (23). This is consistent with CD1a trafficking to the early endocytic pathway. CD1a has also been detected in Birbeck granules of Langerhans cells (LCs), an organelle in the endosomal pathway, although the mechanism of translocation is still unclear.

CD1 lipid antigens are extracted and solubilised from membranes or aggregates and loaded into the antigen groove following exchange with the resident ligand. This is

facilitated by chaperone proteins. Microsomal triglyceride transfer protein (MTP) is a chaperone protein that was identified as an important cellular protein in the transfer of lipids between vesicles (24). MTP is plays a major role in loadng of endogenous lipids in the CD1d groove in the ER, and a lack of MTP leads to decreased CD1d expression, antigen presentation and trafficking (25).

Sphingolipid activator proteins (SAP) are involved in the degradation of glycosphingolipids in the lysosome. There are five SAPs thought to be involved in lipid transfer, saposins A-D and GM2AP (4). These molecules activate different enzymes involved in the hydrolysis of different glycolipids or form complexes with lipids, lifting them for processing. Mice deficient in the saposin precursor, prosaposin, or SAP itself showed reduced CD1b and CD1d and so were unable to activate CD1d-restricted T cells (26, 27). Furthermore, these mice lacked natural killer T cells due to the lack of lipids loaded onto CD1d in the thymus necessary for positive selection. Therefore saposins are important in the transfer and loading of various lipid antigens into CD1d, as well as Group 1 CD1 molecules, with each saposin demonstrating unique lipid antigen specificity.

1.1.3 CD1 lipid antigens

Exogenous lipid antigens are loaded onto CD1 molecules when they intersect and co-localise in the endosomal pathway. Following phagocytosis, lipid antigens and CD1 molecules in phagosomes can directly enter the endosomal pathway or lipids are released from phagosomes and enter the endosomal pathway separately, where they encounter recycled CD1 (21). They can also enter the endosomal pathway through receptor mediated lipid uptake, binding to receptors such as mannose receptor and DC-SIGN (28). Lipids are sorted to the different compartments of the endosomal

pathway based on the chain length of the hydrophobic tails, with longer tails penetrating further down the pathway, co-localising with cognate CD1 molecules (29).

Lipid antigens are held in the antigen binding groove through interactions of the hydrophobic acyl chains with a cleft rich in non-polar amino acid residues and made from an A' and an F' pocket (analogous to MHC class I counterparts). The structure of the A' and F' pockets, as well as the degree of opening of the groove, determines the repertoire of lipids able to bind to a specific CD1 isoform (5). The α1 and α2 helices interact to form the edges of the groove that lies above a 6-strand β-pleated sheet floor. A partial roof is formed over the pockets through contacts between the α1 and α2 helices of the CD1 heavy chain, due to the presence of bulky amino acids at specific positions (5). The entrance of the CD1 groove is narrower than MHC class I proteins but has a larger volume with more depth. This allows lipid antigens to bind deeper into the groove than peptides in MHC molecules, which sit more superficially (5). The roof also prevents access along the entire length of the binding groove and lipid access to the pockets is restricted to the F' portal. The F' portal also provides stability for the bound lipid through hydrogen bonds and contains residues than can be ionised at low pH, as found in endosomal compartments, resulting in partial opening of the cleft to facilitate lipid exchange and binding (2). Many CD1 lipid antigens are composed of hydrophobic acyl chains which bind into the hydrophobic pockets, and a polar head group which is exposed from the antigen binding groove and can interact with TCR (5).

The A' and F' pockets of CD1a extend out from the centre, running between the α1 and α2 helices, with hydrophobic residues forming interconnected tunnels to bind acyl chains (8). The F' pocket is not as deep as the A' pocket but it connects the A' pocket with the outer surface of CD1a (30). While the A' pocket is formed between the α1 and

α2 helices in a U-shaped curve with a closed end, the F' pocket is long and straight but opens out as it meets the binding groove surface. This affects the size of acyl chain accommodated by the two pockets, with the constricted A' pocket acting as a 'molecular ruler'. Defining the crystal structures of a self sulphatide lipid antigen (31) and a mycobacterial lipid, dideoxymycobactin (DDM) (30) bound to CD1a have increased our understanding of lipid interaction with CD1a. Sulphatides are composed of a sphingosine base with a C18 acyl chain that is N-amide linked to a fatty acid chain varying from C14 to C24 in length. The C18 sphingosine acyl chain binds into the A' pocket, following its curve, while the variable fatty acid chain sits in the F' pocket and protrudes into the T cell recognition surface with the head group (31).

DDM is a mycobacterial lipopeptide composed of a fatty acid chain linked to a hydrophilic head group. The head group contains a peptide backbone with 4 chiral centres and forms two segments, the N-aryl branch and the lysine branch (30). In contrast with self-sulphatide antigen, DDM contains only one acyl chain of shorter overall length, and a different hydrophilic head group (30). Despite these differences between the lipids, the overall conformation of CD1a and the key residues in the binding cleft remains the same. The acyl chains of both the lipopeptide and sphingosine base are inserted into the A' pocket. Some differences in the conformation of bound antigen can be seen across the A'-F' junction, demonstrating how structurally different antigens can bind CD1a and activate T cells (30). The sphingosine chain of the sulphatide antigen forms a curve across the A'-F' junction, to accommodate its longer chain length and positioning the headgroup higher up in the F' pocket and closer to the TCR contact site (30). The lysine branch of DDM however binds straight across the A'-F' junction. Interestingly, the lysine branch appears to follow a similar path to the fatty acid chain of the sulphatide, while the N-aryl branch sits in a similar

position to the sulphatide headgroup, to interact with TCR (30). CD1a has the smallest antigen binding groove of the CD1 isoforms, restricting the size of its lipid ligands. It also differs from other CD1 isoforms as it provides access to the cleft via a portal on the side directly above the F' pocket, while other CD1 isoforms provide access between the A' and F' pockets (32). This is due to interactions between the α1 and α2-helices of CD1a protein above the A' pocket forming a roof-like structure which blocks access to the A' pocket.

The generation of CD1a tetramers and dextramers has facilitated the investigation of CD1a, lipid and TCR interactions (33). More recently, CD1a has been shown to bind and present headless hydrophobic lipids antigens, such as fatty acids, squalene and triacylglyceride (34), oily lipids produced by the sebaceous gland. They demonstrated that CD1a is able to bind a number of different lipids but that while some antigenic lipids were able to engage the TCR of an autoreactive CD1a-restricted T cell clone, others, with large carbohydrate or charged headgroups, were antagonistic to the same TCR and inhibited T cell activation (34). Apolar lipids seem to displace the inhibitory lipids with polar headgroups that interfere with CD1a-TCR interaction. This raises the question of how TCR are able to interact with CD1a bound to headless antigens. Studies from CD1a and other CD1 isoforms have shown that binding of lipid antigens internally can alter the outer conformation of CD1 (35, 36), modifying TCR docking sites. CD1b also bind small endogenous lipids that are sequestered deep in the binding groove. A second exogenous lipid can then also fit into the groove and interact with TCR (37). This explains how CD1b can present lipids with large variations in size, from C12 to C80 (29). This is unlikely to be possible for CD1a which has the smallest volume of antigen binding groove while CD1b has the largest.

1.1.4 CD1-restricted T-cells

CD1-restricted T cells recognise both self and foreign antigens via TCR interactions and are involved in bridging the innate and adaptive arms of the immune system during infection. They have also been implicated in the pathology of some diseases, but have a protective role in others. CD1d-restricted T cells, also known as natural killer T cells (NKTs) expressing an invariant TCR chain (Vα24-Jα18 with Vβ11 in humans; Vα14-Jα18 with Vβ2 in mice), and natural killer (NK) cell associated molecules (CD56, CD161), are known as invariant NKT cells (iNKT). They have been extensively studied due to their unique marker, the invariant TCR, and because CD1d is the only isoform expressed in mice. On stimulation, iNKT cells are rapidly activated to release pre-formed cytokines and recruit adaptive immune cells (38). The stimulation threshold for these cells is also lower than conventional T cells as they do not always require classical co-stimulation and can be stimulated by cytokines alone (38). CD1-restricted T cells are therefore able to respond rapidly to stimuli and generate an effective immune response to clear the pathogen.

Mycobacterial lipid antigens have been identified that bind to each of the CD1 isoforms and stimulate a strong T cell response and production of interferon (IFN)-γ and tumour necrosis factor (TNF)-α, key cytokines in the resolution of tuberculosis (TB) infection. The response to mycobacterial infection has been studied with all CD1 isoforms. Intravenous administration of BCG stimulated early iNKT expansion followed by contraction in the lungs using a mouse model (39). iNKT cells are also involved in granuloma formation and production of granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ and TNF-α, which may contribute to the clearance of *M. tuberculosis* (40, 41). However, CD1d or Jα18 knockout mice do not show any impairment in resolution of infection. This suggests a certain amount of redundancy in

the CD1d-restricted immune response to *M. tuberculosis*, which may enhance the early tissue-specific response (42, 43).

M. tuberculosis infection has also been studied in the context of Group I CD1 using a humanised SCID mouse model with human Group I CD1 genes under the control of endogenous promoter. This mouse model was shown to have the same CD1 tissue pattern expression as humans (44). Using this model, *M. tuberculosis* infection was shown to generate a CD1-restricted T cell response with cytotoxic activity and production of IFN-γ and TNF-α (45). The development of Group I CD1 tetramers has also allowed analysis of polyclonal Group I CD1-restricted T cell response in unrelated individuals. Using CD1a dextramers, DDM-specific CD1a-restricted T cells were detected *ex vivo* in individuals with active tuberculosis or positive tuberculin skin test (33). Tetramer analysis also identified CD1c-restricted T cells specific for phosphomycoketide in patients with latent tuberculosis *ex vivo*, a lipid antigen found in infection-competent Mycobacterial species but not environmental ones (46).

M. tuberculosis infection of humanised transgenic Group I CD1 mice or *M. tuberculosis* lipid exposure showed key differences in Group I CD1-restricted T cell responses compared to iNKT responses (45). While iNKT respond rapidly on primary infection but show reduced response and even anergy on secondary exposure, Group I CD1-restricted T cells had a slower primary response but responded rapidly when exposed again (45). This suggests that Group I CD1-restricted T cells behave more like conventional T cells than iNKT, with a more adaptive antigen-experienced phenotype. T cells responding to all four CD1 isoforms recognise mycobacterial lipids, which is suggestive of a role in resolving *M. tuberculosis* infection.

Although *M. tuberculosis* is the primary pathogen studied in relation to Group I CD1-restricted T cells, iNKT cells are also stimulated by other bacteria, including *S. pneumonia, S. typhimurium* and *B. burgodorferi*, as well as viral and fungal infection (43, 47). Hepatitis B-specific T and B cell responses were reduced in mouse models lacking iNKT or CD1d, resulting in impaired viral clearance (48). CD1d expression was also upregulated on hepatocytes in hepatitis C infection and hepatic NKT cells produced IFN-γ and some interleukin (IL)-13 on stimulation (49). Asperamide B, a fungal lipid, was shown to directly stimulate iNKT cell IFN-γ and IL-4 production (50). Activation of CD1-restricted T cells by lipid antigens from various different micro-organisms suggest that they play an important role in the effector immune response against pathogens.

CD1-restricted cells have also been implicated in disease pathogenesis, such as allergic hypersensitivity and autoimmune disease. Asperamide B is a glycolipid found in *Aspergillus fumigatus*, a commonly found fungus, and is associated with allergic sensitisation and severe asthma (50). Intranasal treatment with Asperamide B of BALB/c mice induced airway hyper-reactivity (AHR) and airway inflammation, associated with infiltration of macrophages, Th2 cells and IL-4 and IL-13 producing iNKT cells (50). The AHR was dependent on iNKT activity as it was not seen in mice lacking CD1d but could be reversed by adoptive transfer of iNKT cells (50, 51), suggesting that iNKT cells are involved in driving the hyper-reactive immune response in allergic asthma. Similarly, development of allergic contact dermatitis (ACD) is also influenced by iNKT cells, as CD1d expression on keratinocytes and iNKT numbers are elevated in ACD lesions (52). More recently, lipids, particularly phospholipids, at the pollen surface were shown to bind CD1d and CD1a and were recognised by T-cells, with highest responses seen in sensitive subjects at the peak of the allergic season

(53). As well as being expressed on LCs, CD1a is also expressed on dermal, gut and lung DCs. These are all mucosal surfaces, at the interface between host and environment, and so are key sites for allergic sensitisation. The role of CD1a in allergen associated inflammation is not known and presentation of allergenic lipid antigens on CD1a may contribute, particularly in local responses.

As key components in bridging the innate and adaptive immune systems, iNKT cells have a pro-inflammatory role in a number of different diseases. However, a suppressive role has also been suggested for iNKT cells, acting as regulatory cells and inducing T cell tolerance. The initial cytokine environment is important in shaping the response as interaction with IL-10 producing cells result in iNKT cells with a regulatory phenotype, while interaction with IL-12 producing cells induces pro-inflammatory iNKT cell responses (54). iNKT cells regulate T cell-mediated ACD, as mice lacking iNKT cells exhibit increased contact hypersensitivity (CHS) and enhanced priming of $CD8^+$ effector T cells compared to wild-type mice, which is normalised by adoptive transfer of iNKT cells (55). NKT cells also have a potent anti-metastatic effect and induce tumour cell death through expression of cell death-inducing molecules, such are perforin, FasL and TRAIL (56).

In autoimmune diseases, CD1d-restricted T cells have both a pathogenic and protective role depending on the disease, by activating either effector or regulatory T cells. Graves' disease and Hashimoto's thyroiditis, inflammatory thyroid diseases, are associated with large infiltrations of Group I $CD1^+$ APCs and CD1-restricted T cells during acute and chronic stages, indicating role for the CD1-restricted T cell response in the development of autoimmune thyroiditis (57). However activation of iNKTs with α-Galactosyl-Geramide (αGalCer) had a protective effect in a mouse model of Graves' disease (58). The frequency of NKT cells is reduced in a number of autoimmune

diseases, such as systemic sclerosis, multiple sclerosis, type I diabetes and systemic lupus erythematosus (59-62). The transfer of rat pancreatic islets into mice pre-treated with anti-CD4 to minimise rejection, resulted in graft survival for over 100 days in wild type mice but led to early graft rejection in mice lacking iNKT cells. Graft tolerance was restored by reconstitution with iNKT cells, identifying iNKT cells as key regulators of tolerance (63). Administration of αGalCer to non-obese diabetic (NOD) mice, a model of autoimmune diabetes, stimulates NKT secretion of Th1 and Th2 cytokines, but repeated injections increases Th2 type regulatory but not Th1-type NKTs (64). The role of CD1-restricted T cells in autoimmune diseases remains conflicting some ways, partly due to the dual function of NKTs but also due to the differences in animal models and experiment parameters.

Recent studies of human polyclonal autoreactive CD1-restricted T cells have increased our understanding of these cells in steady-state conditions, particularly the Group I CD1-restricted T cells. Using CD1-transfected cell lines as APC (K562 cell line; myelogenous leukaemic cell line expressing minimal MHC class I and II), polyclonal CD1-restricted T cells were detected in the peripheral blood (65). CD1a-restricted autoreactive T cells were detected at a significantly higher frequency (0.02-0.4% of memory T cells in peripheral blood) in blood and skin than the other CD1-restricted autoreactive T cells (65, 66). These autoreactive cells were shown to express skin homing markers, including cutaneous lymphocyte antigen (CLA), suggesting a role for them in the skin, where CD1a expression is high, particularly on LCs.

CD1a-restricted autoreactive clones showed diverse TCR and were CD4, CD8 or double negative T cells (66). When comparing cord blood and adult blood, CD1a-restricted autoreactive T cells were detected at birth with naïve phenotype but showed

an increase in percentage of CD45RO+ memory cells in adults, similar to conventional T cells (66). Cytokine analysis revealed secretion of a number of cytokines, including IFN-γ, TNF-α, IL-13, IL-2 and IL-22 (65). IL-22 is produced by restricted subsets of T cells, often in combination with other cytokines. A newly emerging T cell subset, Th22, secretes IL-22 in the absence of IL-17, and is thought to be involved in the immune response in the skin. After sorting T cells based on surface expression of chemokine receptors in Th1, Th17 or Th22 subsets, CD1a-restricted autoreactive T cells were identified within the Th22 subsets (65). Therefore CD1a is a molecular target for some Th22 cells and CD1a-restricted autoreactive T cells may form a subgroup of Th22 cells found in the blood and skin of healthy individuals (65).

While a number of self-lipid antigens recognised by CD1 molecules have now been identified, those involved in autoimmune diseases are still not clear. Interestingly, it has been shown that some TCRs have dual reactivity and are able to interact with Group I CD1 molecules presenting self-lipids as well as microbial antigens. Self-antigens generated a less potent response than foreign antigens, with weaker TCR recognition, and may prime CD1-restricted T cells to respond more rapidly during the initial immune response to foreign antigens (67).

1.2 Allergic hypersensitivity

Allergy is the disease caused by an exaggerated or inappropriate response to environmental antigens that are otherwise innocuous, in patients who have a genetic predisposition (68). It is classed as a Type I hypersensitivity reaction, from the Gell and Coombs classification to identify the nature of immune reactions that cause tissue injury and disease, although in practice many disorders will involve more than one type. Many of the immune cells and mediators involved in the allergic response

evolved originally to generate an effective immune response against parasites, and heal and restore the resulting damaged tissue (69). In an allergic reaction, this inflammatory immune response is activated against environmental allergens in genetically susceptible individuals.

Allergen sensitisation during first encounter with an allergen involves recruitment and priming of allergen-specific naïve T cells. APC include DC, basophils, macrophages, mast cells, eosinophils and innate lymphoid cells (ILC) and can be skewed towards a Th2 bias by mediators released from surrounding epithelial cells, such as GM-CSF, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 (70). The APC then present allergen peptides to conventional naïve T cells resulting in Th2 differentiation. This requires co-stimulatory molecules, chemokines, such as CCL17 and CCL19, and Th2 cytokines, such as IL-4 and TSLP, produced by neighbouring cells (70). Th2 cells then produce cytokines, such as IL-4, IL-5 and IL-13. Th2 cytokines are important in the activation of eoinosphils (IL-5) and promote class switching of allergen-specific B cells to immunoglobulin (Ig)E (IL-4, IL-13, BAFF) (70). Class switching is the process where B cells change the class of immunoglobulin heavy chain expressed from IgM to IgA, IgG or IgE by somatic recombination (68). This process is influenced by the surrounding environment and phenotype and bias of interacting cells. Both Th2 and B cells undergo clonal expansion and induction of a memory population (68). Allergen-specific IgE bind to FcεR1 on the surface of mast cells and eosinophils. Subsequent allergen exposure results in the release of inflammatory mediators from mast cells and eosinophils in response to cross-linking of allergen on specific IgE held on the surface of the cells (68). These mediators induce local and systemic reactions through interactions with specific receptors, causing the symptoms of allergy and anaphylaxis. Widely seen effects include systemic vasodilation, bronchial smooth muscle

contraction and increased vascular permeability, leading to hypotension, restricted breathing, angioedema and tissue infiltration of leucocytes (68, 70). Some of the common mediators and their effects are detailed in Table 1-1.

Class	Mediators	Physiological Effects
Preformed mediators	Histamine, Proteases, including tryptase, chymase and carboxypeptidase; serotonin; heparin; hydrolases; peroxidase; phospholipase; major basic protein	Vasodilation; Angiogenesis; Mitogenesis; Pain; Protein processing; Lipid hydrolysis; Tissue damage; Inflammation
Lipid mediators	Leukotreines B4, C4; Prostaglandins E2, D2; Platelet activating factor	Leucocyte chemotaxis; vasodilation; bronchoconstriction; platelet activation
Cytokines	TNF-α; TGF-β; IFN-γ; IFN-α; IL-1; IL-4; IL-5; IL-13; IL-25	Inflammation – leucocyte migration and proliferation and class-switching
Chemokines	CXCL8; CCL3; CCL2; CCL17; CCL13; CCL5; CCL11; CCL19	Chemoattraction and tissue infiltration of leucocytes
Growth Factors	CSF; GM-CSF; VEGF	Vasodilation; angiogenesis

Table 1-1: Major mast cell mediators and their effects *(71)*.

The allergic response can be divided into two stages: early and late phase. The early phase is mediated by histamine, lipid mediators and other inflammatory mediators as described above, leading to the immediate symptoms of allergy, often within minutes (68). The late phase response develops in the following 2-12 hours after allergen exposure. While mast cells still play a key role in the late phase response, cytokines and chemokines secreted in the early phase response drive the migration of allergen-specific T cells to the site of allergen exposure (68). Allergen presentation by local APCs activates T cells, which can be enhanced by IgE facilitated antigen presentation.

IgE facilitated antigen presentation involves the uptake of allergen by APCs via IgE bound to low affinity FcεRII (CD23), which is then processed and presented on MHC class II to T cells (72). Although Th2 cells are accepted as the key T cell subtype involved in allergy, Th17, Th22 and Th9 cells have all been shown to contribute at different levels and sites (73, 74). IL-9 produced by Th9 for example is important in mast cell maturation (75) and recruitment of eosinophils to the site of allergen exposure (76) and activated to further the inflammatory response. The late phase response can take up to two days to fully resolve.

Genome wide association studies (GWAS) have identified a number of genetic factors that contribute to the risk of developing atopy. Many of the susceptible loci identified contain genes involved in Th2 differentiation, Th2 cytokine or IgE production, such as signal transducer and activator of transcription (STAT) 6, IL-33 and FcεRα1 (77, 78). This further implicates the Th2 cells in allergic disease and may influence the severity of the disease (70).

1.2.1 Venom allergy

Stinging insects of the order hymneoptera, including vespidae (wasps), apidae (bees) and formicidae (ants) can cause allergic reactions in sensitised individuals. These reactions are against allergens found in the venom and most currently known venom allergens are glycoproteins. The major allergens in *Apis mellifera* venom are phospholipase A2 (Api m 1), hyaluronidase (Api m 2) and acid phosphatase (Api m 3) and in *Vespula vulgaris* they are phospholipase A1 (Ves v 1), hyaluronidase (Ves v 2) and antigen 5 (Ves v 5). An Apis mellifera sting injects 50µg on average of venom protein (79). The *Vespula* species however releases less venom per sting, between 1.7-3.1µg of venom protein (79).

Reactions to venom stings can vary from mild to severe. The most common reaction is a painful localised swelling and erythema around the sting site, lasting 2 days, caused by the body's response to venom components. In allergic individuals, a large localised reaction is seen, with swelling at the site of the sting greater than 10cm and lasting longer than 24 hours (80). The swelling may involve the rest of the limb but without systemic reactions and has an immunological basis. The prevalence of large localised reactions ranges from 2.4-26.4% (80). Some will go on to experience mild systemic reactions, including skin, gastrointestinal, respiratory, cardiovascular and reactions (Table 1-2) (81). Unusual reactions, involving the neurologic, renal, ocular and haematological systems have also been reported.

Organ	Symptoms	Cause
Skin	Urticarial rash; Pruritus; Erythema; angio-oedema	Vasodilation; Increased vascular permeability
Gastrointestinal	Abdominal pain; Vomiting; Diarrhoea	Smooth muscle contraction; Mucosal oedema
Respiratory	Airway obstruction; Laryngeal oedema	Bronchospasm; Stridor
Cardiovascular	Systemic hypotension; Myocardial ischaemia; Syncope	Vasodilation

Table 1-2: Systemic reaction to insect stings (81)

More severe systemic reactions, or anaphylaxis, include swelling of the throat or mouth, leading to difficulty in swallowing, breathing and speaking, generalised hives and erythema and syncope. The prevalence of systemic anaphylaxis reactions is between 0.3-7.5%, but is lower in children and high in beekeepers (82). Fatalities however are relative low, estimated at 0.25 deaths per million per year, which may be

underestimated due to misdiagnosis (83). Systemic anaphylaxis is IgE mediated in the majority of cases (82). Short-term sensitising IgG antibodies and complement activation of IgG-venom complexes may occasionally play role, as well as mast cell mediator release in patients with mastocytosis (82).

As with most allergic disease, a diagnosis of venom allergy is based on a detailed history. This includes the number and site of sting reactions, the symptoms experienced, their severity and time taken from sting. It is also important to determine the environment and activities prior to the sting that may confound a diagnosis of venom allergy. Risk factors for venom allergy and the severity of the reaction should also be assessed to contribute to the diagnosis and help with future management. The risk of developing a severe reaction to insect venom can be influenced by a number of factors and an increased risk is associated with a lower number of stings in beekeepers (due to tolerance induction), shorter time between stinging episodes, increased severity of previous stings, older patients, cardiovascular disease, especially those treated with β-blockers, the type of insect (bee more than wasp) and elevated baseline levels of serum tryptase (mastocytosis) (82). Previous sensitisation to venom allergens is also major risk factor for the development of severe systemic reactions and can be determined by *in vitro* IgE testing and/or skin prick testing (84).

Identifying the specific allergen causing an allergic reaction can be complicated by the presence of multiple other allergens at the time and site of the reactions. Diagnostic tests are available to narrow down the specific allergen. These should be performed at least two weeks after an allergic episode (85). Skin tests include skin prick tests (SPT), intradermal tests and patch test. SPT is the most widely used and involves adding a drop of allergen, saline (negative control) or histamine (positive control) solutions on the skin (86). The skin is then pricked through the solutions using a sterile

lancet or needle. After 15-20 minutes, a raised wheal is expected to the positive control in all patients and to the allergen in sensitised patients, with a weal size 2-3mm greater than the negative control considered as positive (86). The size of the wheal however does not correlate with severity of clinical reactions seen (87).

A negative result despite a strong clinical history may indicate further investigation with lower concentration of allergen by intradermal testing which is more sensitive but less specific (86). Intradermal tests involve injecting approximately 20µl of allergen solution with a needle into the dermis. As with SPT, signs of an allergic reaction are evaluated after 15-20 minutes from the size of the wheal (80).

Venom allergen specific IgE levels can also be measured to confirm a clinical diagnosis of venom allergy. Serum allergen specific IgE levels are evaluated by radio-labelled or fluorescent immunoassay. A positive diagnostic test result however is not predictive of clinical reactions or severity (87). Equally, reports suggest that more than 30% of those with systemic reactions to insect venom are still negative by skin testing (88). The tests provide confirmation of a clinical history and help identify the specific allergen.

Differentiating between wasp and bee venom allergy can be challenging as it can be difficult to identify the particular stinging insect during a reaction. *In vitro* testing has its own drawbacks, in that 30-50% of patients show double positivity, despite true double sensitisation being relatively rare (89). This double positivity may be due to sequence homology of allergens, such as hyalurondinase (90). In addition, carbohydrate side chains linked to allergen glycoproteins have been reported to bind IgE and are cross-reactive. As such they are known as cross-reactive carbohydrate determinants (CCD) and play a role in double positivity to wasp and bee venom by *in vitro* testing (89).

Serum from patients allergic to one insect contain carbohydrate specific IgE which may cross-react with carbohydrate chains on allergens from a different insect venom (89). Current understanding of cross-reacting carbohydrate specific IgE is that they do not play a role in clinical hypersensitivity as they are unable to cross-link IgE effectively (91, 92). Most current tests use whole venom preparations, but the use of recombinant venom allergens is now thought to increase the sensitivity and specificity of the tests, providing a more robust tool in the diagnosis of wasp or bee venom allergy.

Treatment of an allergic reaction following insect sting varies according to the severity of the reaction. Localised reactions are treated with anti-histamines or topical steroids, with analgesics to mediate pain (93). Oral corticosteroids may be prescribed for large localised reactions. Self-injectable adrenaline is given to patients who have previously experienced severe reactions or have active asthma (93). Other risk factors mentioned earlier are also taken into consideration (82). Patients experiencing symptoms of airway obstruction and hypotension should administer 0.3mg for adults and 0.15mg for children of adrenaline intramuscularly. Adrenaline acts via α1 and β1 and β2-adrenergic receptors to reverse the effects of mediators of anaphylaxis. α1 adrenergic receptor mediated vasoconstriction reverses the vasodilation, alleviating hypotension, erythema, urticaria and angioedema (94). Interaction with β1-adrenergic receptors increases heart rate and stroke volume, resulting in greater myocardial output, while β2-adrenergic receptors mediate bronchodilation in the lungs, improving breathing, and suppresses the release of further mediators by mast cells and eosinophils (94).

1.2.2 Venom Immunotherapy

Allergen specific immunotherapy (SIT) is the only disease modifying treatment that desensitises the patient to an allergen. As well as increasing tolerance to allergen upon subsequent exposure (both early and late phase responses), SIT decreases the

risk of novel sensitisations to other allergens (95) and the development of asthma in allergic rhinitis patients (96). Both localised and systemic reactions are reduced and patients report a significantly improved quality of life following SIT (97). Meta-analysis studies demonstrate that venom immunotherapy is effective in the treatment of venom allergy and has a high success rate when compared to other SIT, such as grass pollen or food allergen SIT (98, 99). Follow-up studies of patients post venom immunotherapy (VIT) has also demonstrated long-term efficacy (100).

SIT involves repeated administration of the specific allergen subcutaneously, sublingually or epicutaneously, resulting in allergen desensitisation and induction of clinical tolerance. SIT treatment protocols are divided into two phases; induction and maintenance phase. The induction phase involves the administration of gradually increasing doses of allergen for 8-28 weeks, until the maintenance dose (usually 100µg for VIT) is achieved (101). The starting dose administered is between 1000-10,000 fold less than the maintenance dose, depending on the severity of previous reactions as well as other concurrent risk factors (101). The duration of SIT varies from 3 to 10 years and may vary between patients. Systemic reactions are a risk of SIT and are seen mostly in the induction phase and in VIT a greater risk of systemic reactions is seen with bee venom than wasp venom (102, 103).

Accelerated protocols of immunotherapy are now also available. Rush SIT uses a reduced induction phase of 4-7 days and ultrarush protocols achieve maintenance dose in 1-2 days. They are associated with an increased risk of local and systemic adverse reactions, although accelerated protocols of VIT seem to be relatively well tolerated (102, 104). To minimise the risk of adverse reactions, some studies have also evaluated the use of anti-IgE monoclonal antibody (Omalizumab) or anti-

histamines (105) or investigated prognostic markers, such as increased basophil allergen sensitivity by CD63 expression (103).

A number of contraindications for SIT have been suggested, including (106):

- Asthma
- Autoimmune disorders
- Malignant neoplasias
- β-blockers
- ACE inhibitors
- Monoamine oxidase inhibitors
- Cardiovascular disease
- Pregnancy
- Immunodeficiency
- Chronic infections or inflammatory disorders
- Immunosuppressive drugs

However, the evidence for contraindication is not always robust, and SIT may still be indicated, especially in patients with a very high risk of severe systemic reactions to future stings.

SIT is administered either subcutaneously or sublingually (SLIT), although subcutaneous administration is usually favoured. Oral administration of allergen has been effective in improving symptoms of a number of inhalant allergens, such as grass pollen in allergic rhinitis and house dust mite in asthma patients and is associated with fewer adverse events (107, 108). Subcutaneous administration is favoured for VIT as it more closely resembles natural insect stings. However, studies have demonstrated effective reduction in symptoms and adverse reaction following sublingual venom

immunotherapy (109, 110), which may be an alternative for those experiencing large localised reactions to subcutaneous SIT.

Our current understanding of the mechanisms by which SIT induces clinical tolerance suggests that SIT induces distinct immunological components, altering the phenotype of the immune response and leading to peripheral tolerance. Both allergic and non-allergic individuals respond to the same T cell epitopes of bee venom phospholipase A2 (PLA2), indicating that it is the quality or quantity of the immune response that differs between the two groups (111). It as yet unclear however which components are actually required and necessary for allergen desensitisation, in part due to differences in study protocols, allergens used, administration routes and outcome measures, which prevent comprehensive analysis. Common features identified include the modulation of T and B cells, mast cells and eosinophils and inflammatory mediators.

DCs are important APC in priming and activating Th2 cells in allergy. DCs are able to affect the quality of the T cell response by driving T cells towards a suppressor phenotype. While DCs generate a Th2 bias during allergy, SIT induces a switch towards a more regulatory phenotype. Tolerogenic DCs have been shown to produce IL-10 and are elevated after SIT (112). IL-10 expressing DCs can then induce the formation of T regulatory cells (113) which will contribute to desensitisation to the allergen.

Immunotherapy modulates the T cell response in several different ways. Firstly, it induces expansion of local and peripheral IL-10 producing $CD4^{+}CD25^{+}$ T regulatory cells (T regs) and T regulatory 1 cells (Tr1) (114, 115). These regulatory cells are allergen specific and can suppress effector cell function, inhibiting allergen-specific proliferation and cytokine production. This is mediated through the production of

suppressor cytokines such as IL-10 and transforming growth factor (TGF) β (116), or through cell-cell interaction of surface inhibitory molecules (117). It has been reported that T reg function is impaired in allergic individuals, but can be restored with SIT (118).

As well as increasing the regulatory component of the immune system, SIT also suppresses the Th2 response while enhancing the Th1 response. This is seen in a shift from IL-4 producing to IFN-γ producing $CD4^+$ and $CD8^+$ allergen-specific T cells (119). Following bee venom immunotherapy, stimulation of T cells with PLA2 induced production of Th1 and Th2 cytokines, although Th1 cytokines were not detected prior to immunotherapy and Th2 cytokine levels significantly decreased post immunotherapy, suggesting a switch from Th2 to Th1 bias (120). Decreased IL-4 and increase IL-10 mRNA was also reported in cutaneous biopsies following SIT (121).

Effector cells, such as mast cells, eosinophils and basophils are also affected by SIT. Mast cell numbers were significantly reduced in the skin post immunotherapy and a decrease in tryptase levels was also reported, suggesting impaired function (122, 123). SIT is also associated with reduced basophil numbers and decreased IgE-mediated histamine release (124, 125). A significant decrease in basophil activation markers (CD63, CD203c) and Th2 cytokine production was also observed (126). Levels of inflammatory mediators, such as leukotriene C4, released by effector cells on degranulation are reduced after immunotherapy (127). Chemokine and cytokine levels, such as IL-8, MCP-1 and RANTES, are also reduced, decreasing the recruitment and activation of inflammatory cells (128). The decrease in number and activation threshold of effector cells plays an important role in the long-term clinical effectiveness of SIT and reduction of subsequent allergic reactions.

The effect of SIT on B cells and antibody production was demonstrated by the discovery that serum factors able to inhibit allergic skin reactions were in fact IgG (129). While levels of allergen-specific IgE do not necessarily change, IgA, IgG1 and IgG4 levels are increased following immunotherapy (99). IgG4 antibodies in particular seem to play a key role as removing them restores allergen-induced T cell activation and proliferation (112). Allergen-specific IgG4 captures allergen, competing with FcεRI-bound IgE, and so preventing IgE cross-linking. It has also been shown to inhibit facilitated antigen presentation, reducing the late phase response, and decrease basophil and mast cell allergen-specific degranulation (130, 131). However the correlation of IgG4 levels with clinical effectiveness differs between studies. IgG4 functional assays, such as the inhibition of IgE-allergen interactions, correlated well with clinical effectiveness of grass pollen immunotherapy, suggesting that IgG4 function rather than levels can be used as a marker of clinical response (132).

The suppressor cytokines IL-10 and TGF-β play an important role in inducing allergen tolerance. Indeed, blocking IL-10 reverses the inhibitory effect and an in increase in effector cell proliferation and activation is seen (133). Levels of both cytokines at the mRNA and protein level, are increased in the skin and blood of patients following immunotherapy, with IL-10 detected as early as Day 2 (112, 116, 133). IL-10 has a number of direct and indirect effects on effector cells. While not directly responsible for IgG4 class-switching, IL-10 prevents IL-4 induced IgE class switching and directly stimulates IgG1 class-switching (134). Co-stimulatory molecules and MHC molecules are downregulated on APC, preventing effective T cell activation (135).

TGF-β also has a variety of effects of a number of different cells contributing to desensitisation. FcεRI levels were reduced on LCs in the skin by TGF-β (136). Transcription factors and inhibitory molecules associated with regulatory T cells, such

as FOXP3, RUNX and CTLA-4, are upregulated by TGF-β, increasing the number and phenotype of suppressor T cells (137). In B cells TGF-β induces class-switching to IgA, suggesting a role for this isotype in clinical effectiveness post-immunotherapy (116).

Analysis of gene expression of PBMCs pre-, during and post-immunotherapy revealed changes in a number of genes associated with cell differentiation, cell-cell interactions, signalling and signal transduction, transcription and ion transport (138, 139). This further indicates that the variety of cells and soluble factors modulate the immune response during immunotherapy via different mechanisms, in order to suppress, regulate, render anergic or delete effector cells.

1.3 Skin

The skin is a large and complex organ that plays a crucial role in the host defence against the environment and microorganisms. It provides a mechanical barrier, preventing water loss and entry of potentially harmful factors (140). As one of the first lines of defence against pathogens, the skin also acts as an active barrier with an extensive immune network (140). Figure 1-2 shows the structure of the skin and the different cellular components in the different layers. Immune homeostasis in the skin requires interaction of components of the immune system, structural components, such as keratinocytes, fibroblasts and the extracellular matrix, and microorganisms that colonise the surface and outer layers of the skin. These interactions are highly regulated and so dysregulation can result in inflammation of the skin.

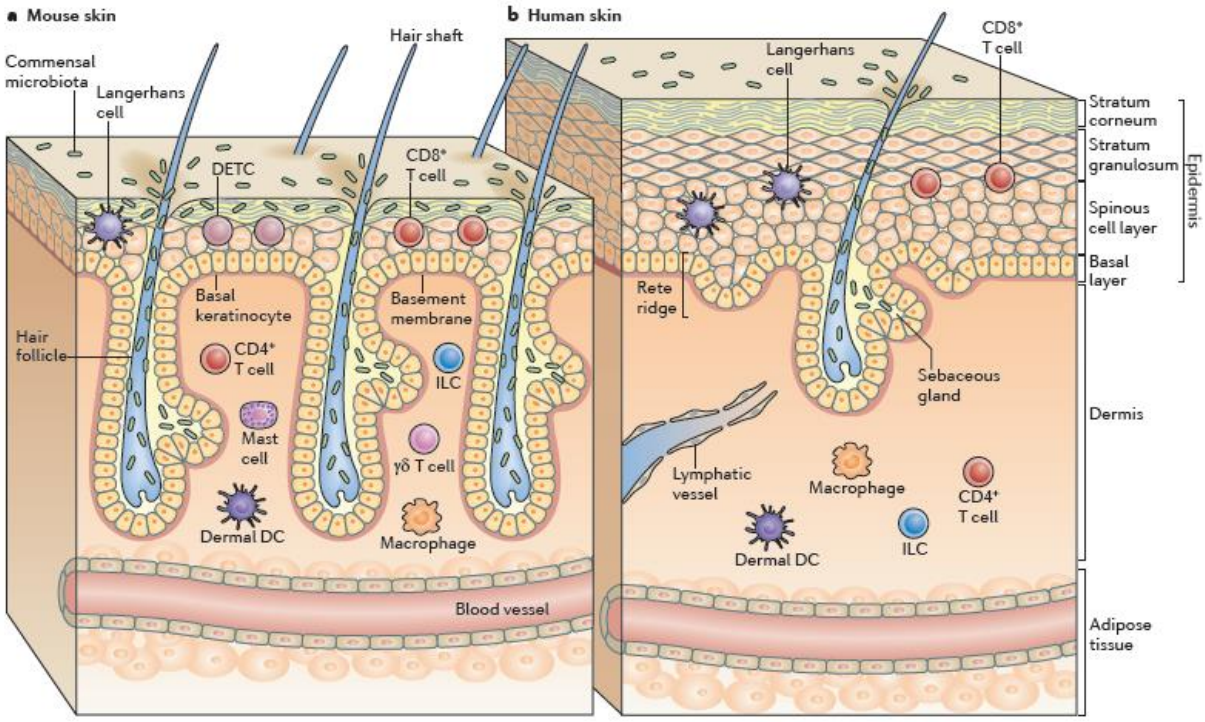


Figure 1-2: Structure and cellular components of the skin in mice and humans *(140)*.

The dermis is formed of two main layers rich in fibroblasts and fibrocytes, with a lower reticular layer and upper papillary layer that produce elastin and collagen fibres arranged in parallel to the skin surface and decrease in thickness down the dermis (141). The dermis also contains blood and lymphatic vessels, hair follicles, allowing the trafficking of leucocytes to and from the skin, and sebaceous and eccrine glands and nerves. The adipose tissue below the dermis contains larger blood vessels and nerves and connects the skin via connective tissue to underlying muscle and bone (141).

The top layer of the skin, the epidermis, is composed predominantly of keratinocytes and is divided into four layers (141). The stratum basale, the bottom layer above the basement membrane, is a layer of melanocytes and keratinocytes and undifferentiated stem cells that constantly divide and renew the epidermis. The newly generated cells

move to the stratum spinosum, pushing existing cells further up, and differentiate, changing shape and producing keratin (140). In the stratum granulosum, the keratinocytes lose their nuclei and actively produce large amounts of late differentiation keratins and lipids, which provide the structure and water-proof nature of the skin. The stratum corneum is composed of corneocytes which are terminally differentiated anucleate keratinocytes that form a flattened network of cells, providing a framework for intercellular lipid lamellae (142).

1.3.1 Barrier Function of the Skin

Corneocytes in the stratum corneum are surrounded by a cornified envelope of structural proteins that are linked to intracellular bundles of keratin filaments providing strength. The envelope is attached to the intercellular lamella through the interaction of lipids, which forms the lipid envelope (143). The cornified protein and lipid envelopes and lamellar lipids form the epidermal permeability barrier (EPB) which provides protection from dehydration and impedes barrier permeability. Lipids and proteins in the lipid lamellae are delivered from lamellar granules found in keratinocytes of the granular layer (143). Lamellar granules contain stacks of lipid lamellae which are released when the granules fuse with the cell membrane during keratinocytes differentiation into corneocytes. Formation of the EPB requires cross-linking of envelope proteins with extracellular lipid polymers and tight packing of corneocytes through interactions of desmosomes (143).

The intercellular lamellae form a multilamellar intact matrix of the stratum corneum, rich in ceramides, as well as cholesterol, free fatty acids and cholesterol sulphate, with unsaturated acyl chains allowing tight and ordered packing of corneocytes with less fluidity (143). These lipids are important in minimising transepidermal water loss (TEWL) as their removal with solvents is associated with barrier dysfunction (144).

Corneocytes are continuously shed from the surface of the stratum corneum in a process called desquamation, which involves cell renewal at the basal layer of the epidermis and shedding at the surface. Desquamation requires breakdown of cellular interactions while still maintaining protection from harmful environmental factors. The extracellular matrix contains numerous hydrolytic proteins involved in the breakdown of extracellular desmosome adhesion proteins, including serine proteases, such as kallikrein-related proteases KLK5, KLK7 and KLK14, and cysteine proteases, such as cathepsin L2 (143). Regulation of these enzymes is critical to desquamation while maintaining barrier integrity, which is achieved through protease inhibitors and production of inactive precursors of the proteases (145). Some protease inhibitors, such as cystatin protease inhibitors A and M/E and lymphoepithelial Kazal-type 5 serine protease inhibitor (LEKTI) also inhibit exogenous enzyme (143, 146, 147).

Tissue injury is associated with increased protease activity, mRNA and activity of enzymes involved in lipid synthesis and epidermal proliferation (143). Detecting breaches in the skin and breakdown of barrier function is crucial to initiate repair or healing and an immune response to infiltrating pathogens or allergens, and the skin has effective mechanisms to rapidly detect and respond to any changes. Acute barrier disruption causes increased TEWL and is associated with changes to stimulate barrier repair (143).

The skin has a pH gradient across the stratum corneum of decreasing pH from the inner to outer layers, with an average pH of pH5.4-5.9 (143). This acidic environment is maintained by endogenous factors, such as urocanic acid production by deamination of histidine, free fatty acids generated by secretary phospholipase A2 (sPLA2) hydrolysis of phospholipids, and a non-energy dependent sodium-protein

exchanger (148). Exogenous sources, including microbial metabolites are also thought to contribute to the acidic environment.

The acidic environment is important in maintaining permeability barrier function of the skin as well as stratum corneum integrity and cohesion. Exposing the stratum corneum to neutral pH or blocking acidification (sPLA2 or non-energy dependent sodium-proton exchanger) is associated with a decrease in β-glucocerebrosidase activity but can be recovered by incubation in acidic buffer (149). This is an enzyme with low optimum pH involved in lipid processing, resulting in defective lipid organisation and metabolism and so increased TEWL. Exposure to neutral pH also results in rapid activation of serine proteases leading to desmosome degradation and disruption of cell-cell interactions (150). Many serine proteases, such as KLK5 and KLK7, show optimum activity at neutral pH (151), while other protease inhibitors, such as LEKTI, show optimum activity at acidic pH (147), leading to altered desmosome protein degradation (152).

Regulation of pH is therefore very important and changes are seen in tissue injury. Tape stripping experiments of mice skin resulted in increased pH and serine protease activity (143, 149, 150). These serine proteases also trigger protease activated receptor 2 (PAR2; G-protein coupled receptor) signalling cascades, leading to an innate inflammatory response, inhibition of lamellar granule secretion and promotion of terminal differentiation of keratinocytes (153). This enhances cornification and recruits inflammatory mediators to initiate tissue repair restoring the barrier.

The epidermis also has a calcium gradient, with low levels in the basal layer which increases progressively towards the outer stratum granulosum and declines again in the stratum corneum (154). Calcium levels regulate many of the proteins of the

cornified envelope (filaggrin, involucrin, loricrin) at the transcriptional level, and so regulate the different stages of keratinocyte differentiation (155, 156). Low calcium levels due to acute barrier disruption by tape-stripping and the use of organic solvents or detergents is associated with formation and secretion of lamellar granules, resulting in the release of their lipid contents, as well as inhibition of terminal differentiation towards the outer surface while stimulating proliferation of keratinocytes in the basal layer (154, 157). Replacing the cells and intercellular lipid lamellae of the epidermal barrier will contribute to barrier repair. Interestingly, changes in calcium gradient and pH are associated opposing effects on keratinocyte terminal differentiation. It has been suggested that this may allow the rapid transition of newly generated keratinocyte through the epidermis to the stratum corneum, and through the differentiation pathway, allowing regeneration of the epidermal barrier (143).

The importance of the barrier function of skin is highlighted by the association of skin inflammatory disease with barrier dysfunction. Atopic dermatitis (AD) is an inflammatory skin disorder, characterised by defective epidermal barrier function (143). Corneocytes in AD skin were shown to be smaller compared to normal skin, which affects skin permeability and barrier integrity (158). AD is also strongly associated with loss of function mutation is the pro-filaggrin gene (159). Pro-filaggrin is cleaved into filaggrin, a polypeptide found in the cornified epithelium of corneocytes, is important in the aggregation of keratin filaments into bundles, promoting the collapse and flattened shape of the cells (160). Breakdown products of filaggrin are found in the extracellular matrix and are natural moisturising factors, preventing corneocyte water loss and having anti-microbial effects (161). *FLG* mutations result in impaired cornified envelope and increased water loss (159).

Defects in adhesion proteins involved in the assembly of desmosomes are also associated with inflammatory skin condition. Mice lacking DSG3 or desmocollin-1 have fragile skin (143) and decreased levels of claudin-1 were reduced in AD patients compared to controls (162). AD patients also have increased expression of serine proteases KLKs. A 4bp addition in KLK7 gene increases the half-life of the mRNA, leading to increased transcription, enzyme expression and breakdown of adhesion proteins (163), and KLK7 transgenic mice also develop AD-like lesions (164). Mutations in genes encoding protease inhibitors, such as cystatin A, are also associated with AD, due to decreased mRNA stability and reduced levels of the inhibitor in the epidermis (165, 166). This will lead to increased endogenous and exogenous protease activity. Defects in adhesion protein expression or breakdown and malformation of desmosomes affects corneocyte cohesion, so that they are unable to preserve barrier integrity.

Defects in the epidermal permeability barrier, due to smaller corneocytes, degradation or malformation of desmosomes, changes in pH and calcium levels and lipid content, leaves the skin exposed to penetrating environmental factors, such as bacteria and allergens. Some of these can cause further deterioration of barrier integrity. Der p1, a major house dust mite allergen, is a cysteine protease that has been shown to cause desmosome degradation and activate PAR2 signalling (167). Although this causes breakdown of the barrier, inflammatory skin disorders are only seen in genetically susceptible individuals, such as those with *FLG* mutations (143). *Staphylococcus aureus* colonisation is relatively common in AD skin, and as well as stimulating an immune response, this interferes with lipid formation and desmosome formation (143, 168). Therefore infiltrating allergens and bacteria contribute to the breakdown of barrier integrity seen in inflammatory skin conditions.

1.3.2 Immune system of the skin

The skin has an extensive immune network with components of both the innate and adaptive immune systems. The various cells reside in distinct regions of the skin, although it is by no means a static tissue and cells traffic continuously in and out, particularly during inflammatory conditions.

Antigen Presenting Cells

As a one of our major interfaces with the environment, the skin provides the first line of immunological defence, encountering microorganisms first, and so has a number of different APCs to sample the environment and initiate an immune response. Both skin-resident and bone-marrow derived migratory DC populations have been identified, although under inflammatory conditions, circulatory DCs were shown to re-populate many skin-resident populations (140). Several myeloid DC subsets have now been identified, based on surface marker expression, in the circulation, lymph nodes and skin-resident (primarily in the dermis) (Figure 1-3). While $CD14^{-}CD141^{hi}CD11c^{lo}$ DCs are potent activators, $CD14^{+}CD1a^{-}$ DCs are weak stimulators but were shown to initiate B cell responses, and $CD14^{-}CD1a^{+}CD1c^{+}$ DCs produce regulatory IL-10 (140, 169, 170). Although there is some overlap or redundancy between subsets, this suggests that the subsets have different properties and roles in tissue homeostasis or skin inflammation. Indeed, different subsets of DCs are elevated in patients with AD or psoriasis, two different inflammatory skin conditions (171). Plasmacytoid DCs are not detected in the skin in the steady state but migrate rapidly during skin inflammation (172).

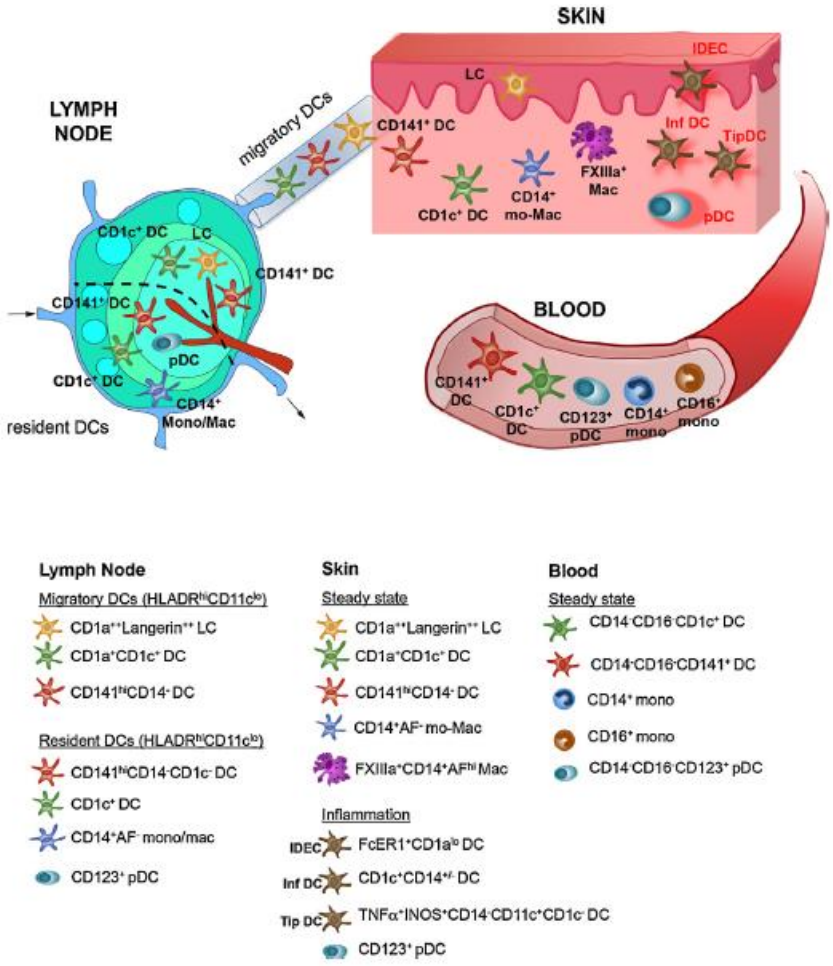


Figure 1-3: Distribution of human dendritic cells, monocytes and macrophages in skin, blood and lymph nodes (140).
Changes during inflammation are indicated in red text.

Skin-resident macrophages are found deeper in the dermis than DCs and in the perivascular areas (173). As with other APCs, they are important in surveying the skin, detecting pathogens and providing an early immune response. Macrophage subsets, M1 and M2 are both found in the skin and dysregulation has been associated with skin inflammation. Pro-inflammatory M1 cells are increased in both psoriatic and atopic dermatitis skin, suggesting that while M1 cells are involved in skin inflammation, they may play a broad role (174, 175). M2 cells on the other hand, have a regulatory phenotype, producing IL-10 and TGF-β, and were shown to play a role in the resolution of skin inflammation (176). M2 cells also promote immune suppression by removing

antigen from the skin, preventing presentation to T cells. They show increased phagocytosis and promote angiogenesis by secreting VEGF-like factors, so that antigen is degraded or trafficked away from the skin (177). Langerhans cells are discussed in more detail below.

Mast cells

Mast cells contain pre-formed granules containing inflammatory mediators and so are able to provide immediate response to challenge in the skin. In addition they produce de-novo synthesised mediators such as prostaglandins, and are a source of phospholipases such as PLA2G3. As with many other cell types in the skin, mast cells can have both pro- and anti-inflammatory effects. Mast cell granules contain many mediators of inflammation, pro-inflammatory cytokines and chemokines to recruit leucocytes and further potentiate inflammation. They have been shown to induce M1 cells, recruit NK and NKT cells in Dengue fever and enhance IgE and IL-4 production in atopic dermatitis in the skin (140, 178).

Innate Lymphoid Cells

ILCs are CD45+ haematopoietic cells derived from a common lymphoid progenitor but lack rearranged antigen specific receptors (179). As with T cells, ILCs are divided into three groups based on transcription factor, surface marker and cytokine expression and are thought to have different effector functions (180). All three ILC groups are detected in the skin. AD is an inflammatory skin disease associated with a type II immune response and increased numbers of ILC2s were detected in the skin of AD patients compared to healthy controls, and they produce abundant Th2 cytokines IL5 and IL13 when stimulated with combinations of IL-25, IL-33 and TSLP (181). ILC3 are known to produce IL-17 on stimulation, and as such have been implicated in the

pathogenesis of psoriasis, an IL-17 associated skin inflammatory condition (182). ILCs are in the ideal location to detect insults to the skin and serve as the bridge between the innate and adaptive arms of the immune system.

T cells

There are a large number of T cells resident in the skin in the steady-state, nearly twice as many as in the circulation (183). During infection or inflammation, circulating effector T cell infiltrate the dermis, driven by cytokines and chemokines. Once the infection has been resolved, T cell infiltration declines as chemokine levels and expression of skin homing markers decrease (140). In the circulation, the memory T cell response generated includes central memory T cells (Tcm), that patrol lymphoid organs and migrate to tissues upon antigen encounter, and effector memory T cells (Tem), that do not express lymph node homing markers (CD62L or chemokine (C-C motif) receptor (CCR)7) but patrol the blood and non-lymphoid tissue to provide rapid effector function (184). More recently, $C103^{+}CD8^{+}$ tissue-resident T cells (Trm) were identified as T cells that were found in tissue a substantial amount of time after antigen clearance (185). Trm are long-lived cells that are derived from KLRG1- precursors that enter the skin during the effector phase of the immune response (185). The development and localisation of Trm in the skin is governed by a number of factors, including chemokines (CXCL9 and CXCL10), cytokines (TGF-β and IL-15), surface markers (CD69 and CD103) and infiltration of the epidermis (185). Trm provide rapid immune response on subsequent exposure to antigen and can also be found throughout non-involved skin tissue (186). Removing Tcm demonstrated that Trm are able to provide better long-term protection against infection than Tcm (186). Tcm are also important however and T cell migration into the skin following antigen challenge was impaired in older skin, contributing to infection in the skin during aging (187).

Two distinct populations of γδ T cells have been identified in the skin of normal mice. In the epidermis, these T cells are Vγ5Vδ1+ with invariant chain TCR (also known as dendritic epidermal T cells; DETC) and are derived from thymic precursors that migrate to the skin from the thymus and are maintained by limited *in situ* proliferation (188). The dermal γδ T cell population is enriched with Vγ4Vδ4+ T cells, which unlike Vγ5Vδ1+ T cells in the epidermis, express a skin homing marker CCR6, suggesting they were recruited previously during an inflammatory event (189). Indeed, compared to epidermal γδ T cells, dermal γδ T cells express markers of activation and are highly motile. Expansion of γδ T cells is driven by IL-23 and induces IL-17A production (189). The IL-17 axis is thought to play a key role in the induction of psoriasis, and it has been shown that dermal γδ T cells are important producers of pathogenic IL-17, as TCRδ knockout mice were resistant to TLR-7 agonist induced psoriasis (190).

The human counterpart of Vγ4δ4+ T cells in the mouse were identified as Vγ9δ2+ dermal T cells. Elevated numbers of these cells were detected in skin biopsies from psoriatic patients while decreased levels were seen in the blood (191). The expression of CLA and CCR6 on cells in the blood suggest that they traffic into the skin under inflammatory conditions (192). As in mice, dermal γδ T cells in humans are also driven by IL-23 to produce IL-17 (192). γδ T cells have also been implicated in the immune response to infection such as mycobacterial and staphylococcal infection, by recruiting immune cells and driving the subsequent cell-mediated immune response (140). During mycobacterial infection, TCRδ knockout mice showed reduced neutrophil skin infiltration and reduced bacteria in draining lymph nodes (193).

B cells

The phenotype and function of skin associated B cells are not as well characterised as other immune cells in the skin. A recent study demonstrated tissue-resident B cells as well as migratory B cells trafficking through uninflamed skin, but with accumulation in inflamed skin, driven by CCR6-chemokine ligand (CCL)20 interactions (194). Skin B cells were distinct from lymph node B cells, expressing higher levels of MHC class II, CD1 and CD80/86. Together with the high expression of IgM, which is associated with a more innate-like phenotype, this suggests a role for skin B cells in antigen processing and presentation to T cells at the site of inflammation (194). An increase in antibody titres in the skin was also detected during inflammation, which may be important in rapid resolution of local inflammation (194).

1.3.3 Langerhans cells

LCs are migratory tissue DCs, localised in stratified squamous epithelium, including the basal and suprabasal layer of the epidermis of the skin, and respiratory, digestive and urogentital epithelia. LC are distinct from other DC subsets in that they express significantly higher levels of CD1a and a C type lectin, Langerin (CD207), as well as FcεRI, DEC-205, E-cadherin and CD36 (195). Ten years post-tissue allograft, LCs in the epidermis were still recipient-derived cells with mitotic capacity, suggesting that LCs self-renew in the epidermis throughout life (196).

Langerin is internalised by receptor-mediated endocytosis from the cell surface into an LC-specific organelle of the endosomal recycling pathway, called Birbeck granules (195). While the exact function of this organelle remains unclear, it is thought to be involved in receptor-mediated endocytosis and antigen processing and presentation. Interestingly, co-expression of CD1a with Langerin has been demonstrated in Birbeck

granules in Langerhans cell histiocytosis (197) which could indicate a role for Langerin in lipid antigen presentation by CD1a. Using anti-langerin antibodies before or after pulsing with *M. leprae* sonicates, *Hunger et al* showed that LCs present lipid antigens to T cells in a CD1a-restricted and Langerin dependent manner (198). Studies have demonstrated an important role for Langerin in the biogenesis of Birbeck granules, as expression of Langerin cDNA in fibroblasts led to the formation of Birbeck granules, while blocking endocytosis results in their breakdown (199, 200). Although LCs are thought to largely be self-renewing within the skin, they can be re-populated by circulating monocytes under inflammatory conditions (201, 202). Re-population of LCs after UV treatment was shown to occur in two waves. Gr-1^{hi} monocytes are the first to enter the skin and differentiate into LCs in an Id2 independent manner (201). However, these cells are short-lived. A second wave of Id2 dependent repopulation by non-monocytic, bone marrow-derived cells provide long-term LCs. The short-term LCs are induced during inflammatory conditions, while long-term LCs are generated in the steady-state (201).

In the steady-state, immature LCs form a dense network throughout the epidermis where they are in an ideal position to detect foreign stimuli, by scanning the environment with their extended dendrites (203). When they encounter antigen, under inflammatory conditions, activated LCs process the antigen and migrate by decreasing E-cadherin and increasing CCR7 expression, to draining lymph nodes (DLN) where they interact with T-cells (204). During migration, they acquire a mature phenotype with increased expression of MHC and co-stimulatory molecules CD86 and CD40, and cytokine IL-12p40/70 production, stimulating IFN-γ production from T cells (205). Skin-derived DCs were also shown to cross-present viral and self-antigens to induce a potent $CD8^+$ T cell response (206).

However, a number of studies have now suggested that skin-derived DCs, including LCs are not able to effectively present antigens to $CD8^+$ T cells and rather suggest a role in antigen transport to DCs in the lymph node. Herpes simplex virus (HSV) -1 and self-antigen cross-presentation by LCs did not prime naïve $CD8^+$ T cells (207) and mouse HSV-2 in vagina epithelia was unable to trigger a protective $CD4^+$ Th1 response (208). These and other experiments suggest a tolerogenic role for LCs in some circumstances (see below).

Ultraviolet radiation (UVR) decreases LC numbers in the epidermis and prevent effective T cell priming. DNA-damaged intact LC lymph node migration was shown to be required to induce T regs and effector T cell anergy (209). UVR treatment stimulates LC IL-10 production via RANK-L signalling, which increases T reg activity promoting further IL-10 production through $CD4^+$ T (210) cell-LC interaction (211). TGF-β is well-characterised as an immunosuppressive cytokine. It is required for LC development, as there is an absence of LCs in *Tgfb1* knockout mice or downstream signalling molecules such as Runx3 (212). TGF-β was shown to promote LC antigen presentation and co-stimulation and is important in the steady-state for self-antigen transport to draining lymph nodes (DLN) (213). LC may also exert a tolerogenic effect through the stimulation of regulatory T cells or γδ T cells in the skin (214, 215).

Mouse models of LC depletion however, have provided conflicting results, as three different groups generated such models using different methods (216). Constitutive deletion of epidermal LCs from birth, using mice expressing diphtheria toxin under the control of human Langerin gene locus (langerin-DTA), resulted in increased CHS as measured by ear swelling following oxalazone administration (217). This suggests a regulatory role for LCs in inducing peripheral tolerance.

Two different groups generated an inducible model of LC depletion by generating mice that express human diphtheria toxin receptor (DTR) together with mouse Langerin (Langerin-DTR) (218, 219). Treatment with diphtheria toxin results in ablation of all Langerin expressing cells. One group found no difference while the other found diminished but not absent ear swelling. The differences between the two langerin-DTR models may be due to the time of administration of the allergen, as langerin+ dermal cells recover sooner than LCs and may affect the response. A key difference between the langerin-DTR and langerin-DTA models is that the former deletes all langerin+ cells while the latter specifically deletes LCs, which may explain the discrepancy in CHS response (216). More recently, an LC-specific langerin-DTR model has been developed, and these mice do experience enhanced CHS reactions, suggesting that LC are not involved in generating peripheral tolerance from birth, but have a suppressive effect at the time of immunisation, although this may depend on antigen type (220).

The dual function of LCs in the steady-state and under inflammatory conditions is still ambiguous, due to the complexities of the various different DC subset of the skin, and due to the sensitivity of mouse models to small variations in technical administration. It is clear though that LCs play a role in maintaining tissue homeostasis and can have a tolerogenic role in the steady-state, driven by interactions with commensals, but are still able to produce an effective immune response in inflammatory conditions.

1.3.4 T helper 22 cells

T helper 22 (Th22) cells are a recently defined T cell subset, driven by TNF-β, IL-6 and dermal DCs and characterised by the secretion of IL-22 in the absence of IL-17, which is seen with Th17 cells (221). IL-22 is an IL-10 family cytokine that signals via a heterodimeric receptor composed of the IL-10R α-chain and IL-22R chain, which is

expressed on non-immune cells. Therefore IL-22 acts primarily within local non-lymphoid tissue (221). Th22 cells may have both a protective and harmful effect in the skin. They stimulate the release of antimicrobial peptides (S100A7), chemokines (chemokine (C-X-C motif) ligand (CXC)9, CXCL10, CXCL11), cytokines (IL-7, IL-15 and IL-32), complement factors (C1s, C1r) by keratinocytes (222). Secretion of multiple immune mediators contributes to the innate defence and was shown to provide protection against Gram-negative bacteria (223) and during acute liver inflammation (224).

Th22 cells are also associated with epithelial repair by inducing keratinocyte proliferation while inhibiting their differentiation, promoting tissue remodelling and allowing rapid wound healing after injury (222). However, this increased proliferation and downregulation of genes associated with terminal differentiation was shown to contribute epithelial hyperplasia seen in psoriasis (225). Increased numbers of Th22 and elevated levels of IL-22 and CCL27 (chemokine for CCR10, expressed on Th22) were described in the skin of AD patients, with levels of Th22 corresponding to disease severity (226, 227). A common feature of AD skin is the colonisation by bacteria such as *S. aureus*, which induce a strong response by Th22 cells, suggesting that they may contribute to skin inflammation in AD (228).

IL-22 also acts synergistically with other cytokines in the induction of pro-inflammatory immune response. Th22 also produce TNFα, which stimulates chemokine, cytokine and complement production from keratinocytes (222). *In vitro* keratinocyte culture with a combination of recombinant IL-22 and TNFα synergistically enhanced keratinocyte stimulation in the presence of both cytokines (222). Similar pro-inflammatory synergistic effects were seen with IL-22 and IL-17 in human bronchial epithelial cells (229). Th22 cells and IL-22 have a dual role in the immune response, providing effector

immune responses in concert with other cytokines in inflammatory conditions and a protective or regenerative role in steady-state conditions.

1.4 Phospholipase

Phospholipids are a class of lipids abundantly found in all living organisms, and consist of a headgroup, phosphoric acid and diacylglyceride (two fatty acid chains covalently bonded to glycerol), of varying chain lengths and saturation. Phospholipids have various functions, including structural roles as the primary component of biomembranes due to their zwitterionic properties, and physiologically active roles as ligands for receptors and substrates for enzymes. Phospholipases are enzymes that hydrolyse phospholipids and are categorized into four major groups based on the position at which they cleave phospholipids (Figure 1-4). Phospholipase A1 (PLA1) and phospholipase A2 (PLA2) cleave the carboxylic ester bonds of phospholipids at different positions, removing an acyl chain. Phospholipase C (PLC) and phospholipase D (PLD) are both phosphodiesterases that cleave phospholipids before and after the phosphate group respectively, modifying the polar headgroup.

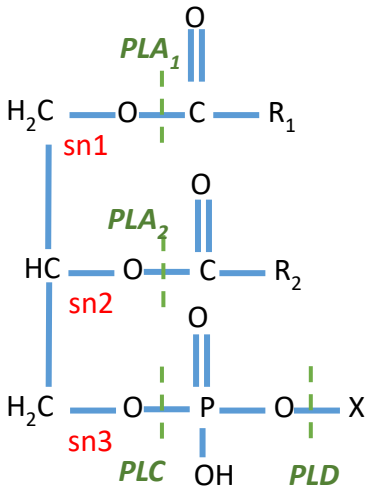


Figure 1-4: Cleavage sites of phospholipase groups.

PLA1 cleaves at the sn-1 position; PLA2 cleaves at the sn-2 position; PLB cleaves at sn-1 and sn-2; PLC cleaves before phosphate group; PLD cleave after phosphate group.

Each major phospholipase group is then subdivided into groups. There are now 16 PLA2 groups based on sequence homology (Table 1-3).

Type	Group	Subgroup
sPLA2	G1	A; B
	GII	A; B;C; D; E; F
	GIII	
	GV	
	GXI	A; B
	GXII	A; B
	GXIII	
	GIV	
cPLA2	GVI	A(α); B(β); C(γ); D(δ); E(ε); F(ζ)
iPLA2	GVII	A(β); B(γ); C(δ); D(ε); E(ζ); F(η)
PAF-AH	GVIII	A(Lp-PLA2); B (PAF-AH II)
Ly-PLA2	GXV	A (α1); B (α2); β
Ad-PLA2	GXVI	

Table 1-3: Phospholipase A2 superfamily *(230)*.

Groups I and II include PLA2 homologous to old and new world snake venom respectively, while insect venom-like PLA2 are in Group III. These three groups are all secretory PLA2 (sPLA2) (230). Group IV was the first cytosolic PLA2 (cPLA2) group identified, although others soon followed, including platelet activating factor-acetylhydrolases (PAF-AH) and lysosomal PLA2 (LyPLA2). Most PLA2 species are

calcium dependent but calcium independent PLA2s have also been identified (iPLA2; Group VI). The most recent PLA2 discovery, Group XVI, was found in adipose tissue (Ad-PLA2) (230). As new PLA2 species are discovered, new groups or subgroups are created depending on their similarity to existing PLA2. Expression of each of the different subgroups varies between species, tissues, cells and type of pathology, including inflammation. Each phospholipase can also act on a number of phospholipids, with a certain amount of overlap between them. PLA2 cleaves phospholipids at the *sn*-2 position releasing fatty acids (FA) and lysophospholipids (LPL) (Figure 1-5).

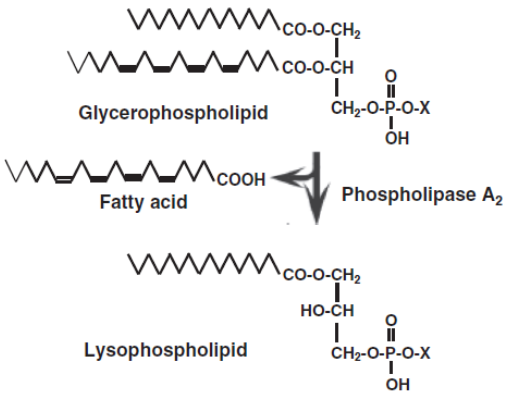


Figure 1-5: PLA2 activity on phospholipids .
PLA2 cleaves phospholipids at the sn-2 position to yield fatty acids and lysophospholipids *(231)*.

Phospholipases play a key role in many biological processes such as lipid metabolism, signal transduction and maintaining cell membranes. cPLA2 activity is regulated through calcium interaction with a calcium binding loop, resulting in enzyme localisation to the plasma membrane (230). Although calcium is not directly required for enzymatic activity, PLA2 interaction with phospholipid membrane is important. Enzymatic activity is greater if the phospholipid substrate forms an aggregate such as a bilayer rather than monomers as interfacial recognition and adsorption is crucial for

enzyme activity (232). cPLA2 also interact with lipid 2nd messengers phosphaticylinositol 4,5-bisphophate (PIP2) and ceramide-1-phophate (C1P) which enhance membrane penetration and binding time (230). Phosphorylation sites are also key to inducing the conformational changes required to allow interaction of the active sit in the correct orientation with the membrane (230).

cPLA2 are key regulators of eicosanoid biosynthesis pathway. cPLA2 cleavage of phospholipid releases fatty acids, including arachidonic acid (AA). AA is further catabolised via two different pathways leading to the generation of prostaglandins, thromboxanes, leukotrienes and other inflammatory lipid mediators (Figure 1-6), which have diverse effects (233). cPLA2 group IV deficient mice have impaired eicosanoid biosynthesis and are resistant to models of inflammatory disease such as collagen induced arthritis and experimental and autoimmune encephalitis (234, 235). LPL, the second substrate of PLA2 activity, also contribute to the inflammatory response acting via G-protein coupled receptors (GPCR) on immune cells to stimulate proliferation, differentiation, chemotaxis, antibody production and cytokine section (236).

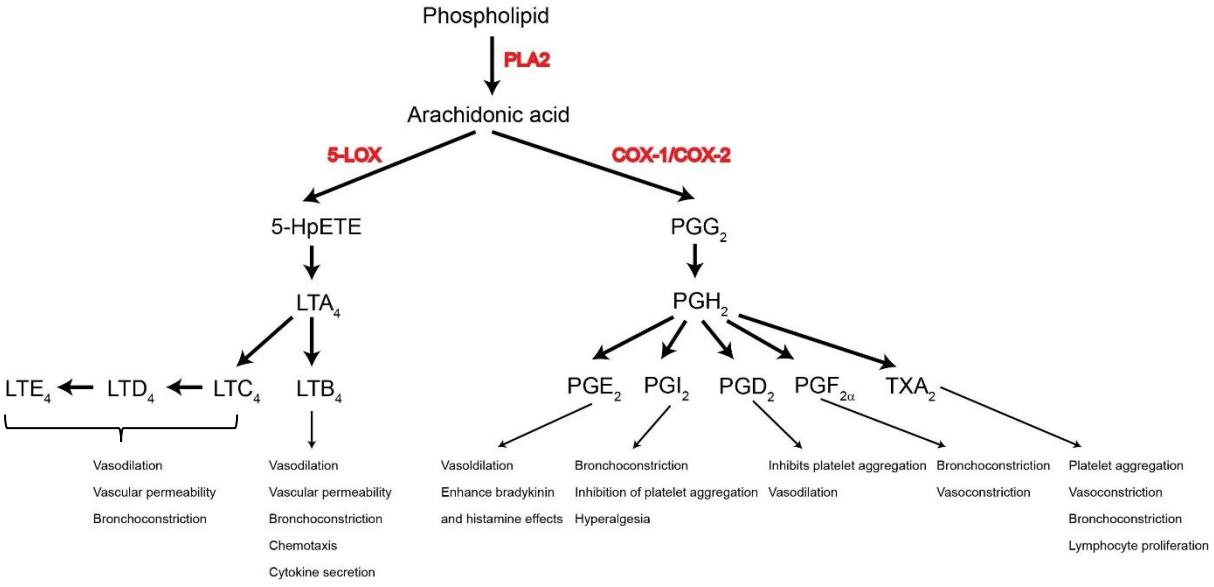


Figure 1-6: Eicosanoid biosynthesis pathway and effects (233).

1.4.1 Phospholipase and disease

FA and LPL products of PLA2 are found in lipid ligands eluted from CD1 molecules and CD1d molecules presenting LPL have been shown to activate NKT cells (48, 237). Blocking PLA2 in antigen-presenting cells inhibits NKT cell clone activation, indicating that PLA2 activity is a key source of lipid antigen of CD1d (237). Lysophospholipids have also been shown to act as adjuvants, which may be through the activation of NKT cells (237, 238). This suggests a role for PLA2 in CD1-mediated immune response. PLA2 can be activated by endogenous sources in response to stress and inflammation (cytokines, hormones, hypoxia) (239), as well as by exogenous sources. Therefore the immune response generated by CD1-restricted T-cells could be autoreactive or pathogenic.

sPLA2s have been implicated in the antimicrobial and inflammatory immune response. Although cPLA2s are thought to be the key initiators of the eicosanoid biosynthesis pathway, some sPLA2 groups are also able to contribute. sPLA2 group IIA is found at high levels in tears where it has an antimicrobial effect on both Gram negative and positive bacteria by acting on the cell membrane (230, 240). Group IV sPLA2 transgenic mice or exogenous administration was protective for *S. aureus* and *L. monocytogenes* infection (240). Other groups are able to prevent adenovirus infection of host cells through cleavage of host phospholipid, although the exact mechanisms are unknown (241). Bee venom PLA2 and Group X sPLA2 recognise and block HIV virus entry by steric inhibition of chemokine receptors (242, 243).

Elevated PLA2 level and activity has been detected in several inflammatory diseases, such as rheumatoid arthritis, Crohn’s disease and acute pancreatitis, as well as in fluid from patients with allergic rhinitis and asthma after antigen or histamine challenge (148, 230, 239). Greatest differences were seen during the late phase response and

correspond to increases in lysophospholipid and eicosanoid levels (244). As with other inflammatory disease, increased sPLA2 activity was detected in bronchoalveolar lavage (BAL) fluid of asthmatic patients following antigen challenge (245). This contributes to the increase of lipid mediators by generating AA through hydrolysis of phospholipids or upregulating expression and activity of other enzymes in the eicosanoid biosynthesis pathway, as well as cPLA2. Deficiency of group V and X sPLA2 was associated with a reduction in AHR and asthma (246, 247). However *Cpla2-/-* mice also showed no increase in bronchoconstriction or airway response to methacholine compared to *Cpla2*+/+, and Group V sPLA2 null mice exhibited an increased autoantibody driven arthritic response, implicating more than one PLA2 in disease (248, 249).

Pro-inflammatory stimuli, such as cytokines or LPS, induce sPLA2 expression from immune and non-immune cells, which have a number of different effects. Group IIA sPLA2 deficient mice developed fewer signs of arthritis in models of the disease, suggesting a pro-inflammatory role for the enzyme (249). Several immune cells, including mast cells, basophils, T-cells (particularly Th2) and eosinophils, express PLA2. PMA induced proliferation and IL-2 production by T cells was shown to be dependent on sPLA2 expression, showing that these enzymes regulate the differentiation and activation of T cells (250). PLA2 in turn can have effects on immune cells. Group III sPLA2 has been shown to promote dendritic cell maturation and so improve their capacity to stimulate T-cells (251). This effect required the catalytic site of the enzyme suggesting that it was mediated by the products of enzymatic activity, and resulted in upregulation of key transcription factors in immune regulation, such as NF-κB and NFAT (251, 252). Some PLA2s have also been shown to induce production of several cytokines and chemokine (148). These effects of PLA2 contribute in part to

inflammation and can be attributed to the actions of lipid mediators generated by the enzyme.

Mast cells, key mediators of allergic reactions, also produce PLA2. A recent study showed that group III sPLA2 is the main PLA2 in mast cells that are released by exocytosis on activation. *Pla2g3-/-* mice developed less ear swelling and mast cell degranulation when challenged with allergen compared to wild type, although mast cell numbers were unchanged, indicating a role for sPLA2G3 in mast cell maturation but not migration into tissues (253). Co-culture experiments with fibroblasts demonstrated that mast cell dependent sPLA2G3 initiated fibroblast PGD2 synthesis which was dependent on fibroblast L-PGDS. PGD2 binds and activates its receptor DP1 on mast cells, triggering mast cell maturation (253). Therefore sPLA2G3 contribute to inflammation and allergic disease by stimulating mast cell maturation and activation.

In the skin, PLA2 have a number of different roles as discussed earlier, including maintaining the permeability barrier, skin pH, wound healing and membrane remodelling. Increased levels of eicosanoids have been measured in inflammatory skin disease such as psoriasis and ACD and Group IID sPLA2 is also raised in the epidermis of psoriatic lesions (254). UVR exposure is also associated with elevated PGE2 levels as well as cPLA2 levels and activity, as measured by phosphorylation (255). Furthermore, group II sPLA2 transgenic mice develop inflammation in the skin, characterised by hyperplasia, hyperkeratosis and alopecia, with similar lesions to psoriatic skin (256). However, it is unclear if the enzymes have an active role in disease pathogenesis.

A major immunostimulatory allergen in bee venom is a group III PLA2. The human group III sPLA2 (PLA2G3) composed of unique C-terminal and N-terminal domains surrounding a central sPLA2 domain shows greatest homology to bee venom PLA2. PLA2G3 may play a role in atherosclerosis as transgenic mice expressing human PLA2G3 (*Pla2g3-Tg* mice) showed changes in plasma lipoprotein (257). This can be a risk factor for atherosclerosis and *Pla2g3*-Tg mice developed more severe atherosclerotic lesions when fed a high fat diet. These mice also developed inflammation with age, particularly in the skin (252). The dermatitis was characterised by acanthosis, hyperkeratosis, an influx of inflammatory cells and an increase of pro-inflammatory cytokines, slightly skewed towards a Th2 phenotype (252). Therefore PLA2G3 may be important in inflammatory responses in the skin, as increased levels of PLA2 products are associated with inflammatory skin disorders, such as atopic dermatitis and psoriasis. The homology of PLA2G3 with bee venom PLA2 suggests that they both work on similar substrates and produce similar products.

Corticosteroids are commonly used to treat inflammatory disorders, including allergic and skin conditions, although the exact mechanisms of immunosuppression are still unclear. Glucocorticoids were shown to suppress group II sPLA2 production by inhibiting mRNA synthesis or post-transcriptional expression (258). The associated reduction in eicosanoid biosynthesis and initiation of an inflammatory response may be a possible mechanism by which glucocorticoids reduce inflammation.

1.4.2 Phospholipase protein interactions

There are some effects of PLA2 that cannot be attributed to its enzymatic activity. Group I sPLA2 was shown to have antimicrobial effects independent of enzymatic activity. Some isoforms of PLA2 are known to induce mast cell degranulation, exocytosis of cells, including eosinophis and macrophages, and cytokine production

by macrophages, monocytes and mast cells (259). These effects appear to be independent of PLA2 enzymatic activity as they were not inhibited using catalytically inactive PLA2 (231). PLA2s are known to interact with cell surface receptors, including M-type receptor which belongs to the mannose receptor family (part of the C-type lectin superfamily) (259). M-type receptor was discovered on muscle cells but has now been identified throughout the body on various different tissues (260). Other members of this family, which are structurally similar, have also been shown to bind PLA2 and are involved in antigen internalisation and processing via clathrin-coated pits, as well as playing a role in both the innate and adaptive immune responses (261).

Activation of the receptor may be involved in the development of endotoxic shock, as receptor knockout mice survive longer following LPS administration and have reduced serum TNF-α and lL-1β (262). Binding of PLA2 to the receptor inhibits its enzymatic activity, and so M-type receptor may be important in the regulation of PLA2 activity by inhibiting enzymatic activity, as well as internalising and degrading the enzyme (259). Naturally occurring inhibitors in insects and mammals, such as lung surfactant protein SP-A, were shown to have similarities with M-type receptor in the PLA2-binding region (263). However, once internalised into the endocytic pathway, receptor-PLA2 uncoupling may allow the enzyme to function intracellularly, where they would encounter different, unexposed lipid species. M-type receptor has been studied in more depth recently due to the observation that reduced receptor expression is seen in many cancers. Studies using M-type receptor knockout mice and overexpression in cancer cell lines have shown that M-type receptor is important in cellular senescence, promoting apoptosis and blocking transformation (264). This effect is mediated by JAK2 signalling and generation of reactive oxidative species and could be a potential target for therapy (264).

A second receptor was discovered on brain membranes and so were called N-type receptor and have a neurotoxic effect. Interaction with PLA2 is calcium dependent (although only low levels) via the surface region surround the hydrophobic channel (259). Different PLA2 groups bind M-type and N-type receptor with different affinity and so have different functions. Bee venom PLA2 binds N-type but not M-type receptors, while group IB and IIA sPLA2 have low affinity for N-type receptors (259). Membrane receptors may also act to increase local enzyme concentration by sequestering PLA2 and leading to increased activity and lipid mediator release.

While PLA2 has been studied extensively in both insects and mammals, a lot less is known about PLA1. Phospholipid hydrolysis by PLA1 generates FA and LPL, similar to PLA2, and so PLA1 may also generate lipid mediators by initiating the eicosanoid biosynthesis pathway. PLA1 species have also been identified in humans, although in much fewer numbers than PLA2. These enzymes appear to be more substrate specific, as three PLA1s identified in the pancreas were specific for different lipid substrates (265). They may play a role in initiating an immune response by generating lipid mediators when exposed to substrate, which are sequestered under physiological conditions. Phosphatidylserine which is only exposed on cell surface of apoptotic cells and platelets, is cleaved by phosphatidylserine specific PLA1 to generate lysophosphatidylserine, a potent mast cell activator (265). Therefore, although there are no current receptors known for PLA1, they may have similar pro-inflammatory effects as PLA2 through catalytic activity.

2 Aims and Objectives

The capacity of T cells to recognise lipid antigens presented by CD1 molecules has been well-established for some time now. However our understanding of the physiological role of these cells during diseases is still limited and much of the data focuses on CD1d-restricted T cells. Most studies on Group I CD1-restricted T cells are based on clones, due to the lack of specific markers and expression in mice. Translational research of CD1a-restricted T cells will also require the study of polyclonal T cells.

Recent studies have demonstrated that polyclonal CD1a-restricted T cells are found in the skin and respond to skin-derived lipids, suggesting a role for these non-conventional T cells in the cutaneous immune response. In order to investigate this further, we chose to focus on an antigen that is delivered below the stratum corneum (and so is unaffected by variations in barrier integrity (266)), is associated with T cell infiltration, and is delivered into the skin at a defined site and time, with little variations in amount and penetration of antigen delivered between individuals. We therefore used wasp and bee venom as antigens and allergy as a measure of their function in disease.

We hypothesised that wasp and bee venom contain CD1a ligands and that CD1a-restricted T cells contribute to the local skin response and to allergic disease. This hypothesis was tested by investigating the following aims:

Chapter 4 Identify CD1a-restricted T-cells in blood and skin of healthy donors and investigate if they respond to wasp and bee venom.

Chapter 5 Investigate CD1a-restricted T-cells in wasp and bee venom allergy and during clinical tolerance induction.

Chapter 6 Characterise the phenotype and functions of PLA2 responsive CD1a-restricted T cells

3 Materials and Methods

3.1 Reagents

Sigma-Aldrich: *Vespula spp*. venom (V4879), *Apis mellifera* venom (V43375), Apis mellifera venom phospholipase A2 (V9279), Arachidonic acid (A3555), Mastoparan (M212), Fatty acid C16:1 (P9417), Fatty acid C18:1 (O1008).

Lipids from Avanti polar lipids: Phosphatidylcholine C14:0 (850345), C16:0 (850355), C18:0 (850365), C20:0 (850368), C14:1 (850346), C16:1 (850358), C18:1 (850375), C20:1 (850396), Lysophosphatidylcholine C16:1 (845885), Lysophosphatidylcholine C18:1 (845875). Lysophosphatidylethanolamine C16:1 (846715), Lysophosphatidyl-ethanolamine C18:1 (846725).

sPLA2G3 (Novus Biologicals; H00050487-001)

CD1a, CD1b and CD1c recombinant proteins: NIH tetramer facility and gift from Demin Li (Level 5, JR Hospital, Oxford)

Flow Cytometry Antibodies (dilutions):

Biolegend: Langerin APC (1:100); CLA PB (1:100); CD45RO PE-Cy7 (1:50); IL-17A APC (1:100); CD1d PE (1:50).

BD Biosciences: CD1a PE (1:50); CD3 PerCP (1:50); CD4 FITC (1:25); CD8 APC (1:100); CD56 PE (1:50); TCR Vβ panel (1:50).

Ebioscience: TCRα/β APC (1:100); IL-22 PE-Cy7 (1:100); CD1b APC (1:100); CD1c FITC (1:50);

Life Technologies: Live/Dead® Fixable violet dead cell stain (1:50)

3.2 PBMC isolation from blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation. Heparinised blood was diluted 1:1 with RPMI supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin and Hepes (Life Technologies, CA, USA) (RHepes) before layering over 15ml Lymphoprep (Nycomed, Roskilde, Denmark) and centrifuged for 20 minutes at 800g with the brake switched off. The buffy coat layer of PBMCs was removed and washed twice in RHepes at 450g.

3.3 Donor PBMC isolation

PBMCs were isolated from healthy adult donors (age range 25-50 years) with a previous history of bee or wasp sting, and from wasp and bee allergic individuals with a history of anaphylaxis under local ethics approval (CO2.291 and 09/H0606/71). T cells were isolated using CD3 MACs bead separation (Miltenyi Biotec, Germany) for *ex-vivo* assays. Or, PBMCs were expanded by culturing with feeder cells for 14-20 days (1000 patient PBMCs plus $0.2x10^6$ irradiated donor PBMCs (3000 rad) and $0.4x10^6$ transformed B-cell line per well of a round-bottom 96-well plate in RPMI supplemented with 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 5% human serum (Sigma-Aldrich, MO, USA), non-essential amino acids, HEPES, sodium pyruvate and 2-mercaptoethanol (Life Technologies, CA, USA) in the presence of 1nM IL-2 (PeproTech, NJ, USA) (T cell medium) and 50ng/ml anti-CD3 antibody (OKT3). After cell numbers had expanded, T cells were isolated using CD3 MACs beads (Miltenyi Biotec, Germany).

3.4 Monocyte-derived Dendritic (mDC) Cell culture

$CD14^+$ cells were isolated from PBMCs by MACS cell separation (Miltenyi Biotec Inc., San Diego, California, USA) according to the supplier's instructions. $CD14^+$ cells were cultured in 6-well plates at 0.2-0.5 x 10^6 cells/ml in complete medium in the presence of 50ng/ml GM-CSF (PeproTech, London, UK) and 1000U/ml IL-4 (PeproTech, London, UK). At day 3, half the media was removed and replaced with fresh media and cytokines at above concentrations. CD1a expression was checked by flow cytometry on day 5-6.

3.5 Langerhans-like Cell culture

In vitro LC-like cells: $CD14^+$ cells were isolated from human buffy coats from the NHS Blood Bank (John Radcliffe Hospital, Oxford, UK) by MACS cell separation (Miltenyi Biotec Inc., San Diego, California, USA) according to the supplier's instructions. $CD14^+$ cells were cultured in 6-well plates at 0.2-0.5 x 10^6 cells/ml in complete medium in the presence of GM-CSF (100ng/ml; PeproTech, London, UK), IL-4 (250ng/ml; PeproTech, London, UK) and TGF-β1 (10ng/ml; PeproTech, London, UK). At days 2 and 4, cultures were re-plated in the presence of the above cytokines to generate cells (267) which were at least 10-15% $CD1a^+CD207^+$ by flow cytometry. The in vitro LC-like cells were incubated with 10µg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32 and L243 respectively) for one hour before co-culture with T cells, to minimise HLA-restricted responses.

LCs: Human cord blood were obtained from CFAR Virology Core Laboratory at UCLA (Los Angeles, CA). $CD34^+$ human progenitor cells were isolated in Harvard using MACS cells separation (Miltenyi Biotec) according to suppliers' instructions. $CD34^+$ cell cultures were established in the presence of stem cell factor (25ng/ml; R&D

Systems), GM-CSF (200U/ml; Sanofi US Services, Inc.) and TGF-β1 (1ng/ml; R&D Systems) to increase CD1a expression (198). LC were harvested at day 12. The LC quality of the cells was checked by flow cytometry, and found to consist of 30-40% $CD1a^+CD207^+$ cells.

3.6 Cell isolation from skin

Skin samples from abdominoplasty and face lift surgeries were obtained from Nuffield Health, The Manor Hospital, Oxford, from patients with informed consent. Samples were cut up as finely as possible and incubated overnight at 37°C 5% CO_2 in 10µg/ml collagenase in R10. The following day, 2-3ml cold 10mM PBS/EDTA was added and clumps were broken up by repeated pipetting. The sample was then passed through a 70µm nylon strainer and washed through with 50ml PBS/EDTA, before passing through a 40µm nylon strainer. After washing for 20 minutes at 300g and 4°C, cells were resuspended in 25ml R0 and then layered over 15ml Lymphoprep (Nycomed, Roskilde, Denmark) and spun for 20 minutes at 2000RPM with the centrifuge brake switched off. The buffy coat layer of cells was removed and resuspended in RPMI supplemented with 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 10% foetal calf serum (FCS) (Sigma-Aldrich, MO, USA), non-essential amino acids, HEPES, sodium pyruvate and 2-mercaptoethanol (Life Technologies, CA, USA) (R10).

3.7 Isolation of $CD1a^+$ cells from skin

After removing subcutaneous fat, skin sections were cut into ~5mm-wide pieces and cultures in 2mg/ml dispase solution at 4°C overnight. The epidermis was then separated from the dermis and cultured in complete media for 5d. Migratory adherent cells were harvested and enriched by density gradient centrifugation, and $CD1a^+$ cells

were isolated using MACs cells separation (Miltenyi Biotec). The $CD1a^+$ cells were incubated with 10µg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32 and L243 respectively) for 1h before co-culture with T cells, to minimise HLA-restricted responses.

3.8 Skin blisters

Skin blisters were performed on healthy volunteers with informed consent at The Dermatology clinic at The Churchill Hospital, Oxford. 200mmHg negative pressure was applied to the skin using a 1cm diameter suction cup (Eschman VP25) to induce dermo-epidermal separation and blister formation. 24-48 hours later, blister fluid and cells were isolated by aspiration (268, 269). Cells were then stained and sorted by FACS.

3.9 *Ex-vivo* and Cultured ELISpots

T-cells were isolated from PBMCs by $CD3^+$ MACS cell separation according to supplier's instructions. For *ex-vivo* ELISpots, T-cells were left to rest for 3-5 days in T-cell medium. Cultured ELISpots were performed to enrich for antigen-specific CD1a-restricted cells. 2.5 x 10^6/ml T-cells were cultured in in 24-well plates at 2ml/well. Pulsed (1µg/ml antigen), irradiated (5000 rad) and washed (twice) 0.2 x 10^6 K562-CD1a were added per well. Cells were cultured at 37ºC 5% CO_2 for 10-14 days, re-plating as required. For both *ex-vivo* and cultured ELISpots, cells were washed twice in RHepes and returned to a clean well overnight in RPMI supplemented with 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 5% human serum (Sigma-Aldrich, MO, USA), non-essential amino acids, HEPES, sodium pyruvate and 2-mercaptoethanol (Life Technologies, CA, USA) (R5*) to rest prior to ELISpot on the following day.

96-well multiscreen IP-plates (Millipore Corp; Bedford, MA; USA) were coated with anti-IFN-γ, GM-CSF or IL-13 antibody (Mabtech AB, Nacka, Sweden) overnight at 4^{o}C. The plates were washed 6 times with RPMI-1640 and blocked for 1hr at 37^{o}C with RPMI-1640 supplemented with 2mM L-glutamine, 100IU/ml penicillin and 100µg/ml strep and 10% human serum (R10*). The plates were washed again before unpulsed or antigen-pulsed (24h) and washed antigen presenting cells, were added in 100µl R5*, followed by T-cells in 100µl R5*. T-cells were added in the absence of APC as negative control and with 20ng/ml *Phorbol* 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) and 10ng/ml Ionomycin (Sigma-Aldrich, St Louis, MO, USA) as a positive control. Plates were incubated overnight at 37^{o}C at 5% CO_2 overnight. The plates were then washed 6 times with phosphate buffered saline (PBS)-Tween20 0.05% and incubated for 2 hours at room temperature (RT) with 1µg/ml biotin-linked anti-IFN-γ, GM-CSF or IL-13 monoclonal antibody (mAb) (Mabtech AB). The plates were washed again 6 times with PBS-Tween20 0.05% and incubated at RT for 1 hour with 1µg/ml streptavidin alkaline phosphatase (ALP) (Mabtech AB). Following further washes with PBS-Tween 0.05% and distilled water, spots were visualised using an ALP conjugate substrate kit (Biorad, Hercules, CA, USA) and counted using an automated ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic). Results were expressed as spots per number of cells added per well after subtracting the background.

3.10 Cytokine ELISA

IFN-γ and IL-10 levels were measured in supernatants by ELISA (Human Read-Set-Go!®, EBioscience; CA, USA) and IL-13 (Human Ultrasensitive IL-13 ELISA kit; Life Technologies, CA, USA). 96-well plates (Nunc Maxisorp) were coated with 100µl/well coating antibody, diluted 1:250 in coating buffer (Ebioscience). After washing 5 times

with PBS-Tween20 0.05% the plates were incubated for one hour at RT with 200µl/well 1%BSA/PBS (Ebioscience). Pre-coated ELISA strips were used for IL-13 (Life Technologies). Standards containing known cytokine levels, controls and samples were added in duplicates and incubated for 2 hours at RT (Life Technologies) or overnight at 4ºC (Ebioscience). Plates were then washed 5 times with PBS-Tween20 0.05% and incubated with biotinylated cytokine-specific antibody for 1 hour at RT (Ebioscience and Life Technologies). Following a further 5 washes with PBS-Tween20 0.05%, plates were incubated with streptavidin-horse radish peroxidase (HRP) for 30minutes at RT (Ebioscience and Life Technologies). Plates were then developed after a final wash, using 3,3',5,5'-Tetramethylbenzidine (TMB) (Ebioscience) for 15 minutes or stabilised chromogen for 30minutes (Life Technologies) at RT in the dark. The reaction was stopped using sulphuric acid. Cytokine levels were quantified using a microtitre plate reader, read at 450nm (Bio-Rad iMark™ Microplate Reader, CA, USA).

3.11 Multi-plexed bead array

Unpulsed or PLA2 exposed (1µg/ml) K562 or K562-CD1a cells were cultured with polyclonal T cell lines (enriched for CD1a and PLA2-specificity) at a K562 cell/T cell ratio of 1:5 for 24h. Concentrations of human cytokines in the supernatants were measured using a Bio-Plex™ Pro Human Cytokine TH1/Th2 Assay kit (Bio-Rad) with magnetic beads. All steps were performed at RT with shaking at 850rpm. 50µl beads were added per well and washed twice, followed by 50µl of standards, blanks and sample supernatants and incubated for 30 minutes. Following three washes, 25µl detection antibody was then added per well for 30 minutes. After washing unbound antibody, 50µl streptavidin-PE was added for 10 minutes. Following a final three

washes, beads were resuspended in 125µl assay buffer for 30 seconds. Results were obtained with a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA).

3.12 CD1a-plate bound assay

Purified CD1a, CD1b or CD1c proteins and anti-CD11a antibodies (R&D Serotec) were added on NUNC plates at 10µg/ml and 2.5µg/ml respectively for 24h at 4^{o}C, and unbound proteins were washed with PBS-Tween 20 0.05%. Wasp and bee venoms and their protein and lipid extracts, other allergen SPT preparation, DDM, arachidonic acid, and lipids (phosphatidylcholine, lysophosphatidylcholine, lysophosphatidyl-ethanolamine and fatty acids) were loaded at 10µg/ml in PBS for 24-48h at RT. After washing 10µg/ml anti-CD1a or isotype control antibody were added to appropriate wells for 1 hr RT. Following a final wash, T cells were then added to the wells at 100,000 cells/well.

3.13 Quantitative real time polymerase chain reaction (qPCR)

Unpulsed or PLA2 exposed (1µg/ml) K562 or K562-CD1a cells were cultured for 6h with polyclonal T cell lines (enriched for CD1a and PLA2-specificity) at a K562 cell/T cell ratio of 1:5. Following RNA extraction using Qiagen RNeasy® mini kit (Qiagen, Manchester, UK), and reverse transcription (Life Technologies), qPCR were performed using the following gene expression assays (Applied Biosystems): *IFNG* (Hs00989291_m1), *IL13* (Hs00174379_m1) and *IL22* (Hs01574154_m1). Reactions were performed in a 7500 Fast Thermal Cycler (Applied Biosystems).

3.14 HPLC-(electrospray ionisation)-quadrupole time of flight mass spectrometry

The analyses were performed using an Agilent Technologies 6520 ESI-QTof coupled with HPLC (Agilent Technologies 1200). Lipids were separated using a 3µm x 150mm

x 2mm diol column (Varian) in Harvard as previously described (270). Spectra were collected in positive mode from 100 to 3,000 *m/z* at 1 spectrum/s. Internal calibrations were performed using calibrants at 121.050873 *m/z* and 922.009798 *m/z* that were coinfused into the spray chamber and used to monitor the stability of ion signal. Data were analysed using MassHunter Workstation Qualitative Analysis Software (Agilent Technologies). HPLC-MS was performed at Harvard Medical School.

3.15 Phospholipase activity

3.15.1 Phospholipase A2 activity

Phospholipase A2 activity was measured using a colorimetric assay from Cayman Chemicals, according to manufacturer's instructions. 10µl samples were added per well in a flat bottom 96-well plate, plus 5µl assay buffer (containing Ca^{2+}) were incubated for 1 hour at RT with a substrate solution. Substrates used have a thiol group attached at the sn-2 position of the phospholipid and included arachidonyl thio-PC, heptanoyl thio-PC, diheptanoyl thio-PC and palmityl thio-PC. In the presence of PLA2, cleavage of the substrate at the *sn*-2 position resulted in release of the thiol group. After 1 hour incubation, 10ul DNTB/EGTA was added, which reacts with free thiol groups producing a coloured precipitate, which was measured at 415nm wavelength (Bio-Rad iMark™ Microplate Reader, CA, USA).

3.15.2 Phospholipase A1 activity

PLA1 activity in wasp and bee venom were detected using site specific substrate analysis (EnzChek® Phospholipase A1 Assay Kit, Life Technologies, Paisley, UK) according to manufacturers' instructions. In a flat-bottom 96-well plate, 50ng wasp venom (Sigma-Aldrich, MO, USA) ((1µg/ml in reaction buffer) was incubated for 30 minutes at RT with substrate solution. The substrate used in

glycerophosphoethanolamines with a BODIPY® FL acyl chain in the *sn*-1 position, with dinitrophenyl quencher head group. Cleavage of BODIPY® FL pentanoic acid results in decreased quenching and so increased PLA1-dependent fluorescence emission. Fluorescence intensity was measured at 515nm using a microplate reader (Clariostar®; BMG Labtech).

3.16 Flow Cytometry

0.5-1 x 10^6 cells were washed twice in FACs buffer (PBS, 0.5% BSA, 2mM EDTA, pH7.2) and cells were incubated for 20 minutes at 4^{o}C with live/dead stain (Invitrogen) and relevant antibodies. List of antibodies and titrations used is found in Chapter 3.1. Cells were then washed twice in FACs buffer and acquired using CyAn™ (DakoCytomation) and then analysed using FlowJo software (Tree star, Ashland, Oregon).

3.16.1 Intracellular Cytokine Staining (ICS)

Unpulsed or PLA2 exposed (1µg/ml) K562 or K562-CD1a cells were cultured in T cell medium with polyclonal T cell lines (enriched for CD1a and PLA2-specificity) at a K562 cell/T cell ratio of 1:5 for 5h in the presence of Brefeldin A (1µg/ml). After culture, cells were washed and stained with APC-conjugated CD8 antibody (BD Biosciences) in FACs buffer for 20 minutes at 4^{o}C. After washing twice, cells were permeabilised using Cytoperm/Cytoperm™ solution (BD Biosciences) for 10-20 minutes at 4^{o}C. The cells were washed again twice using Perm/Wash™ solution (BD Biosciences) and then incubated for a further 20 minutes at 4^{o}C with anti-cytokine antibodies (IL-17A and IL-22; Biolegend and E-Bioscience respectively). Following a final wash step, cells were acquired using CyAn™ (DakoCytomation) and then analysed using FlowJo software (Tree star, Ashland, Oregan).

3.16.2 IFN-γ secretion assay

1-10 x 10^6 T-cells were stimulated with 0.5-5 x 10^6 K562-CD1a (pulsed with antigen overnight, irradiated (5000 rad) and washed) in 24-well plates for 6 hours at 37ºC in 5% CO_2. Cells were then washed in cold PBS, containing 0.5% bovine serum albumin (BSA) and 2mM EDTA (MACs buffer) and labelled with anti-IFN-γ mAb conjugated to anti-CD45 mAb (Miltenyi Biotec, Germany) for 5 minutes on ice. 10ml of warm R5* (37ºC) was then added and cells were slowly rotated at 37ºC for 45 minutes. The cells were then washed in MACs buffer at 4ºC and stained with PE-conjugated anti-IFN-γ mAb (Miltenyi Biotec), CD3 PerCP (BD Biosciences) and Live/Dead® Fixable violet dead cell stain (Life Technologies) for 10 minutes at 4ºC. After washing in MACs buffer, cells were resuspended in R5* and sorted one cell per well of 96-well round bottom plate on a MoFlo™ (Beckman Coulter, Fullerton, CA, USA). T cell lines were expanded with rapid expansion mix of feeders.

3.16.3 Rapid expansion method of feeders

Rapid expansion method of feeders used PBMCs from 1 donor, 2 transformed B-cell lines and anti-CD3 antibody to expand T-cell lines or clones. T-cells were added to 96-well round bottom plate (1-1000cells/well) in T-cell medium, together with 0.2 x 10^6 irradiated (3000 rad) PBMCs, 0.04 x 10^6 irradiated (3000 rad) B cell line and 50ng/ml anti-CD3 antibody (OKT3; Ebioscience, CA, USA). Cells were transferred to flat bottom plates after 2-3 weeks and split as necessary.

3.17 Statistics

Statistical analyses were performed using GraphPad Prism 6. Cohort of healthy donors investigated for CD1a-restricted wasp venom, bee venom and PLA2-specific responses (Chapter 4) were analysed using one-tailed Wilcoxon matched-pairs signed

rank test. A non-parametric test, comparing two paired groups (in the presence or absence of antigen) was used, due to uncertainty in the distribution of the data. Polyclonal T cell response of cultured cells (Chapter 4) and allergic and healthy donors investigated for *ex-vivo* CD1a-reactive wasp venom, bee venom, and PLA2-specific responses (Chapter 5) were analysed using one-tailed Mann-Whitney tests. This non-parametric test was used due to the relatively small sample size, with a one-tailed analysis as an increase had been hypothesised. Changes detected over the course of immunotherapy were analysed using one-tailed Wilcoxon matched-pairs signed rank test. This non-parametric test, comparing two paired groups, in the presence and absence of antigen, was used, due to the relatively small sample size and uncertainty of distribution of the data. All other data were analysed using one-tailed unpaired t-tests, due to sufficient sample size and as the spread of the two groups being compared was varied (parametric test assumes both groups do not have same distribution).

4 Identification of wasp and bee venom responsive CD1a-restricted T cells

4.1 Introduction and Aims

Extensive evidence for the important role of peptide-MHC complexes in T cell activation evolved into a widespread belief that peptides are the only common and natural target of human T cell responses. Therefore, until recently, nearly all human clinical studies of T cell action in autoimmune, allergic and infectious diseases were targeted at peptide antigens. For example, most candidate antigens for human T cell mediated autoimmune diseases are proteins (271). Subunit vaccines (272) and diagnostic tests (273) rely on defined peptide motifs, and mouse models of autoimmunity start with protein and peptide antigen vaccination. However, the discovery of the function of CD1a, CD1b, CD1c and CD1d proteins (274, 275) as antigen presenting molecules expands the biochemical spectrum of natural antigens for T cells to include many types of lipids (276-278).

CD1 proteins are conserved among mammals (279) and are expressed at high density on thymocytes and professional APCs in the periphery, including LCs, B cells, macrophages and myeloid dendritic cells (280). In cells, CD1 proteins bind and display hundreds of molecular species of self-sphingolipids, phospholipids and acylglycerides (281), and more than 20 types of stimulatory lipid antigens for T cells are now known (282). The molecular basis by which lipids are recognized by T cells is well established through crystal structures of CD1, CD1 bound to lipid and CD1-lipid bound to a TCR (283-285). The alkyl chains of lipids are sequestered within the grooves of CD1 proteins, allowing the carbohydrate, sulphate, phosphate and other polar elements to protrude and interact with TCRs.

Despite the wealth of molecular and cell biological data showing that mammalian αβ T cells can recognize lipids, translational research to determine the roles of CD1-restricted lipid antigens *in vivo* or during diseases that are commonly seen by physicians is limited. Most reported studies have focused on CD1d and CD1d-restricted T cells, known as NKT cells, because CD1d is the only CD1 isoform expressed in commonly used mouse models (286). Yet CD1a, CD1b and CD1c differ from CD1d, and from one another, in their trafficking and tissue distribution, suggesting that they exert different physiological roles (279). Notably, CD1a, unlike CD1b and CD1c, has been known for decades as a phenotype specific marker of human epidermal LC (280). In addition to studies of guinea pigs (287, 288) and transgenic mice (45), the functions of CD1a, CD1b and CD1c have been studied in humans during tuberculosis (289), seasonal allergy (53) and other diseases (290). However, human studies rely mostly on T cell clones whose functions change over time during *in vitro* culture and may not represent the natural populations of T cells *in vivo*.

To study polyclonal autoreactive human T cells *ex vivo*, APCs that lack detectable surface expression of MHC proteins (K562 cells) were engineered to express CD1a, CD1b, CD1c or CD1d proteins at high density (K562-CD1). K562-CD1 cells provide lipid autoantigens, and such MHC^{low} $CD1^{high}$ APCs largely avoid MHC alloreactivity (65). Therefore, T cells from groups of randomly chosen or unrelated donors can be tested for antigen responses, allowing cohort studies and quantitation of CD1 autoreactivity in healthy subjects. Three recent studies show that CD1a autoreactive T cells are present in the peripheral blood of nearly all humans tested, defining CD1a autoreactive T cells as a distinct component of the human immune system (34, 65, 66). These studies also found that the rates of T cell autoreactivity are higher to CD1a, as compared to CD1b, CD1c and CD1d. In addition to studies of

peripheral blood, recent studies implicate CD1a autoreactive T cells as functioning in organ-specific immunity in the skin. CD1a is the major human CD1 protein expressed at high density on LC (280), and CD1a autoreactive T cells express skin-specific homing receptors (CLA, CCR4, CCR6, CCR10) and home to the skin in large numbers (65). In addition, skin-specific oils, including squalene, function as autoantigens presented by CD1a (34).

Venoms act within the skin to cause pain or death by transfer to highly unrelated organisms, so typically involve multiple active substances, that typically function by co-opting host responses and so have a long history as a tool to understand natural host inflammatory responses. In particular, venom-induced immune responses have been relevant beyond venom reactivity and have provided broader immunological insights in to novel pathways of inflammation and tolerance (291-293). Because bee and wasp venom responses are localized initially in skin, where CD1a is so abundantly expressed and because the lipidic content of the bee and wasp venoms was unexplored, bee and wasp venom were used as a model system to investigate novel pathways of CD1a-dependent inflammation in the skin. The key aims within this chapter were to identify polyclonal venom-responsive CD1a-restricted T cells in blood and skin of healthy individuals, characterise their phenotype based on cytokine secretion, and investigate their development using cord blood.

After initial studies documented a CD1a-mediated response in individual T cell clones and among cohorts of patients, mechanistic studies surprisingly tracked the antigenic substance to venom phospholipase. By investigating venom-derived proteins, an unexpected mechanism was discovered by which venom phospholipases create lipid neoantigens *in vivo*. Therefore the aims for this chapter were expanded to include the investigation of phospholipase responsive CD1a-restricted T cells.

4.2 Results

4.2.1 Responses of T cells to CD1 molecules and venom

Stinging insects in the genera vespula (wasp) and apis (bee) generate a local intradermal inflammatory response. To investigate a possible role of CD1, CD3+ T cells from healthy adult individuals were isolated and mixed with wasp venom-treated and irradiated K562 APCs, which were mock transfected or transfected with either CD1a, CD1b, CD1c or CD1d. As expected based on previous studies (34), low but detectable alloreactive background responses were observed, of ~ 1 in 2,000 T cells after 12-14 days in culture to mock-transfected target cells in donor C1175, which is representative of three donors (Figure 4-1 left panel). In the absence of venom, T cells showed responses to K562-CD1a cells at rates higher than to mock transfected cells or cells expressing other CD1 isoforms, confirming the presence of CD1a autoreactive T cells (34, 65, 66). Further, polyclonal responses to wasp venom above background levels were detected, but were seen only when using CD1a-expressing cells as targets ($P<0.05$, Figure 4-1A, representative of three donors). These responses demonstrate that a T cell response to wasp venom is mediated by CD1a.

Apis mellifera (honeybee or bee) venom also causes skin immune responses and shares certain mechanisms of immune action with wasp venom (294). For example, patients that develop hypersensitivity towards wasp venom can also show reactivity to bee venom (295). Therefore, the CD1-restricted T cell response to bee venom was investigated next and the pattern of response was similar to that of wasp venom, including augmentation of the baseline autoreactive response by venom in the presence of CD1a (Figure 4-1B). Thus, this pilot study provided proof of principle for

CD1a-mediated human cellular responses to wasp and bee venom among human polyclonal T cells.

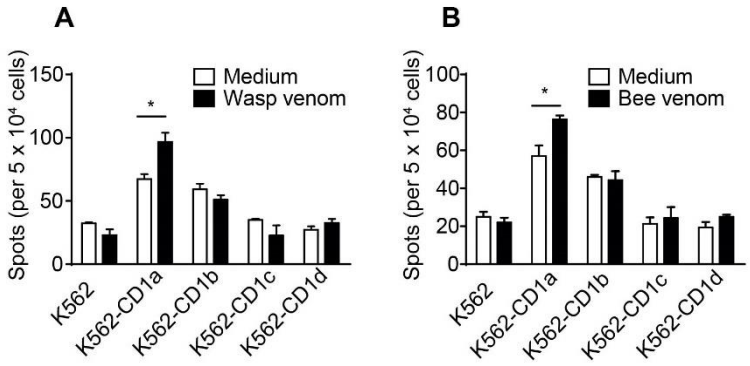


Figure 4-1: *Vespula spp* and *Apis mellifera* venoms induce a preferential activation of CD1a-restricted T cells among the group1-CD1 reactive cells.

T cells were isolated by CD3 MACS beads from healthy donor PBMC and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562-CD1a) or CD1b (K562-CD1b) or CD1c (K562-CD1c) or CD1d (K562-CD1d) or an empty vector (K562) in the presence of venom. CD1 reactivity was then examined by IFN-γ ELISpot with transfected or untransfected K562 cells either in the presence or absence of *Vespula spp* (A) *or Apis mellifera* (B) venoms. Representative data for one donor (C1175) of three are shown. Data were mean of triplicate measurements ± SEM. * $P < 0.05$.

The total IFN-γ response of fresh polyclonal T cells consisted of three identifiable components: a response to K562, an increment in response to CD1a and a further increment to added venom, consistent with CD1a presentation of venom antigens. Expansion of T cells with antigen *ex vivo* offered the opportunity to more definitively establish the molecular mechanisms involved. T cells were cultured in IL-2 for two weeks in the presence of wasp venom treated K562-CD1a cells to permit the outgrowth of responder cells. ELISpot studies clearly confirmed reactivity to CD1a alone as well as to CD1a plus wasp venom, but no response to venom was seen in the absence of CD1a. A similar pattern of response was seen to T cells treated with K562-CD1a and bee venom.

Venom concentration titrations showed that polyclonal T cell IFN-γ responses continued to rise with increasing venom concentrations up to 1µg/ml, indicating that the responses seen were mediated by the venoms. At higher concentrations however, T cell responses decreased, possibly due to the toxicity of venom on cells (Figure 4-2A). Both autoreactive and venom-induced polyclonal T cell responses were also dependent on number of CD1a molecules, as increasing the K562:T cell ratio resulted in increasing T cell responses, implying that CD1a is required for these responses (Figure 4-2B).

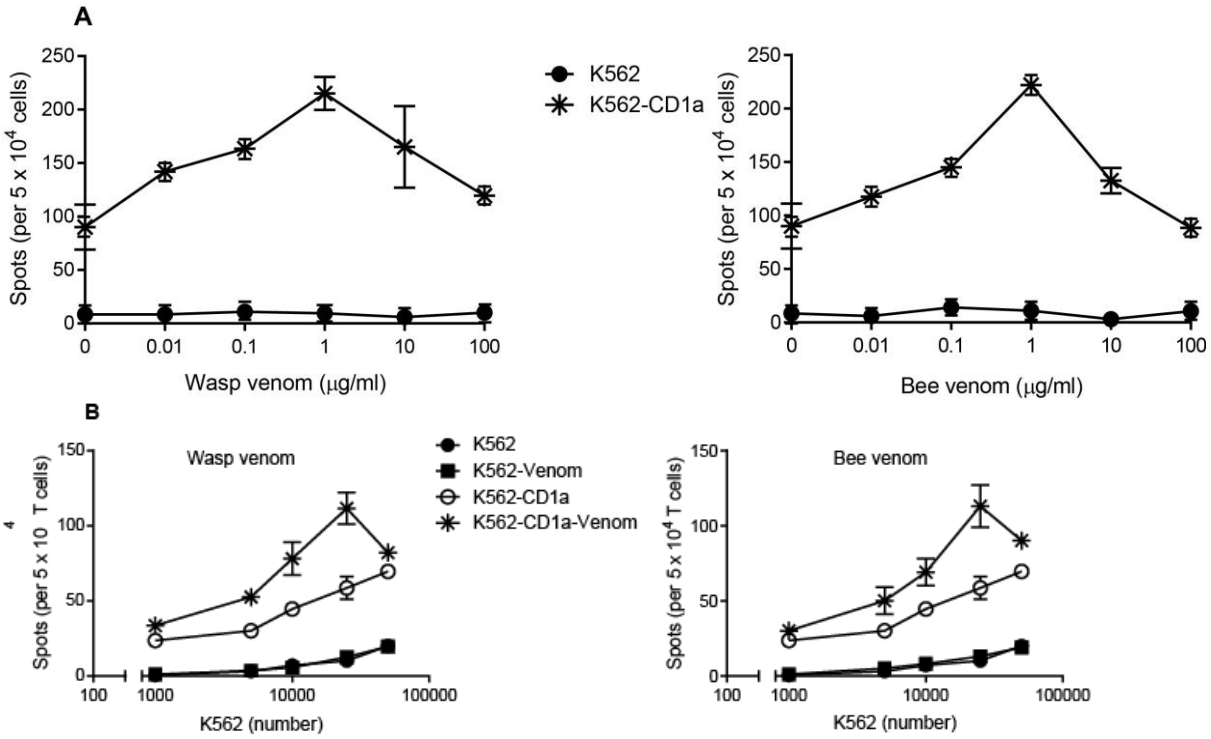


Figure 4-2: Optimisation of IFN-γ ELISpot assay to investigate T cell responses to *Vespula spp.* and *Apis mellifera* venoms and K562-CD1a.

T cells were isolated by CD3 MACS beads from healthy donor PBMC and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562-CD1a) in the presence of venom. CD1 reactivity was then examined by IFN-γ ELISpot with transfected or untransfected K562 cells either in the presence or absence of *Vespula spp* (A) or *Apis mellifera* (B) venoms, with increasing concentrations of venoms (top panels) and varied K562/T cells ratio (bottom panels). Representative data for one donor (C347) of three are shown. Data were mean of triplicate measurements ± SEM.

4.2.2 CD1a-restricted venom responses in a healthy cohort

Next this pattern of wasp venom responses was tested in a larger cohort of 21 healthy individuals. Measuring mean IFN-γ spots for all donors, a higher (P<0.0001) response to CD1a transfectants as compared to untransfected target cells was observed (Figure 4-3B, left panel).

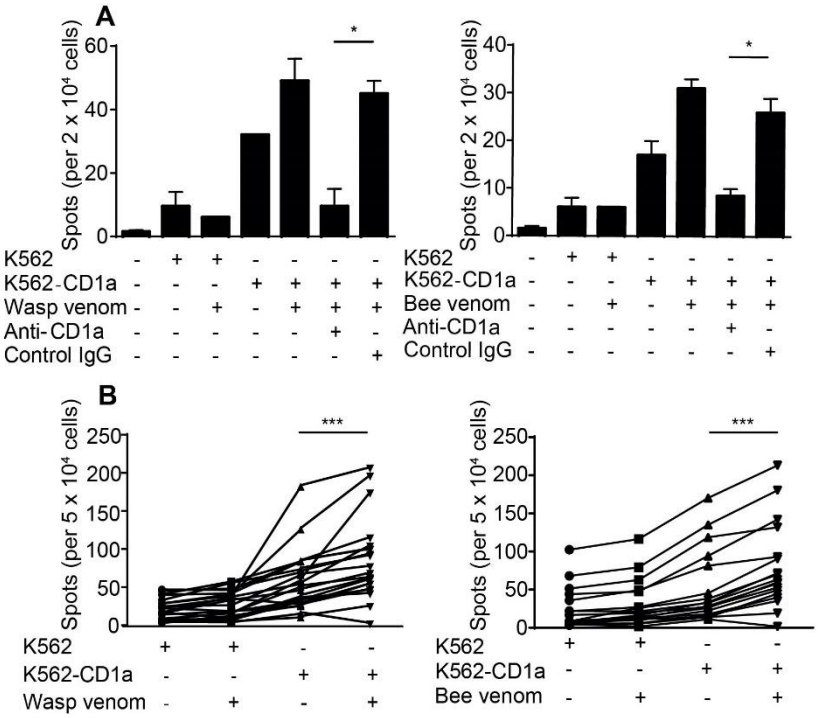


Figure 4-3: CD1a-restricted reactivity to *Vespula spp* and *Apis mellifera* venoms is found among polyclonal T cells from blood of healthy donors.

T cells were isolated by CD3 MACS beads from healthy donor PBMCs and cultured for 12-14 d with IL-2 and irradiated K562 transfected with CD1a (K562-CD1a) or an empty vector (K562) in the presence of venom. CD1a reactivity was examined by IFN-γ ELISpot with transfected or untransfected K562 cells either in the presence or absence of *Vespula spp* or *Apis Mellifera* venom, and was inhibited by anti-CD1a (donor C1098) (A). CD1a-restricted venom-specific responses were measured in 21 donors for *Vespula spp* or *Apis Mellifera* venom (B). Data were mean of triplicate measurements ± SEM. * P < 0.05, *** P < 0.001.

Similar to the pilot studies, higher spot numbers were detected when wasp venom was added, but only when CD1a was present (P<0.001). Further, anti-CD1a mAb blocked the response to background levels (P<0.05), proving that CD1a is required (Figure

4-3A left panel). The pattern of IFN-γ production to bee venom was dependent on CD1a expression (P<0.001) and similar in magnitude to that seen with wasp venom (Figure 4-3, right panels).

K562 cells are transformed cells that represent useful tools which bypass MHC alloreactivity. The two types of primary human skin APCs that express CD1a are myeloid dendritic cells and LCs. To investigate whether these cell types mediate the venom response similarly to K562 cells, monocytes were differentiated with GM-CSF and IL-4 to produce monocyte-derived dendritic cells (mDCs), which mimic key features of myeloid DCs, and cells were activated *in vitro* with cytokines to mimic LC ("*in vitro* LC"), including high CD1a expression (Figure 4-4A) (267, 296-298). Venoms did not affect mDC or LC-like cell maturation (Appendix 8.1).

Both primary cell types were able to mediate wasp venom and bee venom responses which were abrogated in the presence of anti-CD1a (Figure 4-4). The intensity of response correlated with the venom concentration, indicating the response observed was mediated by the venoms. At higher concentrations, the responses began to decrease due to toxicity, although this occurred at lower doses than with K562s (Figure 4-2), as primary cells are less robust than immortalised cell lines. Overall this study showed that CD1a autoreactivity and CD1a-dependent venom reactivity are common in humans. This pattern of combined CD1a autoreactivity and antigen-dependent reactivity is reminiscent of invariant NKT cells, which are activated weakly by CD1d and strongly by CD1d with lipid antigen (299).

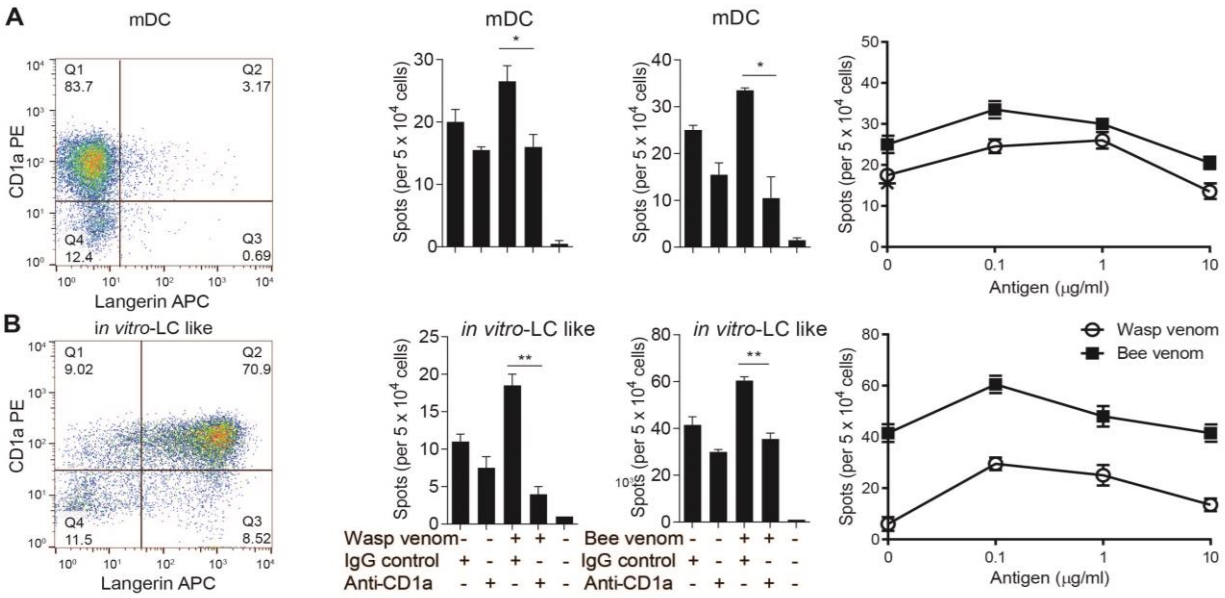


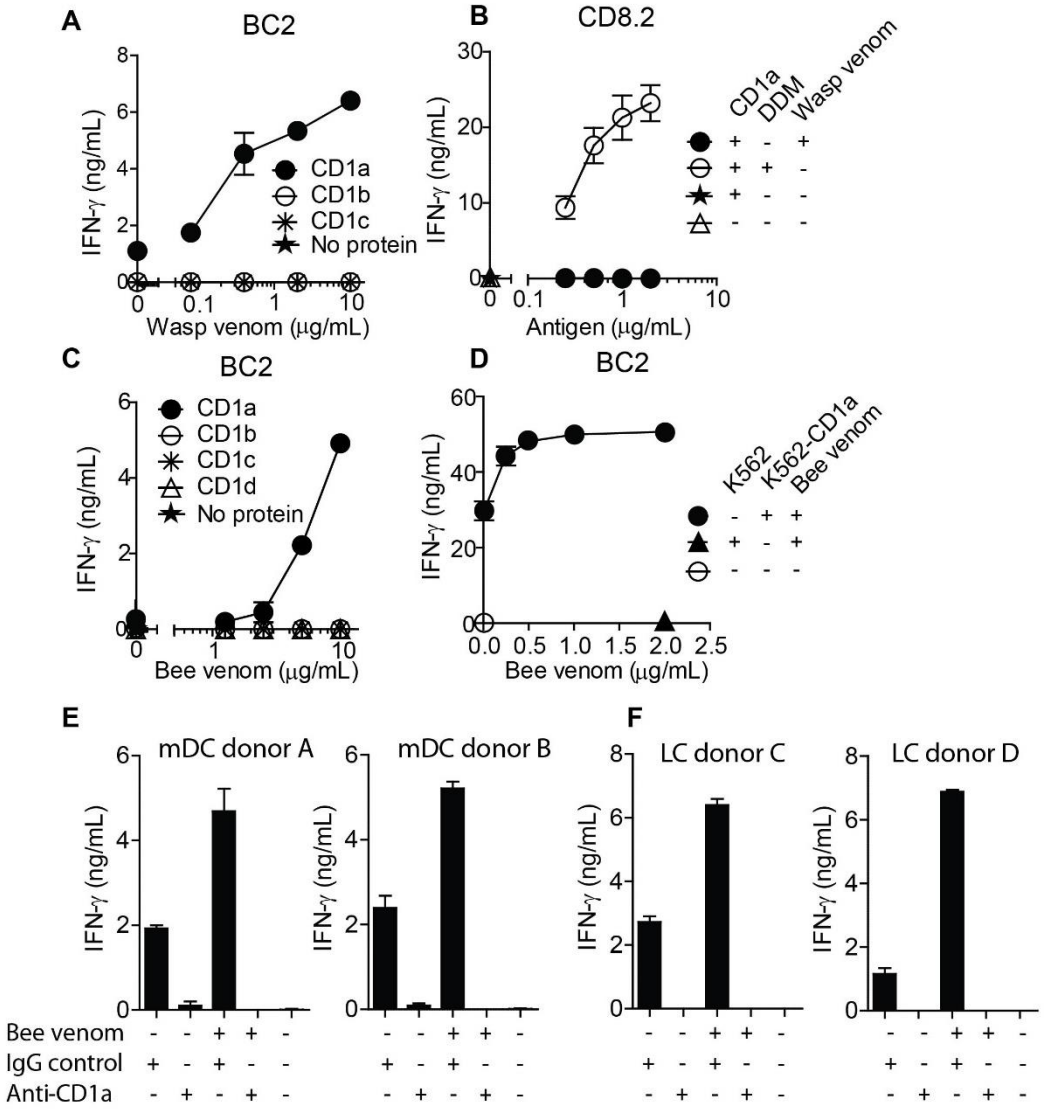
Figure 4-4: mDC and LC-like cells mediate T cell reactivity to *Vespula spp* and *Apis mellifera* venoms.

Monocyte-derived DC (mDC) (A) or *in vitro*-LC-like cells (B) derived from CD14+ cells were pulsed with 1μg/ml wasp venom or bee venom and incubated with the cultured T cells in the presence or absence of anti-CD1a antibody or isotype control. IFN-γ production was measured by cultured IFN-γ ELISpot. Representative data for one donor (C556) of three are shown. Data were mean of triplicate measurements ± SEM. * P < 0.05, **P<0.01.

4.2.3 Isolation of stimulatory factors from venom

To neutralize diverse predators or prey, natural venoms are usually high potency toxins that have multiple mechanisms of action (300). Repeated exposure of wasp and bee venoms to lymphocytes resulted in apparent toxicity and cell death based on monitoring of T cells by trypan blue exclusion. Long-term T cell lines could not be generated by direct exposure to venom *in vitro*, existing panels of CD1a-reactive T cell clones were screened for responses to *Vespula* species (wasp) and *Apis mellifera* (bee) venoms. These experiments were performed in collaboration with Prof. Branch Moody at Harvard Medical School. To minimize toxicity and interrogate the mechanism of venom action, a switch was made from K562-CD1a activation assays (cellular assays) to assays in which biotinylated CD1a proteins are coated onto streptavidin

plates (plate assays). In the plate assay, CD1a proteins are pulsed with venom-derived products, washed and then exposed to T cells. This method diminishes venom exposure to APCs and responding cells, thereby minimizing toxicity. Also, the plate method might favour detection of stimulants that act by forming complexes with CD1 proteins in preference to indirect effects of venom on APCs or T cells that do not involve binding to CD1. The T cell clone BC2 showed a dose-dependent increase in IFN-γ secretion when cultured in the presence of CD1a proteins pre-treated with wasp venom (Figure 4-5A). The response was likely to be specific antigen recognition rather than a general mitogenic activation because no response was seen when plates were coated with CD1b, CD1c (Figure 4-5A) or CD1d (data not shown), or when venom-treated CD1 proteins were exposed to another T cell line, CD8-2, which normally recognizes dideoxymycobactin lipopeptides presented by CD1a (301) (Figure 4-5B). Furthermore, a similar dose-dependent recognition by T cell clone BC2 of bee venom was observed in the presence of CD1a proteins (Figure 4-5C).



Experiments performed by Elvire Bourgeois, Harvard Medical School

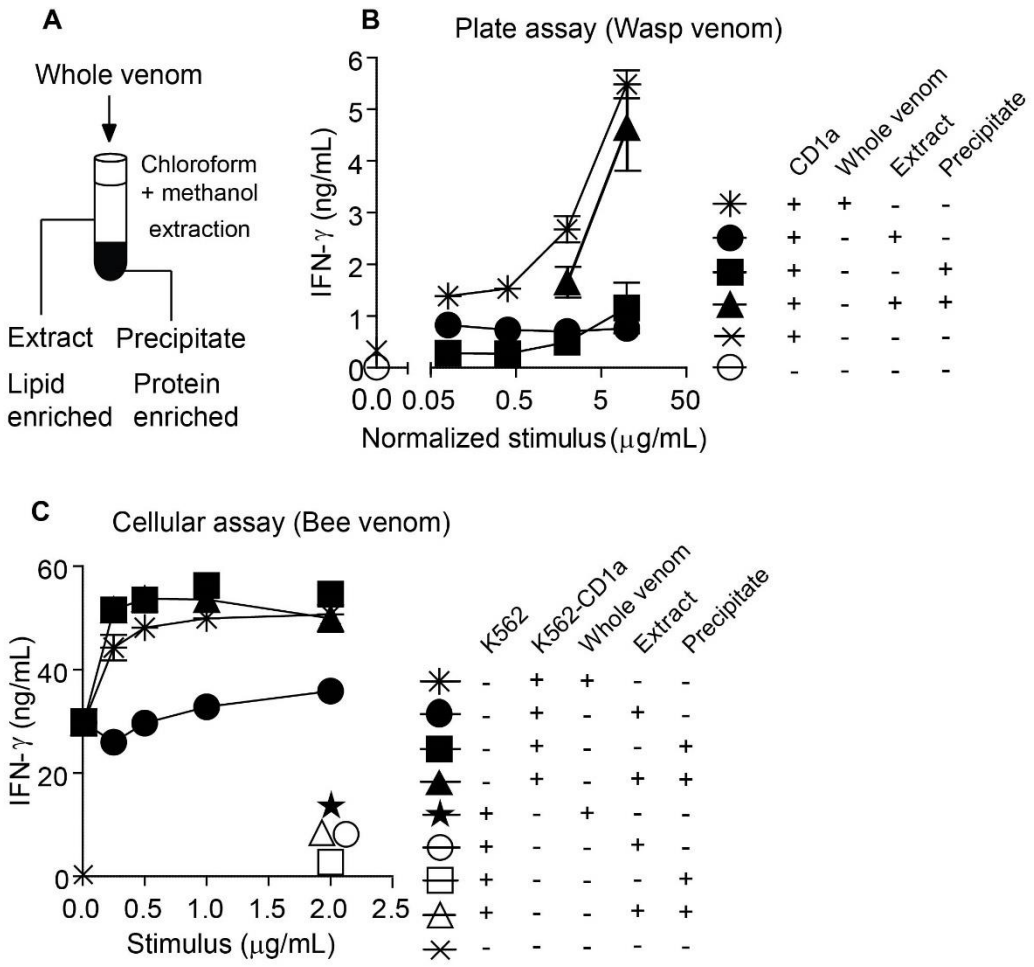
Figure 4-5: The auto-reactive CD1a-restricted T cell clone BC2 is activated by wasp and bee venoms.

Biotinylated CD1a, CD1b and CD1c and CD1d proteins were coated on streptavidin plates. After washing, wasp (A and B), bee (C) venoms and dideoxymycobactin (DDM) (B) were added to the wells for 24 to 48 h. After washing, BC2 cells (A and C) or CD8.2 cells (B) were added, and the supernatants were collected 24 h later and IFN-γ quantified. (D) BC2 cells were co-cultured for 24 h with universal target cells (K562) as APCs, transfected with CD1a (K562-CD1a) or empty vector (K562) and bee venom. IFN-γ in supernatants was measured by ELISA. Data are representative of three separate experiments. (E) mDC and (F) LC were incubated with IgG control or anti-CD1a antibodies for 1 hour at 37C. Bee venom was then added at 2μg/mL for 1 h. BC2 cells were then added at a 1:25 APC:T cell ratio. Two representative donors out of three are shown.

Using cellular K562 assays, a CD1-dependent and dose-dependent response to bee venom was again observed, but this time with significant CD1a autoreactivity. The higher responses seen in cellular assays might be explained if venom acts on the APC in some way or if lipid autoantigens are provided by K562 cells (Figure 4-5D). In addition to using monocyte-derived dendritic cells, an alternative system for the generation of LC-like cells was also used and so CD34+ progenitors were sorted and activated with relevant cytokines, which are well established to serve as a model of LC function (198). The cells express CD1a at moderate to high levels (data not shown and (296, 297, 302)) and when added to cultures mediated CD1a and venom dependent responses of BC2 at a ratio of 1 APC to 25 T cells (Figure 4-5E-F). Thus, both transformed and primary CD1a+ cells were used to formally show that T cells can be activated by bee and wasp venom, and the primary cells validated BC2 as a reagent that mimics the pattern of patient responses seen *ex vivo* (Figure 4-3). Thus, this T cell clone could be used for the subsequent assays needed to isolate and identify stimulatory molecule(s) in venom.

4.2.4 Fractionation of venom to discover antigens

To fractionate natural venoms and discover the stimulatory substance, *Apis mellifera* (bee) and a mix of *Vespula* species (wasp) venoms were treated with chloroform and methanol to create a two-phase mixture. Lipids partition into the organic solvents (extract), whereas proteins, nucleotides and other polar molecules are excluded (precipitate) (Figure 4-6A). Nearly all CD1-presented antigens are lipids, so it was surprising that the lipidic extract failed to activate BC2 cells (Figure 4-6B). Likewise, the protein-enriched precipitate failed to activate T cells. However, T cell activation was detectable when the extract and precipitate were recombined. This result excluded the possibility that chloroform and methanol somehow destroyed the capacity of the whole venom to activate BC2, and it suggested that multiple compounds within the venom acted in concert to generate T cell response. An analogous experiment performed using the K562-CD1a cellular assay showed different results, which provided possible explanations for the unexpected pattern. In contrast to the plate assay, venom precipitate applied to intact K562-CD1a APCs was sufficient to activate T cells. Again, extracted lipids alone failed again to activate T cells (Figure 4-6C). These seemingly contradictory results might be explained if venom does not provide an activated lipid antigen, but instead provides a protein that precipitates in organic solvents and then is capable of producing antigenic substances when combined with venom-derived or K562-derived lipids.



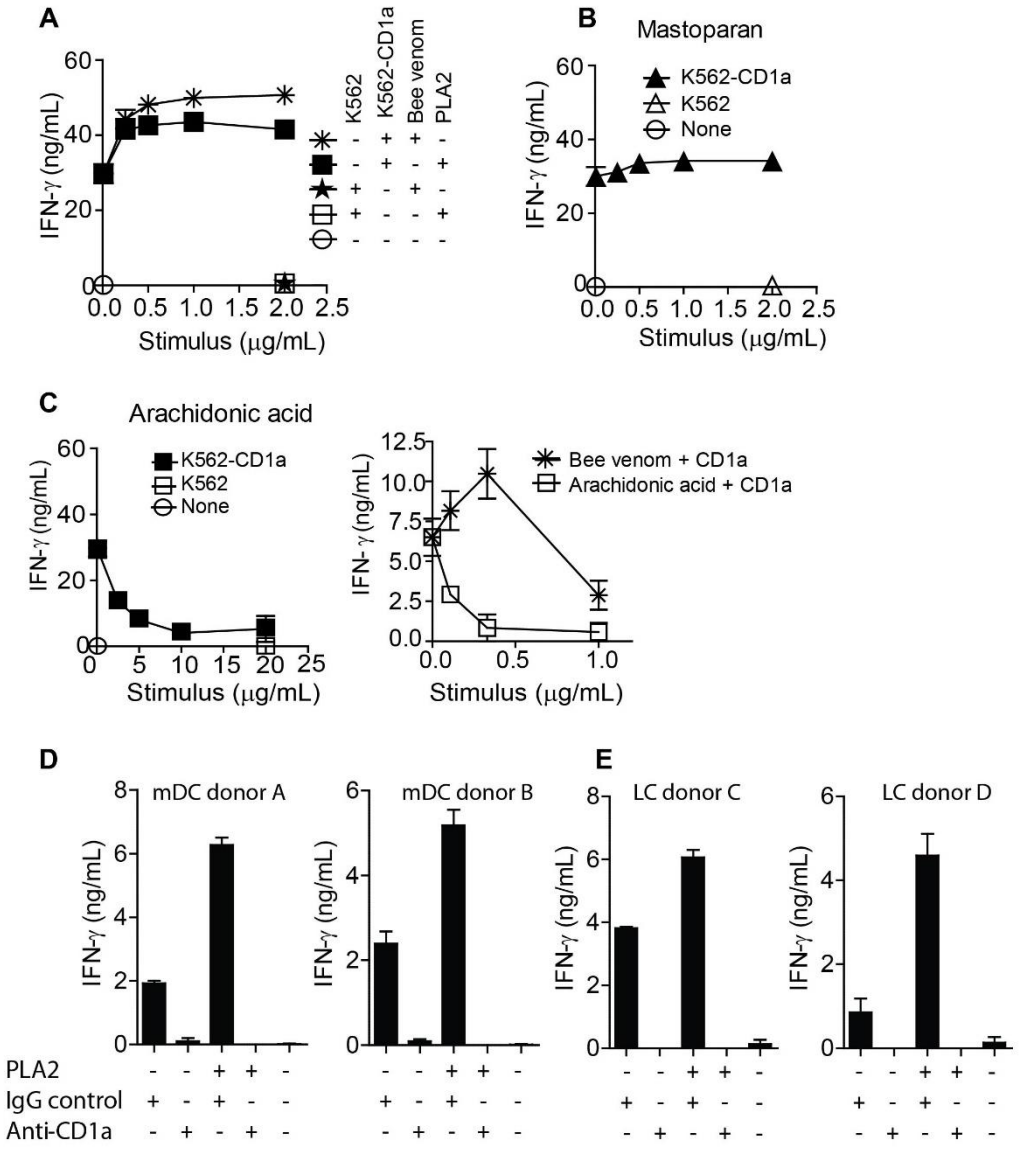
Experiments performed by Elvire Bourgeois, Harvard Medical School

Figure 4-6: The co-incubation of both proteins and lipids from wasp and bee venoms triggers BC2 cells activation when presented by CD1a protein.

Protein and lipid fractions of bee and wasp venoms were separated by organic solvent extraction using chloroform and methanol (A). CD1a proteins were coated on streptavidin plates. After washing, wasp or bee venoms and their extracts were added to the wells for 24 to 48 hours. After washing, BC2 cells were added, supernatants were collected after 24 h and IFN-γ quantified (B). BC2 cells (C) (generated as in Fig 1) were co-cultured with universal target cells transfected with CD1a (K562 CD1a) or empty vector (K562) and bee or wasp venom or their extracts. Supernatants were collected after 24 h and IFN-γ in supernatants was measured by ELISA (C). Data are representative of three separate experiments. Data are representative two separate experiments.

4.2.5 Phospholipase A2 activates T cells

In considering protein factors that might generate antigens for CD1a, it is well established that wasp and bee venoms contain phospholipase A1 (PLA1) and A2 (PLA2) respectively. Phospholipases are major components of venom that have been extensively studied in regard to their antigenicity towards MHC-restricted T cells and B cells (303, 304). Viral phospholipases can promote activation of NKT cells by CD1d (48). Based on these preliminary experiments with recombined protein and lipid fractions, phospholipases were investigated next, to determine if they generate CD1a ligands by acting on venom-derived lipids (Figure 4-5) or the K562 membrane phosphodiacylglycerols (Figure 4-6) cleaving ester bonds and generating free fatty acids, and lysophospholipids. Purified venom PLA2 from *Apis mellifera* strongly activated BC2 T cells (Figure 4-7A). No T cell activation was seen when PLA2 was exposed to T cells with K562 cells lacking CD1a (Figure 4-7A). Therefore, the mechanism of PLA2 on T cells is indirect and requires CD1a.



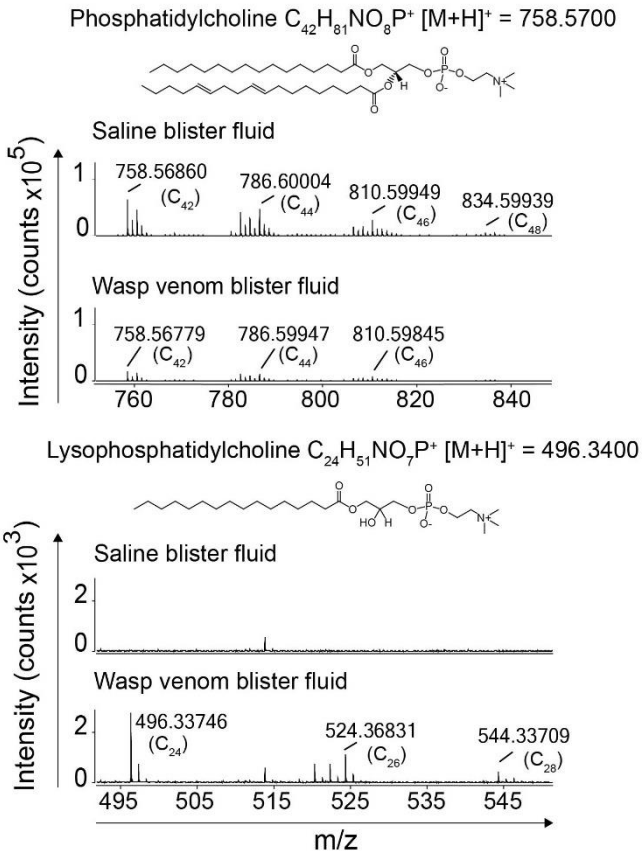
Experiments performed by Elvire Bourgeois at Harvard Medical School

Figure 4-7: Bee venom PLA2 alone induces a CD1a-restricted T cell response by releasing free fatty acids.

BC2 cells were co-cultured for 24 h with universal target cells (K562) transfected with CD1a (K562 CD1a) or empty vector (K562) and bee venom or bee venom PLA2 (A), mastoparan (B) or arachidonic acid (C). IFN-γ in supernatants was measured by ELISA. In (C) right panel, biotinylated CD1a proteins were coated on streptavidin plates. After washing, bee venom or arachidonic acid were added to the wells for 24 to 48 h. After washing, BC2 cells were added for 24 h, and supernatant was sampled for IFN-γ quantification by ELISA. Data are representative of three separate experiments. (D) mDC and (E) LC were incubated with IgG control or anti-CD1a antibodies for 1 h at 37C. PLA2 was then added at 2 µg/mL for 1 h. BC2 cells were then added at a 1:25 APC:T cell ratio. Two representative donors out of three are shown.

4.2.6 Mechanisms of PLA2 action

Another well-known polypeptide present in bee venom that binds lipids and enhances PLA2 activity is mastoparan (305, 306). However, mastoparan acting alone (Figure 4-7B) did not activate BC2. PLA2 releases large amounts of lipid mediators of inflammation through downstream action of cyclooxygenases, which create arachidonic acid metabolites. Therefore, testing another candidate mechanism of action relating to PLA2, we hypothesized that free arachidonic acid might bind to CD1a as an antigen or act indirectly as cyclooxygenase substrate to stimulate BC2 via receptors other than the TCR. However, no arachidonic acid-mediated stimulation was observed and dose dependent inhibition of BC2 activation by arachidonic acid in K562 cellular and plate assays when added at high absolute dose (Figure 4-7C), ruling out both hypotheses. Finally, mDC (Figure 4-7D) and LC (Figure 4-7E), act as APC and substrate of the PLA2 to activate BC2 cells in a CD1a-dependent manner, suggesting that neo-antigens can be generated in primary APCs and that neither mDCs or LCs express inhibitory factors.



Data by Elvire Bourgeois, Harvard Medical School

Figure 4-8: Mass spectrometry tracings of blister fluid after infection of saline or venom.

Healthy donor epidermis was injected with 10µg of wasp venom or saline. Skin blisters were raised and 24h later the blister fluid was sampled. Lipids were extracted with chloroform, methanol and water using the Bligh-Dyer method and analysed on the mass spectrometer quadruple time of flight. Positive mode EIC-MS detected ions at m/z 758.5686, 786.6000, 810.5994 and 834.5993, corresponding to the homologous series of phosphatidylcholine $C_{42}H_{81}NO_8P^+$ (16:0/18:2), $C_{44}H_{85}NO_8P^+$ (18:0/18:2), $C_{46}H_{85}NO_8P^+$ (18:2/20:2) and $C_{48}H_{85}NO_8P^+$ (20:4/20:2). Positive mode EIC-MS detected ions at m/z 496.3374, 524.3683 and 544.3370, corresponding to the homologous series of lysophosphatidylcholine $C_{24}H_{51}NO_7P^+$ (16:0), $C_{26}H_{55}NO_7P^+$ (18:0), and $C_{28}H_{51}NO_7P^+$ (20:4). Data from one representative donor out of two are shown.

4.2.7 Venom generates lysophospholipids *in vivo*

Membrane phospholipids and free fatty acids are both abundant in blood and tissue. However phospholipids largely self-assemble into bilayer membrane structures, and free fatty acids exist mainly as cytosolic metabolites or lipoprotein structures generated during gastrointestinal absorption. Thus, lysophospholipids and free fatty acids are normally stored in macromolecular clusters and do not reach high concentration as free molecules in the extracellular space, where CD1a lipid loading is thought to occur (307). Furthermore, although extracellular lipids, including fatty acids, accumulate in the stratum corneum and sebaceous glands, they are not detectable in normal dermis. Accordingly, most models of self or altered self-lipid display emphasize release of free fatty acids and other antigens into the extracellular space in the proximity of CD1+ epidermal LC (34, 65, 308). Injection of bee and wasp venoms into skin might locally generate such cleavage of intact phosphodiacylglycerides to lysolipids and free fatty acids, so we tested this in a human model for skin immune responses using suction cup blisters (181). This method uses low pressure, sustained (~60 min) suction to produce extracellular blister fluids that are captured for immunological and biochemical analysis before or after antigen challenge (181, 268, 269).

After injection of 10µg of venom, which mimics a wasp sting, or saline vehicle, 2 mm into the skin at two sites in the arm of one patient, two blister fluid samples were obtained. After extracting the lipids from blister fluids using a mixture of chloroform, methanol and water, the presence of substrates and products of the wasp venom PLA was assessed by mass spectrometry using sensitive quadrupole time of flight (QToF) methods (Figure 4-8B). Focusing on the main natural substrate for PLA2, the phosphatidylcholine series were monitored for key ions with differing fatty acid content

corresponding to $C_{42}H_{81}NO_8P^+$ (16:0/18:2), $C_{44}H_{85}NO_8P^+$ (18:0/18:2), $C_{46}H_{85}NO_8P^+$ (18:2/20:2) and $C_{48}H_{85}NO_8P^+$ (20:4/20:2) (Fig. 5B). The masses of detected ions (*m/z* 758.5686, 786.6000, 810.5994, 834.5993) matched these PC variants within the mass accuracy of the detector. Comparing saline and venom injected sites, a two-fold lower intensity was detected for signals corresponding of all molecular variants of PC in the PLA2 injected skin samples, indicating that the enzyme substantially consumed local PC (Figure 4-8B). In a second patient, signals were reduced after venom injection for all PC species except PC18:1 18:1 (data not shown). Conversely, ions matching the lysophosphatidylcholine series, $C_{24}H_{51}NO_7P^+$ (16:0), $C_{26}H_{55}NO_7P^+$ (18:0), and $C_{28}H_{51}NO_7P^+$ (20:4), were readily detected in wasp venom blister fluid with high signal, but were not detected in the blister fluid from saline injection sites. Thus, lysolipids derive from PLA itself and not some aspect of the suction blister model. Signals corresponding to the expected masses of free fatty acids were measured in the positive and negative mode in these samples, but could not detect them. Mass spectrometry detection of fatty acids is less sensitive than detection of anionic phospholipids, so we cannot derive any direct conclusions about fatty acid concentrations in these *in vivo* experiments. Nevertheless, these results demonstrate that the injection of venom alters the local lipid content in ways that are predicted from the specificity of PLA2 and that the cleavage reactions needed to generate fatty acid and lysophospholipid antigens do occur.

4.2.8 Direct measure of wasp and bee venom PLA bioactivity

In individual donors and cohorts (Figure 4-3) and *in vitro* studies with clones (Figure 4-5), strikingly similar responses were noted to bee and wasp venom, which are currently thought to differ in their PLA subtypes, as described above. However if fatty acids are the CD1a-lipid ligand, similar immunological responses are expected, as fatty acids will be generated whether PLA1 or PLA2 cleave acyl chains from phospholipids at *sn*-1 or *sn*-2 respectively. Interestingly, PLA2 has been found in venom of the neotropical social wasps *Polybia paulista* (309, 310) and *Agelaia pallipes pallipes* (311). This led us to investigate whether PLA2 activity is also exerted by *Vespula* species of wasp venom, and furthermore whether there were shared specificities between bee and wasp venom phospholipase. Using *sn-2* thiol-labeled PLA2 substrates (arachidonyl thio-PC, heptanoyl thio-PC, diheptanoyl thio-PC and palmitoyl thio-PC), PLA2 activity was observed with both bee and wasp venom indicating that wasp venom contains PLA2 activity in addition to its known PLA1 activity, and that specificities are partially shared with bee venom PLA2 (Figure 4-9A). We confirmed the PLA2 activity of wasp venom could be inhibited by manoalide, a previously known PLA2 inhibitor (Figure 4-9B). Taken together, these data suggest that both wasp and bee venom phospholipase A2s can generate common CD1a fatty acid ligands.

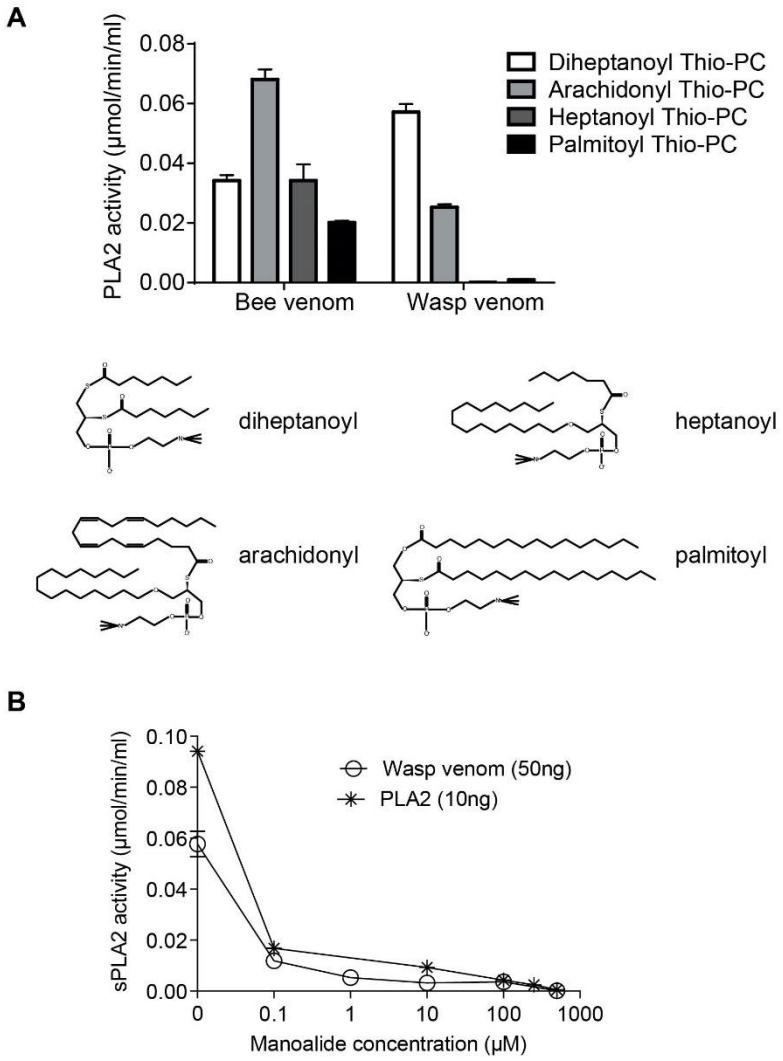


Figure 4-9: *Vespula spp* and *Apis mellifera* venom PLA2 activity.

Whole wasp venom and its protein and lipid extracts were analysed for PLA1 and PLA2 activity using a fluorescent biochemical assay (A). (A) PLA2 activity was detected as free thiol groups released from thiol-labelled lipid substrates through addition of DNTB/EGTA and measuring colour generation at 415nm using *Apis mellifera* venom with four phosphatidylcholine-based substrates of varying acyl chain lengths and saturation, but the same headgroup. *Vespula spp* venom has PLA2 activity for substrates diheptanoyl-phosphatidylcholine and arachidonyl-phosphatidylcholine ($P<0.001$ compared to no enzyme control). (B) Using lipid substrate with a thiol group at the sn2 position, manoalide inhibits PLA2 activity in *Vespula spp* venom detected using DNTB/EGTA which produces a coloured precipitate and measured at 415nm. Data were mean of triplicate measurements ± SEM.

4.2.9 PLA2 induced polyclonal responses *ex vivo*

Having identified PLA2 as sufficient to generate CD1a presented antigens *in vitro* using T cell clones, we next sought to determine if bee venom PLA2 is sufficient to activate polyclonal T cells *ex vivo* in cellular assays. As with whole venoms (Figure 4-1), higher IFN-γ producing cells were detected with the addition of 1μ/ml bee venom PLA2 but only in the presence of CD1a and not other CD1 isoforms (Figure 4-10). This response was mediated both by CD1a and PLA2 as the intensity of the response correlated with PLA2 concentrations and amount of CD1a (number of K562-CD1a cells) molecules, although similar toxic effects were seen at higher concentrations, as with whole venom (Figure 4-11). Significantly ($P<0.001$) higher CD1a-restricted responses were observed in the presence of PLA2 in 18 donors (Figure 4-12A). More detailed testing of an individual responder showed that anti-CD1a antibodies prevented the response to CD1a and PLA2 ($P<0.05$), confirming the essential role of CD1a (Figure 4-12B left panel).

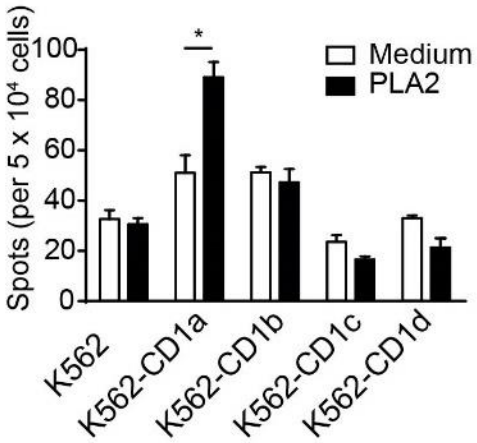


Figure 4-10: CD1a-restricted reactivity to *Apis mellifera* venom PLA2 is found among polyclonal T cells from blood of healthy donors.

CD3+ cells were isolated by magnetic beads from healthy donor PBMC and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562 CD1a) or CD1b (K562 CD1b) or CD1c (K562 CD1c) or CD1d (K562 CD1d) or an empty vector (K562) in the presence of *Apis mellifera* venom PLA2. CD1 reactivity was then examined by IFN-γ ELISpot with transfected or untransfected K562 cells either in the presence or absence of *Apis mellifera* venom PLA2 (1µg/ml). Data from one representative donor (C1175) of three are shown. Data were mean of triplicate measurements ± SEM. * $P < 0.05$.

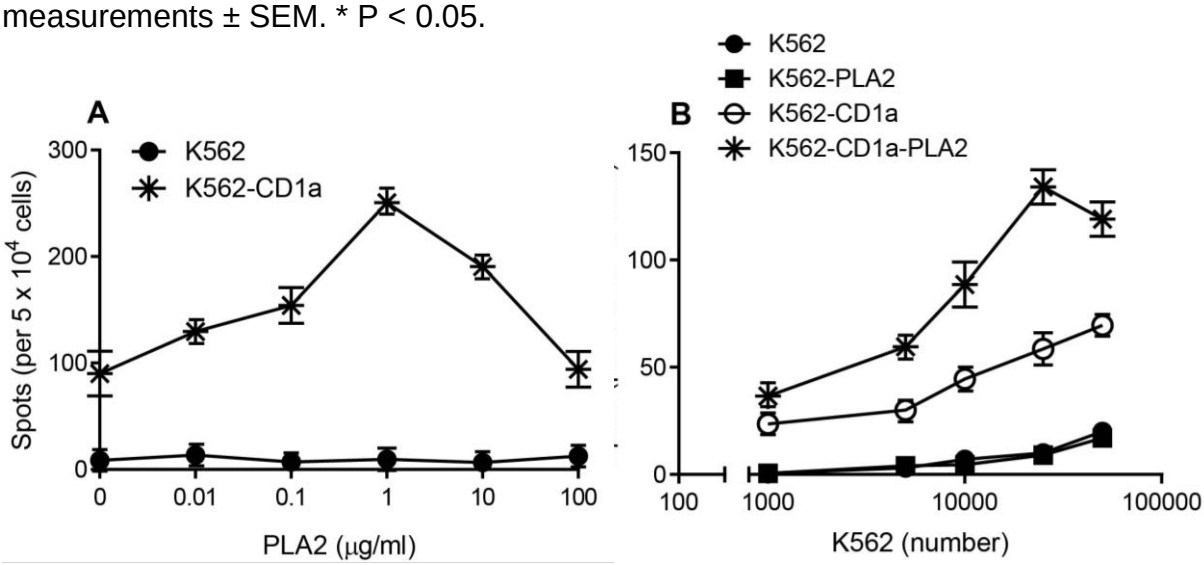


Figure 4-11: Optimisation of IFN-γ ELISpot assay to analyse T cell responses to K562-CD1a *Apis mellifera* venom PLA2 in the context of K562-CD1a.

T cells were isolated by CD3 MACS beads from healthy donor PBMC and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562-CD1a) in the presence of *Apis mellifera* venom PLA2. CD1a reactivity was then examined by IFN-γ ELISpot with untransfected (K562) or CD1a transfected (K562-CD1a) cells either in the presence or absence of *Apis mellifera* venom PLA2, with increasing concentrations of venoms (A) and increasing K562/T cell ratio (B). Representative data for one donor (C347) of three are shown. Data were mean of triplicate measurements ± SEM.

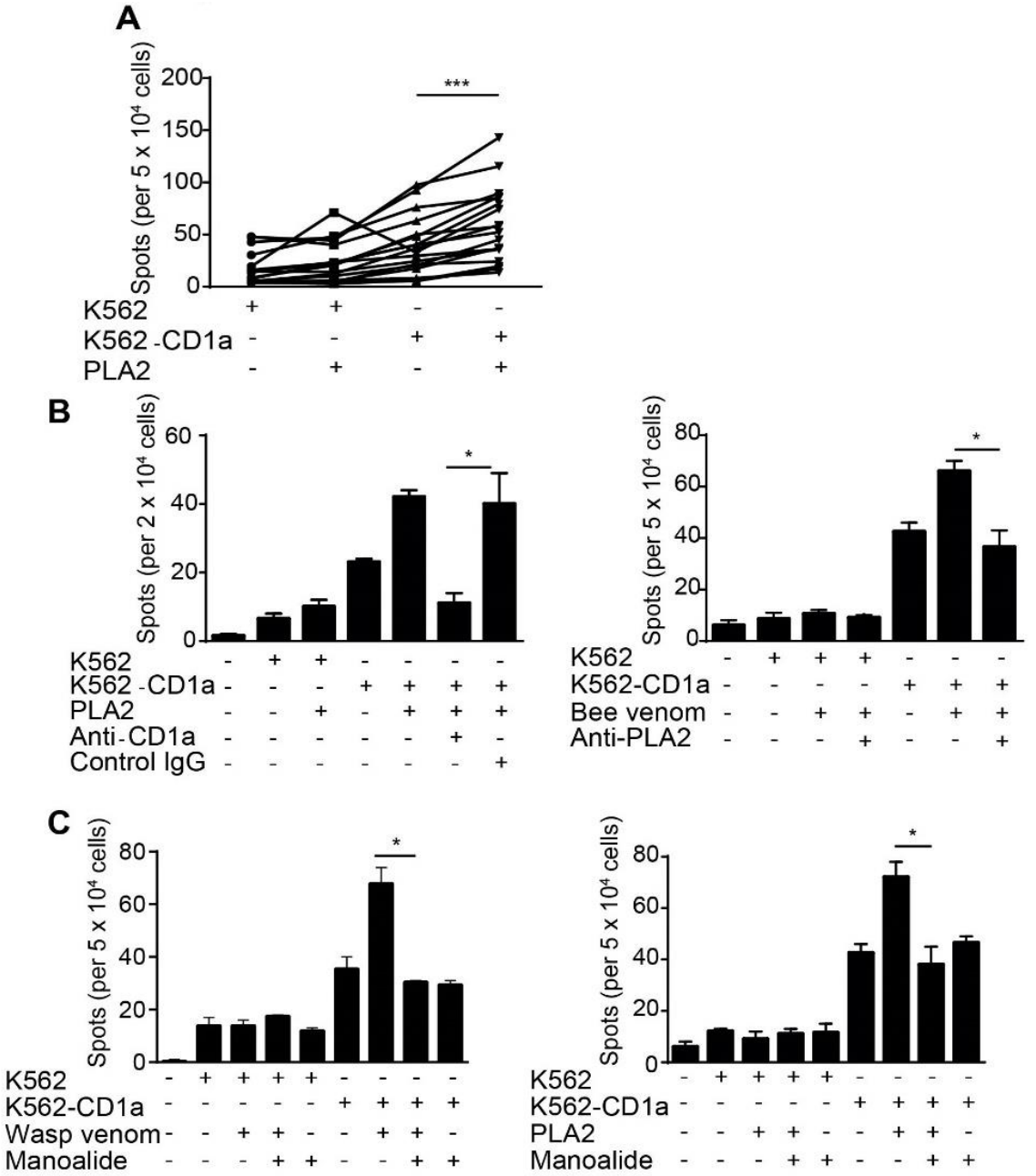


Figure 4-12: *Apis mellifera* venom PLA2 induce a preferential activation of CD1a-restricted T cells among the group1-CD1 reactive cells.

T cells were isolated by CD3 MACS beads from healthy donor PBMCs and cultured for 12-14 d with IL-2 and irradiated K562 transfected with CD1a (K562-CD1a) or an empty vector (K562) in the presence of *Apis mellifera* venom PLA2. CD1 a reactivity was then examined by IFN-γ ELISpot with transfected or untransfected K562 cells either in the presence of absence of *Apis mellifera* venom (1µg/ml). Venom PLA2 specific CD1a-restricted T cell responses were examined in the presence or absence of anti-CD1a for donor C1098, and anti-PLA2-neutraising antibodies, and manoalide (specific PLA2 inhibitor) for wasp venom in donor C559 and venom PLA2 in donor C334. CD1a-restricted PLA2-specific responses were measured in 18 donors for *Apis mellifera* venom PLA2 (B). Data were mean of triplicate measurements ± SEM. * $P < 0.05$, ***$P<0.001$.

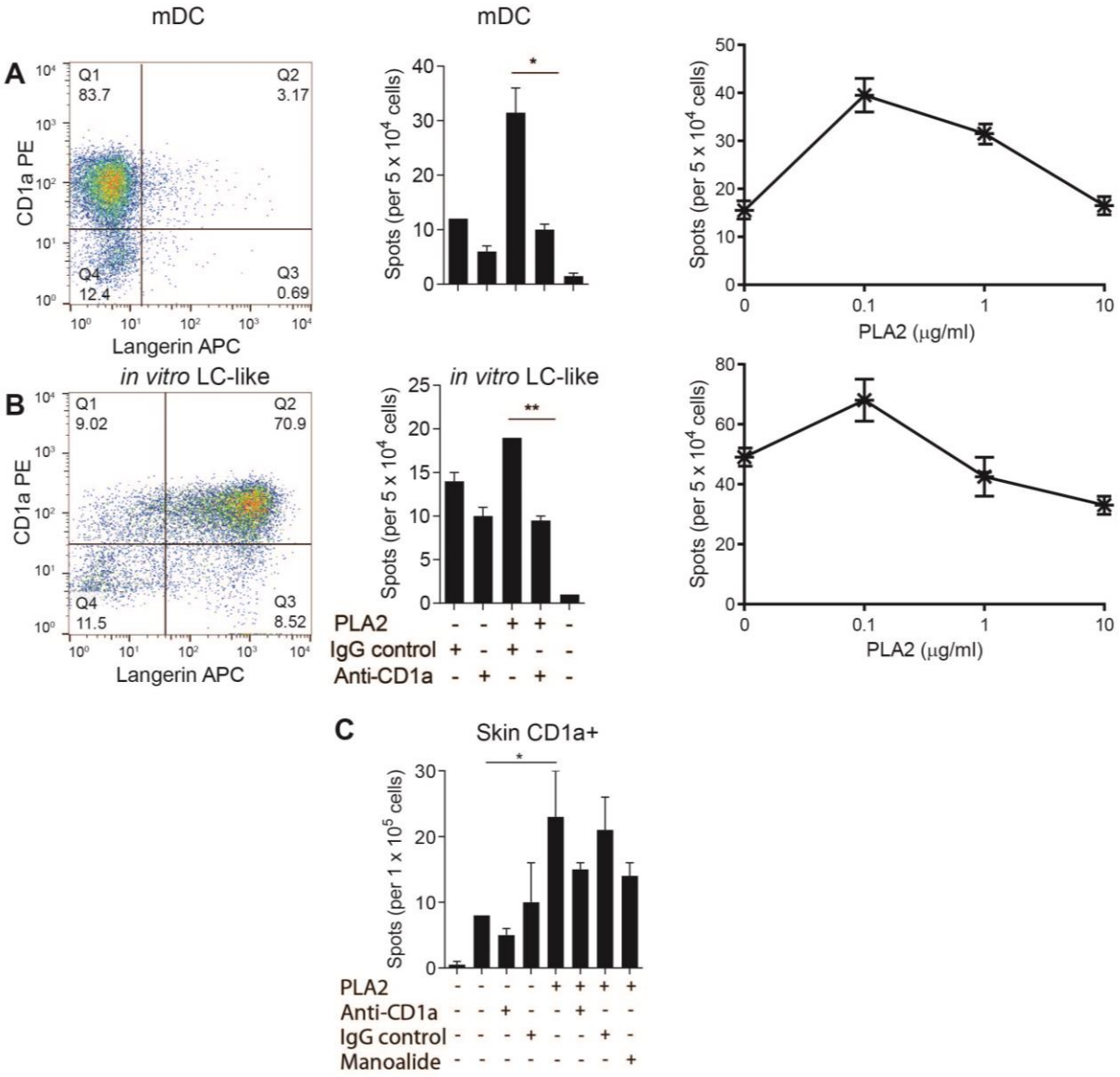


Figure 4-13: mDC and LC-like cells mediate T cell reactivity to *Apis mellifera* venom PLA2.

Monocyte-derived DC (mDC) (A) or *in vitro*-LC-like cells (B) derived from CD14+ cells, or freshly isolated skin CD1a+ cells (C) were pulsed with 100ng/ml *Apis mellifera* venom PLA2 and incubated with the cultured T cells in the presence or absence of anti-CD1a antibody, isotype control or manoalide. IFN-γ production was measured by cultured IFN-γ ELISpot. Representative data from three (A and B) or two (C) are shown. Data were mean of triplicate measurements ± SEM. * P < 0.05, **P<0.01.

4.2.10 PLA2 is the essential component of venom for CD1a ligand generation

Collectively these studies showed that PLA2 was sufficient to recapitulate the response to venom, so we next sought to determine if PLA2 was necessary, or instead whether other active compounds in venom could generate a response. Inhibition of IFN-γ production was observed in response to the venoms treated with neutralizing antibodies that bind PLA2 ($P<0.05$) (Figure 4-12B right panel). Further, using manoalide a blockade of response was noted to wasp and PLA2 to background levels seen with CD1a alone ($P<0.05$, Figure 4-12C) and not to lower levels, which might be expected in the event of non-specific toxicity to cells. Lastly, monocyte-derived DC and LC-like cells derived *in vitro*, as well as $CD1a^+$ cells isolated directly *ex vivo* from skin could induce a response by PLA2-specific polyclonal T cells in a CD1a-dependent and PLA-dependent manner (Figure 4-13). PLA2 did not affect mDC or LC-like cell maturation (Appendix 8.1). We conclude that PLA2 is a necessary factor involved in venom dependent activation of CD1a-reactive T cells.

4.2.11 Skin T cells recognise PLA2 derived lipid antigens in a CD1a dependent manner

Having demonstrated the presence of PLA2 responsive CD1a-restricted T cells in the blood, we sought to determine if they play a role in the skin. Venom antigens are delivered directly into the skin, and the presence of these cells at the site of the sting was investigated. Cutaneous lymphocyte antigen (CLA) is a well-known skin homing marker and when T cells were separated into CLA+ and CLA- fractions, although some autoreactivity was seen in the CLA- fraction, a response to PLA2 was restricted to CLA+ cells (Figure 4-14). Whilst low responses were seen in the absence of CD1a, some CD1a autoreactivity was observed, and these responses were increased in the presence of bee venom ($P<0.05$) or bee venom PLA2 ($P<0.05$) and abrogated by a CD1a blocking antibody (Figure 4-14).

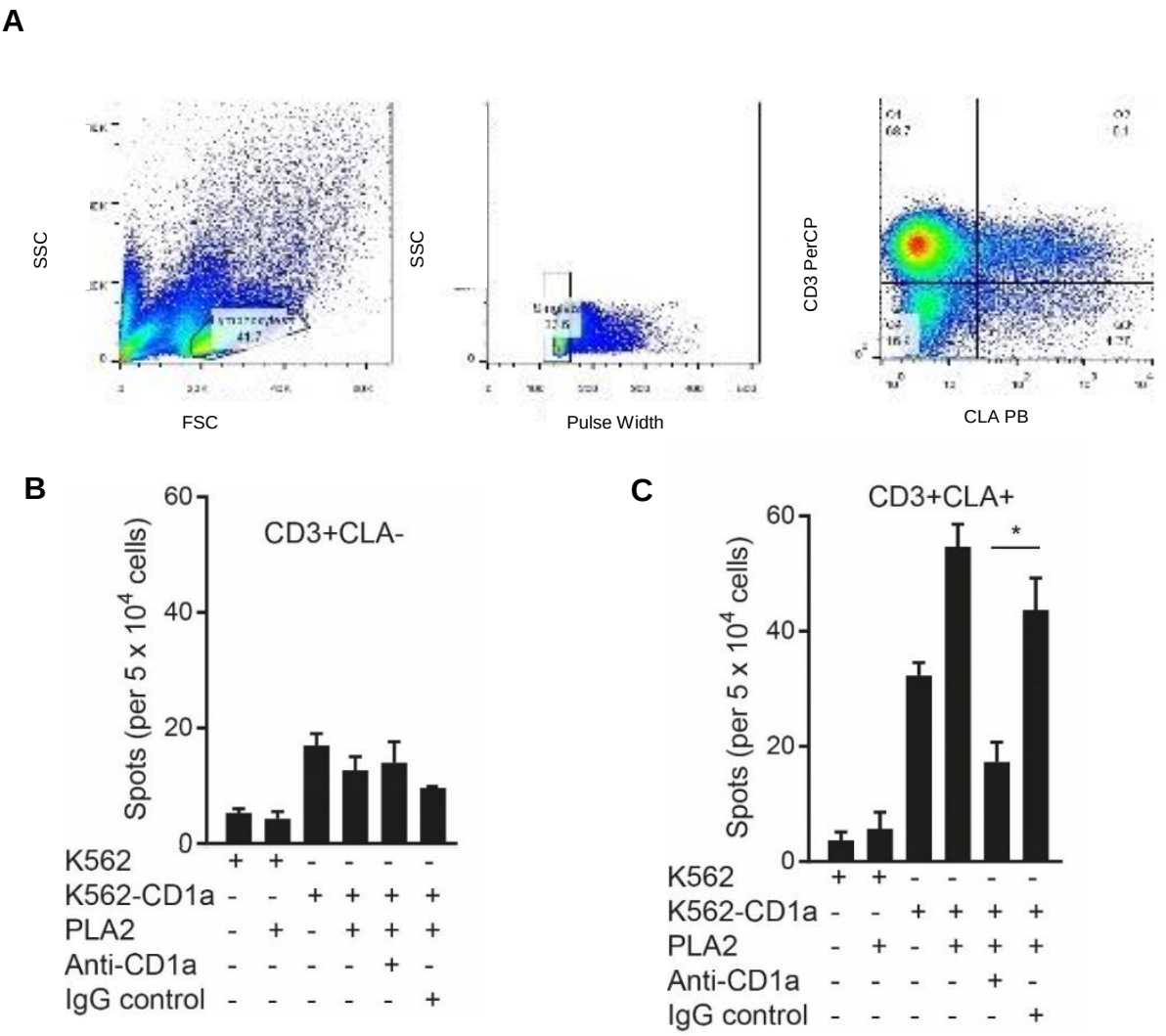


Figure 4-14: CD1a-restricted T cells expressing skin homing markers respond to PLA2.

Following separation (sorting; A) and polyclonal expansion by rapid expansion method to increase cells numbers), CD3+CLA- (B) T cell or CD3+CLA+ (C) T cell response to PLA2 was examined by IFN-γ ELISpot using CD1a transfected K562 cells as APCs in the presence or absence of *Apis mellifera* venom PLA2, anti-CD1a antibody or isotype control. Representative data from 1 of 3 separate donors shown. Data are mean of triplicate results ± SEM. * $P < 0.05$.

T cells isolated from human skin using manual and enzymatic digestion followed by FACS, responded similarly to blood derived T cells in a PLA2 dependent manner (Figure 4-15). This shows that PLA2 responsive CD1a-restricted T cells home to the skin, and can be found in the skin itself, suggesting that they may play a role in the local immune response following a bee or wasp sting, where CD1a levels are high.

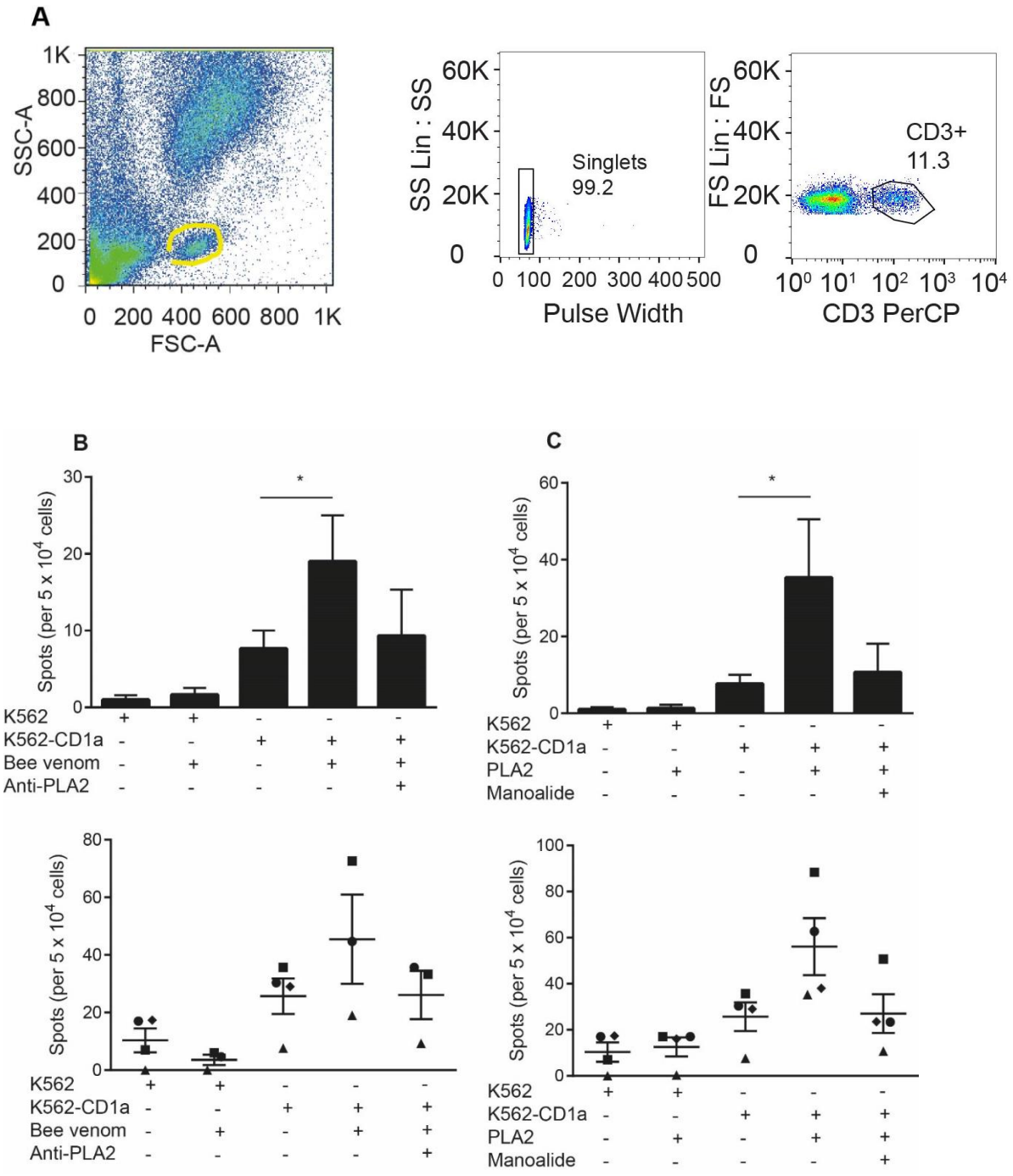


Figure 4-15: CD1a restricted reactivity to Apis *mellifera* venom PLA2 are found in human skin.

CD3+ T cells were isolated (sorting strategy A), following manual and enzymatic digestion of human skin. Following polyclonal expansion to increase cell numbers by rapid expansion method, CD1 reactivity was examined by IFN-γ ELISpot with untransfected (K562) or transfected (K562-CD1a) either in the presence or absence of *Apis mellifera* venom (B) or *Apis mellifera* venom PLA2 (1µg/ml) (C). PLA2 activity was blocked using anti-PLA2 antibody or PLA2 inhibitor manoalide. Representative data from 4 donors shown. Data are mean of duplicate results ± SEM. * $P < 0.05$.

4.2.12 Cytokine profile of CD1a-restricted PLA2 responsive T cells

Previous studies investigating CD1a-restricted T cells have focussed on primarily on pathological conditions, particularly *Mycobacterium tuberculosis* infection (312). They are known to produce common T cell cytokines such as IFN-γ, which was used here initially to identify the presence of these cells in blood and skin. In order to understand the potential effector functions of PLA2 responsive CD1a-restricted T cells, a panel of cytokines were studied following stimulation with CD1a and PLA2, by cytokine bead array, which included cytokines typically associated with Th1, Th2, Th17 and T-regulatory responses. In response to PLA2, T cells upregulated IFN-γ, IL-2 and GM-CSF in a CD1a-dependent manner, which can be considered somewhat promiscuous T cell cytokines. Cytokine bead array data showed that they also produced TNF-α, which has been demonstrated in previous studies in the context of *M. tuberculosis* antigens (Figure 4-16), and has led to the belief that they are implicated in the resolution of TB infection. Interestingly, some Th2 cytokines, IL-5 and IL-13, were also upregulated, suggesting that these cells produce both Th1 and Th2 cytokines. However they produced little IL-17 and IL-10, cytokines associated with Th17 and T regulatory responses respectively.

IL-22 production by polyclonal CD1a-restricted T cells in response to PLA2 was detected by RT-qPCR ($P<0.05$) (Figure 4-17). IL-22 acts on non-haematopoietic epithelial cells, including keratinocytes in the skin, stimulating keratinocyte differentiation, proliferation and survival (222). This provides further evidence for the role of PLA2 responsive CD1a-restricted T cells in the immune response in the skin by influencing the local environment.

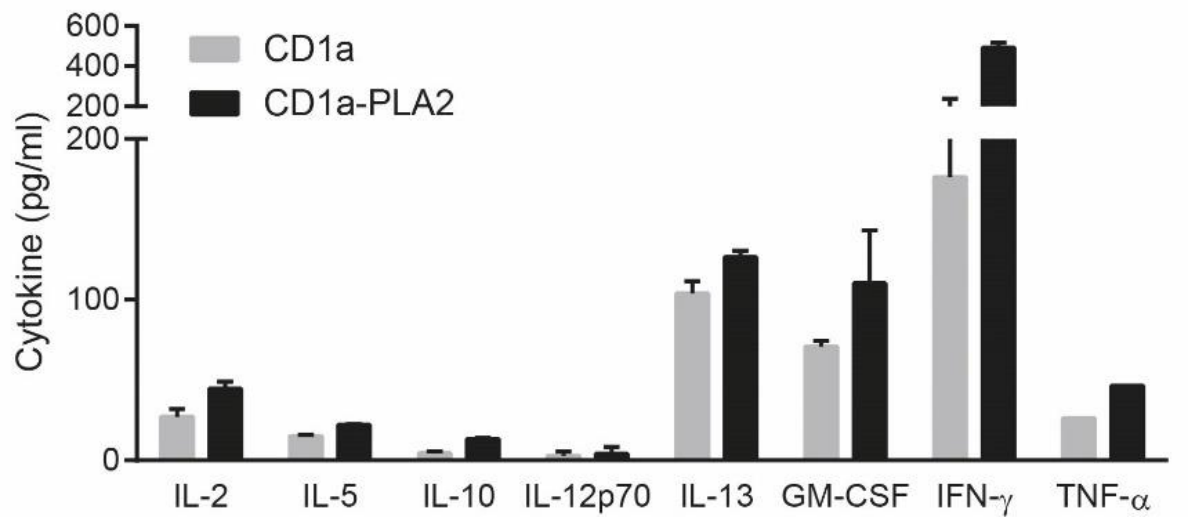


Figure 4-16: Cytokine profile of CD1a-dependent *Apis mellifera* venom PLA2 responsive T cells.

CD3+ T cells were isolated by MACs bead separation from PBMCs and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a in the presence of *Apis mellifera* venom PLA2. CD1a reactivity was then examined by cytokine bead array assay with CD1a transfected or untransfected K562 cells either in the presence or absence of *Apis mellifera* venom PLA2. Representative data from 2 separate donors. Data are mean of duplicate results ± SEM, above the response to untransfected K562.

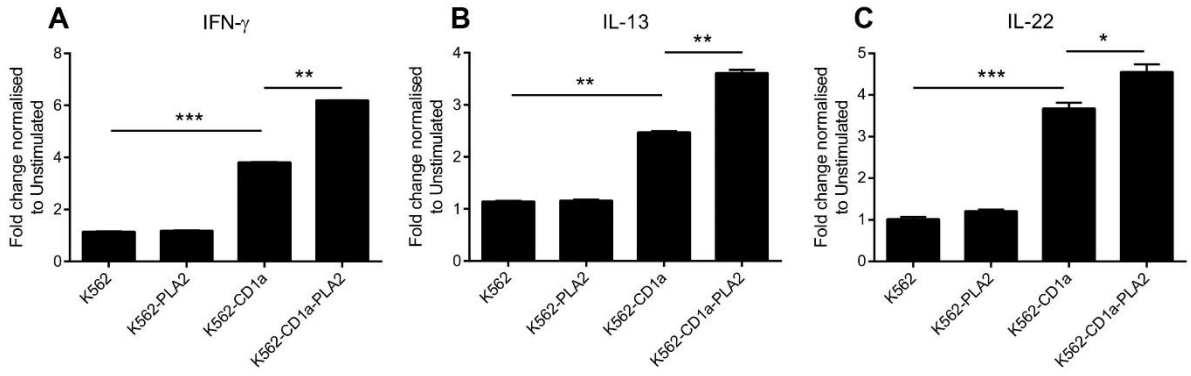


Figure 4-17: IL-22, IFN-γ and IL-13 production by RT-qPCR by CD1a-reactive T cells in response to *Apis mellifera* venom PLA2.

CD3+ T cells were isolated by MACs bead separation from PBMCs and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562 CD1a) in the presence of *Apis mellifera* venom PLA2. Real-time PCR analysis of upregulation of IFN-γ (A), IL-13 (B) and IL-22 (C) encoding genes in T cells, following 6h co-culture with CD1a transfected or untransfected K562 cells either in the presence or absence of *Apis mellifera* venom PLA2, at a K562/T cell ration of 1:10; results were normalised to GAPDH and unstimulated cells. Representative data from one of two donors shown. * P<0.05; ** P<0.01; *** P<0.001.

4.2.13 Venom and phospholipase responsive T cells in cord blood

CD1a autoreactive T cells have been detected in newborns and develop from a naïve phenotype at birth and increase in memory phenotype in adults, indicating age dependent expansion, similar to conventional T cells (66). To determine if the same dynamics are observed with CD1a-reactive T cells responding to venom of PLA2, cord blood and healthy adult blood T cells were isolated and cultured for 12-14 days in the presence of IL-2 and K562-CD1a with *Vespula spp* venom, Apis mellifera venom or *Apis mellifera* venom PLA2, to permit outgrowth of responder cells. CD1a reactivity was then determined by IFN-γ ELISpot in the presence of untransfected or CD1a transfected K562, in the presence of wasp venom, bee venom, or bee venom PLA2. CD1a-autoreactive T cells were detected in cord blood samples as well as healthy adult blood (Figure 4-18A), as described in published studies (66). While these studies showed comparable frequencies of autoreactive cells in adult and cord blood, here a higher frequency was observed in adult blood than cord blood ($P<0.001$) (Figure 4-18). The culture of T cells for 12-14 days prior to ELISpot analysis enriches for CD1a-reactive T cells and may explain the difference to previous studies. Venom (Figure 4-18 B and C) and PLA2 (Figure 4-18D) responsive CD1a-reactive T cells were observed at low frequencies in cord blood and significantly higher in adult blood ($P<0.01$ and $P<0.001$ respectively). This suggests that CD1a-restricted T cells are present at birth but undergo antigen driven expansion with age through increased exposure.

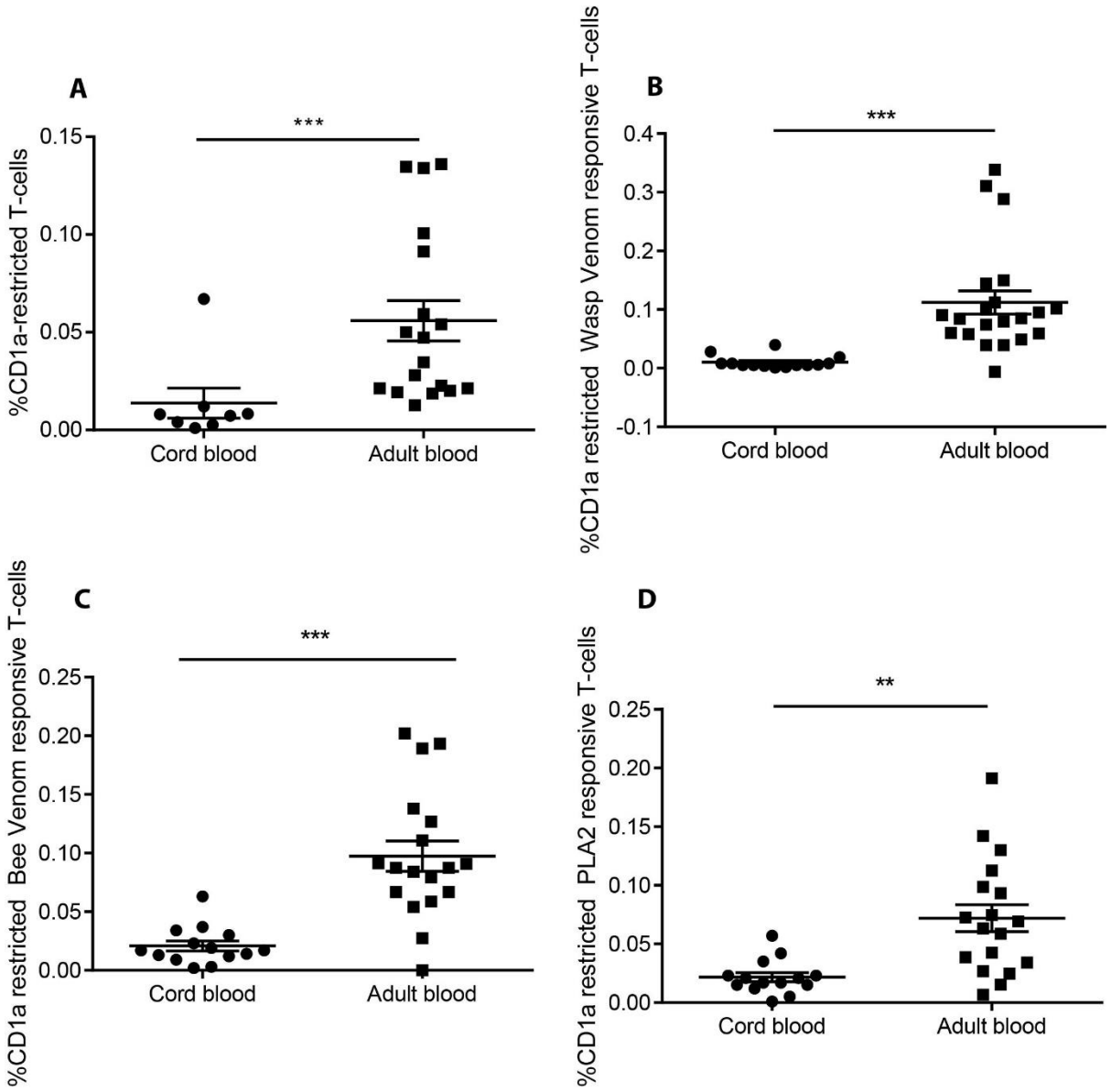


Figure 4-18: Comparison of venom and phospholipase responsive T cells in cord blood and healthy adult blood.

CD3+ cells were isolated by magnetic beads from healthy adult or cord blood PBMC and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562 CD1a) in the presence of *Vespula spp.* venom, *Apis mellifera* venom or *Apis mellifera* venom PLA2. CD1 reactivity was then examined by IFN-γ ELISpot with CD1a transfected or untransfected K562 cells either in the presence or absence of *Vespula spp.* venom, *Apis mellifera* venom or *Apis mellifera* venom PLA2. The frequency of CD1a-autoreactive T cell response above responses to untransfected K562 (A), and CD1a-reactive T cell responses to *Vespula spp.* Venom (B), *Apis mellifera* venom (C) or *Apis mellifera* venom PLA2 (D) above the autoreactive response was compared between adult (n=18) and cord blood (n=14). ** $P < 0.01$, *** $P < 0.001$.

4.2.14 Human phospholipase also generates CD1a lipid antigen recognised by T cells

Phospholipases are widely found in organisms, from insects to mammals. Humans express a broad range of phospholipases, including 16 groups of sPLA2. sPLA2G3 is expressed in the kidney, lungs, heart and PBL, including mast cells, and shares the most homology with bee venom PLA2. Having demonstrated that venom PLA2 induces CD1a-reactive T cell stimulation, we then sought to determine if this was limited to venom PLA2, or could be replicated by host PLA2. IFN-γ ELISpot analysis was performed of polyclonal T cells expanded to enrich for CD1a-reactive bee venom PLA2 responsive T cells as previously described, in the presence of untransfected or CD1a transfected K562 and venom PLA2 or sPLA2G3 (Figure 4-19). As with bee venom PLA2 (Figure 4-3), higher IFN-γ producing cells were detected with the addition of 1µg/ml sPLA2G3 but only in the presence of CD1a. This response was both CD1a and PLA2-dependent as it was abrogated almost down to background levels by blocking CD1a ($P<0.01$) or down to autoreactive levels by blocking sPLA2G3 activity using manoalide ($P<0.05$). This pathway of PLA2 induced activation of CD1a-reactive T cells is therefore not unique to venom PLA2, but can also be generated by host PLA2, suggesting a physiological function in immune homeostasis or dysregulation.

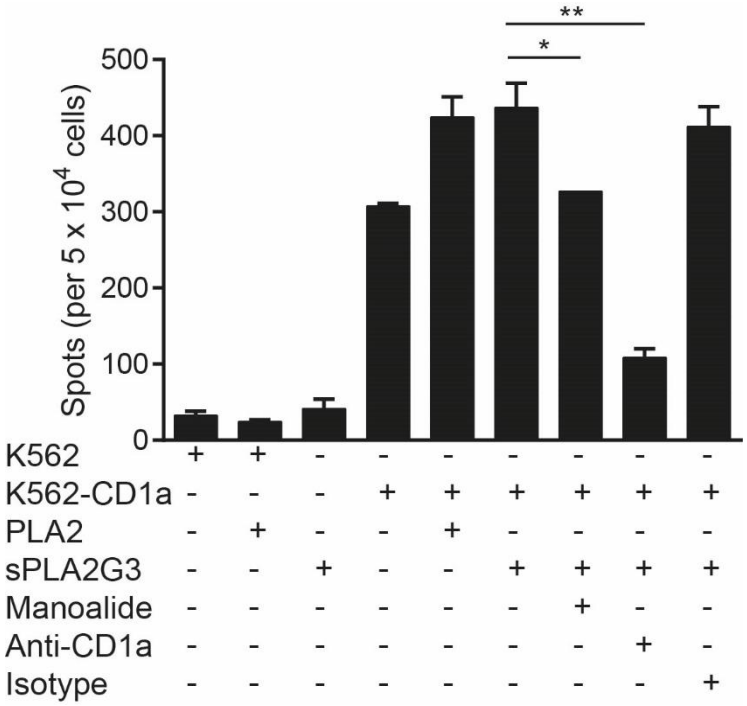


Figure 4-19: CD1a-restricted reactivity to *Apis mellifera* PLA2 is replicated by human sPLA2G3 in polyclonal T cells from blood of healthy donors.

CD3+ cells were isolated by magnetic beads from healthy donor PBMC and cultured for 12-14 days with IL-2 and irradiated K562 cells transfected with CD1a (K562 CD1a) or an empty vector (K562) in the presence of *Apis mellifera* venom PLA2. CD1 reactivity was then examined by IFN-γ ELISpot with untransfected (K562) or transfected (K562-CD1a) cells either in the presence or absence of *Apis mellifera* venom PLA2 or human sPLA2G3 (1µg/ml). CD1a reactivity was confirmed using an anti-CD1a antibody or isotype control, and PLA2 activity was inhibited by manoalide. Representative data from one of two donors are shown. Date are mean ± SEM of triplicate measurements. * $P < 0.05$, ** $P < 0.01$.

4.3 Discussion

Using venoms as a model system, our data identify a new mechanism of *in vivo* antigen processing by which phospholipases cause local alterations in lipid content and conversion of non-antigenic substances to smaller lipids, which have CD1-mediated T cell antigenicity. Specifically, wasp and bee venom phospholipases were identified, which act as the key proteins that are necessary and sufficient to cleave common cell membrane phosphodiacylglycerides, to release free fatty acid and lysophospholipids (34). Polyclonal T cells they may be responding to these and other lipids as well, and it is notable that CD1d presents lysophospholipids to NKT cells (48, 313, 314). CD1a-restricted T cells are present at birth but undergo antigen driven expansion with age through increased exposure, as shown here with increased frequencies in adult versus cord blood. Because the CD1a dependence of lipid-specific T cell responses is observed in polyclonal T cells and among many unrelated human donors, these data suggest that phospholipases are important in CD1a biology, providing a potential molecular pathway underlying previous observations of T cell responses to fatty acids, which may be important for skin barrier sensing and inflammation (34).

Recent studies of CD1a function have found that CD1a autoreactive T cells and CD1a proteins are both abundantly present within adjacent compartments of the skin (34, 65). The presence of bee venom and PLA2 responsive CD1a-restricted T cells in the skin and within skin-homing population of T cells demonstrated here, provides further evidence for these cells in cutaneous immunity. The diverse cytokines produced in response to PLA2, including both Th1 and Th2 cytokines, will affect the local environment in the skin and influence the adaptive immune response generated. Although IL-22 is mostly secreted together with IL-17 by Th17 cells, a recently

described Th22 subset does so in the absence of IL-17. *De Jong et al.* (65) discovered that CD1a autoreactive T cells produce IL-22 in the absence of IL-17, which was also seen here in response to PLA2. Determining the presence and function of CD1a-restricted T cells and PLA2 in IL-22 influenced inflammatory skin diseases, such as psoriasis, will be important in increasing our understanding of the disease pathophysiology.

In general, there exists a physical separation of CD1a proteins, which are mainly expressed on LC in the epidermis, and natural autoantigens such as fatty acids, wax esters and squalene, which are concentrated more superficially in the sebum and cornified epithelium. The observations suggested a near neighbour model in which intact skin prevents direct contact of autoantigens from CD1a, but skin breach through infection or injury deposits antigenic substances more deeply within the skin so that antigens come into contact with CD1a expressing epidermal LC (65, 308, 315). Considering bee or wasp sting as a variant of this model, phospholipases are normally injected up to 2 mm deep into the skin in a process that can be meaningfully mimicked by needle injection (316). Several aspects of these data fulfil predictions that phospholipase injection might lead to antigen generation beneath cornified epithelia in proximity to CD1a proteins. Responses to venom and phospholipase are detected most strongly in response to the particular CD1 isoform (CD1a) that is most extensively expressed in the skin. Also, although free fatty acids could not be detected *ex vivo* by mass spectrometry, phospholipid and lysophospholipid concentrations are demonstrated to change in suction blisters in a process that is dependent on PLA itself. Thus, bee or wasp stings might represent a means to locally remodel lipids and generate neoantigens in proximity to CD1a proteins, mimicking an evolutionarily conserved system of barrier sensing.

Venoms injure and thereby neutralize diverse predators and prey through transfer of toxic substances that act on many types of animals or insects encountered in the wild. Thus, venoms are typically comprised of many toxic substances with parallel actions, and act by co-opting conserved neurological or immunological mechanisms in the stung animal. We speculate that injection of venom PLA represents a means to co-opt, through overstimulation, the natural effects of endogenous mammalian PLAs in barrier sensing and immune response. Mammalian PLA2 enzymes exist as lysosomal, cytosolic and secreted (sPLA2) forms. The sPLA2 superfamily is divided into 16 groups based on disulphide bridge structure and sPLA2 enzymes act in lipid homeostasis, host defence, and inflammation (317). Human sPLA2G3, which has the closest homology to bee venom PLA2 (318, 319), was able to replicate the CD1a-restricted response seen with bee venom PLA2. Other evidence to suggest that mammalian PLAs might have CD1 or lipid mediated effects on T cells includes a recent study of mammalian PLAs from hepatitis virus that activate NKT cells (48, 314) and the role of thymic PLA in controlling NKT cell selection (320). Free fatty acids were identified as natural skin antigens for CD1a earlier this year (34). Collectively, these studies provide multiple lines of evidence that PLAs control various aspects of T cell response in different organs.

The clinical study of bee and wasp sting responses represents a model system to study the basic role of PLAs because it is characterized by single, discreet exposures and there exists practical means to mimic a stinging exposure and study the response *in vivo* and *ex vivo*. Having established a role for CD1a and PLA in the normal response to venom, future studies will likely examine the role in hypersensitivity and immunotherapeutic systems and take advantage of information about

lysophospholipids and free fatty acids as natural pathways that could be considered for diagnosis through skin testing and antigen-based immunotherapy.

5 Wasp and bee venom responsive CD1a-restricted T-cells in venom allergic patients

5.1 Introduction

Vespula (wasp) and apis (bee) stings cause different clinical reactions in individuals, ranging from the expected localised cutaneous inflammation that is directly mediated by venom and seen in virtually all humans, through to systemic host response anaphylaxis and death in venom-allergic individuals (82). The secondary allergic immune responses are mediated by the crosslinking of allergen-specific IgE antibodies bound to mast cells, eosinophils or basophils by allergens, resulting in the release of inflammatory mediators, such as histamine, tryptase and leukotrienes (99). The generation of protein specific IgE antibodies is dependent on specific antigen recognition by T cells, thought to be presented by APC such as dermal DCs and LCs in the skin in the case of venom allergy. Skewing of the peptide-specific T cell responses towards a type 2 response, influenced by cytokines, co-stimulatory signals, and cell-cell interactions, further influences peptide-specific B cell responses and class-switching to IgE production (99). As well as mediating the degranulation of mast cells and basophils, IgE can also mediate antigen presentation, through complex formation of allergen, IgE and low affinity IgE receptor (FcεRII or CD23) (321). This complex is then be internalised and processed and allergen peptides are then presented to CD4+ T cells. The allergic immune response is then driven further, even at low antigen concentrations, with Th2 cell expansion and further B cell class switching to IgE (322). Indeed, venom allergic individuals have elevated frequencies of venom peptide-specific T cells circulating in peripheral blood (120, 293, 323, 324).

During venom-specific immunotherapy, repeated exposure to the venom in a controlled environment leads to allergen desensitization and clinical tolerance

induction. Successful immunotherapy has been associated with a rise in allergen-specific IgG antibodies (IgG4) (325) and a decrease in Th2 cytokines from peptide-specific CD4+ T cells (326). This transition from a Th2 cytokine profile bias affects IgE class switching and eosinophil differentiation and survival, which are Th2 cytokine-dependent (123). Regulatory T cells have also been implicated in successful desensitisation, with an increase in peptide antigen-specific CD4+CD25+ cells and IL-10, potentially facilitating IgG4 production (293, 327). Allergen-specific IgG4 antibodies are also known as blocking antibodies as they compete with IgE for epitope binding, neutralising the allergen, and preventing cross-linking on IgE at mast cell surface. They have been also shown to inhibit facilitated antigen presentation. However the underlying mechanisms by which allergen immunotherapy achieves clinical improvement in allergic patients are still not fully understood.

CD1a is expressed on thymocytes and several APC in the periphery, but most CD1a expression is found in the skin, where epidermal Langerhans cells (LC) express CD1a at particularly high density. Indeed, CD1a has long been used as a marker of LC (280). Polyclonal CD1a-autoreactive T cells are a distinct and normal component of the T cell repertoire and are found in most humans (65, 66). They were identified in both peripheral blood and skin, expressing skin homing markers, such as cutaneous lymphocyte antigen (CLA) (65). Moreover, comparison of CD1a autoreactive T cells to several cell types and organs found that antigenic oils specifically accumulate in the skin (34). The colocalization of CD1a autoreactive T cells, CD1a proteins and CD1a-presented antigens within the skin suggests possible roles in inflammatory skin disease or homeostatic immunity of the skin.

While the recognition of lipid antigens by T cells is well-established in healthy donors (34, 65, 66, 328), the role of CD1-reactive lipid antigens and T cells in disease is largely

unstudied. Nevertheless, CD1-reactive T cells have been implicated in the host defence against pathogens, autoimmune and anti-tumour responses (1, 329). Studies have shown that CD1d-restricted T cells may also contribute to the immune response in allergy. For example, mice deficient in natural killer T cells (NKT) restricted to CD1d, did not develop allergen-induced airway hyper-reactivity (51). Lipids, particularly phospholipids, at the pollen surface were shown to bind CD1d and CD1a and were recognised by T cells with highest responses seen in sensitive subjects at the peak of the allergic season (53). However, most studies have focused on CD1d-restricted lipids, as only CD1d is expressed in mice, but new tools, including MHC^{low} $CD1^{high}$ human APCs now allow cross-sectional clinical studies in humans (65, 328)

Because wasp and bee venom are normally injected near the site of CD1a expression, wasp and bee venom were investigated for the presence of CD1a-presented antigens using T cell clones and polyclonal peripheral blood derived T cells from healthy individuals (328). Venom contains a number of different active proteins and peptides designed to paralyse prey or parasite hosts or deter predators by causing pain. Some of the proteins, including phospholipase and hyaluronidase, are well known to induce protein-specific B and T cell responses leading to clinical allergic reactivity in susceptible individuals. However, in the previous chapter, CD1a autoreactive cells responding to neolipid antigens generated by wasp venom phospholipase A1 (PLA1) and bee venom phospholipase A2 (PLA2) were found within the skin (328). This suggested that T cell responses reactive to CD1a may contribute to the ensuing inflammatory response, but the numbers or role of CD1a-reactive cells has not been investigated in venom allergic disease.

The main aim of this chapter was to detect *ex-vivo* CD1a-restricted responses to wasp and bee venom, and bee venom PLA2, and to characterise these responses in the

blood of allergic and non-allergic individuals. As in the previous chapter, K562 cells with low surface expression of MHC Class I and II, but transfected to express CD1a, were used as APC to study CD1a-reactive polyclonal human T cells. This allowed comparison of polyclonal CD1a-reactive T cell responses to venom in unrelated non-allergic and venom allergic individuals, while minimising MHC alloreactive responses. The frequency and phenotype of these responses based on cytokine production (IFN-γ, GM-CSF and IL-13) were studied to determine if they were involved in the immunopathology of allergic disease. The cross-reactivity of the wasp and bee venom responses were also evaluated, to identify a potential common antigen. Changes in CD1a-restricted responses to wasp venom were investigated over the course of immunotherapy in blood derived T cells (post polyclonal expansion due to limited cell numbers) in 8 allergic individuals. The final aim of this chapter was to determine whether serum from wasp venom allergic individuals post immunotherapy (with elevated venom-specific IgG4) exerted a blocking effect on PLA2 activity and so reduced CD1a responses.

5.2 Results

5.2.1 Increased bee venom-responsive CD1a-reactive T cells in venom allergic individuals

Adult individuals with a history of anaphylaxis to bee venom, and a positive skin prick test or raised bee venom specific IgE antibodies, as well as healthy controls were recruited. To understand the role of lipid antigens in venom allergy, first cytokine production by venom-responsive CD1a-reactive T cells isolated *ex vivo* from our allergic and control cohorts was investigated. Low *ex-vivo* IFN-γ, GM-CSF and IL-13 responses were detected to mock transfected K562s, in the presence or absence of bee venom. In the absence of venom, background responses to K562 cells were detected at a rate of ~6 per 10,000, which is similar to that previously reported (65, 328). T cell autoreactive responses to K562-CD1a in the absence of bee venom were higher than to mock transfected K562, at ~ 1:350 T cells, in agreement with previous studies showing circulation of CD1a autoreactive T cells *in vivo*. This response is thought to be due to recognition of K562-derived lipids (65, 66). However in the presence of bee venom, significantly increased responses were observed only in the presence of CD1a transfected cells, consistent with the conclusion that CD1a mediates the response. Strong evidence for an essential role of CD1a was found when the IFN–γ response were reduced nearly to background levels by a blocking anti-CD1a antibody (OKT6) but not isotype control (Figure 5-1A left panel). Similar patterns of response were seen with GM-CSF (Figure 5-1A middle panel) and IL-13 Figure 5-1A right panel) in response to bee venom and CD1a expressing targets. The frequency of IFN-γ ($p<0.01$; Figure 5-1B left panel), GM-CSF ($p<0.001$; Figure 1B middle panel) and IL-13 ($p<0.05$; Figure 5-1B right panel) responding T cells in the presence of K562-CD1a and bee venom was greater in a panel of bee venom allergic

(BVA) than non-allergic individuals (NA) (Figure 5-1B). These responses show that T cell responses to bee venom are in part mediated by CD1a, and are increased in BVA compared to NA.

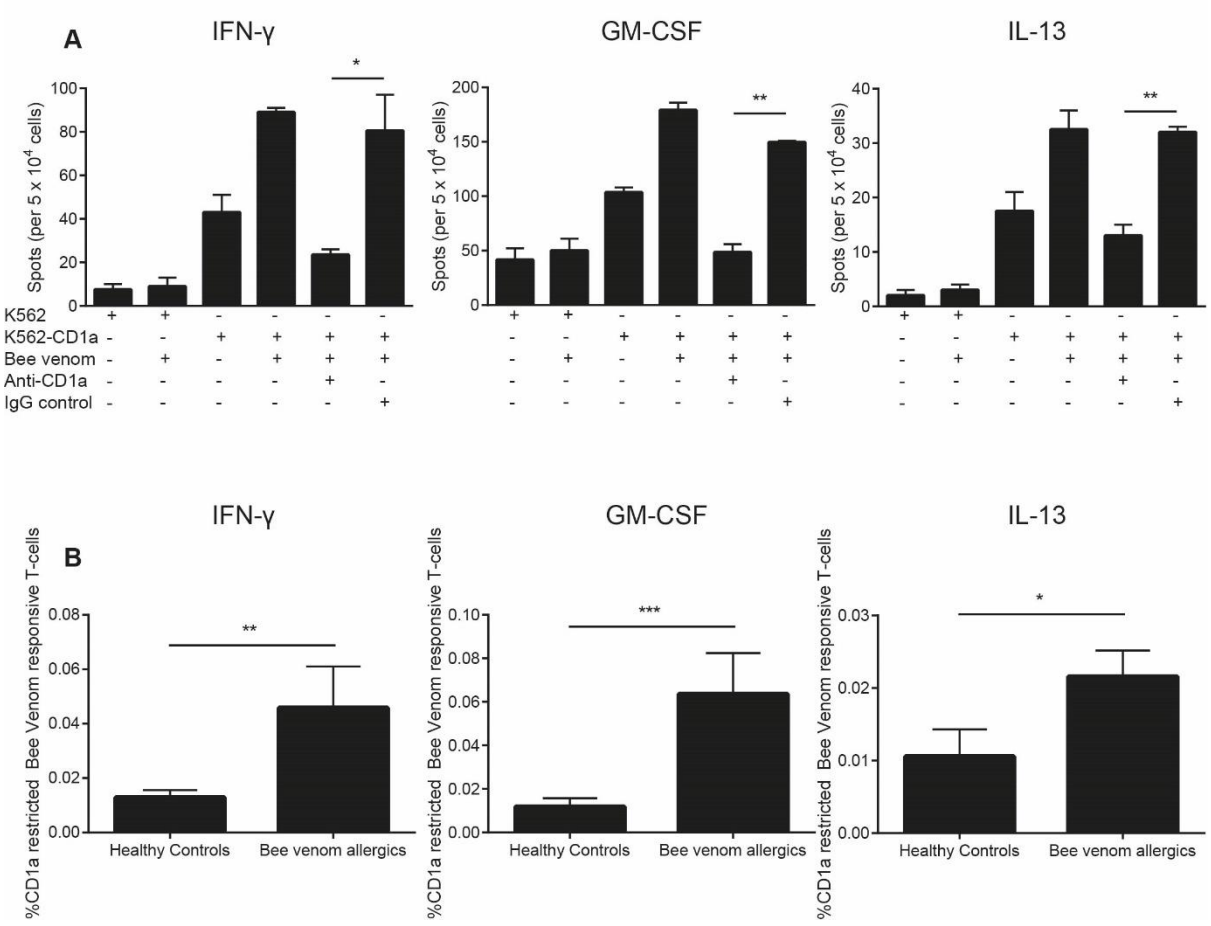


Figure 5-1: Bee allergic individuals show increased levels of bee venom responsive CD1a-reactive T cells compared to non-allergic individuals.

CD3+ T cells were isolated from peripheral blood by magnetic bead separation. (A) CD1a reactivity was examined by ELISpot with K562 or K562-CD1a in the presence or absence of bee venom (1µg/ml) and/or 10µg/ml anti-CD1a mAb (OKT6). Data from 1 donor of 13 are shown and are mean of duplicate measurements ± SEM (B) The frequency of CD1a-reactive T cells responsive to bee venom above the auto-reactive response was compared between non-allergic (n=8) and bee allergic individuals (n=5). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

5.2.2 Bee venom PLA2 reproduces the CD1a-reactive whole venom response in allergic individuals

Phospholipase (PLA) is known to be an important target for peptide-specific T cells in venom allergic individuals (120, 293, 323, 324). Having shown in Chapter 1 that PLA2 in bee venom can generate CD1a lipid antigens for recognition by CD1a-reactive T cells in cultured assays of T cells derived from healthy donors (328), we sought to determine if the increased T cell responses to bee venom in allergic individuals was also generated by PLA2 itself or whether other pathways were important in allergy. In the presence of PLA2 and K562-CD1a, *ex-vivo* T cells produced IFN-γ, GM-CSF and IL-13 (Figure 5-2A). Responses were CD1a-reactive as the T cell responses to PLA2 were abrogated in the presence of a blocking anti-CD1a antibody but not an isotype control (Figure 5-2A). The frequency of IFN-γ (ns; Figure 2B left panel), GM-CSF ($p<0.05$; Figure 5-2B middle panel) and IL-13 ($p<0.05$; Figure 5-2B right panel) producing T cells in the presence of K562-CD1a and PLA2 above the autoreactive response, was greater in bee venom allergic than non-allergic individuals. Thus, the increase in IFN-γ, GM-CSF and IL-13 producing CD1a-reactive T cells in bee venom allergic individuals was similar in magnitude and pattern to that observed with PLA2 and whole bee venom.

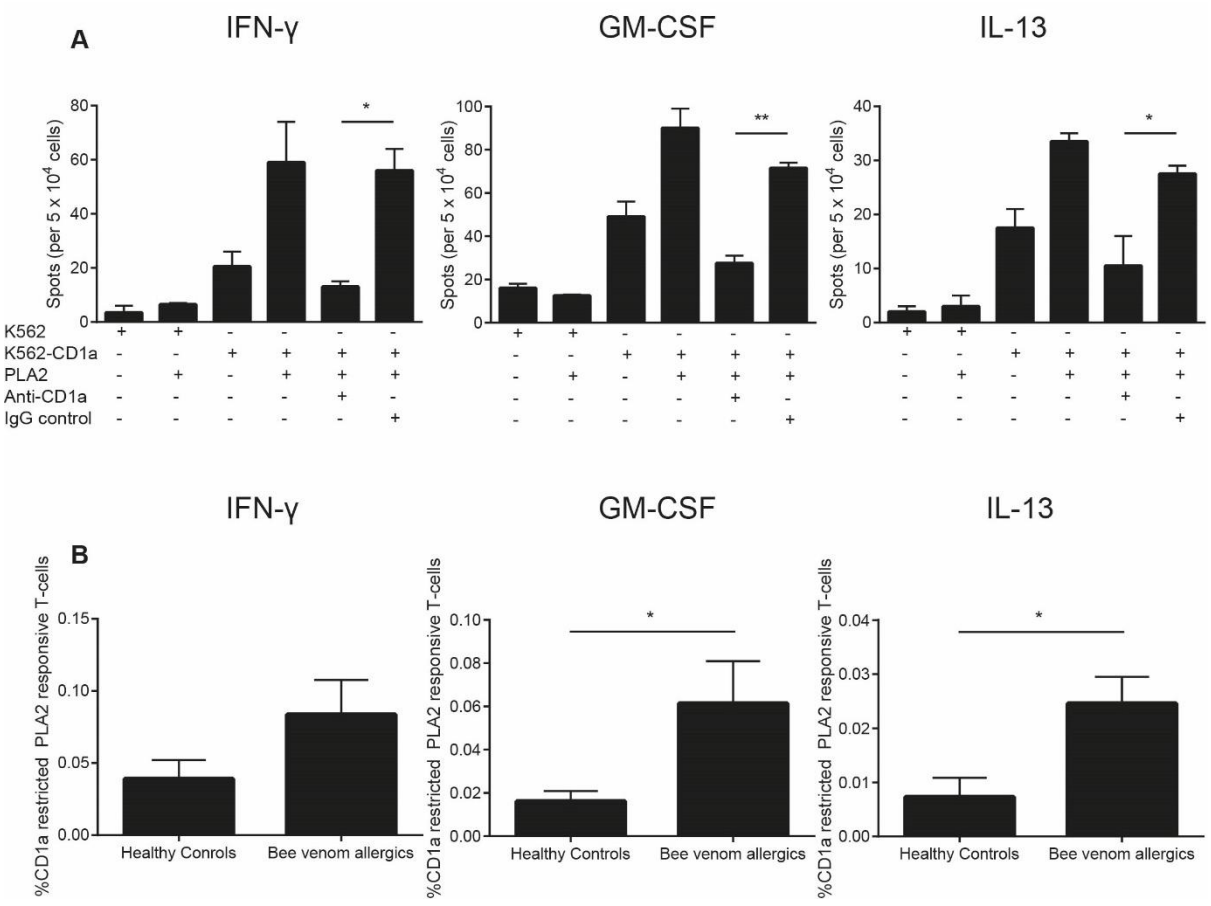


Figure 5-2: Bee allergic individuals show increased frequencies of CD1a-reactive T cell responsive to bee venom PLA2 compared to non-allergic individuals.

CD3+ T cells were isolated from peripheral blood by magnetic bead separation. (A) CD1a reactivity was examined by ELISpot with K562 or K562-CD1a in the presence or absence of bee venom PLA2 (1µg/ml) and/or 10µg/ml anti-CD1a mAb (OKT6). Data from 1 donor of 14 are shown and are mean of duplicate measurements ± SEM. (B) The frequency of CD1a-reactive T cells responsive to bee venom PLA2 above the autoreactive response was compared between non-allergic (n=9) and bee allergic individuals (n=5). * $p<0.05$; ** $p<0.01$.

5.2.3 Increased CD1a reactivity to wasp venom in allergic individuals

Separately, human T cell responses to wasp venom and CD1a were also investigated. Adult wasp allergic individuals with a history of anaphylaxis to wasp venom, and a positive skin prick test or raised wasp venom specific IgE antibodies were recruited. In the presence of wasp venom and K562-CD1a, T cell responses were observed, which were not seen in the absence of CD1a expression, absence of venom or after treating with anti-CD1a blocking antibody. Patterns of IFN-γ, GM-CSF and IL-13 were similar in detailed testing of one individual (Figure 5-3A). As with bee venom allergy, increased in IFN-γ ($p<0.05$; Figure 5-3B left panel), GM-CSF ($p<0.05$; Figure 5-3B middle panel) and IL-13 were all significant ($p<0.05$; Figure 5-3B right panel) when comparing each of these cytokines between a cohort of wasp venom allergic (WVA) and non-allergic individuals (NA). Thus human CD1a-mediated responses to bee and wasp venom are both elevated in allergic individuals and are similar in frequency, strength and cytokine pattern.

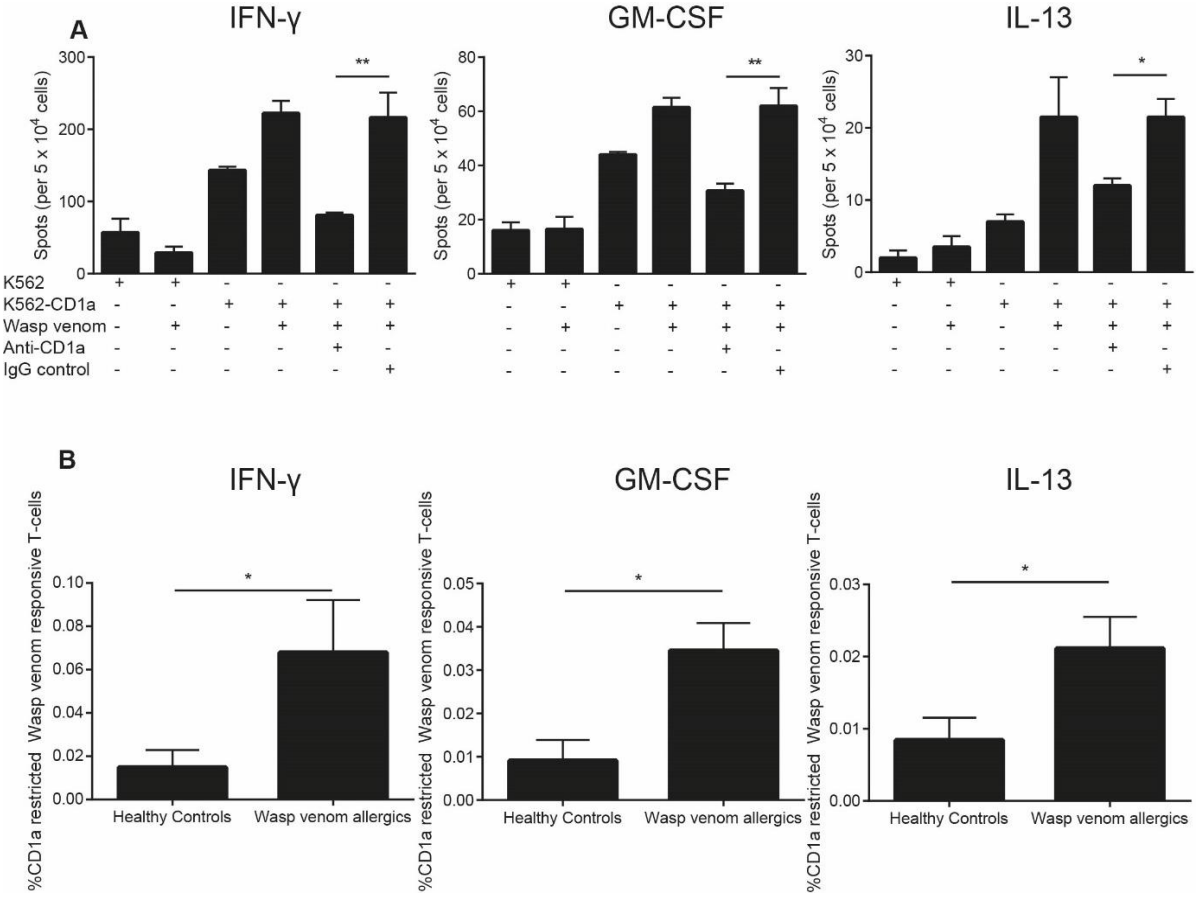


Figure 5-3: Wasp allergic individuals show increased levels of wasp venom responsive CD1a-reactive T cells compared to non-allergic individuals.

CD3+ T cells were isolated from peripheral blood by magnetic bead separation. (A) CD1a reactivity was examined by ELISpot with K562 or K562-CD1a in the presence or absence of wasp venom (1μg/ml) and/or 10μg/ml anti-CD1a mAb (OKT6). Data from 1 donor of 18 are shown and are mean of duplicate measurements ± SEM. (B) The frequency of CD1a-reactive T cells responsive to wasp venom above the auto-reactive response was compared between non-allergic and (n=9) wasp allergic individuals (n=9). * $p<0.05$; ** $p<0.01$.

5.2.4 Cross reactivity of wasp and bee venom responsive CD1a- reactive T cells.

Prior studies completed outside the context of CD1a have shown that individual patients with allergy to bee or wasp venom show a predisposition to show cross-sensitisation and cross-reactivity (330). To investigate whether wasp and bee allergic individuals respond to the same ligands presented by CD1a, we cultured CD3+ T cells from adult individuals with wasp-venom pulsed, bee venom-pulsed or bee PLA2-pulsed and irradiated K562-CD1a APCs for 10-14 days. The resulting T cell lines derived with one antigen were then tested for cross-reactivity to all three antigens. T cells cultured in the presence of wasp venom and CD1a were enriched for wasp venom responsive CD1a-reactive T cells. However these cells were also able to respond to bee venom and bee PLA2. (Figure 5-4A left panel). Similarly, T cells cultured in the presence of bee venom (Figure 5-4A middle panel) or PLA2 (Figure 5-4A right panel) and CD1a were also able to respond to wasp venom.

The simplest explanation is the T cells lines are responding to the same or similar antigens in each case. This favoured interpretation is also supported by a prior study, which dissected the role of wasp and bee venom PLA (328). This study and other studies of the specificity of wasp and bee venom PLA, demonstrated that PLA acts to generate free fatty acids and lysophospholipids (328), both of which are known as antigens for CD1a or CD1d (32, 237). Last, it is notable that the absolute response to CD1a of human T cell lines generated by repeated exposure to CD1a is higher (Figure 5-4), and in some cases more than 10-fold higher than seen directly *ex vivo* (Figure 5-1-3). This result is not expected if venoms are providing foreign lipid antigens but is expected if T cells are responding to self-lipid antigens that are more efficiently liberated by wasp-derived enzymes.

Separate studies of *ex vivo* polyclonal T cell responses among allergic individuals found that T cells from wasp venom allergic individuals (Figure 5-4B left panel) responded to wasp venom, bee venom and bee PLA2 and *vice versa* (Figure 5-4B, right panel). Having shown that bee PLA2 can reproduce the response seen to whole venom, this further suggests that wasp and bee venom responsive CD1a-reactive T cells may respond to the same ligands, generated by PLA2.

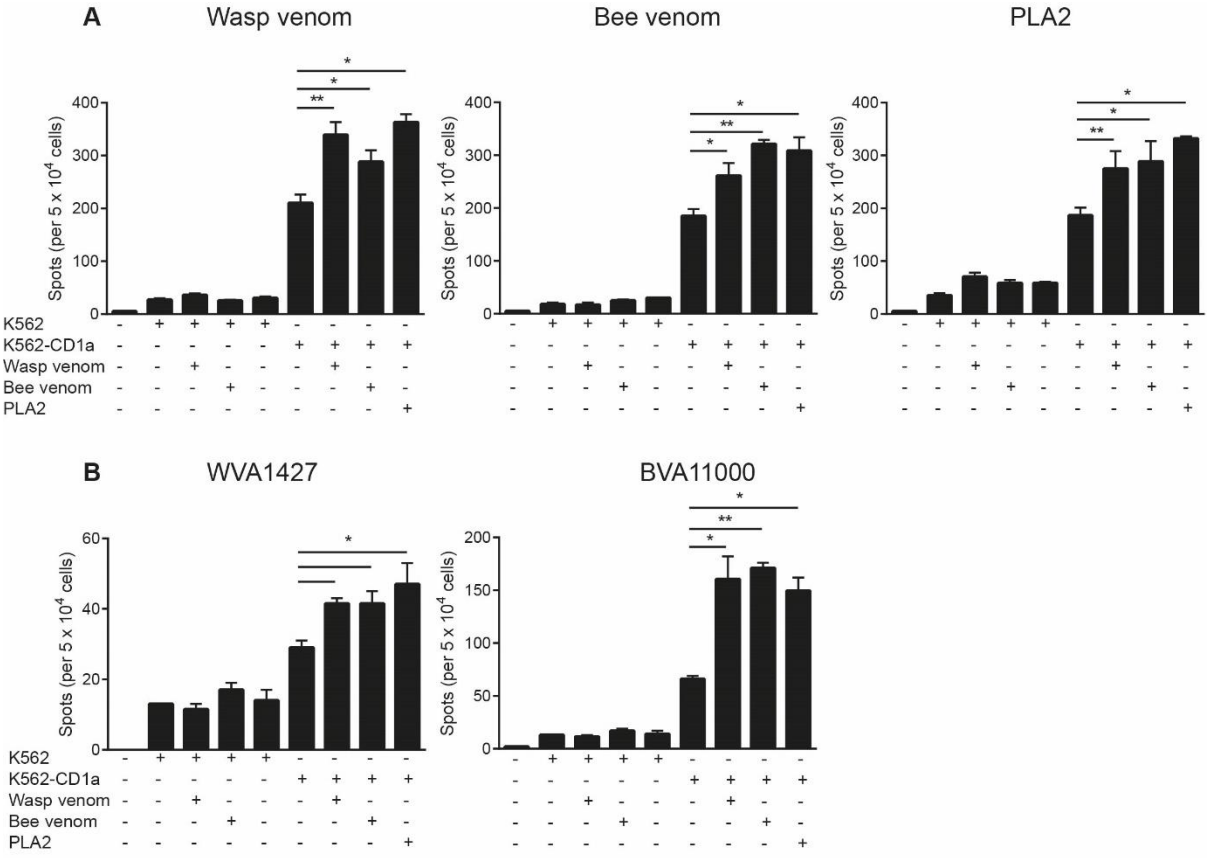


Figure 5-4: Cross-venom CD1a-reactive T cell responses.

T cell lines were generated by culturing CD3+ T cells from healthy adult human peripheral blood for 10-14 days in the presence of wasp venom-pulsed, bee venom-pulsed or bee PLA2-pulsed irradiated K562-CD1a cells. (A) CD1a reactivity of antigen enriched T cell lines from wasp venom allergic (left panel) or bee venom allergic (middle and right panels) individuals was examined by IFNγ-ELISpot in response to K562-CD1a cells in the presence of wasp venom, bee venom or bee PLA2. (B) CD1a reactivity of *ex-vivo* T cells from a wasp (WVA) and a bee venom allergic (BVA) individual was examined by IFNγ-ELISpot in response to K562-CD1a cells in the presence of wasp venom, bee venom or bee PLA2. Data are representative of 3 donors and the mean of duplicate measurements ± SEM. * $p<0.05$; ** $p<0.01$.

5.2.5 Venom immunotherapy modulates CD1a- reactive T cell frequency and function

Subcutaneous immunotherapy is a highly effective treatment for patients with systemic allergic venom reactions (99). However, the underlying mechanisms are still not fully understood. Having shown that CD1a-reactive T cells respond to venom-derived antigens, their involvement in clinical tolerance induction and desensitisation was investigated. T cells isolated from peripheral blood of 9 wasp venom allergic individuals were analysed, taken at weekly intervals for 8 weeks, just prior to antigen administration. Polyclonal T cell responses were examined by ELISpot analysis of IFN-γ release to K562 or K562-CD1a in the presence or absence of wasp venom. Initially, with increasing allergen dose, we observed induction of IFN-γ producing wasp venom responsive CD1a-reactive T cells ($p<0.05$), reaching a peak at week 5 of immunotherapy (Figure 5-5A). However the frequency of the cells then fell by week 6 and reached pre-immunotherapy levels by week 8 (Figure 5-5A), despite continued increases in antigen dose administered. IL-13 and IL-10 secretion was measured in ELISpot supernatant by ELISA. A similar increase in IL-13 levels was detected above the autoreactive response, in the presence of K562-CD1a and wasp venom, peaking at week 4-5 ($p<0.05$), and falling by week 8 (Figure 5-5B), suggesting the loss of functional Th2 responses. IL-10 secretion by wasp venom responsive CD1a-reactive T cells did not significantly vary over the course of immunotherapy (Figure 5-5C). Previous published studies from the same cohort showed that the successful immunotherapy associated with wasp venom-specific IgG4 production and induction of wasp venom peptide-specific T cells at similar frequencies to the lipid-specific T cells presented herein (293). Overall these data demonstrate that CD1a-reactive T-cell responses to wasp venom are initially induced during immunotherapy, but IFN-γ

and IL-13 responses are reduced towards the end of immunotherapy despite increasing antigen dose.

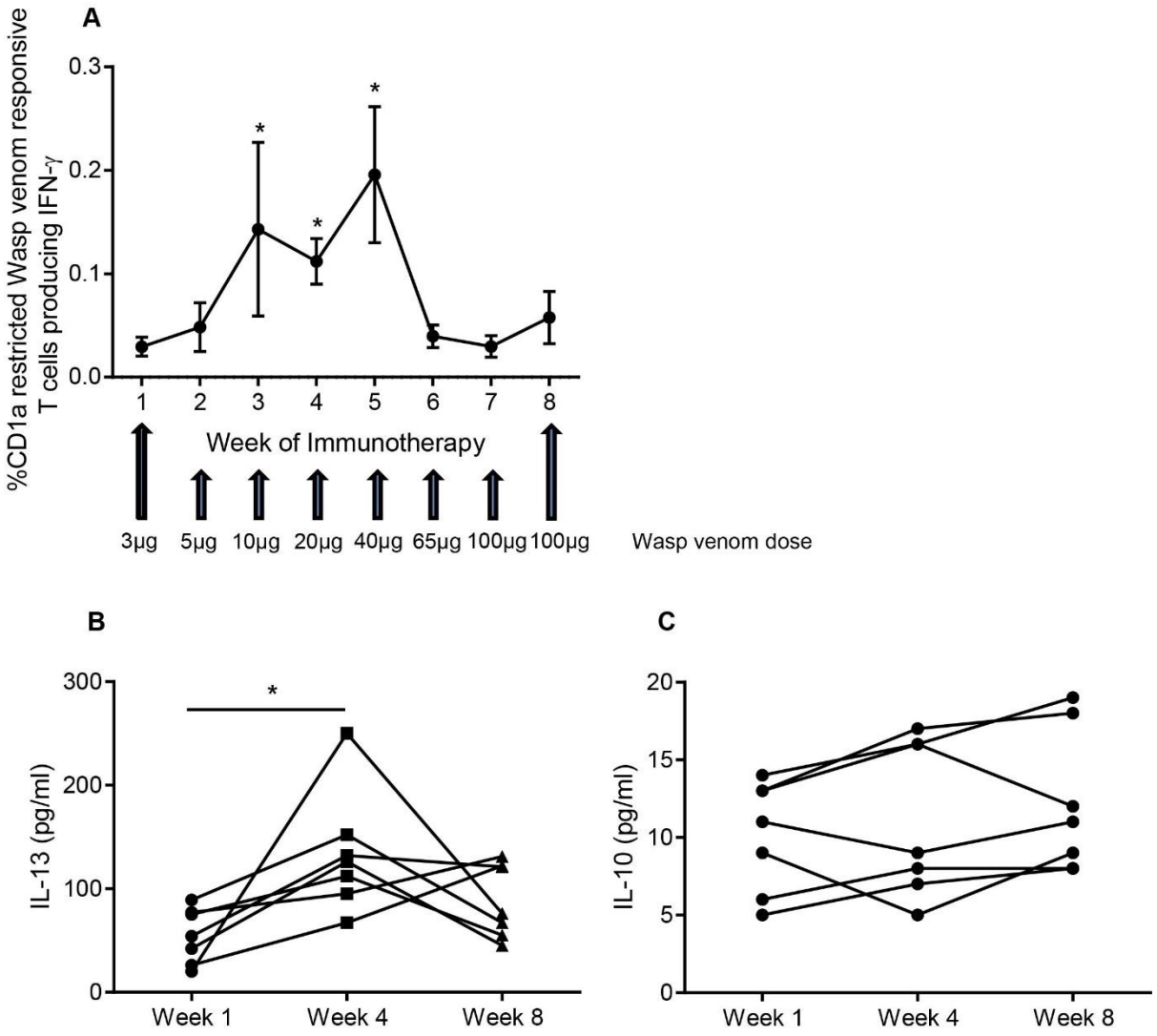


Figure 5-5: Longitudinal cytokine responses to immunotherapy in wasp venom allergic patients.

CD3+ T cells were isolated from peripheral blood by magnetic bead separation from wasp venom allergic individuals (n=21), following polyclonal PBMC expansion. (A) Polyclonal wasp venom responsive CD1a-reactive T cell frequencies were measured in wasp venom allergic individuals by IFN-γ ELISpot. IL-13 (B) and IL-10 (C) secretion in response to wasp venom by CD1a-reactive T cells were measured by ELISA.

5.2.6 Effect of serum from wasp venom allergic individuals post immunotherapy on PLA2 activity and CD1a reactivity

The induction of clinical tolerance through venom immunotherapy is associated with a rise in allergen-specific IgG4 blocking antibodies. The blocking antibodies have a protective effect, modulating the allergic immune response (132, 325). Since an increase in blocking antibodies to wasp venom PLA (Ves v 1) has been demonstrated post immunotherapy (293, 325), we sought to determine if these antibodies may also have a blocking effect on PLA2 activity and PLA2 induced CD1a-reactivity, resulting in decreased responsiveness post immunotherapy. The effect of serum from WVA post immunotherapy (week 12) or non-allergic individuals (NA) on WV PLA2 activity was measured using a biochemical sPLA2 activity assay, as described earlier (Figure 5-6A). An increase in PLA2 activity was observed in the presence of patient serum (both NA and WVA) compared to WV alone, confirming that PLA2 is found in human serum, as reported in previous studies (331). However, there were no significant differences in PLA2 activity in the presence WVA compared to NA serum, either at neat or 1:2 dilution of serum (Figure 5-6A), or throughout the course of immunotherapy (Figure 5-6B), suggesting that blocking antibodies to WV PLA does not inhibit PLA activity. This was confirmed using patient serum in a cellular assay. T cell lines were used to investigate WV induced CD1a reactivity by IFN-γ ELISpot in the presence or absence of NA or WVA (post immunotherapy; week 12) serum. However no significant differences were detected in the CD1a-restricted response in the presence of NA compared to WVA serum, in the presence or absence of WV (Figure 5-6C). This suggests that IgG blocking antibodies to WV PLA do not block PLA activity or affect PLA ability to generate CD1a ligands.

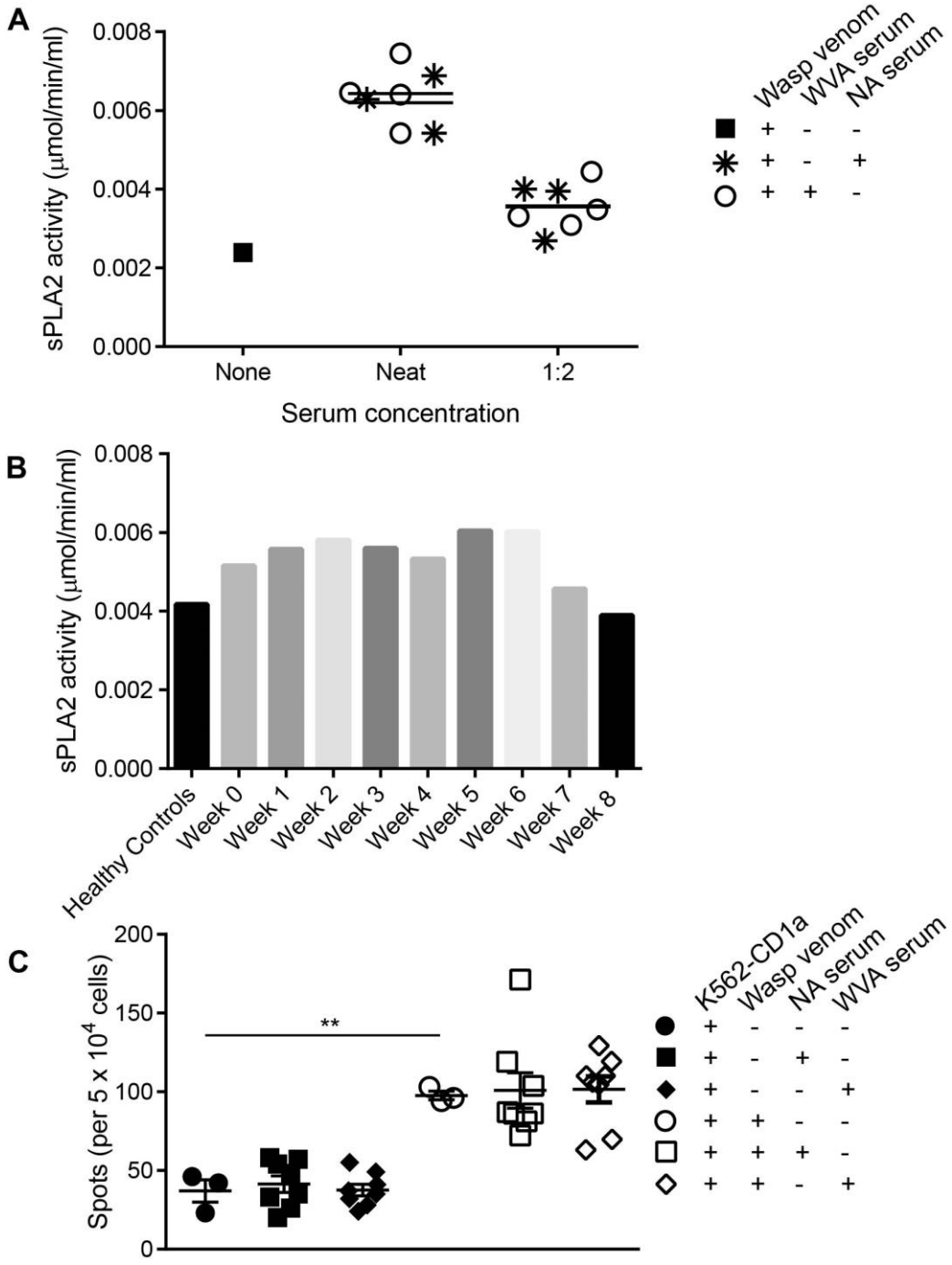


Figure 5-6: Effect of serum from wasp venom allergic individuals post immunotherapy on PLA2 activity and wasp venom responsive CD1a reactivity.

sPLA2 activity was measured in wasp venom in the presence or absence of serum (at 2 dilutions) from non-allergic (NA; n=4), wasp venom allergic individuals (WVA; n=4) post immunotherapy (A), as well as from WVA throughout immunotherapy (n=12) (B). The effect of serum from allergic patients on the CD1a restricted response to wasp venom was evaluated by IFN-γ ELISpot of CD3+ T cells from 3 healthy adults peripheral blood, cultured for 10-14 days in the presence of wasp venom-pulsed irradiated K562-CD1a cells (C). ELISpots were performed in serum-free media, other than added patient serum. ** P<0.01

5.3 Discussion

Venom allergy is an important cause of anaphylaxis, with a prevalence of 0.3-7.5% in the population (80) and has been studied as a model of hypersensitivity and inflammation. Venom protein- and peptide-specific responses have been relatively well characterised in venom allergy (293), and allergic individuals are known to have raised levels of allergen-specific Th2, B cells and IgE (332, 333), with infiltration of allergen-specific T cells in the skin and increased production of Th2 cytokines (333). Having shown in Chapter 1 that CD1a-reactive T cells from blood and skin of healthy individuals respond to wasp and bee venom (328), this chapter shows that venom allergic individuals have a higher frequency of venom responsive IFN-γ, GM-CSF and IL-13 producing CD1a-reactive T cells. IL-13 is associated with type 2 immune responses and allergic inflammation, particularly through the promotion of IgE class switching, which may be one way in which CD1a-reactive T cells promote allergic disease. In the skin, IL-13 upregulates keratinocyte expression of matrix metalloprotease (MMP) 9 and chemokine ligand (CCL) 22 (334, 335). MMP is involved in tissue remodelling and so facilitates infiltrating cell migration (334). CCL22 acts as a chemoattractant for T cells expressing chemokine (C-C motif) receptor (CCR) 4. CCR4 is predominantly expressed on Th2 cells (336) and is important for skin infiltration in disease (337). Indeed, IL-13 stimulated keratinocytes were shown to preferentially attract CCR4+CD4+ cells (335).

GM-CSF has a wide range of different effector functions, altering T cell responses directly or indirectly, by influencing APC. In the skin, GM-CSF is produced by and influences many cells, including enhancing keratinocyte growth and LCs recruitment and maturation (338, 339). It can therefore act as a link between the innate and adaptive immune response, and so could contribute to the allergic inflammation in

venom allergy. IFN-γ is a multifunctional cytokine that is important in skin inflammation (340, 341). It is known to upregulate keratinocyte expression of human leukocyte antigen, cell adhesion proteins and chemokines. This is further enhanced by Th2 cytokines IL-13 and IL-4 (335), facilitating leukocyte infiltration and peptide presentation, allowing IFN-γ to further drive the allergic response. GM-CSF is also used in the culture of mDC and *in vitro* LC-like cells from $CD14^+$ PBMCs to upregulate CD1a expression.

Insects inject venom to kill prey, parasitize or repel predators and so contain different toxic substances, including PLA. Venom PLA injected into the skin cleaves phospholipid substrates from the skin or from venom itself (previous chapter and (328)). Products of venom PLA activity, including free fatty acids and lysophospholipids, were recently shown to bind CD1a, found on nearby LCs in the epidermis (32, 34, 328) and indeed the skin is abundant in CD1a-binding lipids (34). These neolipids generated by PLA activity are recognised by CD1a-reactive T cells (previous chapter and (328)). PLA2 responsive CD1a-reactive T cells in BVA were shown in this chapter to be increased compared to NA. Through the initiation of IL-13, GM-CSF, IFN-γ secretion and potentially other cytokines, PLA2 may help drive the allergen peptide-specific Th2 response and protein-specific IgE response. Indeed elegant studies have shown that PLA-derived peptides represent an important target for T cells from venom allergic individuals and can be used to modulate clinical reactivity (120, 323, 324).

CD1a expression is highly restricted to certain tissues, with the most abundant expression in the epidermis. Natural ligands for CD1a, such as wax esters, free fatty acids and squalene are found in the stratum corneum, separating antigen presenting molecule and ligand under steady state conditions. Therefore, current models (65)

emphasize local release of self-antigens that can augment T cell responses to CD1a, suggesting that venom injected into the skin triggers the cleavage of intact self-phospholipids into activating forms. The identity of antigens was solved in other studies (32, 34, 328) but these results do show that treatment of T cells with active venoms increases the observed CD1a reactivity.

In considering the cross reactivity among insect species, having found in Chapter 4.2.8 (Figure 4-9A) that in addition to having its well-known PLA1 activity, wasp venom PLA also has a degree of PLA2 activity so that the two venoms would be expected to have similar effects on host phospholipids (328). CD1a-reactive T cells responding to WV were shown here to also recognise BV and *vice versa*. This further suggests that wasp and bee venom PLA may act on similar substrates and generate similar CD1a reactive lipid antigens, resulting in a cross-venom CD1a-reactive T cell response. Indeed it is known that some individuals with bee venom allergy are also allergic to WV suggesting that common pathways of allergenicity may be involved (330). This raises the possibility that future diagnostic or immunotherapeutic approaches may target the shared CD1a-reactive response to modulate the subsequent allergic process and provides a specific rationale to consider PLA and the lipid products of PLA, namely fatty acids and lysolipids, as immunodiagnostic and immunotherapeutic targets. Having established raised frequencies of WV responsive CD1a-reactive T cells in venom allergy, further studies could focus on their influence on the peptide specific immune response, including allergen specific B cells and IgE production.

Immunotherapy provides an opportunity to understand the mechanisms involved in generating and maintaining long term clinical tolerance. This is likely to be important in the future treatment of a number of immune disorders, including allergy and autoimmunity (342, 343). Within days of commencing immunotherapy, a decrease in

mast cell and basophil activation and degranulation has been reported (124, 344). Allergen-specific effector T and B cells shifting towards a regulatory phenotype appears to be important, inducing effector T cell tolerance, decreasing pro-inflammatory cytokines from mast cells, decreasing eosinophil function and activity, and directing allergen-specific antibody isotype from IgE to IgG4 (99, 327). During the first 4 weeks of immunotherapy the induction of wasp venom responsive CD1a-reactive T cells producing IFN-γ and IL-13 was observed, providing further evidence that CD1a-reactive T cells respond to wasp venom and are generated through antigen exposure. However a reduction in IFN-γ and IL-13 producing CD1a-reactive T cells was seen in response to wasp venom from week 4 of immunotherapy despite increasing antigen dose. Whether this reduction in IFN-γ and IL-13 producing CD1a-reactive T cells is sustained beyond week 8 is unknown, however it is noteworthy that it coincides with the induction of clinical tolerance, suggesting that wasp venom responsive CD1a-reactive T cells may play a role in allergic disease. It is possible that wasp venom responsive CD1a-reactive T cells become less responsive or even anergic, with continued exposure to increasing doses of allergen. Although the frequency of IL-10 producing cells did not change, the reduction of IL-13 producing cells may result in greater modulatory effect of IL-10. *Ex-vivo* analysis with greater cell numbers would allow analysis of the IL-13:IL-10 ratio.

Venom immunotherapy is associated with increases allergen-specific antibody isotypes, other than IgE, including IgA, IgG1 and IgG4. Concentrations of IgG4 antibodies to Ves v 1 (WV PLA1) and Api m 1 (BV PLA2) has been shown to correlate with clinical tolerance induction in WVA and number of stings and duration of beekeeping (99, 293). These antibodies bind the same allergens as sensitising IgE antibodies, and have a protective effect through blocking IgE-allergen binding, and so

are termed blocking antibodies. However, the data here suggest that these antibodies do not block the enzymatic activity of the allergen, PLA. Therefore even after induction of clinical tolerance, CD1a ligands are still generated by venom PLA, which can activate T cells, indicating that it is the reduction in frequency and/or phenotype of venom responsive CD1a restricted T cells that may contribute to desensitisation and clinical tolerance.

It is interesting to note that during a sting, venom is delivered directly into the epidermis and dermis where CD1a-expressing cells are present. In contrast, during immunotherapy, venom is delivered subcutaneously, where there are few CD1a-expressing cells. This leads to the prediction that successful immunotherapy may partly depend on site of delivery. This is indeed the case, as subcutaneous or oral delivery of grass pollen lead to systemic clinical tolerance. It is also of note that peptide-based immunotherapy can produce clinical efficacy, consistent with the possibility that it is the lipids that contribute to the allergic process (324). Although there was an increase in frequency of venom responsive T cells in the first few weeks of immunotherapy, it is possible that immunotherapy achieves clinical tolerance in part by bypassing CD1a and therefore modulating the phenotype of the subsequent peptide-specific T cell and IgE responses.

In summary, we have shown an increased frequency of venom responsive CD1a-retricted T cells in allergic individuals, suggesting a role for these cells in allergic disease. The loss of responses seen during immunotherapy despite increasing antigen doses, suggests that CD1a-reactive responses may shape the subsequent peptide-specific and IgE responses. The findings have implications for mechanisms of allergy and of clinical tolerance induction, with relevance to many forms of hypersensitivity. Whereas all current approaches focus on the role of proteins or

peptides in T cell response, these findings support a broad new view in which CD1a and non-peptide antigens could participate in allergy. Because many known allergens are non-specific small molecules, the proof of principle for a role of CD1a in allergic responses could promote generally new approaches to immunodiagnosis and treatment.

6 Characterisation of PLA2 responsive CD1a-reactive T cells

6.1 Introduction and Aims

Since the discovery of their role in antigen presentation, our knowledge of CD1 and CD1-restricted T cells has grown significantly. While we now know that CD1d-restricted T cells are important in bridging the innate and adaptive arms of the immune system and providing rapid response to insults (38), there is still much that is not known about Group I CD1-restricted T cells. Due to a lack of specific markers, studies have previously relied on CD1a-restricted T cell clones to model how they function in the immune system. Increasing our understanding of their phenotype, including surface marker expression, the specific antigens to which they respond, and the quality of the response to stimulation, will in turn shed light on their physiological role.

Studies based on large T cell clone libraries have identified CD1a-restricted T cells in the CD4, CD8 and DN T cell repertoire (65, 66) and in both the α/β and γ/δ T-cell repertoire (53, 65). Interestingly, all γ/δ CD1a-restricted T cells clones to date have been Vδ1+ T cells. Vδ1+ T cells, which are enriched in tissue epithelia, particularly in the intestine, were shown to proliferate and exhibit cytolytic activity in response to self-antigen in the context of CD1c (345).

Investigating how CD1a-restricted T cells respond to stimulation will help to further our understanding of their function. Studies to date have demonstrated proliferation of T cell clones and cytokine production in response to CD1a-antigen (66). The discovery that mycobacterial lipids are ligands for all CD1 isoforms has perhaps led to a bias in the spectrum of cytokines studied, particularly those crucial to the resolution of TB infection. IFN-γ, TNF-α and IL-2 secretion has been detected on stimulation of CD1a-restricted T cells with mycobacterial lipids, as well as self-ligands (41, 289, 296, 312). More recently, *de Jong et al* observed that autoreactive CD1a-restricted T cells homing

to the skin produced a number of different cytokines, including those classically considered as Th1 (IFN-γ), Th2 (IL-13, IL-4) and Th22 (IL-22 in the absence of IL-17) cytokines (65). This study focussed on the polyclonal T cell response and so it is not certain that the same cells produced all cytokines. However, pollen phospholipid-specific CD1a-restricted T cell clones also produced functionally distinct cytokines, IL-4, IL-10. TGF-β and IFN-γ, on stimulation (53). This indicates a very varied role for these cells in different immune responses.

The number of known lipid antigens for CD1a and for CD1a-dependent T cell stimulation has been growing steadily. Mycobacterial lipid, DDM, activation of CD1a-restricted T cells indicates that they are involved in generating a protective immune response against pathogens. On stimulation, they secrete IFN-γ and TNF-α, two key cytokines in resolving mycobacterial infection and granuloma formation, and CD1a deficiency was recently shown to be associated with susceptibility to mycobacterial infection (17, 301). CD1a-restricted T cells also respond to pollen-derived lipid antigens, which were elevated during the allergic season (53). Together with the results from Chapter 5 suggests that they may be involved in the allergic immune response. CD1a-restricted T cells may also have a homeostatic role through the recognition of self-antigens such as sulphatides and oily skin lipids (31, 34). CD1-restricted T cell clones expressing a single TCR were able to respond to both self and foreign antigens, although foreign antigens were more potent agonists then self-antigens (67). Low intensity stimulation by self-antigens were proposed to prime the T cells allowing them to respond rapidly to inflammatory stimuli, in the absence of immunological memory (67). Therefore the discovery of novel CD1a-ligands could contribute to our understanding of the function of the responding cells.

The use of CD1a-restricted T cell clones has been critical to investigate their phenotype and function. Further investigation into the mechanisms of activation, signalling pathways, co-stimulatory molecules and transcription factors will identify potential effector functions. It may also lead to the discovery of a unique marker or markers to identify these T cells from a heterogeneous population. T cell clones are most commonly generated using limiting dilution assays, where a T cell suspension is seeded in the first well of a round-bottom 96-well plate and then double diluted down and across the plate. This is done in the presence of feeder cells (irradiated PBMCs form three donors), IL-2 and PHA. The aims of this chapter however, were to generate PLA2-responsive CD1a-restricted T cell lines. Therefore the IFN-γ secretion assay was used to sort one T cell per well. T cell lines generated were then tested for PLA2 specificity, as well as against other stimuli, and their phenotype was characterised by examining surface marker expression and cytokine production.

6.2 Results

6.2.1 Generation of PLA2 responsive CD1a-reactive T lines

In order to investigate PLA2 responsive CD1a reactive T cells more closely, T cell clones were generated using the IFN-γ secretion assay and FACS. Peripheral blood T cells were stimulated under various conditions were individually sorted based on CD3+ and IFN-γ+ followed by expansion with feeder cells (Figure 6-1). As well as identifying PLA2-responsive CD1a-restricted T cells, this method will also identify CD1a autoreactive and non-specifically activated T cells. Therefore it was important to screen all the clones generated, which was done using CD1a plate bound assays, where recombinant CD1a coated onto plates were pulsed with antigen, washed and then exposed to T cell clones. T cell reactivity was examined by analysing IFN-γ secretion in supernatants by ELISA. Of all the lines generated, two CD1a-restricted T cell lines strongly responsive to bee venom PLA2 from two different donors were identified (Figure 6-2).

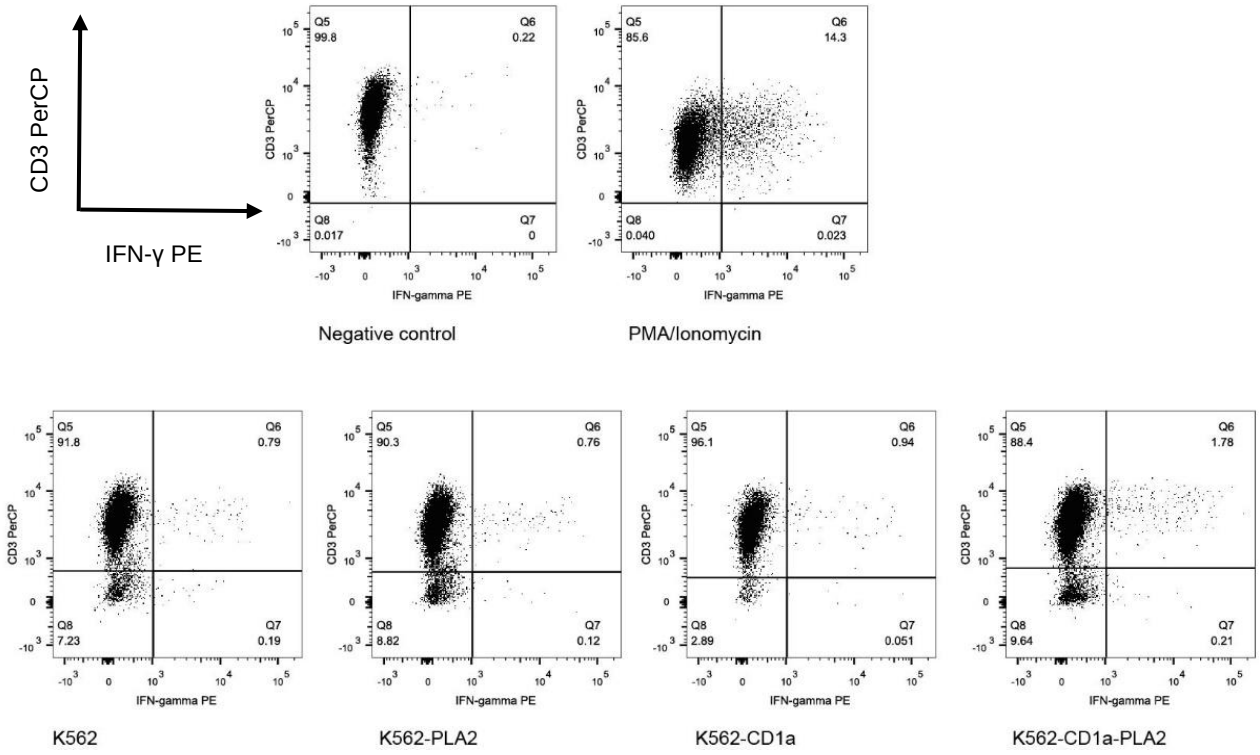


Figure 6-1: Flow cytometry gating strategy to generate PLA2 responsive CD1a-restricted T cell lines.

T cells were isolated from PBMCs by CD3+ bead separation and used in the IFN-γ secretion assay after resting for 3-5 days. T cells were stimulated for 6h with PMA and ionomycin, or untransfected or CD1a transfected K562 cells, in the presence or absence of *Apis mellifera* venom PLA2, and then incubated with a bispecific anti-IFN-γ and anti-CD45 antibody for a further 45 minutes. Any IFN-γ secreted was held on the cell surface and detected with a second R-phycoerythrin (PE)-conjugated anti-IFN-γ antibody. Stimulated cells secreting IFN-γ were sorted by flow cytometry (gating on lymphocytes, singlets, live cells and CD3+IFN-γ+).

Similar to the BC2 cell (Figure 4-7A) no activation was seen on PLA2 exposure in the absence of CD1a but CD1a reactivity in the absence of PLA2 was observed, which is characterised as an autoreactive response, and was inhibited by an anti-CD1a blocking antibody (Figure 6-2A). Increased IFN-γ secretion to PLA2 above autoreactive levels was detected in a dose dependent manner (Figure 6-2B). As with BC2 cells, this response was dependent on CD1a and PLA2 activity as they were abrogated by anti-CD1a blocking antibody or inhibition of PLA2 enzymatic activity with manoalide.

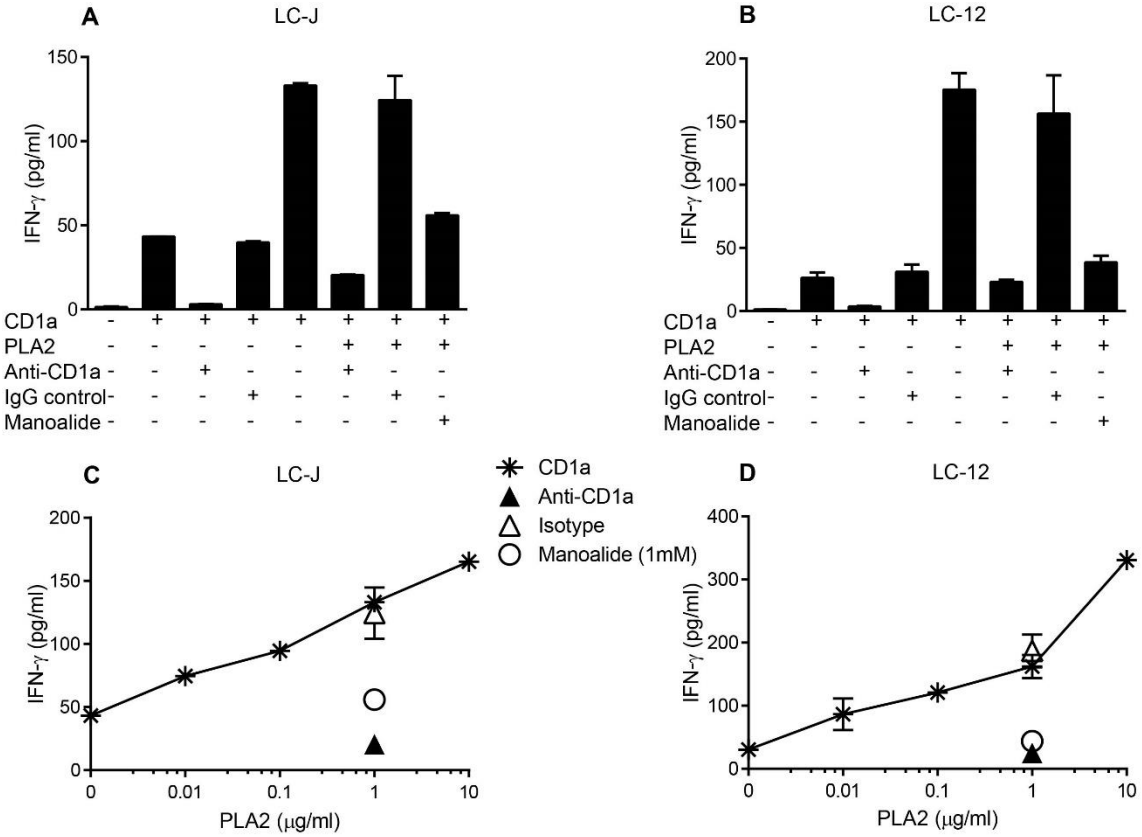


Figure 6-2: PLA2 stimulates LC-J and LC-12 cell lines in a CD1a restricted manner.

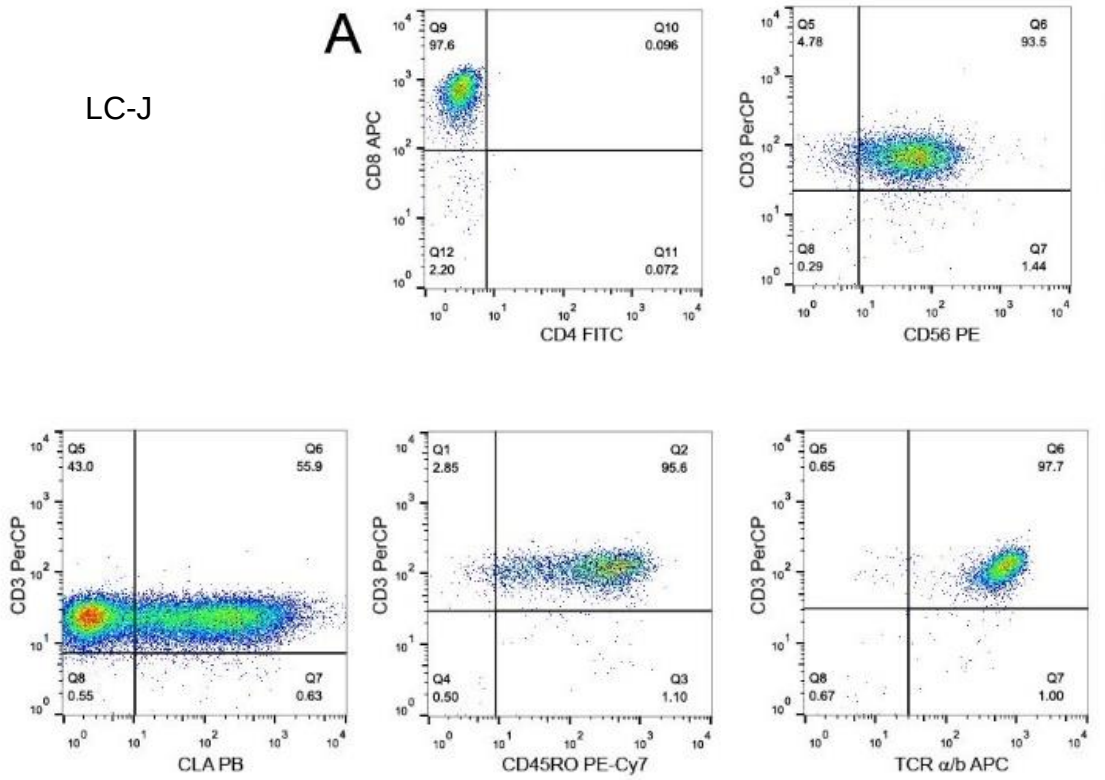
CD1a proteins were coated onto Nunc Maxisorp plates. After washing PLA2 was added for 48h. After further washing LC-J (A and C) or LC-12 (B and D) clones were added, and supernatants collected after 24h were analysed by IFN-γ ELISA. Data are representative of three separate experiments. Data are mean of duplicate results ± SEM.

6.2.2 Surface marker expression and cytokine profile of PLA2 responsive CD1a-reactive T cell lines

Understanding the phenotype and cytokine profile of the cell lines may provide insight into their effector functions. Both lines were memory (CD45RO+) CD8+ single positive α/β T cells. CD3 and CD56 co-expression was also observed (Figure 6-3), which is characteristic of NKT cells and is suggestive of an innate function. Skin homing marker expression (CLA) although detectable, was variable throughout the population (Figure

6-3). Repeated culture and stimulation of T cell lines can affect their phenotype and expression of surface markers over time. T cell stimulation is known to downregulate co-stimulatory molecules expression such as CD3, CD4 and CD8.

LC-J



LC-12

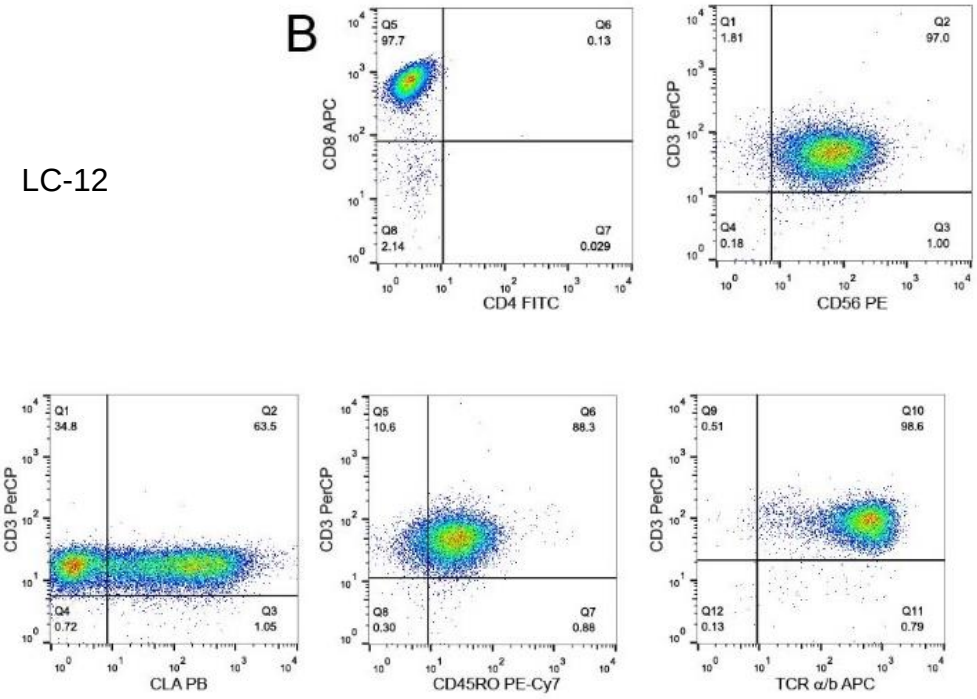
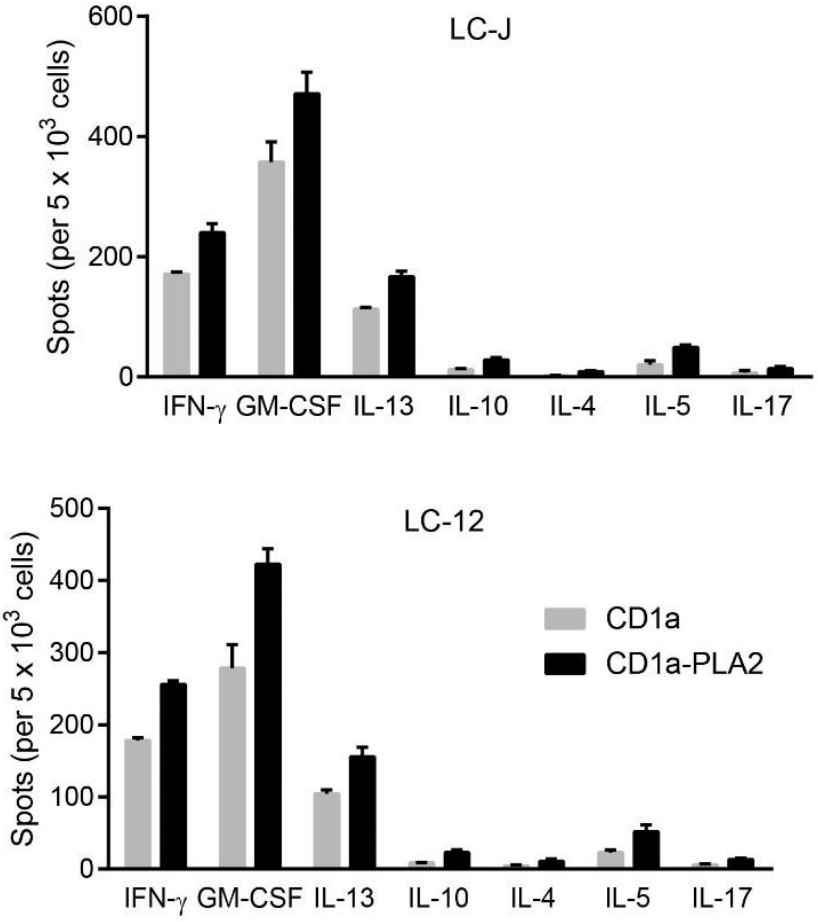


Figure 6-3: Surface expression of co-stimulatory molecules and other markers on LC-J and LC-12 lines.

Flow cytometric analysis of CD3, CD56, CD4, CD8, CD45RO, CLA and TCR αβ of LC-J (A) and LC-12 (B) clones.

The cytokines produced by T cells can be indicative of their effector function during an immune response. As demonstrated with polyclonal CD1a-restricted T cells previously (65) and here in Chapter 4.2.12 (Figure 6-4), both cell lines also produced cytokines classically associated with a Th1 (IFN-γ) and Th2 (GM-CSF and IL-13) response by ELISpot analysis (A). This was in both in response to CD1a alone and CD1a plus PLA2. IL-22 was also investigated because it is restricted to a limited subset of T cells (Th17 and Th22), there is growing evidence for a role in skin immunity (221, 222). Recently, autoreactive CD1a-restricted T cells were also shown to produce IL-22 (65). PMA and ionomycin stimulation induced IL-22 but not substantial IL-17A production (Figure 6-4), which together with the expression of skin homing marker CLA support the idea that CD1a-restricted T cells are involved in skin immunity.

A


LC-J
Spots (per 5 x 10^3 cells)
600
400
200
0
IFN-γ
GM-CSF
IL-13
IL-10
IL-4
IL-5
IL-17
LC-12
Spots (per 5 x 10^3 cells)
500
400
300
200
100
0
CD1a
CD1a-PLA2
IFN-γ
GM-CSF
IL-13
IL-10
IL-4
IL-5
IL-17

B

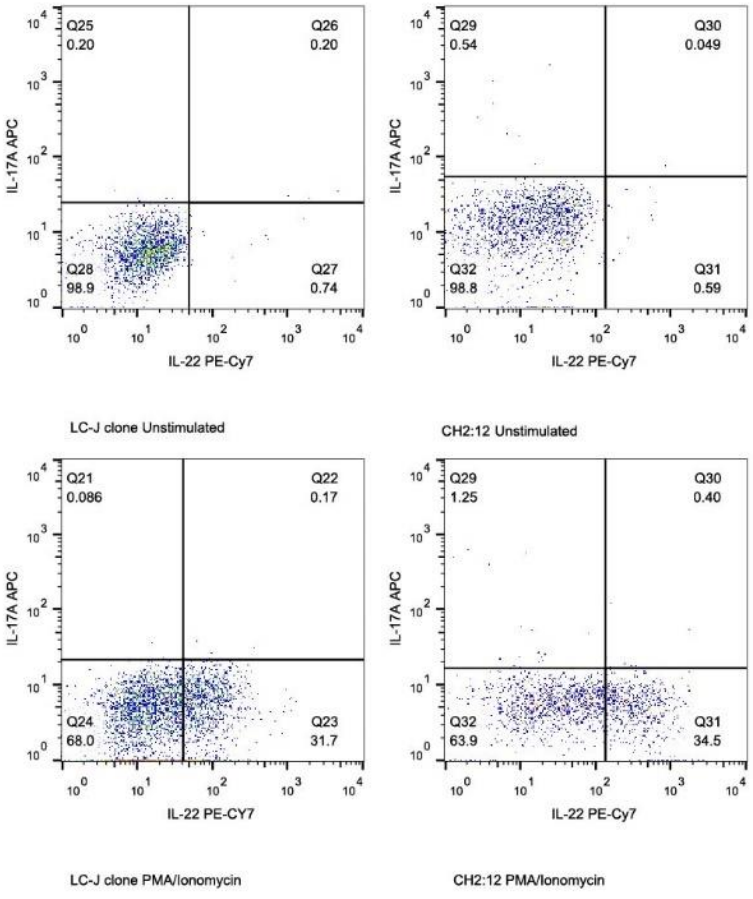
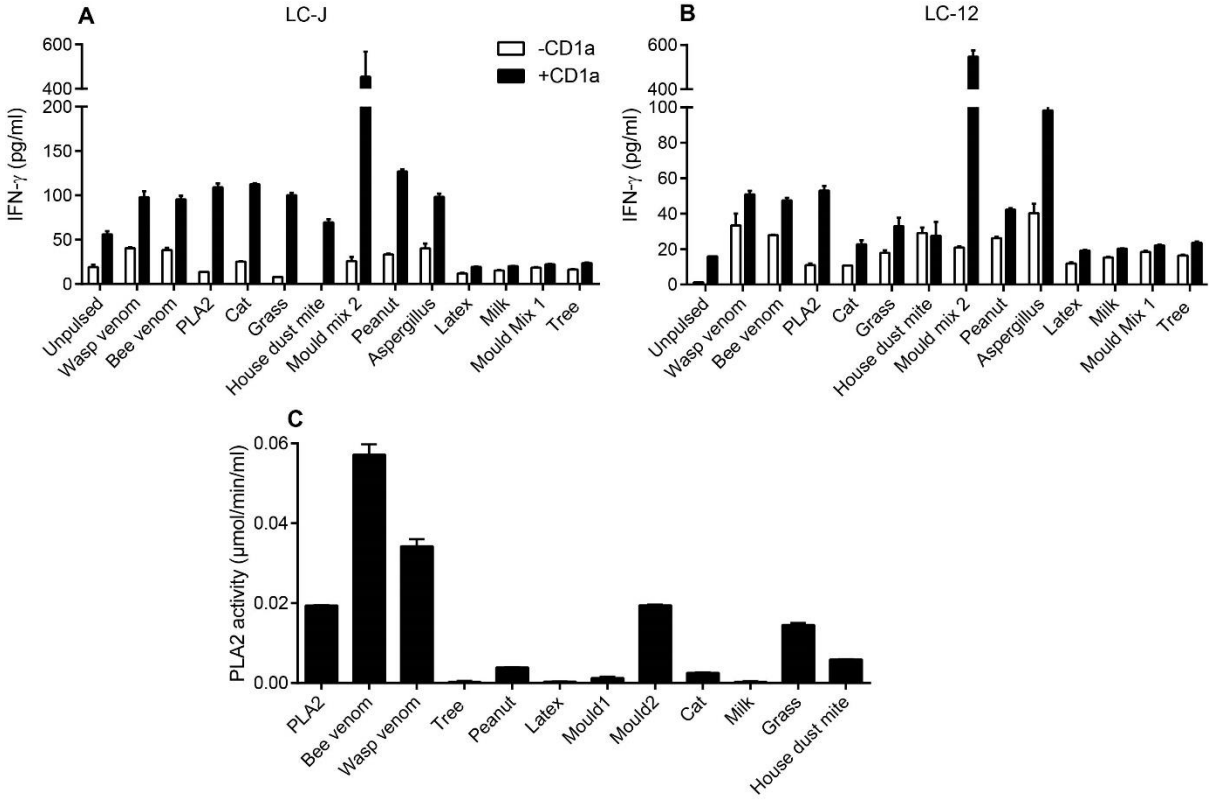

Q25
0.20
Q26
0.20
Q28
98.9
Q27
0.74
IL-17A APC
IL-22 PE-Cy7
LC-J clone Unstimulated
Q29
0.54
Q30
0.049
Q32
98.8
Q31
0.59
IL-17A APC
IL-22 PE-Cy7
CH2:12 Unstimulated
Q21
0.086
Q22
0.17
Q24
68.0
Q23
31.7
IL-17A APC
IL-22 PE-CY7
LC-J clone PMA/Ionomycin
Q29
1.25
Q30
0.40
Q32
63.9
Q31
34.5
IL-17A APC
IL-22 PE-Cy7
CH2:12 PMA/Ionomycin

Figure 6-4: Cytokine production of PLA2 responsive CD1a-reactive T cell line by ELISpot analysis.

PLA2-responsive CD1a-restricted T cell line phenotype was investigated by cytokine ELISpot analysis with K562 or K562-CD1a in the presence or absence of *Apis Mellifera* venom PLA2 (1µg/ml) (A). Data are mean of duplicate experiments ± SEM. Intracellular cytokine staining of IL-17A and IL-22 in T cell lines without stimulation or in response to stimulation with PMA and ionomycin (B). Data are representative of two separate experiments and are mean ± SEM.

6.2.3 PLA2 responsive CD1a-restricted T cell lines respond to different PLA2 containing allergens

Hymenoptera venom induces CD1a-restricted T cells which is mediated by venom PLA2, as demonstrated in Chapter 4 and these cells are elevated in allergic individuals indicating a role in allergic disease. Phospholipases have also been detected in other allergens (346). To investigate a possible role of CD1a-restricted T cells in other allergies, PLA2 activity in skin prick test (SPT) allergen preparation was measured and



their ability to stimulate LC-J and LC-12 cell lines was determined using the CD1a pate bound assay (Figure 6-5).

Figure 6-5: J and LC-12 lines react in a CD1a-restricted manner to numerous allergens.

CD1a proteins were coated on plates and different skin prick test allergen preparations were added after washing for 48h. Following another wash, LC-J (A) and LC-12 (B) lines were added for 24h, and supernatant was sampled for IFN-γ quantification by ELISA. Data are representative of three separate experiments. Using lipid substrate with a thiol group at the sn2 position, PLA2 activity in various SPT allergen preparations was detected using DNTB/EGTA which produces a coloured precipitate and measured at 415nm (C). Data were mean of triplicate measurements ± SEM.

sPLA2 activity was detected in numerous different SPT allergen preparations, although with differences in levels of activity (Figure 6-5C). These allergens stimulated IFN-γ production by T cell lines in CD1a-restricted manner (Figure 6-5 A and B). Some allergens, including wasp and bee venom, induced low levels of IFN-γ release in the absence of CD1a, but this was increased in the presence of CD1a. Wasp and bee venom contain several toxic mediators, such as mastoparan, which can adhere to the plate and cause toxic damage to T cells, resulting in IFN-γ release either due to cell stimulation or cell death. It is interesting to note that the level of sPLA2 activity of an allergen did not correlate with the intensity of the CD1a-restricted T cell response observed. For example, while mould mix 2 had similar sPLA2 activity as 1µg/ml of bee venom PLA2, the CD1a-restricted T cell response was 4 times greater. This may suggest that the PLA2 have differential substrate preferences which lead to differential generation of CD1a ligands.

6.2.4 Identifying the lipid antigens of PLA2 responsive CD1a-restricted T cell lines

Characterising the lipid antigens of CD1a that stimulate T cells will increase our understanding of the range of lipids able to interact with CD1a, CD1a binding specificity and requirements, and help to predict and identify novel ligands in the future. A recent study showed that fatty acid, products of phospholipase activity, are ligands for CD1a (34). To identify the specific PLA2 products that activate T cell lines, whole phospholipids of varying acyl chain lengths and saturation were used with and without PLA2 in the CD1a plate bound assay (Figure 6-6A). Phosphatidylcholine was used as the substrate with chain length between C14 and C20 with no or one double bond, as this headgoup, chain length and saturation are commonly found on membranes in the skin, and so would be available substrate during a sting event (347, 348).

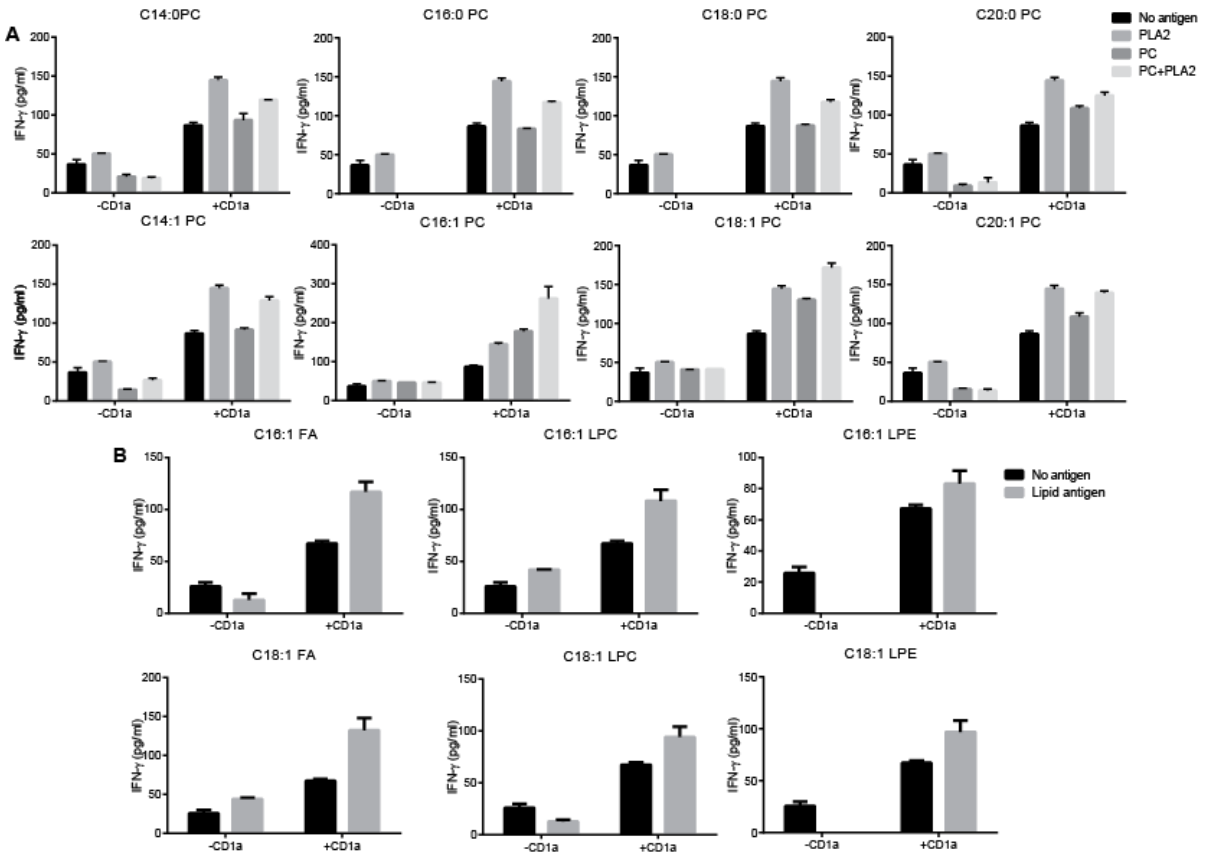


Figure 6-6: PLA2-responsive CD1a-restricted T cells lines respond to products of PLA2 activity depending on acyl chain length and saturation.

CD1a proteins were coated on plates. Then, either PLA2 and/or phospholipid (phosphatidylcholine PC) (A), or products of PLA2 activity (fatty acid FA; lysophosphatidylcholine LPC; lysophosphoethanolamine LPE) (B), of varying acyl chain length and saturation, were added after washing for 48h. Following another wash, LC-12 line was added for 24h, and supernatant was sampled for IFN-γ quantification by ELISA. Data are representative of three separate experiments.

As expected based on previous experiments, low responses were observed in the absence of CD1a, confirming reactivity to CD1a, and an autoreactive response above background was also detected, which was further elevated in presence of PLA2 (Figure 6-6A). While no increase above autoreactivity was detected to substrate alone, the addition of C16:1 or C18:1 PC with PLA2 resulted in an increased response above PLA2 alone. Products of PLA2 activity with 16 or 18 carbon chain length and with one double bond were able to bind CD1a and interact with the autoreactive TCR. Interestingly, increasing or decreasing the chain length, or removal or the double bond, caused a slight reduction in response compared to PLA2 alone. It is possible that PLA2 products generated by cleavage of lipid membrane compete for binding in the CD1a antigen groove with PLA2 products generated by cleavage of exogenous PC. If the TCR is able to interact with PLA2 product of exogenous PC, an increase in response is observed but if the TCR has lower affinity to the PLA2 product of exogenous PC than membrane phospholipids, a reduction in IFN-γ secretion is detected. This suggests that while CD1a is able to bind lipids of different chain length and saturation, as have been previously demonstrated in elution studies (32), TCR interaction and T cell activation is dependent on the acyl chain length and saturation.

FA presented by CD1a were shown to activate BC2 cells (34). To confirm that FA products of PLA2 activity were the lipids responsible for LC-12 cell line activation,

purified FA and LPC with C16:1 and C18:1 acyl chains were used in CD1a plate bound assays. Surprisingly, both FA and LPC replicated the response seen to PLA2 and PC (Figure 6-6B), providing further evidence that interaction of the acyl chain within the CD1a groove is critical to TCR interaction and T cell activation, and that the polar headgroup has little involvement. This was confirmed with experiments using lysophosphatidylethanolamine (LPE), which has an ethanolamine headgroup but with C16:1 or C18:1 acyl chain, and a similar response to LPC was observed. These studies demonstrate the importance of acyl chain length and saturation but not headgroup in activation of CD1a-restricted T cells.

6.2.5 T cell line reactivity to different CD1 isoforms

To confirm that the LC-J and LC-12 cell lines were restricted to CD1a, plate and cellular assays were performed using CD1a, CD1b, C1c or CD1d proteins or transfected K562 cells (Figure 6-7).

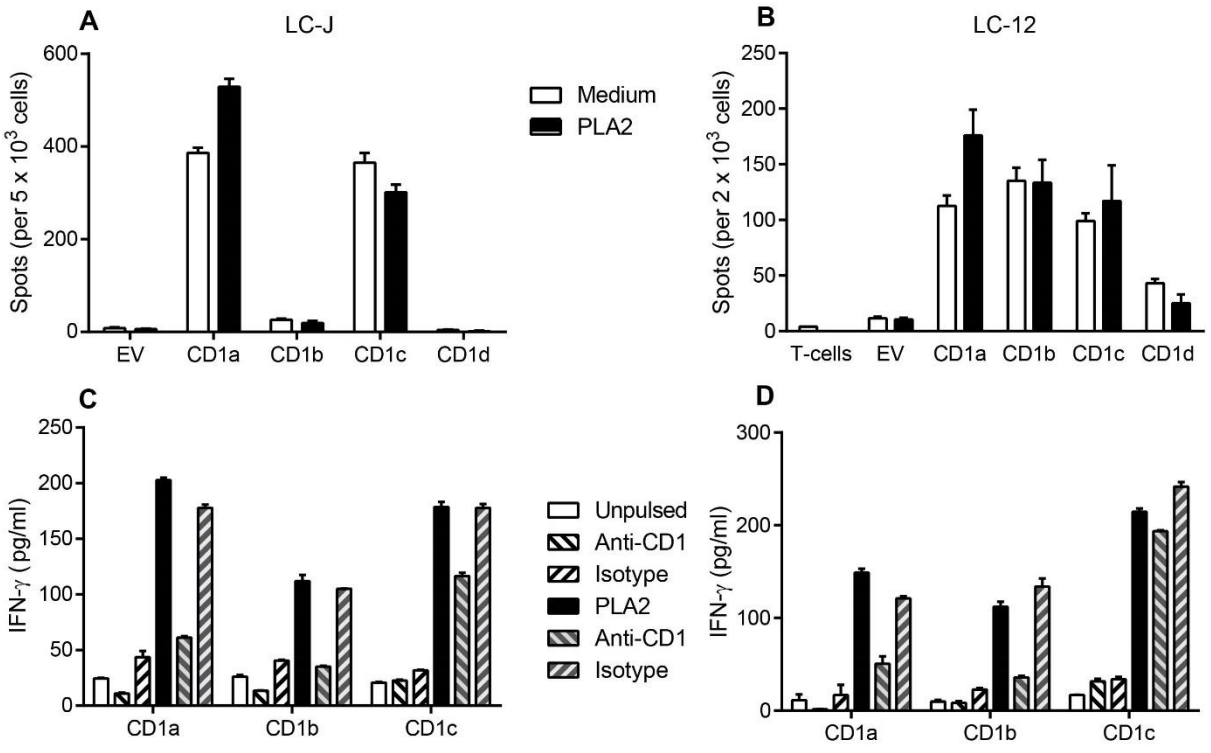
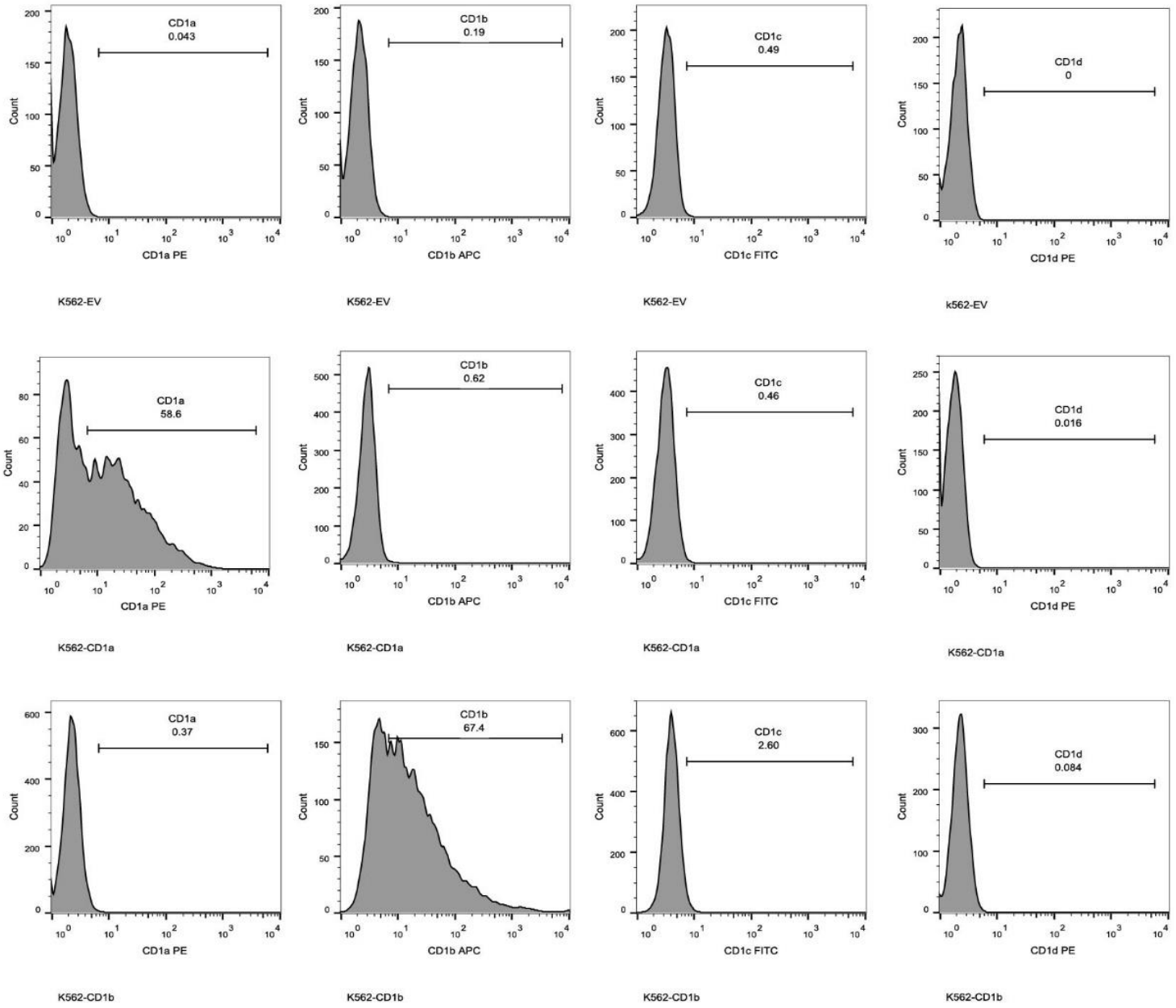


Figure 6-7: LC-J and LC12 lines react to more than 1 CD1 isoform.

LC-J and LC12 line reactivity to different CD1 isoforms were investigated by cellular (A and B) and plate assays (C and D). LC-J line (A) and LC-12 line (B) responses to untransfected or CD1a, CD1b, CD1c or CD1d transfected K562 cells in the presence of absence of *Apis mellifera* venom PLA2 were analysed by IFN-γ ELISpot. LC-J line (C) and LC-12 line (D) responses to recombinant CD1a, CD1b or CD1c coated on plates pulsed with *Apis mellifera* venom PLA2, and in the presence or absence of the corresponding anti-CD1 antibodies or isotype control, were examined by sampling supernatant for IFN-γ quantification by ELISA. One of two separate experiments shown. Data represents mean of duplicate results ± SEM.

Having found previously that both lines could be inhibited by a blocking CD1a antibody, it was surprising to discover that they were also able to respond to other group 1 CD1 isoforms to varying extents (Figure 6-7). In the cellular assay, both lines showed responses to K562-CD1a, K562-CD1b and K562-CD1c cells in the absence of PLA2 at higher rates then to mock transfected cells, although LC-J cell line showed low responses to CD1b compared to LC-12 clone (Figure 6-7A and B). Interestingly, the response to PLA2 was only seen in the context of CD1a. However, both cell lines reacted to a similar extent to CD1a, CD1b and CD1c exposed to bee venom PLA2 in the plate assay, which was inhibited with the relevant blocking antibody (Figure 6-7C and D). An obvious explanation for this would be cross-contamination of the transfected K562 cells or that the lines contained an oligoclonal population of different TCR specificity. K562contamination was ruled out by flow cytometry staining. (Figure 6-8). T cell clonality was verified by staining with an antibody panel for the different TCR Vβ chains, which confirmed that both populations expressed only one TCR, suggesting that they are clones (Figure 6-9). However we will go on to confirm single CDR3 expression using TCR sequencing.


CD1a
0.043
Count
CD1a PE
K562-EV
CD1b
0.19
Count
CD1b APC
K562-EV
CD1c
0.49
Count
CD1c FITC
K562-EV
CD1d
0
Count
CD1d PE
k562-EV
CD1a
58.6
Count
CD1a PE
K562-CD1a
CD1b
0.62
Count
CD1b APC
K562-CD1a
CD1c
0.46
Count
CD1c FITC
K562-CD1a
CD1d
0.016
Count
CD1d PE
K562-CD1a
CD1a
0.37
Count
CD1a PE
K562-CD1b
CD1b
67.4
Count
CD1b APC
K562-CD1b
CD1c
2.60
Count
CD1c FITC
K562-CD1b
CD1d
0.084
Count
CD1d PE
K562-CD1b

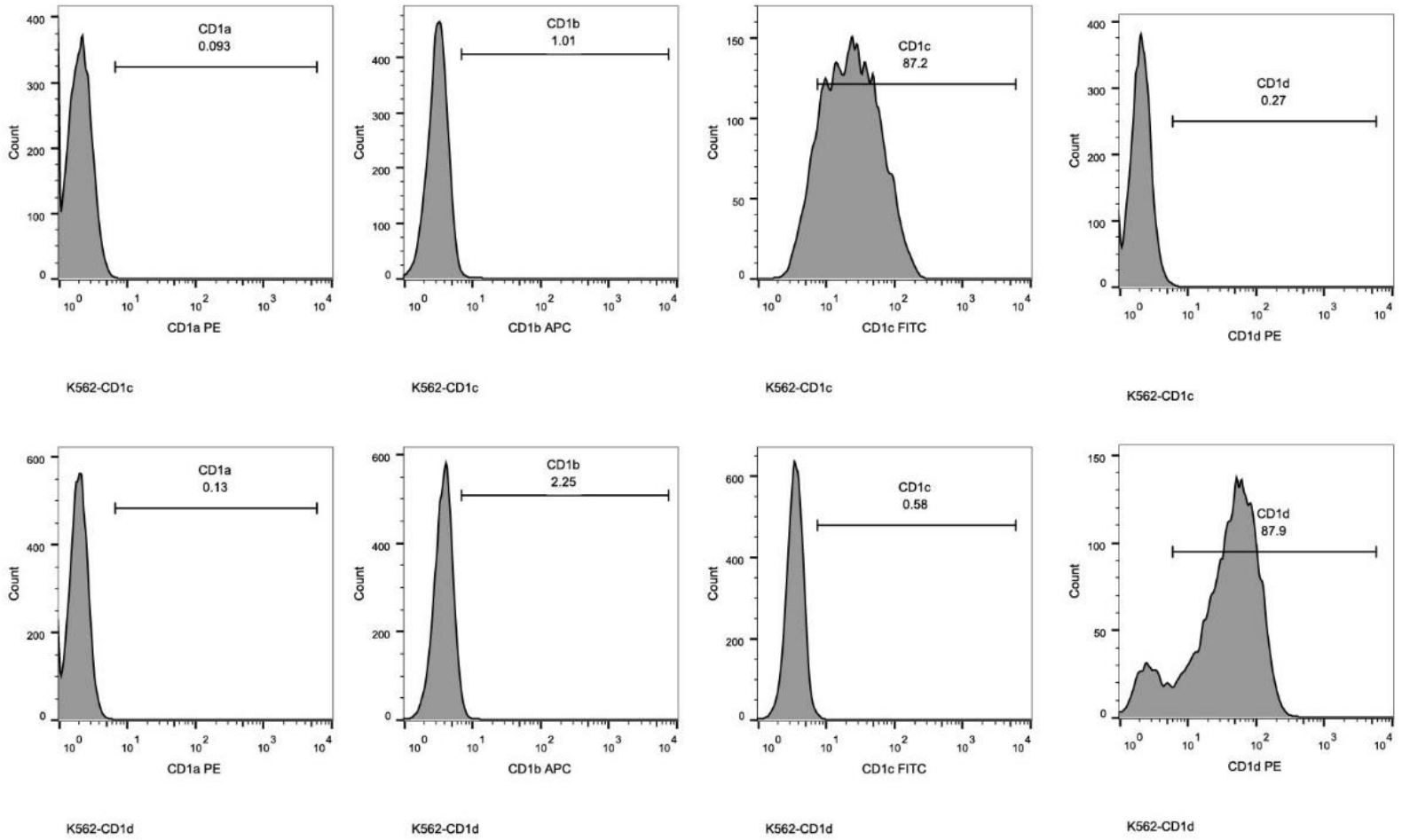
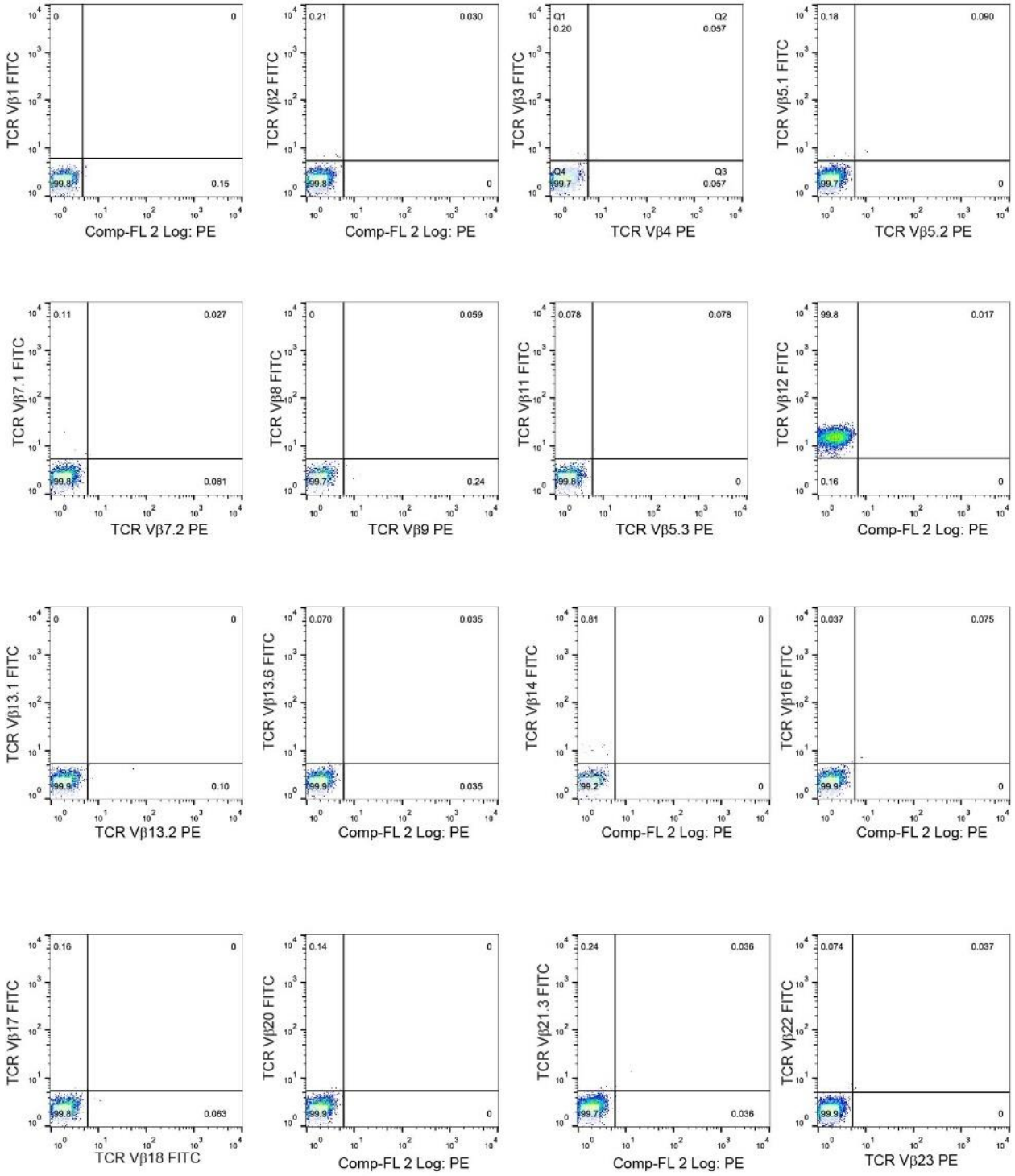


Figure 6-8: CD1a, CD1b CD1c and CD1d expression of untransfected and transfected K562 cell lines.
Untransfected and transfected K562 cells were analysed by flow cytometry for CD1a, CD1b, CD1c and CD1d expression.

A


TCR Vβ1 FITC
Comp-FL 2 Log: PE
TCR Vβ2 FITC
Comp-FL 2 Log: PE
TCR Vβ3 FITC
TCR Vβ4 PE
TCR Vβ5.1 FITC
TCR Vβ5.2 PE
TCR Vβ7.1 FITC
TCR Vβ7.2 PE
TCR Vβ8 FITC
TCR Vβ9 PE
TCR Vβ11 FITC
TCR Vβ5.3 PE
TCR Vβ12 FITC
Comp-FL 2 Log: PE
TCR Vβ13.1 FITC
TCR Vβ13.2 PE
TCR Vβ13.6 FITC
Comp-FL 2 Log: PE
TCR Vβ14 FITC
Comp-FL 2 Log: PE
TCR Vβ16 FITC
Comp-FL 2 Log: PE
TCR Vβ17 FITC
TCR Vβ18 FITC
TCR Vβ20 FITC
Comp-FL 2 Log: PE
TCR Vβ21.3 FITC
Comp-FL 2 Log: PE
TCR Vβ22 FITC
TCR Vβ23 PE

B

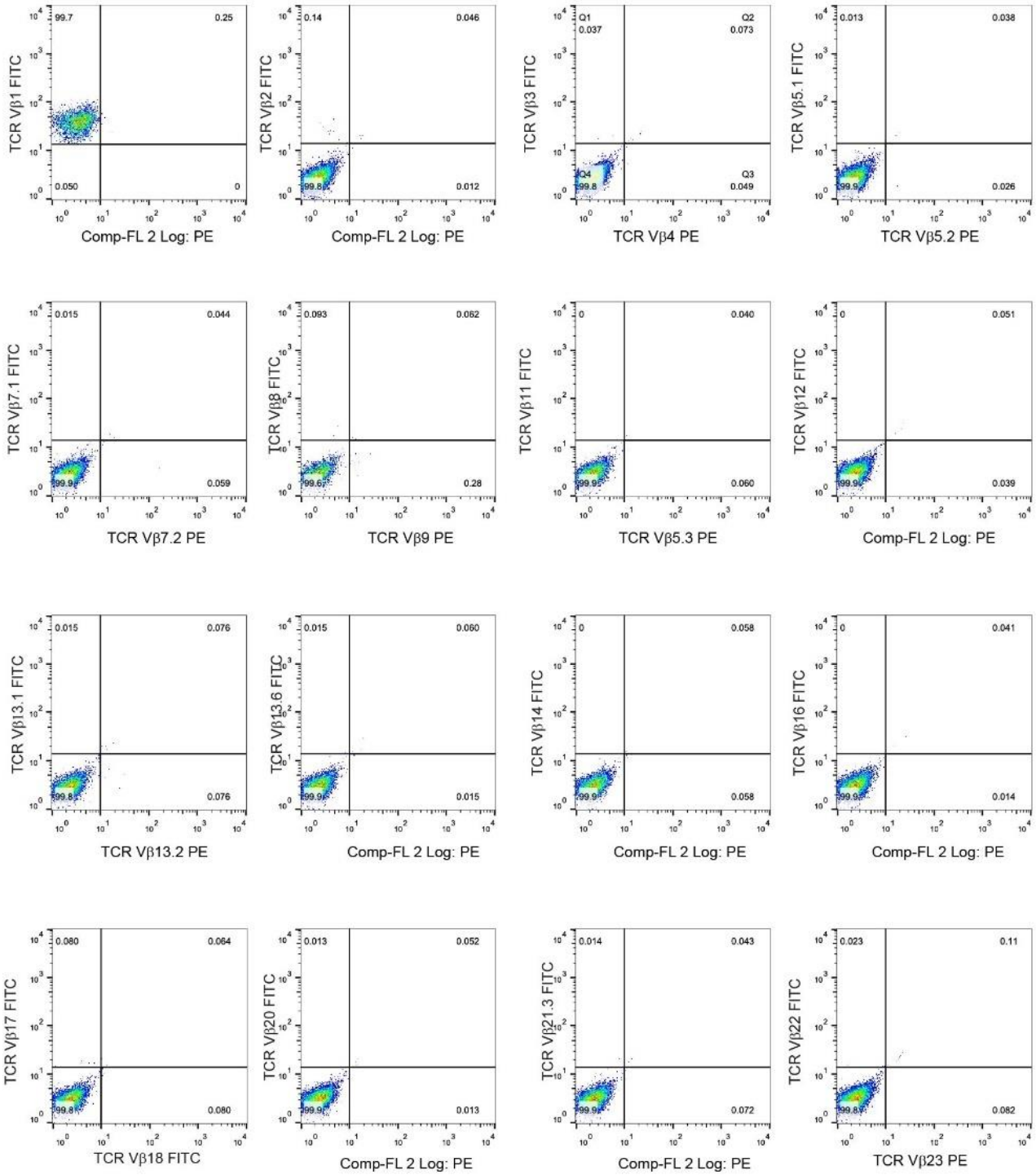


Figure 6-9: Flow cytometry analysis of TCR Vβ chain expression of LC-J and LC12 cell lines.
LC-J (A) and LC-12 (B) cell lines were stained with a panel TCR Vβ antibodies and analysed by flow cytometry to determine clonality.

6.3 Discussion

T cells responding to PLA2 derived antigens presented by CD1a are found in most individuals and represent a novel pathway of T cell activation described in Chapter 4. However, we do not yet understand their function within the immune system and characterisation of their phenotype will contribute to our understanding of physiological function. Evaluating the specific phenotype of these cells is complicated within a heterogeneous polyclonal T cell population and so T cell clones or lines were used. Here, two T cell line clonal populations are described, generated by IFN-γ secretion assay that showed CD1a autoreactivity but increased response to PLA2 which was dependent on its catalytic activity. Both LC-J and LC-12 cell lines were CD3+CD8+CD56+CD45RO+αβ+ T cells, with variable CLA expression. CD56 is also expressed on NKT cells and is associated with an innate phenotype within the immune system suggesting that, like NKT cells, CD1a-restricted T cells may be involved in bridging the innate and adaptive arms of the immune system. The cells also express CD45RO, indicating previous antigen encounter. As PLA2 reactivity is dependent on its enzymatic activity, it is possible that circulating T cells may have previously encountered products of an endogenous phospholipid by allergen or endogenous PLA2. Memory cells are able to respond more rapidly than naïve cells and so may help provide a first line response to insult. Interestingly, the majority of CD1a-restricted T cell clones generated (19 out of 22 in total) were $CD8^+$ single positive. CD1 molecules are structurally similar to MHC Class I, which may explain the bias of CD8+ TCR recognition of CD1a. However, previously reported libraries were composed predominantly of CD4 and DN T cells (65, 66). It is possible that the method of generating T cell clones used here resulted in selection for CD8+ CD1a-restricted T cells, either by PLA2 itself or perhaps the K562 cell line. Using the limiting dilution

assay would remove this bias, although the percentage of PLA2-responsive CD1a-restricted T cells within all clones generated would be extremely low.

Cytokine secretion by the T cell lines was similar to the polyclonal T cell response and previously reported autoreactive and pollen-specific CD1a-restricted responses (53, 65). Stimulation was characterised by strong IFN-γ, GM-CSF and IL-13 secretion, with elevated levels in the PLA2-induced compared to autoreactive response. The production of both Th1 and Th2 cytokines by a clonal population of cells is interesting and could imply a multi-functional role in the immune response, where the local environmental or inflammatory conditions influence which response dominates. ICS or fluorospot analysis could be used to determine whether the same cell expresses both Th1 and Th2 cytokines on stimulation or if cells within a clonal population have a pre-determined bias. IL-22 production in the absence of IL-17 is consistent with previous findings (65), that has identified autoreactive CD1a-restricted T cells as potential subsets of Th22 cells, and together with the expression of skin homing marker, CLA, is further evidence for a role in skin immunity.

Phospholipases have now been identified in bacteria, fungi, insects, reptiles and mammals (230) and some of these species are responsible for allergic reactions. T cell responses were shown here to be restricted to PLA2-containing allergens in the context of CD1a, although this requires confirmation by inhibiting PLA2 activity and blocking CD1a. In the previous chapter, an increase in PLA2-responsive CD1a-restricted T cells was observed in bee venom allergic individuals compared to healthy controls and similar patterns were reported for pollen phospholipid-specific CD1a-restricted T cells (53). These data provide further evidence for CD1a-restricted T cell involvement in several other allergies too. The LC-J and LC-12 cell lines were generated using bee venom PLA2 and it is interesting that they are able to respond to

PLA2 from other species, suggesting a certain amount of homology and substrate cross-reactivity. The level of PLA2 activity detected however did not correspond to the intensity of the T cell response, which was shown to be dose-responsive to purified PLA2 (Figure 6-2). The PLA2 activity assay only measure sPLA2 activity so some allergens may also contain other phospholipase groups that generate more potent CD1a-ligands or have alternate substrate preferences. The allergen preparations may also contain some lipids that contribute to CD1a-restricted T cell activation.

Recent studies have demonstrated that fatty acid (FA) stimulates autoreactive CD1a-restricted T cells (34), which is consistent with the data shown here. The data here show that LPL, the second product of PLA2 activity also stimulates CD1a-restricted T cells. Changes in the headgroup of lysophospholipid (LPL) did not affect T cell stimulation but the acyl chain length and saturation were crucial and restricted to a narrow margin. Therefore TCR binding to CD1a-lipid is dependent on the interaction of the acyl chain within the A' pocket of the CD1a antigen groove. While the TCR interacts with the exposed headgroup of lipopeptide (DDM and self-sulfatide) presented on CD1a, the headgroup does not appear to be important in the TCR interactions of PLA2-responsive CD1a-restricted T cells. This is consist with observations from the resolution of the crystal structure of CD1a bound to apolar lipids interacting with TCR (32). Using tetramer analysis and TCR from a known autoreactive CD1a T cell clone, hundreds of lipids were eluted from CD1a-lipid-TCR complexes, confirming that CD1a is able to bind and present a number of different lipids to the TCR. Removing the lipid ligand abrogated subsequent T cell activation, indicating that lipid loading is required for TCR interaction (32). Lipids eluted from both CD1a-lipid and CD1a-lipid-TCR complexes were termed 'permissive' ligands, while those only identified in CD1a-lipid eluate were termed 'non-permissive' ligands, as they prevented

autoreactive TCR engagement (32). At least three lipids commonly found in cellular membranes were identified in this way, indicative of a common docking mode.

Not all lipids were headless, and LPL, composed of fatty acid chain, phosphate group and a polar headgroup, were also able to engage other autoreactive TCR (32). Therefore both products of phospholipase activity can stimulate CD1a-restricted T cells. iNKT cells can also recognise LPL presented on CD1d and this was also shown to be dependent on PLA2 activity, although the source of PLA2 was endogenous (237). The strength of individual iNKT cell clone response to LPL was varied, suggesting that conformational changes caused by LPL binding to CD1d affect interaction with different TCR.

Previous studies of TCR interaction with peptide-MHC complexes found that MHC class I restricted TCR bind diagonally across the binding groove, with complementarity determining region (CDR) 1 and 2 interacting with MHC α-helix and CDR3 making contact with peptide (285). In contrast, TCR interaction with CD1d and α-galactosylceramide (α-GalCer; a well-characterised CD1d ligand) is parallel to and at the far end of the antigen binding cleft, above the F' pocket. CDR2β and CDR3α loops provide the majority of the contact of TCR with CD1d and lipid binding is mediated by CDR1α and CDR3α loops (285). Similarly, modelling of TCR-CD1a-DDM complex suggests parallel orientation of the TCR, which encounters mostly CD1a surface above the A' pocket; while the N- and C- terminus of the α1 and α2 helices contact via CDRs of the α-chain (30). At the F' pocket where the ligand is exposed, CDRs of the TCR β-chain mediate contact with the exposed lipidic headgroup (30).

TCR engagement of CD1a bound to apolar lipids however was orthogonal across the antigen binding cleft and at the extreme end, docking onto the roof-like structure (32).

The α- and β-chains of the TCR interact with the α1- and α2-helices of CD1a respectively, mediated primarily by CDR3α and CDR3β loops (32). As such, there is no interaction with the lipid antigen in the cleft, as its solvent exposure is restricted to the other end, at the F' pocket. Together with the large roof-like structure (larger than other CD1 isoforms) this allows TCR to interact only with the CD1a protein and explains how TCR can engage CD1a complexed with a diverse array of lipids. Indeed non-permissive ligands disrupt the architecture and electrostatic properties of the roof-like structure, which together with the more exposed polar headgroup, inhibited autoreactive TCR engagement, causing diminished tetramer staining. This study demonstrates how CD1a-restricted T cells are able to respond to phospholipase generated lipid neoantigens presented on CD1a.

T cell reactivity to more than one CD1 isoform has as yet not been described, but was observed with the two cell lines here, despite expressing a single TCR Vβ chain. Although TCR sequencing is required to confirm the clonal nature of these cells, recognition of multiple CD1 isoforms by a clonal population is an interesting finding. Dual TCR α- or β-chain expression on T cells has been reported, which may explain the promiscuous reactivity of the TCR (349, 350). Abrogation of the responses by anti-CD1 antibodies suggests that these are true TCR-CD1 interactions and not non-specific experimental artefacts. Interestingly, although TCR reactivity appeared to be promiscuous, this was restricted to Group I CD1 molecules and CD1d did not elicit a response, implying recognition of a conserved region of the Group I CD1 molecules, which is different in CD1d. This is potentially a new and exciting finding of CD1a-restricted T cells which will have important implications for their functions.

7 Discussion

The discovery of CD1a-restricted autoreactive T cells as normal part of the T cell repertoire (65, 66) has generated considerable interest in the function these cells, both in the steady state and disease. There is accumulating evidence for a role for CD1a-restricted T cells in the skin, where they co-localise with high density to CD1a-expressing cells and produce IL-22. These T cells are inherently autoreactive but their contributions to inflammatory responses, particularly in the skin, are not yet understood.

In order to further our understanding of CD1a-restricted T cells, this thesis aimed to address a number of questions about the frequency and phenotype of the cells and their role in cutaneous immunity and allergic disease. Using wasp and bee venom as a model system, polyclonal CD1a-restricted T cells were studied in blood and skin and compared between healthy and allergic individuals. The T cell phenotype was also investigated, to understand their function in health and disease. This led to the discovery of a novel pathway of inflammation through the generation of CD1a-restricted neo-lipid ligands by venom phospholipases that stimulate T cells and generate an immune response.

Insect stings were an ideal candidate antigen to investigate CD1a-restricted T cell responses in inflammatory skin disease. Wasp and bee venoms are relatively well defined antigens in allergy, and have long been associated with T cell infiltration and in the pathogenesis of allergic reactions. Unlike other allergens exposed to the skin, venom antigens are delivered directly into the skin, below the stratum corneum, at a defined site and time. Allergic reactions to venom are also less affected by variations

in antigen exposure or penetration, or in host barrier integrity. House dust mite, for example, or microbial exposure will vary between individuals, and antigen penetration is affected by the influence of host genetics on barrier integrity, such as *FLG* mutations. Wasp venom reactivity however is independent of *FLG* mutations (266) and so direct comparisons between individuals is possible.

The presence of wasp and bee venom responsive polyclonal CD1a-restricted T cells was established here in blood and skin of most healthy donors tested who had been previously exposed to hymenoptera venom. The frequency of cells was low and only detectable by cultured ELISpot and not *ex-vivo*. A range of frequencies was observed between individuals, possibly due to the number of stings and time since last sting, although *de Jong et al* (65) also observed a similar differences in autoreactive CD1a-restricted T cells between individuals. CD1a expression can vary in individuals due to transcriptional regulation by an SNP in the 5'UTR (14). It is not known however whether the frequency of CD1a-restricted T cells is affected by CD1a expression in an individual, which may have contributed to the differences in T cell responses seen.

The responses to venom were shown to be dependent on phospholipase, which is present in both wasp and bee venom, and is specifically dependent on its enzymatic capacity. Venom phospholipase was active in the skin when injected, where CD1a expression is high. One of the products of phospholipase activity, a free fatty acid (C18:1; oleic acid) was recently shown to stimulate a CD1a-restricted autoreactive T cell clone (34). Although the phospholipid source may be in the skin, serum or venom, plate bound assays demonstrated that membrane phospholipids provide sufficient substrate to generate CD1a ligands and activate T cells. Therefore, during a stinging event, insect phospholipase injected into the skin can hydrolyse skin-derived or venom-derived phospholipids, releasing neo-antigens such as free fatty acids, which

are loaded onto nearby CD1a molecules on Langerhans cells or dermal DCs and presented to dermal T cells (Figure 7-1).

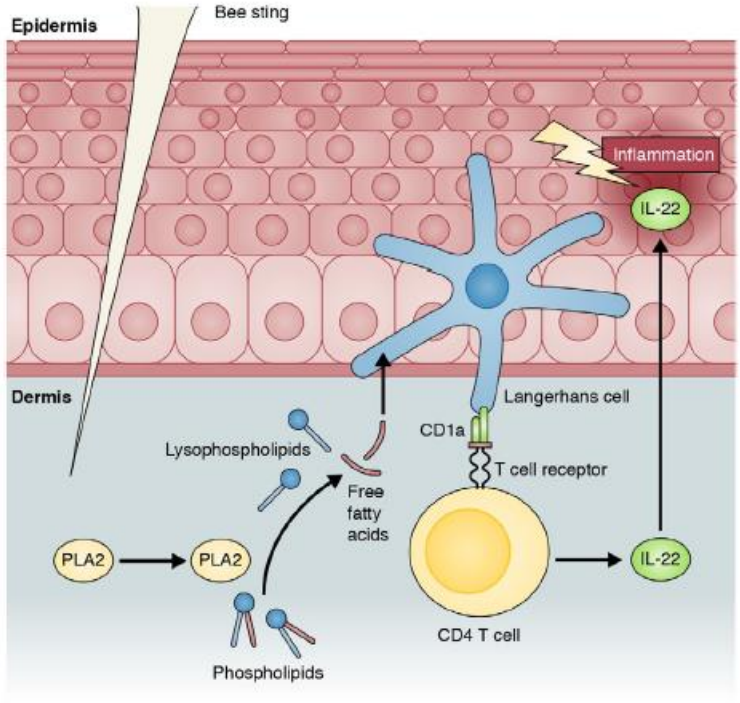


Figure 7-1: Mechanism of CD1a-restricted T cell activation by venom phospholipase-derived antigens (351).

Bee venom PLA2 injected into human skin cleaves ubiquitous phospholipids into free fatty acids and lysophospholipids that function as neoantigens. These neoantigens are presented by epidermal LCs and dermal DCs on CD1a proteins and at their surface to skin-resident dermal CD1a-reactive T cells. These T cell produce cytokines including IL-22 that contribute to the inflammatory response (351).

Venom and phospholipase responsive CD1a-restricted T cells were detected here at higher frequencies in allergic compared to non-allergic individuals. This is line with previous studies showing elevated pollen-specific CD1a-restricted T cells in allergic individuals, particularly during the allergic season, and together indicate a role for CD1a-restricted T cells in allergic reactions. Upon activation by phospholipase, these cells were shown here to produce Th2 cytokines (IL-13, GM-CSF), which could contribute to the local environment and promote a Th2 bias and drive the allergic

reaction. PLA2-responsive CD1a-restricted T cells also secreted Th1 cytokines, including IFN-γ, which upregulate MHC class II on keratinocytes and so these cells may contribute to enhancing the immune response by supporting presentation of allergen peptides to conventional T cells. It is also possible that during an allergic reaction *in vivo*, the local Th2 environment may lead to a Th2 bias of CD1a-restricted T cells, inhibiting IFN-γ production. Bee venom PLA2 hydrolysis of membrane phospholipids was recently shown to induce potent Th2 responses, through the production of LPL and induction of IL-33 secretion (352). IL-33 promotes Th2 differentiation by signalling through IL-33 receptor (ST2) on T cells (352).

PLA2 is found in number of other allergens, including house dust mite and *Aspergillus fumigatus* (346, 353) and sensitisation to these allergens is commonly seen in AD and allergic bronchopulmonary aspergillosis patients respectively. HDM-derived PLA2 is then able to act in conjunction with endogenous PLA2 to further degrade the skin barrier. CD1a-restricted T cells may also be involved in skin inflammation associated with AD. Using the same mechanisms as venom PLA2, HDM-derived PLA2 may generate neo-lipid ligands, presented by CD1a on LCs to T cells. The impaired permeability barrier associated with AD may also allow increased penetration of HDM, cleavage of phospholipids and CD1a-restricted T cell activation, compared to healthy skin.

The production of IL-22 by CD1a-restricted T cells in response to PLA2 is suggestive of a role in skin homeostasis and/or pathogenesis of inflammatory skin disorders, such as AD or psoriasis. As discussed in detail in Chapter 1.3.4, IL-22 has both pro- and anti-inflammatory effects, including production of antimicrobial molecules, and keratinocyte proliferation and differentiation. Further investigation of the function of CD1a-restricted T cells in inflammatory skin disease may provide insight into disease

pathogenesis or reveal potential targets for therapy. IL-22 production by CD1a-restricted T cells was not studied in this thesis in wasp and bee venom allergic individuals but will be an important avenue for future work. This may provide further evidence for these cells in the local inflammatory response in an allergic reaction and their influence on systemic allergy.

Hymenoptera allergy immunopathology is an ideal model system to decipher the mechanisms involved in the immunopathogenesis of allergy and as well as clinical tolerance induction. Most studies to date have focussed on the peptide-specific T and B cell responses. The pattern of lipid-specific CD1a-restricted T cell response observed here over the course of wasp venom immunotherapy and clinical tolerance was very similar to the peptide-specific response (293). The rise in frequency of the T cells at the start of immunotherapy suggests that they are initially present in the circulation, allowing for *in vitro* expansion. The reduction in CD1a-restriced T cell response back to pre-therapy levels after week 5, despite the administration of increasing doses wasp venom, suggests modulation of these cells that previously were contributing to allergic disease. Venom antigen is delivered where CD1a-expressing cells are not present and so it is possible that immunotherapy achieves clinical tolerance in part by bypassing CD1a and therefore modulating the phenotype of the subsequent peptide-specific T cell and IgE responses. Peptide-specific regulatory T cells and IgG4 may also contribute to the reduction in CD1a-restricted T cell response. Ex-vivo analyses in the future would allow further investigation but would require larger cell numbers. It is unlikely that lipid-specific T cells cause all aspects of allergic disease and peptide-specific responses are of course crucial to the allergic response. It is possible however, that CD1a-restricted T cells in the skin promote peptide-specific Th2 cells through the production of cytokines, which in turn

promote venom protein specific IgE production and mast cell degranulation associated with allergy. Understanding the mechanisms of desensitisation is important in the treatment of allergic disease but will also contribute to the use of this approach in the treatment of other diseases, such as autoimmune diseases.

Most CD1-restricted TCR studied to date, bind to lipid-CD1 complexes through interactions with an exposed polar headgroup of the lipid and the CD1 molecule itself. However, CD1a-restricted autoreactive T cell clones that respond to small apolar lipids lacking charged headgroups, such as free fatty acids, were inhibited by lipids with polar headgroups (34). This suggests that TCR interacts with the CD1a molecules itself. Studies from CD1a and other CD1 isoforms have shown that that binding of lipid antigens internally can alter the outer conformation of CD1a (35, 36), modifying TCR docking sites. Recent resolution of the crystal structure of CD1a bound to apolar lipids interacting with TCR provided further evidence, demonstrating that the acyl chain binds deep in the A' pocket which is not exposed to the TCR but obstructed by the formation of a roof-like structure by interaction of the α1- and α2-chains of CD1a (32). The TCR docking site was on this roof-like structure, which is larger in CD1a than other CD1 isoforms. They were also able to demonstrate CD1a-TCR in complex with lipid antigens that contain a polar headgroup, LPL, composed of fatty acid chain and a polar headgroup (32). The fatty acid chain of LPL followed a similar path to the free fatty acid, binding in the A' pocket. The polar headgroup protruded from the F' pocket at the opposite end of CD1a from the TCR docking site, preventing interaction of TCR and headgroup (32). Functional studies with T cell lines in Chapter 6 confirmed that autoreactive T cells are able to respond to both free fatty acids and LPL, and that the TCR does not interact with headgroup, as changes in headgroup did not affect T cell stimulation. Functional responses were affected by the chain length and saturation of

acyl chain. FA or LPL chain length of C16 or C18 with one double bound appeared to provide optimal stimulation. These are commonly found chain lengths within the skin (347, 348) and so could be presented by resident APCs to CD1a-restricted T cells following an insect sting or a breach in skin barrier integrity.

FA and ceramides are major components of the stratum corneum where they form part of the permeability barrier preventing TEWL. FA are also important in maintaining the acidic pH of the skin to regulate protease activity involved in the breakdown of junctions between corneocytes (254). A number of different endogenous PLA2s have been identified in healthy skin, primarily in the upper layer, and are secreted by keratinocytes and other cells in a differentiation-dependent manner (354). These enzymes produce FA by hydrolysing membrane phospholipids, which are CD1a ligands and are a potential source for the generation of autoantigens in normal skin for CD1a-restrcted autoreactive skin-homing T cells.

The lipid content of the skin varies in the different layers according to its function. The stratum corneum is rich in long to medium length ceramides, which are important in maintaining barrier function, as a reduction in acyl chain length below C12 results in increased barrier permeability (355). Ceramides of the stratum corneum are mostly C14-C20 in length (355), within the range of the fatty acid shown to bind CD1a and stimulate T cells. This suggests that the epidermis is enriched with natural CD1a ligands and these ligands are in close proximity to CD1a, as LCs are located here with dense CD1a expression. T cells however are located in the dermis, minimising direct contact with autoantigen and CD1a in the steady state. Autoreactive CD1a T cells shown to be normal component of T cell repertoire (65) therefore separation of TCR and CD1a prevents constant activation. CD1a expression is also highly regulated throughout the body and is restricted to Langerhans cells, thymocytes and myeloid

DCs. Circulating PBMCs do not express CD1a, CD1b or CD1c but do express CD1d, and mDCs cultured in human serum rather than FCS also develop the same profile (356). Factors in human serum, including immunoglobulins and polar organic lipids such as cardiolipin, were shown to regulate CD1 expression at the gene transcriptional level, which was mediated by FcγRII (CD32a) and peroxisome proliferator-activated receptor (PPAR) respectively (356, 357). Tight regulation and restricted expression of CD1a minimises continuous activation of CD1a-restricted T cells recognising autoantigens, including products of phospholipase activity.

A break in skin barrier however could result in lipids from the epidermis entering the dermis and LC migration into dermis. Inflammation in the skin and defects in barrier integrity are associated with altered lipid profile (347, 358, 359). Filaggrin deficiency is strongly associated with AD and a breakdown in barrier integrity. Filaggrin insufficient skin exhibit an accumulation of fatty acids, disorganisation of intracellular lamellae lipids and increased permeability (360). Elevated PLA2 levels and changes in location within the skin have also been demonstrated during inflammatory skin disease (354). Group X sPLA2 is located in the epidermis of healthy skin, but disappears from here in psoriatic skin and appears at relatively high levels in the dermis (354).

A breach of the skin and breakdown of barrier integrity due to infection, injury or inflammation, therefore can lead to elevated PLA2 activity and higher concentrations of PLA2-derived lipids (Figure 7-2). These neoantigens are presented by dermal DCs or LCs from the epidermis that show increased migration into the dermis during inflammation (140), where they co-localise with T cells. Therefore CD1a-restricted T cells recognising products of phospholipase activity may play an important role in sensing barrier integrity (Figure 7-2). This may have potential therapeutic implications and PLA2 could be a therapeutic target. Interestingly, corticosteroids, commonly used

in the treatment of inflammatory skin conditions, have been shown to inhibit PLA2. The exact mechanisms of corticosteroid-induced immune suppression is not fully understand, and inhibition of this pathway may contribute. Although T cells are located primarily in the dermis, in skin conditions such as AD, they can also be found in the epidermis. Therefore during inflammatory skin conditions, LCs in the epidermis may present PLA2 products to epidermal T cells as well as dermal T cells.

Although the primary function of this pathway may be in barrier sensing, insect phospholipases co-opt this mechanism to generate an immune response. Insect venom of many different species have all evolved to include phospholipases, possibly due to their toxicity or ability to cause pain via neurotoxic PLA2 receptors, which would be helpful to evade predators or catch prey. Some wasp species are parasitic and lay their eggs inside the host and the proteins injected are critical to successful parasitism, of which phospholipase would be important in breaking down surrounding tissue (361). While the CD1a-restricted T cell response to phospholipase products may function in barrier sensing and allergy, it is also possible that it evolved to detect and respond to parasites and pathogens (bacteria, virus, fungi), many of which also have phospholipases.

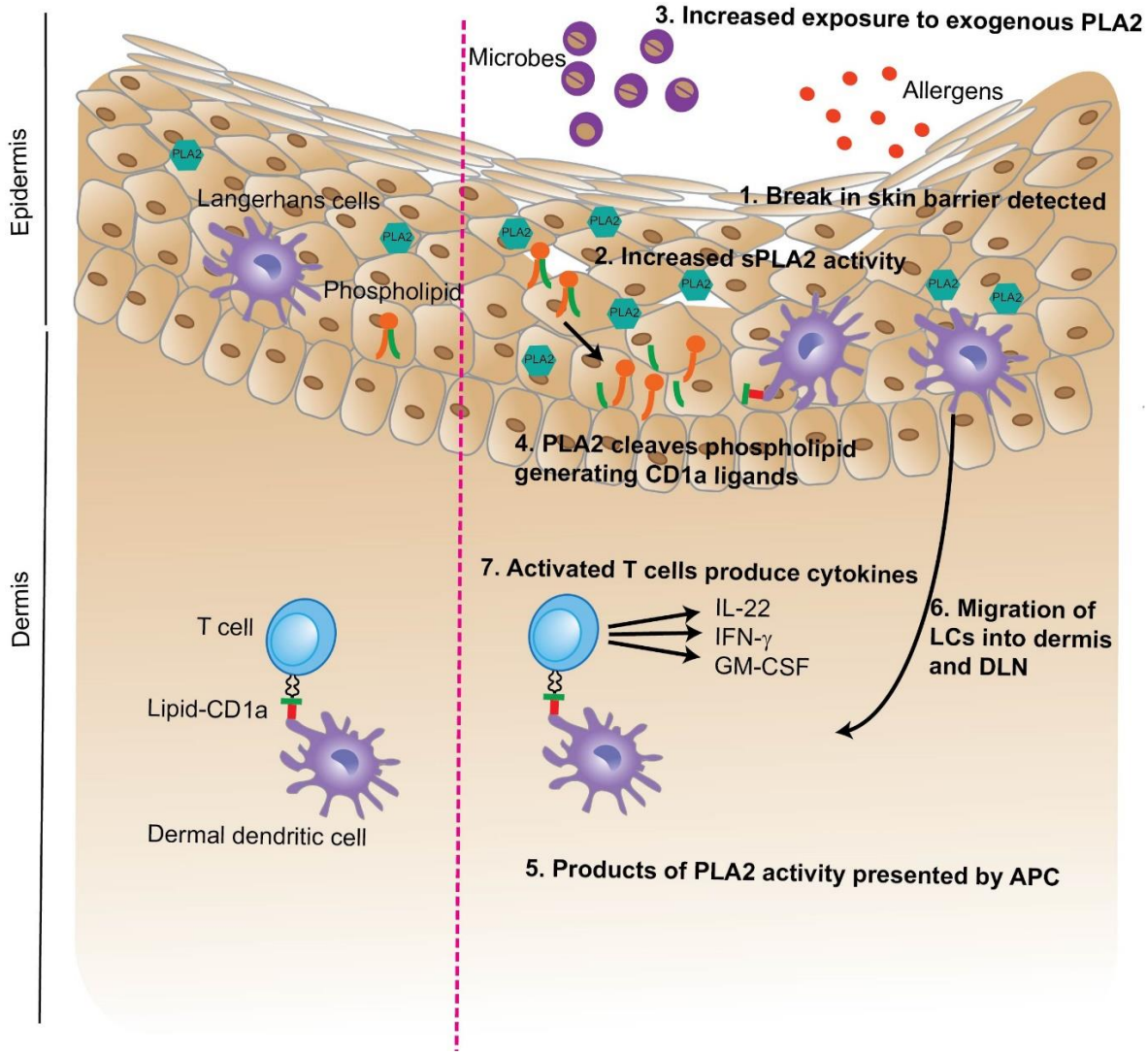


Figure 7-2: Proposed mechanism of CD1a-restricted T cell detection of break in skin barrier.

1) Breakdown of skin barrier integrity due to physical insult or chemical damage, or genetic defects in proteins key to maintaining the barrier. This breach results in many changes in the skin, such as change in pH and calcium level, increased TEWL, altered lipid profile and decreased protease inhibitor activity. 2) All of which causes increased sPLA2 secretion and activity. 3) Breach in skin barrier also results in increased exposure to environmental antigens, including microbes and allergens, containing PLA2. 4) Increased endogenous and exogenous PLA2 activity leads to increased phospholipid cleavage and production of CD1a ligands. FA and LPL are presented by skin APCs, including 5) dermal DCs or 6) migrating LCs to CD1a-restricted T cells in the dermis. 7) Stimulated T cells produce cytokines, such as IFN-γ, IL-22 and GM-CSF, and initiate an immune response through the recruitment of other immune cells to repair the breach to the skin barrier.

Using lines to further investigate the physiological role PLA2-responsive CD1a-restricted T cells, identified a number of interesting observations. As described previously, the lines expressed skin homing markers and produced IL-22, indicative of involvement in cutaneous immunity. They also produced a number of Th1 and Th2 cytokines, suggesting that they have a diverse role in the immune system that is driven by the local environment and cytokine milieu. Both lines studied here were able to respond to multiple CD1 isoforms with varying intensities, which has previously never been reported. T cells have been identified that express more than one TCR providing multiple antigen specificities (349, 350). Although TCR sequencing is required to confirm that they are clones and not cell lines, this is an interesting finding and will have implications for their function *in vivo*.

We have demonstrated the presence of polyclonal venom-responsive CD1a-restricted T cells in blood of healthy individuals. PLA2 was identified as the venom component responsible for generating CD1a ligands, which is a novel pathway of inflammation and antigen presentation to non-conventional T cells. PLA2-responsive T cells were also identified in the skin, expressed skin homing markers and IL-22, suggesting that they may play a role in cutaneous immunity. These cells may be involved in sensing breaches to the skin barrier via this novel pathway, as CD1a-restricted T cell responding to venom PLA2 also responded to an endogenous PLA2. Elevated venom PLA2 responsive CD1a-restricted T cell levels were demonstrated in allergic individuals and found changes over the course of immunotherapy, implicating them in the development of allergic disease and identifies PLA2 as a potential therapeutic target.

7.1 Future Work

There are some areas that require further investigation to provide more definitive conclusions and to answer further questions that the results have raised. Having demonstrated the presence of venom and PLA2-responsive CD1a-restricted T cells in the blood and skin, the next step would be to investigate their function *in vivo*, which could be done using humanised mice expressing human Group I CD1 molecules, and studying responses to venom or PLA2 challenge. This would help answer the question of whether PLA2-responsive CD1a-restricted T cells are directly involved in the pathophysiology of allergic disease. It would be interesting to study the cytokine response *in vivo* and if the same broad range of cytokines are produced. Investigating CD1a-restricted T cells in *Pla2g3-Tg* mice may also help determine their physiological role. We have also identified here a potential homeostatic role for CD1a-restricted T cells in the skin by detecting breaches in barrier integrity. This requires further investigation, using the humanised mouse model and studying diseases associated with breakdown of barrier function, such as AD or psoriasis in humans. This pathway of PLA2-induced T cell dependent inflammation identifies new targets for therapy, such as inhibition of PLA2. Further investigation of PLA2 inhibitors using disease models of AD, psoriasis or allergic inflammation could provide novel treatments for inflammatory skin disease.

This study focused on IFN-γ, IL-13 and GM-CSF production to measure frequencies of CD1a-reactive T cells in allergic compared to non-allergic individuals. Other cytokines produced would be interesting to investigate. IL-22 in particular would be worth investigating due to its known function in the skin and as it is secreted by CD1a-reactive T cells. In order to investigate whether these cells are directly involved in allergic disease, comparison of CD1a-restricted T cells and IgE levels (total and PLA2

specific) in allergic individuals, or IgG4 levels during immunotherapy, could provide further insight. Although we have demonstrated venom-specific CD1a-restricted T cells in venom allergy, and pollen-specific CD1a-restricted T cells have been reported during the allergic season (53), it is not known whether this pathway is involved in other allergies. Having demonstrated that CD1a-reactive T cells respond to a number of PLA2-containing allergens, frequencies of these cells could be measured in allergic individuals, as was done here with venom allergic individuals.

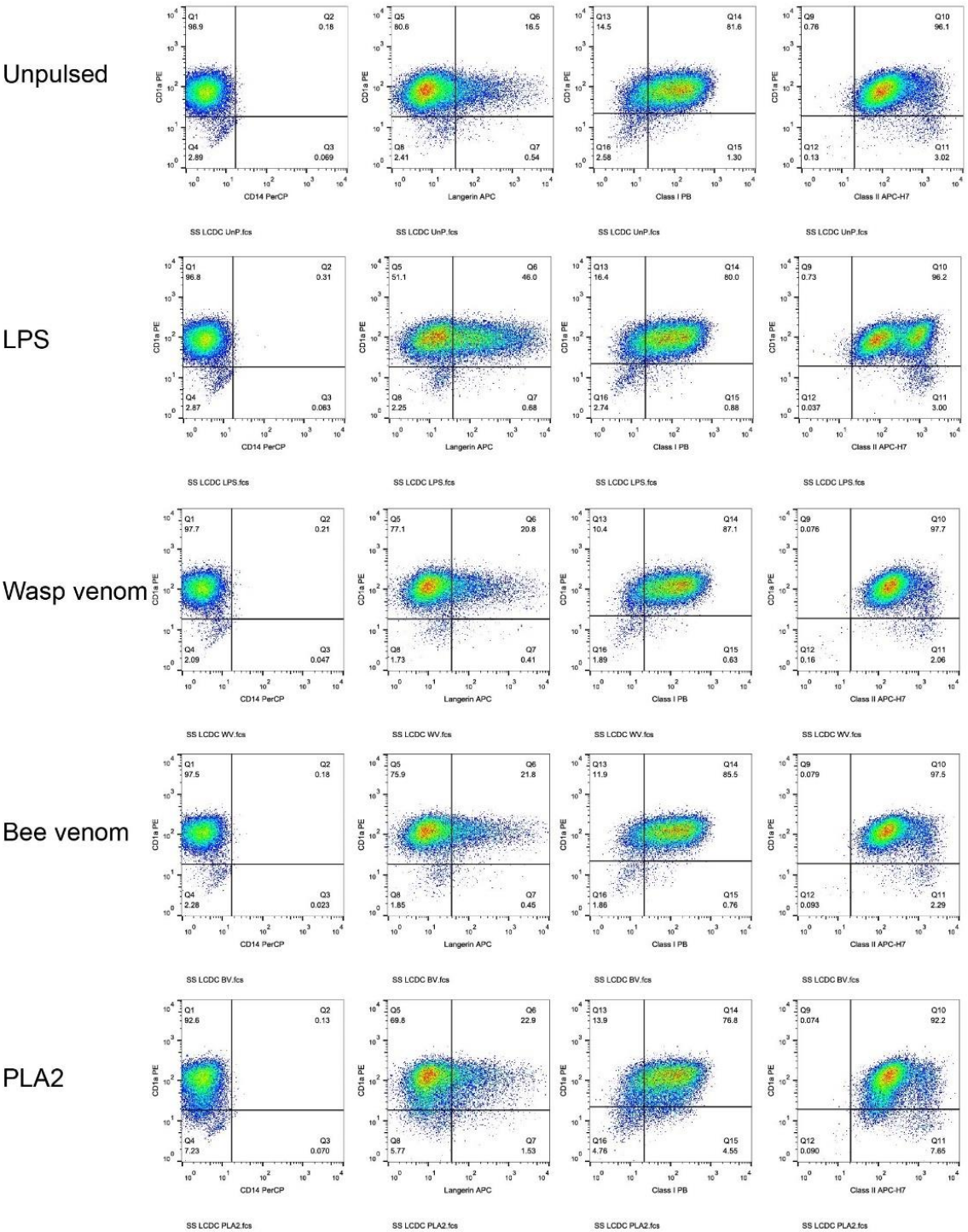
In order to fully characterise the PLA2 responsive CD1a-reactive lines generated, additional experiments are required, including TCR sequencing to determine clonality, experiments with known foreign CD1a antigens such as DDM, and target cell lysis assays. ICS and Fluorospot analysis would be useful to determine if each cell is producing both Th1 and Th2 cytokines or their expression is restricted. Further investigation of the range of lipids recognised by PLA2-responsive CD1a-reactive cells would enhance our understanding of their function. Studies using squalene and triglycerides or fatty acids with longer chain length and more saturation, may provide further insights into lipid-CD1a interactions.

Lipid antigen presentation by LCs on CD1a was shown to require Langerin (198). It would be interesting to investigate this further in the context of PLA2-generated ligands. Interestingly, both Langerin and PLA2 receptors are C-type lectins. Although they are from different families, one could hypothesise that PLA2 binds to Langerin and is then internalised by receptor-mediated endocytosis and co-localises with CD1a in Birbeck granules, where it has access to previously unexposed phospholipids. Or, PLA2 binding at the surface bring it in proximity to CD1a, and so as neo-antigens are generated, they can be exchanged onto CD1a for presentation at the cell surface. These ideas are the foci of ongoing work in the laboratory.

8 Appendix

8.1 Antigen presentation and co-stimulatory molecule expression on mDC and LC-like cells following antigen exposure

A



B

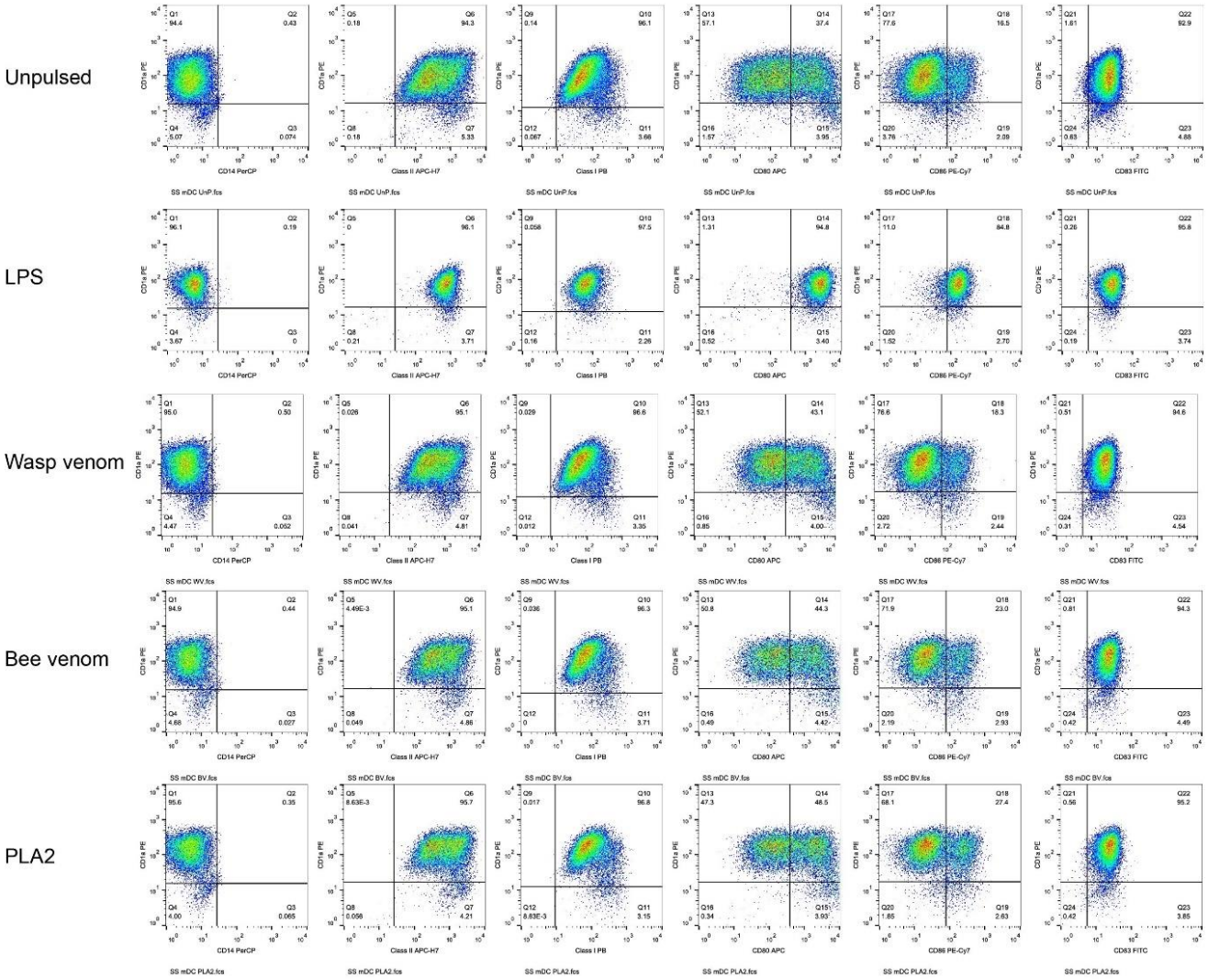


Figure 8-1: Expression of key surface markers on mDC and LC-like cells is not affected by *Vespula spp.* venom, *Apis mellifera* venom, and *Apis mellifera* venom PLA2.

mDC (A) and LC-like cells (B), generated from CD14+ cell isolated by MACs bead separation from PBMCs, were pulsed for 24h with 100ng/ml *Vespula spp.* venom, *Apis mellifera* venom or *Apis mellifera* venom PLA2, or 1µg/ml LPS. Cells were then washed and stained for flow cytometry analysis.

9 References

1. Brigl, M., Brenner, M.B. 2004. CD1: antigen presentation and T cell function. *Annual review of immunology*. 22:817-90
2. De Libero, G., Mori, L. 2010. How the immune system detects lipid antigens. *Progress in lipid research*. 49:120-7
3. Salio, M., Silk, J.D., Cerundolo, V. 2010. Recent advances in processing and presentation of CD1 bound lipid antigens. *Current opinion in immunology*. 22:81-8
4. Hava, D.L., Brigl, M., van den Elzen, P., Zajonc, D.M., Wilson, I.A., Brenner, M.B. 2005. CD1 assembly and the formation of CD1-antigen complexes. *Current opinion in immunology*. 17:88-94
5. Moody, D.B., Zajonc, D.M., Wilson, I.A. 2005. Anatomy of CD1-lipid antigen complexes. *Nature reviews Immunology*. 5:387-99
6. Salomonsen, J., Sørensen, M.R., Marston, D.A., Rogers, S.L., Collen, T., van Hateren, A., Smith, A.L., Beal, R.K., Skjødt, K., Kaufman, J. 2005. Two CD1 genes map to the chicken MHC, indicating that CD1 genes are ancient and likely to have been present in the primordial MHC. *Proceedings of the National Academy of Sciences of the United States of America*. 102:8668-73
7. Blumberg, R.S., Gerdes, D., Chott, A., Porcelli, S.A., Balk, S.P. 1995. Structure and Function of the CD1 Family of MHC-like Cell Surface Proteins. *Immunological reviews*. 147:5-29
8. Moody, B.D. T cell activation by CD1 and lipid antigens: Springer Science & Business Media; 2007. 4-21 p.
9. Yang, Z., Wang, C., Wang, T., Bai, J., Zhao, Y., Liu, X., Ma, Q., Wu, X., Guo, Y., Zhao, Y. 2015. Analysis of the reptile CD1 genes: evolutionary implications. *Immunogenetics*. 67:337-46
10. Han, M., Hannick, L.I., DiBrino, M., Robinson, M.A. 1999. Polymorphism of human CD1 genes. *Tissue antigens*. 54:122-7
11. Golmoghaddam, H., Pezeshki, A.M., Ghaderi, A., Doroudchi, M. 2011. CD1a and CD1d genes polymorphisms in breast, colorectal and lung cancers. *Pathology oncology research : POR*. 17:669-75
12. Caporale, C.M., Notturno, F., Pace, M., Aureli, A., Di Tommaso, V., De Luca, G., Farina, D., Giovannini, A., Uncini, A. 2011. CD1A and CD1E gene polymorphisms are associated with susceptibility to multiple sclerosis. *International journal of immunopathology and pharmacology*. 24:175-83
13. Caporale, C.M., Papola, F., Fioroni, M.A., Aureli, A., Giovannini, A., Notturno, F., Adorno, D., Caporale, V., Uncini, A. 2006. Susceptibility to Guillain-Barre syndrome is associated to polymorphisms of CD1 genes. *Journal of neuroimmunology*. 177:112-8
14. Seshadri, C., Shenoy, M., Wells, R.D., Hensley-McBain, T., Andersen-Nissen, E., McElrath, M.J., Cheng, T.-Y., Moody, D.B., Hawn, T.R. 2013. Human CD1a deficiency is common and genetically regulated. *The Journal of Immunology*. 191:1586-93
15. Chang, C.-C.J., Wright, A., Punnonen, J. 2000. Monocyte-derived CD1a+ and CD1a− dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. *The Journal of Immunology*. 165:3584-91
16. Cernadas, M., Lu, J., Watts, G., Brenner, M. 2009. CD1a expression defines an interleukin-12 producing population of human dendritic cells. *Clinical & Experimental Immunology*. 155:523-33

17. Seshadri, C., Thuong, N.T.T., Yen, N.T.B., Bang, N.D., Chau, T.T.H., Thwaites, G.E., Dunstan, S.J., Hawn, T.R. 2014. A polymorphism in human CD1A is associated with susceptibility to tuberculosis. *Genes and immunity*. 15:195-8
18. Bendelac, A., Lantz, O., Quimby, M.E., Yewdell, J.W. 1995. CD1 recognition by mouse NK1positive T lymphocytes. *Science*. 268:863
19. Sugita, M., Porcelli, S.A., Brenner, M.B. 1997. Assembly and retention of CD1b heavy chains in the endoplasmic reticulum. *The Journal of Immunology*. 159:2358-65
20. Bauer, A., Hüttinger, R., Staffler, G., Hansmann, C., Schmidt, W., Majdic, O., Knapp, W., Stockinger, H. 1997. Analysis of the requirement for β2-microglobulin for expression and formation of human CD1 antigens. *European journal of immunology*. 27:1366-73
21. Moody, D.B., Porcelli, S.A. 2003. Intracellular pathways of CD1 antigen presentation. *Nature reviews Immunology*. 3:11-22
22. Cernadas, M., Cavallari, M., Watts, G., Mori, L., De Libero, G., Brenner, M.B. 2010. Early recycling compartment trafficking of CD1a is essential for its intersection and presentation of lipid antigens. *Journal of immunology*. 184:1235-41
23. Sugita, M., Grant, E.P., van Donselaar, E., Hsu, V.W., Rogers, R.A., Peters, P.J., Brenner, M.B. 1999. Separate pathways for antigen presentation by CD1 molecules. *Immunity*. 11:743-52
24. Hussain, M.M., Shi, J., Dreizen, P. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *Journal of lipid research*. 44:22-32
25. Brozovic, S., Nagaishi, T., Yoshida, M., Betz, S., Salas, A., Chen, D., Kaser, A., Glickman, J., Kuo, T., Little, A., Morrison, J., Corazza, N., Kim, J.Y., Colgan, S.P., Young, S.G., Exley, M., Blumberg, R.S. 2004. CD1d function is regulated by microsomal triglyceride transfer protein. *Nature medicine*. 10:535-9
26. Kang, S.J., Cresswell, P. 2004. Saposins facilitate CD1d-restricted presentation of an exogenous lipid antigen to T cells. *Nature immunology*. 5:175-81
27. Winau, F., Schwierzeck, V., Hurwitz, R., Remmel, N., Sieling, P.A., Modlin, R.L., Porcelli, S.A., Brinkmann, V., Sugita, M., Sandhoff, K., Kaufmann, S.H., Schaible, U.E. 2004. Saposin C is required for lipid presentation by human CD1b. *Nature immunology*. 5:169-74
28. Engering, A., Geijtenbeek, T.B., van Vliet, S.J., Wijers, M., van Liempt, E., Demaurex, N., Lanzavecchia, A., Fransen, J., Figdor, C.G., Piguet, V., van Kooyk, Y. 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *Journal of immunology*. 168:2118-26
29. Moody, D.B., Briken, V., Cheng, T.Y., Roura-Mir, C., Guy, M.R., Geho, D.H., Tykocinski, M.L., Besra, G.S., Porcelli, S.A. 2002. Lipid length controls antigen entry into endosomal and nonendosomal pathways for CD1b presentation. *Nature immunology*. 3:435-42
30. Zajonc, D.M., Crispin, M.D., Bowden, T.A., Young, D.C., Cheng, T.Y., Hu, J., Costello, C.E., Rudd, P.M., Dwek, R.A., Miller, M.J., Brenner, M.B., Moody, D.B., Wilson, I.A. 2005. Molecular mechanism of lipopeptide presentation by CD1a. *Immunity*. 22:209-19
31. Zajonc, D.M., Elsliger, M.A., Teyton, L., Wilson, I.A. 2003. Crystal structure of CD1a in complex with a sulfatide self antigen at a resolution of 2.15 A. *Nature immunology*. 4:808-15
32. Birkinshaw, R.W., Pellicci, D.G., Cheng, T.Y., Keller, A.N., Sandoval-Romero, M., Gras, S., de Jong, A., Uldrich, A.P., Moody, D.B., Godfrey, D.I., Rossjohn, J. 2015. alphabeta T cell antigen receptor recognition of CD1a presenting self lipid ligands. *Nature immunology*.

33. Kasmar, A.G., Van Rhijn, I., Magalhaes, K.G., Young, D.C., Cheng, T.-Y., Turner, M.T., Schiefner, A., Kalathur, R.C., Wilson, I.A., Bhati, M. 2013. Cutting edge: CD1a tetramers and dextramers identify human lipopeptide–specific T cells ex vivo. *The Journal of Immunology*. 191:4499-503
34. de Jong, A., Cheng, T.Y., Huang, S., Gras, S., Birkinshaw, R.W., Kasmar, A.G., Van Rhijn, I., Pena-Cruz, V., Ruan, D.T., Altman, J.D., Rossjohn, J., Moody, D.B. 2014. CD1a-autoreactive T cells recognize natural skin oils that function as headless antigens. *Nature immunology*. 15:177-85
35. McCarthy, C., Shepherd, D., Fleire, S., Stronge, V.S., Koch, M., Illarionov, P.A., Bossi, G., Salio, M., Denkberg, G., Reddington, F. 2007. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *The Journal of experimental medicine*. 204:1131-44
36. Manolova, V., Kistowska, M., Paoletti, S., Baltariu, G.M., Bausinger, H., Hanau, D., Mori, L., De Libero, G. 2006. Functional CD1a is stabilized by exogenous lipids. *European journal of immunology*. 36:1083-92
37. Garcia-Alles, L.F., Versluis, K., Maveyraud, L., Vallina, A.T., Sansano, S., Bello, N.F., Gober, H.J., Guillet, V., de la Salle, H., Puzo, G., Mori, L., Heck, A.J., De Libero, G., Mourey, L. 2006. Endogenous phosphatidylcholine and a long spacer ligand stabilize the lipid-binding groove of CD1b. *The EMBO journal*. 25:3684-92
38. Brennan, P.J., Brigl, M., Brenner, M.B. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nature reviews Immunology*. 13:101-17
39. Chiba, A., Dascher, C.C., Besra, G.S., Brenner, M.B. 2008. Rapid NKT cell responses are self-terminating during the course of microbial infection. *Journal of immunology*. 181:2292-302
40. Guidry, T.V., Olsen, M., Kil, K.S., Hunter, R.L., Jr., Geng, Y.J., Actor, J.K. 2004. Failure of CD1D-/- mice to elicit hypersensitive granulomas to mycobacterial cord factor trehalose 6,6'-dimycolate. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*. 24:362-71
41. Rothchild, A.C., Jayaraman, P., Nunes-Alves, C., Behar, S.M. 2014. iNKT cell production of GM-CSF controls Mycobacterium tuberculosis. *PLoS pathogens*. 10:e1003805
42. Kawakami, K., Kinjo, Y., Uezu, K., Yara, S., Miyagi, K., Koguchi, Y., Nakayama, T., Taniguchi, M., Saito, A. 2002. Minimal contribution of Valpha14 natural killer T cells to Th1 response and host resistance against mycobacterial infection in mice. *Microbiology and immunology*. 46:207-10
43. Siddiqui, S., Visvabharathy, L., Wang, C.R. 2015. Role of Group 1 CD1-Restricted T Cells in Infectious Disease. *Frontiers in immunology*. 6:337
44. Lockridge, J.L., Chen, X., Zhou, Y., Rajesh, D., Roenneburg, D.A., Hegde, S., Gerdts, S., Cheng, T.Y., Anderson, R.J., Painter, G.F., Moody, D.B., Burlingham, W.J., Gumperz, J.E. 2011. Analysis of the CD1 antigen presenting system in humanized SCID mice. *PloS one*. 6:e21701
45. Felio, K., Nguyen, H., Dascher, C.C., Choi, H.J., Li, S., Zimmer, M.I., Colmone, A., Moody, D.B., Brenner, M.B., Wang, C.R. 2009. CD1-restricted adaptive immune responses to Mycobacteria in human group 1 CD1 transgenic mice. *The Journal of experimental medicine*. 206:2497-509
46. Ly, D., Kasmar, A.G., Cheng, T.Y., de Jong, A., Huang, S., Roy, S., Bhatt, A., van Summeren, R.P., Altman, J.D., Jacobs, W.R., Jr., Adams, E.J., Minnaard, A.J., Porcelli, S.A., Moody, D.B. 2013. CD1c tetramers detect ex vivo T cell responses to

processed phosphomycoketide antigens. *The Journal of experimental medicine*. 210:729-41
47. Brigl, M., Bry, L., Kent, S.C., Gumperz, J.E., Brenner, M.B. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nature immunology*. 4:1230-7
48. Zeissig, S., Murata, K., Sweet, L., Publicover, J., Hu, Z., Kaser, A., Bosse, E., Iqbal, J., Hussain, M.M., Balschun, K., Rocken, C., Arlt, A., Gunther, R., Hampe, J., Schreiber, S., Baron, J.L., Moody, D.B., Liang, T.J., Blumberg, R.S. 2012. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nature medicine*. 18:1060-8
49. Durante-Mangoni, E., Wang, R., Shaulov, A., He, Q., Nasser, I., Afdhal, N., Koziel, M.J., Exley, M.A. 2004. Hepatic CD1d expression in hepatitis C virus infection and recognition by resident proinflammatory CD1d-reactive T cells. *Journal of immunology*. 173:2159-66
50. Albacker, L.A., Chaudhary, V., Chang, Y.J., Kim, H.Y., Chuang, Y.T., Pichavant, M., DeKruyff, R.H., Savage, P.B., Umetsu, D.T. 2013. Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity. *Nature medicine*. 19:1297-304
51. Akbari, O., Stock, P., Meyer, E., Kronenberg, M., Sidobre, S., Nakayama, T., Taniguchi, M., Grusby, M.J., DeKruyff, R.H., Umetsu, D.T. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nature medicine*. 9:582-8
52. Balato, A., Zhao, Y., Harberts, E., Groleau, P., Liu, J., Fishelevich, R., Gaspari, A.A. 2012. CD1d-dependent, iNKT-cell cytotoxicity against keratinocytes in allergic contact dermatitis. *Experimental dermatology*. 21:915-20
53. Agea, E., Russano, A., Bistoni, O., Mannucci, R., Nicoletti, I., Corazzi, L., Postle, A.D., De Libero, G., Porcelli, S.A., Spinozzi, F. 2005. Human CD1-restricted T cell recognition of lipids from pollens. *The Journal of experimental medicine*. 202:295-308
54. Taniguchi, M., Tashiro, T., Dashtsoodol, N., Hongo, N., Watarai, H. 2010. The specialized iNKT cell system recognizes glycolipid antigens and bridges the innate and acquired immune systems with potential applications for cancer therapy. *International immunology*. 22:1-6
55. Goubier, A., Vocanson, M., Macari, C., Poyet, G., Herbelin, A., Nicolas, J.F., Dubois, B., Kaiserlian, D. 2013. Invariant NKT cells suppress CD8(+) T-cell-mediated allergic contact dermatitis independently of regulatory CD4(+) T cells. *The Journal of investigative dermatology*. 133:980-7
56. Nakagawa, R., Nagafune, I., Tazunoki, Y., Ehara, H., Tomura, H., Iijima, R., Motoki, K., Kamishohara, M., Seki, S. 2001. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. *Journal of immunology*. 166:6578-84
57. Roura-Mir, C., Catalfamo, M., Cheng, T.Y., Marqusee, E., Besra, G.S., Jaraquemada, D., Moody, D.B. 2005. CD1a and CD1c activate intrathyroidal T cells during Graves' disease and Hashimoto's thyroiditis. *Journal of immunology*. 174:3773-80
58. Nagayama, Y., Watanabe, K., Niwa, M., McLachlan, S.M., Rapoport, B. 2004. Schistosoma mansoni and alpha-galactosylceramide: prophylactic effect of Th1 Immune suppression in a mouse model of Graves' hyperthyroidism. *Journal of immunology*. 173:2167-73

59. Bosma, A., Abdel-Gadir, A., Isenberg, D.A., Jury, E.C., Mauri, C. 2012. Lipid-antigen presentation by CD1d(+) B cells is essential for the maintenance of invariant natural killer T cells. *Immunity*. 36:477-90
60. Sumida, T., Sakamoto, A., Murata, H., Makino, Y., Takahashi, H., Yoshida, S., Nishioka, K., Iwamoto, I., Taniguchi, M. 1995. Selective reduction of T cells bearing invariant V alpha 24J alpha Q antigen receptor in patients with systemic sclerosis. *The Journal of experimental medicine*. 182:1163-8
61. Gombert, J.M., Herbelin, A., Tancrede-Bohin, E., Dy, M., Carnaud, C., Bach, J.F. 1996. Early quantitative and functional deficiency of NK1+-like thymocytes in the NOD mouse. *European journal of immunology*. 26:2989-98
62. Illes, Z., Kondo, T., Newcombe, J., Oka, N., Tabira, T., Yamamura, T. 2000. Differential expression of NK T cell V alpha 24J alpha Q invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. *Journal of immunology*. 164:4375-81
63. Jiang, X., Kojo, S., Harada, M., Ohkohchi, N., Taniguchi, M., Seino, K.I. 2007. Mechanism of NKT cell-mediated transplant tolerance. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*. 7:1482-90
64. Sharif, S., Arreaza, G.A., Zucker, P., Mi, Q.S., Sondhi, J., Naidenko, O.V., Kronenberg, M., Koezuka, Y., Delovitch, T.L., Gombert, J.M., Leite-De-Moraes, M., Gouarin, C., Zhu, R., Hameg, A., Nakayama, T., Taniguchi, M., Lepault, F., Lehuen, A., Bach, J.F., Herbelin, A. 2001. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nature medicine*. 7:1057-62
65. de Jong, A., Pena-Cruz, V., Cheng, T.Y., Clark, R.A., Van Rhijn, I., Moody, D.B. 2010. CD1a-autoreactive T cells are a normal component of the human alphabeta T cell repertoire. *Nature immunology*. 11:1102-9
66. de Lalla, C., Lepore, M., Piccolo, F.M., Rinaldi, A., Scelfo, A., Garavaglia, C., Mori, L., De Libero, G., Dellabona, P., Casorati, G. 2011. High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells. *European journal of immunology*. 41:602-10
67. Vincent, M.S., Xiong, X., Grant, E.P., Peng, W., Brenner, M.B. 2005. CD1a-, b-, and c-restricted TCRs recognize both self and foreign antigens. *Journal of immunology*. 175:6344-51
68. Galli, S.J., Tsai, M., Piliponsky, A.M. 2008. The development of allergic inflammation. *Nature*. 454:445-54
69. Yazdanbakhsh, M., Kremsner, P.G., van Ree, R. 2002. Allergy, parasites, and the hygiene hypothesis. *Science*. 296:490-4
70. Barnes, P.J. 2011. Pathophysiology of allergic inflammation. *Immunological reviews*. 242:31-50
71. Brown, J., Wilson, T., Metcalfe, D. 2008. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clinical & Experimental Allergy*. 38:4-18
72. Wilcock, L.K., Francis, J.N., Durham, S.R. 2006. IgE-facilitated antigen presentation: role in allergy and the influence of allergen immunotherapy. *Immunology and allergy clinics of North America*. 26:333-47, viii-ix
73. Souwer, Y., Szegedi, K., Kapsenberg, M.L., de Jong, E.C. 2010. IL-17 and IL-22 in atopic allergic disease. *Current opinion in immunology*. 22:821-6
74. Soroosh, P., Doherty, T.A. 2009. Th9 and allergic disease. *Immunology*. 127:450-8

75. Matsuzawa, S., Sakashita, K., Kinoshita, T., Ito, S., Yamashita, T., Koike, K. 2003. IL-9 enhances the growth of human mast cell progenitors under stimulation with stem cell factor. *Journal of immunology*. 170:3461-7
76. Louahed, J., Zhou, Y., Maloy, W.L., Rani, P.U., Weiss, C., Tomer, Y., Vink, A., Renauld, J., Van Snick, J., Nicolaides, N.C., Levitt, R.C., Haczku, A. 2001. Interleukin 9 promotes influx and local maturation of eosinophils. *Blood*. 97:1035-42
77. Nakamura, Y., Kubo, M., Tamari, M., Hirota, T. 2014. Genome-wide association studies of allergic diseases. *Genome*.
78. Weidinger, S., Gieger, C., Rodriguez, E., Baurecht, H., Mempel, M., Klopp, N., Gohlke, H., Wagenpfeil, S., Ollert, M., Ring, J. 2008. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. *PLoS Genetics*. 4:e1000166
79. Hoffman, D.R., Jacobson, R.S. 1984. Allergens in hymenoptera venom XII: how much protein is in a sting. *Annals of allergy*. 52:276-8
80. Krishna, M., Ewan, P., Diwakar, L., Durham, S., Frew, A., Leech, S., Nasser, S. 2011. Diagnosis and management of hymenoptera venom allergy: British Society for Allergy and Clinical Immunology (BSACI) guidelines. *Clinical & Experimental Allergy*. 41:1201-20
81. Sampson, H.A., Munoz-Furlong, A., Campbell, R.L., Adkinson, N.F., Jr., Bock, S.A., Branum, A., Brown, S.G., Camargo, C.A., Jr., Cydulka, R., Galli, S.J., Gidudu, J., Gruchalla, R.S., Harlor, A.D., Jr., Hepner, D.L., Lewis, L.M., Lieberman, P.L., Metcalfe, D.D., O'Connor, R., Muraro, A., Rudman, A., Schmitt, C., Scherrer, D., Simons, F.E., Thomas, S., Wood, J.P., Decker, W.W. 2006. Second symposium on the definition and management of anaphylaxis: summary report--Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *The Journal of allergy and clinical immunology*. 117:391-7
82. Biló, B.M., Rueff, F., Mosbech, H., Bonifazi, F., Oude-Elberink, J.N.G., the, E.I.G.o.I.V.H. 2005. Diagnosis of Hymenoptera venom allergy. *Allergy*. 60:1339-49
83. Mosbech, H. 1983. Death caused by wasp and bee stings in Denmark 1960-1980. *Allergy*. 38:195-200
84. Golden, D.B., Marsh, D.G., Freidhoff, L.R., Kwiterovich, K.A., Addison, B., Kagey-Sobotka, A., Lichtenstein, L.M. 1997. Natural history of Hymenoptera venom sensitivity in adults. *Journal of allergy and clinical immunology*. 100:760-6
85. Goldberg, A., Confino-Cohen, R. 1997. Timing of venom skin tests and IgE determinations after insect sting anaphylaxis. *The Journal of allergy and clinical immunology*. 100:182-4
86. Heinzerling, L., Mari, A., Bergmann, K.C., Bresciani, M., Burbach, G., Darsow, U., Durham, S., Fokkens, W., Gjomarkaj, M., Haahtela, T., Bom, A.T., Wohrl, S., Maibach, H., Lockey, R. 2013. The skin prick test - European standards. *Clinical and translational allergy*. 3:3
87. Warrington, R. 2006. Lack of Correlation between Severity of Clinical Symptoms, Skin Test Reactivity, and Radioallergosorbent Test Results in Venom-Allergic Patients. *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology*. 2:62-7
88. Golden, D.B., Kagey-Sobotka, A., Norman, P.S., Hamilton, R.G., Lichtenstein, L.M. 2001. Insect sting allergy with negative venom skin test responses. *Journal of allergy and clinical immunology*. 107:897-901
89. Hemmer, W., Focke, M., Kolarich, D., Wilson, I.B., Altmann, F., Wöhrl, S., Götz, M., Jarisch, R. 2001. Antibody binding to venom carbohydrates is a frequent cause for

double positivity to honeybee and yellow jacket venom in patients with stinging-insect allergy. *Journal of allergy and clinical immunology*. 108:1045-52
90. Lu, G., Gonzalez, M., Qian, N., Soldatova, L. 1996. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy. *Journal of allergy and clinical immunology*. 98:588-600
91. van der Veen, M.J., van Ree, R., Aalberse, R.C., Akkerdaas, J., Koppelman, S.J., Jansen, H.M., van der Zee, J.S. 1997. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *Journal of Allergy and Clinical Immunology*. 100:327-34
92. van Ree, R., Aalberse, R.C. 1999. Specific IgE without clinical allergy. *Journal of allergy and clinical immunology*. 103:1000-1
93. Sicherer, S.H., Leung, D.Y. 2015. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects in 2014. *The Journal of allergy and clinical immunology*. 135:357-67
94. Schoessler, S. 2013. Epinephrine the drug of choice for anaphylaxis. *NASN School Nurse*. 28:52-
95. Pajno, G., Barberio, G., De Luca, F., Morabito, L., Parmiani, S. 2001. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. *Clinical & Experimental Allergy*. 31:1392-7
96. Möller, C., Dreborg, S., Ferdousi, H.A., Halken, S., Høst, A., Jacobsen, L., Koivikko, A., Koller, D.Y., Niggemann, B., Norberg, L.A. 2002. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *Journal of Allergy and Clinical Immunology*. 109:251-6
97. Oude Elberink, J.N., De Monchy, J.G., Van Der Heide, S., Guyatt, G.H., Dubois, A.E. 2002. Venom immunotherapy improves health-related quality of life in patients allergic to yellow jacket venom. *The Journal of allergy and clinical immunology*. 110:174-82
98. Ross, R.N., Nelson, H.S., Finegold, I. 2000. Effectiveness of specific immunotherapy in the treatment of hymenoptera venom hypersensitivity: a meta-analysis. *Clinical therapeutics*. 22:351-8
99. Ozdemir, C., Kucuksezer, U., Akdis, M., Akdis, C. 2011. Mechanisms of immunotherapy to wasp and bee venom. *Clinical & Experimental Allergy*. 41:1226-34
100. Karagol, H.I.E., Bakirtas, A., Yilmaz, O., Topal, E., Arga, M., Demirsoy, M.S., Turktas, I. 2015. Long-term follow-up of re-sting reactions in children with moderate to severe venom hypersensitivity. *European journal of pediatrics*. 1-6
101. Cox, L., Nelson, H., Lockey, R., Calabria, C., Chacko, T., Finegold, I., Nelson, M., Weber, R., Bernstein, D.I., Blessing-Moore, J. 2011. Allergen immunotherapy: a practice parameter third update. *Journal of Allergy and Clinical Immunology*. 127:S1-S55
102. Sturm, G., Kränke, B., Rudolph, C., Aberer, W. 2002. Rush Hymenoptera venom immunotherapy: a safe and practical protocol for high-risk patients. *Journal of allergy and clinical immunology*. 110:928-33
103. Korošec, P., Žiberna, K., Šilar, M., Dežman, M., Čelesnik Smodiš, N., Rijavec, M., Kopač, P., Eržen, R., Lalek, N., Bajrović, N. 2015. Immunological and clinical factors associated with adverse systemic reactions during the build-up phase of honeybee venom immunotherapy. *Clinical & Experimental Allergy*.
104. Brehler, R., Wolf, H., Kütting, B., Schnitker, J., Luger, T. 2000. Safety of a two-day ultrarush insect venom immunotherapy protocol in comparison with protocols of

longer duration and involving a larger number of injections. *Journal of allergy and clinical immunology*. 105:1231-5
105. Reimers, A., Hari, Y., Müller, U. 2000. Reduction of side-effects from ultrarush immunotherapy with honeybee venom by pretreatment with fexofenadine: a double-blind, placebo-controlled trial. *Allergy*. 55:483-7
106. Pitsios, C., Demoly, P., Bilò, M., Gerth van Wijk, R., Pfaar, O., Sturm, G., Rodriguez del Rio, P., Tsoumani, M., Gawlik, R., Paraskevopoulos, G. 2015. Clinical contraindications to allergen immunotherapy: an EAACI position paper. *Allergy*.
107. Dahl, R., Kapp, A., Colombo, G., De Monchy, J.G., Rak, S., Emminger, W., Riis, B., Grønager, P.M., Durham, S.R. 2008. Sublingual grass allergen tablet immunotherapy provides sustained clinical benefit with progressive immunologic changes over 2 years. *Journal of Allergy and Clinical Immunology*. 121:512-8. e2
108. Corzo, J., Carrillo, T., Pedemonte, C., Plaza Martin, A., Martín Hurtado, S., Dige, E., Calderon, M. 2014. Tolerability during double-blind randomized phase I trials with the house dust mite allergy immunotherapy tablet in adults and children. *Journal of Investigative Allergology and Clinical Immunology*. 24:154-61
109. Patriarca, G., Nucera, E., Roncallo, C., Aruanno, A., Lombardo, C., Decinti, M., Pascolini, L., Milani, M., Buonomo, A., Schiavino, D. 2008. Sublingual desensitization in patients with wasp venom allergy: preliminary results. *International journal of immunopathology and pharmacology*. 21:669-77
110. Severino, M.G., Cortellini, G., Bonadonna, P., Francescato, E., Panzini, I., Macchia, D., Campi, P., Spadolini, I., Canonica, W.G., Passalacqua, G. 2008. Sublingual immunotherapy for large local reactions caused by honeybee sting: a double-blind, placebo-controlled trial. *Journal of Allergy and Clinical Immunology*. 122:44-8
111. Carballido, J., Carballido-Perrig, N., Kägi, M., Meloen, R., Wüthrich, B., Heusser, C., Blaser, K. 1993. T cell epitope specificity in human allergic and nonallergic subjects to bee venom phospholipase A2. *The Journal of Immunology*. 150:3582-91
112. Nouri-Aria, K.T., Wachholz, P.A., Francis, J.N., Jacobson, M.R., Walker, S.M., Wilcock, L.K., Staple, S.Q., Aalberse, R.C., Till, S.J., Durham, S.R. 2004. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *The Journal of Immunology*. 172:3252-9
113. Akbari, O., DeKruyff, R.H., Umetsu, D.T. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nature immunology*. 2:725-31
114. Meiler, F., Zumkehr, J., Klunker, S., Ruckert, B., Akdis, C.A., Akdis, M. 2008. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *The Journal of experimental medicine*. 205:2887-98
115. Francis, J.N., Till, S.J., Durham, S.R. 2003. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *The Journal of allergy and clinical immunology*. 111:1255-61
116. Jutel, M., Akdis, M., Budak, F., Aebischer-Casaulta, C., Wrzyszcz, M., Blaser, K., Akdis, C.A. 2003. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *European journal of immunology*. 33:1205-14
117. Akdis, M., Verhagen, J., Taylor, A., Karamloo, F., Karagiannidis, C., Crameri, R., Thunberg, S., Deniz, G., Valenta, R., Fiebig, H. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-

specific T regulatory 1 and T helper 2 cells. *The Journal of experimental medicine*. 199:1567-75
118. Ling, E.M., Smith, T., Nguyen, X.D., Pridgeon, C., Dallman, M., Arbery, J., Carr, V.A., Robinson, D.S. 2004. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet*. 363:608-15
119. Schuerwegh, A.J., De Clerck, L.S., Bridts, C.H., Stevens, W.J. 2001. Wasp venom immunotherapy induces a shift from IL-4-producing towards interferon-gamma-producing CD4+ and CD8+ T lymphocytes. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 31:740-6
120. Akdis, C.A., Akdis, M., Blesken, T., Wymann, D., Alkan, S.S., Müller, U., Blaser, K. 1996. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *Journal of Clinical Investigation*. 98:1676
121. Nasser, S.M., Ying, S., Meng, Q., Kay, A.B., Ewan, P.W. 2001. Interleukin-10 levels increase in cutaneous biopsies of patients undergoing wasp venom immunotherapy. *European journal of immunology*. 31:3704-13
122. Dugas-Breit, S., Przybilla, B., Dugas, M., Arnold, A., Pfundstein, G., Kuchenhoff, H., Rueff, F. 2010. Serum concentration of baseline mast cell tryptase: evidence for a decline during long-term immunotherapy for Hymenoptera venom allergy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 40:643-9
123. Akdis, C.A., Akdis, M. 2011. Mechanisms of allergen-specific immunotherapy. *Journal of Allergy and Clinical Immunology*. 127:18-27
124. Novak, N., Mete, N., Bussmann, C., Maintz, L., Bieber, T., Akdis, M., Zumkehr, J., Jutel, M., Akdis, C. 2012. Early suppression of basophil activation during allergen-specific immunotherapy by histamine receptor 2. *Journal of Allergy and Clinical Immunology*. 130:1153-8. e2
125. Shim, J.Y., Kim, B.S., Cho, S.H., Min, K.U., Hong, S.J. 2003. Allergen-specific conventional immunotherapy decreases immunoglobulin E-mediated basophil histamine releasability. *Clinical & Experimental Allergy*. 33:52-7
126. Siegmund, R., Vogelsang, H., Machnik, A., Herrmann, D. 2000. Surface membrane antigen alteration on blood basophils in patients with Hymenoptera venom allergy under immunotherapy. *The Journal of allergy and clinical immunology*. 106:1190-5
127. Jutel, M., Muller, U.R., Fricker, M., Rihs, S., Pichler, W.J., Dahinden, C. 1996. Influence of bee venom immunotherapy on degranulation and leukotriene generation in human blood basophils. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 26:1112-8
128. Akoum, H., Duez, C., Vorng, H., Fahy, O., Wallaert, B., Tonnel, A.B., Tsicopoulos, A. 1998. Early modifications of chemokine production and mRNA expression during rush venom immunotherapy. *Cytokine*. 10:706-12
129. Cooke, R.A., Barnard, J.H., Hebald, S., Stull, A. 1935. Serological evidence of immunity with coexisting sensitization in a type of human allergy (hay fever). *The Journal of experimental medicine*. 62:733-50
130. Visco, V., Dolecek, C., Denepoux, S., Le Mao, J., Guret, C., Rousset, F., Guinnepain, M.T., Kraft, D., Valenta, R., Weyer, A., Banchereau, J., Labecque, S. 1996. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. *Journal of immunology*. 157:956-62

131. Flicker, S., Steinberger, P., Norderhaug, L., Sperr, W.R., Majlesi, Y., Valent, P., Kraft, D., Valenta, R. 2002. Conversion of grass pollen allergen-specific human IgE into a protective IgG(1) antibody. *European journal of immunology*. 32:2156-62
132. Shamji, M., Ljørring, C., Francis, J., A Calderon, M., Larche, M., Kimber, I., Frew, A., Ipsen, H., Lund, K., Würtzen, P. 2012. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy*. 67:217-26
133. Akdis, C.A., Blesken, T., Akdis, M., Wuthrich, B., Blaser, K. 1998. Role of interleukin 10 in specific immunotherapy. *The Journal of clinical investigation*. 102:98-106
134. Jeannin, P., Lecoanet, S., Delneste, Y., Gauchat, J.F., Bonnefoy, J.Y. 1998. IgE versus IgG4 production can be differentially regulated by IL-10. *Journal of immunology*. 160:3555-61
135. Akdis, C.A., Blaser, K. 2001. Mechanisms of interleukin-10-mediated immune suppression. *Immunology*. 103:131-6
136. Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., Wahl, S.M. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of experimental medicine*. 198:1875-86
137. Klunker, S., Chong, M.M., Mantel, P.Y., Palomares, O., Bassin, C., Ziegler, M., Ruckert, B., Meiler, F., Akdis, M., Littman, D.R., Akdis, C.A. 2009. Transcription factors RUNX1 and RUNX3 in the induction and suppressive function of Foxp3+ inducible regulatory T cells. *The Journal of experimental medicine*. 206:2701-15
138. Davis, L.S., Bhutani, S., Barnett, S.R., Khan, D.A. 2011. Early gene expression changes with rush immunotherapy. *Clinical and molecular allergy : CMA*. 9:12
139. Niedoszytko, M., Bruinenberg, M., de Monchy, J., Weersma, R.K., Wijmenga, C., Jassem, E., Elberink, J.N. 2011. Changes in gene expression caused by insect venom immunotherapy responsible for the long-term protection of insect venom-allergic patients. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*. 106:502-10
140. Pasparakis, M., Haase, I., Nestle, F.O. 2014. Mechanisms regulating skin immunity and inflammation. *Nature Reviews Immunology*. 14:289-301
141. Proksch, E., Brandner, J.M., Jensen, J.M. 2008. The skin: an indispensable barrier. *Experimental dermatology*. 17:1063-72
142. Madison, K.C. 2003. Barrier function of the skin: "la raison d'etre" of the epidermis. *The Journal of investigative dermatology*. 121:231-41
143. Cork, M.J., Danby, S.G., Vasilopoulos, Y., Hadgraft, J., Lane, M.E., Moustafa, M., Guy, R.H., Macgowan, A.L., Tazi-Ahnini, R., Ward, S.J. 2009. Epidermal barrier dysfunction in atopic dermatitis. *The Journal of investigative dermatology*. 129:1892-908
144. Abrams, K., Harvell, J.D., Shriner, D., Wertz, P., Maibach, H., Maibach, H.I., Rehfeld, S.J. 1993. Effect of organic solvents on in vitro human skin water barrier function. *The Journal of investigative dermatology*. 101:609-13
145. Caubet, C., Jonca, N., Brattsand, M., Guerrin, M., Bernard, D., Schmidt, R., Egelrud, T., Simon, M., Serre, G. 2004. Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. *The Journal of investigative dermatology*. 122:1235-44
146. Kato, T., Takai, T., Mitsuishi, K., Okumura, K., Ogawa, H. 2005. Cystatin A inhibits IL-8 production by keratinocytes stimulated with Der p 1 and Der f 1:

biochemical skin barrier against mite cysteine proteases. *The Journal of allergy and clinical immunology*. 116:169-76
147. Deraison, C., Bonnart, C., Lopez, F., Besson, C., Robinson, R., Jayakumar, A., Wagberg, F., Brattsand, M., Hachem, J.P., Leonardsson, G., Hovnanian, A. 2007. LEKTI fragments specifically inhibit KLK5, KLK7, and KLK14 and control desquamation through a pH-dependent interaction. *Molecular biology of the cell*. 18:3607-19
148. Triggiani, M., Granata, F., Giannattasio, G., Marone, G. 2005. Secretory phospholipases A2 in inflammatory and allergic diseases: not just enzymes. *The Journal of allergy and clinical immunology*. 116:1000-6
149. Hachem, J.P., Crumrine, D., Fluhr, J., Brown, B.E., Feingold, K.R., Elias, P.M. 2003. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. *The Journal of investigative dermatology*. 121:345-53
150. Hachem, J.P., Man, M.Q., Crumrine, D., Uchida, Y., Brown, B.E., Rogiers, V., Roseeuw, D., Feingold, K.R., Elias, P.M. 2005. Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing enzymes and profound alterations of barrier function and stratum corneum integrity. *The Journal of investigative dermatology*. 125:510-20
151. Ekholm, I.E., Brattsand, M., Egelrud, T. 2000. Stratum corneum tryptic enzyme in normal epidermis: a missing link in the desquamation process? *The Journal of investigative dermatology*. 114:56-63
152. Ovaere, P., Lippens, S., Vandenabeele, P., Declercq, W. 2009. The emerging roles of serine protease cascades in the epidermis. *Trends in biochemical sciences*. 34:453-63
153. Hachem, J.P., Houben, E., Crumrine, D., Man, M.Q., Schurer, N., Roelandt, T., Choi, E.H., Uchida, Y., Brown, B.E., Feingold, K.R., Elias, P.M. 2006. Serine protease signaling of epidermal permeability barrier homeostasis. *The Journal of investigative dermatology*. 126:2074-86
154. Elias, P., Ahn, S., Brown, B., Crumrine, D., Feingold, K.R. 2002. Origin of the epidermal calcium gradient: regulation by barrier status and role of active vs passive mechanisms. *The Journal of investigative dermatology*. 119:1269-74
155. Elias, P.M., Ahn, S.K., Denda, M., Brown, B.E., Crumrine, D., Kimutai, L.K., Komuves, L., Lee, S.H., Feingold, K.R. 2002. Modulations in epidermal calcium regulate the expression of differentiation-specific markers. *The Journal of investigative dermatology*. 119:1128-36
156. Elsholz, F., Harteneck, C., Muller, W., Friedland, K. 2014. Calcium--a central regulator of keratinocyte differentiation in health and disease. *European journal of dermatology : EJD*. 24:650-61
157. Mao-Qiang, M., Mauro, T., Bench, G., Warren, R., Elias, P.M., Feingold, K.R. 1997. Calcium and potassium inhibit barrier recovery after disruption, independent of the type of insult in hairless mice. *Experimental dermatology*. 6:36-40
158. Kashibuchi, N., Hirai, Y., O'Goshi, K., Tagami, H. 2002. Three-dimensional analyses of individual corneocytes with atomic force microscope: morphological changes related to age, location and to the pathologic skin conditions. *Skin research and technology : official journal of International Society for Bioengineering and the Skin (ISBS) [and] International Society for Digital Imaging of Skin (ISDIS) [and] International Society for Skin Imaging (ISSI)*. 8:203-11
159. Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-

Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S., McLean, W.H. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature genetics*. 38:441-6
160. Stout, T.E., McFarland, T., Mitchell, J.C., Appukuttan, B., Stout, J.T. 2014. Recombinant filaggrin is internalized and processed to correct filaggrin deficiency. *The Journal of investigative dermatology*. 134:423-9
161. Rawlings, A.V., Harding, C.R. 2004. Moisturization and skin barrier function. *Dermatologic therapy*. 17 Suppl 1:43-8
162. De Benedetto, A., Rafaels, N.M., McGirt, L.Y., Ivanov, A.I., Georas, S.N., Cheadle, C., Berger, A.E., Zhang, K., Vidyasagar, S., Yoshida, T., Boguniewicz, M., Hata, T., Schneider, L.C., Hanifin, J.M., Gallo, R.L., Novak, N., Weidinger, S., Beaty, T.H., Leung, D.Y., Barnes, K.C., Beck, L.A. 2011. Tight junction defects in patients with atopic dermatitis. *The Journal of allergy and clinical immunology*. 127:773-86.e1-7
163. Weidinger, S., Baurecht, H., Wagenpfeil, S., Henderson, J., Novak, N., Sandilands, A., Chen, H., Rodriguez, E., O'Regan, G.M., Watson, R., Liao, H., Zhao, Y., Barker, J.N., Allen, M., Reynolds, N., Meggitt, S., Northstone, K., Smith, G.D., Strobl, C., Stahl, C., Kneib, T., Klopp, N., Bieber, T., Behrendt, H., Palmer, C.N., Wichmann, H.E., Ring, J., Illig, T., McLean, W.H., Irvine, A.D. 2008. Analysis of the individual and aggregate genetic contributions of previously identified serine peptidase inhibitor Kazal type 5 (SPINK5), kallikrein-related peptidase 7 (KLK7), and filaggrin (FLG) polymorphisms to eczema risk. *The Journal of allergy and clinical immunology*. 122:560-8.e4
164. Hansson, L., Backman, A., Ny, A., Edlund, M., Ekholm, E., Ekstrand Hammarstrom, B., Tornell, J., Wallbrandt, P., Wennbo, H., Egelrud, T. 2002. Epidermal overexpression of stratum corneum chymotryptic enzyme in mice: a model for chronic itchy dermatitis. *The Journal of investigative dermatology*. 118:444-9
165. Lee, Y.A., Wahn, U., Kehrt, R., Tarani, L., Businco, L., Gustafsson, D., Andersson, F., Oranje, A.P., Wolkertstorfer, A., v Berg, A., Hoffmann, U., Kuster, W., Wienker, T., Ruschendorf, F., Reis, A. 2000. A major susceptibility locus for atopic dermatitis maps to chromosome 3q21. *Nature genetics*. 26:470-3
166. Vasilopoulos, Y., Cork, M.J., Teare, D., Marinou, I., Ward, S.J., Duff, G.W., Tazi-Ahnini, R. 2007. A nonsynonymous substitution of cystatin A, a cysteine protease inhibitor of house dust mite protease, leads to decreased mRNA stability and shows a significant association with atopic dermatitis. *Allergy*. 62:514-9
167. Jeong, S.K., Kim, H.J., Youm, J.K., Ahn, S.K., Choi, E.H., Sohn, M.H., Kim, K.E., Hong, J.H., Shin, D.M., Lee, S.H. 2008. Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *The Journal of investigative dermatology*. 128:1930-9
168. Jinnestal, C.L., Belfrage, E., Back, O., Schmidtchen, A., Sonesson, A. 2014. Skin barrier impairment correlates with cutaneous Staphylococcus aureus colonization and sensitization to skin-associated microbial antigens in adult patients with atopic dermatitis. *International journal of dermatology*. 53:27-33
169. Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P.S., Wang, X.N., Malinarich, F., Malleret, B., Larbi, A., Tan, P., Zhao, H., Poidinger, M., Pagan, S., Cookson, S., Dickinson, R., Dimmick, I., Jarrett, R.F., Renia, L., Tam, J., Song, C., Connolly, J., Chan, J.K., Gehring, A., Bertoletti, A., Collin, M., Ginhoux, F. 2012. Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity*. 37:60-73

170. Chu, C.C., Ali, N., Karagiannis, P., Di Meglio, P., Skowera, A., Napolitano, L., Barinaga, G., Grys, K., Sharif-Paghaleh, E., Karagiannis, S.N., Peakman, M., Lombardi, G., Nestle, F.O. 2012. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *The Journal of experimental medicine*. 209:935-45
171. Guttman-Yassky, E., Lowes, M.A., Fuentes-Duculan, J., Whynot, J., Novitskaya, I., Cardinale, I., Haider, A., Khatcherian, A., Carucci, J.A., Bergman, R., Krueger, J.G. 2007. Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis. *The Journal of allergy and clinical immunology*. 119:1210-7
172. Nestle, F.O., Conrad, C., Tun-Kyi, A., Homey, B., Gombert, M., Boyman, O., Burg, G., Liu, Y.J., Gilliet, M. 2005. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *The Journal of experimental medicine*. 202:135-43
173. Wang, X.N., McGovern, N., Gunawan, M., Richardson, C., Windebank, M., Siah, T.W., Lim, H.Y., Fink, K., Li, J.L., Ng, L.G., Ginhoux, F., Angeli, V., Collin, M., Haniffa, M. 2014. A three-dimensional atlas of human dermal leukocytes, lymphatics, and blood vessels. *The Journal of investigative dermatology*. 134:965-74
174. Sugaya, M., Miyagaki, T., Ohmatsu, H., Suga, H., Kai, H., Kamata, M., Fujita, H., Asano, Y., Tada, Y., Kadono, T., Okochi, H., Sato, S. 2012. Association of the numbers of CD163(+) cells in lesional skin and serum levels of soluble CD163 with disease progression of cutaneous T cell lymphoma. *Journal of dermatological science*. 68:45-51
175. Fuentes-Duculan, J., Suarez-Farinas, M., Zaba, L.C., Nograles, K.E., Pierson, K.C., Mitsui, H., Pensabene, C.A., Kzhyshkowska, J., Krueger, J.G., Lowes, M.A. 2010. A subpopulation of CD163-positive macrophages is classically activated in psoriasis. *The Journal of investigative dermatology*. 130:2412-22
176. Mantovani, A., Biswas, S.K., Galdiero, M.R., Sica, A., Locati, M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. *The Journal of pathology*. 229:176-85
177. Kataru, R.P., Jung, K., Jang, C., Yang, H., Schwendener, R.A., Baik, J.E., Han, S.H., Alitalo, K., Koh, G.Y. 2009. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood*. 113:5650-9
178. St John, A.L., Rathore, A.P., Yap, H., Ng, M.L., Metcalfe, D.D., Vasudevan, S.G., Abraham, S.N. 2011. Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. *Proceedings of the National Academy of Sciences of the United States of America*. 108:9190-5
179. Salimi, M., Ogg, G. 2014. Innate lymphoid cells and the skin. *BMC dermatology*. 14:18
180. Kim, B.S. 2015. Innate lymphoid cells in the skin. *The Journal of investigative dermatology*. 135:673-8
181. Salimi, M., Barlow, J.L., Saunders, S.P., Xue, L., Gutowska-Owsiak, D., Wang, X., Huang, L.C., Johnson, D., Scanlon, S.T., McKenzie, A.N., Fallon, P.G., Ogg, G.S. 2013. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *The Journal of experimental medicine*. 210:2939-50
182. Pantelyushin, S., Haak, S., Ingold, B., Kulig, P., Heppner, F.L., Navarini, A.A., Becher, B. 2012. Rorγt+ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *The Journal of clinical investigation*. 122:2252-6

183. Clark, R.A., Chong, B., Mirchandani, N., Brinster, N.K., Yamanaka, K., Dowgiert, R.K., Kupper, T.S. 2006. The vast majority of CLA+ T cells are resident in normal skin. *Journal of immunology*. 176:4431-9
184. Schenkel, J.M., Masopust, D. 2014. Tissue-resident memory T cells. *Immunity*. 41:886-97
185. Mackay, L.K., Rahimpour, A., Ma, J.Z., Collins, N., Stock, A.T., Hafon, M.-L., Vega-Ramos, J., Lauzurica, P., Mueller, S.N., Stefanovic, T. 2013. The developmental pathway for CD103+ CD8+ tissue-resident memory T cells of skin. *Nature immunology*. 14:1294-301
186. Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C., Kupper, T.S. 2012. Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. *Nature*. 483:227-31
187. Agius, E., Lacy, K.E., Vukmanovic-Stejic, M., Jagger, A.L., Papageorgiou, A.P., Hall, S., Reed, J.R., Curnow, S.J., Fuentes-Duculan, J., Buckley, C.D., Salmon, M., Taams, L.S., Krueger, J., Greenwood, J., Klein, N., Rustin, M.H., Akbar, A.N. 2009. Decreased TNF-alpha synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. *The Journal of experimental medicine*. 206:1929-40
188. O'Brien, R.L., Born, W.K. 2015. Dermal γδ T cells–What have we learned? *Cellular immunology*.
189. Gray, E.E., Suzuki, K., Cyster, J.G. 2011. Cutting Edge: Identification of a Motile IL-17–Producing γδ T Cell Population in the Dermis. *The Journal of Immunology*. 186:6091-5
190. van der Fits, L., Mourits, S., Voerman, J.S., Kant, M., Boon, L., Laman, J.D., Cornelissen, F., Mus, A.-M., Florencia, E., Prens, E.P. 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *The Journal of Immunology*. 182:5836-45
191. Cai, Y., Shen, X., Ding, C., Qi, C., Li, K., Li, X., Jala, V.R., Zhang, H.-g., Wang, T., Zheng, J. 2011. Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity*. 35:596-610
192. Mabuchi, T., Singh, T.P., Takekoshi, T., Jia, G.F., Wu, X., Kao, M.C., Weiss, I., Farber, J.M., Hwang, S.T. 2013. CCR6 is required for epidermal trafficking of gammadelta-T cells in an IL-23-induced model of psoriasiform dermatitis. *The Journal of investigative dermatology*. 133:164-71
193. Sumaria, N., Roediger, B., Ng, L.G., Qin, J., Pinto, R., Cavanagh, L.L., Shklovskaya, E., Groth, B.F.d.S., Triccas, J.A., Weninger, W. 2011. Cutaneous immunosurveillance by self-renewing dermal γδ T cells. *The Journal of experimental medicine*. 208:505-18
194. Geherin, S.A., Fintushel, S.R., Lee, M.H., Wilson, R.P., Patel, R.T., Alt, C., Young, A.J., Hay, J.B., Debes, G.F. 2012. The skin, a novel niche for recirculating B cells. *The Journal of Immunology*. 188:6027-35
195. Merad, M., Ginhoux, F., Collin, M. 2008. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nature reviews Immunology*. 8:935-47
196. Kanitakis, J., Morelon, E., Petruzzo, P., Badet, L., Dubernard, J.M. 2011. Self-renewal capacity of human epidermal Langerhans cells: observations made on a composite tissue allograft. *Experimental dermatology*. 20:145-6
197. Dziegiel, P., Dolilńska-Krajewska, B., Dumańska, M., Wecławek, J., Jeleń, M., Podhorska-Okołów, M., Jagoda, E., Fic, M., Zabel, M. 2007. Coexpression of CD1a, langerin and Birbeck's granules in Langerhans cell histiocytoses (LCH) in children:

ultrastructural and immunocytochemical studies. *Folia Histochemica et Cytobiologica*. 45:21-5
198. Hunger, R.E., Sieling, P.A., Ochoa, M.T., Sugaya, M., Burdick, A.E., Rea, T.H., Brennan, P.J., Belisle, J.T., Blauvelt, A., Porcelli, S.A., Modlin, R.L. 2004. Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *The Journal of clinical investigation*. 113:701-8
199. Valladeau, J., Clair-Moninot, V., Dezutter-Dambuyant, C., Pin, J.-J., Kissenpfennig, A., Mattéi, M.-G., Ait-Yahia, S., Bates, E.E., Malissen, B., Koch, F. 2002. Identification of mouse langerin/CD207 in Langerhans cells and some dendritic cells of lymphoid tissues. *The Journal of Immunology*. 168:782-92
200. Mc Dermott, R., Ziylan, U., Spehner, D., Bausinger, H., Lipsker, D., Mommaas, M., Cazenave, J.-P., Raposo, G., Goud, B., de la Salle, H. 2002. Birbeck granules are subdomains of endosomal recycling compartment in human epidermal Langerhans cells, which form where Langerin accumulates. *Molecular biology of the cell*. 13:317-35
201. Seré, K., Baek, J.-H., Ober-Blöbaum, J., Müller-Newen, G., Tacke, F., Yokota, Y., Zenke, M., Hieronymus, T. 2012. Two distinct types of Langerhans cells populate the skin during steady state and inflammation. *Immunity*. 37:905-16
202. Merad, M., Manz, M.G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I.L., Cyster, J.G., Engleman, E.G. 2002. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nature immunology*. 3:1135-41
203. Flacher, V., Bouschbacher, M., Verronese, E., Massacrier, C., Sisirak, V., Berthier-Vergnes, O., de Saint-Vis, B., Caux, C., Dezutter-Dambuyant, C., Lebecque, S., Valladeau, J. 2006. Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. *Journal of immunology*. 177:7959-67
204. Geissmann, F., Dieu-Nosjean, M.C., Dezutter, C., Valladeau, J., Kayal, S., Leborgne, M., Brousse, N., Saeland, S., Davoust, J. 2002. Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. *Journal of experimental medicine*. 196:417-30
205. Stoitzner, P., Holzmann, S., McLellan, A.D., Ivarsson, L., Stossel, H., Kapp, M., Kammerer, U., Douillard, P., Kampgen, E., Koch, F., Saeland, S., Romani, N. 2003. Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. *The Journal of investigative dermatology*. 120:266-74
206. He, Y., Zhang, J., Donahue, C., Falo, L.D., Jr. 2006. Skin-derived dendritic cells induce potent CD8(+) T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity*. 24:643-56
207. Allan, R.S., Smith, C.M., Belz, G.T., van Lint, A.L., Wakim, L.M., Heath, W.R., Carbone, F.R. 2003. Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. *Science*. 301:1925-8
208. Zhao, X., Deak, E., Soderberg, K., Linehan, M., Spezzano, D., Zhu, J., Knipe, D.M., Iwasaki, A. 2003. Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *The Journal of experimental medicine*. 197:153-62
209. Simon, J.C., Tigelaar, R.E., Bergstresser, P.R., Edelbaum, D., Cruz, P.D., Jr. 1991. Ultraviolet B radiation converts Langerhans cells from immunogenic to tolerogenic antigen-presenting cells. Induction of specific clonal anergy in CD4+ T helper 1 cells. *Journal of immunology*. 146:485-91

210. Schwarz, A., Noordegraaf, M., Maeda, A., Torii, K., Clausen, B.E., Schwarz, T. 2010. Langerhans cells are required for UVR-induced immunosuppression. *The Journal of investigative dermatology*. 130:1419-27
211. Beissert, S., Ullrich, S.E., Hosoi, J., Granstein, R.D. 1995. Supernatants from UVB radiation-exposed keratinocytes inhibit Langerhans cell presentation of tumor-associated antigens via IL-10 content. *Journal of leukocyte biology*. 58:234-40
212. Fainaru, O., Woolf, E., Lotem, J., Yarmus, M., Brenner, O., Goldenberg, D., Negreanu, V., Bernstein, Y., Levanon, D., Jung, S., Groner, Y. 2004. Runx3 regulates mouse TGF-beta-mediated dendritic cell function and its absence results in airway inflammation. *The EMBO journal*. 23:969-79
213. Kaplan, D.H., Li, M.O., Jenison, M.C., Shlomchik, W.D., Flavell, R.A., Shlomchik, M.J. 2007. Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. *The Journal of experimental medicine*. 204:2545-52
214. Lutz, M.B., Dohler, A., Azukizawa, H. 2010. Revisiting the tolerogenicity of epidermal Langerhans cells. *Immunology and cell biology*. 88:381-6
215. Seneschal, J., Clark, R.A., Gehad, A., Baecher-Allan, C.M., Kupper, T.S. 2012. Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. *Immunity*. 36:873-84
216. Kaplan, D.H., Kissenpfennig, A., Clausen, B.E. 2008. Insights into Langerhans cell function from Langerhans cell ablation models. *European journal of immunology*. 38:2369-76
217. Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., Shlomchik, M.J. 2005. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity*. 23:611-20
218. Bennett, C.L., van Rijn, E., Jung, S., Inaba, K., Steinman, R.M., Kapsenberg, M.L., Clausen, B.E. 2005. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *The Journal of cell biology*. 169:569-76
219. Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhe, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D., Saeland, S., Davoust, J., Malissen, B. 2005. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity*. 22:643-54
220. Bobr, A., Olvera-Gomez, I., Igyarto, B.Z., Haley, K.M., Hogquist, K.A., Kaplan, D.H. 2010. Acute ablation of Langerhans cells enhances skin immune responses. *Journal of immunology*. 185:4724-8
221. Witte, E., Witte, K., Warszawska, K., Sabat, R., Wolk, K. 2010. Interleukin-22: a cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection. *Cytokine & growth factor reviews*. 21:365-79
222. Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F., Odorisio, T., Traidl-Hoffmann, C., Behrendt, H., Durham, S.R., Schmidt-Weber, C.B., Cavani, A. 2009. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *The Journal of clinical investigation*. 119:3573-85
223. Aujla, S.J., Chan, Y.R., Zheng, M., Fei, M., Askew, D.J., Pociask, D.A., Reinhart, T.A., McAllister, F., Edeal, J., Gaus, K., Husain, S., Kreindler, J.L., Dubin, P.J., Pilewski, J.M., Myerburg, M.M., Mason, C.A., Iwakura, Y., Kolls, J.K. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nature medicine*. 14:275-81

224. Zenewicz, L.A., Yancopoulos, G.D., Valenzuela, D.M., Murphy, A.J., Karow, M., Flavell, R.A. 2007. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity*. 27:647-59
225. Boniface, K., Bernard, F.-X., Garcia, M., Gurney, A.L., Lecron, J.-C., Morel, F. 2005. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *The Journal of Immunology*. 174:3695-702
226. Nograles, K.E., Zaba, L.C., Shemer, A., Fuentes-Duculan, J., Cardinale, I., Kikuchi, T., Ramon, M., Bergman, R., Krueger, J.G., Guttman-Yassky, E. 2009. IL-22–producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17–producing T H 17 T cells. *Journal of Allergy and Clinical Immunology*. 123:1244-52. e2
227. Homey, B., Alenius, H., Müller, A., Soto, H., Bowman, E.P., Yuan, W., McEvoy, L., Lauerma, A.I., Assmann, T., Bünemann, E. 2002. CCL27–CCR10 interactions regulate T cell–mediated skin inflammation. *Nature medicine*. 8:157-65
228. Niebuhr, M., Scharonow, H., Gathmann, M., Mamerow, D., Werfel, T. 2010. Staphylococcal exotoxins are strong inducers of IL-22: A potential role in atopic dermatitis. *The Journal of allergy and clinical immunology*. 126:1176-83 e4
229. Aujla, S.J., Chan, Y.R., Zheng, M., Fei, M., Askew, D.J., Pociask, D.A., Reinhart, T.A., McAllister, F., Edeal, J., Gaus, K. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nature medicine*. 14:275-81
230. Dennis, E.A., Cao, J., Hsu, Y.H., Magrioti, V., Kokotos, G. 2011. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chemical reviews*. 111:6130-85
231. Murakami, M., Taketomi, Y., Sato, H., Yamamoto, K. 2011. Secreted phospholipase A2 revisited. *Journal of biochemistry*. 150:233-55
232. Winget, J.M., Pan, Y.H., Bahnson, B.J. 2006. The interfacial binding surface of phospholipase A2s. *Biochimica et biophysica acta*. 1761:1260-9
233. Khanapure, S.P., Garvey, D.S., Janero, D.R., Letts, L.G. 2007. Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. *Current topics in medicinal chemistry*. 7:311-40
234. Hegen, M., Sun, L., Uozumi, N., Kume, K., Goad, M.E., Nickerson-Nutter, C.L., Shimizu, T., Clark, J.D. 2003. Cytosolic phospholipase A2alpha-deficient mice are resistant to collagen-induced arthritis. *The Journal of experimental medicine*. 197:1297-302
235. Marusic, S., Leach, M.W., Pelker, J.W., Azoitei, M.L., Uozumi, N., Cui, J., Shen, M.W., DeClercq, C.M., Miyashiro, J.S., Carito, B.A., Thakker, P., Simmons, D.L., Leonard, J.P., Shimizu, T., Clark, J.D. 2005. Cytosolic phospholipase A2 alpha-deficient mice are resistant to experimental autoimmune encephalomyelitis. *The Journal of experimental medicine*. 202:841-51
236. Graler, M.H., Goetzl, E.J. 2002. Lysophospholipids and their G protein-coupled receptors in inflammation and immunity. *Biochimica et biophysica acta*. 1582:168-74
237. Fox, L.M., Cox, D.G., Lockridge, J.L., Wang, X., Chen, X., Scharf, L., Trott, D.L., Ndonye, R.M., Veerapen, N., Besra, G.S., Howell, A.R., Cook, M.E., Adams, E.J., Hildebrand, W.H., Gumperz, J.E. 2009. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS biology*. 7:e1000228
238. Perrin-Cocon, L., Agaugue, S., Coutant, F., Saint-Mezard, P., Guironnet-Paquet, A., Nicolas, J.F., Andre, P., Lotteau, V. 2006. Lysophosphatidylcholine is a natural adjuvant that initiates cellular immune responses. *Vaccine*. 24:1254-63

239. Granata, F., Balestrieri, B., Petraroli, A., Giannattasio, G., Marone, G., Triggiani, M. 2003. Secretory phospholipases A2 as multivalent mediators of inflammatory and allergic disorders. *International archives of allergy and immunology*. 131:153-63
240. Gronroos, J.O., Salonen, J.H., Viander, M., Nevalainen, T.J., Laine, V.J. 2005. Roles of group IIA phospholipase A2 and complement in killing of bacteria by acute phase serum. *Scandinavian journal of immunology*. 62:413-9
241. Mitsuishi, M., Masuda, S., Kudo, I., Murakami, M. 2007. Human group III phospholipase A2 suppresses adenovirus infection into host cells. Evidence that group III, V and X phospholipase A2s act on distinct cellular phospholipid molecular species. *Biochimica et biophysica acta*. 1771:1389-96
242. Kim, J.O., Chakrabarti, B.K., Guha-Niyogi, A., Louder, M.K., Mascola, J.R., Ganesh, L., Nabel, G.J. 2007. Lysis of human immunodeficiency virus type 1 by a specific secreted human phospholipase A2. *Journal of virology*. 81:1444-50
243. Fenard, D., Lambeau, G., Maurin, T., Lefebvre, J.C., Doglio, A. 2001. A peptide derived from bee venom-secreted phospholipase A2 inhibits replication of T-cell tropic HIV-1 strains via interaction with the CXCR4 chemokine receptor. *Molecular pharmacology*. 60:341-7
244. Chilton, F.H., Averill, F.J., Hubbard, W.C., Fonteh, A.N., Triggiani, M., Liu, M.C. 1996. Antigen-induced generation of lyso-phospholipids in human airways. *The Journal of experimental medicine*. 183:2235-45
245. Bowton, D.L., Seeds, M.C., Fasano, M.B., Goldsmith, B., Bass, D.A. 1997. Phospholipase A2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. *American journal of respiratory and critical care medicine*. 155:421-5
246. Munoz, N.M., Meliton, A.Y., Arm, J.P., Bonventre, J.V., Cho, W., Leff, A.R. 2007. Deletion of secretory group V phospholipase A2 attenuates cell migration and airway hyperresponsiveness in immunosensitized mice. *Journal of immunology*. 179:4800-7
247. Henderson, W.R., Jr., Chi, E.Y., Bollinger, J.G., Tien, Y.T., Ye, X., Castelli, L., Rubtsov, Y.P., Singer, A.G., Chiang, G.K., Nevalainen, T., Rudensky, A.Y., Gelb, M.H. 2007. Importance of group X-secreted phospholipase A2 in allergen-induced airway inflammation and remodeling in a mouse asthma model. *The Journal of experimental medicine*. 204:865-77
248. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., Shimizu, T. 1997. Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature*. 390:618-22
249. Boilard, E., Lai, Y., Larabee, K., Balestrieri, B., Ghomashchi, F., Fujioka, D., Gobezie, R., Coblyn, J.S., Weinblatt, M.E., Massarotti, E.M., Thornhill, T.S., Divangahi, M., Remold, H., Lambeau, G., Gelb, M.H., Arm, J.P., Lee, D.M. 2010. A novel anti-inflammatory role for secretory phospholipase A2 in immune complex-mediated arthritis. *EMBO molecular medicine*. 2:172-87
250. Tessier, C., Hichami, A., Khan, N.A. 2002. Implication of three isoforms of PLA(2) in human T-cell proliferation. *FEBS letters*. 520:111-6
251. Perrin-Cocon, L., Agaugue, S., Coutant, F., Masurel, A., Bezzine, S., Lambeau, G., Andre, P., Lotteau, V. 2004. Secretory phospholipase A2 induces dendritic cell maturation. *European journal of immunology*. 34:2293-302
252. Sato, H., Taketomi, Y., Isogai, Y., Masuda, S., Kobayashi, T., Yamamoto, K., Murakami, M. 2009. Group III secreted phospholipase A2 transgenic mice spontaneously develop inflammation. *The Biochemical journal*. 421:17-27

253. Taketomi, Y., Ueno, N., Kojima, T., Sato, H., Murase, R., Yamamoto, K., Tanaka, S., Sakanaka, M., Nakamura, M., Nishito, Y., Kawana, M., Kambe, N., Ikeda, K., Taguchi, R., Nakamizo, S., Kabashima, K., Gelb, M.H., Arita, M., Yokomizo, T., Nakamura, M., Watanabe, K., Hirai, H., Nakamura, M., Okayama, Y., Ra, C., Aritake, K., Urade, Y., Morimoto, K., Sugimoto, Y., Shimizu, T., Narumiya, S., Hara, S., Murakami, M. 2013. Mast cell maturation is driven via a group III phospholipase A2-prostaglandin D2-DP1 receptor paracrine axis. *Nature immunology*. 14:554-63
254. Dan, P., Rosenblat, G., Yedgar, S. 2012. Phospholipase A(2) activities in skin physiology and pathology. *European journal of pharmacology*. 691:1-8
255. Kang-Rotondo, C.H., Miller, C.C., Morrison, A.R., Pentland, A.P. 1993. Enhanced keratinocyte prostaglandin synthesis after UV injury is due to increased phospholipase activity. *The American journal of physiology*. 264:C396-401
256. Grass, D.S., Felkner, R.H., Chiang, M.Y., Wallace, R.E., Nevalainen, T.J., Bennett, C.F., Swanson, M.E. 1996. Expression of human group II PLA2 in transgenic mice results in epidermal hyperplasia in the absence of inflammatory infiltrate. *The Journal of clinical investigation*. 97:2233-41
257. Sato, H., Kato, R., Isogai, Y., Saka, G., Ohtsuki, M., Taketomi, Y., Yamamoto, K., Tsutsumi, K., Yamada, J., Masuda, S., Ishikawa, Y., Ishii, T., Kobayashi, T., Ikeda, K., Taguchi, R., Hatakeyama, S., Hara, S., Kudo, I., Itabe, H., Murakami, M. 2008. Analyses of group III secreted phospholipase A2 transgenic mice reveal potential participation of this enzyme in plasma lipoprotein modification, macrophage foam cell formation, and atherosclerosis. *The Journal of biological chemistry*. 283:33483-97
258. Nakano, T., Ohara, O., Teraoka, H., Arita, H. 1990. Glucocorticoids suppress group II phospholipase A2 production by blocking mRNA synthesis and post-transcriptional expression. *The Journal of biological chemistry*. 265:12745-8
259. Lambeau, G., Lazdunski, M. 1999. Receptors for a growing family of secreted phospholipases A2. *Trends in pharmacological sciences*. 20:162-70
260. East, L., Isacke, C.M. 2002. The mannose receptor family. *Biochimica et biophysica acta*. 1572:364-86
261. Valentin, E., Lambeau, G. 2000. Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. *Biochimica et biophysica acta*. 1488:59-70
262. Hanasaki, K., Yokota, Y., Ishizaki, J., Itoh, T., Arita, H. 1997. Resistance to endotoxic shock in phospholipase A2 receptor-deficient mice. *The Journal of biological chemistry*. 272:32792-7
263. Fisher, A.B., Dodia, C., Chander, A., Beers, M.F., Bates, S.R. 1994. Inhibition of Trimeresurus flavoviridis phospholipase A2 by lung surfactant protein A (SP-A). *Biochimica et biophysica acta*. 1211:256-62
264. Brglez, V., Lambeau, G., Petan, T. 2014. Secreted phospholipases A2 in cancer: diverse mechanisms of action. *Biochimie*. 107 Pt A:114-23
265. Aoki, J., Nagai, Y., Hosono, H., Inoue, K., Arai, H. 2002. Structure and function of phosphatidylserine-specific phospholipase A1. *Biochimica et biophysica acta*. 1582:26-32
266. Aslam, A., Lloyd-Lavery, A., Warrell, D.A., Misbah, S., Ogg, G.S. 2011. Common filaggrin null alleles are not associated with hymenoptera venom allergy in Europeans. *International archives of allergy and immunology*. 154:353-5
267. Geissmann, F., Prost, C., Monnet, J.P., Dy, M., Brousse, N., Hermine, O. 1998. Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood

monocytes into dendritic Langerhans cells. *The Journal of experimental medicine*. 187:961-6
268. Vukmanovic-Stejic, M., Sandhu, D., Sobande, T.O., Agius, E., Lacy, K.E., Riddell, N., Montez, S., Dintwe, O.B., Scriba, T.J., Breuer, J., Nikolich-Zugich, J., Ogg, G., Rustin, M.H., Akbar, A.N. 2013. Varicella zoster-specific CD4+Foxp3+ T cells accumulate after cutaneous antigen challenge in humans. *Journal of immunology*. 190:977-86
269. Vukmanovic-Stejic, M., Agius, E., Booth, N., Dunne, P.J., Lacy, K.E., Reed, J.R., Sobande, T.O., Kissane, S., Salmon, M., Rustin, M.H., Akbar, A.N. 2008. The kinetics of CD4+Foxp3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo. *The Journal of clinical investigation*. 118:3639-50
270. Layre, E., Sweet, L., Hong, S., Madigan, C.A., Desjardins, D., Young, D.C., Cheng, T.Y., Annand, J.W., Kim, K., Shamputa, I.C., McConnell, M.J., Debono, C.A., Behar, S.M., Minnaard, A.J., Murray, M., Barry, C.E., 3rd, Matsunaga, I., Moody, D.B. 2011. A comparative lipidomics platform for chemotaxonomic analysis of Mycobacterium tuberculosis. *Chemistry & biology*. 18:1537-49
271. Klein, L., Kyewski, B., Allen, P.M., Hogquist, K.A. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews Immunology*. 14:377-91
272. Tameris, M.D., Hatherill, M., Landry, B.S., Scriba, T.J., Snowden, M.A., Lockhart, S., Shea, J.E., McClain, J.B., Hussey, G.D., Hanekom, W.A., Mahomed, H., McShane, H. 2013. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet*. 381:1021-8
273. Lalvani, A., Pareek, M. 2010. A 100 year update on diagnosis of tuberculosis infection. *British medical bulletin*. 93:69-84
274. McMichael, A.J., Pilch, J.R., Galfre, G., Mason, D.Y., Fabre, J.W., Milstein, C. 1979. A human thymocyte antigen defined by a hybrid myeloma monoclonal antibody. *European journal of immunology*. 9:205-10
275. Calabi, F., Milstein, C. 1986. A novel family of human major histocompatibility complex-related genes not mapping to chromosome 6. *Nature*. 323:540-3
276. Porcelli, S., Brenner, M.B., Greenstein, J.L., Balk, S.P., Terhorst, C., Bleicher, P.A. 1989. Recognition of cluster of differentiation 1 antigens by human CD4-CD8- cytolytic T lymphocytes. *Nature*. 341:447-50
277. Porcelli, S., Morita, C.T., Brenner, M.B. 1992. CD1b restricts the response of human CD4 - 8 - T lymphoyctes to a microbial antigen. *Nature*. 360:593-7
278. Kronenberg, M., Kinjo, Y. 2005. Infection, autoimmunity, and glycolipids: T cells detect microbes through self-recognition. *Immunity*. 22:657-9
279. Kasmar, A., Van Rhijn, I., Moody, D.B. 2009. The evolved functions of CD1 during infection. *Current opinion in immunology*. 21:397-403
280. Dougan, S.K., Kaser, A., Blumberg, R.S. 2007. CD1 expression on antigen-presenting cells. *Current topics in microbiology and immunology*. 314:113-41
281. Huang, S., Cheng, T.Y., Young, D.C., Layre, E., Madigan, C.A., Shires, J., Cerundolo, V., Altman, J.D., Moody, D.B. 2011. Discovery of deoxyceramides and diacylglycerols as CD1b scaffold lipids among diverse groove-blocking lipids of the human CD1 system. *Proceedings of the National Academy of Sciences of the United States of America*. 108:19335-40
282. Young, D.C., Moody, D.B. 2006. T cell recognition of glycolipids presented by CD1 proteins. *Glycobiology*.

283. Zeng, Z., Castano, A.R., Segelke, B.W., Stura, E.A., Peterson, P.A., Wilson, I.A. 1997. Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. *Science*. 277:339-45
284. Gadola, S.D., Zaccai, N.R., Harlos, K., Shepherd, D., Castro-Palomino, J.C., Ritter, G., Schmidt, R.R., Jones, E.Y., Cerundolo, V. 2002. Structure of human CD1b with bound ligands at 2.3 A, a maze for alkyl chains. *Nature immunology*. 3:721-6
285. Borg, N.A., Wun, K.S., Kjer-Nielsen, L., Wilce, M.C., Pellicci, D.G., Koh, R., Besra, G.S., Bharadwaj, M., Godfrey, D.I., McCluskey, J. 2007. CD1d–lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature*. 448:44-9
286. Godfrey, D.I., Rossjohn, J. 2011. New ways to turn on NKT cells. *The Journal of experimental medicine*. 208:1121-5
287. Hiromatsu, K., Dascher, C.C., LeClair, K.P., Sugita, M., Furlong, S.T., Brenner, M.B., Porcelli, S.A. 2002. Induction of CD1-restricted immune responses in guinea pigs by immunization with mycobacterial lipid antigens. *Journal of immunology*. 169:330-9
288. Hiromatsu, K., Dascher, C.C., Sugita, M., Gingrich-Baker, C., Behar, S.M., LeClair, K.P., Brenner, M.B., Porcelli, S.A. 2002. Characterization of guinea-pig group 1 CD1 proteins. *Immunology*. 106:159-72
289. Moody, D.B., Ulrichs, T., Muhlecker, W., Young, D.C., Gurcha, S.S., Grant, E., Rosat, J.P., Brenner, M.B., Costello, C.E., Besra, G.S., Porcelli, S.A. 2000. CD1c-mediated T-cell recognition of isoprenoid glycolipids in Mycobacterium tuberculosis infection. *Nature*. 404:884-8
290. Zeissig, S., Dougan, S.K., Barral, D.C., Junker, Y., Chen, Z., Kaser, A., Ho, M., Mandel, H., McIntyre, A., Kennedy, S.M., Painter, G.F., Veerapen, N., Besra, G.S., Cerundolo, V., Yue, S., Beladi, S., Behar, S.M., Chen, X., Gumperz, J.E., Breckpot, K., Raper, A., Baer, A., Exley, M.A., Hegele, R.A., Cuchel, M., Rader, D.J., Davidson, N.O., Blumberg, R.S. 2010. Primary deficiency of microsomal triglyceride transfer protein in human abetalipoproteinemia is associated with loss of CD1 function. *The Journal of clinical investigation*. 120:2889-99
291. Gutierrez, D.A., Rodewald, H.R. 2013. A sting in the tale of TH2 immunity. *Immunity*. 39:803-5
292. Bilo, B.M., Bonifazi, F. 2008. Epidemiology of insect-venom anaphylaxis. *Current opinion in allergy and clinical immunology*. 8:330-7
293. Aslam, A., Chan, H., Warrell, D.A., Misbah, S., Ogg, G.S. 2010. Tracking antigen-specific T-cells during clinical tolerance induction in humans. *PloS one*. 5:e11028
294. Muller, U.R., Johansen, N., Petersen, A.B., Fromberg-Nielsen, J., Haeberli, G. 2009. Hymenoptera venom allergy: analysis of double positivity to honey bee and Vespula venom by estimation of IgE antibodies to species-specific major allergens Api m1 and Ves v5. *Allergy*. 64:543-8
295. Egner, W., Ward, C., Brown, D.L., Ewan, P.W. 1998. The frequency and clinical significance of specific IgE to both wasp (Vespula) and honey-bee (Apis) venoms in the same patient. *Clinical & Experimental Allergy*. 28:26-34
296. Porcelli, S., Morita, C.T., Brenner, M.B. 1992. CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. *Nature*. 360:593-7
297. Sallusto, F., Lanzavecchia, A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *Journal of experimental medicine*. 179:1109-18

298. Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Durand, I., Cella, M., Lanzavecchia, A., Banchereau, J. 1997. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. *Blood*. 90:1458-70
299. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H., Taniguchi, M. 1997. CD1d-restricted and TCR-mediated activation of Và14 NKT cells by glycosylceramides. *Science*. 278:1626-9
300. King, T.P., Jim, S.Y., Wittkowski, K.M. 2003. Inflammatory role of two venom components of yellow jackets (Vespula vulgaris): a mast cell degranulating peptide mastoparan and phospholipase A1. *International archives of allergy and immunology*. 131:25-32
301. Moody, D.B., Young, D.C., Cheng, T.Y., Rosat, J.P., Roura-Mir, C., O'Connor, P.B., Zajonc, D.M., Walz, A., Miller, M.J., Levery, S.B., Wilson, I.A., Costello, C.E., Brenner, M.B. 2004. T cell activation by lipopeptide antigens. *Science*. 303:527-31
302. Yakimchuk, K., Roura-Mir, C., Magalhaes, K.G., de Jong, A., Kasmar, A.G., Granter, S.R., Budd, R., Steere, A., Pena-Cruz, V., Kirschning, C., Cheng, T.Y., Moody, D.B. 2011. Borrelia burgdorferi infection regulates CD1 expression in human cells and tissues via IL1-beta. *European journal of immunology*. 41:694-705
303. Aslam, A., Kessler, B., Batycka, M., O'Callaghan, C.A., Misbah, S.A., Warrell, D.A., Ogg, G. 2006. Defining the T cell antigen proteome of wasp venom. *Clin Exp Allergy*. 36:1274-80
304. Sin, B.A., Akdis, M., Zumkehr, J., Bezzine, S., Bekpen, C., Lambeau, G., Akdis, C.A. 2011. T-cell and antibody responses to phospholipase A2 from different species show distinct cross-reactivity patterns. *Allergy*. 66:1513-21
305. Cabrera, M.P., Alvares, D.S., Leite, N.B., de Souza, B.M., Palma, M.S., Riske, K.A., Neto, J.R. 2011. New insight into the mechanism of action of wasp mastoparan peptides: lytic activity and clustering observed with giant vesicles. *Langmuir : the ACS journal of surfaces and colloids*. 27:10805-13
306. Argiolas, A., Pisano, J.J. 1983. Facilitation of phospholipase A2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom. *The Journal of biological chemistry*. 258:13697-702
307. Manolova, V., Hirabayashi, Y., Mori, L., De Libero, G. 2003. CD1a and CD1b surface expression is independent from de novo synthesized glycosphingolipids. *European journal of immunology*. 33:29-37
308. Colonna, M. 2010. Skin function for human CD1a-reactive T cells. *Nature immunology*. 11:1079-80
309. de Oliveira, M.R., Palma, M.S. 1998. Polybitoxins: a group of phospholipases A2 from the venom of the neotropical social wasp paulistinha (Polybia paulista). *Toxicon : official journal of the International Society on Toxinology*. 36:189-99
310. dos Santos, L.D., da Silva Menegasso, A.R., dos Santos Pinto, J.R., Santos, K.S., Castro, F.M., Kalil, J.E., Palma, M.S. 2011. Proteomic characterization of the multiple forms of the PLAs from the venom of the social wasp Polybia paulista. *Proteomics*. 11:1403-12
311. Costa, H., Palma, M.S. 2000. Agelotoxin: a phospholipase A(2) from the venom of the neotropical social wasp cassununga (Agelaia pallipes pallipes) (Hymenoptera-Vespidae). *Toxicon : official journal of the International Society on Toxinology*. 38:1367-79

312. Ulrichs, T., Moody, D.B., Grant, E., Kaufmann, S.H., Porcelli, S.A. 2003. T-cell responses to CD1-presented lipid antigens in humans with Mycobacterium tuberculosis infection. *Infection and immunity*. 71:3076-87
313. Gumperz, J., Roy, C., Makowska, A., Lum, D., Sugita, M., Podrebarac, T., Koezuka, Y., Porcelli, S.A., Cardell, S., Brenner, M.B., Behar, S.M. 2000. Murine CD1d-Restricted T Cell Recognition of Cellular Lipids. *Immunity*. 12:211-21
314. Fox, L.M., Cox, D.G., Lockridge, J.L., Wang, X., Chen, X., Scharf, L., Trott, D.L., Ndonye, R.M., Veerapen, N., Besra, G.S., Howell, A.R., Cook, M.E., Adams, E.J., Hildebrand, W.H., Gumperz, J.E. 2009. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biology*. 7:e1000228
315. Kronenberg, M., Havran, W.L. 2014. Immunology: oiling the wheels of autoimmunity. *Nature*. 506:42-3
316. Cortellini, G., Severino, M., Francescato, E., Turillazzi, S., Spadolini, I., Rogkakou, A., Passalacqua, G. 2012. Evaluation and validation of a bee venom sting challenge performed by a micro-syringe. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*. 109:438-41
317. Murakami, M., Lambeau, G. 2013. Emerging roles of secreted phospholipase A(2) enzymes: an update. *Biochimie*. 95:43-50
318. Valentin, E., Ghomashchi, F., Gelb, M.H., Lazdunski, M., Lambeau, G. 2000. Novel human secreted phospholipase A(2) with homology to the group III bee venom enzyme. *The Journal of biological chemistry*. 275:7492-6
319. Scott, D.L., White, S.P., Otwinowski, Z., Yuan, W., Gelb, M.H., Sigler, P.B. 1990. Interfacial catalysis: the mechanism of phospholipase A2. *Science*. 250:1541-6
320. Paduraru, C., Bezbradica, J.S., Kunte, A., Kelly, R., Shayman, J.A., Veerapen, N., Cox, L.R., Besra, G.S., Cresswell, P. 2013. Role for lysosomal phospholipase A2 in iNKT cell-mediated CD1d recognition. *Proc Natl Acad Sci U S A*. 110:5097-102
321. Holm, J., Willumsen, N., Würtzen, P.A., Christensen, L.H., Lund, K. 2011. Facilitated antigen presentation and its inhibition by blocking IgG antibodies depends on IgE repertoire complexity. *Journal of Allergy and Clinical Immunology*. 127:1029-37
322. Poellbauer, E.-M. 1996. Antigen presentation in allergic sensitization. *Immunology and cell biology*. 74:167-73
323. Akdis, C.A., Blesken, T., Akdis, M., Alkan, S.S., Wüthrich, B., Heusser, C.H., Blaser, K. 1997. Induction and differential regulation of bee venom phospholipase A 2–specific human IgE and IgG 4 antibodies in vitro requires allergen-specific and nonspecific activation of T and B cells. *Journal of allergy and clinical immunology*. 99:345-53
324. Müller, U., Akdis, C.A., Fricker, M., Akdis, M., Blesken, T., Bettens, F., Blaser, K. 1998. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *Journal of allergy and clinical immunology*. 101:747-54
325. Wachholz, P.A., Durham, S.R. 2004. Mechanisms of immunotherapy: IgG revisited. *Current opinion in allergy and clinical immunology*. 4:313-8
326. Wambre, E., DeLong, J.H., James, E.A., Torres-Chinn, N., Pfützner, W., Möbs, C., Durham, S.R., Till, S.J., Robinson, D., Kwok, W.W. 2014. Specific immunotherapy modifies allergen-specific CD4+ T-cell responses in an epitope-dependent manner. *Journal of Allergy and Clinical Immunology*. 133:872-9.e7

327. Akdis, M., Akdis, C.A. 2014. Mechanisms of allergen-specific immunotherapy: Multiple suppressor factors at work in immune tolerance to allergens. *Journal of Allergy and Clinical Immunology*. 133:621-31
328. Bourgeois, E.A., Subramaniam, S., Cheng, T.-Y., De Jong, A., Layre, E., Ly, D., Salimi, M., Legaspi, A., Modlin, R.L., Salio, M. 2015. Bee venom processes human skin lipids for presentation by CD1a. *The Journal of experimental medicine*. 212:149-63
329. Seino, K.i., Motohashi, S., Fujisawa, T., Nakayama, T., Taniguchi, M. 2006. Natural killer T cell-mediated antitumor immune responses and their clinical applications. *Cancer science*. 97:807-12
330. Müller, U., Schmid-Grendelmeier, P., Hausmann, O., Helbling, A. 2012. IgE to recombinant allergens Api m 1, Ves v 1, and Ves v 5 distinguish double sensitization from crossreaction in venom allergy. *Allergy*. 67:1069-73
331. Petrovic, N., Grove, C., Langton, P.E., Misso, N.L., Thompson, P.J. 2001. A simple assay for a human serum phospholipase A2 that is associated with high-density lipoproteins. *Journal of lipid research*. 42:1706-13
332. Romagnani, S. 2000. The role of lymphocytes in allergic disease. *Journal of Allergy and Clinical Immunology*. 105:399-408
333. Maggi, E. 2010. T cell responses induced by allergen-specific immunotherapy. *Clinical & Experimental Immunology*. 161:10-8
334. Purwar, R., Kraus, M., Werfel, T., Wittmann, M. 2008. Modulation of keratinocyte-derived MMP-9 by IL-13: a possible role for the pathogenesis of epidermal inflammation. *Journal of investigative dermatology*. 128:59-66
335. Purwar, R., Werfel, T., Wittmann, M. 2006. IL-13-Stimulated Human Keratinocytes Preferentially Attract CD4+ CCR4+ T cells: Possible Role in Atopic Dermatitis. *Journal of investigative dermatology*. 126:1043-51
336. Bonecchi, R., Bianchi, G., Bordignon, P.P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *The Journal of experimental medicine*. 187:129-34
337. Wakugawa, M., Hayashi, K., Nakamura, K., Tamaki, K. 2001. Evaluation of mite allergen-induced Th1 and Th2 cytokine secretion of peripheral blood mononuclear cells from atopic dermatitis patients: association between IL-13 and mite-specific IgE levels. *Journal of dermatological science*. 25:116-26
338. Heufler, C., Koch, F., Schuler, G. 1988. Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *The Journal of experimental medicine*. 167:700-5
339. Kaplan, G., Walsh, G., Guido, L., Meyn, P., Burkhardt, R., Abalos, R., Barker, J., Frindt, P., Fajardo, T., Celona, R. 1992. Novel responses of human skin to intradermal recombinant granulocyte/macrophage-colony-stimulating factor: Langerhans cell recruitment, keratinocyte growth, and enhanced wound healing. *The Journal of experimental medicine*. 175:1717-28
340. Grine, L., Dejager, L., Libert, C., Vandenbroucke, R.E. 2014. An inflammatory triangle in psoriasis: TNF, type I IFNs and IL-17. *Cytokine & growth factor reviews*. 26:25-33
341. Zaidi, M.R., Merlino, G. 2011. The two faces of interferon-γ in cancer. *Clinical cancer research*. 17:6118-24
342. Xia, W., Wang, J., Xu, Y., Jiang, F., Xu, L. 2014. L-BLP25 as a peptide vaccine therapy in non-small cell lung cancer: a review. *Journal of thoracic disease*. 6:1513

343. Sabatos-Peyton, C.A., Verhagen, J., Wraith, D.C. 2010. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Current opinion in immunology*. 22:609-15
344. Eberlein-Konig, B., Ullmann, S., Thomas, P., Przybilla, B. 1995. Tryptase and histamine release due to a sting challenge in bee venom allergic patients treated successfully or unsuccessfully with hyposensitization*. *Clinical & Experimental Allergy*. 25:704-12
345. Spada, F.M., Grant, E.P., Peters, P.J., Sugita, M., Melian, A., Leslie, D.S., Lee, H.K., van Donselaar, E., Hanson, D.A., Krensky, A.M., Majdic, O., Porcelli, S.A., Morita, C.T., Brenner, M.B. 2000. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *The Journal of experimental medicine*. 191:937-48
346. Mori, K., Sajiki, J. 1997. Phospholipid metabolism in European house dust mite (Acari: Pyroglyphidae). *Journal of medical entomology*. 34:538-43
347. Farwanah, H., Raith, K., Neubert, R.H., Wohlrab, J. 2005. Ceramide profiles of the uninvolved skin in atopic dermatitis and psoriasis are comparable to those of healthy skin. *Archives of dermatological research*. 296:514-21
348. Schröder, J.-M. Fatty acids and inflammatory skin diseases: Springer Science & Business Media; 1999.
349. Padovan, E., Casorati, G., Dellabona, P., Giachino, C., Lanzavecchia, A. 1995. Dual receptor T-cells. Implications for alloreactivity and autoimmunity. *Annals of the New York Academy of Sciences*. 756:66-70
350. Padovan, E., Giachino, C., Cella, M., Valitutti, S., Acuto, O., Lanzavecchia, A. 1995. Normal T lymphocytes can express two different T cell receptor beta chains: implications for the mechanism of allelic exclusion. *The Journal of experimental medicine*. 181:1587-91
351. Van Kaer, L. 2015. Bee venom stirs up buzz in antigen presentation. *The Journal of experimental medicine*. 212:126
352. Palm, N.W., Rosenstein, R.K., Yu, S., Schenten, D.D., Florsheim, E., Medzhitov, R. 2013. Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. *Immunity*. 39:976-85
353. Birch, M., Denning, D.W., Robson, G.D. 2004. Comparison of extracellular phospholipase activities in clinical and environmental Aspergillus fumigatus isolates. *Medical mycology*. 42:81-6
354. Haas, U., Podda, M., Behne, M., Gurrieri, S., Alonso, A., Furstenberger, G., Pfeilschifter, J., Lambeau, G., Gelb, M.H., Kaszkin, M. 2005. Characterization and differentiation-dependent regulation of secreted phospholipases A in human keratinocytes and in healthy and psoriatic human skin. *The Journal of investigative dermatology*. 124:204-11
355. Janusova, B., Zbytovska, J., Lorenc, P., Vavrysova, H., Palat, K., Hrabalek, A., Vavrova, K. 2011. Effect of ceramide acyl chain length on skin permeability and thermotropic phase behavior of model stratum corneum lipid membranes. *Biochimica et biophysica acta*. 1811:129-37
356. Leslie, D.S., Dascher, C.C., Cembrola, K., Townes, M.A., Hava, D.L., Hugendubler, L.C., Mueller, E., Fox, L., Roura-Mir, C., Moody, D.B., Vincent, M.S., Gumperz, J.E., Illarionov, P.A., Besra, G.S., Reynolds, C.G., Brenner, M.B. 2008. Serum lipids regulate dendritic cell CD1 expression and function. *Immunology*. 125:289-301
357. Smed-Sorensen, A., Moll, M., Cheng, T.Y., Lore, K., Norlin, A.C., Perbeck, L., Moody, D.B., Spetz, A.L., Sandberg, J.K. 2008. IgG regulates the CD1 expression

profile and lipid antigen-presenting function in human dendritic cells via FcgammaRIIa. *Blood*. 111:5037-46
358. Motta, S., Monti, M., Sesana, S., Mellesi, L., Ghidoni, R., Caputo, R. 1994. Abnormality of water barrier function in psoriasis. Role of ceramide fractions. *Archives of dermatology*. 130:452-6
359. Di Nardo, A., Wertz, P., Giannetti, A., Seidenari, S. 1998. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta dermato-venereologica*. 78:27-30
360. Vavrova, K., Henkes, D., Struver, K., Sochorova, M., Skolova, B., Witting, M.Y., Friess, W., Schreml, S., Meier, R.J., Schafer-Korting, M., Fluhr, J.W., Kuchler, S. 2014. Filaggrin deficiency leads to impaired lipid profile and altered acidification pathways in a 3D skin construct. *The Journal of investigative dermatology*. 134:746-53
361. Moreau, S.J., Asgari, S. 2015. Venom Proteins from Parasitoid Wasps and Their Biological Functions. *Toxins*. 7:2385-412