Contents

Abstract

The role of CD1a-restricted T cells in the pathogenesis of atopic dermatitis (AD)

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In recent years, T-cells that recognise lipid ligands presented by CD1 molecules have been described. CD1a is highly expressed by Langerhans cells of the epidermis and it has been shown that CD1a-restricted T-cells circulate at high frequencies, infiltrate the skin and recognise natural skin lipids. Epidermal CD1a-expressing cells are enriched in AD lesions, however pathways associated with CD1a presentation and AD have not been studied.

The aim of this project was to investigate the role of CD1a-restricted T-cells in the pathogenesis of AD.

The use of target APCs with absent MHC expression (K562 cells) enabled investigation of polyclonal T cell, and HDM responsive CD1a-reactive T cell line responses from many genetically unrelated donors. These T cells were enriched in the blood and skin in patients with AD, and produced a number of cytokines known to be relevant to AD pathogenesis. Using the skin suction blister technique and antigen challenge system, these cells were found to infiltrate human skin following HDM antigen challenge in AD patients.

In order to define the underlying mechanisms, we fractionated total HDM extract and found that CD1a reactivity resided within the protein fraction of HDM, which could be explained by HDM-derived secretory phospholipase A2 (sPLA2) activity. T cell responses were dependent on HDM-derived sPLA2, which most likely

generates neolipid antigens for presentation via CD1a. T cell lines were also generated to further characterise these cells, and produced a broad range of type 1 and type 2 cytokines on stimulation with HDM and other phospholipase containing allergens.

Filaggrin insufficiency is associated with AD and filaggrin was observed to inhibit HDM-derived sPLA2 activity and T cell responses from the blood and skin *ex vivo*.

In summary, the data identify a novel pathway of AD cutaneous inflammation in which HDM-derived sPLA2 generates antigenic neolipids for presentation to CD1a-reactive T cells, and define sPLA2 inhibition as a novel function of filaggrin, with potential therapeutic implications.

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List of publications

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CD1a-restricted T cells to atopic allergens. *Poster presentation at World Immune Regulation Meeting-VII 2013*

Abbreviations

αGalCer	α-Galactosylceramide
AA	Arachidonic acid
AD	Atopic dermatitis
AHR	Airway hyper-responsiveness
AMP	Antimicrobial peptide
APC	Antigen presenting cell
B2M	Beta-2-microglobulin
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDR	Complementarity-determining region
CLA	Cutaneous leucocyte-associated 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 cell
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
FA	Fatty acid
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box protein P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GWAS	Genome wide association studies
HDM	House dust mite
HLA	Human leukocyte antigen
ICS	Intra-cellular cytokine staining
IFN-γ	Gamma-interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cells

LC	Langerhans cells
LPL	Lysophospholipid
MACS	Magnetic cell separation (Miltenyi Biotec)
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MTP	Microsomal triglyceride transfer protein
NOD	Non-obese diabetic/Nucleotide-binding oligomerisation domain
NK	Natural killer
NKT	Natural killer T cell
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLB	Phospholipase B
PLC	Phospholipase C

PLD	Phospholipase D
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RANTES	regulated on activation normal T-cell expressed and secreted
R0	RPMI, glutamine, penicillin and streptomycin
R10	RPMI, 10% FCS, glutamine, penicillin and streptomycin
RPM	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute -1640 (media formulation)
RT-PCR	Reverse transcriptase polymerase chain reaction
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
sPLA2	Secretory phospholipase A2
SPT	Skin prick test
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation-regulated cytokine
TB	Tuberculosis
T-bet	T-cell-specific T-box transcription factor
Tc	Cytotoxic T cell

TCR	T cell receptor
TEWL	Trans-epidermal 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 lymphopoeitin
UTR	Untranslated region
UVR	Ultraviolet radiation

1 Introduction

Antigen presentation to T cells is essential for the generation of an effective immune response.

Peptide antigens are processed and presented on the cell surface by the highly polymorphic major histocompatibility complex (MHC) Class I or Class II molecules, and are recognised by conventional MHC-restricted T cells. Such peptide-MHC co-recognition is one of the most important immunological discoveries of the twentieth century, and for years it was believed that T cells exclusively recognised peptide antigens within this system such that medical approaches aiming to influence and monitor T cell responses are based on this fundamental principle (1).

However, it has become increasingly recognised that a proportion of the overall T cell repertoire is composed of unconventional populations of T cells. These T cells recognise antigens presented by non-classical non-polymorphic antigen presenting molecules encoded outside of the MHC locus, such as CD1 and MHC class 1-related protein (MR1) molecules (1). The biochemical range of T cell antigens has significantly increased to include lipids and non-peptidic small molecules with our growing knowledge regarding the mechanism of T cell activation by these non-classical antigen presenting molecules (1), indicating a broader model whereby non-peptide antigens may also contribute to the development of clinical disease by being presented for T cell recognition.

In the past 25 years substantial evidence has accumulated that unconventional T-cells recognising bacterial and mammalian lipids presented by CD1 molecules exist ("CD1-restricted T cells"), and have an significant role in the immune response to infection, inflammation, and malignancy (2).

The human CD1 gene family is clustered on chromosome 1, and is composed of 5 relatively nonpolymorphic genes (CD1A-E) which were originally described as *"a novel family of human MHC related genes not mapping to chromosome 6"* (3).

Based on sequence homology, pattern of expression and functional properties the CD1 family is divided into three groups. Group 1 is comprised of CD1a, CD1b and CD1c, Group 2 of CD1d and Group 3 of CD1e. Groups 1 and 2 are cell-surface expressed and bind and present lipids to T cells, with Group 1 CD1 molecules being expressed on antigen-presenting cells (APCs) and thymocytes, and CD1d being expressed on most haematopoietic cells and epithelial cells including keratinocytes and hepatocytes (2). CD1e is expressed intracellularly in the endosomal compartment, and is involved in assisting lipid antigen processing and lipid loading onto CD1 molecules (4, 5).

Structurally, CD1 molecules have an intron/exon structure similar to that of MHC class I genes, consisting of integral membrane proteins comprised of a heavy chain noncovalently linked to β2 microglobulin (β2M) (2). The heavy chain is composed of an extracellular lipid antigen-binding domain, a transmembrane domain, and a cytoplasmic tail. Three α helices make up the extracellular antigen-binding domain – the α1, α2 and α3 helices. The α3 helix, which is closest to the cell membrane, reinforces the more distal α1 and α2 helices through it's association with β2M. The α1 and α2 helices overlie a β-pleated sheet and mediate lipid ligand binding (6).

However in contrast to MHC Class I molecules, CD1 molecules exhibit minimal polymorphism and present lipid antigens, which are bound to a large, deep hydrophobic antigen binding groove, instead of peptide antigens (2). As described, the binding groove has a similar structure to MHC class I, with α 1 and 2 helices overlying a β sheet floor, however it is narrow and hydrophobic, with permits binding of the hydrocarbon lipid tails of presented antigens (7, 8). Two major channels,

designated A' and F' are present in all CD1 family binding grooves. Distinctive ligand binding characteristics and hence presentation of a vast array of lipid antigens to the different CD molecules is conferred by structural difference in these two channels and additional features of the binding grooves (9-12). Combined with the differing cellular expression of the different CD1 isoforms, this suggests defined roles in the immune response for CD1-restricted T cells.

1.1 CD1

1.1(i) CD1 molecules – evolution, assembly and antigen presentation

An important role for CD1 molecules in the immune response is suggested by the observation that CD1 genes are highly conserved throughout evolution.

CD1 genes are present in mammals and birds (13), and have more recently been identified in reptiles (14). The number of CD1 genes differs amongst species (1), which likely reflects selective pressure on immune system function (1). Multiple CD1 molecules have been identified in all mammalian genomes explored to date, with the exception of muroid rodents where deletion of group 1 CD1 genes by chromosomal rearrangement has resulted in mice only expressing CD1d molecules (15). It is thought that evolutionary advantages of maximizing antigen sampling and minimizing any potential adverse effects of genetic mutations are conferred by the presence of group 1 CD1 molecules in humans, in the form of overlapping but non completely redundant antigen recognition systems. Indeed, the proposition of non-redundant immunological functions for each CD1 isoform is consistent with the evolutionary retention of multiple CD1 genes.

Early diversification of the CD1 isoforms, with varied lipid antigen binding and thus distinct roles in the immune response is suggested by the observation that between species, CD1 molecules exhibit a high degree of evolutionary conservation, with the individual CD1 isoforms showing a more similar sequence homology to the same CD1 isoform in other species than to other CD1 isoforms within the same species (16). The discovery of avian CD1 molecules is indicative of the existence of ancestral CD1 genes before the bird–mammal split at least 300 million years ago (1, 15), and additionally increases our understanding of the evolution of the CD1 genes. Whilst human CD1 genes are located separate to the MHC locus on chromosome 6 in a cluster on chromosome 1, the chicken CD1 gene is situated within the chicken MHC class I gene, indicating that the CD1 and MHC class I genes were once associated and that the CD1 system was present in the primordial MHC and therefore is approximately 600 million years old (15). The notion of an important, non-redundant role of CD1 molecules in the immune response is again supported by this discovery of a higher degree of evolutionary conservation than initially understood.

Despite their structural homology to MHC Class I molecules, the human CD1 gene family exhibit only limited allelic diversity (3). Early genomic analysis of the different CD1 isoforms in humans revealed that there was limited sequence diversity in areas encoding the α1 and α2 lipid antigen binding domains (exon 2 and exon 3), with CD1a, CD1d and CD1e having only two variant alleles, and no variant alleles found in CD1b and CD1c (17); in isolated individuals, four additional rare variants of CD1e have additionally since been reported (18, 19). In CD1a, it was found that substitution of tryptophan and isoleucine (allele 1) with cysteine and threonine (allele 2) were due to alterations in amino acid sequence at positions 13 and 51 (17). Allele 1 is present within the population at a frequency of 0.13, allele 2 at a frequency of 0.87 (17). The functional impact of this remains uncertain. No significant susceptibility to lung, breast, or colorectal cancer was detected in one study (20). However, although the

underlying mechanisms remains to be elucidated, other studies have reported an increased risk of Guillain-Barre Syndrome and Multiple Sclerosis (21, 22).

However, more recent data suggest that common genetic variation does exist within the CD1 locus, but that this variation is enriched in the noncoding regions of the gene i.e. outside the protein encoding exons and therefore that it does not affect CD1 protein structure and lipid binding (23), with the recent reported findings of individuals with low or undetectable CD1a expression (23). Such donors did not upregulate CD1a on *in vitro* monocyte-derived dendritic cells at the mRNA or protein level, and were unable to present mycobacterial antigens to a CD1a-resricted T cell clone (23). This functional CD1a deficiency was found to be associated with two common single nucleotide polymorphisms located in the 5' untranslated region of the CD1a gene (23), and it was proposed that such genetic variation in the non coding regions of the CD1a gene may provide a system for achieving *"population level differences in immune responses despite limited structural variation in CD1a proteins"* (23). Phenotypically immature dendritic cells exhibiting enhanced phagocytosis and reduced production of cytokines has also been associated with reduced cell surface expression of CD1a molecules (24, 25). An increased susceptibility to tuberculosis in individuals with such CD1a deficiency has been reported in one study (26), however the significance of this in other diseases remains uncertain.

In order to be presented to CD1-restricted T cells, lipids require processing and loading onto CD1 molecules, which occurs within antigen presenting cells (APCs).

CD1 molecules are synthesised in the endoplasmic reticulum (ER), and folded with β2 microglobulin by ER chaperone proteins, through a rapid association of β2M with the α3 helix (27). β2M-deficient cell lines showed retention of CD1b molecules in the ER (28), and accelerated CD1 heavy chain degradation (29), and defective CD1d-restricted T cell development and stimulation has been reported in mice deficient in

β2M (30). These results illustrate the important role of β2M in the generation of stable CD1 molecules and in the regulation of their exit from the ER and trafficking to the cell surface. As the heavy chain is folded in the ER CD1 molecules are also loaded with endogenous lipids (31). These function as chaperone lipids and additionally impart structural stability until co-localisation of CD1 molecules with antigenic lipids occurs on entry of the CD1 molecules to the secretory and endosomal pathways (8).

CD1 isoforms exit from the ER after undergoing glycosylation within the Golgi apparatus, whereupon they traffic to the plasma membrane through the secretory pathway. At the plasma membrane, through mechanisms dependent on the characteristics of their different cytoplasmic tails, CD1 molecules are then rapidly internalised into the endosomal compartment (32), with different CD1 isoforms being directed to different stages of the endocytic pathway for antigen surveillance. Trafficking of each CD1 molecule is determined by interaction of specific adaptor proteins (AP) with their unique cytoplasmic tail tyrosine-based sorting motif (2). For example, the intracellular localization of CD1b is almost exclusively in the lysosomal compartment, and is mediated by the adaptor protein AP-3 through interactions with the cytoplasmic tyrosine-based motif of CD1b (33). Three adaptor proteins have been reported (34) AP1, AP2 and AP3. It has been shown that AP1 promotes endosomal sorting of CD1 molecules and is expressed in the trans-Golgi network (TGN), AP2 facilitates the internalisation of CD1b, CD1c and CD1d molecules into clathrin-coated vesicles for differential endosomal compartment trafficking and is cell surface expressed, and AP3 delivers CD1b molecules to late endosomes and lysosomes and is expressed at the TGN and in endosomes (34).

Among the CD1 isoforms, CD1a is unusual because it has the shortest cytoplasmic tail and does not have an AP binding tyrosine-based cytoplasmic sorting motif. As a consequence, CD1a is highly expressed on the cell surface and uniquely localises to

the early endocytic recycling compartment (35), where a neutral pH is sufficient for lipid antigen loading (35, 36). CD1a molecules and invariant chain are known to associate at the cell surface (37), where, via a clathrin/dynamin-independent mechanism, they undergo internalisation to the early/sorting endosomes; they reach a Rab 11+ compartment, before rapidly recycling back to the cell surface, a process which is dependent on the small GTPases Rab22, ARF-6 (38) and Arl13b (39). Detection of CD1a molecules within specialised sorting endosomes of Langerhans cells known as Birbeck granules has also been reported, however the mechanism of trafficking into these intracellular compartments has yet to be elucidated (36).

During CD1 lipid loading, resident endogenous lipid ligands are exchanged with CD1 lipid antigens. This process is facilitated by chaperone proteins including sphingolipid activator proteins (SAPs or saposins) which are also involved in lysosomal glycosphingolipid degradation, and are important in the transfer and loading of various lipid antigens onto Group 1 and Group 2 CD1 molecules (40, 41). Five chaperone proteins are believed to be involved in the loading of lipid antigens each of which exhibits unique lipid antigen specificity: saposins A-D and GM2AP (42). These chaperone proteins facilitate lipid antigen processing by complexing with bound lipids or through the activation of enzymes that are involved in glycolipid processing. Lack of iNKT cell selection due to a failure of lipid antigen loading onto CD1d cortical thymocytes has been demonstrated in mice deficient in prosaposin, the saposin precursor, illustrating a crucial role in iNKT cell positive selection in the thymus (43). In addition, more recently, saposins have also been found to modulate human CD1d-restricted iNKT cell reactivity (44), and saposin C was also found to be essential for the stimulation of human CD1b-restricted T cell clones (41).

Uniquely among the CD1 molecules, CD1a does not require lipid transfer proteins for loading. This occurs within the neutral conditions found in the early endosomes, and may also occur at the cell surface (45). The neutral pH requirements of lipid antigen

CD1a loading was demonstrated by experiments that precluded the formation of an endosomal pH gradient by inhibiting vacuolar ATPases. That a low pH was not necessary for CD1a lipid igand loading was confirmed by the absence of an effect on CD1a antigen presentation (36).

Overall, the targeting of each CD1 isoform is directed to a different intracellular endosomal compartment enables antigen surveillance across the endocytic compartments.

1.1(ii) CD1 lipid antigens

Within the endosomal pathway, lipid loading onto CD1 molecules for presentation to CD1-restricted T cells takes place.

Following uptake via a number of different mechanisms (5, 32, 46, 47), lipid antigens intersect with different CD1 molecules in the endosomal compartments (48). For example, CD1 molecules and lipid ligands within phagosomes may enter the endosomal compartment after undergoing phagocytosis (34). Receptor mediated lipid ligand uptake, for example via the LDL and mannose receptors, also plays an important role in lipid ligand endosomal pathway entry (49).

Depending on their bio-physical properties including alkyl chain length and saturation, lipids are sorted into differing endosomal compartments. Specifically, there is trafficking of lipid antigens containing short or unsaturated alkyl chains to the early endocytic recycling compartment, and the late endocytic compartment contains lipids with long saturated tails (5).

In this manner different CD1 isoforms sample a range of different lipid antigens, facilitating antigen surveillance across the endocytic compartments.

As described, the binding groove of CD1 molecules has a structure analogous to MHC class I, and is composed of α 1 and 2 helices overlying a β sheet floor. However, it is more narrow and deep, and is hydrophobic in character which permits binding of lipid antigens' hydrocarbon lipid tails (7), which interact with an apolar amino acid residue enriched groove composed of the A' and F' pocket. The lipid antigen repertoire of each CD1 isoform is dictated by the structure of the A' and F' pockets in addition to additional characteristics of the antigen binding grooves (8). The lipid antigen binding groove overlies a 6-strand β-pleated sheet floor and consists of interactions between the CD1 α 1 and α 2 helices. The inclusion of bulky amino acids at particular amino acid sequence positions means that interactions between the α 1 and α 2 helices additionally form a partial roof over the A' and F' pockets (8). The CD1 antigen binding groove has a narrower entrance and is deeper than MHC Class I molecules, enabling lipid antigens to bind more deeply within the antigen binding groove than peptides presented by MHC Class I molecules (8). Access to the antigen binding groove is also blocked by the CD1 roof, restricting lipid access to the F' portal. This portal is comprised of amino acid residues that are capable of being ionised at the acidic pHs of endo-lysosomal compartments, enabling lipid ligand binding and exchange by causing the antigen binding cleft to open, and has an important role in hydrogen bond mediated stabilisation of bound lipid ligands (6, 50). To date, the majority of described CD1 lipid antigens consist of hydrophobic alkyl chains which bind to the hydrophobic antigen binding groove of the CD1 molecules, and a polar head group which emerges from the antigen binding groove for TCR recognition (8).

Out of the CD1 family, it is known that the CD1a binding groove has the smallest capacity, with the A' and F' pockets being shorter and shallower. These pockets are composed of hydrophobic amino acid residues which generate interconnected channels which bind the hydrophobic alkyl chains of presented lipid ligands. The A'

pocket is connected to the external CD1a surface by the F' pocket, which is more superficial than the A' pocket (51). The A' pocket consists of a closed U-shaped curve; the F' pocket is a straight, elongated channel which widens as it joins the external surface of the antigen binding groove. The constricted A' pocket has been described as a *"molecular ruler"*, and has consequences for the size of alkyl chain that may be bound by the CD1a molecule. The narrow A' channel limits capacity to lipid antigens with short alkyl chains, and the F' channel is wider but shallower than other CD1 isoforms. It is thought that CD1a has less complex antigen loading requirements than other CD1 molecules due to this combination of reduced capacity with increased accessibility, which consequently permits sampling of lipid antigens within early sorting endosomes and at the cell surface (11). CD1a is also different from the other CD1 isoforms as the antigen binding groove is accessed via a portal on the side of the CD1a molecule superior to the F' pocket, whilst access to the other CD1 isoforms is between the A' and F' pockets, which allows the presented lipid head group to protrude centrally from the antigen binding groove for TCR surveillance (52).

Our appreciation of CD1a molecule-lipid ligand interaction has been furthered by resolution of the crystal structures of the self lipid antigen, sulfatide (11), and of the mycobacterial lipid dideoxymycobactin (DDM) (51) in complex with CD1a molecules.

Sulfatide is a sulfate ester of β-D-galactosylceramide, and is a sphingolipid constituent of mammalian brain lipids (11). Sulfatides are comprised of a 18 carbon alkyl chain containing sphingosine base that is N-amide linked to a fatty acid chain that may vary in length from 14-24 carbon atoms. In complex with CD1a, the C18 sphingosine alkyl chain has been shown to bind to the curved A' pocket, and the variable length fatty acid chain is contained within the F' pocket, with the head group protruding from the antigen binding groove for TCR recognition (11).

DDM is an antigenic lipopeptide isolated from *M. tuberculosis* (53, 54), which is composed of a hydrophilic peptidic headgroup acylated at lysine to a single alkyl chain (54). A peptide backbone which is made up of two sections, the lysine branch and the N-aryl branch forms the peptide headgroup (51). An alkyl chain length of 20 carbon atoms with one unsaturation (C20:1) was reported to be optimal for CD1a-restricted T cell activation, although this may vary in carbon chain length and saturation (54). DDM has a different polar head group and contains only a single alkyl chain (51) as opposed to sulfatide, however, the alkyl chains of both the sphingosine base of sulfatide and DDM bind within the A' pocket so that the same key amino acid residues in the antigen binding groove of the CD1a molecule with lipid ligand are seen. The mechanism by which structurally different lipid ligands may be presented by CD1a molecules for T cell recognition is explained by differences in the configuration of lipid ligand binding across the CD1a A'-F' junction (51). Sulfatide's sphingosine alkyl chain curves across this junction, which enables binding of it's longer alkyl chain length and places it's head group more superiorly and in close proximity to the TCR recognition site in the F' pocket (51). In contrast, DDM's lysine branch binds across the A'-F' junction in a straight orientation, whilst the N-aryl branch of DDM lies similarly to the sulfatide headgroup for TCR engagement (51).

There has recently been some exciting progress in this field, with the discovery that CD1a is capable of presenting headless lipid antigens (55), and further enablement of the examination of lipid, CD1a, and TCR interactions by the advent of CD1a tetramers and dextramers (56).

CD1a was recently demonstrated to bind and present fatty acids, triacylglyceride, wax esters and squalene, an oily lipid secreted by the sebaceous gland, which are all headless hydrophobic skin lipids (55). In this study, it was shown that CD1a was capable of binding a large number of different lipid ligands, but whilst small apolar stimulatory lipids were able to stimulate the TCR of an autoreactive CD1a-restricted

T cell clone, other lipids, which structurally contained polar head groups inhibited T cell activation of the same clone (55). Stimulatory apolar, headless lipid ligands were capable of displacing the inhibitory polar head group containing lipid ligands that were non permissive to T cell stimulation (55).

This study raised the question of how the CD1a molecule bound to a headless lipid antigen is able to interact with a TCR, and it was suggested that the activating self-antigens sequestered deep within the CD1a binding groove, such that there might be direct contact only between CD1a and the TCR, so that the CD1a molecule was stabilised in the correct conformation for TCR interaction by binding of the lipid antigen (55, 57). Consequently, a number of hydrophobic substances that fitted within the CD1a antigen binding groove were able to stimulate the CD1a-restricted T cells, rather than single lipid antigens being recognised with a high specificity (55, 57).

A conformational change of CD1 molecules with a consequent modification of TCR docking sites by lipid antigen binding has been demonstrated for CD1a in addition to other CD1 isoforms (45, 58, 59), and CD1b and CD1d molecules have additionally been shown to bind small endogenous spacer lipids that nestle deep within the antigen binding groove. This mechanism facilitates interaction of a second exogenous lipid ligand with the TCR, which additionally binds into the antigen binding groove (60, 61), and describes how lipid antigens with a great variation in size (ranging from 12 to 80 carbon atoms), are able to be presented by the CD1b molecule (62, 63). However in contrast to CD1b which has the largest volume antigen binding groove, as described above CD1a has the smallest volume antigen binding groove of the CD1 molecules hence this mechanism is not likely to be the case for the CD1a molecule.

Of great significance, the crystal structure of TCR-CD1a lysophosphatidylcholine and oleic acid has recently been solved (64). A common docking mode for both lipid ligands was identified, with the TCR docking orthogonally across the long axis of the CD1a binding groove over the A' roof of the CD1a-antigen, with the α and β chains of the TCR located over the α2 and α1 helices, respectively (64). The TCR docked over the A' roof of CD1a, contacting the CD1a molecule itself rather than the stimulatory lipid ligand lysophosphatidylcholine (C18), and was distant from and hence did not interact with the polar head group of this lipid. A second lipid, sphingomyelin (C24) which was not permissive to TCR binding was found to disrupt this TCR-CD1a contact zone by disrupting a triad of amino acids in the A' roof of CD1a. Both lysophosphatidylcholine and sphingomyelin have the same polar head group, but the longer C24 acyl chain of sphingomyelin resulted in this polar head group protruding further from the F' portal of CD1a, which consequently inhibited TCR binding by modifying the electrostatic properties and conformation of the A' roof. It was concluded that in situations when the surface contact zone of CD1a was not modified by nonpermissive ligands, direct contact of the TCR with the CD1a molecule itself may result in CD1a reactivity (64).

1.1(iii) CD1-restricted T cells

CD1-restricted T cells have been reported to bridge the innate and adaptive components of the immune system and to be stimulated by both self and foreign lipid antigens (65).

1.1(iii) –Group 2 CD1-restricted T cells (invariant natural killer T cells)

Group 2 CD1d-restricted T cells include invariant natural killer T cells (iNKT cells), which express an invariant TCR chain (Vα24-Jα18 chain paired with Vβ11 in

humans; Vα14-Jα18 chain paired with Vβ2, Vβ7 and Vβ8.1 in mice) and natural killer cell associated molecules (65). In contrast to non-invariant type 2 (diverse) NKT cells and Group 1 CD1-restricted T cells, iNKT cells have been well described because of the invariant TCR, their unique marker, in addition to the availability of both animal models lacking these cells and of CD1d tetramers to specifically stain them in mice and in humans. These cells have been described as having an *"innate-like"* function, as they are able to rapidly respond to stimuli, with release of pre-formed type 1 and type 2 cytokines and recruitment of adaptive immune cells (66). iNKT cells are additionally capable of being activated by cytokines alone, without the need for co-stimulation and are known have a lower threshold for stimulation than conventional T cells (66), and roles for these cells in multiple clinical settings, such as infection, autoimmunity and cancer, have been described over the years (65).

A role for iNKT cells in mycobacterial responses is well documented, and indeed the anti-mycobacterial response has been examined with each of the CD1 isoforms, and there has been identification of mycobacterial lipid antigens for each CD1 molecule. In a mouse model, iNKT cell expansion with a subsequent reduction in cell number in the lungs was promoted by intravenous BCG injection (67), and a role for iNKT cells in the resolution of *M. tuberculosis* infection has been suggested by their production of IFN-γ, TNF-α and GM-CSF and contribution to granulomata formation (68, 69). However, redundancy in this system is implied by the observation that no significant increase in mortality or bacterial burdens in the lung in *M. tuberculosis* infection is seen in CD1d or Jα18 -/- mice (70, 71).

M. tuberculosis has been extensively studied in iNKT cell immunobiology, however multiple other bacteria including *Sphingomonas*, *S. typhimurium*, *S. pneumonia*, and Chlamydia, in addition to viruses and fungi are also known to activate iNKT cells (65, 72-76). Indeed, the notion of an important contribution of these cells to the effector

immune response to micro-organisms is corroborated by their stimulation by lipid ligand antigens derived from multiple pathogens.

Studies have demonstrated that the fungal lipid Asperamide B may stimulate mouse and human iNKT cell cytokine secretion (77). This glycosphingolipid has been found to be linked to asthma and allergic sensitisation in humans, and is found in the ubiquitous fungus *Aspergillus fumigatus* (77), implicating CD1-restricted T cells in allergic hypersensitivity. Airway hyper-reactivity and an inflammatory infiltrate of IL-4 and Il-13 producing iNKT cells, macrophages, and Th2 cells were induced by treatment with this glycolipid in an *in vivo* mouse model (77), and such airway hypersensitivity was absent in CD1d knockout mice, an effect which could be effectively reversed by adoptive transfer of CD1d tetramer sorted wildtype iNKT cells and was therefore found to require iNKT cells (77, 78). These findings implicate iNKT cells in the pathogenesis of allergic asthma, and indeed these cells have also been shown to play a role in other allergic hypersensitivity conditions including reactivity to pollens (79) and allergic contact dermatitis (80). In allergic contact dermatitis lesions, increased numbers of iNKT cells and enhanced keratinocyte CD1d expression has been reported, and immunostaining revealed that cytotoxic granules localised to iNKT cells in this condition (80). Human CD1-restricted T cell recognition of lipids from pollens has also been recently described (79). In this study, pollen phospholipids were demonstrated to bind CD1d and CD1a molecules, and to elicit IFN-γ and IL-4 CD1-restricted T cell responses in T cell lines from pollen sensitive individuals (79). CD1-restricted T cell responses were found to be maximal in pollen sensitive patients during the apex of the allergic season (79).

With respect to anti-viral responses, mouse models deficient in CD1d molecules or iNKT cells were reported to have impaired viral resolution and abrogated hepatitis B-specific T cell responses, (81), and hepatic iNKT cells were found to produce large amounts of IFN-γ on activation in hepatitis C infection, which was associated with

upregulated hepatocyte CD1d expression (82). iNKT cells are also thought to play a role in anti-herpes virus immunity (83) and have also been shown to influence the inflammatory cell infiltrate and infection outcome during influenza infection, with an increased influenza infection susceptibility having been reported in iNKT cell deficient mice (65).

iNKT cells have also been shown to have an important role in tumour surveillance, promoting tumour cell death and exerting a potent anti-metastatic effect through the expression of cell surface molecules including Fas ligand, which induce cell death (84, 85).

Therefore it may be concluded that iNKT cells have a pro inflammatory role in several diseases, as key players bridging the innate and adaptive arms of the immune system. In addition to this, an immunosuppressive role for these cells has also been proposed, and it is thought that this response is moulded by the primary cytokine environment, with exposure to IL-12 generating pro-inflammatory iNKT cells, and exposure to IL-10 resulting in regulatory iNKT cells (86).

iNKT cells have been postulated to have an immunosuppressive regulatory role in a number of autoimmune diseases, as suggested by reported findings that several autoimmune conditions including multiple sclerosis (87), systemic sclerosis (88), systemic lupus erythematosus (89), and type 1 diabetes (90, 91) exhibit a reduced frequency of iNKT cells, and iNKT cells have also been described as important mediators of immunological tolerance (92). iNKT cells were identified as key regulators of tolerance in one early study where mice deficient in iNKT cells exhibited early graft rejection of transferred rat pancreatic islet cells compared to wild type mice (93); restoration of graft tolerance was successfully achieved by adoptive transfer of iNKT cells into iNKT cell deficient mice (93). Furthermore, α GalCer, a potent agonist of iNKT cells, treatment in a murine model of autoimmune diabetes

(the non-obese diabetic NOD mouse) activated iNKT cells and prevented the onset and recurrence of type 1 diabetes (94). In a mouse model of the autoimmune inflammatory thyroid condition Graves' disease α GalCer was also demonstrated to have a protective effect (95).

In conclusion, it is clear that there is further work to do regarding the mechanism of action of regulatory iNKT cells, however there is convincing evidence that these cells have a dual role, exerting both a pro-inflammatory effect in antimicrobial and antitumour immunity in addition to a tolerogenic function that is important in the prevention of autoimmune diseases and the maintenance of T cell tolerance (96).

1.1(iii) –Group 1 CD1-restricted T cells

As previously stated, Group 1 CD1-restricted T cells have been less well studied due to lack of specific cell markers and limited knowledge of antigen specificity.

However a role for presentation of both self and foreign lipid antigens has been described (11, 53, 97). Indeed, they were the first CD1-restricted T cells to be studied. In 1989 a subset of T-cells in humans directly recognising CD1a and CD1c were described, suggesting a potential role in presentation of endogenous lipids (97), and more recently group 1 CD1 molecules have been reported to bind and present sulfatide (11, 98), the main constituent of mammalian brain lipids.

Like Group 2 CD1d-restricted iNKT cells, there is also compelling evidence that Group 1 CD1-restricted T cells play a role in anti-mycobacterial responses, and these cells have been isolated from *M. tuberculosis* and *M. leprae* infected patients (99, 100). Group 1 CD1-restricted T cells have been reported to recognise both self and mycobacterial lipid antigens, and were found in CD4+, CD8+ and CD4-CD8- (double negative) T cell subsets (101-103). CD1a has been shown to bind and present dideoxymycobactin (DDM), an antigenic lipopeptide derived from M. tuberculosis (53,

54), and CD1b and CD1c molecules are known to bind and present multiple mycobacterial cell wall components to CD1b- and CD1c-restricted T cells (102, 104).

These data suggest that group 1 CD1–restricted T cells may participate in immune responses during mycobacterial infection, and a variety of experimental approaches have been used to investigate the role of Group 1 CD1-restricted T cells in the anti-*M. tuberculosis* response. It has been demonstrated that *M. tuberculosis* infection stimulated a Group 1 CD1-restricted Th1 cytotoxic T cell response, characterised by secretion of TNF-α and IFN-γ (105). These studies utilised a humanised murine model which has been demonstrated to have an analogous pattern of CD1 expression as that found in humans, via endogenous promoter controlled Group I CD1 genes (106). Fundamental differences to iNKT cell anti- *M. tuberculosis* responses were identified in that Group 1 CD1-restricted T cells exhibited delayed primary responses, and more rapid secondary responses, in contrast to iNKT cells which had rapid responses to primary infection and exhibited attenuated responses to secondary exposure (105). These experiments suggest that Group 1 CD1-restricted T cells exhibit a more adaptive antigen-driven phenotype than iNKT cells, similar to conventional T cells (105). The recent advent of Group 1 CD1 tetramers has additionally facilitated investigation of Group 1 CD1-restricted T cell responses. Recently, *ex vivo* DDM-specific CD1a-restricted T cells have been detected in donors with active tuberculosis or a positive tuberculin skin test (56) using CD1a tetramers and dextramers, and CD1b tetramers have been utilised to identify the mycobacterial glycolipid antigen glucose monomycolate specific CD1b-restricted T cells in patients with tuberculosis (107). CD1c tetramer analysis also identified *M. tuberculosis* phosphomycoketide specific CD1c-restricted T cells in patients with latent *M. tuberculosis ex-vivo* (108).

As previously discussed, a CD1a-restricted T cell response to phospholipids in pollens has recently been described (109). Furthermore, it has been shown that, in

addition to being highly expressed in Langerhans cells of the epidermis and by a subset of dermal dendritic cells and thymocytes, CD1a is expressed on subsets of dendritic cell populations at other surface sites, including lung, gut and genital mucosa (110-114). These peripheral sites are all key sites for allergic sensitisation being located at the host-environment interface, so that a role of Group 1 CD1-restricted T cells in other allergic inflammation may prove to be of importance. Interestingly, pulmonary CD1a+ DCs expressing both myeloid and plasmacytoid blood DC antigens (BDCAs) have been described, which exhibited significant plasticity *in vitro* (115).

A role of Group 1 CD1-restricted T cells in organ-specific autoimmune disease is additionally suggested by reported elevated frequencies of Group 1 CD1-restricted T cells and group 1 CD1+ antigen presenting cells in the acute and chronic phases of the autoimmune inflammatory thyroid diseases Graves' disease and Hashimoto's thyroiditis (116). However, whilst several self-lipid ligands have been reported that stimulate a CD1-restricted T cells response in the context of CD1 molecules, their pathogenic role in autoimmune disease remains to be established. Of interest, some Group 1 CD1-restricted TCRs are capable of recognising Group 1 CD1 molecules that present both self-lipid ligands and microbial lipid ligands derived from *M. tuberculosis* and hence have been shown to have dual reactivity. Weaker responses were seen with the self-lipid antigens than the foreign lipid antigens, suggesting that these CD1-restricted T cells may have a role in anti-microbial defence (117).

More recently, an unexpectedly high frequency of self reactive CD1-restricted T cells has been detected in human blood (118, 119), with the highest frequency (0.02-0.4% of peripheral memory T cells) being amongst CD1a-restricted T cells (118), identifying CD1a-autoreactive T cells as *"a normal component of the human αβ T cell repertoire"* (119).

Functionally, CD1a-autoreactive T cells frequently expressed the skin homing markers CLA/CCR4/CCR10, and produced interleukin 22 (IL-22) in response to CD1a on Langerhans cells (119). This suggests a role for these cells in the skin, and this is further supported by the recently published findings that CD1a-autoreactive T cells recognise natural skin oils including fatty acids, triacylglyceride, wax esters and squalene (55) in addition to common membrane phospholipids (64).

Comparisons of CD1a-restricted T cells in umbilical cord and adult human blood suggested that, like conventional T cells, they develop from naïve to memory T cells over time (118). The CD1a-autoreactive T cells were predominantly CD4+, although CD8+ and double negative T cell clones were also identified (118, 119) and secretion of a number of cytokines from these cells was detected, including IFN-γ, TNF-α, IL-2, IL-13, GM-CSF and IL-22 (118, 119). The CD1a-autoreactive T cells expressed diverse T cells receptors (118, 119) another important distinguishing factor from CD1d-restricted iNKT cells. This is in keeping with the generally accepted view that Group 1 CD1-restricted T cells have diverse T cell receptors (117). However, interestingly, the recently identified CD1b-restricted so called 'germline-encoded, mycolyl lipid reactive' (GEM) T cells challenges this opinion, with genetically unrelated patients with tuberculosis being found to have T cells with highly stereotyped TCR α-chains (107). The authors postulate that the interdonor TCR conservation within the CD1-restricted T cell repertoire may prove to be more common than is currently appreciated (107).

1.2 Atopic Dermatitis

AD is a common, chronic, relapsing inflammatory skin condition with a lifetime risk of 20-30% in the UK population (120). Pertinent clinical features include the presence of

icthyosis, significant pruritus with frequent thickening of the skin (also known as lichenification), acute inflammatory flares and an association with a personal, or family history of atopy, although presentation can be highly variable (121). Pathogenesis is complex, with convincing evidence that skin barrier dysfunction, immune dysregulation, and environmental factors play important roles (122). Most patients respond satisfactorily to topical treatments, but a subset develop severe refractory disease and around 10% of patients require one or more systemic agents to achieve adequate disease control.

1.2(i) Definition, diagnosis and epidemiology

The commonest chronic inflammatory skin disease (123, 124), AD is defined by the World Allergy Organisation as *"an inflammatory, chronically relapsing, non-contagious and extremely pruritic skin disease……with demonstrable IgE association"* (125).

Written reports of AD date as far back as the early nineteenth century, and the widely variable clinical phenotype has resulted in a vast nomenclature for this heterogeneous condition (126). Diagnosis is clinical, and is based on the Hanifin and Rajka diagnostic criteria of 1980 (Table 1) (127), which consists of major and minor criteria for diagnosis. These criteria have since been refined by the UK working party, with the generation of reproducible validated evidence-based diagnostic criteria (Table 2) (128-131).

Table 1. Hanifin and Rajka diagnostic criteria for AD (127). For a diagnosis of AD, 3 out of 4 major criteria and 3 minor criteria must be present.

MAJOR CRITERIA	FEATURES

1.) Pruritus	Present as a clinical symptom
2.) Dermatitis	Affecting the flexural surfaces in adults or the face and extensors in infants
3.) Pattern of disease	Chronic or relapsing
4.) Personal of family history of atopy	Cutaneous or respiratory allergy
MINOR CRITERIA	
1.) Facial features	Facial erythema or pallor, hyopigmented patches, infraorbital darkening, infraorbital folds or wrinkles, cheilitis, recurrent conjunctivitis, anterior neck folds
2.) Triggers	Emotional factors, environmental factors, foods, cutaneous irritants
3.) Complications	Susceptibility to cutaneous viral and bacterial infections, impaired cell mediated immunity, immediate skin test reactivity, raised serum IgE, predisposition to keratoconus, anterior subcapsular cataracts.
4.) Others	Early age of onset, dry skin, ichthyosis, keratosis pilaris, hyperlinear palms, hand and foot dermatitis, nipple eczema, white dermatographism, perifollicular

	accentuation

Table 2. UK working party criteria for the diagnosis of AD (130). A diagnosis of AD is made when one major and three or more minor criteria are present.

MAJOR CRITERIA	**FEATURES**
Pruritus	Evidence of itchy skin, or parental report of scratching or rubbing the skin
MINOR CRITERIA	
History of a recurrent dermatitis	Affecting the flexures (including cheeks in children under 10 years)
Personal (or family history) of atopy	Asthma or hay fever (or atopy in first degree relative if under 4 years)
Disease onset	Disease onset under 2 years old (not used if child is under 4 years)
Current active dermatitis	Affecting the flexures (including cheeks, forehead and extensor surfaces if under 4 years)
Dry skin	Personal history of dry skin over the last year

AD is the commonest chronic inflammatory skin disease (123, 124), which, with a reported prevalence of up to 30% in children and up to 10% in adults (132), has a considerable associated morbidity, mortality and economic health burden (120).

Interest has been sparked in AD due to the fact that prevalence has increased two-three fold in industrialised countries in recent decades, although it remains significantly lower in more agricultural areas including Eastern Europe, rural Africa, and China (121). In developed countries, an urban-rural gradient in prevalence has also been reported, and AD has been shown to be more common in higher social class groups (133, 134). Indeed in countries inhabited by groups of similar genetic background, wide variations in prevalence have additionally been observed suggesting that lifestyle and environmental factors play a critical role in AD (135). A higher level of education and income, smaller family sizes, increased usage of antibiotics and high uptake of vaccinations, and migration from farming rural to urban environments as part of the so-called 'western lifestyle' are some of the identified risk factors found to be associated with the increase in atopic disease (136-138). These findings are supported by studies which demonstrated promotion of type 2 immune responses by atopic conditions, whereas type 1 immune responses were driven by infections (139). These immune responses are mutually antagonistic, so that the lack of microbial stimulation in early childhood (and consequent absence of type 1 polarising signals) predisposes to a type 2 mediated allergic phenotype as part of the 'hygiene hypothesis' (137).

1.2(ii) AD onset, clinical and histological features

AD typically affects males and female equally and although it may present later in life and persist into adulthood (122), generally presents during early infancy/childhood (140). Disease onset is within the first year in 60% of cases, and before five years of age in 95% of cases (141, 142). Disease presenting in infancy has a natural history

of gradual improvement in the majority of cases, with around three quarters of children having disease that spontaneously remits before adolescence. However, the remaining quarter will experience AD persisting into adulthood or experience a relapse of symptoms after symptom-free years (142). A family history of atopy and severe disease are the strongest predictive factors for development of persistent disease (143).

Clinically, AD is a complex heterogeneous condition with a wide spectrum of overlapping clinical phenotypes.

The classical clinical picture of childhood AD is that of acute flares of inflammatory, pruritic lesions characterised by erythema, papules, vesicles, and excoriations on a background of dry skin at sites of typical predilection such as the flexures of the elbows, knees, wrists and ankles (144). Icthyosis, debilitating pruritus which frequently results in thickening of the skin with lichenification, papules and nodules over time, acute inflammatory flares associated with a personal or family history of other atopic disease, are key clinical features (121).

This clinical picture varies according to age of onset of AD. Typically, infants experience AD that is often localised to the face, scalp, and extensor aspects of the arms and legs, but disease may be widespread. In adulthood AD lesions frequently localise to the face (often peri-orbital and forehead regions), neck, upper back, upper arms and lower legs, and up to 30% of adults develop atopic hand dermatitis, with considerable associated morbidity (142).

At any age acute flares in disease activity are associated with an exacerbation of pruritis and increased inflammation, and superimposed cutaneous infection manifests itself as ulceration, exudation, and crusting.

The European Task Force for Atopic Dermatitis (ETFAD) developed the **SCOR**ing **A**topic **D**ermatitis (SCORAD) system, which is based on the subjective symptoms of AD, the extent of AD and the intensity of six clinical signs: erythema, swelling, scratch marks, oozing/crusting, dryness and ichenification, and is one of the commonly used approaches to evaluate disease severity in the clinical setting (145).

Histologically, clinically unaffected skin of AD patients is abnormal, containing a sparse perivascular infiltrate of mononuclear cells (146, 147), and Langerhans cells (LC), although to a lesser degree than in AD lesional skin, have surface-bound IgE (148). Histology of acute AD skin lesions demonstrates marked spongiosis (intercellular oedema) and vesicle formation of the epidermis, epidermal hyperplasia, a marked dermal perivascular T cell infiltrate, and monocyte-macrophages, eosinophils and mast cells at various stages of degranulation are abundant (149).

"Chronic" lichenified lesions are typified by an irregularly hyperplastic epidermis with minimal spongiosis, elongation of the rete ridges, and prominent hyperkeratosis. Increased numbers of epidermal IgE-bearing LC numbers can be seen, and macrophages dominate the dermal inflammatory mononuclear cell infiltrate. There is often hyperplasia of the papillary dermis, and chronic AD lesions have an increased number of mast cells. Although rarely seen histologically, it is believed that there are increased numbers of dermal eosinophils as their products including eosinophil major basic protein and eosinophil cationic protein may be detected by immunostaining (121, 149).

1.2(iii) Pathogenesis of Atopic dermatitis

AD is widely accepted to be a complex heterogeneous disease in which innate and adaptive immune responses, in addition to environmental factors, strongly influence an underlying disease susceptibility in genetically predisposed individuals (150).

1.2(iii) – 1 Environmental factors in Atopic dermatitis

Environmental factors are well established to be implicated in AD pathogenesis (122), and are known to predispose genetically susceptible individuals to atopic diseases such as AD.

Migrant studies have demonstrated that children born in areas of low atopic prevalence may acquire the atopic risk of the area that they move to, and as discussed an increasing prevalence of AD in recent decades has additionally been reported, with developed countries having a reported higher prevalence of AD than less developed regions (151). These observations lend support to a role of environmental factors in the development of atopy.

Habitation in developed countries, affluent, urban lifestyles, small family size, high antibiotic use and uptake of vaccinations, stable intestinal microflora, and low helminth infection rates are some of the environmental factors identified as contributing to the increasing prevalence of atopic conditions. Conversely habitation in developing countries, poor sanitation, a rural lifestyle with exposure to livestock, a larger family size, low antibiotic usage and uptake of vaccinations, transient intestinal microflora and high helminth infection rates are more likely to associated with a non-atopic phenotype (152).

The hygiene hypothesis originated from a study where an inverse relationship between birth order and family size, and allergic rhinitis, was reported. It was postulated that an increase in the incidence of allergic disease was due to the increase of smaller households with improved hygiene, which consequently reduced the risk of early childhood infections (153). These observations were lent support by the Th1/Th2 paradigm (154), with the theory that Th1-biased responses, which typically result from repeated microbial exposure and suppress Th2-biased

responses, are reduced by lack of microbial stimulation in early childhood in these households. This enables Th2-biased responses to predominate with resulting atopy in genetically susceptible individuals. However such Th1/Th2 imbalance cannot completely explain the increase prevalence of atopic disease, as there has been a concomitant increase in Th1-mediated autoimmune diseases (155) so that a refinement of the hygiene hypothesis has been propositioned, which postulates that microbial stimuli are important in instigating the regulatory mechanisms that influence the Th1/Th2 balance (156). This 'counter regulation' model is based on regulatory T cell production of IL-10. In this theory frequent infections are thought to increase IL-10 production, with a consequent reduction in allergic and autoimmune conditions (157).

There is also convincing evidence that antigen-specific reactivity to environmental allergen challenge has a role in the pathogenesis of atopic dermatitis. For example, the pathology of atopic dermatitis shares many features with classic delayed-type hypersensitivity, including epidermal oedema and a dominant T cell inflammatory infiltrate. Approximately 80% of individuals with atopic dermatitis have specific IgE which recognise proteins derived from one or more ubiquitous environmental allergens, including house dust mite (HDM), animal dander, pollens and fungal allergens (eg *Aspergillus* spp), with patients often exhibiting immune responses to these allergens (122, 158). Of these, HDM recognised as one of the most important allergens in AD (159), with evidence that homes of AD patients have higher numbers of HDMs than healthy non-atopics (160); positive HDM skin prick tests and HDM specific IgE are additionally found in 40-90% of patients (161, 162), and IgE sensitisation has been reported to correlate to levels of exposure (163, 164).

House dust mites are microscopic arthropods that are known to thrive in warm, moist environments provided by modern homes. Two species predominate: *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, with

Dermatophagoides pteronyssinus being more abundant in temperate/tropical coastal regions and *Dermatophagoides farinae* favouring continental climates. These species coexist in many areas with notable exceptions being the UK and Australasia where *Dermatophagoides pteronyssinus* is known to predominate (165). Allergens from the two species may cross react (166). HDMs have a 2-3 month life cycle and feed on organic detritus including shed human skin scales. They flourish in soft furnishings, particularly mattresses, bedding, carpets and furniture, with reported figures of up to 500 HDMs per 0.1 gram of mattress dust (160). To date 31 groups of allergen have been identified within HDMs (167). Many of these are proteolytic enzymes which facilitate digestion of human skin substrate. They are derived from the mite's digestive tract and are excreted at high concentrations of 0.2 ng/particle in the faeces where they become airborne (168, 169). For example Der p1, a group 1 allergen, is a cysteine protease and has been found to cleave occludin, a protein of intercellular tight junctions (170); allergen groups with α amylase and serine protease activities have additionally been reported (167). HDM allergens are sub-classified according to their molecular weight, with the group 1 and 2 allergens having been most extensively studied and currently considered to be the major allergens, being recognised by over 80% of European mite sensitive patients (171).

HDM allergen peptide-specific type 2 cytokine-producing T cells have been documented in many studies to be present in the peripheral blood of affected individuals (172-177), and more recently, type 2 innate lymphoid cells (ILC2) have also been shown to be enriched in lesional atopic dermatitis skin and to infiltrate after HDM challenge (178). Early reports suggested that development of allergic sensitisation of a genetically susceptible individual may be determined by early environmental exposure to allergens. For example a higher risk of birch pollen sensitisation was reported in Scandinavian children born prior to the birch pollen season than those born after it (179), and children born in the autumn, when HDMs

are highest in number, had a higher risk of HDM allergic sensitisation (180). Although controversial, decreased AD and asthma prevalence have been correlated with reduced exposure to food and HDM allergens in the first year (160, 181), and furthermore HDM avoidance measures have reported to be efficacious in clinically improving AD activity (182, 183), and are advocated in clinical practice in HDM sensitive individuals.

Such sensitization towards environmental allergens have lead to the proposal of the classification of AD into 2 types: the extrinsic, or allergic, form, which arises in the context of increased IgE levels and sensitization to environmental allergens and affects approximately 70% to 80% of adult patients with AD, and the intrinsic, nonallergic form, which is associated with low IgE levels and the absence of detectable allergen sensitization and affects 20% to 30% of adult patients with AD (161). However, although some authors promote this classification, others feel that, at least in infancy, the non-IgE associated intrinsic form of AD may in fact be a transitional phase of the IgE associated extrinsic form of AD (149), and furthermore it is thought that IgE to microbial allergens or autoreactive T cells to autoallergens may exist in intrinsic AD patients, which are not measured routinely (184-186). In addition, revision of the terminology for atopy and atopic diseases by the World Allergy Organisation, defines atopy solely in association with IgE sensitization so that the term 'atopic' should only be used in combination with a positive skin prick test or documented allergen specific serum IgE antibodies (187).

1.2(iii) – 2 Genetics in Atopic dermatitis

AD may be described as a "*genetically complex, familially transmitted disease*" (188). Twin studies have demonstrated concordance rates of 0.72-0.86 in monozygotic and 0.21-0.23 in dizygotic twins indicating a substantial contribution of genetic factors to the development of AD (189, 190), and the existence of AD specific genes is

suggested by the observation that a greater risk to offspring is conferred by parental AD than parental allergic rhinitis or asthma (121, 191).

Two major experimental techniques have been utilised to investigate genetic factors predisposing to AD: Genome-wide linkage analysis and candidate gene analysis.

Genome-wide linkage analysis is aimed at detecting an association of the AD phenotype with a chromosomal region(s). It has been used in several populations to date and has identified several chromosomal regions with linkage to AD (192-199) including regions on chromosomes 1q21 and 17q25 which coincide with regions known to contain psoriasis susceptibility loci, regions on chromosomes 5q31-33 which contain a grouped family of type 2 cytokine genes (including IL-4, IL-5 and IL-13), a region on chromosome 3q21 encoding the costimulatory molecules CD80 and CD86, and chromosomal regions 3p24-22, 4q22, 18q21, 3q14, 11p14, 13q14, 15q14-15 and 17q21 which are uncharacterised at present (188). The so called 'Epidermal differentiation complex' located on chromosome 1q21, which contains a dense cluster of genes and gene families expressed during epidermal terminal differentiation, confers the highest risk (193). Several of the identified loci overlap with previously known susceptibility loci for asthma (200) and allergic sensitisation (201), and additional chromosomal regions have been identified in composite phenotypes, for example AD with increased allergen specific IgE levels (195) and AD and asthma (193). More recently, a multi-ancestry genome-wide association study identified 10 new risk loci robustly associated with AD, bringing the total number of AD risk loci up to 31 (201). These newly identified loci harboured candidate genes with roles in innate host defence, including Langerin, a Langerhans cell specific C-type lectin with mannose binding specificity that binds to microbial carbohydrates and is known to have a role in antigen uptake. Interestingly the majority of the new susceptibility loci comprised candidate genes with functional links to autoimmunity (201). Evidence for autoimmunity/autoreactivity, particularly in the context of

progression to the chronic phase of AD, is a recently emerging field which has generated considerable interest (184).

In contrast to genome-wide linkage analysis, candidate gene studies are restricted to a single gene that has previously been identified as having a possible role in the pathogenesis of AD and investigating the association of polymorphisms of that particular gene with the phenotype of AD.

Candidate genes that have been examined for their role in AD pathogenesis may be broadly classified into genes that are involved in immune system function and genes related to skin barrier function.

With respect to immune system function, many genes of the innate and adaptive immune system have been found to be associated with AD (202). These include mutations in the pattern recognition receptors toll-like receptors (TLR polymorphisms including TLR2, TLR6 and TLR9) and nucleotide-binding oligomerisation domain like receptors (NLR including CARD4, CARD12, CARD15, NALP1 and NALP12) in addition to mutations in anti-microbial peptides (AMPs), which are associated with enhanced susceptibility to cutaneous infections in this condition (202). Considering the adaptive immune system, defined IL-4, IL-13, IL-4 receptor α and IL-13 receptor α polymorphisms have additionally been reported to confer AD susceptibility in specific populations (202), and variants in STAT6, a key transcription factor in IL-4 and IL-13 mediated responses, have been linked with allergic disease (202). Other cytokine and chemokine variants including IL-2, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-18 and IL-31 and RANTES have also been found to be associated with AD (202), in addition to haplotypes of the histamine receptor H4, and polymorphisms of the high affinity IgE receptor FcεR1 which binds to the IgE constant region (202).

In relation to skin barrier function, candidate gene analyses have identified mutations in the serine protease inhibitor kazal-type 5 (SPINK 5) gene, which encodes the

protease inhibitor lymphoepithelial Kazal-type related inhibitor (LEKTI) (203) implicated in Netherton's disease (204), an inflammatory dermatosis which is characterised by eczematous lesions, elevated IgE levels, and skin barrier defects (202). SPINK5 single nucleotide polymorphisms have shown a significant association with AD in some cohorts (205-207). More recently, loss-of-function mutations in the filaggrin (*FLG*) gene (208), and polymorphisms in the gene encoding the tight junction protein Claudin 1 (209) have been described in AD. Such loss-of-function mutations in the filaggrin gene, which encode the important skin barrier protein profilaggrin consititute the strongest genetic association with AD reported to date, and will be discussed in detail in the next section.

1.2(iii) – 3 Role of the skin barrier in Atopic dermatitis

The skin is the largest organ in the body and plays a fundamental role in host defence. As such, it forms a complex mechanical barrier, preventing transepidermal water loss and impeding the entry of allergens, toxic chemicals and infectious organisms, in addition to possessing an extensive immune network as the first line of defence against micro-organisms (210). It may be surmised that there are three mechanisms of barrier defence within the skin: a physical barrier provided by an intact epidermis, an anti-microbial and biochemical barrier, in addition to multiple components of the innate and adaptive immune systems.

Perturbations in epidermal barrier function as determined by enhanced transepidermal water loss (TEWL) is a typical feature in patients with AD (122), and the recent identification of the association of common loss-of-function mutations in the filaggrin (filament aggregating protein) gene with AD (208) has refocused attention on the important role of epidermal barrier dysfunction in the pathogenesis of AD.

In 2002, genome screens established genetic linkage of the *FLG* locus to the epidermal differentiation complex on chromosome 1q21 (211), and two loss-of-function (R501X and 2282del4) mutations in the *FLG* gene were subsequently shown to be the cause of Icthyosis Vulagris (212). This condition is the commonest disorder of keratinisation and is characterised by dry, scaly skin; AD is seen in up to 70% of patients. Soon after this, the association of common loss-of-function mutations in the *FLG* gene with AD was identified by McLean and colleagues (208), with loss-of-function *FLG* mutations being reported in up to 50% of patients with moderate-to-severe AD (213). Robust and reproducible associations have since been described in several studies from around the world, including an association with early onset and persistent disease, allergic sensitisations and elevated IgE, and asthma in the context of AD (214-225). Associations with food allergy and microbial infections have additionally been described (150).

The *FLG* gene encodes the protein profilaggrin, which is a key protein that facilitates terminal differentiation of the epidermis and formation of the skin barrier. The 400 kilodalton protein profilaggrin is the precursor to filaggrin and is expressed in the granular layers of the epidermis, where it forms keratohyalin granules that are visible on light microscopy and consists of 10-12 tandem repeats of filaggrin peptide monomers (226). On terminal differentiation of the epidermis, profilaggrin is cleaved into 37 kilodalton free filaggrin monomers (226) which have a number of roles in epidermal physiology, explaining why a reduction in expression of a single genetic element has such a profound effect on the integrity of the epidermal barrier. During cell compaction free filaggrin binds to and aggregates the keratin cytoskeleton, leading to collapse of the cytoskeleton, which results in flattening of keratinocytes into squames (226). This dense protein-lipid matrix is subsequently heavily cross-linked by transglutaminases resulting in formation of the cornified cell envelope, which lies beneath the membrane of terminally differentiated keratinocytes and is

important for normal skin barrier function, impeding water loss and preventing entry of allergens, infectious organisms and toxic chemicals. The cleaved N-terminal S100-like calcium binding domain of profilaggrin additionally enters the keratinocyte nucleus, and is thought to regulate the terminal differentiation of these cells. Free filaggrin monomers also have an important role in epidermal hydration through the production of filaggrin degradation products such as natural moisturizing factor, in addition to functioning in UV and antimicrobial protection and the regulation of cutaneous pH through acidic breakdown products, with carriers of FLG mutations having been reported to have an elevated skin pH (227, 228). This increases the activity of serine proteases leading to degradation of corneodesmosomes with resultant impaired keratinocyte intercellular adherence, in addition to attenuating the activity of enzymes responsible for ceramide synthesis resulting in a reduced epidermal ceramide content (144). It is speculated that epidermal dysfunction results in penetration of allergens, microbes and irritants into the epidermis with resulting cutaneous inflammation in AD patients with filaggrin mutations (229). Indeed a paracellular barrier abnormality has been reported in the flaky-tail mouse, which lacks murine filaggrin (230), and increased frequencies of allergen specific T cells have been reported in patients with filaggrin loss-of-function mutations, supporting the mechanism whereby barrier dysfunction facilitates enhanced allergen penetration and delivery to antigen specific T cells (231).

Despite strong and compelling evidence for an involvement of loss-of-function mutations in the *FLG* gene in the pathogenesis of AD, impaired skin barrier function in AD is additionally observed independently of *FLG* genotype indicating that other genetic and immunologic factors impact on cutaneous barrier function, and the situation is known to be complex with other factors beyond genetically determined barrier function having also been shown to affect *FLG* gene expression. For example, *FLG* gene expression can also be reduced by means of epigenetic

modification such as DNA methylation state (232), and it has also been shown that the cytokine mileu in the skin is important, with type 2 cytokines and IL-17, IL-22, IL-25 and IL-31 as well as histamine, micro-organisms colonising the skin, and topical and systemic therapies being able to modulate filaggrin expression (228, 233). However the majority of these studies have been undertaken using keratinocyte monolayers with isolated cytokines, and so extrapolation to the *in vivo* relevance of the findings must be taken with caution.

Beyond filaggrin, other defects in the epidermal barrier in AD have additionally been reported. These include variants in other genes located within the epidermal differentiation complex including filaggrin 2 (234), hornerin (235) and the cornified envelope precursor SPRR3 (236), however how these variants affect AD pathogenesis remains to be elucidated. As described above, single nucleotide polymorphisms in the tight junction protein claudin-1 have also been associated with AD, with the claudin-1 level being found to inversely correlate with esoinophils and serum IgE level (209). Tight junction proteins comprise an important diffusion barrier within the skin by regulating the paracellular movement of water, solutes and ions.

Furthermore a decrease in ceramide levels and an abnormal ceramide profile with an increase in short chain ceramides has been reported in in the cornified envelope of both lesional and nonlesional skin in patients with AD (122, 237-243). Long chain ceramides are the predominant water-retaining molecules in the extracellular space of the cornified cell envelope of the epidermis, and are abundant in the healthy stratum corneum. A matrix of structural proteins bound to ceramides provide the barrier function of the cornified cell envelope (122, 244-246), so that a ceramide deficiency affects cutaneous permeability resulting in a significant impairment in the barrier integrity. There are 3 major lipids found in the epidermis: ceramides, cholesterol and free fatty acids which are derived from lamellar bodies present within the granular layer and are present in a ratio of approximately 3:1:1 (202). It has also

been reported that increased sphingomyelin deacylase activity in AD may contribute to free fatty acid production instead of ceramide (247). To further compound this deficiency, ceramidase, which degrades ceramides into sphingosine and fatty acid, is secreted from the colonising bacterial flora on the skin of patients with AD (126).

In summary, there is convincing evidence of a primary defect of epidermal barrier in AD, which may be further compounded by the downstream effects of cutaneous inflammation in this condition.

1.2(iii) – 4 Immune mechanisms in Atopic dermatitis

Despite compelling evidence for a primary barrier defect in the pathogenesis of AD (the "outside-in" hypothesis), there are equally convincing arguments that AD is also a disorder of immune dysregulation (the "inside-out" hypothesis), which proposes that AD is driven by polarised immune pathways that result in a secondary skin barrier defect.

This include the following (summarised from (150)):

1.) *FLG* gene mutations are not present in the majority of AD patients
2.) Most children with AD outgrow their condition, irrespective of *FLG* status
3.) In contrast to Ichthyosis Vulgaris, where the entire skin is affected at birth, individuals with *FLG* mutations with AD develop the condition after birth and have areas of lesional and non lesional skin involvement
4.) In addition to filaggrin, a wide range of differentiation abnormalities (e.g. loricrin, involucrin, corneodesmosin and claudins) can be seen in lesional and nonlesional AD skin
5.) Keratinocyte treatment with type 2 cytokines and IL-17, IL-22, IL-25 and IL-31 downregulates filaggrin expression, resulting in barrier dysfunction.

6.) Genetically engineered mice overexpressing IL-33 or type 2 cytokines in the skin develop spontaneous AD and demonstrate *in vivo* skin barrier defects

7.) Filaggrin expression is restored with anti-inflammatory regimens including topical corticosteroids and calcineurin inhibitors

8.) Resolution of moderate-to-severe AD disease activity with broad spectrum immunosuppressive therapies including ciclosporin and narrow-band UV phototherapy, in addition to the development of specific immune targeted therapies (e.g. dupilumab) which cause resolution of abnormal epidermal responses

9.) In addition, there is evidence that AD skin has a predilection for Th2 immune polarisation independent of *FLG* status, suggesting that AD-specific cutaneous immune signalling exists irrespective of *FLG* gene mutations (248)

Therefore, substantial evidence suggests that altered immune mechanisms greatly contribute to AD pathogenesis. Both innate, and adaptive immune system perturbations have been described in AD, which will be discussed in detail below.

1.2(iii) – 4a.) Innate immune mechanisms in Atopic dermatitis

Pattern recognition receptors

As described above, mutations in the germline-encoded pattern recognition receptors (PRRs) toll-like receptors (TLRs) and nucleotide-binding domain leucine rich repeat receptors (NLRs including NOD1, CARD4, CARD12 and CARD15) have been shown to play a role in the pathogenesis of AD (202).

PRRs may be intracellular, secreted or endocytic and recognise pathogen-associated molecular patterns (PAMPs) located on a broad range of microbial components to initiate host defence mechanisms.

TLRs are PRRs which are known to induce dendritic cell (DC) maturation and inflammation, with Th1 or Th17 adaptive immune responses important for anti-bacterial, -viral, and –fungal defence being stimulated by most TLR ligands (249). Polymorphisms in TLR2, TLR4, TLR6 and TLR9 have been associated with AD (202). Polymorphisms in TLR2 were additionally associated with *S.aureus* infections (250), and a recent study reported that conversion of a Th2 type dermatitis into chronic cutaneous inflammation in a mouse model might involve stimulation of TLR2 on dendritic cells (251), although the mechanisms regulating the progression of acute to chronic AD remain to be elucidated. With respect to TLR4 more severe cutaneous inflammation and AD-like symptoms were observed in mice deficient in TLR4 in an allergen-induced mouse model (252). The TLR9 promoter polymorphism C-1237T has also been associated with the intrinsic form of AD (253). TLR9 is known to promote Th1 IgG responses (254), so that this gain of function mutation may partly explain the reduction in IgE levels and Th2 cytokines observed in intrinsic AD.

NLRs may be further subdivided into the CARD and pyrin subfamilies. CARDs recognise PAMPs, with the resulting signaling cascade causing transcription of genes pivotal to host defence, including proinflammatory cytokines, chemokines and AMPs; the pyrin subfamily includes the N-terminal pyrin-domain containing proteins NALP1-14 (255). CARD4 and CARD15 respond to peptidoglycans, the major bacterial cell wall component of *S.aureus*, and specific polymorphisms of CARD4, CARD12, CARD15, NALP1 and NALP12 have been reported in AD patients (202).

Anti-microbial peptides

Mutations in cutaneous anti-microbial peptides have also been shown to have a role in AD pathogenesis, including several SNPs in human β-defensin 1 (hBD-1), which have been found to be associated with AD (202). hBD-1 is an anti-microbial peptide, the family of which includes the β-defensin family, cathelicidins, psoriasin and ribonuclease (202).

In the epidermis, AMPs are predominantly synthesised in the stratum granulosum, where they are packaged into lamellar bodies prior to being extruded into the stratum corneum (256). As components of innate immunity, AMPs are critical for anti-microbial defence and contain a broad spectrum of antimicrobial activity against a wide range of pathogens including bacteria, viruses and fungi (257). Mechanisms include direct binding to microbial cell wall phospholipids, which results in physical disruption and membrane destabilisation (258) in addition to bridging the innate and adaptive immune systems via chemotaxis of dendritic cells and T cells (259).

Expression of the AMPS hBD-2 and cathelicidin has been reported to be decreased in AD lesions (260), with Th2 cytokines (IL-4 and IL-13), and a reduction in the IL-10 regulated proinflammatory cytokines IFN-γ and TNF-α in AD skin being found to cause inhibition of expression of these AMPs (261-263). This deficiency of keratinocyte production of AMPs in AD causes an increased susceptibility to recurrent bacterial skin infections including *S. aureus* and viral infections (264) in this condition, and this is further exacerbated by increased colonisation of *S. aureus* in AD skin due to Th2 cytokine mediated enhanced expression of adhesion molecules for this bacteria such as fibronectin (265, 266). Furthermore, *S. aureus* further dampens skin barrier integrity by producing high levels of serine proteases that break down the epidermal barrier (267), and secreting virulence factors including α-toxin which result in keratinocyte cell death (268) and promote Th2 inflammation, further amplifying the process (269). In this way increased skin colonisation of *S. aureus* in patients with AD may reduce cutaneous barrier function through multiple

mechanisms. A reduction in the AMPs sphingosine and dermicidin, in addition to IL-8 and inducible nitric oxide synthetase has also been reported in AD patients (261, 270, 271). In addition the serine protease family of kallikreins, which are expressed in higher levels in AD lesional skin (272), have been shown to regulate proteolysis of cathelicidin (273), so that higher kallkrein expression might further compound the decreased expression of cathelicidin in AD patients. Working synergistically, these mechanisms further compromise the anti-microbial capacity of the skin barrier and help to explain the increased incidence of bacterial and other infections in AD patients.

Neutrophils

Reduced levels of chemoattractants including AMPs may also result in reduced numbers of neutrophils in AD. Even in cases of *S. aureus* colonisation and infection, neutrophils are conspicuously absent from AD lesional biopsies. Several mechanisms have been reported to play a role in this, including a reduction in chemotaxis (274), and neutrophils isolated from AD patients have been reported to have reduced CD11b expression, a component of the β_2 integrin Mac-1 which is known to be important for cutaneous neutrophil migration (275). In addition, a number of perturbations in functional responses have been reported in AD neutrophils, including reduced capacity for production of reactive oxygen species and defective phagocytosis (276, 277).

Natural killer (NK) cells

The number of circulating natural killer (NK) cells, other cells of the innate immune system, have also been reported to be significantly reduced in AD patients, which additionally demonstrate a functional impairment in the form of decreased production of IFN-γ and increased apoptosis (278), correlating with the increased susceptibility to infection seen in AD patients. A further impairment in antiviral responses in AD

patients may result from a significantly diminished number of plasmacytoid dendritic cells (pDC) in the skin of AD lesions in comparison to other inflammatory dermatoses (279). pDCs are important for innate immunity, being a critical source of the antiviral type I IFNs (IFNα and IFNβ), and may also be functionally impaired in AD patients as cross-linking of their high affinity IgE receptor (FceR1) has been found to reduce IFN production (280).

Innate lymphoid cells (ILCs)

Derived from a common lymphoid progenitor but with absent rearranged antigen specific receptors (281), ILCs are $CD45^+$ haematopoietic cells which are known to link the innate and adaptive immune systems. ILCs are subdivided into three groups which are believed to have differing effector function on the basis of cytokine production, cell surface marker expression and transcription factor usage (282). All three groups have been identified in the skin and it is the ILC2 subset that have recently been shown to have a role in the pathogenesis of AD, being enriched in lesional AD skin and to infiltrate the skin after HDM challenge and abundantly produce type 2 cytokines on activation with IL-25, IL-33 and TSLP (283).

1.2(iii) – 4b.)Adaptive immune mechanisms in Atopic dermatitis

It is also clear that AD has an adaptive immunological component as demonstrated by the observations that AD did not occur in the absence of T cells in an animal model (284), that primary T cell immunodeficiency disorders often have elevated IgE and eczematoid skin lesions that resolve after effective bone marrow transplantation (285), and the efficacy of anti-T cell targeted therapies in the treatment of AD (132). An extensive body of evidence proves the role of the adaptive immune system in the pathogenesis of AD; areas relevant to this thesis will be reviewed in this section.

Role of Th2 cells and type 2 cytokines in AD

It is widely accepted that acute AD exhibits a Th2-biased immune response, and the Th2 cytokines IL-4, IL-5 and IL-13 have been found to be significantly elevated in both lesional and non-lesional skin in the acute phase of AD (286). A positive correlation between lesional and nonlesional skin expression of Th2 cytokines and AD disease severity has also been demonstrated (150). IL-4 and IL-13 contribute to the differentiation of Th2 cells (149) which are known to play an important role in the initiation of cutaneous inflammation via multiple mechanisms, including upregulation of expression of adhesion molecules such as VCAM-1 on endothelial cells (287), inducing immunoglobulin isotype class switching to IgE through germline transcription of the ε exon, and favouring eosinophil differentiation (288). It has been shown that higher levels of IL-4 and IL-13 in cord blood confer an increased risk of developing AD (289), and that inflammatory dermatoses that satisfy the clinical and histological diagnostic criteria of human AD develop in IL-4 and IL-13 transgenic mice models (290, 291).

Furthermore IL-4 and IL-13 have been reported to have direct effects on epidermal barrier function as detailed in previous sections, downregulating keratinocyte expression of terminal differentiation genes such as filaggrin, loricrin and involucrin (292, 293), in addition to influencing innate immunity by attenuating AMP production (261), and increasing *S. aureus* colonisation by upregulating *S. aureus* adhesion molecules (266). Signalling of both IL-4 and IL-13 cytokines is through the common IL-4 receptor α (IL-4Rα), inhibition of which has recently been identified as a promising therapeutic target and is currently under investigation in ongoing clinical trials (294, 295).

Enhanced eosinophil survival has been demonstrated by treatment with IL-5, an additional Th2 cytokine, and indeed eosinophilia and increased eosinophilic cationic

protein are recognised disease activity markers in AD (296, 297). A new Th2 cytokine IL-31 has also been implicated in AD (298, 299), having been shown to play a role in transgenic mice models (300), in addition to correlating with AD disease severity (301).

The mechanism of Th2 polarisation in acute AD remains to be fully established. Evidence suggests that dendritic cell (DC) function, a deficiency of Th1 cells, co-stimulatory factors and production of cytokines and chemokines are all important in determining the outcome of the Th2 cell response in AD (202). It is postulated that an impaired skin barrier in addition to cytokine and chemokine secretion by keratinocytes (discussed later in this section) may modify the epidermal microenvironment, with the resultant recruitment of DCs which are induced to express high affinity IgE receptors. These DCs then respond to thymic stromic lymphopoietin (TSLP) and histamine, ultimately initiating a Th2 immune response (149, 302) composed of ILCs, T cells, and IgE production (229), with ensuing cutaneous inflammation. The skin microbiome is also known to influence the adaptive T cell response, for example it has been shown that staphylococcal super antigen can promote a cytokine milieu which allows Th2 cells to respond to allergen-derived peptides presented by keratinocytes (303).

Role of Th1 cells and type 1 cytokines in AD

Despite the fact that acute AD is closely associated with a Th2-biased immune response, in chronic disease, there is substantial evidence implicating both Th1 and Th2 cytokines (121, 122, 188), where increased numbers of cells expressing mRNA of IL-5, GM-CSF, IL-12 and IFNγ are reported (121). There is additionally convincing evidence that AD is a biphasic inflammation, with a Th1 predominance in the chronic phase (304). Two phases of HDM induced lesions were reported in an approach that used the atopy-patch test technique: a first phase dominated by IL-4 producing Th2

cells, followed by a second phase (after 24-48h) dominated by IFNγ producing Th1 cells (305). This transition may be attributable to the predominant DC type (306), and is thought to be modulated by local IL-12 production by infiltrating DCs and/or eosinophils (126).

IFNγ, a characteristic Th1 cytokine, has a vital role in the immune response, with it's biological effects mediated through the JAK-STAT signalling pathway (307). IFNγ activates T-box transcription factor (T-bet) protein, which is an inhibitory transcription factor for Th2 responses (308), thereby inhibiting IgE synthesis, Th2 cell proliferation, and T cell expression of the IL-4 receptor (121). IFNγ is also a potent inducer of Th1 antiviral responses, through the activation of macrophages and the increased expression of MHC and antigen processing compartments, and genetic variations in IFNγ, the IFNγ receptor, and Interferon regulatory factor have been reported in AD patients with eczema herpeticum (309, 310). Within the skin, IFNγ is additionally known to exert direct effects on keratinocytes including induction of HLA Class II and ICAM-1 expression with consequent enhancement of the immune response by facilitating presentation of allergen peptides to conventional T cells (311), production of high levels of the potent chemoattractants monocyte-chemotactic protein 1 and RANTES (188), and upregulation of Fas, with T cell mediated Fas ligand induced apoptosis thought to contribute to the spongiosis seen in AD (312). IFNγ is also known to be a potent inducer of tissue remodelling by regulation of plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (tPA) (313, 314). Chronic lichenification – thickened plaques with increased markings is also a typical feature of chronic AD lesions, and the skin of IFNγ knockout mice demonstrates a reduction in dermal thickening (315), suggesting a role for IFNγ in this process.

IFNγ induced T-bet activation additionally upregulates expression of the IL-12 receptor (202). IL-12 is an additional Th1 cytokine that is elevated in chronic AD

lesions (316), and positively correlates with the level of IFNγ (317). IL-12 induces IFNγ production and the development of Th1 cells and NK cells. Interestingly, chronic AD lesion expression of IL-12 mRNA could be downregulated by corticosteroid treatment (318). It is thought that a deficiency of IL-12 in acute AD results in attenuated inhibition of IL-4 and IL-18, thereby increasing IgE levels and eosinophils promoting a Th2-biased immune response (319).

In chronic AD, high levels of GM-CSF and IL-5 in the skin also enhance eosinophil, monocyte-macrophage and Langerhans cell survival (188), which may contribute to persistent skin inflammation (122).

Role of other Th subsets in AD:Th17 cells, Th22 cells, Th9 cells and Treg cells

In addition to the role of Th1 and Th2 cells and their cytokines in AD, other Th subsets have been shown to play a role in the pathogenesis of AD.

Th17 cells are characterised by the production of high levels of IL-17A and Il-17F (320). Multiple studies have reported increased Th17 cells in acute AD (321, 322), where triggering by staphylococcal enterotoxin B has been found to cause high expression of this cytokine (202). Th17 cells contribute to cutaneous inflammation through a number of mechanisms including promotion of a Th2 response, interaction with eosinophils, induction of B cells to produce antigen-specific IgE (323), and upregulation of AMPs in keratinocytes (324, 325), and a deficiency of Th17 cells in chronic AD may play a role in the susceptibility to bacterial infection observed in this condition (326). IL-17 has also been reported to downregulate filaggrin expression, compounding epidermal barrier dysfunction in AD (228).

The recently defined Th subset Th22 cells, which secrete IL-22 (327), have also been found to be increased in AD. This cytokine may also be produced from Th17 and NK cells and the IL-22 receptor (a IL-22R1/IL-10R2 dimer) is expressed on the

cell surface of cells of non-haematopoietic origin, particularly epithelial cells including keratinocytes (328). The frequency of IL-22 producing cells was found to positively associate with AD severity in the skin of AD lesions (329). IL-22 expression may be induced by *S. aureus* staphyloccoal enterotoxin B and α-toxin (330), and has been found to act synergistically with IL-17 in the upregulation of AMP production by keratinocytes (331). IL-22 has also been reported to exert additional effects in the skin, promoting epidermal hyperplasia that can be found in AD (332, 333), and downregulating epidermal differentiation and filaggrin expression, thereby exacerbating the skin barrier impairment seen in AD (334).

Initially denoted a Th2 cytokine, IL-9 is now believed to be primarily produced by Th9 cells, another novel subpopulation of Th cells, which are involved in the regulation of inflammation in inflammatory and allergic diseases (335), and the level of IL-9 has been reported to be increased in the peripheral blood of children with AD and to positively correlate with AD severity (336). Polymorphisms in the IL-9 and IL-9 receptor genes additionally associate with AD (337), however the role of IL-9 and Th9 cells in AD remains to be elucidated and is an area for further study.

Murine models of autoimmunity additionally suggest a role for T regulatory (Treg) cells in the pathogenesis of AD, with adoptive transfer of Tregs preventing disease development (338). More recently it has been demonstrated that in immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, where a deficiency of Tregs results from mutations in the forkhead box protein (Fox)-P3 gene, patients develop allergy related symptoms including elevated IgE and dermatitis (339).

In the context of atopy, numerous clinical settings have been shown to provide evidence for allergen-specific Th2 effector function Treg inhibition (340-342), and it has also been reported that Treg function is deficient in atopic individuals (343), and

that the Treg population increases with glucocorticoid treatment (344). Tregs are $CD4^+CD25hi$, and use IL-10 and TGF-β as secreted cytokines and cell surface molecules such as CTLA-4, PD-1,TGF-βR and IL-10R to supress Th1 and Th2 cell function (345). Tregs can be further subdivided into several subtypes (345), and it has been demonstrated that the Tr1 (inducible) subset infiltrates AD lesional skin (343), which is thought to lead to a dysregulation of disease-causing effector T cells in this condition due to a reduction in the number of $FoxP3^+CD4^+CD25^+$ (natural) Tregs (346). Allergic immune responses are thought to be highly dependent on the proportion of Tr1 cells, Th2 cells and Th1 cells, with a fine balance between allergen-specific Th2 and Tr1 cells believed to determine allergic outcome (343). The presence of bacterial superantigens is known to significantly affect this balance, having been shown to reduce the immunosuppressive activity of Tregs, thus enabling expansion of activated T cells in AD patients (347). Treg frequency and function in the peripheral blood of patients with AD has been reported to be normal in some studies (346, 348), and increased in others (347, 349) such that a definitive role for Tregs in the pathogenesis of AD remains to be established.

$CD8^+$T cells

Despite AD being immunopathologically characterised by a predominance of effector T helper $CD4^+$ T cells (350), $CD8^+$ T cells have been demonstrated to play a role in AD pathogenesis, with a significant proportion of T cells infiltrating AD lesions being $CD8^+$ (351). It has additionally been shown that despite the generally accepted association of the production of type 1 cytokines with $CD8^+$ T cells and although initially believed to be restricted to $CD4^+$ T cells, the Th1/Th2 effector cell subsets may be applied to $CD8^+$ T cells in the context of atopy, and Tc1 subsets which produce IFNγ and IL-2, and Tc2 subsets which produce IL-4 and IL-5 have been described (352, 353). Tc2 $CD8^+$ T cells from AD patients have been shown to induce B cell production of IgE *in vitro* (354), and several studies have showed increased

numbers of such cells in the peripheral blood of atopic patients (355, 356). Interestingly, distinct IL-22-producing $CD4^+$ and $CD8^+$ T-cell populations were recently reported to be significantly increased in AD skin, and it was the $IL\text{-}22^+CD8^+$ subset that correlated with AD disease severity (329).

Memory T cells and skin homing

In the steady state, many T cells are known to reside in the skin, with figures of nearly twice that present in the circulation reported (357). Circulating effector T cells additionally infiltrate the dermis in the advent of infection or inflammation in response to cytokines and chemokines, and cutaneous T cell numbers reduce on resolution of the infection/inflammation with diminishing skin homing marker expression and chemokine levels (210). The memory T cell compartment is composed of two populations within the circulation: central memory T cells (Tcm) that patrol the lymphoid organs and on antigen encounter traffic to the tissues, and effector memory T cells (Tem), that exhibit rapid effector function and patrol non-lymphoid tissue and blood (358). Tcm express high levels of the lymph node homing markers CCR7 and CD62L, which are necessary for T cell extravasation through high endothelial venules (359), and are believed to have a high replicative capacity but a low effector function (360). They express low levels of skin homing markers, such as CLA, a carbohydrate ligand which binds the adhesion molecule E-selectin, and are thought to participate in prolonged antigen specific responses (361). In contrast Tem cells express high levels of tissue-specific homing markers such as CLA, low levels of CCR7 and CD62L, and are have rapid effector function but diminished replicative ability. In AD, the frequency of CLA^+CD4^+ Th2 cells in the peripheral blood has been reported to be increased compared to healthy controls (362). In AD skin, CLA^+CD8^+ T cells have additionally been reported (363).

More recently, tissue-resident T cells (Trm) have also been identified as T cells resident in the tissue for a prolonged time following antigen clearance (364). During the effector phase of an immune response, Trm infiltrate the skin, and are long lived T cells that are $CD8^+CD103^+$ (364). Multiple factors determine their cutaneous localisation and development, including cytokines (IL-15 and TGF-β), chemokines (CXCL9 and CXCL10), and cell surface markers (CD103 and CD69) (364). On ensuing antigen exposure, Trm cells exhibit a rapid immune response and these cells have been shown to give superior long-term immunity to infection than central memory T cells (365). Their role in AD remains to be established.

γδ T cells

The above discussion has focussed on T cells expressing αβ TCRs.

Indeed, in contrast to mice, where $Vγ5Vδ1^+$ dendritic epidermal T cells comprise over 90% of epidermal T cells (366, 367) and the dermal T cell population is enriched with $Vγ4Vδ4^+$ T cells (368), in human skin γδ T cells constitute only a minor fraction of the total T cell population in the epidermis (1–10%) and the dermis (2–9%) (369). The human equivalent of murine dermal $Vγ4Vδ4^+$ T cells has recently been identified in the form of dermal $Vγ9Vδ2^+$ T cells, which have been found to be elevated in psoriatic skin (370), to express CLA and CCR6 suggesting skin trafficking in the setting of cutaneous inflammation, and are known to produce IL-17 on stimulation with IL-23 (371). γδ T cells have also been found to be increased in numbers in the skin of patients with Langerhans cell histiocytosis, melanoma, leprosy, cutaneous leishmaniasis, and chronic cutaneous lupus erythematosus, which suggest a role in a broad range of cutaneous pathologies (369), in addition to having roles in epithelial cell integrity (372), wound healing (373), and antimicrobial defence (374). A role for γδ T cells in the pathogenesis of allergic disease has additionally been postulated (375), and in the context of AD, a significantly increased percentage of circulating

Vγ9Vδ2^{+} T cells has been reported in children, with a positive correlation with clinical score severity (375). A mechanistic link remains to be established.

Chemokines in Atopic Dermatitis

Chemokines are small molecules that function to regulate lymphocyte migration into tissues and activate and direct effector cells to the site of tissue damage and are produced by many cell types in response to physical insult or infection. Selective activity of different chemokines is achieved by expression of chemokine receptors on particular cell populations, so that many T cell subsets express unique patterns of homing molecules for preferential recruitment to target tissues. Composition of the inflammatory infiltrate in AD is defined by these molecules (376).

Phenotypic classification of Th1 (expressing CXCR-3 and CCR5) and Th2 subsets (expressing CCR4, CCR8, and CRTH2) (377-379) by selective expression of chemokine receptors is possible, and a number of chemokines have been reported to be upregulated in lesional AD skin including the CCR4 ligands CCL17 (TARC) (380) and CCL22 (MDC) (380) which reportedly correlate with AD disease severity, the CCR8 ligand CCL1 (379), and the CCR5 ligands CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (381). CCR6 and CCR10 are also thought to be important skin-homing chemokine receptors, and their ligands (CCL20/MIP-3α and CCL27/CTACK respectively) have additionally been reported to be upregulated in AD patients (376, 382) with the latter correlating with AD disease severity. Interestingly, the newly defined Th22 subset of T cells have been shown to express high levels of CCR10 (329). In addition CCL18-driven recruitment of memory T cells and increased serum and lesional cutaneous levels of CCL18 were reported in AD (383). Of interest, this chemokine has been shown to be significantly upregulated by allergen exposure and staphylococcal superantigen (384).

A number of other chemokines and cytokines have also been demonstrated to be important in AD having a chemo attractant effect on other cell types including CCL11 (eotaxin) and CCL13 (monocyte chemotactic protein 4) which in combination with CCL5 (RANTES) contribute to the chemotaxis of CCR3 expressing eosinophils (121), and TSLP which is produced at high levels by keratinocytes in patients with AD and promotes Th2 polarisation by stimulating DCs, with a resultant increase in CCL17 (TARC) and CCL22 (MDC) production (385). In addition to T cells, inflammatory DC subsets are also recruited by chemokines such as CCL17 and CCL22 (386). In acute AD skin lesions, elevation of IL-16, a Langerhans cell-derived cytokine that is a potent chemoattractant for CD4+ T cells in addition to contributing to recruitment of eosinophils and macrophages, has also been demonstrated (387).

Keratinocyes

As the predominant cell type in epidermis, a key role of keratinocytes in the initiation and perpetuation of cutaneous inflammation is becoming increasingly recognised.

In addition to their crucial role in the maintenance of the epidermal barrier and anti-microbial immunity discussed earlier in this chapter, keratinocytes are a recognised source of a number of chemokines pertinent to AD following mechanical stimulation such as scratching or exposure to proinflammatory cytokines. These include TSLP as discussed above, which has been demonstrated to be expressed in increased quantities in AD skin (385) and has been described as a “master switch” in the development of DC-mediated Th2 allergic inflammation (388). Activated keratinocytes in AD also express high levels of CCL5 (RANTES) (389) with resultant eosinophil and T cell migration and activation (390), and in chronic AD the presence of the pro-inflammatory cytokines IFNγ and TNFα leads to the increased keratinocyte production of CCL2 (MCP-1) and CCL5 (RANTES), resulting in further T cell, DC and eosinophil recruitment (390). Other examples of keratinocyte-derived chemokines

reported to be elevated in AD that are chemotactic for T cells include CCL11 (eotaxin) (391), CCL17 (380), CCL20 (382), and CCL27 (376).

Keratinocytes have additionally been shown to produce numerous cytokines with varied immunomodulatory functions, notably the epithelial cytokines IL-25 and IL 33, which alongside TSLP are known to play a role in promoting Th2 polarisation in atopic inflammation and have been reported to be increased in AD patients (392, 393). Interestingly, polymorphisms in the ST2 gene which encodes the IL-33 receptor and is expressed by keratinocytes have been associated with AD (394). In AD lesions keratinocytes have additionally been shown to express IL-31RA (299), and overexpression of IL-31 in a transgenic mouse model has been reported to induce itch-scratch behaviour (300) and thereby may play a role in promoting AD development.

Furthermore, activated keratinocytes are capable of efficiently processing and presenting antigen to $CD4^+$ and $CD8^+$ memory T cells to induce functional T cell responses, and have been shown to upregulate HLA Class II and ICAM-1 in the inflammatory setting (311), which may play a role in AD. IFNγ activated keratinocytes have also been shown to upregulate Fas on the keratinocyte cell surface, and T cell Fas ligand-induced apoptosis of keratinocytes is believed to play a role in the spongiosis seen in AD (395).

Finally it is interesting to note that a recent systematic review found that the prevalence of IgE autoreactivity to epithelial cell targets varied from 18-91.4% in patients with AD (184), suggesting that autoantibodies to antigens expressed by keratinocytes may be important in AD pathogenesis.

Antigen presenting cells (APC) in AD

In their role as professional APCs, DCs are sentinels of the immune system, and are crucial for initiation of the host immune response (396). Immature DCs are found in tissues, where they express numerous receptors including complement receptors, Fc receptors and C-type lectins that can recognise cytokines secreted from infected or damaged cells or PAMPs released by microorganisms, and continuously survey the local environment for the presence of pathogens. When these are detected, DCs become activated and mature, migrating to regional draining lymph nodes where they are capable of priming naïve T cells. On maturation, DCs lose their ability to take up antigen and MHC antigen presenting molecules and T cell costimulatory molecules (e.g. CD80 and CD86) are upregulated, enabling priming of naïve T cells and interaction with antigen experienced T cells. DCs are divided into two main categories based on their origin, phenotype and function: myeloid DC (mDC) and plasmacytoid DC (pDC, which have been discussed previously and are important for anti-viral responses in the skin). mDCs can be further subdivided into Langerhans cells (LC) and epidermal DCs (also known as inflammatory dendritic epidermal cells) which together constitute the epidermal APC population. The corresponding dermal population is made up of dermal DCs and tissue macrophages (121).

Increased numbers of LCs, epidermal DCs and dermal DCs have been demonstrated in AD patients, which as a hallmark express the high affinity IgE receptor (FcεR1) on their surface (121, 148, 397, 398). This is thought to be preserved by elevated IgE levels, and receptor expression increases with disease severity and facilitates antigen processing and presentation to T cells by enabling cellular uptake and internalisation of IgE and IgE bound allergens (399). These DCs also contribute to expansion of the pool of T cells, by migrating to the regional lymph nodes to stimulate naïve T cells. The number of epidermal and dermal Langerin-FcεR1^{+} DCs increases following antigen challenge or onset of inflammation due to other factors, and, a major amplifier of atopic inflammation, these inflammatory DCs

have been shown to release large quantities of proinflammatory chemokines and cytokines after allergen challenge *in vitro* (399). Furthermore, studies have suggested that signalling within DCs is different in AD patients, with DCs isolated from the skin of atopics demonstrating PLC-γ tyrosine phosphorylation-dependent mobilisation of calcium on cross linking of FcεR1, an effect which was not observed in non-atopics (126). An important interaction with keratinocyte chemokine production such as TSLP has additionally been described in AD (detailed above), and indeed *in vitro* studies have demonstrated that DCs isolated from AD patients have the potential to drive either Th1, Th2 or Th17 immune responses, suggesting that the local micromilieu impacts on their T cell polarisation *in vivo* (400).

Langerhans cells are the dominant APC of the epidermis, and may be distinguished from other subsets of DCs by the expression of langerin (CD207), in addition to significantly higher levels of CD1a, FcεR1, E-cadherin, DEC-205 and CD36 (401). Langerin is a C-type lectin that is internalised into Birbeck granules (401), which are specialised organelles of the early endosomal recycling compartment thought to be involved in atypical antigen processing and presentation and are a characteristic feature of Langerhans cells. Langerin has been shown to be a potent inducer of cell membrane superimposition and zippering, leading to birbeck granule formation (402, 403), and interestingly CD1a has been reported to co-localise with langerin in Birbeck granules (404), suggesting a role for langerin in CD1a-mediated lipid antigen presentation. Indeed, it has also been reported that Langerhans cells presented M. leprae lipid antigens to T cell clones derived from a leprosy patient in a CD1a-restricted and langerin dependent manner (405). In AD, the clinical importance of these cells is supported by the observation that the presence of epidermal $FcεR1^{+}CD1a^{+}$ Langerhans cells was required to provoke a positive atopy patch test reaction in an experimental model of aeroallergen-induced patch test reactions on atopic skin (406).

Role of B cells and IgE production

B cells are additionally known to have an important role in AD pathogenesis, through their role as APCs and production of immunoglobulins (Ig).

B cells are professional APCs, and are efficient at processing and presenting antigen to $CD4^+$ T cells via MHC class II. When activated by B cells, cytokine production by $CD4^+$ T cells stimulates the production of B cell IgM antibodies; co-stimulation of the B cell provided by the interaction of CD40 on B cells and CD40L on T cells is additionally crucial for this event. Cytokine production by T cells at this time determines the class switching from IgM to other classes of immunoglobulins, with Th2 cytokines promoting switching to IgE, and Th1 cytokines promoting switching to IgG so that B cell activation and proliferation is dependent on antigen exposure, co-stimulation and Th cell cytokine release. During clonal expansion, the affinity of the BCR is refined by the process of somatic hypermutation (407), and B cells with mutated variable Igs with a high affinity for the antigen are selected, some of which will go on to differentiate into antibody secreting plasma cells. Small numbers of these cells will persist in the periphery and produce antigen specific antibodies (408).

B cell production of IgE is a hallmark of allergic disease, and FcεRs, IgE specific Fc receptors, are expressed by a wide range of cells including DCs, B cells, mast cells, basophils, eosinophils and keratinocytes. FcεRs can bind IgE with high (FcεR1) or low (FcεR2) affinity, and recognition of antigen by receptor-bound IgE leads to receptor aggregation, internalisation of receptor-IgE-antigen complex and cellular activation (409). Following internalisation, FcεR expressing cells may present antigens to T cells so that cells expressing specific IgE may play an important role in T cell priming and the expansion of antigen-specific T cell responses in AD. The role of IgE in T cell priming is supported by the observation that T cell-mediated late phase reactions after repeated antigen challenge of the skin can be inhibited by anti-

IgE therapy (410), however although currently approved for the treatment of asthma and chronic spontaneous urticaria, the therapeutic benefit of anti-IgE therapy in AD has not been conclusively established (132).

As discussed previously, AD patients frequently demonstrate elevated total and allergen-specific IgE, and are more likely to have a persistent, elevated IgE level and to be sensitised to multiple allergens (411), and it has been reported that there is an age dependency in the pattern of sensitised allergens, with sensitivity to food allergens predominating originally (commonly cow's milk, egg white, soy bean, wheat and peanut), followed by sensitivity to aeroallergens (such as house dust mite, cat dander, grass, weed and mould pollens) with age (411). Avoidance of allergens in sensitive individuals has been demonstrated in some studies to result in a clinical improvement of skin lesions (412). On this basis AD has been subdivided on the basis of elevated serum total and allergen specific IgE, with extrinsic AD patients (70-80%) having increased total and allergen specific IgE, and intrinsic AD patients having normal levels of IgE, suggesting that elevated allergen specific IgE is important but not crucial for the development of AD. However, it has also been shown that intrinisic AD patients can develop specific IgE to common microorganisms in AD, including *M. sympodalis* and *S. aureus* superantigens (185, 413) suggesting significant complexity in this system.

Role of other cells in the pathogenesis of AD: eosinophils and mast cells

Eosinophils also play an important role in the pathogenesis of AD, and although absent in normal skin, tissue and peripheral eosinophilia has been shown to correlate with severity of disease (414), and is found in the vast majority of patients with AD (415). In addition, following allergen patch testing, cutaneous eosinophil infiltration has been reported and has been associated with dermatitis (416). Eosinophils are recruited to the site of inflammation in AD by a myriad of cytokines and chemokines,

with IL-5, abundant in AD lesions as a Th2 cytokine, being one of the key cytokines for eosinophil differentiation, maturation, survival and migration (417). Other type 2 cytokines, namely IL-4 and IL-13 increase eosinophil recruitment and survival through the expression of CCL26 (eotaxin-3) by keratinocytes (418); IL-4 induced CCL11 (eotaxin-1) expression by dermal fibroblasts (419) amongst other cells is also potently chemotactic and levels have been reported to be increased in the blood and AD skin, and to correlate with AD disease activity. CCL5/RANTES is another potent eosinophil chemoattractant (420), and has been found at high levels in the blood and skin of AD patients (421, 422). These chemokines bind to the chemokine receptor CCR3, which is expressed preferentially on eosinophils, and has been shown to be essential for eosinophil recruitment (423). Upon activation, eosinophils degranulate and granular dermal deposits of toxic proteins including eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil major basic protein (MBP) have been reported in AD, with a correlation between levels of these proteins and disease activity (414). Eosinophils also produce a wide variety of proinflammatory cytokines and chemokines, including IL-16 which has been shown to promote exacerbation of AD and to be present at significantly higher levels in AD patients (424, 425).

Mast cells are additionally implicated in the pathogenesis of AD through their ability to become activated via FCεR1-bound allergen specific IgE, and animal models have demonstrated that the development of AD-like skin lesions requires these cells (426). Activation of mast cells results in the release of immediate and delayed inflammatory mediators: histamine, prostaglandins and leukotrienes released from preformed granules are key mediators of immediate type 1 hypersensitivity reactions, but delayed release of a large number of cytokines and chemokines can also result in late phase reactions in conjunction with the adaptive immune system, and can be seen in AD (427). Mast cells constitute one of the main cell types that produce IL-4

and IL-13 and are also a key source of CCL1 which has been found at high levels in the serum of AD patients (379), and a role in activation and migration of T cells, Th2 polarisation, DC recruitment and migration to lymph nodes, B cell development and IgE synthesis, eosinophil recruitment, the anti-microbial response, and production of pro-inflammatory cytokines and growth factors by keratinocytes has been described for mast cell derived cytokines and chemokines (202). A role in the development of pruritus and secondary epidermal barrier defect in AD has also been reported for mast cell derived histamine and tryptase (428). Indeed the number of mast cells has been found to be increased in chronic AD lesions (429), and AD severity in mice has been shown to associate with mast cell degranulation (430).

Summary

In summary it can be seen that the pathogenesis of AD is hugely complex, and that this condition may occur in individuals with an underlying genetic predisposition and is strongly influenced by numerous environmental factors in addition to a complicated interplay between multiple components of the innate and adaptive immune response and the epidermal barrier. Adaptive immunological pathways considered to be involved in the different stages of AD are summarised in the figure below (from (150)), where it can be seen that nonlesional AD skin is infiltrated by immune cells that produce cytokines such as IL-4 and IL-13, contributing to an impaired epidermal barrier. Barrier defects result in penetration of the skin barrier by epicutaneous allergens that encounter epidermal LCs and dermal DCs to stimulate Th2 and Th22 cells involved in the pathogenesis of acute AD. Smaller increases in the Th1 and Th17 immune axes may also be found in the acute stage. Lesions of chronic AD are characterised by a progressive activation of the Th2 and Th22, in addition to Th1 pathways, with IL-22 inducing epidermal hyperplasia, the Th2 pathway downregulating AMPs, and the Th2 and Th22 pathways downregulating epidermal differentiation genes such as filaggrin and loricrin throughout nonlesional, acute, and

chronic AD skin. Type 1 cytokines additionally exert numerous cutaneous effects that have been discussed in detail above. It is thought that IL-31 contributes to itch in patients with AD (150).

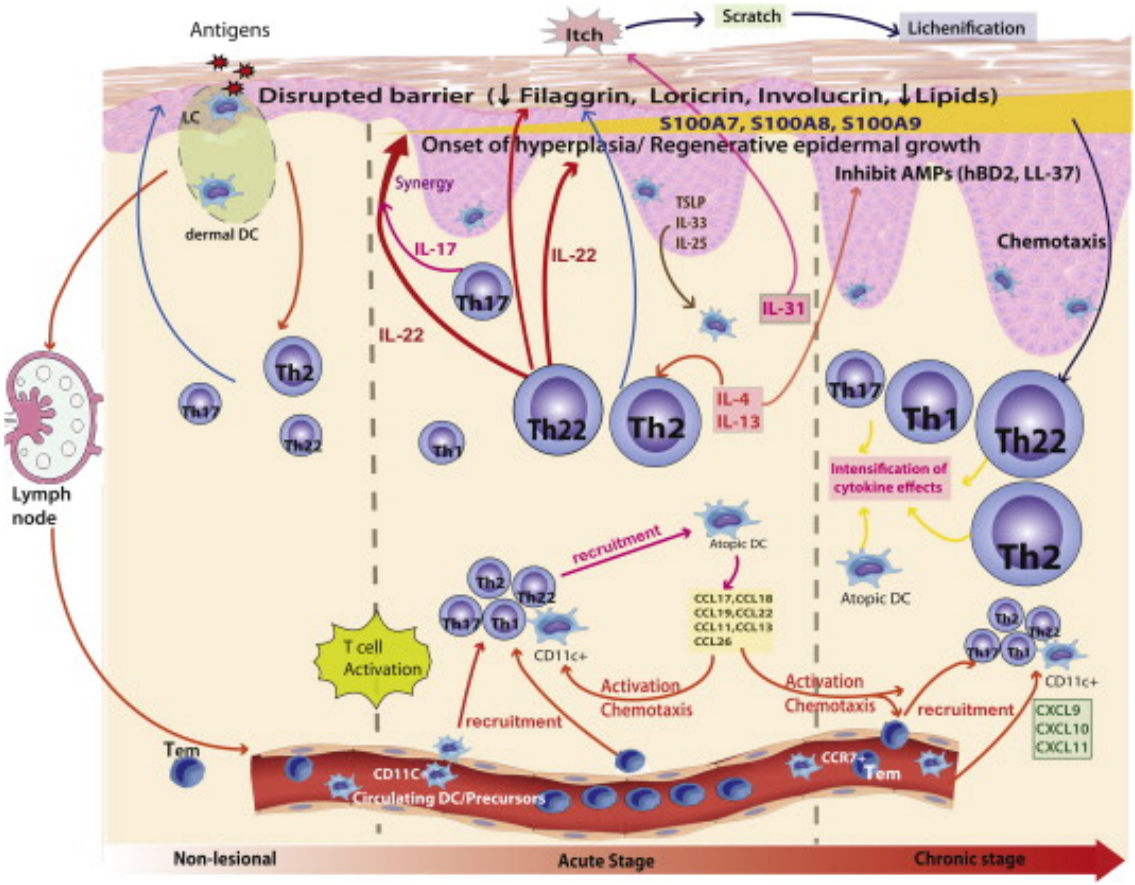


Figure 1.1: Immunologic pathways involved in the pathogenesis of AD. From (150).

1.2(iv) Treatment options in Atopic dermatitis

Current treatment strategies in AD are centred on repairing the defective skin barrier and optimising skin hydration (liberal emollients, including ceramide dominant emollients) and topical immunosuppressants to reduce cutaneous inflammation in

the form of corticosteroids, which remain the mainstay of treatment, and calcineurin inhibitors (122).

The anti-inflammatory effect of corticosteroids is mediated through target cell expression of cytoplasmic glucocorticoid receptors GCR. Ligand-bound GCR then binds to various transcription factors such as NF-κB and activator protein-1 to inhibit the transcription of multiple genes encoding many proinflammatory cytokines, chemokines and adhesion molecules (122). Interestingly, corticosteroids are also thought to have phospholipase inhibition activity (431) which has direct relevance to the studies presented within this thesis. Unwanted side effects of corticosteroids occur through the process of transactivation, where GCR-ligand dimers bind to glucocorticoid response elements located in the promoter regions of target genes (122). Calcineurin inhibitors, such as tacrolimus and pimecrolimus have a direct anti-T cell effect, binding with high affinity to the 12 kDa macrophilin and inhibiting the phosphatase activity of calcineurin, a calcium-dependent serine/threonine phosphatase. This means that the transcription factor the Nuclear Factor of Activated T cells protein (NF-ATp) does not undergo dephosphorylation and thus is unable translocate to the nucleus and hence does not activate transcription of various Th1 and Th2 cytokine genes (122). This class of drugs have shown the most promise for the long-term maintenance therapy of AD due to their encouraging safety profile (188).

Identification and treatment of exacerbating factors is also very important, in the form of occlusive bandages and gloves to break the itch-scratch cycle, allergen and irritant avoidance, and antibiotic treatment for concurrent infection. UV light therapy may be a useful treatment modality in selected cases of chronic recalcitrant AD, with both UVB and psoralen/UVA being utilised, and has been shown to significantly decrease dermal Ig-E binding cells including mast cells and DCs and to inhibit LC migration out of the epidermis (122).

Systemic immunosuppressant treatment is indicated for severe refractory disease, with oral corticosteroids such as prednisolone, oral calcineurin inhibitors such as ciclosporin, and antimetabolites including azathioprine, methotrexate and mycophenolate mofetil which each carry their own side effect profile, limiting their clinical utility.

More recently, due to the advances in knowledge regarding the pathogenesis of AD discussed above, and to the safety and efficacy of Dupilumab in phase I and phase II clinical trials, there has been renewed interest in the use of biologics for the treatment of AD (132). These include anti-IL-4, anti-IL-13, anti-IL-12/23, anti-IL-22, anti-IL-31 and anti-TSLP directed treatments which are all in varying stages of development and clinical trials (132). Dupilumab, a monoclonal antibody directed against the α subunit of the IL-4 receptor which is shared with the IL-13 receptor so that inhibition blocks both pathways, is currently regarded to be the most promising therapy, with phase III clinical trials under development (132, 295).

1.3 Phospholipase

1.3(i) Introduction

Phospholipases (PLAs) are enzymes that hydrolyse phospholipids (432), a lipid class which are plentiful in all living organisms as a major component of cell membranes, and which are composed of a phosphorylated headgroup and diacylglyceride – two fatty acid chains comprised of varying carbon chain lengths and saturation covalently bonded to glycerol.

Phospholipases play an important role in many key biological processes such as signal transduction, lipid metabolism, and the maintenance of cell membranes and are divided into four groups depending upon the position at which they hydrolyse

phospholipids: A, B, C and D (432). Phospholipase A1 (PLA1) and phospholipase A2 (PLA2) release an alkyl chain by hydrolysing the carboxylic ester bonds of phospholipids at the *sn-1* and *sn-2* positions respectively. Phospholipase B (PLB) cleaves at both sn-1 and sn-2 positions, and phospholipase C (PLC) and phospholipase D (PLD) hydrolyse phospholipids before and after the phosphorylated head group (Figure 1.2).

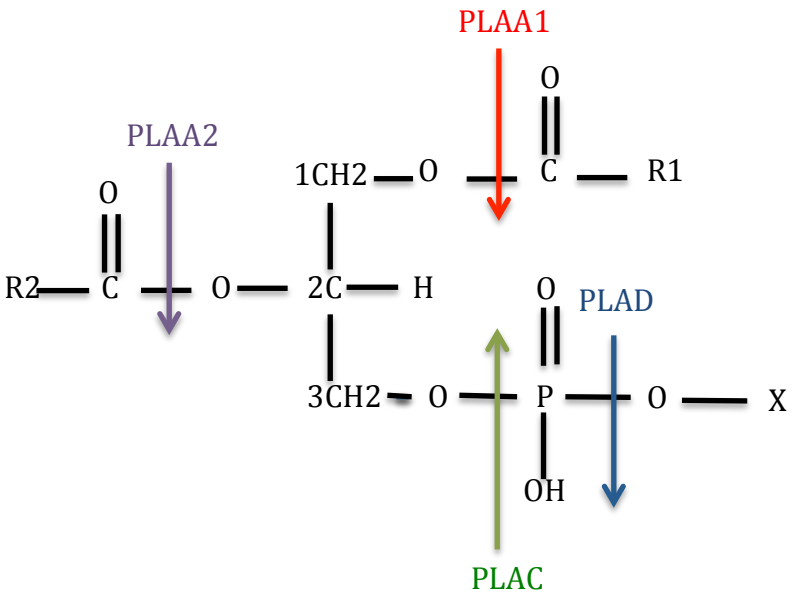


Figure 1.2: Phospholipid cleavage sites of the phospholipase groups. PLA1 hydrolyses at the *sn-1* position; PLA2 hydrolyses at the *sn-2* position; PLB hydrolyses at both *sn-1* and *sn-2*; PLC hydrolyses before the phosphate group; PLD hydrolyses after the phosphate group.

Each phospholipase group is further subdivided into several subgroups, with expression of each subgroup varying between species, tissues and cells. Each phospholipase may hydrolyse a number of different phospholipids, with a degree of overlap between them.

Phospholipase A2 (PLA2) has been extensively studied, and hydrolyses phospholipids at the *sn*-2 position to generate fatty acids and lysophospholipids (Figure 1.3) (433), and may be secretory (sPLA2), cytosolic (cPLA2) or inducible (iPLA2) (433).

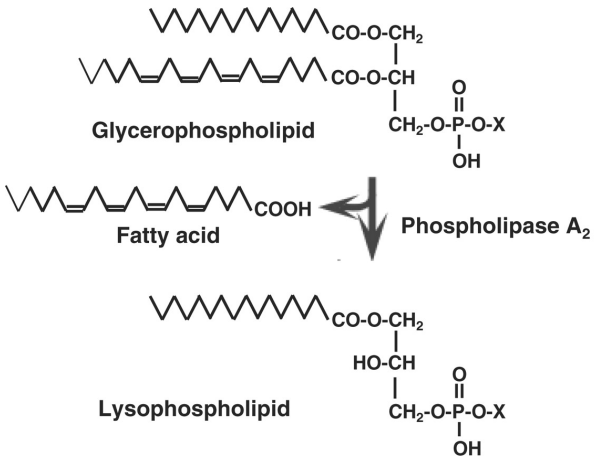


Figure 1.3: PLA2 enzymatic action on phospholipids. PLA2 hydrolyses phospholipids at the sn-2 position to produce free fatty acids and lysophospholipids (From (433)).

Subdivided on the basis of sequence homology, 16 human PLA2 groups have been identified to date (Figure 1.4) (434).

Type	Group and subgroup
sPLA2	**GI** – A, B, **GII** – A, B, C, D, E, F, **GIII**, **GV**, **GIX**, **GX** **GXI** – A, B, **GXII** – A, B,

	GXIII, **GXIV**
cPLA2	**GIV** – A (α), B (β), C (γ), D (δ), E (ε), F (ζ)
iPLA2	**GVI** – A (β), B (γ), C (δ), D (ε), E (ζ), F (η)
PAF-AH	**GVII** – A (Lp-PLA2), B (PAF-AH II), **GVIII** - A (α1), B (α2), β
Ly-PLA2	**GXV**
Ad-PLA2	**GXVI**

Figure 1.4: Phospholipase A2 superfamily (from (434)).

Groups I-III are all secretory sPLA2s which were the first PLA2 type to be discovered (435), with groups I and II sPLA2s including PLA2 homologous to snake venom from the old and new world, and group III sPLA2 insect venom-like PLA2 (434). The first cytosolic PLA2 (cPLA2) group to be discovered was group IV PLA2, with other cytosolic PLA2 groups including lysosomal PLA2 (Ly-PLA2) and the platelet activating factor-acetylhydrolases (PAF-AH) being identified soon after. To date, calcium dependency is a feature of the majority of identified PLA2 species, however inducible group VI PLA2s that are calcium independent have additionally been identified (434). Ad-PLA2, which is localised to adipose tissue, is the most recently identified PLA2 (group XVI) (434), and new PLA2 groups and subgroups are generated on the discovery of new PLA2 species depending on their homology to known PLA2s. Each PLA2 is capable of hydrolysing several phospholipid substrates,

and PLA2 subgroup expression differs amongst species, tissues, and cells, in addition to pathological condition (433).

1.3(ii) Phospholipase in disease

The various PLA2s are known to contribute to the inflammatory immune response through a number of mechanisms.

As dominant players in the eicosanoid biosynthesis pathway, cPLA2s hydrolyse phospholipids to release free fatty acids, which include arachidonic acid. Arachidonic acid is then further processed through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways to produce end product inflammatory mediators including prostaglandins and leukotrienes, which have widespread effects in the inflammatory setting (Figure 1.5) (436).

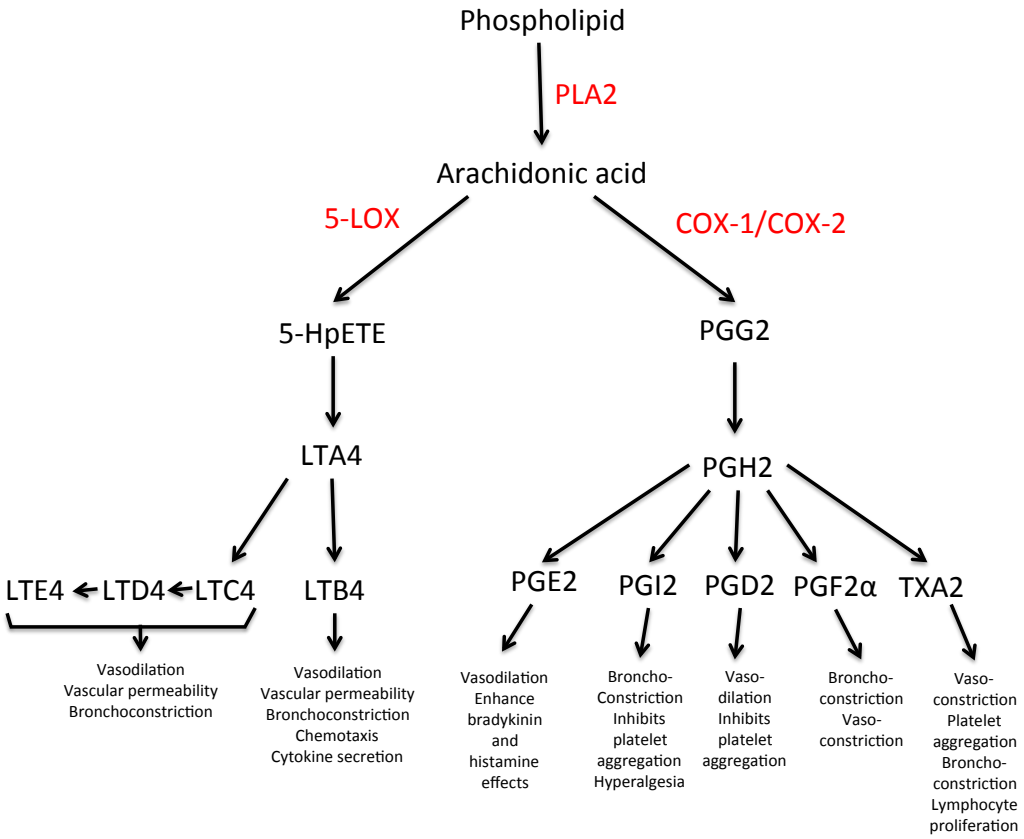


Figure 1.5: Eicosanoid biosynthesis pathway and downstream effects. Modified from (436).

Compromised eicosanoid synthesis is seen in mice deficient in group IV cPLA2, and these mice exhibit resistance to murine inflammatory disease models including experimental and autoimmune encephalitis and collagen induced arthritis (437, 438). A role in inflammation is also known for lysophospholipids through their action on receptors which are expressed on multiple cells of the immune system including T-cells, B-cells and macrophages, with receptor-ligand engagement resulting in cellular differentiation, proliferation, cytokine and antibody production, and chemotaxis (439).

A role in the inflammatory immune response is also known for secretory PLA2s, with some sPLA2 groups additionally playing a part in the eicosanoid pathway. These enzymes are also known to have a role in antimicrobial defence, for example, bee venom sPLA2 and Group X sPLA2 have been shown to neutralise HIV virus and to inhibit viral entry into host cells and to inhibit viral replication (440, 441) and interestingly a strong association with the development of AIDs had been described for polymorphisms is sPLA2G3 (442), which has a high homology to bee venom sPLA2; some sPLA2 subgroups have been shown to inhibit host cell adenovirus infection (443). High quantities of group IIA sPLA2 has also been detected in tears, where, through its action on the bacterial cell membrane, it exerts an antimicrobial action (434, 444, 445), and is believed to be important in protection from group B streptococcus infection (GBS) (446), and overexpression of human group II PLA2 protected transgenic mice from *S. aureus*, *E. coli* and GBS infections (446-448).

Multiple pro-inflammatory stimuli are known to stimulate cellular sPLA2 expression, and many cells of the immune system secrete PLA2 including mast cells, eosinophils, T cells (especially Th2 T cells) and basophils. Indeed, a role for sPLA2

has been demonstrated for PMA mediated activation and proliferation of T cells, suggesting that sPLA2s have a role in T cell activation and differentiation (449). In addition, promotion of Th2 immunity has been shown by group V sPLA2, which has been described as a *"Th2-prone sPLA2"* and is thought to contribute to the recruitment of effector Th2 cells (442). PLA2 has also been shown to have numerous effects on other cells of the immune system. For example, dendritic cell activation (450), maturation and increased expression of immunoregulatory transcription factors including NFAT has been demonstrated to be induced by group III sPLA2, an effect that appears to mediated by products of sPLA2 activity as it was dependent on the enzyme being active (451, 452). Some PLA2s have also been shown to cause exocytosis of cells including eosinophils and macrophages (453, 454), in addition to stimulating cytokine and chemokine secretion (453, 454). A role in macrophage activation (455) and phagocytosis (456) has also been reported, and PLA2 is also abundantly produced by mast cells, described with respect to their role in AD pathogenesis earlier in this chapter. The major mast cell PLA2 was recently reported to be group III sPLA2, and it has been demonstrated that this enzyme is secreted on mast cell activation by exocytosis, and that, in comparison to wild type mice, *PLA2g3*$^{-/-}$ mice exhibited attenuated mast cell degranulation and ear swelling following antigen challenge (457). Facilitation of mast cell maturation via a paracrine prostaglandin D_2 (PGD_2) pathway was reported (457), which has lead to designation of this sPLA2 group as an *"anaphylactic sPLA2"* (442).

Increased sPLA2 activity and enzyme levels have also been reported in numerous inflammatory conditions, including rheumatoid arthritis (458), psoriasis (459) and Crohn's disease, in addition to in bronchoalveolar fluid following challenge with histamine or allergen in sufferers of asthma and allergic rhinitis (434, 453, 454, 460, 461). Elevated sPLA2 levels correlated with increased eicosanoid and lysophospholipid levels (462), and mediated an increase in inflammatory lipid

mediators. Genetically modified mice that are deficient in group V and group X sPLA2 exhibit less airway hyperresponsiveness (463, 464) and in a murine asthma model are protected from inflammation of the airways (442), and group IIA sPLA2 deficient mice had fewer signs of arthritis in a murine model of autoimmune erosive inflammatory arthritis (465).

A role for PLA2 in the human CD1-mediated immune response is additionally suggested by the fact that PLA2 fatty acid and lysophospholipid products may be eluted from human CD1 molecules, and presentation of lysophospholipids by CD1d molecules has been reported to stimulate NKT cells (81, 466). Plasma derived inflammation-associated lysophospholipids have additionally been identified as ligands for CD1d-restricted T-cells in myeloma patients (467). Inhibition of antigen presenting cell PLA2 was also found to significantly reduce activation of one iNTK cell clone (466), implying that PLA2 activity may be an important source of lipid antigen generation for CD1d molecules. Furthermore, a role as immune adjuvants has been demonstrated for lysophospholipids, which might act via the stimulation of iNKT cells (466, 468). In addition, our laboratory has recently reported that bee venom derived PLA can promote the generation of CD1a ligands which are recognised by T cells (469), and the crystal structures of TCR-CD1a lysophosphatidylcholine and oleic acid, products of sPLA2 activity, have recently been solved (64).

Exogenous PLAs are additionally known to be immunogenic. IgE specific to PLA1 in wasp venom and PLA2 in bee venom has been well documented and these proteins have been identified as some of the dominant T-cell antigens in the context of allergy (470), however it is not clear to what extent PLAs may be present in other allergens such as house dust mite and pollens which are known to cause a clinical exacerbation of AD. As recently reported with venoms (469), a potential novel role of

allergen derived PLAs may therefore be the generation of CD1a ligands from either endogenous or exogenous substrates.

Multiple roles of PLA2 within the skin have been described, including involvement in the maintenance of the epidermal barrier and cutaneous pH, remodelling of cellular membranes and wound healing (471). Elevated eicosanoid levels have been detected in inflammatory dermatoses, namely atopic and contact dermatitis and psoriasis, and increased levels of group IIA and IID sPLA2 have also been reported in the psoriatic epidermis (471). Cutaneous inflammation (472), with hyperplasia and hyperkeratosis analagous to that seen in the skin lesions of psoriasis patients has been shown in group II sPLA2 transgenic mice, and transgenic mice expressing human group III sPLA2, which is known to have the greatest homology to bee venom sPLA2 (473), develop inflammation over time (452), including a dermatitis characterised by hyperkeratosis and acanthosis, with an associated inflammatory cell infiltrate (452). A deregulated plasma lipoprotein profile and increased atherosclerotic plaques on ingestion of an atherogenic diet has additionally been reported in these mice (473).

It may therefore be postulated that group III sPLA2 may have a role in cutaneous inflammation, and indeed the homology of group III sPLA2 with bee venom PLA2 implies that these two enzymes might hydrolyse similar phospholipid substrates with the consequent production of similar fatty acid and lysophospholipid products. Furthermore, amelioration of contact dermatitis in patients treated with a topical sPLA2 inhibitor has also been described (474), supporting the involvement of PLA2 activity in skin inflammation and a possible therapeutic impact of its inhibition. Indeed, corticosteroids, which remain the mainstay of treatment for AD, have also been shown to inhibit the secretion of sPLA2 (475), and the resultant decreased eicosanoid synthesis might therefore contribute to the anti-inflammatory action of these drugs.

1.4(iii) Phospholipase receptor interactions

Binding of sPLA2s to sPLA2 receptors has also been described, suggesting that not all of the physiological function of these enzymes is dependent on their enzymatic activity (476). Indeed, several of their immunological effects are thought to be independent of their catalytic action, including cytokine and chemokine production, degranulation of mast cells, and exocytosis of eosinophils, macrophages, and neutrophils, which are thought to be mediated by cell surface receptors (454).

It is known that sPLA2s are able to bind to sPLA2 cell surface receptors, with direct evidence of such receptors in mammals first being reported for sPLA2 receptors identified on cellular membranes in the brain (476). These receptors were found to exert a neurotoxic effect, and were designated the N (neuronal) -type receptor (476).

The M (muscle) -type receptor has additionally been described (476). This receptor is a member of the mannose receptor family, which belong to the C-type lectin superfamily (476). The M-type receptor was first identified on skeletal muscle cells but has since been found on multiple tissues (477). This receptor is thought to play a role in endototoxic shock pathogenesis, with M-type receptor knockout mice exhibiting lower levels of IL-1β and TNF-α in the serum and enhanced survival following administration of lipopolysaccharide (478). A role in regulating PLA2's enzymatic activity has additionally been proposed, in addition to the internalisation and breakdown of PLA2, as M-type receptors are known to have endocytic properties, and M-type receptor binding of PLA2 has been demonstrated to inhibit PLA2 enzymatic activity (476). It has also been postulated that uncoupling of PLA2-receptor may occur after endocytic pathway internalisation, enabling hydrolysis of different intracellular lipid substrates, and that membrane PLA2 receptors might function to sequester PLA2, concentrating this enzyme at the cell surface of target cells (476). Interestingly, an M-type PLA2 receptor has also been shown to be

expressed by podocytes of the kidney and is thought to be a pathogenic target for auto-antibodies in patients with some forms of membranous nephropathy (479). It is noteworthy that membranous nephropathy can recur after transplantation suggesting systemic immune response involvement (480). It is also of interest that it has recently been observed that expression of the M-type receptor is decreased in many cancers, and gain and loss of function studies *in vitro* and *in vivo* have demonstrated that the M-type receptor acts as a tumour suppressor by promoting apoptosis and inhibiting cellular transformation and therefore has a role in tumour senescence (481).

The various sPLA2 subgroups have different binding affinities for the PLA2 M-type and PLA2 N-type receptors and are consequently thought to have differing functions, for example group IB and group IIA sPLA2s exhibit low binding affinities for PLA2 N-type receptors, whereas solely PLA2 N-type receptor binding has been demonstrated for bee venom sPLA2 (476).

Furthermore, PLA2 has also been shown to bind additional members of the mannose receptor family, which are structurally similar to the M-type receptor (477, 482, 483). These receptors are known to have a role in antigen uptake and processing, in addition to being involved in innate and adaptive immune responses (477, 482, 483). It might therefore be speculated that this mechanism could lead to co-localisation of PLA2 with CD1 molecules in the endocytic pathway and therefore have a potential role in lipid antigen loading onto CD1 molecules.

2 Aims and Objectives

As discussed in the introduction, our understanding of the role of CD1-restricted T cells in the pathogenesis of clinical disease remains limited, despite the well-described ability of these cells to recognise CD1 molecule presented lipid ligands. Group 2 CD1d-restricted iNKT cells are the focus of the majority of the published literature, with most of the data on group 1 CD1-restricted T cells being restricted to studies on a limited number of T cell clones, due to the absence of murine group 1 CD1 expression and of a specific marker for these cells. Studies on polyclonal T cells will also be important in order to advance our understanding of these cells as a whole.

It has recently shown that CD1a-restricted T cells are present at higher frequencies than previously considered and can infiltrate the skin and recognise natural skin lipids (55, 118, 119). Langerhans cells of the epidermis express constitutively high levels of CD1a, and so may play a role in the presentation of CD1a ligands to CD1a-restricted T cells. An altered lipid profile has been described in lesional AD skin, which may affect CD1a antigen presentation (237-239), and indeed enrichment of $CD1a^+$ cells within AD lesions has been reported (386). This poses the question: could CD1a-restricted T cells contribute to the cutaneous inflammation seen in AD?

We therefore postulated that these cells might play a role in the pathogenesis of AD, and sought to test the hypothesis that CD1a-reactive T cells contribute to the response to house dust mite in AD in human blood and skin.

This hypothesis was tested by investigating the following aims:

Chapter 4 Identification of HDM responsive CD1a-reactive T cells in the peripheral blood of AD patients and controls

Chapter 5 Identification of HDM responsive CD1a-reactive T cells in the skin of AD patients and controls

Chapter 6 Phenotypic and functional characterisation of HDM responsive CD1a-reactive T cell lines

Chapter 7 Investigation of the role of phospholipase in the generation of CD1a ligands

3 Materials and Methods

3.1 Reagents

ANTIGENS: Dermatophagoides pteronyssinus Soluprick SQ solution 10 HEP (ALK-Abello) (P0700, M1047, L0751) 0.07-14μg/ml, Felis Domesticus Soluprick SQ solution ALK 555 10 HEP (Cat hair) (ALK-Abello), Canis familiaris Soluprick SQ solution ALK 553 10 HEP (Dog hair) (ALK-Abello), 6-Grasses (Avena/Dactylis/Poa/Festuca/Lolium/Phleum) Soluprick SQ solution ALK 299 10 HEP (ALK-Abello), Candida albicans Soluprick SQ solution (ALK-Abello), Malassezia fufur (Mycology Laboratory, Medical School, National and Kapodistrian University of Athens), Dermatophagoides pteronyssinus HDM extract XPB70D3A2.5 (Greer) 0.1-10 μg/ml, Vespula *spp.* Venom (Sigma-Aldrich V4879), Apis mellifera venom phospholipase A2 (Sigma-Aldrich V9279).

Sigma-Aldrich: Fatty acid C16:0 (PO500), Fatty acid C16:1 (P9417), Fatty acid C18:1 (O1008), Lysophosphatidylcholine C18:0 (L2131).

Lipids from Avanti polar lipids: Phosphatidylcholine C18:1 (850375), Lysophosphatidic acid C18:1, Lysophosphatidylcholine C18:1 (845875), Lysophosphatidylcholine C16:0.

CD1a recombinant protein: NIH tetramer facility and gift from Demin Li (Level 5, JR Hospital, Oxford). Anti-CD1a antibody OKT6 10-20 μg/ml (in house), Purified Mouse IgG1 k Isotype control 20 μg/ml (BD Pharmingen), L243/W6/32/IVD12 10-20 μg/ml (in house). Anti-CD3 antibody 50ng/ml (OKT3; Ebioscience, CA, USA), Manoalide BML-E11177-001 (Enzo life sciences)

FLOW CYTOMETRY ANTIBODIES (dilutions):

Biolegend: Langerin APC (1:100), CLA PB (0.5:100), CD1d PE (1:50), Brilliant Violet 650 CD3 (0.5:100), PE/Dazzle 594 TCR γ/δ (1:100), CD194 (CCR4) PE/Cy7 (1:100), CD196 (CCR6) FITC (1:100), CCR10 PE (1:100), CD8 PerCP (1:100), CD80 APC (0.5:100), CD86 PeCy7 (1:100), IgG Isotype Ctrl PE (1:100), IgG1, κ Isotype Ctrl PE/Dazzle 594 (1:100), IgG2a, κ Isotype Ctrl Brilliant Violet 650 (0.5:100), IgG2b, κ Isotype Ctrl PE/Cy7 (1:100), IgG1, κ Isotype Ctrl PE/Cy7 (1:100), IgG1, κ Isotype Ctrl PerCP (1:100), IgM, κ Isotype Ctrl Pacific Blue (0.5:100), IgG2b, κ Isotype Ctrl FITC (1:100), IgG1, κ Isotype Ctrl PE/Dazzle™ 594 (1:100).

BD Biosciences: CD1a PE (1:50), CD3 PerCP (1:50), CD8 APC (1:100), CD56 PE (1:50), HLA-DR APC-H7 (1:100), CD14 PerCP (1:100), CD14 APCCy7 (1:100), CD3 FITC (1:50), CD83 FITC (1:50), CD4 PeCy7 (1:100), CD19 FITC (1:50),

Ebioscience: TCR αβ APC (0.5:100), CD1b APC (1:100), CD1c FITC (1:50), IgG1 K Isotype Control APC (0.5:100), γδ TCR PE (1:100).

Invitrogen: Live/Dead PB (1:1000), Live/Dead Aqua (1:1000).

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 Tecnhologies) (RHepes) before layering over 15ml Lymphoprep (Nycomed, Roskilde, Denmark) in a 50ml Falcon tube and being centrifuged for 20

minutes at 2000 rpm with the centrifuge brake switched off. The buffy coat layer of PBMCs was aspirated and washed twice in RHepes at 1800 rpm for 10 minutes and 1500 rpm for 10 minutes, and then resuspended in RHepes with 10% foetal calf serum (R10).

3.3 Isolation of human T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult donors and atopic dermatitis patients under local ethics approval (09/H0606/71). Atopic dermatitis was diagnosed using the UK refinements of the Hanifin and Rajka diagnostic criteria and disease severity was assessed using SCORAD. Donors were aged 18-67 with disease severity SCORAD range 5-70. Patients were using topical corticosteroids, but were not on systemic immunosuppression nor topical calcineurin inhibitors. Adult (18-67 years) patients with moderate severity psoriasis who were not on systemic therapy were recruited. T-cells were purified from ficollized peripheral blood mononuclear cells using CD3 MACs beads (Miltenyi Biotec, Germany). PBMCs were resuspended in 80 µl MACS buffer (0.5% bovine serum albumin (BSA)/PBS with 0.2mM EDTA) per 10 million cells and incubated with 20 µl CD3 Microbeads per 10 million cells for 15 minutes at 2-8°C. Cells were then washed in 2ml of MACS buffer per 10 million cells at 300g, and resuspended in 500ul Macs buffer per 10 million cells. LS columns on a MACs magnet were washed with 3ml of MACS buffer and then the cell suspension was passed through the LS column and washed 3 times with 3ml MACs buffer. The column was then removed from the magnet, 5ml buffer was added and magnetically labelled cells were flushed out by pushing the plunger into the column. Enriched cells were washed and resuspended in T cell medium (RPMI supplemented with 2mM L-glutamine, 100iu/ml penicillin, 100µg/ml streptomycin, and 5% heat-inactivated human serum, HEPES, non-

essential amino acids, sodium pyruvate and 2-mercaptoethanol (Life Technologies, CA, USA) in the presence of 1nM IL-2 (PeproTech, London, UK)).

CD3 enrichment was verified by flow cytometric staining pre- and post-separation.

3.4 Monocyte-derived Dendritic (mDC) culture

Autologous mDCs were differentiated from $CD14^+$ monocytes. $CD14^+$ human cells were isolated from PBMCS using MACS cell separation (Miltenyi Biotec Inc., Germany) according to the supplier's instructions. These cells were then cultured in 6-well plates at 0.2-0.5 x 10^6 cells/ml in complete medium (RPMI supplemented with 2mM L-glutamine, 100iu/ml penicillin, 100µg/ml streptomycin, and 10% foetal calf serum (FCS) (Sigma-Aldrich), HEPES, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol (Life Technologies)) containing 50ng/ml GM-CSF (PeproTech, London, UK) and 1000iu/ml IL-4 (PeproTech, London, UK). After 4 days, CD1a expression was confirmed by Flow Cytometry, and the differentiated mDCS were used as antigen presenting cells for the ELISpot assay. The *in vitro* mDCs were incubated with 10 µg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32, L243 and IVD12) for one hour prior to co-culture with T-cells, in order to minimize HLA-restricted responses.

3.5 Langerhans-cell like culture

In vitro LC-like cells: Autologous LC-like cells were generated *in vitro* using $CD14^+$ monocytes. $CD14^+$ human cells were isolated from PBMCS using MACS cell separation and cultured as described (484, 485) in complete medium in the presence

of IL-4 (250 ng/ml; PeproTech, London, UK), GM-CSF (100 ng/ml; PeproTech, London, UK) and TGF-β1 (10 ng/ml; PeproTech, London, UK) in 6-well plates at 0.2-0.5 x 10^6 cells/ml. At days 2 and 4, cultures were re-plated in the presence of the above cytokines to generate to generate cells which were at least 24.9-26.5% $CD1a^+CD207^+$ by flow cytometry. The *in vitro* LC-like cells were incubated with 10 μg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32 and L243 respectively) for one hour prior to co-culture with T-cells, in order to minimize HLA-restricted responses.

LCs: Human cord blood was obtained from the NHS cord blood bank (London, UK). $CD34^+$ human progenitor cells were isolated using MACS cells separation (Miltenyi Biotec) according to supplier's instructions. $CD34^+$ cell cultures were established in the presence of stem cell factor (25ng/ml; PeproTech, London, UK), GM-CSF (200U/ml; PeproTech, London, UK), and TGF-β1 (1 ng/ml; PeproTech, London, UK) to increase CD1a expression (405). LCs were harvested at day 12, and the LC quality was checked by flow cytometry. The cord-blood derived LCs were incubated with 10 μg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32, L243 and IVD12) for one hour prior to co-culture with T-cells, in order to minimize HLA-restricted responses.

3.6 Skin blisters

Skin blisters were performed on healthy volunteers and AD patients with informed consent with ethics approval at the Dermatology clinic at the Churchill Hospital, Oxford. Donor epidermis was injected with 0.7 μg of *Dermatophagoides pteronissimus* HDM extract (ALK-Abello) or normal saline. After 30 minutes, 200mmHg negative pressure was applied to the skin using a 1cm diameter suction

cap (Eschman VP25) for approximately 1h, to induce dermo-epidermal separation and blister formation (178). Blister fluid was isolated by needle aspiration at 24 hours and the cells were separated by centrifugation. The blister fluid phase was immediately stored at -20^{o}C. Cytokines were measured by multiplex bead array in the MRC laboratory of Molecular Biology, Cambridge,UK.

3.7 Skin blister T cell isolation

Blister fluid was isolated by needle aspiration at 24 hours and the cells were separated from the blister fluid by centrifugation. Blister derived T cells were then FACS sorted using a MoFlo^{TM} (Beckman Coulter, Fullerton, CA, USA) or Aria III (Becton Dickinson, USA) cell sorter and expanded using the rapid expansion method described below. Specifically, T-cells were plated out at 100-150 cells/well into T-cell media in a round-bottom 96-well plate and 50ng/ml of anti-CD3 (OKT3) antibody and irradiated PBMC and EBV transformed B-cells at 150-200,000 cells/well and 40,000 cells/well respectively were added. Blister T-cells were examined and split regularly and on expansion were maintained at a density of approximately 0.5-1 million cells/ml. CD1a reactivity was determined by ELISpot at approximately 3 weeks.

3.8 Isolation of CD1a+ cells from skin

Skin samples from abdominoplasty surgeries were obtained from Nuffield Health, the Manor Hospital, Oxford, from patients with informed consent and ethics approval. Samples were finely diced and incubated overnight at 37^{o}C 5% CO_2 in 1 mg/ml collagenase in R10. The following day, 2-3 ml cold 10mM PBS/EDTA was added and clumps were dispersed by repeated pipetting. The sample was then passed through

a 70 µm nylon cell strainer and washed through with 50 ml PBS/EDTA, before passing through a 40 µm nylon cell strainer. Samples were washed for 20 minutes at 300g and 4°C, and then cells were resuspended in 25 ml R0 and layered over 15 ml Lymphoprep (Nycomed, Roskilde, Denmark) and spun for 20 minutes at 2000 rpm with the centrifuge brake switched off. The buffy coat layer of cells was collected and resuspended in complete medium. $CD1a^+$ cells were isolated using MACs beads separation (Miltenyi Biotec) according to supplier's instructions, and $CD1a^+$ cell enrichment was confirmed using flow cytometry The $CD1a^+$ cells were incubated with 10 µg/ml anti-HLA-ABC and anti-HLA-DR blocking antibodies (W6/32, L243 and IVD12) for one hour prior to co-culture with T-cells, in order to minimize HLA-restricted responses.

3.9 *Ex-vivo* ELISpots

CD1a reactivity was assessed by IFN-γ, IL-13 and GM-CSF ELISpot (Mabtech AB, Sweden). T cells were isolated from PBMCs by $CD3^+$ MACs cell separation according to supplier's instructions, and left to rest for 3 days in T cell medium.

On day 1 of the ELISpot, T cells were washed twice in RHepes and returned to a fresh plate in R5* (RPMI supplemented with 2mM L-glutamine, 100iu/ml penicillin, 100µg/ml streptomycin, and 5% heat-inactivated human serum, HEPES, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol (Life Technologies)) at 0.5-1 x 10^6 cells/ml. APCs were pulsed with HDM extract (7µg/ml for K562 cells and 3.5µg/ml for mDCs, LC-like cells and cord blood derived LCs unless otherwise stated) or 1µg/ml purified bee venom PLA2 (Sigma) overnight, or other allergens of interest. ELISpot plates (96-well multiscreen IP-plates Millipore Corp., MA, USA) were coated with anti-IFN-γ, anti-GM-CSF or anti-IL-13 antibody overnight at 4°C

(Mabtech AB, Sweden): after pre-treatment with 50 µl/well 35% ethanol for one minute, and washing six times with distilled water, ELISpot plates were coated with 70 µl/well capture antibody (MabTech) at 15µg/ml (anti-IFN-γ, anti-GM-CSF) or 10µg/ml (anti-IL-13) overnight at 4°C according to supplier's instructions.

On day 2 of the ELISpot, antigen-pulsed APCs were washed three times in RHepes and re-suspended in R5* ready for plating out. The ELISpot plates were washed six times with RPMI and blocked for 1h at 37^{o}C with RPMI supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin plus 10% human serum (R10*). The ELISpot plates were then washed twice again with RPMI before 5-50,000 T-cells were added in 100 µl R5* per well to which 25,000 K562 or primary cells in 100 µl R5* were added. Wells were set-up in duplicate or triplicate. Phorbol 12-myristate 13-acetate 20 ng/ml (PMA) (Sigma-Aldrich, St Louis, MO, USA) and Ionomycin 10 ng/ml (Sigma-Aldrich, St Louis, MO, USA) was included as a positive control, and T-cells alone in the absence of APC was included as a negative control.

On day 3 of the ELISpot plates were developed. After overnight incubation at 37°C and 5% CO2, plates were washed 6 times in PBS-Tween 0.05% and incubated with 50 µl /well 1 µg/ml of biotin-linked anti-IFN-γ, anti-GM-CSF or anti-IL-13 monoclonal antibody (Mabtech AB, Sweden) for 2 h at room temperature. Plates were then washed again 6 times with PBS-Tween 0.05%, and incubated for a further 1 hour with 50 µl /well 1 µg/ml streptavidin-alkaline phosphatase (Mabtech AB, Sweden) at room temperature. Following a further 4 washes with PBS-Tween 0.05% and 3 washes with distilled water, spots were visualized using an alkaline phosphatase conjugate substrate kit at 100 µl /well (Biorad, Hercules, CA, USA) and enumerated using an automated ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic, Germany). Results were expressed as spots per number of cells added per well. In some experiments 10 -20 μg/mL anti-CD1a blocking antibody (OKT-6), or 10 -20 μg/mL IgG1 isotype control, or 10 μM manoalide, an inhibitor of PLA2 that acts

by covalently modifying lysine residues (Enzo Life Sciences, NY, USA), or 0.5 μg/mL Filaggrin monomers were added at to K562 or primary cells before addition of T cells.

The frequency of the HDM reactive T cell response was determined in polyclonal T-cell cultures derived from healthy controls (HC) and atopic dermatitis patients (AD) by calculating the mean number of spots/well in the K562-CD1a-pulsed ELISpot wells and subtracting the mean number of spots/well in the K562-EV-pulsed ELISpot wells. The frequency of the CD1a-autoreactive T cell response was determined by calculating the mean number of spots/well in the K562-CD1a ELISpot wells and subtracting the mean number of spots/well in the K562-EV ELISpot wells.

For the Varicella zoster virus (VZV) T-cell responses, T-cells were washed and re-suspended in R5* and 1 vial ($10^{3.3}$ PFU/0.5ml) of Varilrix (live VZV vaccine, GlaxoSmithKline) was resuspended in 500μl R5*. 50μl of the reconstituted virus was incubated with 100,000 T-cells overnight. 50μl R5* was incubated with 100,000 T-cells overnight as a negative control. IFNγ and IL13 production were determined by ELISpot as above.

3.10 Cytokine ELISA

IFNγ cytokine levels were measured in supernatants post-CD1a plate bound assay by ELISA kit (Human Ready-Set-Go, eBioscience) following manufacturer's instructions. 96-well plates (Nunc Maxisorp) were coated with 100 µl /well coating antibody (purified anti-human IFNγ Ebioscience; diluted 1:250 in coating buffer) overnight at 4°C. After washing the plates 5 times with 0.05% PBS-Tween20, the plates were incubated for one hour at RT with 200 µl 1X ELISA diluent (Ebioscience). The plates were washed again 5 times with 0.05% PBS-Tween20 and standards

containing known cytokine levels, controls and samples were added in duplicates and incubated for 2 hours at room temperature. Plates were then washed 5 times and incubated for 1 h at room temperature with 100 µl /well biotinylated anti-human IFNγ antibody (Ebioscience, diluted 1:250). After 5 further washes with 0.05% PBS-Tween20, plates were incubated for a further 30 minutes at room temperature with 100 µl /well secondary antibody (streptavidin horseradish peroxidase (HRP) conjugated; diluted 1:250). Following a final wash step, 100 µl /well of substrate solution (3,3',5,5'-Tetramethylbenzidine TMB) was added, which was converted by antibody-bound enzyme to product a coloured product. The reaction was stopped using 50 µl /well sulphuric acid. The intensity of the colour corresponded to the cytokine content and was detected using a microtitre plate reader, read at 450nm (Bio-Rad iMark^{M} Microplate Reader, CA, USA)

3.11 CD1a plate bound assay

Purified CD1a protein and anti-CD11a antibodies (R&D Serotec) were added to Nunc plates at 10 µg/ml and 2.5 µg/ml respectively for 24 hours at 4^{o}C. Unbound CD1a protein was washed off with sterile PBS, and 200 µl citrate buffer pH 3.4 was added to all wells for 20 minutes at room temperature. Plates were then washed a further 3 times with sterile PBS and 100 µl HDM extract was added to appropriate wells. 100 µl 'negative control' (ALK-Abello) was added to CD1a-unpulsed wells. Plates were then incubated at room temperature for 48 hours. After washing 4 times with sterile PBS, 100µl 10 µg/ml anti-CD1a or isotype control antibody were added to appropriate wells for 1 hour at room temperature. Following a final 3 washes with sterile PBS, T cells were added to the wells at 100,000 cells/well. Plates were incubated overnight at 37°C and 5% CO2, and the following day supernatants were

recovered and IFNγ production was determined by IFNγ cytokine ELISA (Human Ready-Set-Go, eBioscience).

3.12 Quantitative real time polymerase chain reaction (qPCR)

T cells were added to Nunc plates prepared as per the CD1a plate bound assay above. Plates were incubated for 6-8 hours at 37°C and 5% CO2 and T cells were recovered by centrifugation. Following mRNA extraction using Qiagen Turbocapture mRNA kit (Qiagen, Manchester, UK) and reverse transcription (Life Technologies), qPCR was performed using the following Taqman gene expression assays (Applied Biosystems): *GAPDH* (Hs02758991_g1), *IFNG* (Hs00989291_m1), *IL13* (Hs00174379-m1) *IL10* (Hs00961622_m1), *IL17A* (Hs00174383_m1) and *IL22* (Hs01574154_m1). Reactions were performed in a 7500 fast thermal cycler (Applied Biosystems).

3.13 Secretory Phospholipase A2 activity

PLA2 activity in HDM and blister fluids was detected using a site specific substrate kit (Cayman Chemicals, MI, USA), according to manufacturer's instructions. In a flat-bottom 96-well plate, 10 µl (0.7 µg) HDM extract (ALK) or allergen of interest, or blister fluid plus 5 µl assay buffer (25 mM Tris-HCL, pH 7.5, with 10 mM $CaCl_2$, 100 mM KCl and 0.3 mM Triton X-100) and 10 µl 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), were incubated at room temperature with 200 µl substrate solution (1.66 mM diheptanoyl thio-PC). For inhibitor/filaggrin studies, HDM/blister fluid was

incubated with the appropriate concentration of manoalide (Enzo Life Sciences) or filaggrin (synthesised in Poland) for 30 minutes at 37^{o}C prior to plating out. For heat-inactivation, allergens were heat-inactivated using a heat-block for 4 hours at 95°C prior to plating out. Diheptanoyl Thio-PC has a thiol group attached at the sn-2 position, cleavage of which In the presence of PLA2 results in release of the thiol group, which reacts with DTNB to produce a coloured precipitate. This is measured with a spectrophotometer over time (415nm) to give a measure of PLA2 activity (Bio-Rad iMark™ Microplate Reader, CA, USA).

3.14 IFNγ secretion assay

K562-CD1a cells were pulsed with 7 µg/ml HDM extract (ALK) overnight prior to irradiation (5000 rad) and incubation with polyclonal *ex vivo* T cells (at a ratio of 0.5: 1 K562:T cells in 24 well plates) for 6 hours at 37°C in 5% CO_2. T cells alone were used as a negative control and T-cells stimulated with 1µg/ml Staphylococcal Enterotoxin B (Sigma-Aldrich) were used as a positive control. Cells were washed in 10 ml cold MACS buffer and centrifuged at 300xg for 10 minutes at 4-8°C before being resuspended in 80 µl cold R5* and being labelled with 20 µl anti-IFNγ mAb conjugated to anti-CD45 mAb (Miltenyi Biotec, Germany). The suspension was mixed well and incubated on ice for 5 minutes before the addition of 10 ml of warm R5* (37°C) and incubation for 45 minutes at 37°C with slow rotation of the tubes during this IFNγ-secretion period. Tubes were then put on ice and washed at 4°C with cold MACs buffer by being centrifuged at 300xg for 10 minutes at before being resuspended in 80 µl cold MACs buffer and the addition of 20 µl IFNγ Detection Antibody (PE conjugated anti-IFNγ mAb (Miltenyi Biotec)), together with CD3 and L_D antibodies and other antibodies of interest, and being incubated on ice for 10 minutes. Cells were then washed with 10 ml of cold MACS buffer and centrifuged at

300xg for 10 minutes at 4-8°C before being resuspended in R5* for FACS sorting. $CD3^{+}IFN\gamma^{+}$ cells after co-incubation with K562-CD1a cells pulsed with HDM were sorted at 1-10 cells per well in 96-well round bottomed plates on a MoFlo™ (Beckman Coulter, Fullerton, CA, USA) or Aria III (Becton Dickinson, USA) cell sorter. T cell lines were expanded with the rapid expansion method of feeders, described below.

3.15 Rapid expansion method (feeders)

FACs sorted T-cells were expanded using the rapid expansion method. T cells were plated out at 1 or 10 cells/well into T cell media in a round-bottomed 96-well plate and 50ng/ml anti-CD3 (OKT3; Ebioscience, CA, USA) antibody and irradiated (3000 rad) PBMCs from one donor and B-cells from an EBV transformed B-cell line at 150-200,000 cells/well and 40,000 cells/well respectively were added. T-cell lines were examined and fed regularly and transferred to flat-bottomed plates and split as necessary with T cell medium. On expansion, T cell lines were maintained at a density of approximately 0.5-1 million cells/ml. CD1a reactivity was determined by ELISpot at approximately 3 weeks.

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 30 minutes at $4^{o}C$ with live/dead stain (Invitrogen) and relevant antibodies. The list of antibodies and titrations used can be found in Chapter 3.1. Cells were then washed twice in FACs buffer and data was acquired using CyAn™ (DakoCytomation) and Fortessa (Becton Dickinson, USA)

flow cytometers and then analysed using FlowJo software (Tree star, Ashland, Oregon).

3.17 HDM lipid extraction

HDM lipids were extracted with chloroform, methanol and water using a modified Bligh-Dyer method (486). HDM extract was resuspended in a solution of (4:2:1) methanol:chloroform:sample and vortexed before being heated for 30 minutes at 37-40°C. 2 volumes of chloroform and 3 volumes of water were added and the sample was vortexed again and centrifuged at 2-3000 RPM for 5 minutes resulting in separation of the aqueous and organic phases. This process was repeated twice on the aqueous phase to achieve maximal yield. The organic phase was then aspirated, dried by dessication, weighed and dissolved in 0.5% PBS-Tween. The aqueous phase was aspirated, centrifuged at 3000 RPM for 5 minutes and protein-enriched precipitates were resuspended in sterile PBS and concentration determined by Nanodrop.

3.18 Viral cell lines

HLA-A*0201-restricted GILGFVFTL (influenza A matrix), NLVPMVATV (CMV pp65) and GLCTLVAML (EBV BMLF1)-specific T cells were generated by Dr. Mariolina Salio as previously described (487). Briefly, 3×10^6 to 5×10^6 PBMCs were pulsed as a pellet for 1 h at 37 °C with 100 μM of individual peptides containing the following T cell epitope regions: GILGFVFTL, NLVPMVATV and GLCTLVAML. Cells were cultured in R10 at 2×10^6 cells per well in a 24-well Costar plate. IL-2 was added to a final concentration of 100 U/ml on day 3. The cultures were restimulated after 14 d

using HLA-A*0201-positive BCLs pulsed with the appropriate peptides. For the functional assays, cells were pulsed with 200 ng/ml peptide and incubated with 10 μM manoalide, 0.5 μg/mL filaggrin monomers or 0.5 μg/mL Sumo recombinant protein control before IFNγ ELISpot as above. ELISpots were performed using a ratio of 40,000: 5,000 APC cells (JY) : T-cells.

3.19 Filaggrin synthesis

Filaggrin was synthesised by Ewa Podobas and Andrzej Dziembowski (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw). The expression plasmid was constructed using a sequence- and ligation-independent cloning (SLIC) method (488). Nucleotide sequence encoding the 7th filaggrin repeat domain was cloned into pET28 vector using BamHI and XhoI restrictions sites. The construct contains N-terminal His6-tagged SUMO protein sequence which can be enzymatically cleaved by SUMO protease. The construct was transformed into E. coli BL21-CodonPlus-RIL and propagated overnight in LB liquid media containing kanamycin (50 μg/ml) and chloramphenicol (37.5 μg/ml) at 37°C. The bacterial cultures were diluted 1:50 in autoinduction media (Formedium AIM- Super Broth) supplemented with kanamycin and chloramphenicol and incubated for 48 h at 18°C with shaking. The cells were harvested by centrifugation (10 min, 5000 × g, 4°C). The bacteria pellet was mixed with lysis buffer (10 mM Tris pH 8, 150 mM NaCl, 10 mM imidazole) supplemented with protease inhibitors cocktail and lysed by sonication. The cell lysate was clarified by centrifugation (45 min, 70000 × g, 4°C). The following described purification procedure was performed on an ÄKTA™xpress chromatography system. The supernatant after centrifugation was loaded on Ni–NTA Agarose column (Qiagen). Unbound material was washed from the column with lysis

buffer. Enriched proteins were subjected to on-column cleave by SUMO protease (20 μg protease for 5 ml resin) in elution buffer (10 mM Tris pH 8, 150 mM NaCl, 300 mM imidazole) for 8 h at 10°C. Realised protein was further purified by combinations of desalting and Ni-NTA Agarose columns equilibrated with the lysis buffer. Flow though fractions containing the purified protein were collected and automatically loaded into a pre-equilibrated column with buffer (10mM Tris pH 8, 150mM NaCl) Superdex 200 column (GE Healthcare). Fractions containing the purified filaggrin were collected and analyzed by SDS-PAGE. Identical purification from cells without the expression plasmid were processed in parallel as a control. In both cases the same fractions from gel filtration column were collected. Experiments were performed in parallel using control SUMO protein.

3.20 Filaggrin genotyping

Filaggrin genotyping was conducted by Padraic Fallon (Trinity Biomedical Sciences Institute, Trinity College, Dublin, Ireland). The *FLG* mutations R501X and 2282del4 were genotyped using Taqman allelic discrimination assays (Life Technologies), as previously described(208). R501X was screened using forward primer 5' CAC TGG AGG AAG ACA AGG ATC G 3', reverse primer 5' CCC TCT TGG GAC GCT GAA 3' and the probes VIC-CAC GAG ACA GCT C and 6-FAM-CAT GAG ACA GCT CC. 2282del4 was screened using forward primer 5' CCA CTG ACA GTG AGG GAC ATT CA 3', reverse primer 5' GGT GGC TCT GCT GAT GGT GA 3' and the probes 6-FAM- CAC AGT CAG TGT CAG GCC ATG GAC A and VIC-AGA CAC ACA GTG TCA GGC CAT GGA CA alleles. Assays were performed in 384-well plates with each reaction comprising of 20ng DNA, 2.5 μl Universal PCR master mix and 0.125 μl 40X assay mix in a final reaction volume of 6 μl. Assays were run on an Applied Biosystems 7900HT Fast Real-Time PCR system under the following conditions: 1

cycle at 50°C for 2 minutes followed by 1 cycle at 95°C for 10 minutes then 40 cycles of 95°C 15 sec; 60°C 1 minute. Samples heterozygous for the 2282del4 mutation were also confirmed by Sanger sequencing using the published primers RPT1P7 (5' – AAT AGG TCT GGA CAC TCA GGT - 3') and RPT2P1 (5' – GGG AGG ACT CAG ACT GTT T - 3') (489). PCR conditions were 94°C for 5 min; 35 cycles of 94°C for 40 s, 57°C for 1 min, 72°C for 2 min; final extension step at 72°C for 7 min. PCR clean up and sequencing was performed by Source Bioscience plc.

3.21 Statistics

Statistical analyses were performed using GraphPad Prism 6. Cohorts of healthy donors and atopic dermatitis patients investigated for HDM responsive CD1a-reactive T cell responses were analysed using the one-tailed unpaired and paired t-tests, Chi-squared test and Pearson correlation. All other T cell responses and data were analysed using the one-tailed unpaired t-test.

4 Identification of HDM responsive CD1a-reactive T cells in the peripheral blood

Introduction and aims

Antigen recognition via their T cell receptors is essential for T cells to mount a successful immune response.

As discussed in the introduction, conventional MHC-restricted T cells recognise peptide antigens which are processed and presented on the cell surface by highly polymorphic MHC molecules. Peptide-MHC co-recognition is one of the most important immunological discoveries to date, and it was believed for decades that T cells exclusively recognised peptide antigens within this system, such that almost all medical approaches aiming to influence and measure T cell responses in the context of allergy, infection, autoimmune disease and immunisation are based on this fundamental principle (1).

However, it is increasingly becoming recognised that unconventional populations of T cells additionally make up an important constituent of the overall T cell repertoire, including CD1-restricted T cells, which recognise lipid antigens in the context of non-polymorphic CD1 molecules.

CD1 molecules are highly conserved in evolution (490), and are expressed at high levels by peripheral professional antigen presenting cells, such as myeloid dendritic cells and Langerhans cells (491). These molecules are known to bind and present

many different lipid species (492) with over twenty different stimulatory T cell lipid ligands having been identified to date (493). Crystallography has established the molecular basis of T cell lipid recognition, demonstrating that the alkyl chains of bound lipid ligands bind within the hydrophobic antigen binding grooves of CD1 molecules, and protrusion of the lipid polar moiety for TCR recognition (7, 9, 494). Based on sequence homology, pattern of expression and functional properties, the CD1 family is divided into five isoforms- Group 1 consisting of CD1a, CD1b and CD1c, Group 2 of CD1d and Group 3 of CD1e. Each CD1 molecule differs in it's intracellular trafficking and tissue expression, suggesting distinct physiological roles (490).

However, whilst the ability of T cells to recognise lipid antigens presented by CD1 molecules is well established, our understanding of the role of these cells in disease remains in it's infancy. The majority of the published data focuses on group 2 CD1d-restricted iNKT cells, with most of the studies on group 1 CD1-restricted T cells being restricted to reports on a limited number of T cell clones, due to a lack of specific markers for these cells and the absence of group 1 CD1 expression in mice. During *in vitro* culture, The phenotype and function of cell clones changes with time and therefore these cells may not be physiologically representative of *in vivo* T cell populations, thus studies on *ex vivo* polyclonal T cells are additionally important to further our understanding of the function of this class of unconventional T cells as a whole.

CD1a is an MHC Class I-like antigen presenting molecule that is highly expressed by Langerhans cells of the epidermis in addition to a subset of dermal dendritic cells, and subsets of dendritic cell populations at other surface sites (110-114), and interest in the patho-physiological role of CD1a-restricted T cells has been sparked by the recent identification of CD1a-restricted T cells as a normal component of the T cell repertoire (118, 119). CD1a presents self (11, 97), and foreign (54) lipids to T cells,

and CD1a expressing Langerhans cells of the epidermis are well situated to detect skin barrier compromise through the presentation of CD1a ligands to CD1a-reactive T cells. Indeed lesional cutaneous atopic tissue has been shown to have an altered lipid profile (495-499) that may influence CD1a-mediated T cell activation, and furthermore $CD1a^+$ cells are enriched within AD lesions (500). Recent studies have reported that CD1a-reactive T cells are present in the blood at high frequencies, express skin homing markers and can infiltrate human skin and recognise natural skin lipids (501-503). These cells produce cytokines that cause skin disease, such as IFN-γ, IL-13 and IL-22 (501), yet a defined role for CD1a-restricted T cells in human skin disease remains to be established.

We therefore postulated that CD1a-restricted T-cells might contribute to AD pathogenesis, and sought to test the hypothesis that CD1a-reactive T cells contribute to the response to HDM in AD.

The key aims of this chapter were to identify and quantify HDM responsive CD1a-reactive T cells in the peripheral blood of healthy controls and individuals with AD, to characterise their functional phenotype as determined by cytokine secretion, and to study the development of these cells by examining responses in cord blood samples. Studies used APCs with absent surface MHC expression (K562 cells), which had been engineered to express high levels of CD1a molecules (K562-CD1a), and were kindly donated by Branch Moody (Harvard). This system bypasses MHC alloreactivity, thereby allowing parallel testing of many unrelated donors under equivalent conditions (119). HDM responses were examined in clinically relevant dose ranges, and anti-CD1a antibody was used to confirm CD1a specificity of the HDM responsive CD1a-reactive T cell response. Polyclonal T cell responses to other CD1 isoforms were also investigated, and autologous *in-vitro* derived APCs, and an additional HDM source were also used to examine CD1a reactivity. The dynamics of the HDM responsive CD1a-reactive T cell activity was studied, and

experiments were conducted to examine responses in the skin homing fraction of peripheral blood.

4.1 Recognition of HDM extract by *ex-vivo* CD1a-reactive T cells in clinically relevant dose ranges

To investigate if *ex-vivo* CD1a-reactive T cells from healthy individuals and AD patients were able to recognise HDM, PBMC-derived polyclonal T cells were isolated by MACs bead separation (Figure 4.1).

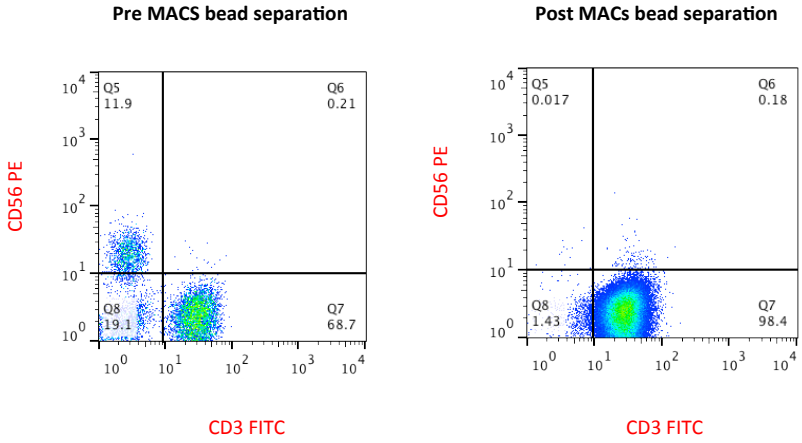


Figure 4.1. Human blood T cell isolation. T cells were isolated by MACS bead separation from PBMCs. Representative FACS plots are shown pre- and post- T-cell isolation. Plots were gated on live cells and singlets.

Ex-vivo polyclonal T cells were then incubated with K562 target cells transfected with CD1a molecules (K562-CD1a) in the presence or absence of whole HDM extract purchased from ALK. K562 cells are HLA^{low} and thereby largely bypass alloreactivity and allow parallel testing with T cells from many unrelated donors under equivalent conditions.

Both type 1 and type 2 cytokines have been shown to play a role in the pathogenesis of AD, and therefore both IFNγ and IL-13 responses were examined (312, 504-506).

As seen previously (55, 119, 469), a background response to K562 cells at a rate of ~1 in 10,000 was observed. Above this background the autoreactive response can be seen as an increased IFNγ response to K562-CD1a cells, which is thought to reflect the stimulation of CD1a autoreactive T cells by endogenous K562 ligands (Figure 4.2A, donor AD9500). Furthermore, there was significantly increased T cell activation against K562-CD1a target cells pulsed overnight with HDM extract. Importantly, no response above background was seen against K562-vector control cells pulsed with HDM, demonstrating that the response to HDM is CD1a-mediated.

CD1a-dependent GM-CSF and IL-13 production on stimulation with HDM was also observed by polyclonal T cells from the same donor (Figure 4.2B).

The CD1a-restricted T cell response to HDM titrated to 0.07μg/ml HDM extract (Figure 4.2C), equivalent to approximately 0.3% of the doses administered *in vivo* during maintenance immunotherapy (up to 21μg) (507).

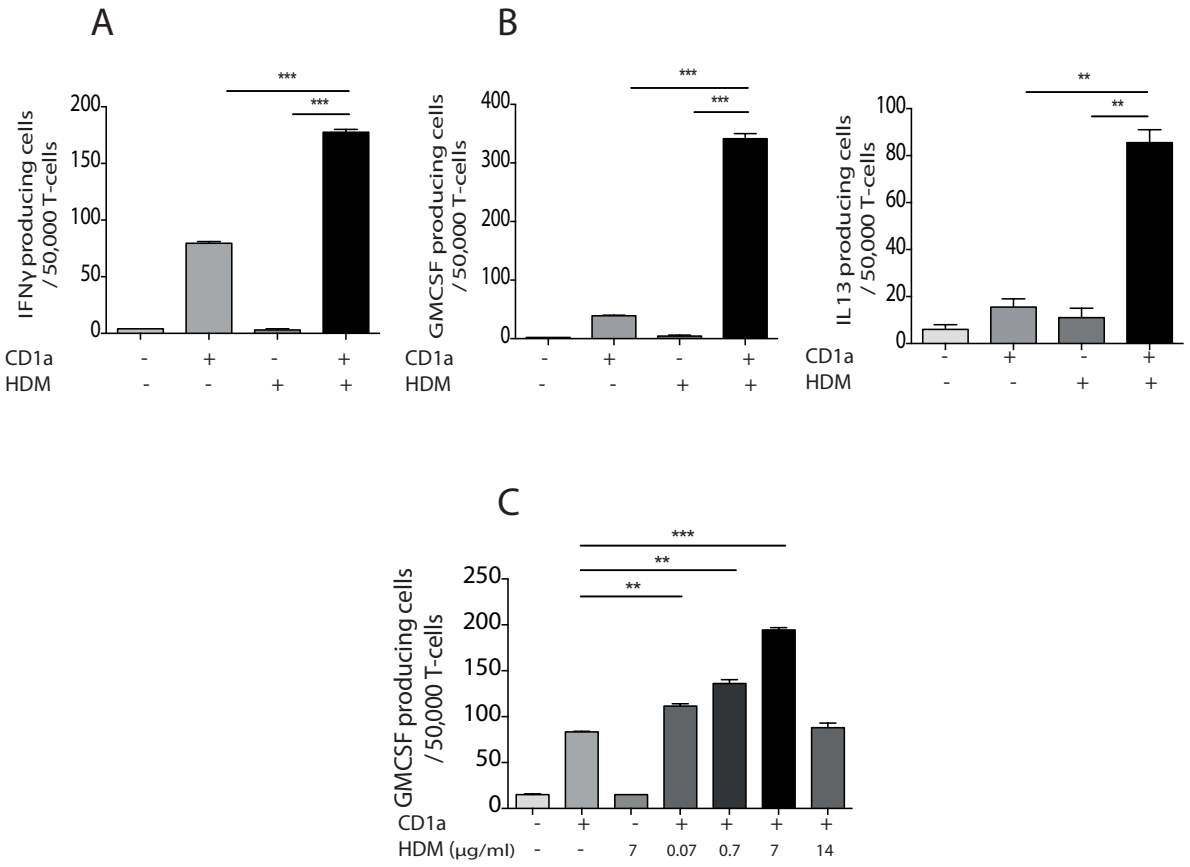


Figure 4.2. Circulating CD1a-reactive HDM-responsive T cells produce IFNγ, GM-CSF and IL-13. T cells were isolated by CD3 MACS beads from AD donor PBMC (AD9500) and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract. IFN-γ (A), GM-CSF (B, left) and IL-13 (B, right) production was measured by ELISpot. The titration of the GM-CSF response to decreasing HDM concentrations is also shown (C). Data representative of at least three donors for each experiment are shown. Bars represent standard error. ** P<0.01; ***P<0.001.

4.2 The *ex-vivo* autoreactive and HDM responsive CD1a-reactive T cell response demonstrates CD1a specificity

4.2(i) The *ex-vivo* autoreactive and HDM responsive CD1a-reactive T cell response is inhibited by anti-CD1a antibody

In order to assess the CD1a specificity of the autoreactive and HDM-reactive T cell response, IFNγ, GM-CSF and IL-13 ELISpot with HDM-pulsed K562-CD1a cells incubated with anti-CD1a antibody or isotype control were performed (Figure 4.3). The response was inhibited by an anti-CD1a antibody but not by isotype control, confirming CD1a dependence (Figure 4.3).

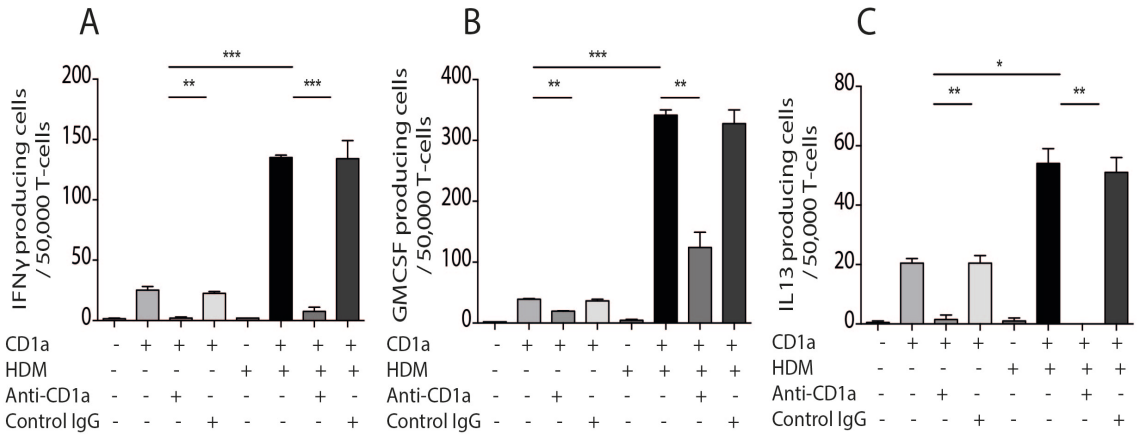
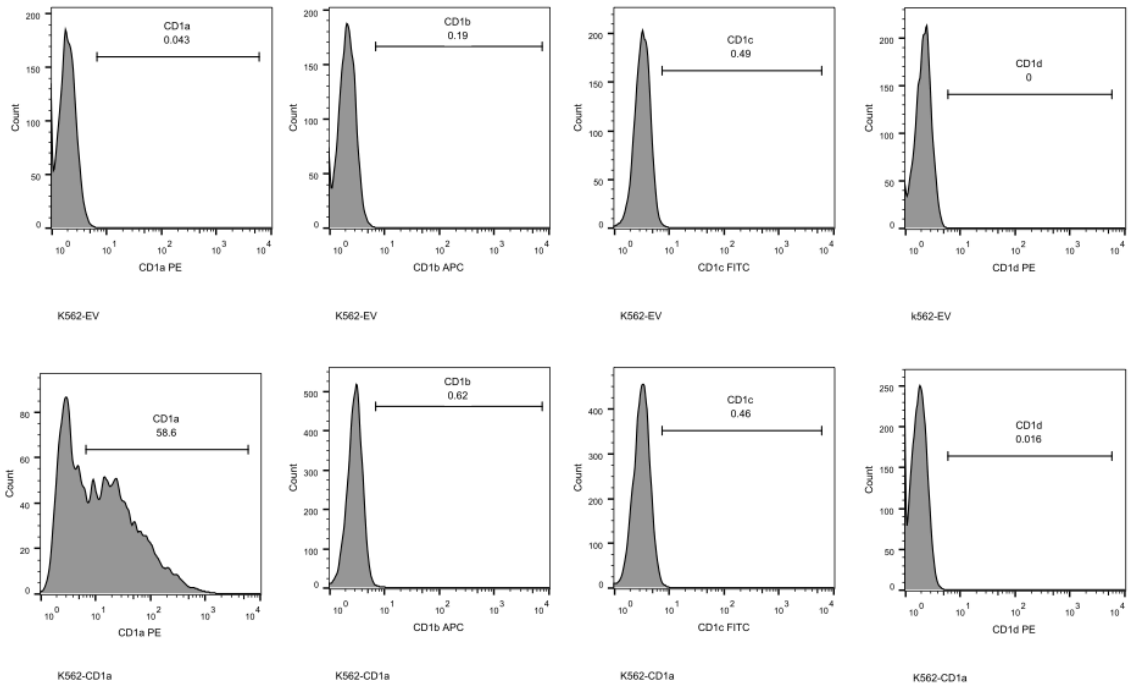


Figure 4.3. The *ex-vivo* autoreactive and HDM-reactive T cell response is abrogated by anti-CD1a antibody. T cells were isolated by CD3 MACS beads from donor PBMCs (AD9500) and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract. IFN-γ (A), GM-CSF (B, left) and IL-13 (C) production was measured by ELISpot in the presence or

absence of anti-CD1a antibody and isotype control. Data representative of at least three donors for each experiment are shown. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001.

4.2(ii) *Ex-vivo* polyclonal T cells respond at highest levels to K562 cells expressing CD1a

To examine whether polyclonal T cells derived from the blood additionally exhibited responses to other CD1 isoforms, IFNγ, ELISpots with HDM-pulsed K562-CD1a, K562-CD1b, K562-CD1c and K562-CD1d cells were performed. K562-CD1a, -CD1b, -CD1c and –CD1d expression was confirmed using flow cytometry (Figure 4.4).



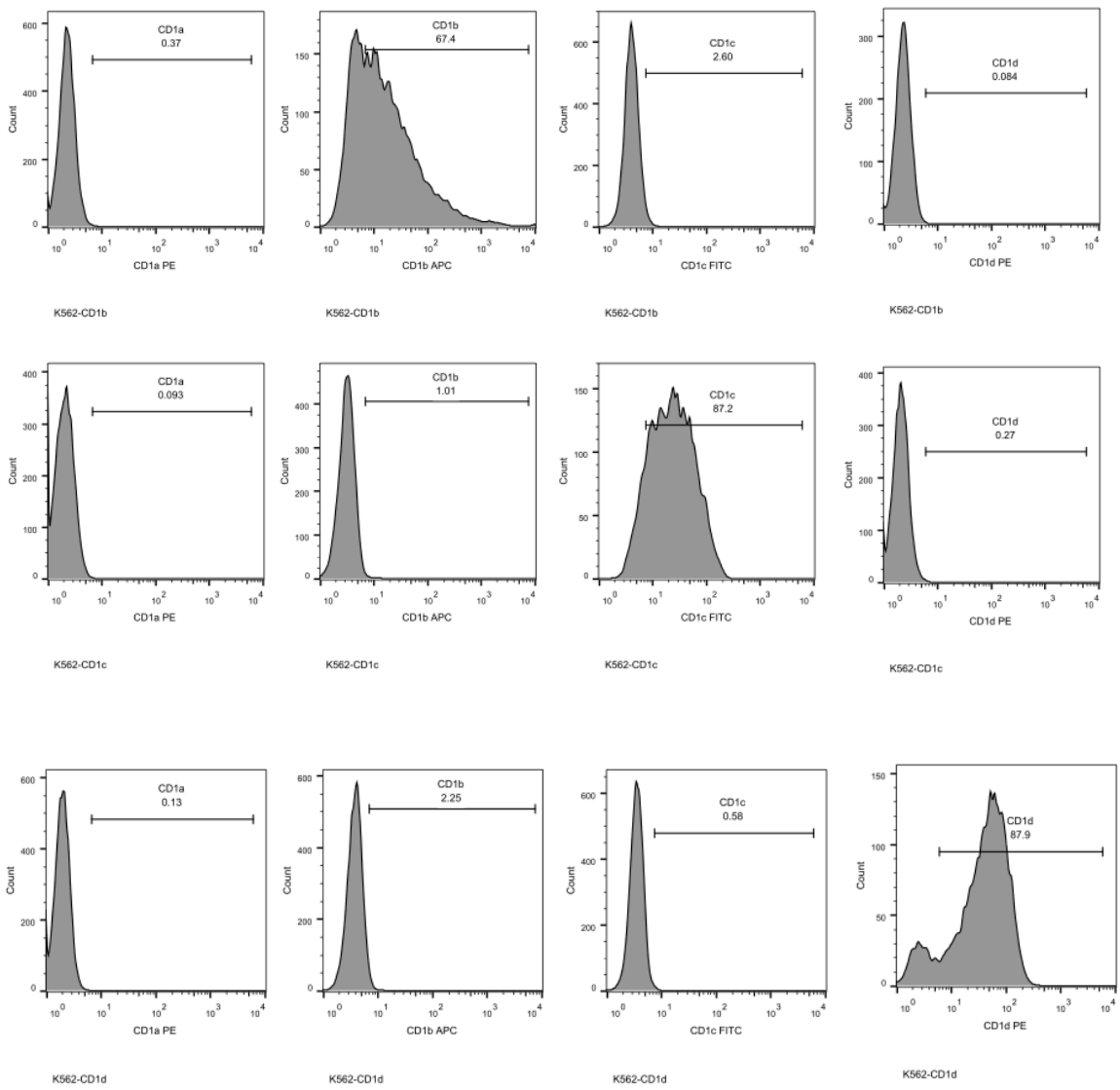


Figure 4.4. K562-CD1a, -CD1b, -CD1c and CD1d expression. Expression of CD1a, CD1b, CD1c and CD1d molecules was examined by flow cytometry in K562-EV cells (top panel), K562-CD1a cells (second panel), K562-CD1b cells (third panel), K562-CD1c cells (forth panel) and K562-CD1d cells (bottom panel). Plots gated on live cells and singlets.

As previously reported (119), polyclonal T cells were found to respond at the highest rates to unpulsed K562 cells expressing CD1a compared to the other CD1 isoforms (Figure 4.5A, B). The HDM-responsive T cell reactivity was also predominantly

directed at K562-CD1a cells, with marginal responses to CD1c and CD1d observed in some donors (4.B).

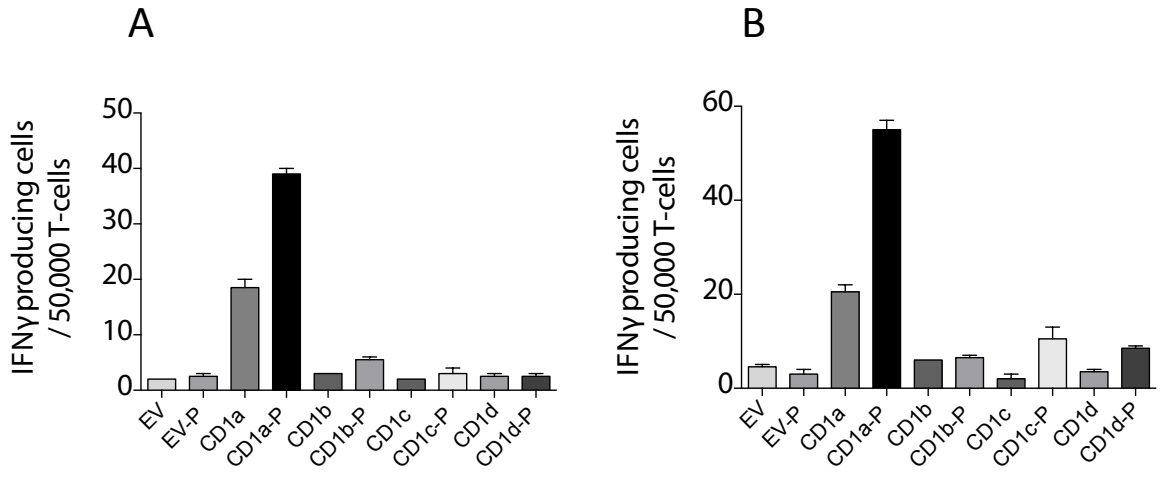


Figure 4.5. E-vivo polyclonal T cells respond at highest rates to K562-CD1a. T cells were isolated by CD3 MACS beads from donor PBMCs (A – donor 1, B – donor 2) and incubated overnight with EV-, CD1a-, CD1b, CD1c- and CD1d-transfected K562 cells pulsed with HDM extract. IFN-γ production was measured by ELISpot. Data representative of at least three donors for each experiment are shown. Bars represent standard error.

4.3 Polyclonal T cells respond in a CD1a dependent manner to primary autologous in-vitro derived CD1a positive antigen presenting cells

K562-CD1a cells are transfected APC that have the advantage of being able to be used with any donor. However these cells might not mimic CD1a mediated

presentation by native CD1a^{+} APCs including myeloid dendritic cells and Langerhans cells (LC).

To investigate whether these cells additionally mediate the CD1a-reactive HDM response, monocytes were therefore differentiated with GM-CSF and IL-4 to produce monocyte-derived myeloid dendritic cells (mDCs) and also activated *in vitro* with cytokines to mimic LCs (LC-like cells) (508-511).

Flow cytometry analysis demonstrated satisfactory differentiation, with high CD1a expression in mDCs, and high CD1a and Langerin expression in LC-like cells (Figure 4.6).

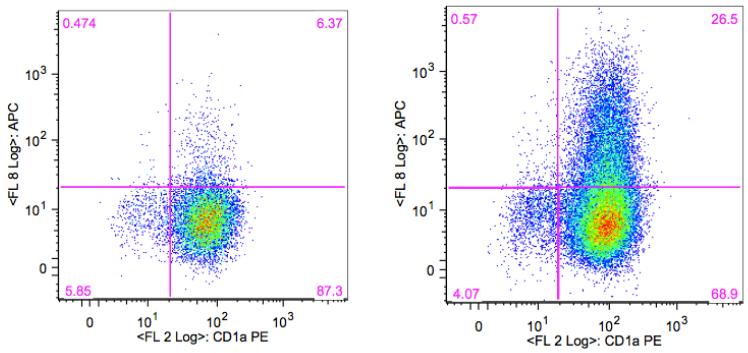


Figure 4.6. CD1a and Langerin expression by mDCs and LC-like cells. Monocytes were differentiated into mDCs (left) and LC-like cells (right) as described. Representative FACS plots of CD1a and Langerin expression, with CD1a PE (x axis) and Langerin APC (y axis). Representative FACS plots; gated on live cells and singlets.

Pulsing overnight with HDM did not affect mDC or LC-like cell maturation (Figure 4.7), excluding contamination of the extract with TLR ligands and other PAMPs, which may lead to T cell activation.

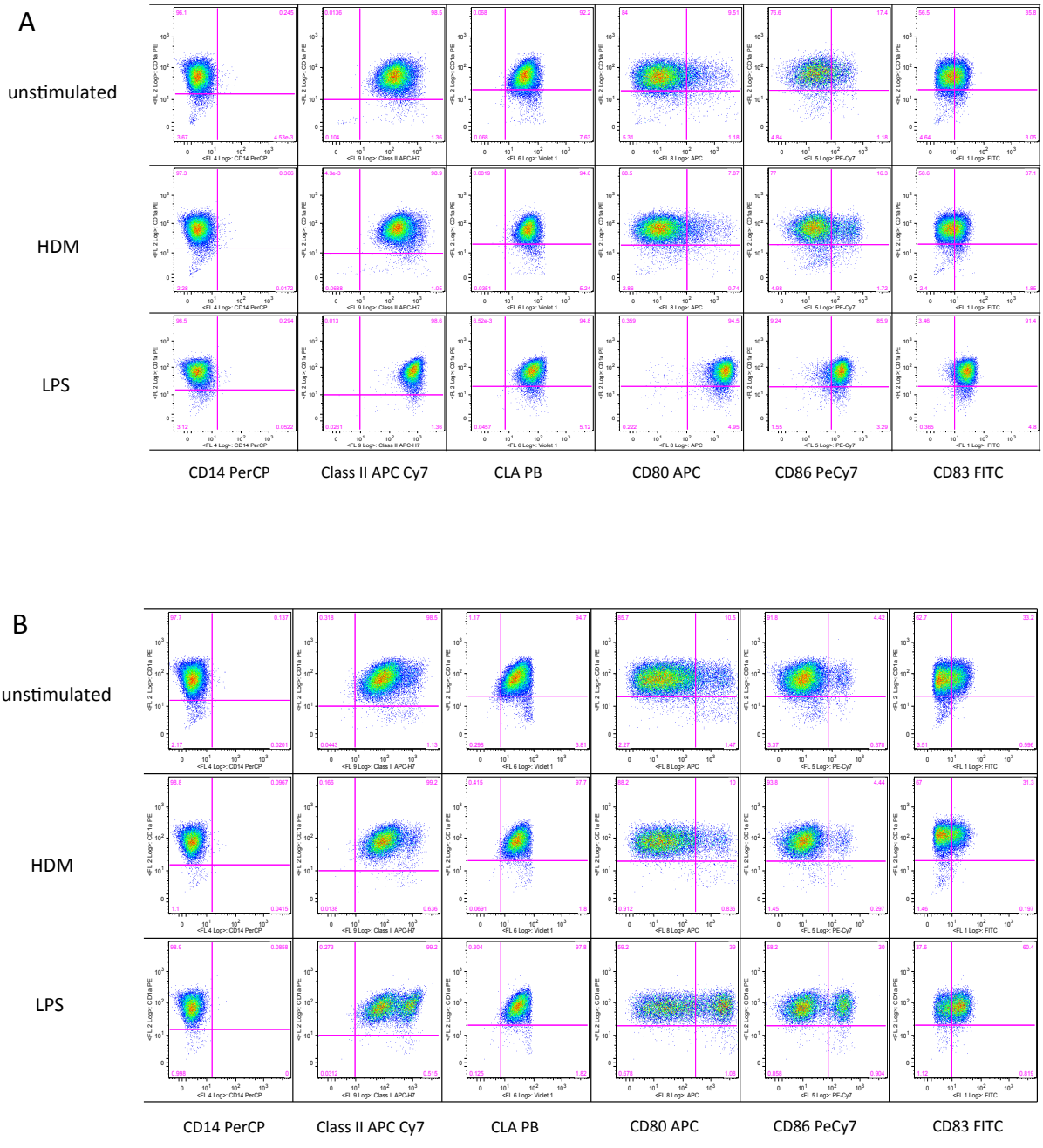


Figure 4.7. Expression of key surface markers on mDC and LC-like cells is not affected by HDM treatment. mDC (A) and LC-like cells (B), generated from CD14+

cells isolated by MACS bead separation were pulsed overnight with HDM. Cells were washed and stained for flow cytometry analysis. CD1a PE on y axis. Representative FACS plots; gated on live cells and singlets.

Similar to prior studies that compared K562 cells, mDCs and LC-like cells side by side, we found that both mDCs and LC-like cells mediated the HDM response of polyclonal autologous T cells in a CD1a- restricted manner (Figure 4.8).

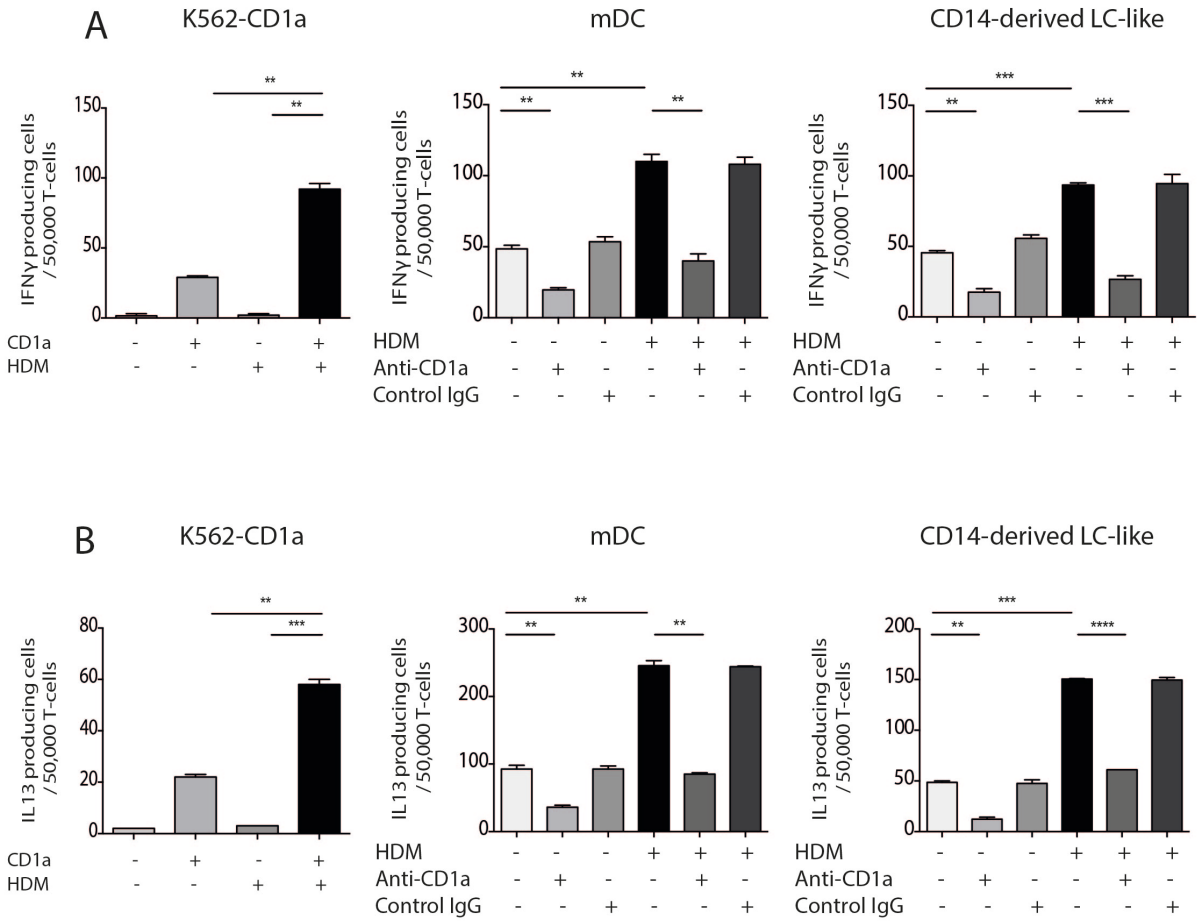


Figure 4.8. Peripheral T cells also respond in a CD1a-restricted manner to mDCs and LC-like cells. CD1a-expressing K562 (left), *in vitro* derived mDC (middle) or LC-like cells (right) were pulsed with HDM extract overnight and incubated with autologous peripheral blood T cells from donor AD9501A. IFNγ (A,

top panel) and IL- 13 (B, bottom panel) production was measured by ELISpot in the presence or absence of anti-CD1a antibody or isotype control. Data representative of at least three donors for each experiment. Bars represent standard error. ** P<0.01; ***P<0.001;****P<0.0001.

In addition to using monocyte derived LC-like cells, Langerhans cells were differentiated from cord-blood derived CD34+ progenitor cells as described (405) to provide an alternate system of primary CD1a+ cell generation. Flow cytometry demonstrated good CD1a and Langerin expression (Figure 4.9).

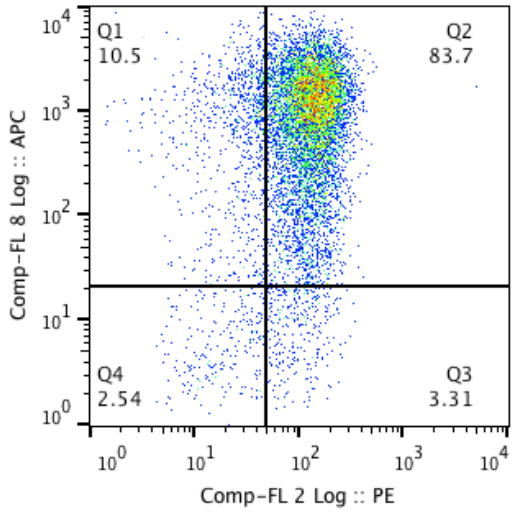


Figure 4.9. CD1a and Langerin expression by cord blood derived LCs. Langerhans cells were differentiated from cord-blood derived CD34+ progenitor cells as described. Representative FACS plot of CD1a and Langerin expression, with CD1a PE (x axis) and Langerin APC (y axis), after gating on live cells and singlets.

These cells additionally mediated CD1a and HDM dependent responses of *ex-vivo* polyclonal T cells (Figure 4.10A, B).

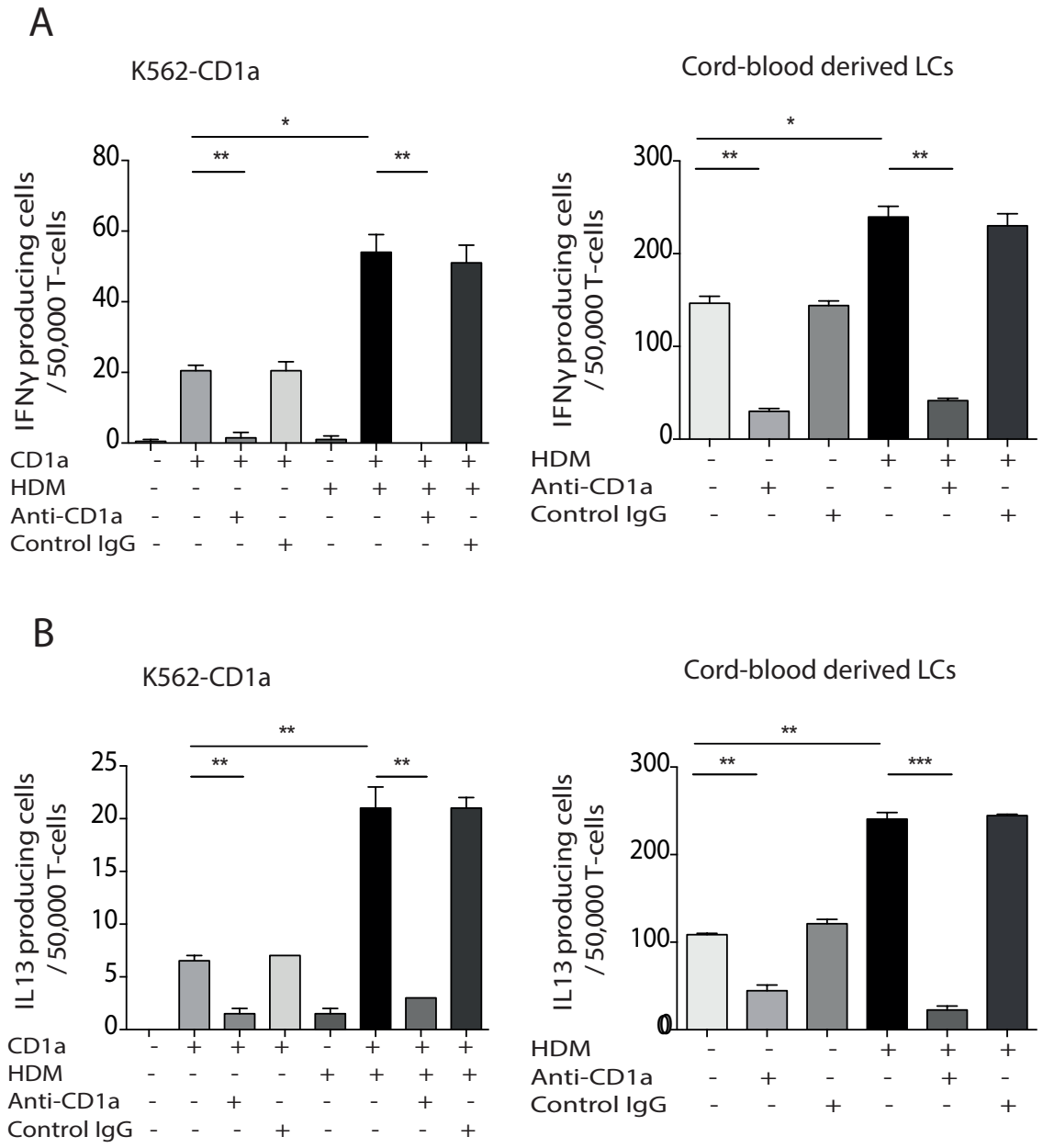


Figure 4.10. Peripheral blood T cells also respond in a CD1a-restricted manner to cord blood derived LCs. CD1a-expressing K562 (left), or *in vitro* cord blood derived LCs (right) were pulsed with HDM extract overnight and incubated with peripheral blood T cells from donor AD229 in the presence of HLA Class I and II blocking antibodies. IFNγ (A, top panel) and IL- 13 (B, bottom panel) production was measured by ELISpot in the presence or absence of anti-CD1a antibody or isotype

control. Data representative of at least three donors for each experiment. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001

Overall, these data show that HDM responsive CD1a-reactive T cells exist in the peripheral blood of healthy individuals and patients with AD at high frequencies and produce IFNγ, GM-CSF and IL-13 in response to HDM challenge at clinically relevant doses.

4.4 HDM responsive CD1a-reactive T cells are enriched in the blood of AD patients

Responses to HDM in a larger cohort of healthy adult donors and individuals with atopic dermatitis were subsequently examined, and compared to those in the cord blood of neonates, which represent a control for naïve polyclonal T cells.

Significantly higher CD1a-reactive *ex vivo* T cell IFNγ responses to HDM in individuals with atopic dermatitis compared to healthy non-atopic controls were observed. However, there were no differences in the autoreactive CD1a-restricted responses between AD patients and controls (Figure 4.11A).

The frequencies of cytokine producing cells were also significantly higher in adult donors than in cord blood samples. These data are consistent with an acquired antigen-driven expansion of HDM responsive CD1a-reactive T cells with age (Figure 4.11A, B), in agreement with previous published reports (512).

In addition, the production of IL-13 by CD1a-reactive T cells was also significantly elevated *ex vivo* in atopic dermatitis patients, confirming type 1 and type 2 cytokine induction (Figure 4.11B).

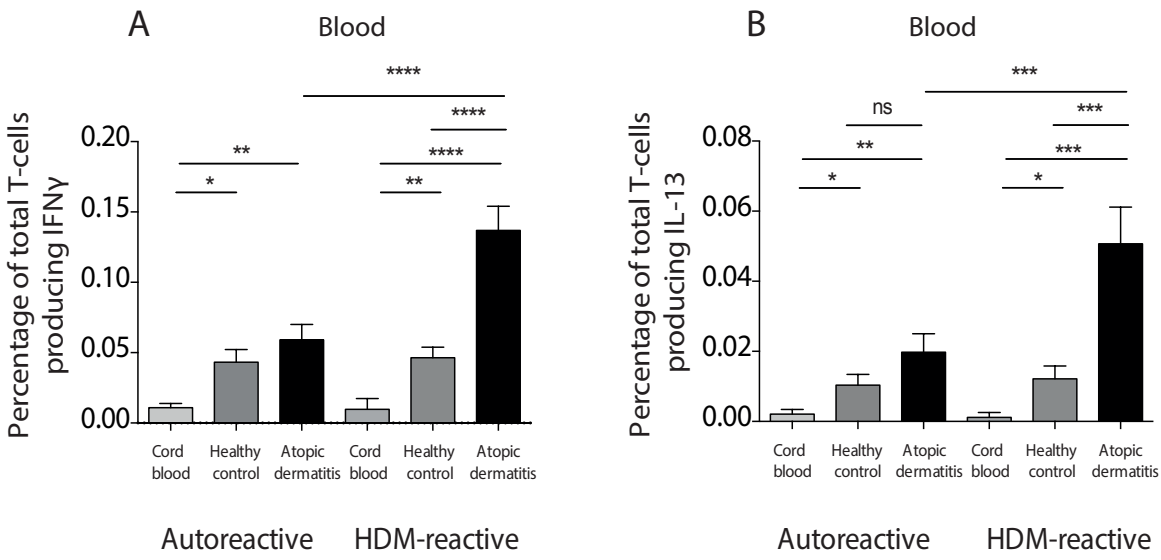


Figure 4.11. HDM-responsive CD1a-reactive T cells are enriched in blood of AD patients. (A, B) T cells derived from the peripheral blood of healthy controls (HC), patients with atopic dermatitis (AD) or from cord blood were incubated with CD1a-transfected K562 or untransfected K562 cells in the presence or absence of HDM extract. IFNγ (A) and IL-13 (B) production was measured by ELISpot and expressed as percentage of responding T cells (A n=30 HC, 24 AD, 10 cord blood; B n=20 HC, 17 AD, 10 cord blood). Auto-reactive is the response to CD1a-K562 in the absence of HDM. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001;****P<0.0001.

Using dual-colour FluoroSpot, we showed while the majority of the type 1 and type 2 cytokine-producing cells are unique subsets, a mean of 7% (range 6-12%) of CD1a-reactive HDM-responsive T cells could produce both IFNγ and IL-13 (Figure 4.12).

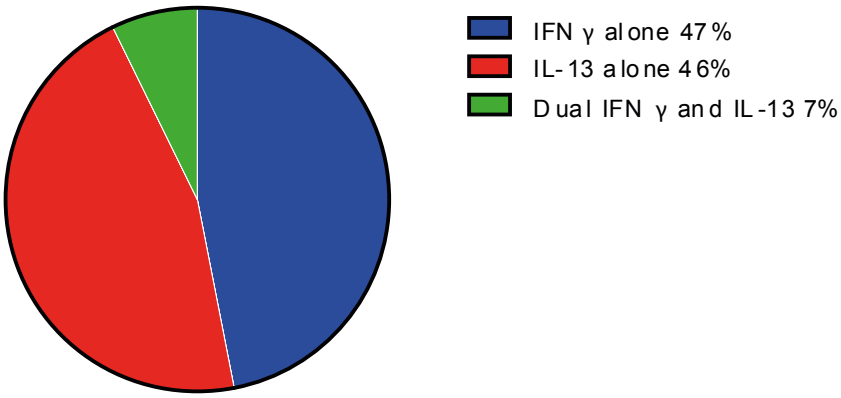


Figure 4.12. Dual–colour IFNγ and IL-13 production by HDM-responsive CD1a-reactive T cells. Dual–colour IFNγ and IL-13 production was measured by FluoroSpot to determine type 1 and type 2 cytokine producing subsets. n=4

As a further control, a cohort of patients with psoriasis, a non-atopic skin disease were included. There was no increase in the IFNγ or IL-13 producing HDM responsive CD1a-reactive T cells in patients with psoriasis, suggesting that this enrichment is specific to AD patients (Figure 4.13A, B).

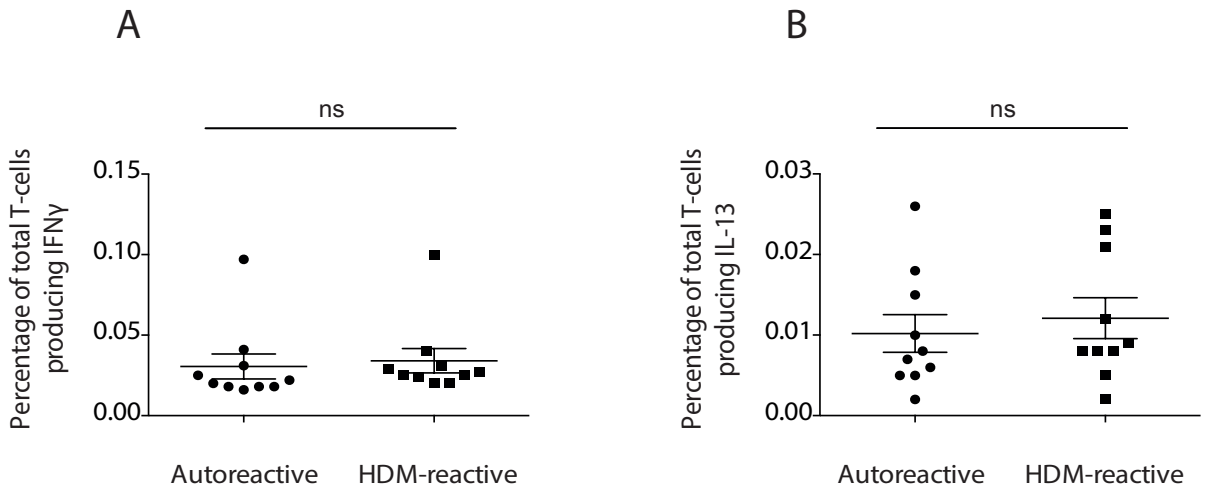


Figure 4.13. HDM responsive CD1a-reactive T cells are not enriched in patients with psoriasis. T cells were isolated by CD3 MACS beads from healthy donor or psoriasis PBMC and incubated overnight with CD1a-transfected K562 or untransfected K562 cells pulsed with HDM total extract. IFNγ (A) and IL-13 (B) production was measured by ELISpot. n=10.

Given the association between filaggrin null mutations and moderate-severe atopic dermatitis, AD patients and controls were genotyped for the two commonest mutations found in Europeans (2282del4 and R501X). Frequencies of HDM responsive CD1a-restricted T cells were significantly increased in those individuals with filaggrin null mutations compared to those with wild-type filaggrin (contingency chi-squared 4.38, P<0.05, Figure 4.14).

Genotype	Frequency of HDM-responsive CD1a-reactive T cells <0.2% of total T cells	Frequency of HDM-responsive CD1a-reactive T cells >0.2% of total T cells
Wild-type *FLG*	33	2
Heterozygous *FLG* mutations	4	2

Figure 4.14. Frequency of *flg* mutations compared to the presence of elevated HDM-responsive CD1a-reactive T cells. The number in each cell refers to the number of patients per group. Contingency chi-squared 4.38, P<0.05, two-sided.

Furthermore, the percentage of IL-13 producing HDM responsive CD1a-reactive T cells significantly correlated with disease severity (r^2=0.445, P<0.001) and there was a significant correlation between fold increase in HDM-responsive T cells and total IgE (r^2=0.588, P<0.05) and HDM-specific IgE (r^2=0.502, P<0.05).

Overall, these data showed that HDM responsive CD1a-reactive T cells increase in frequency in adults, show effector function, and are enriched in the circulation of patients with atopic dermatitis.

4.5 Dynamics of the *ex-vivo* HDM responsive CD1a-reactive T cell response

To examine the dynamics of the *ex-vivo* HDM responsive CD1a-reactive T cell response within donors, *ex-vivo* IFNγ ELISpots examining CD1a reactivity to HDM were performed on T cells form the same donor on three separate occasions, over a time frame of 20 months. The *ex-vivo* HDM-responsive CD1a-reactive T cell response was found to be preserved over time, and reproducible within patients (Figure 4.15 A, B, C).

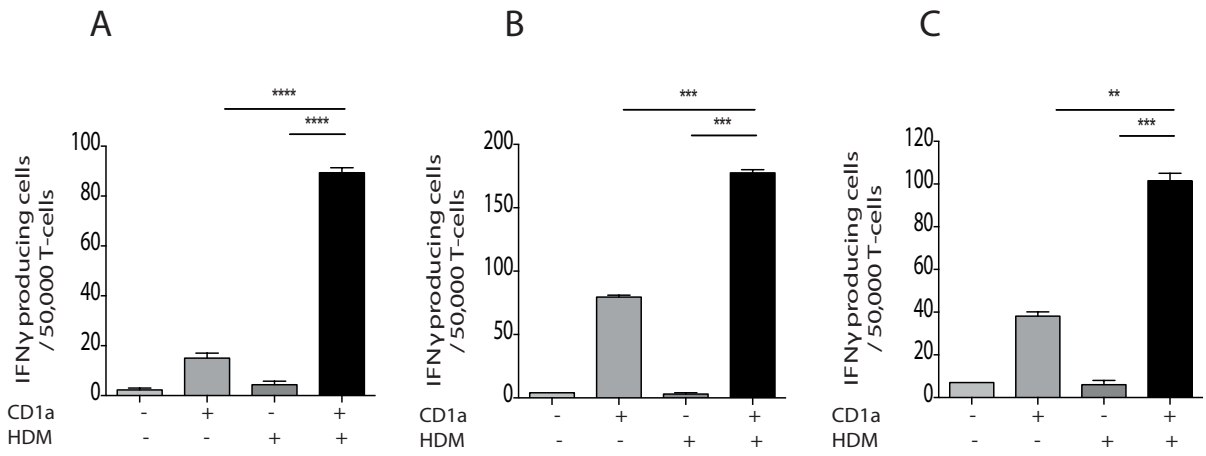


Figure 4.15. The *ex-vivo* HDM-responsive CD1a-reactive T cell response is reproducible within patients. Reproducibility of the *ex-vivo* HDM-responsive CD1a-reactive T cell response was examined by performing repeat IFNγ ELISpots for CD1a reactivity on the same donor on three separate occasions. A - April 2013,

B - August 2013 and C - December 2014. Bars represent standard error. Data are representative of three experiments. ** P<0.01; ***P<0.001;****P<0.0001.

4.6 Polyclonal T cells respond to HDM derived from an additional source

To validate the specificity of the CD1a restricted HDM response using an alternative source of allergen, IFNγ and IL13 ELISpots were repeated using HDM extract purchased from Greer.

Significant IFNγ and IL13 HDM-responsive CD1a-reactive responses to both the ALK and Greer HDM extract were observed in a dose dependent manner (Figure 4.16A, B, C, D). Similarly to the ALK HDM allergen, these Greer HDM responses could be abrogated by anti-CD1a antibody but not by isotype control (Figure 4.17A, B), confirming CD1a dependence.

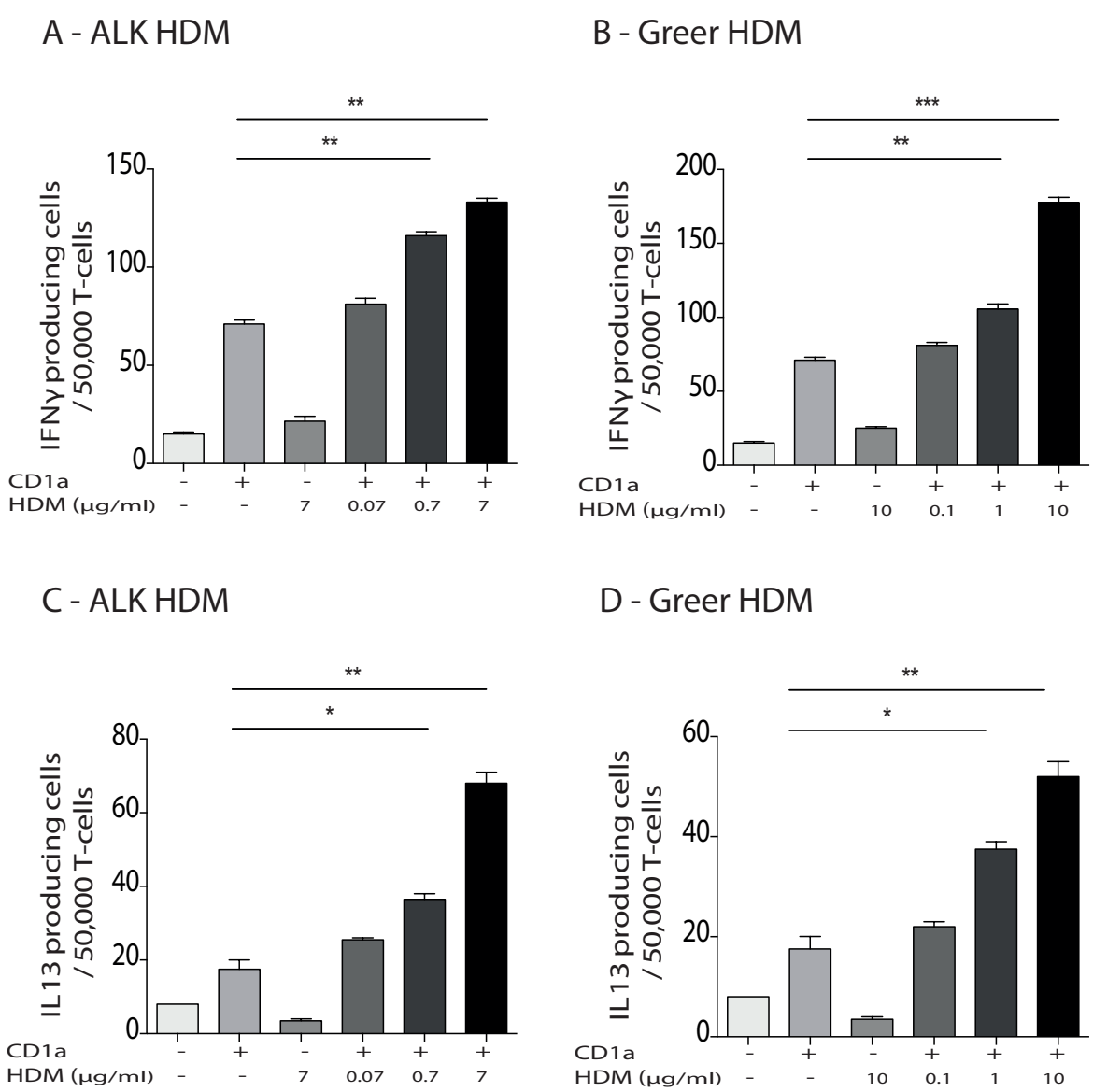


Figure 4.16. Circulating HDM responsive CD1a-reactive T cells produce IFNγ and IL-13 in response to Greer HDM. T cells were isolated by CD3 MACS beads from donor PBMCs (A, B – donor AD1516; C, D – donor AD4) and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with ALK (A, C) and Greer (B, D) HDM extract. IFN-γ (A, B) and IL-13 production (C, D) was measured by ELISpot at different HDM concentrations as labelled. Data representative of at least three donors for each experiment are shown. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001

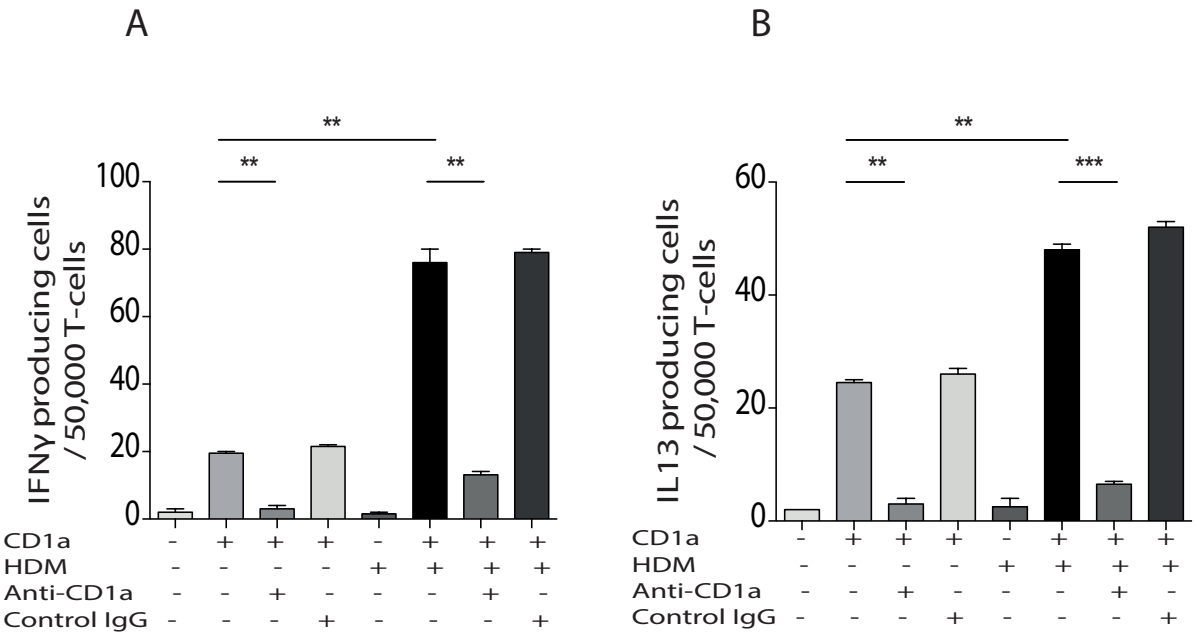


Figure 4.17. The *ex-vivo* autoreactive and Greer HDM responsive CD1a-reactive T cell response is also abrogated by anti-CD1a antibody. T cells were isolated by CD3 MACS beads from donor PBMC (A – donor AD1519, B – donor AD4) and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract. IFN-γ and IL-13 (B) production was measured by ELISpot in the presence or absence of anti-CD1a antibody and isotype control. Data representative of at least three donors for each experiment are shown. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001.

When cohorts of AD patients and healthy controls was examined, individuals with AD had significantly higher frequencies of cytokine producing Greer HDM responsive CD1a-reactive T cells *ex vivo* when compared to healthy controls, as was observed in the case of ALK HDM responsive CD1a-reactive T cells (Figure 4.18A, B). In addition, there was a significant correlation between the percentages of both IFNγ and IL 13 Greer HDM responsive CD1a reactive T cells and ALK HDM responsive CD1a reactive T cells (Figure 4.19A, B; IFNγ – r2= 0.8182, P <0.0001; Il 13 - r2= 0.9789, P <0.0001).

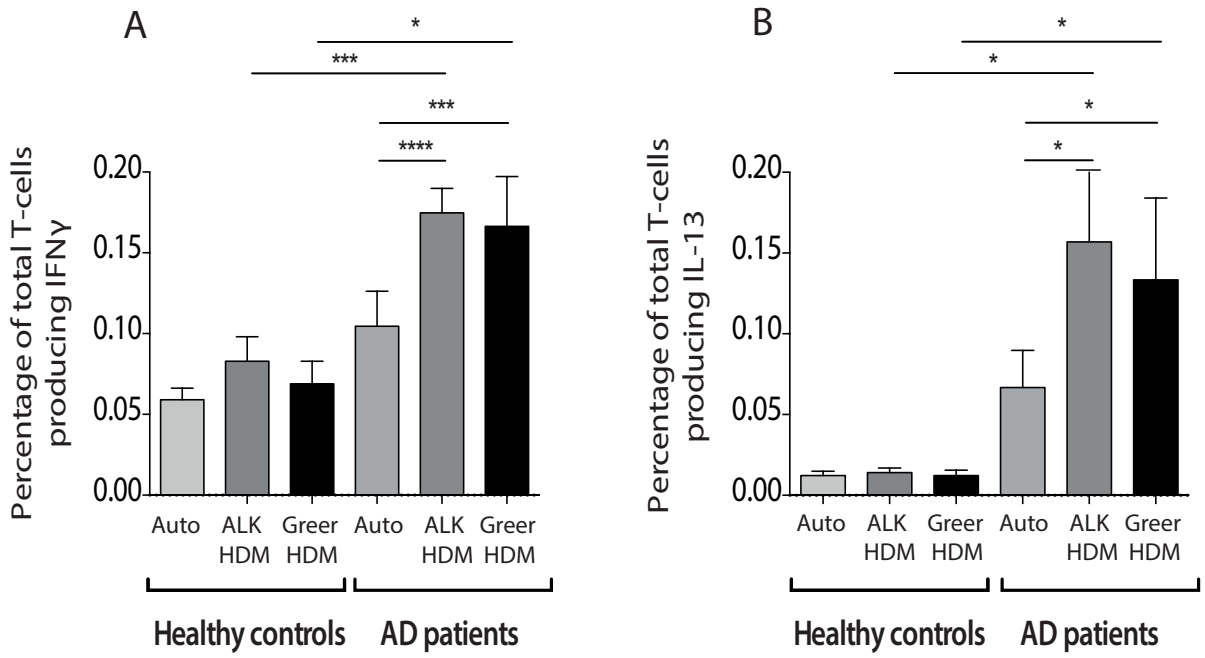


Figure 4.18. Greer HDM-responsive CD1a-reactive T cells are enriched in blood of atopic dermatitis patients. (A,B) T cells derived from the peripheral blood of healthy controls and patients with atopic dermatitis were incubated with CD1a-transfected K562 or untransfected K562 cells in the presence or absence of ALK or Greer HDM extract. IFNγ (A) and IL-13 (B) production was measured by ELISpot and expressed as percentage of responding T cells (A n=6 HC, 8 AD; B n=4 HC, 6 AD). "Auto" is the autoreactive is the response to CD1a-K562 in the absence of HDM, "ALK HDM" is the HDM response to ALK HDM extract, "Greer HDM" is the HDM response to Greer HDM extract. Bars represent standard error. * P<0.05; ***P<0.001;****P<0.0001.

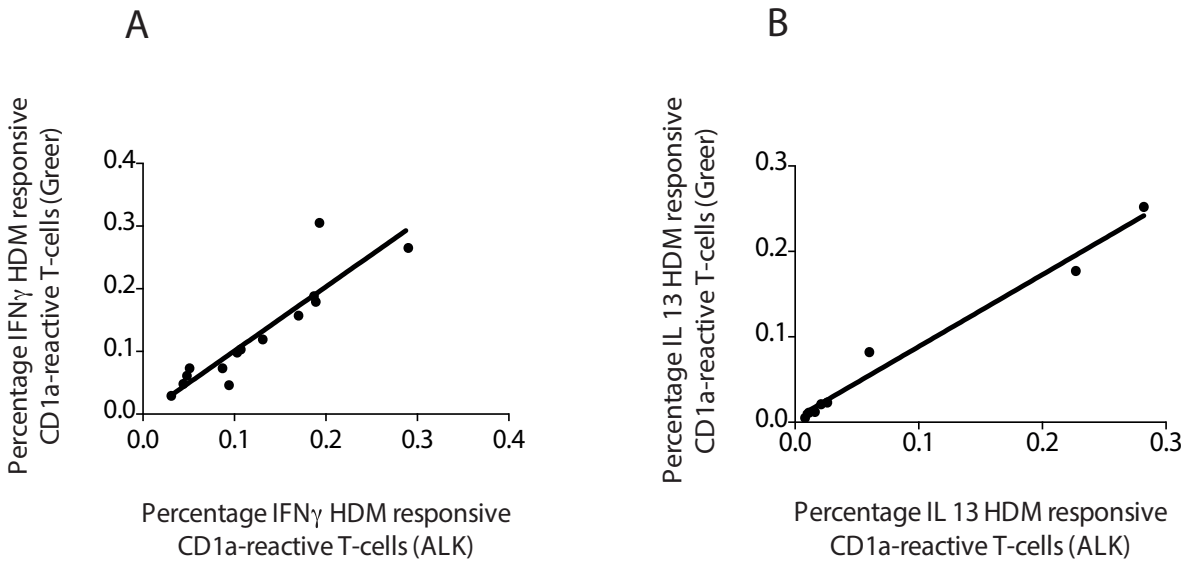


Figure 4.19. A correlation between the Greer and the ALK HDM responsive CD1a-reactive T cell response is observed. A significant correlation between the percentage of Greer HDM responsive CD1a-reactive T cells and ALK HDM responsive CD1a reactive T cells is observed on performing linear regression and calculation of the Pearson correlation coefficient (A - IFN-γ producing cells: r2= 0.8182, P <0.0001; B - IL 13 producing cells: r2= 0.9789, P <0.0001).

The significant correlation between the percentage of IFN-γ and IL-13 Greer HDM reactive CD1a-reactive T-cells and ALK HDM reactive CD1a-reactive T-cells further validates the CD1a reactive HDM T cell response, which therefore further suggests an involvement of HDM responsive CD1a-reactive T-cells in the pathogenesis of AD.

4.7 HDM responsive CD1a-reactive T cell responses are enriched in the skin homing fraction of peripheral blood T cells

To examine whether the HDM responsive CD1a reactive T cell response is enriched in the skin homing fraction of peripheral blood T cells, PBMC-derived *ex-vivo* polyclonal T cells were isolated by MACs bead separation. Polyclonal T cells were then sorted by Flow cytometry into two populations: CD3 positive Cutaneous Lymphocyte Antigen (CLA) positive T cells, and CD3 positive CLA negative T cells. CLA expression or absence was confirmed by Flow cytometry (Figure 4.20) prior to IFN-γ ELISpot for CD1a reactivity.

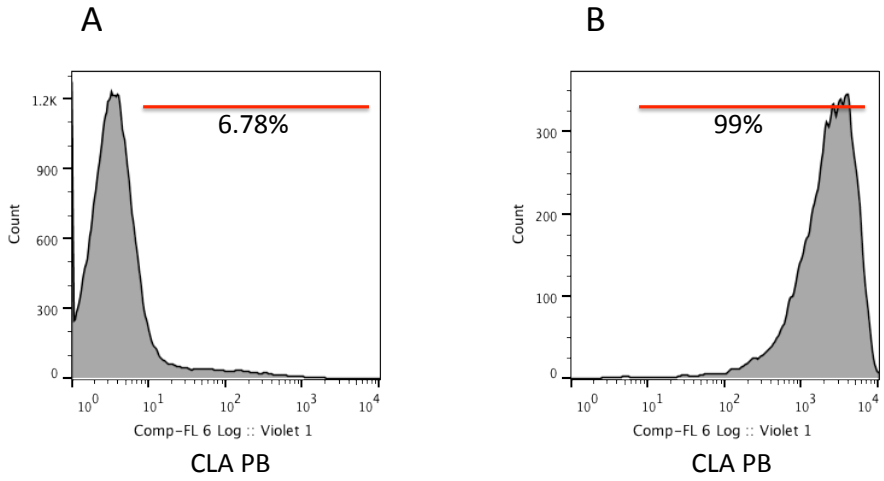


Figure 4.20. CD3+CLA- and CD3+CLA+ populations post FACS sorting. T cells were isolated from PBMCs by MACS bead separation and subsequently sorted into CD3+CLA- (Figure 4.20A) and CD3+CLA+ (Figure 4.20B) populations. Representative FACS plots post FACS sort. Plots were gated on live cell, singlets and CD3 positive cells.

HDM responsive CD1a reactive T cell responses were found to be enriched in the CLA positive T cell fraction (Figure 4.21), in concordance with published data that CD1a autoreactive T cells home to the skin and express skin homing markers on ex-vivo analysis (119).

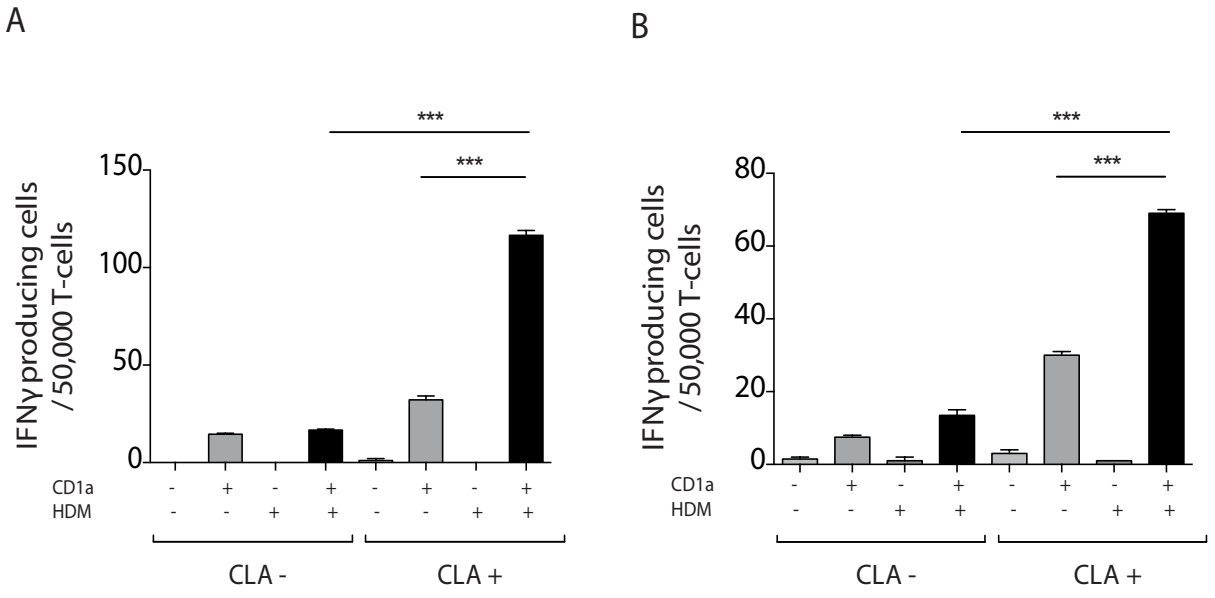


Figure 4.21. **HDM responsive CD1a reactive T cell responses are enriched in the skin homing fraction of peripheral blood**. T cells were isolated from PBMCs by MACS bead separation and subsequently sorted into CD3+CLA- and CD3+CLA+ populations prior to IFN-γ ELISpot for CD1a reactivity. Figure 15A donor AD9500, Figure 15B donor AD9501A. Data representative of three donors. Bars represent standard error. ***P<0.001.

Chapter 4 Summary

Overall these data show that HDM responsive CD1a-reactive T cells exist in the peripheral blood at high frequencies and produce IFNγ, GM-CSF and IL-13 in response to HDM challenge at clinically relevant doses in AD patients. This HDM responsive-CD1a-reactive T cell response was shown to be CD1a-dependent, and polyclonal T cells responded at highest rates to K562 cells expressing CD1a. The cells increased in frequency with age, and were enriched in the circulation of patients with AD. The conserved HDM response to a variety of $CD1a^{+}$ APCs demonstrates

that it is not an artefact of the K562 system, and the fact that the response was reproducible within donors over time, and preserved to HDM allergen derived from two separate sources further validates the specificity. Dual-colour Fluorospot demonstrated that the majority of type 1 and type 2 cytokine producing cells were unique subsets, and frequencies of HDM responsive CD1a-reactive T cells were found to be significantly elevated in individuals with filaggrin mutations. Finally, HDM responsive CD1a-reactive T cells were found to be enriched in the skin homing fraction of peripheral blood.

This is the first time that HDM responsive CD1a-reactive T cells have been described in the literature, and the finding that they are enriched in the circulation of patients with AD suggests that they might contribute to disease pathogenesis. This could be through a number of mechanisms, for example the cytokines produced by these cells are known to have a direct effect on keratinocytes, in addition to broader effects on the innate and adaptive immune systems.

As detailed in the introduction, IFN-γ is known to contribute to acute and chronic AD pathogenesis, exerting direct effects on keratinocytes including induction of HLA Class II and ICAM-1 expression with consequent facilitation of the peptide-specific adaptive immune response by enhancing the presentation of allergen peptides to conventional T cells (513), production of high levels of the potent chemoattractants monocyte-chemotactic protein 1 and RANTES (188), and upregulation of Fas, with T cell mediated Fas ligand induced apoptosis thought to play a role in the spongiosis seen in AD (312). IFN-γ is also believed to be involved in tissue remodelling seen in AD (313-315). IL-13 has additionally been reported to have direct effects on epidermal barrier function as detailed previously, attenuating keratinocyte expression of terminal differentiation genes such as filaggrin, loricrin and involucrin (292, 293), in addition to influencing innate immunity by downregulating AMPs (261), and increasing *S. aureus* colonisation by upregulating *S. aureus* adhesion molecules

(266). GM-CSF is also known to exert a broad range of immunological effects, and may have a direct or indirect effect on T cells through modulation of antigen presenting cells. In the skin, GM-CSF is known to prolong eosinophil, monocyte-macrophage and Langerhans cell survival (188), recruitment and maturation (514, 515), and may therefore link the innate and adaptive immune responses, and may further result in the persistent cutaneous inflammation seen in chronic AD (122).

The finding of increased frequencies in adult samples, compared to cord blood samples suggests that HDM responsive CD1a-reactive T cells are present at birth and likely undergo acquired antigen driven T cell expansion with age with increasing antigen exposure, in agreement with previous published data on CD1a-restricted T cells (512). HDM responsive CD1a-reactive T cell responses were seen in multiple unrelated donors, suggesting that they have an important role CD1a immunobiology and the immune response to HDM. The finding that the frequencies of HDM responsive CD1a-reactive T cells were significantly elevated in individuals with filaggrin mutations further suggests a role for these cells in AD pathogenesis, given the extensive knowledge of the importance of epidermal barrier defects, and filaggrin mutations, in the field of AD. This association is further explored in subsequent chapters.

Again in concordance with the published literature on CD1a-reactive T cells (119), responses were detected most strongly to the CD1 isoform CD1a, which is most highly expressed in the skin. The natural accumulation of CD1a autoantigens, CD1a proteins and CD1a autoreactive T cells (55, 119) point to a natural organ specific function in the skin, and the presence of HDM responsive CD1a-reactive T cells with the skin-homing (CLA^+) fraction of peripheral blood T cells further suggests a role for these cells in cutaneous immunology.

Investigation of the presence of these cells in skin in AD patients will be important to further establish a link to disease pathogenesis, and this will be addressed in Chapter 5: Identification of HDM responsive CD1a-reactive T cells in the skin. It will also be important to further functionally phenotype these cells in terms of cell surface marker expression and cytokine production, which will be the focus of Chapter 6: Phenotypic and functional analysis of HDM responsive CD1a-reactive T cell clones. Finally, it will be key to attempt to establish the CD1a ligand within HDM, i.e. to determine the CD1a binding fraction of HDM, and this will be concentrated on in Chapter 7: Investigation of the role of phospholipase in the generation of CD1a ligands.

5 Identification of HDM responsive CD1a-reactive T cells in the skin

Introduction and aims

As described, it has recently been shown that CD1a-restricted T cells circulate at high frequencies, express skin addressins (such as CLA, CCR4, CCR6 and CCR10) and are able to infiltrate the skin and to recognise skin lipids (55, 119, 512). These *"headless"* self-lipids which naturally accumulate in the skin and sebum and include fatty acids and wax esters, were recently shown to be presented by CD1a for recognition by T cells (55). Furthermore, our laboratory has also recently identified that fatty acids generated by phospholipase activity in wasp and bee venom can be recognised by CD1a-reactive T cells (516). In a novel mechanism of antigen detection, T cell receptor recognition of such headless lipid antigens can occur without direct TCR-lipid contact, and has been shown to be mediated by permissive structural changes in the CD1a molecule on lipid binding (517). Furthermore, pollen- and venom- specific CD1a-restricted T cells have additionally been identified in allergic individuals (109, 469), implying a role for these cells in the pathogenesis of atopic disease. CD1a is predominantly expressed in the skin, particularly by epidermal Langerhans cells and it is noteworthy that CD1a protein is well established as a Langerhans cell marker (491). The natural accumulation of autoantigens, CD1a proteins and skin-homing CD1a-restricted T cells points to a organ-specific function in skin, yet a defined role for this pathway in human skin disease remains to be established.

In chapter 4 we established that HDM responsive CD1a-reactive T cells were enriched in the circulation in patients with AD, where they responded to HDM in clinically relevant dose ranges, suggesting that they may play a role in clinical AD. Furthermore, HDM responsive CD1a-reactive T cell responses were enriched within the skin homing fraction of peripheral blood. We therefore sought to identify these cells at the site of pathology in AD: the human skin.

The aims of this chapter were to identify and quantify HDM responsive CD1a-reactive T cells in the skin of healthy controls and patients with AD, and to examine their functional phenotype in terms of cytokine production. This was investigated using the skin suction blister technique, a method which is well established in the laboratory (283). As in the previous chapter the study of polyclonal HDM responsive CD1a-reactive T cells from multiple unrelated donors was facilitated by the use of K562 cells with absent surface expression of MHC Class I and II transfected to express CD1a molecules as APCs, in addition to using primary CD1a+ APCs to examine HDM responsive CD1a-reactive T cell responses. The HDM antigen challenge system was used to examine the HDM responsive CD1a-reactive T cell response *in vivo*, in order to assess the infiltration of these cells into the skin after antigen challenge. The correlation of the frequency of the HDM responsive CD1a-reactive T cell response with HDM skin prick test score and total/HDM specific IgE was investigated, and frequencies of these cells in the skin compared to the blood were established.

5.1 Establishment of skin suction blisters and an antigen challenge system

To examine whether HDM-responsive CD1a-reactive T cells were also present within in the skin of patients with AD and healthy controls, skin suction blisters were used to isolate T cells from skin.

This method can be performed before or after antigen challenge and is well established in the laboratory (283). Low pressure, sustained suction for approximately 60 minutes is used to induce dermo-epidermal separation and blister formation (Figure 5.1), with the production of extracellular blister fluids that are captured by aspiration 24 hours later and used for immunological and biochemical analysis.

Cells and fluid can be directly aspirated from the skin and used in functional assays in this approach, without the need for prolonged processing with potentially confounding treatments such as dispase and collagenase, thus conferring an advantage over conventional skin biopsies.

On aspiration of blister fluid and cells, multiple cell populations can be identified on flow cytometry (Figure 5.2), including CD3 positive T cells (2A), CD45 negative populations including keratinocytes (2A), CD1a positive populations (2B) including $CD1a^{+}$ $Langerin^{+}$ Langerhans cells (2C), $CD1a^{+}$ $Langerin^{-}$DC populations (2C), and innate lymphoid cell populations (2D).

CD3 positive T cells are subsequently sorted, and expanded using the rapid expansion technique before being tested for CD1a reactivity by IFNγ and IL-13 ELISpot.

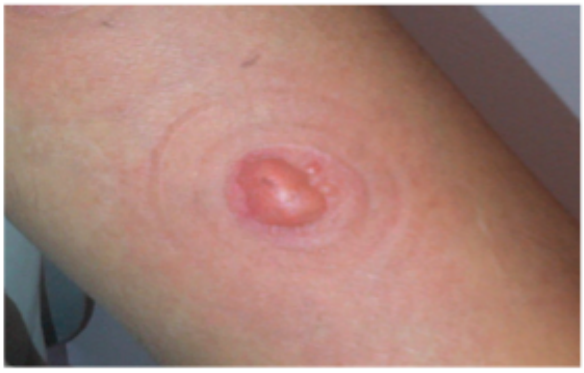


Figure 5.1. Example of skin suction blister raised after 60 minutes of 200mmHg negative pressure.

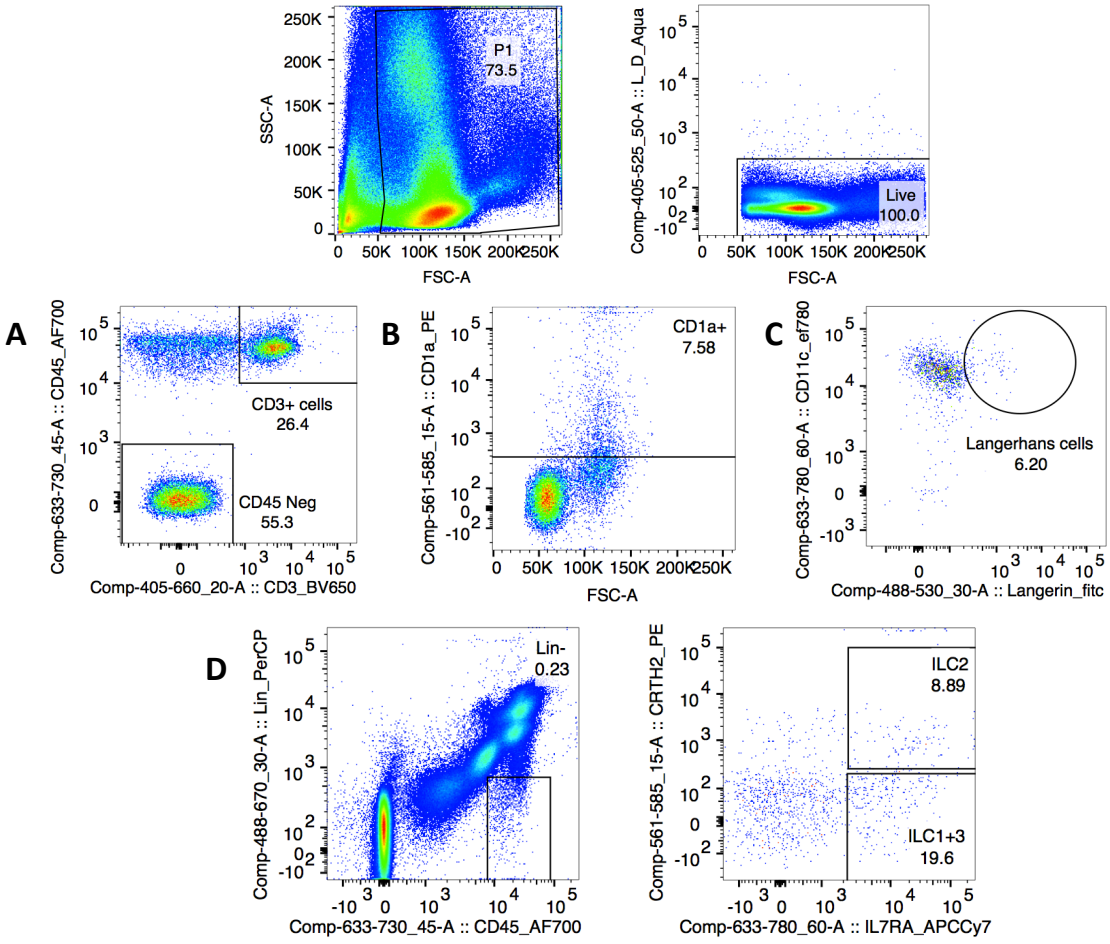


Figure 5.2. Flow cytometry analysis of blister cells. Multiple cell types can be identified including CD3-positive T cells (A), CD45 negative populations including keratinocytes (A), CD1a positive populations (B) including CD1a-positive Langerin-

positive Langerhans cells (C), CD1a-positive Langerin-negative DC populations (C), and innate lymphoid cell populations (D). Plots gated on singlets and live cells; C gated on CD1a+ cells; D (right) gated on lineage negative CD45 positive cells.

5.2 HDM responsive CD1a-reactive T cells are enriched in the skin of AD patients

HDM-responsive CD1a-reactive T cells were found to be present in the skin and produce IFNγ, GM-CSF and IL-13 (Figure 5.3A, B, C).

Furthermore the IL-13 producing T cells were enriched in the skin of patients with atopic dermatitis compared to skin of healthy controls (Figure 5.4) and the frequency correlated with SCORAD disease severity (r^2=0.67, P<0.01), showing that T cell infiltration in atopic dermatitis correlates both with disease outcome and type 2 cytokine production in humans *ex vivo*. There was no enrichment of IFNγ or GM-CSF producing T cells in the skin of atopic dermatitis patients.

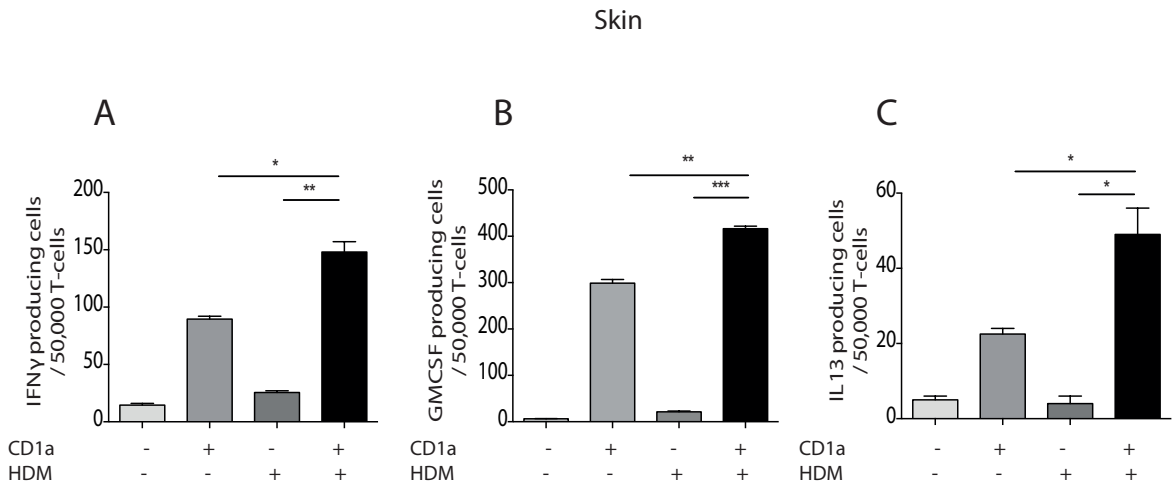


Figure 5.3. HDM-responsive CD1a-reactive T cells are present in the skin of atopic dermatitis patients. Skin blister T cell lines were incubated with CD1a-transfected K562 or untransfected K562 cells in the presence or absence of HDM extract. IFNγ (A, left), GM-CSF (B, middle) and IL-13 (C, right) production was measured by ELISpot. Data are representative of at least three separate donors for each experiment. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001.

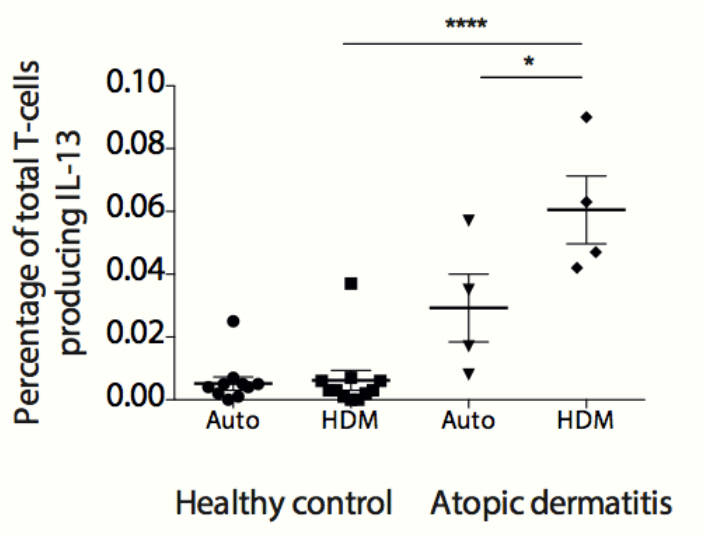


Figure 5.4. HDM-responsive CD1a-reactive T cells are enriched in the skin of atopic dermatitis patients. Skin blister T cells were isolated from unchallenged skin of healthy controls (n=10) or patients with atopic dermatitis (n=4) and incubated with CD1a-transfected or untransfected K562 cells in the presence or absence of HDM extract. IL-13 production was measured by ELISpot and expressed as percentage of T cells. Bars represent standard error. * P<0.05; ****P<0.0001.

5.3 Skin blister derived polyclonal T cells additionally respond in a CD1a dependent manner to primary autologous in-vitro derived and cord blood derived CD1a-positive antigen presenting cells

To investigate whether other CD1a+ APCs additionally mediate the skin blister CD1a-reactive HDM T cell response, monocytes were differentiated with GM-CSF and IL-4 to produce monocyte-derived myeloid dendritic cells (mDCs) (508-511), and Langerhans cell-like cells were differentiated from cord-blood derived CD34+ progenitor cells as described (405), to provide an alternate system of CD1a+ cell generation.

As shown in the previous chapter, flow cytometry analysis demonstrated differentiation, with high CD1a expression in mDCs (Figure 4.6), and high CD1a and Langerin expression in cord-blood derived LC-like cells (Figure 4.9).

As previously observed with blood-derived T cells, polyclonal skin blister-derived T cells reacted to mDCs (Figure 5.5A) and cord-blood derived LC-like cells (Figure 5.5B, C) in a HDM responsive CD1a-restricted manner.

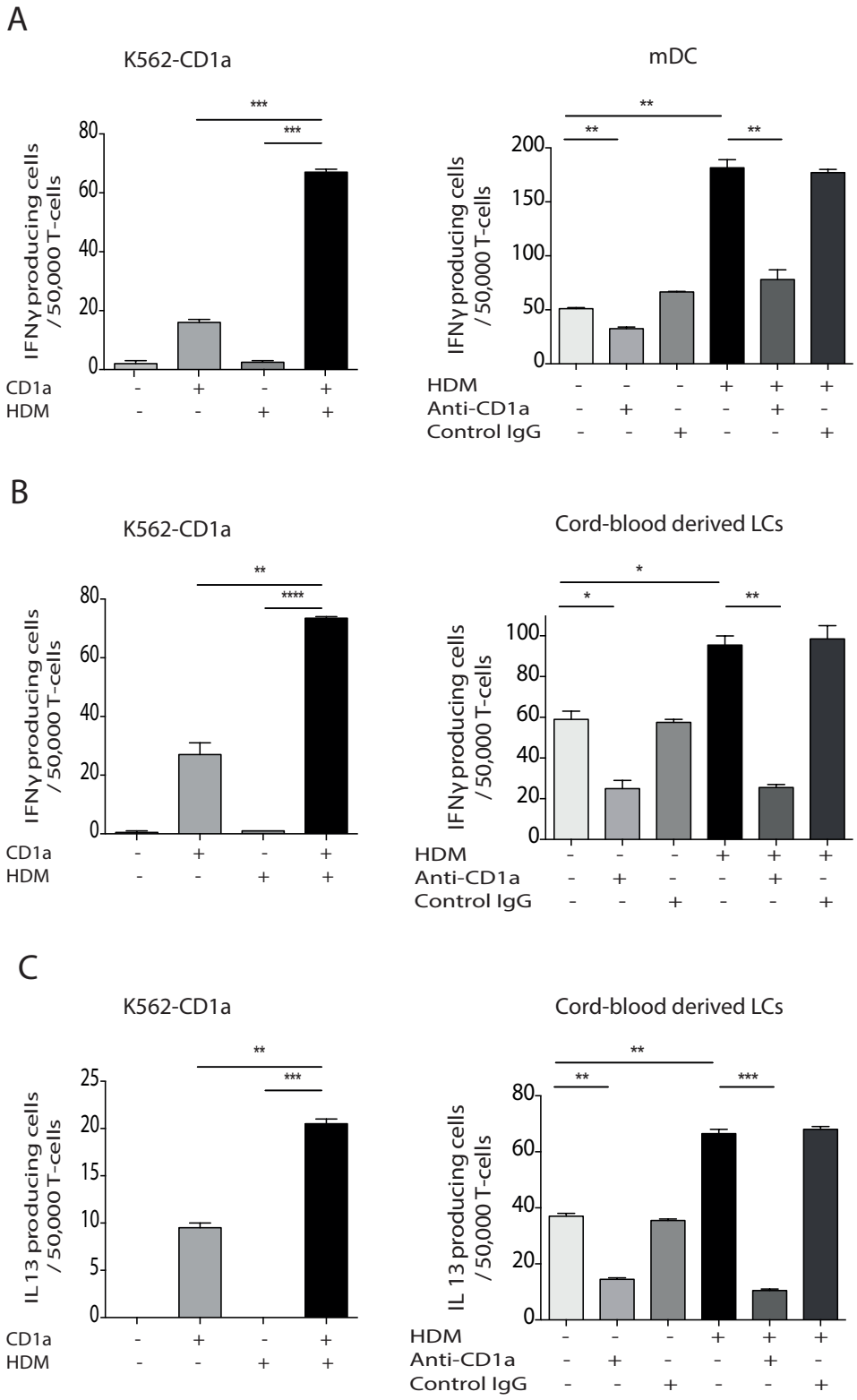


Figure 5.5. Skin blister-derived T cells also respond in a CD1a-restricted manner to mDCs and cord-blood derived LC-like cells. CD1a-expressing K562 (A, B, C left), *in vitro* derived mDC (A, right) or cord-blood derived LC-like cells (B, C

right) were pulsed with HDM extract overnight and incubated with autologous skin blister-derived T cells from donor AD9005 (5A) and non-autologous skin blister-derived T cells from donor AD229 (5B, C). IFNγ (A,B) and IL- 13 (C) production was measured by ELISpot in the presence or absence of anti-CD1a antibody and isotype control and the presence of HLA Class I and II blocking antibodies. Data representative of at least three donors for each experiment. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001;****P<0.0001.

5.4 HDM responsive CD1a-reactive T cells infiltrate the skin after HDM challenge

To investigate the HDM responsive CD1a-reactive immune response *in vivo*, donor skin was challenged intra-epidermally with 0.7μg HDM extract and skin infiltration of HDM-responsive CD1a-reactive T cells at 24 hours was assessed. A dose at 3% of the amount administered during the maintenance phase of subcutaneous immunotherapeutic approaches (up to 21μg) was chosen to assure safety and attempt to mimic low dose exposures (507).

Skin infiltration of IFNγ, GM-CSF and IL-13-producing HDM-responsive CD1a-reactive T cells at 24 hours was noted, suggesting that the cells infiltrate early and may therefore contribute to the ensuing inflammation (Figure 5.6A). Furthermore in patients with atopic dermatitis, there was significantly greater infiltration of IFNγ-producing HDM-responsive CD1a-reactive T cells after HDM challenge than in healthy controls (Figure 5.6B). In contrast, there was no enrichment for varicella zoster virus-specific T cells in the skin after HDM challenge, suggesting that the enrichment is specific to HDM-responsive T cells (Figure 5.6B, right panel).

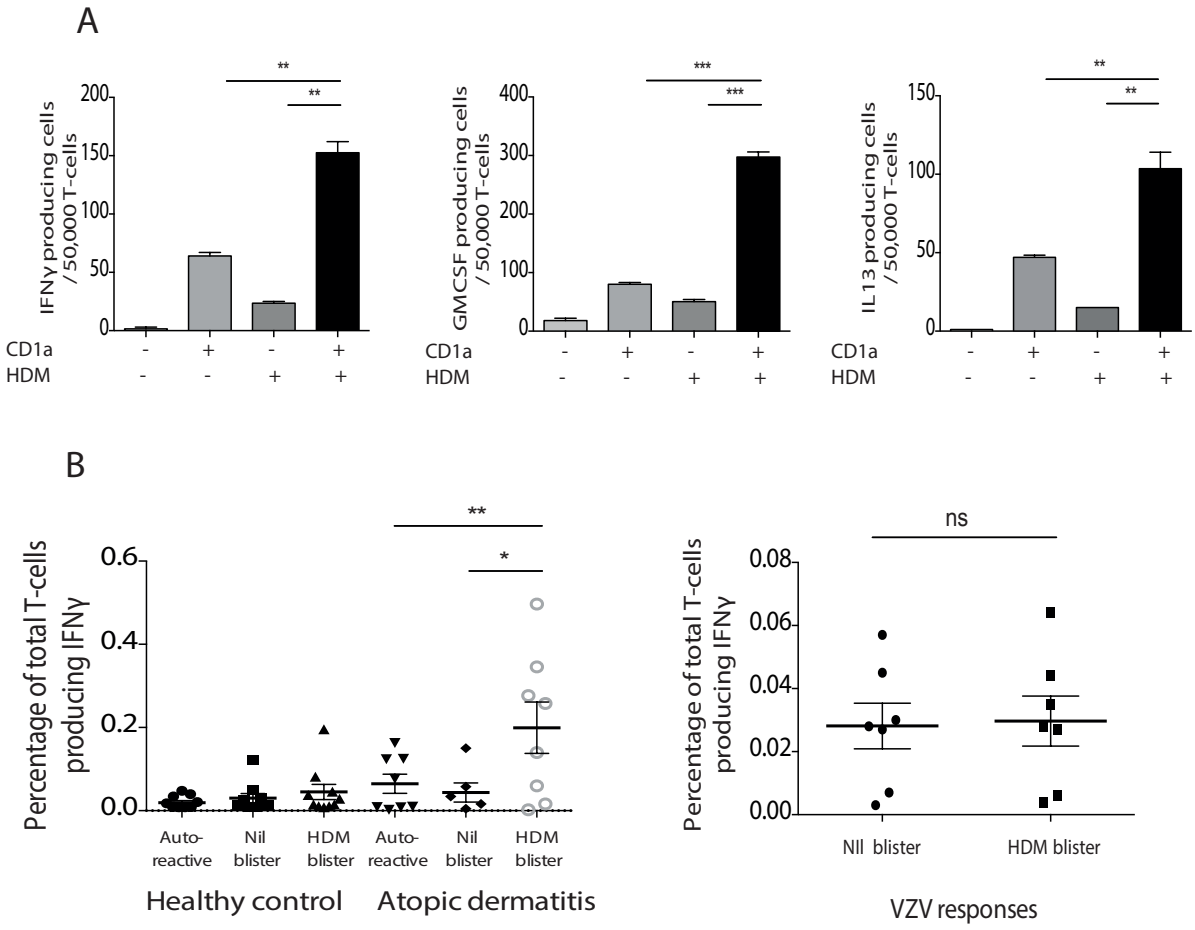


Figure 5.6. HDM-responsive CD1a-reactive T cells infiltrate skin after HDM challenge. Skin blister T cells were isolated from donor AD206 24 hours after HDM skin challenge, expanded and incubated with CD1a-transfected or untransfected K562 cells in the presence or absence of HDM extract. IFNγ (A, left), GM-CSF (A, middle) and IL-13 (A, right) production by donor T cells was measured by ELISpot. The data are representative of at least three separate donors for each experiment. (B, left panel) Overall frequencies of HDM-responsive CD1a-reactive IFNγ-producing T cells infiltrating human skin after saline or HDM skin challenge were compared between healthy controls (n=10) and atopic dermatitis patients (n=8). Autoreactive refers to responses to unpulsed K562-CD1a cells. (B, right panel) Skin blister cells derived from HDM-challenged or unchallenged skin were incubated with live

attenuated varicella zoster virus and IFNγ production was measured by ELISpot. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001.

5.5 Frequencies of HDM responsive CD1a-reactive T cells correlate with skin prick test score, total and HDM IgE

As discussed in the introduction, approximately 80% of individuals with atopic dermatitis have elevated specific IgE recognising proteins derived from one or more ubiquitous environmental allergens, including house dust mite (HDM) (158). The relationship between the frequency of both type 1 and type 2 cytokine producing HDM responsive CD1a-reactive T cells with skin prick test score (Figure 5.7A, B, C) and total and HDM specific IgE levels (Figure 5.8) was therefore examined.

A significant correlation between the percentage of IL 13 producing HDM responsive CD1a-reactive T cells and skin prick test score was observed (Figure 5.7A r2= 0.3423, P=0.0357), in addition to positive trends between the percentages of both IFNγ– (Figure 5.7B) and IL13- (Figure 5.7C) producing HDM responsive CD1a-reactive T cells and a positive response to the HDM skin prick test.

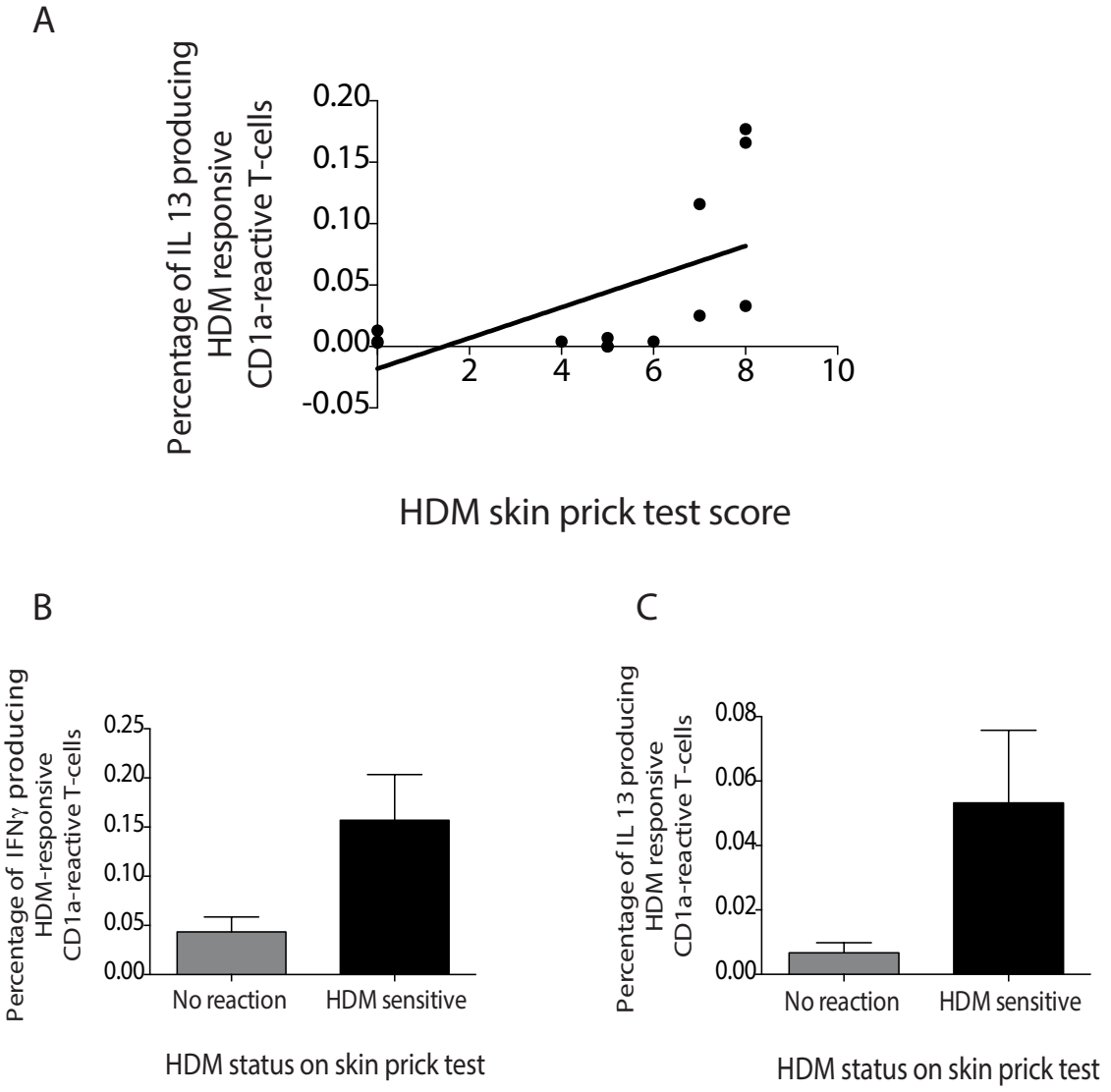


Figure 5.7. A positive correlation between the percentage of IL 13 producing HDM responsive CD1a-reactive T cells and skin prick test score is observed. On performing linear regression and calculation of the Pearson correlation coefficient, there is a significant correlation between the percentage of IL 13 producing HDM responsive CD1a-reactive T cells and skin prick test score (5.7A n=14, r2= 0.3423, P=0.0357). Positive trends between the percentages of both IFNγ– (n=18, 5.7B) and IL13- (n=14, 5.7C) producing HDM responsive CD1a-reactive T cells and a positive result to HDM skin prick test can also be seen.

A significantly increased percentage of both IFNγ– and IL-13-producing HDM responsive CD1a-reactive T cells in donors with elevated total (Figure 5.8A, B) and HDM-specific (Figure 5.8C) IgE was also observed.

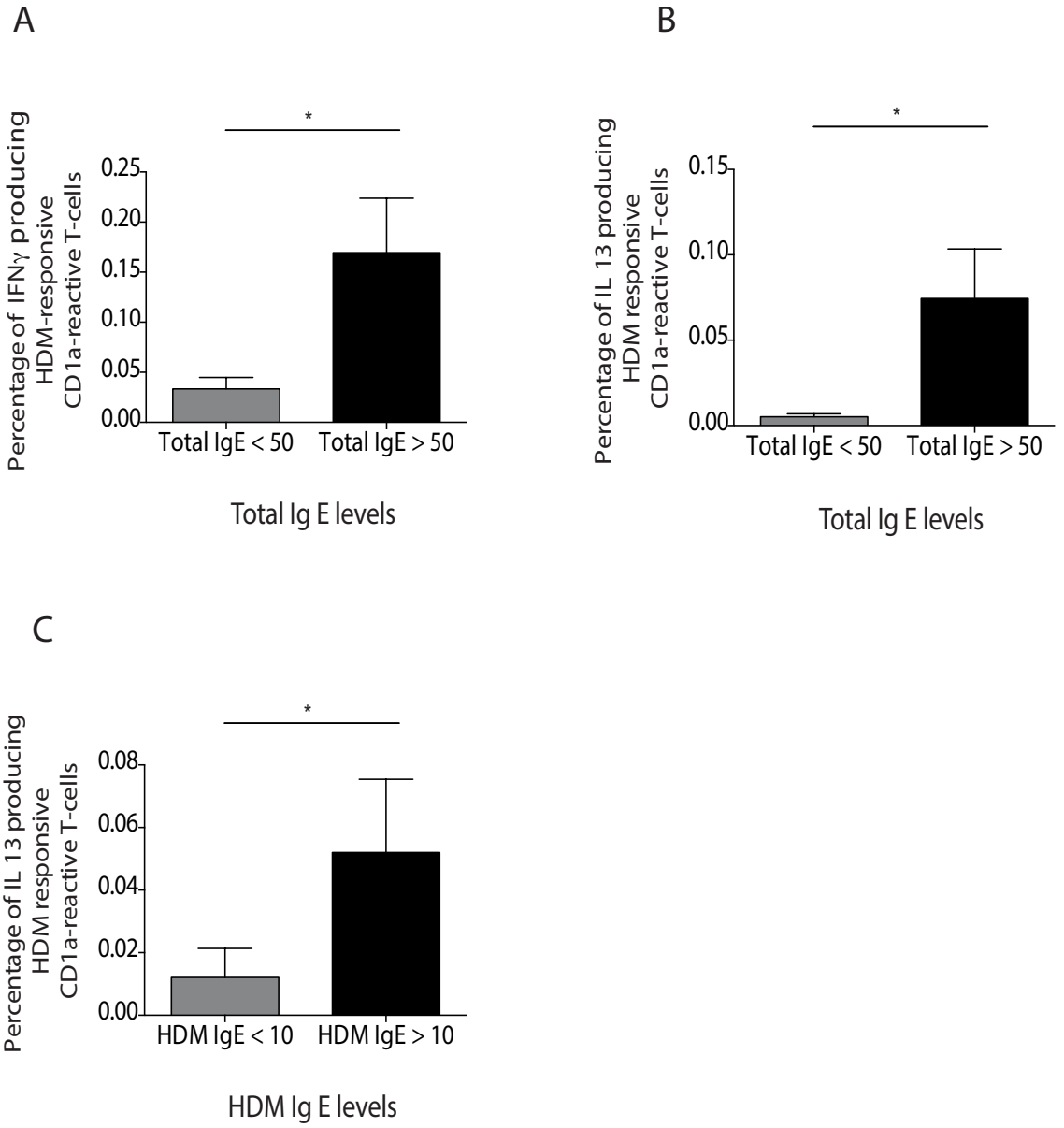


Figure 5.8. Frequencies of both IFNγ – and IL 13-producing HDM responsive CD1a-reactive T cells are associated with elevated total and HDM specific IgE. A significantly increased percentage of both IFNγ– and IL-13-producing HDM responsive CD1a-reactive T cells in donors with elevated total (Figure 5.8A, B) and

HDM-specific (Figure 5.8C) IgE can be seen. n=18 IFNγ–, n=14 IL13. Bars represent standard error. * P<0.05.

5.6 HDM responsive CD1a-reactive T cells are enriched in the HDM skin blister fluid compared to the blood

When percentages of both IFNγ– (Figure 5.9A) and IL-13 (Figure 5.9B) producing HDM responsive CD1a- reactive T cells were compared between the blood and the HDM skin blister fluid, these cells were found to be significantly elevated in the HDM skin blister fluid (Figure 5.9A), showing that there is an enrichment of these cells in the skin.

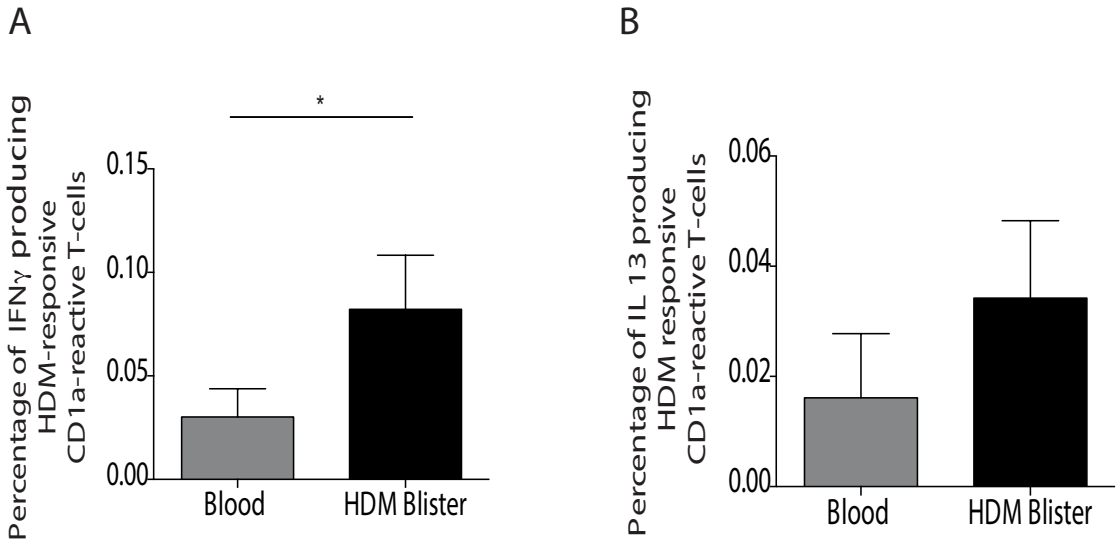


Figure 5.9. HDM responsive CD1a-reactive T cells are enriched in the HDM skin blister fluid compared to the blood. A significantly increased percentage of IFNγ producing HDM responsive CD1a-reactive T cells in the skin blister fluid compared to the blood was observed (Figure 5.9A). A similar trend for IL 13

producing cells can be seen (Figure 5.9B). Percentages of HDM responsive CD1a-reactive T cells corrected for the CD1a-autoreactive response. n=18 IFNγ, n=14 IL13. Bars represent standard error. * P<0.05.

5.7 Type-2 cytokines are elevated in AD skin blister fluid following HDM challenge

As described in the introduction, type 2 cytokines can influence filaggrin and anti-microbial peptide expression in the skin.

The type 2 cytokine content of skin blisters derived from patients with atopic dermatitis and healthy controls was therefore investigated using multiplex bead array, with sampling at non-lesional skin, and after HDM challenge. These studies showed that after HDM challenge of non-lesional skin in atopic dermatitis patients, the concentrations of the type 2 cytokines IL-4, IL-5, IL-13, in addition to GM-CSF within skin blister fluid were significantly increased compared to healthy donors (Figure 5.10).

Experiments performed by Helen Jolin, Andrew Mckenzie laboratory (MRC Laboratory of molecular Biology, Cambridge, UK).

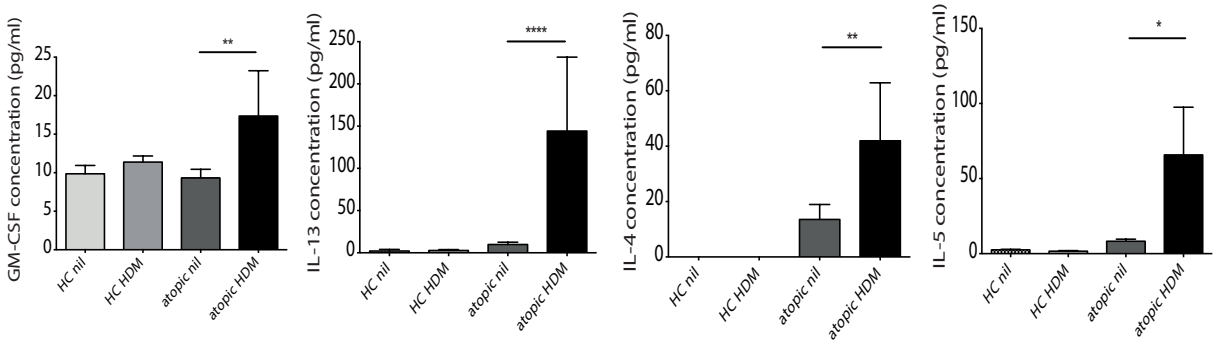


Figure 5.10. Concentrations of type 2 cytokines and GM-CSF was measured in skin blister fluid by multiplex bead array. Bars represent standard error. * P<0.05; ** P<0.01; ****P<0.0001.

Chapter 5 Summary

Overall, by using the skin suction blister technique and antigen challenge system, these data demonstrate that HDM-responsive CD1a-reactive T cells are present within the skin, and are enriched in the skin of patients with AD compared to the skin of healthy controls. These cells were additionally shown to respond in a CD1a-reactive manner to other $CD1a^+$ APCs, again illustrating that these findings are not an artefact of the K562 system. Furthermore, after intra-epidermal HDM allergen challenge, HDM responsive CD1a-reactive T cells infiltrate the skin rapidly in AD patients and associate with the production of type 2 cytokines *in vivo*, suggesting that they infiltrate early and may contribute to disease pathogenesis. Such infiltration after HDM challenge was found to be specific to HDM responsive CD1a-reactive T cells, and was not found in VZV-specific T cells. The frequency of HDM responsive CD1a-reactive T cells was also found to correlate with skin prick test score, total and HDM specific IgE, and an increased frequency of these cells was found in HDM blister fluid compared to peripheral blood, indicating that these cells may play a role in the cutaneous inflammation seen in clinical AD.

As detailed in the introduction and summary to chapter 4, the cytokines produced by these cells, namely IFN-γ, GM-CSF, and IL-13, are known to exert multiple effects on the innate and adaptive immune systems, in addition to having direct effects on keratinocytes, which may provide a mechanistic link between HDM responsive CD1a-reactive T cells and AD.

In addition, correlation of the frequency of these cells with skin prick test score, total and HDM IgE further implicates them in AD pathogenesis, with the role of allergen reactivity and elevated total and HDM specific IgE in extrinsic AD having been discussed in detail in the introduction. It is particularly interesting to note that both Langerhans cells and dermal DCs have been reported to be present in increased numbers in AD. Both of these APCs express CD1a and therefore might be speculated to play a part in lipid antigen presentation to HDM responsive CD1a-reactive T cells, and have additionally been shown to express elevated levels of the high affinity IgE receptor (FcεR1) in AD patients (121, 148, 397, 398). Expression of FcεR1 would be preserved by high IgE levels in AD, and might be postulated to contribute to the capture and internalisation of HDM before processing and presenting to HDM responsive CD1a-reactive T cells in AD skin.

As discussed, CD1a is expressed at high levels by epidermal Langerhans cells, in addition to populations of dermal DCs and DC populations residing at other surface sites (518-522), which are all key locations for allergic sensitisation. Furthermore CD1a-reactive T cells have been reported to express skin homing markers, to infiltrate the skin, and to recognise natural cutaneous lipids (55, 119), suggesting a possible role in the pathogenesis of inflammatory skin disease or cutaneous homeostasis. CD1a autoreactive T cells predominantly localise to the dermis and CD1a proteins are highly expressed by Langerhans cells of the epidermis (55, 119), and a proposed model is that these near neighbours cross the dermal-epidermal junction to interact with each other (119, 523), which may be relevant to the HDM responsive CD1a-reactive T cell response. Furthermore, Langerin^{-}CD1a^{+}FcεR1^{+} inflammatory DCs, the number of which increases in the epidermis and dermis following antigen challenge in AD may contribute to and amplify the HDM responsive CD1a-reactive T cell response (144, 386, 399). This is the first occasion that HDM responsive CD1a-reactive T cells have been isolated from human skin, providing a

pathogenic link by localising these cells to the site of disease in AD thereby implicating them in disease pathogenesis. The observation of an increased frequency of these cells in HDM blister fluid compared to peripheral blood also concentrates them at the site of cutaneous inflammation in this disease. Our laboratory has additionally recently identified that insect venoms process human skin lipids for presentation via CD1a (469), and given that CD1a reactivity has now been demonstrated in three classes of allergens relevant to humans namely insect venoms (469) , pollens (109) and HDM, this suggests that the CD1a pathway may be part of a broader hypersensitivity system in atopic disease.

The above studies were conducted using heterogeneous populations of polyclonal T cells derived from the blood (results chapter 4), or skin blister fluid (results chapter 5). However, whilst it is important to examine polyclonal responses to investigate the contribution of the HDM responsive CD1a-reactive T cell response as a whole, the study of CD1a-restricted T cell clones and lines has additionally been very important in the phenotypic and functional characterisation of these cells. We therefore wanted to go on to attempt to generate HDM responsive CD1a-reactive T cell lines and clones to enable further characterisation of these cells.

6 Phenotypic and functional analysis of HDM responsive CD1a-reactive T cell lines

Introduction and aims

Our understanding of CD1 immunobiology has greatly advanced since CD1 molecules were first identified as having a role in the presentation of lipid antigens to CD1-restricted T cells. For example, it is now widely accepted that immune stimuli induce a rapid response from group 2 CD1d-restricted iNKT cells, which play an important role in bridging the innate and adaptive immune responses (66). However, less is known about group 1 CD1-restricted T cells because of the lack of specific markers for these cells and the absence of group 1 CD1 expression in mice, and consequently studies examining their immunological role have centred on data generated from a limited number of CD1-restricted T cell clones. Our comprehension of their patho-physiological function will be enhanced by furthering our knowledge of the phenotype of CD1-restricted T cells, including their cytokine profile on immunological stimulation, cell surface marker expression, and antigen reactivity.

CD1a-restricted T cells have been identified in the CD4+, CD8+, and double negative components (118, 119), and although predominantly in the αβ repertoire, both αβ and γδ CD1a-restricted T cells have been reported (109, 119).

Advancement of our appreciation of their patho-physiological role will result from the study of the CD1a-restricted T cell response to immunological stimuli. Lipid antigen presentation by CD1a molecules has been reported to stimulate T cell cytokine secretion and proliferation (118, 119). As discussed, identification of mycobacterial

lipid ligands for each CD1 molecule has been reported, and indeed mycobacterial lipid ligands in addition to self lipid antigens have been reported to induce IL-2, IFN-γ, and TNF-α production by group 1 CD1-restricted T cells (69, 100, 101, 104). More recently, a wide range of cytokines have been detected as being produced by CD1a-restricted T cells, including IL-2, IL-22 and type 1 (IFN-γ) and type 2 (IL-4 and IL-13) cytokines, in a study which examined skin homing polyclonal autoreactive CD1a-restricted T cells (119). When stimulated, CD1a-autoreactive T cell clones recently identified by *de Lalla et al*. (118) and pollen reactive CD1a-restricted T cell clones (79, 109) have also been reported to secrete a wide range of cytokines, including IFN-γ, TNF-α, GM-CSF, IL-4, IL-5, IL-10 and TGF-β (79, 109, 118). These studies indicate a heterogeneous role for CD1a-restricted cells in the immune response through the secretion of a wide spectrum of functionally disparate cytokines.

Our knowledge of the immunological role of CD1a-restricted T cells would also be greatly enhanced by the identification of novel lipid ligands for these cells. As covered in the introduction a dual reactivity against self (11) and exogenous lipid ligands (54) has been described (64, 109, 469). For example, production of IFN-γ and TNF-α on stimulation of DDM-specific CD1a-restricted T cells with DDM, a mycobacterial lipopeptide has been demonstrated, suggesting a protective role in the anti-mycobacterial T cell response, and increased mycobacterial infection susceptibility associated with CD1a deficiency in a recent study (26). Elevated frequencies of pollen- and insect venom specific CD1a-restricted T cells have also recently been reported in allergic individuals (79, 109, 469), which in combination with the results in Chapters 4 and 5 suggest a role for these cells in the pathogenesis of atopic disease. CD1a-autoreactive T cells have additionally been shown to be stimulated by self lipid ligands including sulphatide and hydrophobic skin lipids (11, 55), suggesting a role in cutaneous homeostasis and skin barrier immunity (55).

The study of the immunological phenotype of CD1a-restricted T cells has been made possible by the generation of CD1a-restricted T cell lines and clones, and identification of their T cell effector function(s) will be further enhanced by the examination of their activation mechanisms, transcription factor and signalling pathway usage, co-receptors and co-stimulatory molecule expression, and cytokine production. Furthermore, the identification of a unique marker(s) for these cells, enabling detection from heterogenous T cell populations, may be bourne out of this approach. The aims of this chapter were therefore to generate HDM responsive CD1a-reactive T cell lines by using the IFNγ secretion assay to sort 1-10 T cells per well of a 96-well round bottomed tissue culture plate. HDM responsive CD1a-reactive T cell lines generated were then tested for HDM reactivity, in addition to other antigens and CD1 isoforms to examine antigen specificity. The phenotype of HDM responsive CD1a-reactive T cell lines was examined through the study of cytokine secretion to immunological stimuli and cell surface marker expression.

6.1 The IFNγ assay as a tool for generating HDM responsive CD1a-reactive T cell lines

In order to examine HDM responsive CD1a-reactive T cells in further detail, HDM responsive CD1a-reactive T cell lines were generated using the IFNγ secretion assay.

Peripheral blood, and skin blister derived T cells were either unstimulated or stimulated using K562-EV, K562-EV pulsed with HDM, K562-CD1a, K562-CD1a pulsed with HDM or staphylococcal enterotoxin B as a positive control. A CD1a-reactive T cell response was observed (Figure 6.1), and T cells that produced IFNγ

on stimulation with K562-CD1a pulsed with HDM were sorted followed by expansion with the rapid expansion method.

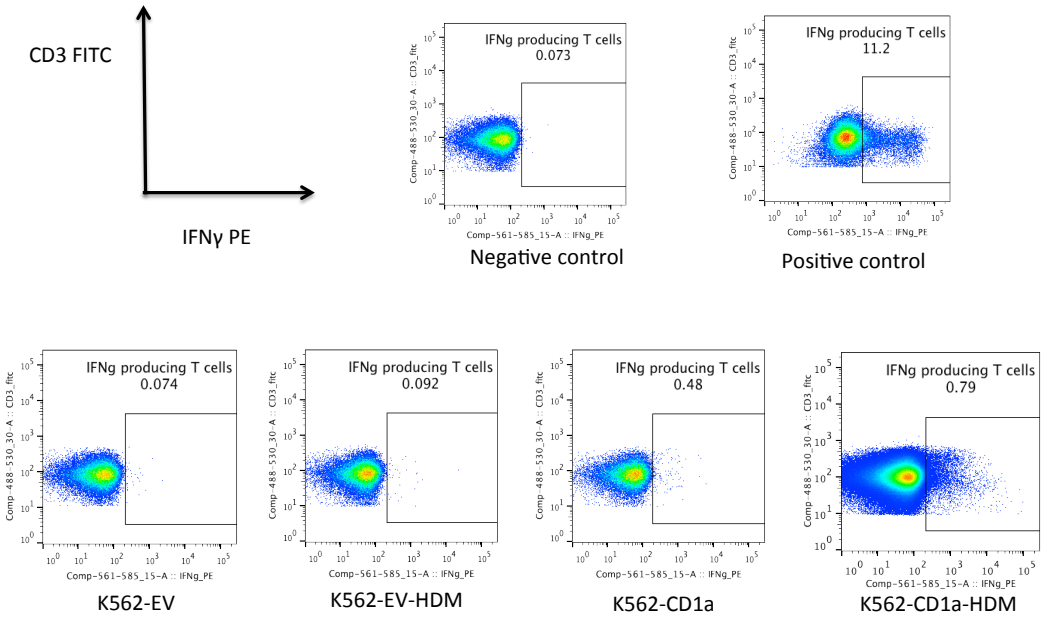


Figure 6.1. Flow cytometry gating strategy for the generation of HDM responsive CD1a-reactive T cell lines using the IFNγ secretion assay. T cells were isolated from PBMCs by CD3+ magnetic separation or expanded following FACs sorting from skin blister fluid. Polyclonal T cells were assayed for IFNγ production against K562-EV, K562-EV pulsed with HDM, K562-CD1a and K562-CD1a pulsed with HDM. T cells alone, and T cells stimulated with staphylococcal enterotoxin B were included as negative and positive controls, respectively. CD3+, IFNγ-producing cells on stimulation with K562-CD1a pulsed with HDM were sorted by flow cytometry into round-bottomed 96 well plates at 1 or 10 cells per well, and expanded using the rapid expansion method. Representative plot from donor AA9005. Plots gated on lymphocytes, singlets, live cells and CD3+ cells.

All lines generated were subsequently screened for CD1a reactivity using IFN-γ ELISpot, and promising lines were further expanded using the rapid expansion

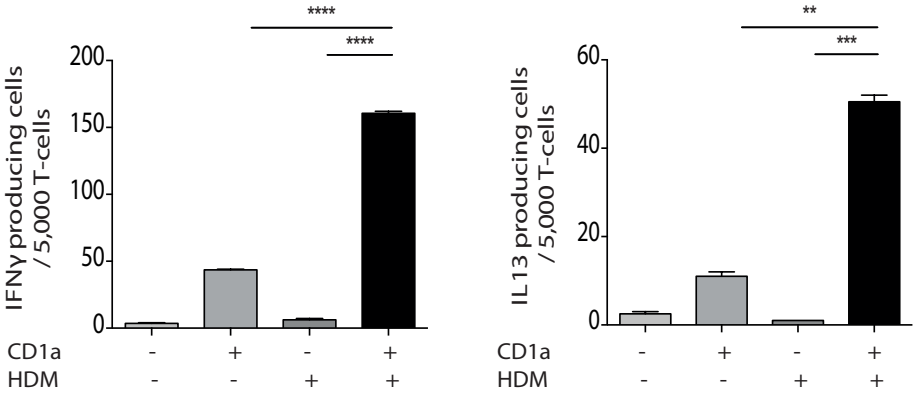
method. Screening for CD1a reactivity was performed after every cycle of expansion. Of all lines generated, five CD1a-reactive T cells lines strongly responsive to HDM were identified: AH5(1), HH11 and H+8 (generated from peripheral blood of donor AD9500), AH5(2) (generated from peripheral blood of donor AD9501A) and AA9005 (generated from skin blister fluid of donor AA9005).

6.2 HDM stimulates T cell lines in a CD1a dependent manner

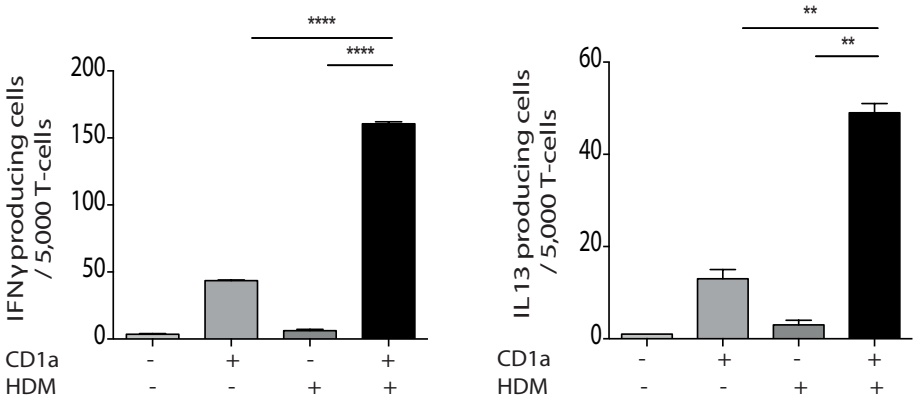
6.2(i) HDM responsive CD1a-reactive T cell lines produce type 1 and type 2 cytokines in a CD1a dependent manner

In order to investigate type 1 and type 2 cytokine production in the context of CD1a reactivity, IFNγ and IL-13 ELISpot were performed using K562-EV, K562-CD1a, K562-EV pulsed with HDM and K562-CD1a pulsed with HDM as described previously (Figure 6.2 A, B, C, D, E). The response was inhibited by an anti-CD1a antibody but not by isotype control, confirming CD1a dependence (Figure 6.3 A, B, C, D, E).

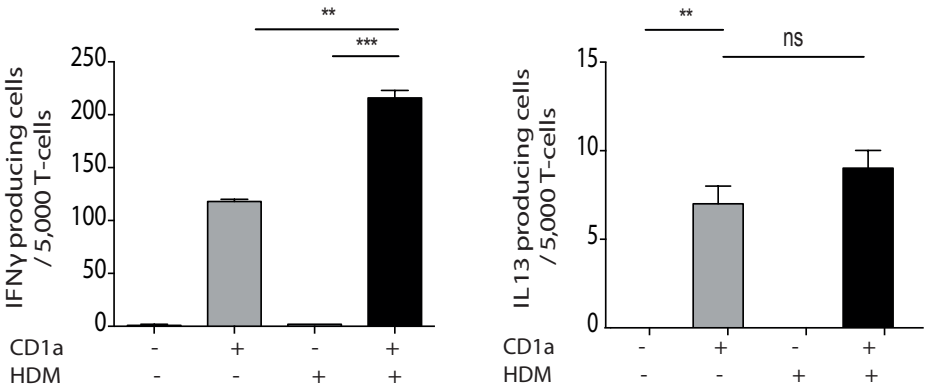
A : AH5(1)



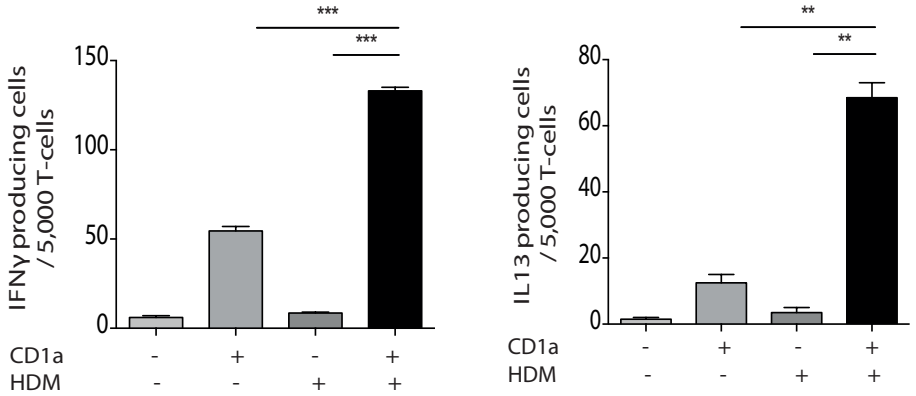
B: HH11



C: H+8



D: AH5(2)



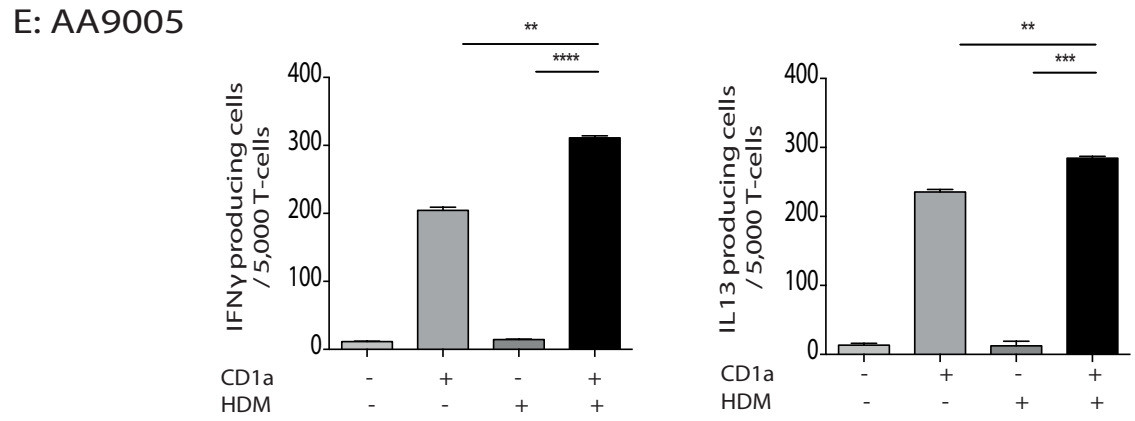
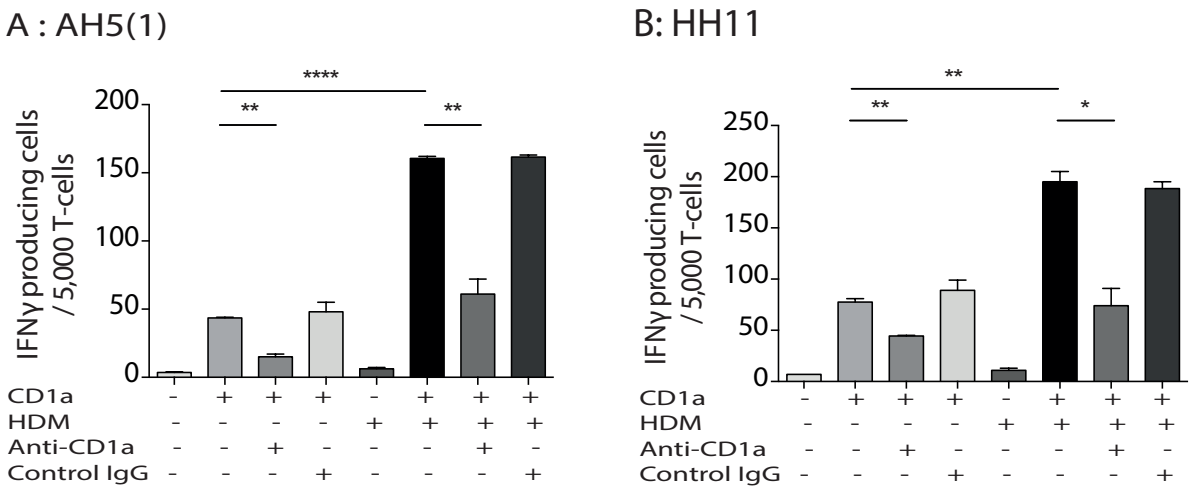


Figure 6.2. HDM responsive CD1a-reactive T cell lines generated using the IFNγ secretion assay produce IFNγ and IL-13. T cell lines were generated using the IFN-γ secretion assay described above and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract. IFN-γ and IL-13 production was measured by ELISpot. Data representative of at least three separate experiments are shown. Bars represent standard error. ** P<0.01; ***P<0.001, ****P<0.0001.



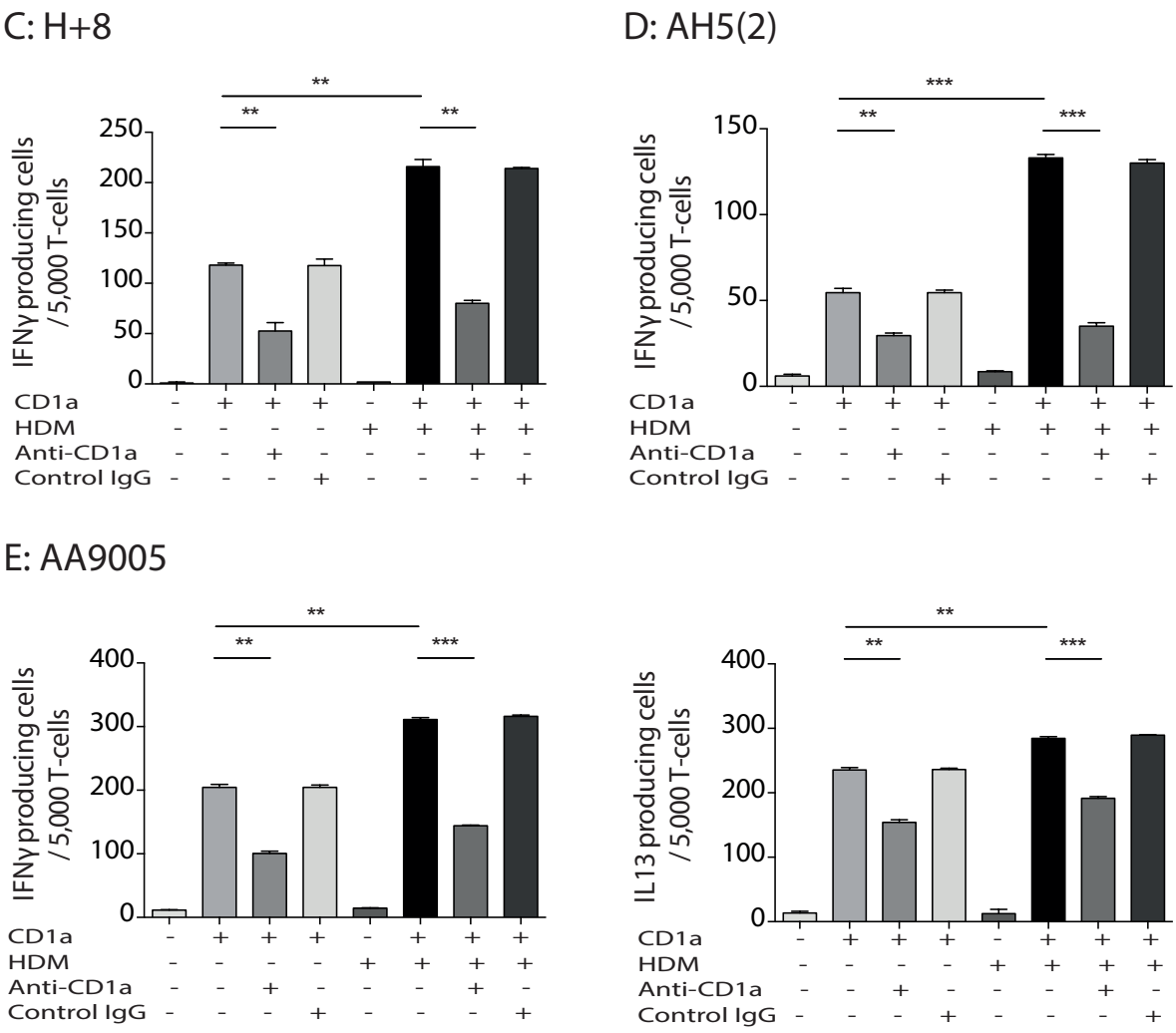


Figure 6.3. The autoreactive and HDM responsive CD1a-reactive T cell line response is abrogated by anti-CD1a antibody. T cell lines were generated using the IFN-γ secretion assay described above and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract. IFN-γ and IL-13 production was measured by ELISpot in the presence or absence of anti-CD1a antibody and isotype control. Data representative of at least three experiments are shown. Bars represent standard error. * P<0.05; ** P<0.01; ***P<0.001, ****P<0.0001.

A dose titrated response to HDM was additionally observed (Figure 6.4 A, B).

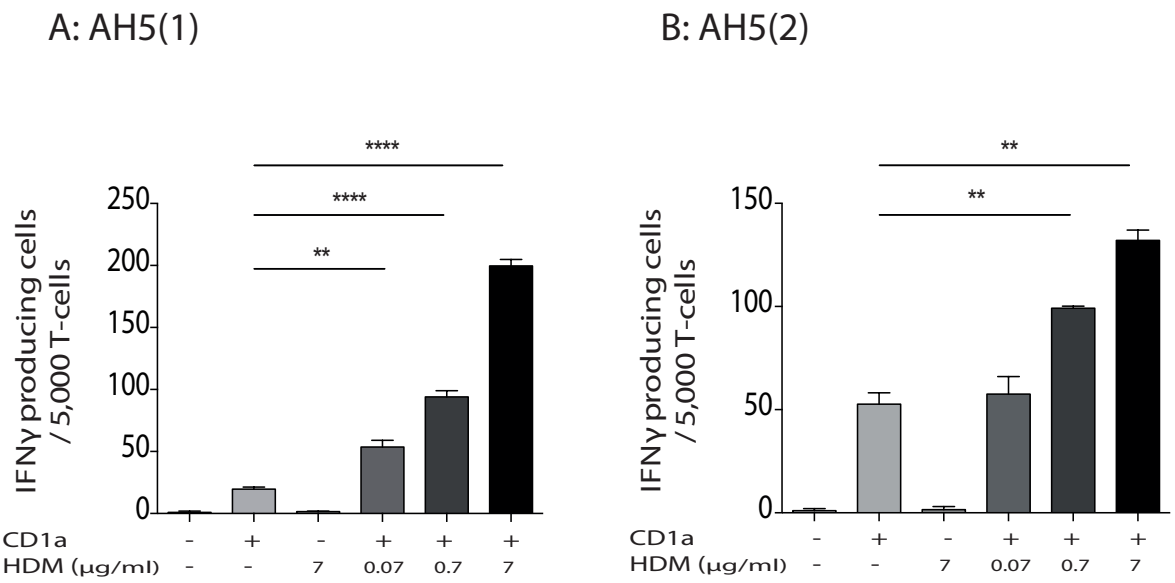


Figure 6.4. HDM responsive CD1a-reactive T cell lines generated using the IFNγ secretion assay respond to HDM in a dose dependent manner. T cell lines generated using the IFN-γ secretion assay were incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract at increasing HDM concentrations. Data representative of at least two experiments for each T cell line are shown. Bars represent standard error. ** P<0.01; ***P<0.001

Dual-colour IFNγ/IL13 Fluorospot was performed using the HDM responsive CD1a-reactive T cell lines AH5(1) and AH5(2) to determine the type 1 and type 2 cytokine producing subsets of these T cell lines (Figure 6.5). The majority of the type 1 and type 2 cytokine-producing cells were unique subsets, with a range of 2-10% of CD1a-reactive HDM-responsive T cells producing both IFNγ and IL-13 (Figure 6.5).

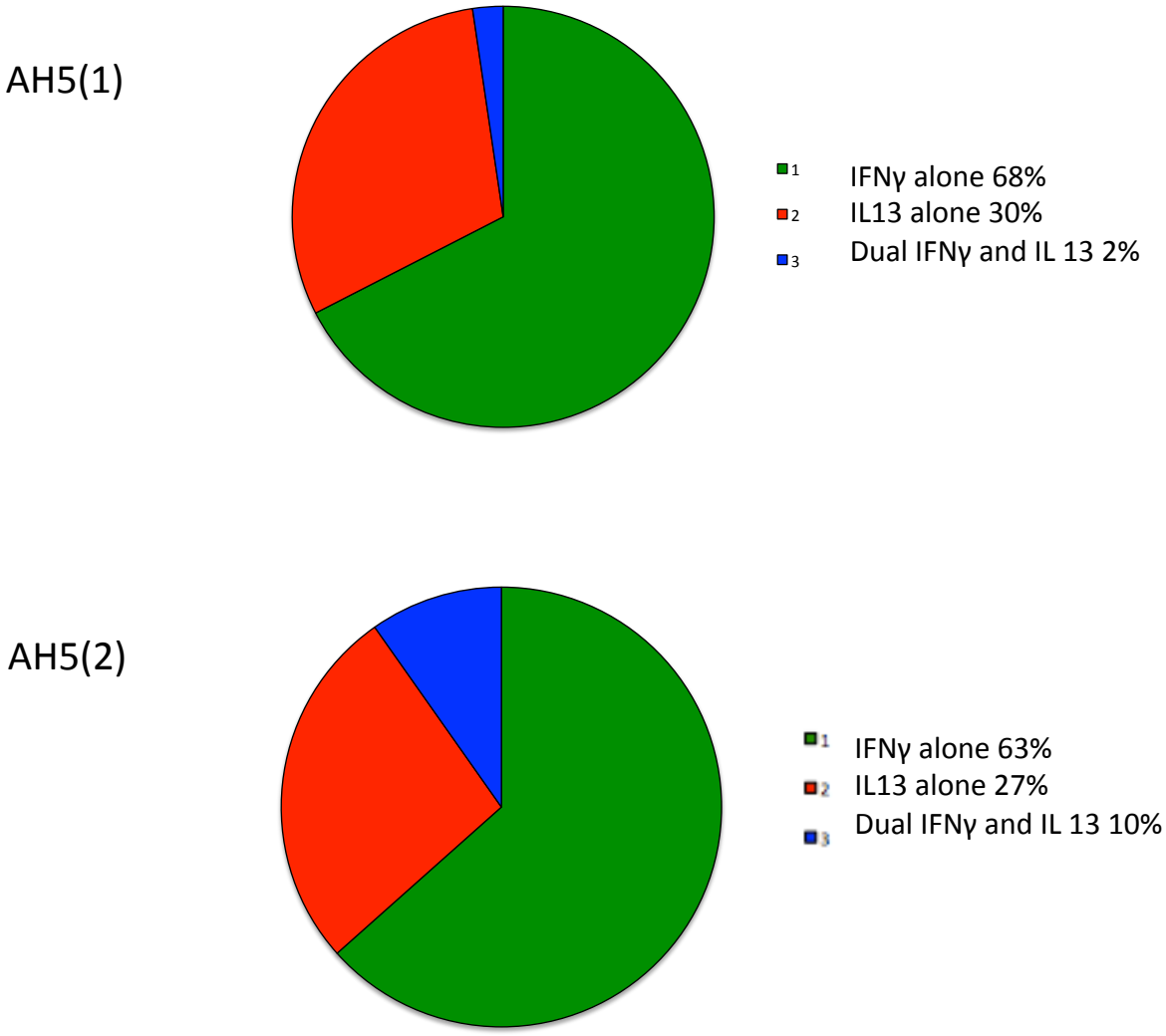


Figure 6.5. Dual–colour IFNγ and IL-13 production of HDM-responsive CD1a-reactive T cell lines. Dual–colour IFNγ and IL-13 production were measured by FluoroSpot to determine type 1 and type 2 cytokine producing subsets.

6.2(ii) HDM responsive CD1a-reactive T cell lines respond to primary CD1a positive APC in a CD1a dependent manner

To investigate whether primary CD1a positive APCs additionally mediate HDM responsive CD1a-reactive T cell line responses, autologous monocytes were differentiated with GM-CSF and IL-4 to produce monocyte-derived myeloid dendritic

cells (mDCs) and also activated *in vitro* with cytokines to mimic LCs (LC-like cells) (508-511). As shown in chapter 4, flow cytometry analysis demonstrated differentiation, with high CD1a expression in mDCs, and high CD1a and Langerin expression in LC-like cells (Figure 4.6). Pulsing overnight with HDM did not affect mDC or LC-like cell maturation (Figure 4.7).

Langerhans cell-like cells were also differentiated from cord-blood derived CD34+ progenitor cells as described (405) to provide an alternate system of primary CD1a+ cell generation. Flow cytometry demonstrated CD1a and Langerin expression (Figure 4.9).

Furthermore, as an additional system for the generation of CD1a+ APC, CD1a+ cells were isolated from human skin using magnetic separation. Flow cytometry demonstrated successful enrichment, with the majority of the CD1a positive cells being Langerin positive Langerhans cells (Figure 6.6).

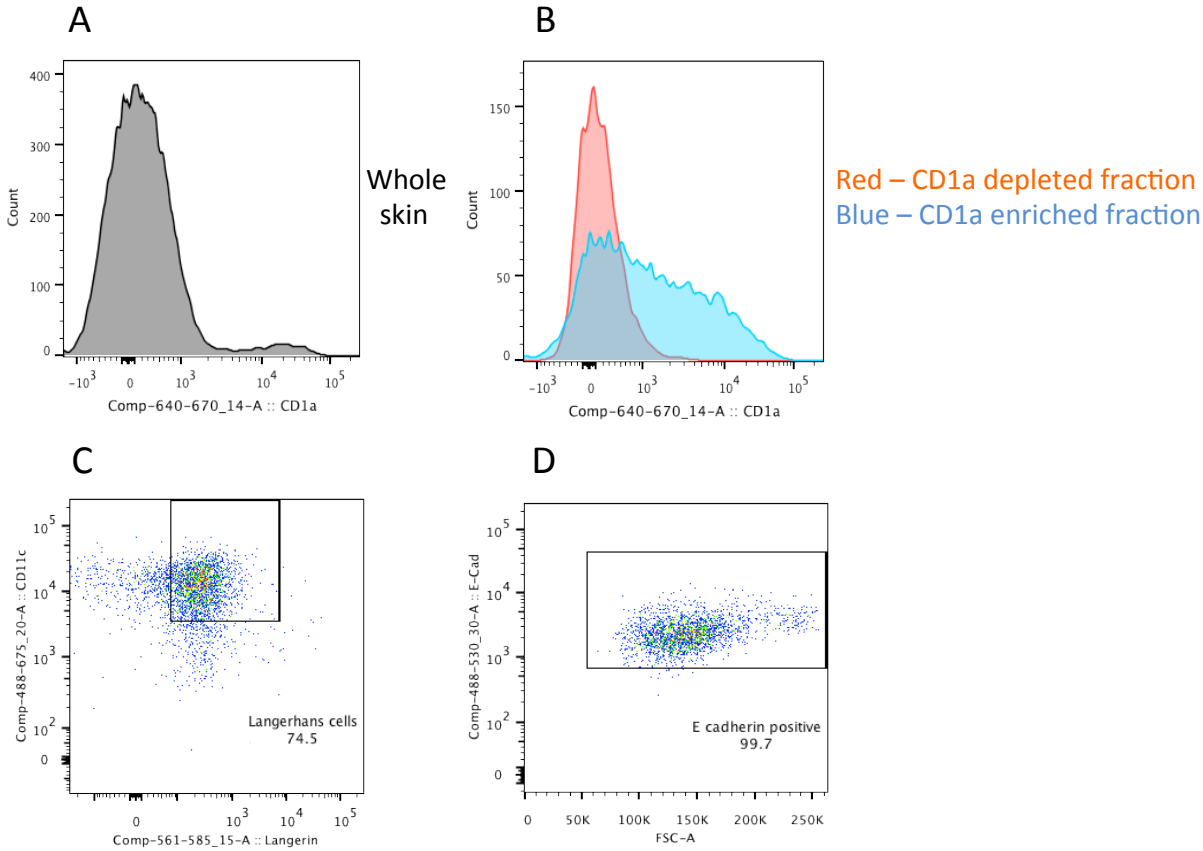
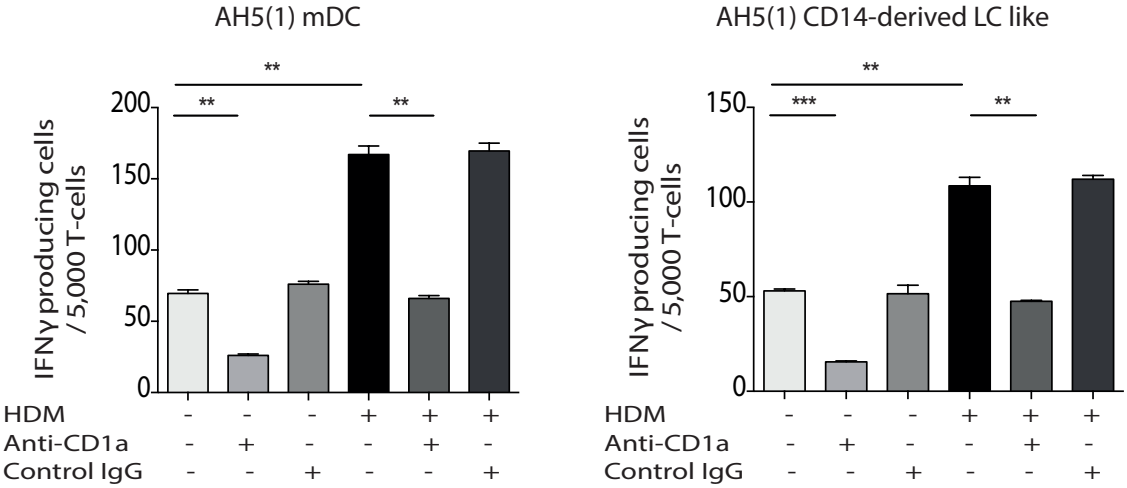


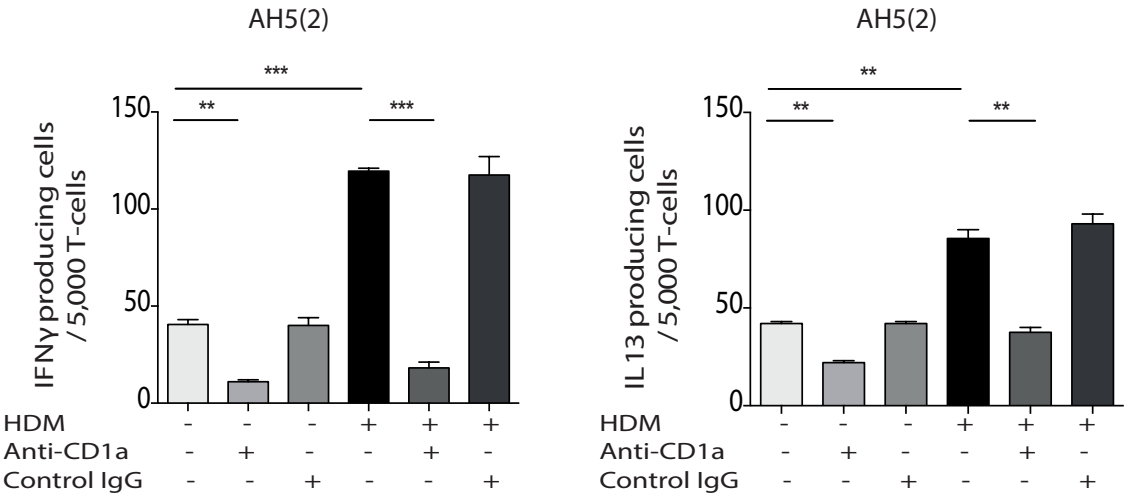
Figure 6.6. CD1a+ cell isolation from human skin. CD1a+ cells were isolated from human skin by MACS bead separation. Representative histograms pre- (A, whole skin) and post- CD1a+ cell isolation (B Red-CD1a depleted fraction, Blue – CD1a enriched fraction) documenting CD1a positive cells (gated on live cells and singlets). Plot C gated on CD1a positive cells illustrating Langerin positive Langerhans cells in the CD1a enriched fraction, which also stain positive for E-cadherin (Plot D gated on plot C).

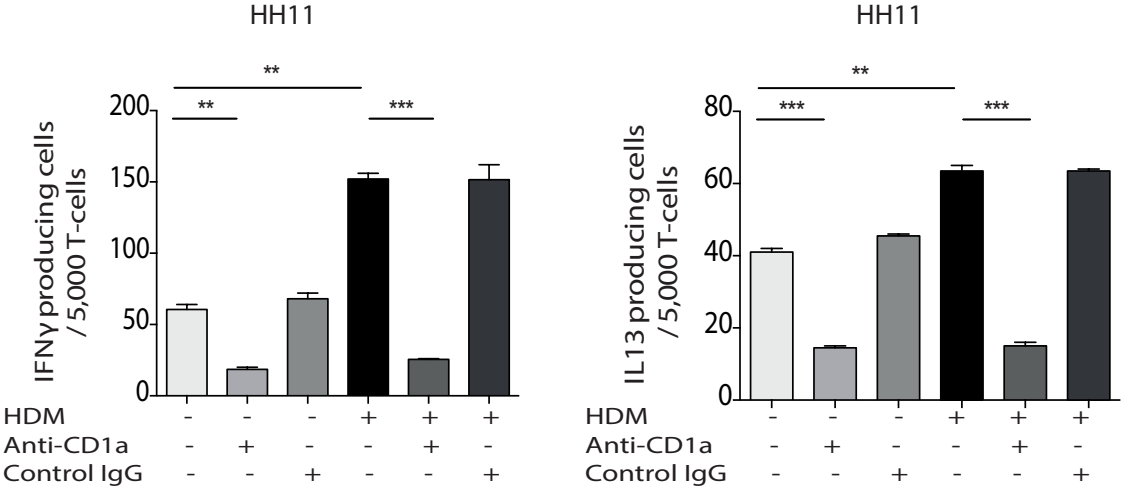
HDM responsive CD1a-reactive T cell lines responded to the different types of CD1a+ APCs in IFNγ and IL 13 ELISpot assays (Figure 6.7 A,B,C).

A mDC, CD14-derived LC-like



B Cord-blood derived LCs





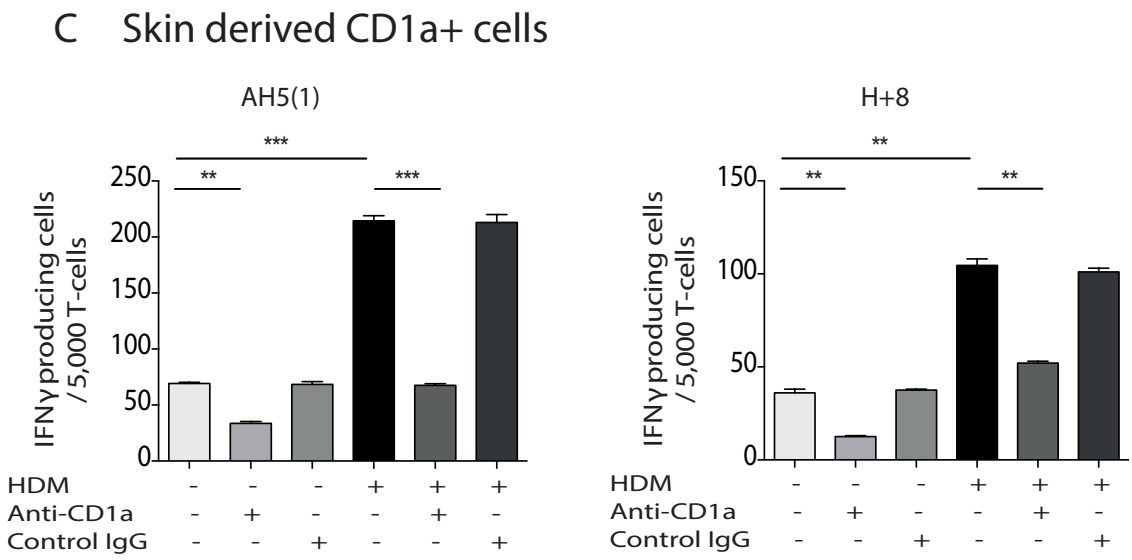


Figure 6.7. HDM responsive CD1a-reactive T cell lines also respond in a CD1a-restricted manner to mDCs, LC-like cells, cord blood derived LCs and skin derived CD1a+ cells. CD1a-expressing *in vitro* derived mDC or LC-like cells (A), cord blood derived LCs (B) and skin derived CD1a+ cells (C) were pulsed with HDM extract overnight and incubated with HDM responsive CD1a-reactive T cell lines generated by the IFNγ secretion assay. IFNγ and IL- 13 production was measured by ELISpot in the presence or absence of anti-CD1a antibody, isotype control and HLA Class I and II blocking antibodies. Bars represent standard error. ** P<0.01; ***P<0.001.

6.3 IFNγ ELISA CD1a plate bound assay results

The CD1a plate bound assay was also used to examine CD1a dependency of selected HDM responsive CD1a-reactive T cell lines. In this assay, recombinant CD1a protein is coated onto plates which are then pulsed with HDM extract and washed prior to co-incubation with the T cell lines. T cell reactivity was then

examined by IFNγ ELISA of T cell line supernatants. No T cell stimulation was seen on HDM exposure in the absence of CD1a protein (Figure 6.8). The CD1a autoreactive response can be seen as CD1a reactivity to unpulsed CD1a protein (i.e. in the absence of HDM) (Figure 6.8). Increased IFNγ secretion in response to CD1a protein pulsed with HDM extract above the autoreactive response can additionally be seen (Figure 6.8).

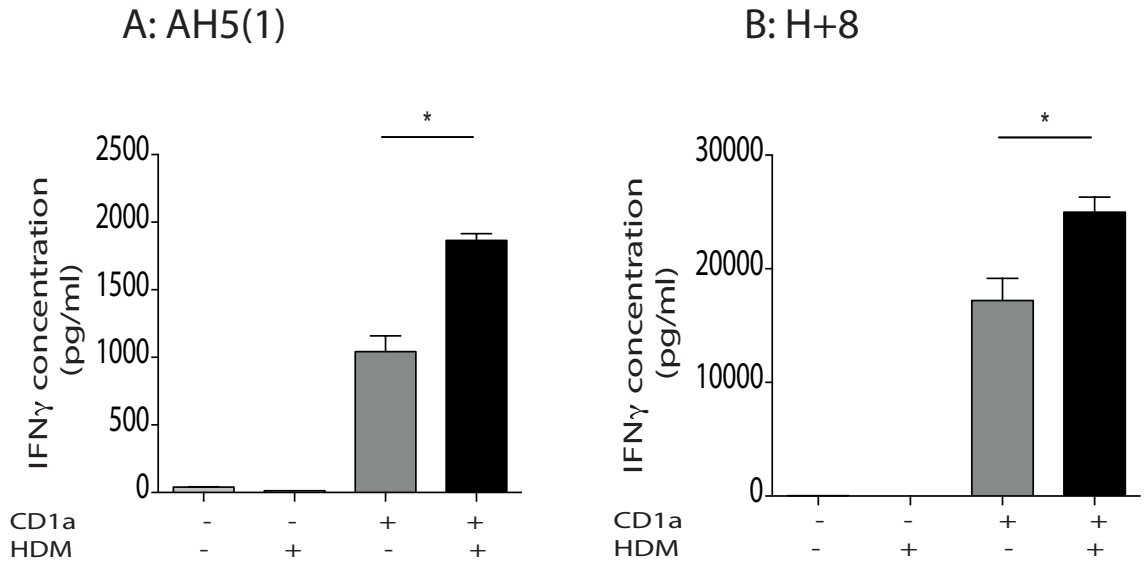


Figure 6.8. HDM stimulates HDM responsive CD1a-reactive T cell lines in the CD1a plate bound assay. CD1a protein was coated onto Nunc maxisorp plates. After washing HDM was added for 48 hours. Plates were washed again, and AH5(1) (A, left) and H+8 (B, right) T cell lines were added. Supernatants were collected after 24 hours and analysed using IFNγ ELISA. Data are representative of three separate experiments. Bars represent standard error. * P<0.05.

Both the CD1a autoreactive and HDM reactive CD1a plate bound assay responses were dependent on CD1a as they could be abrogated by anti-CD1a blocking antibody (Figure 6.9).

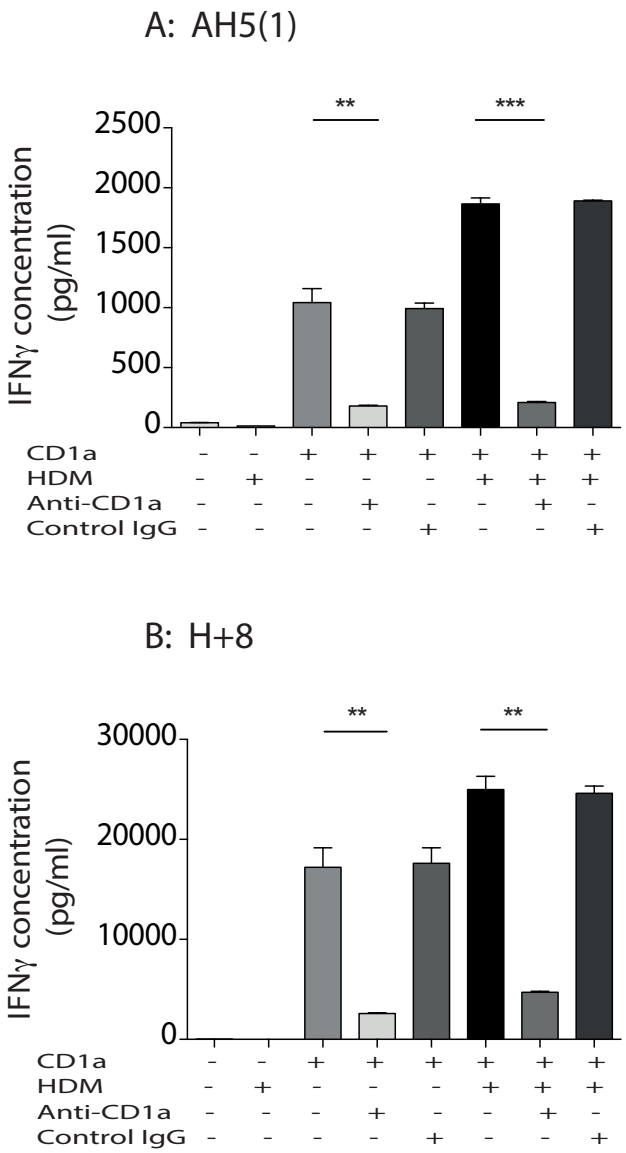


Figure 6.9. The HDM responsive CD1a-reactive T cell line response observed in the CD1a plate bound assay is CD1a dependent. CD1a protein was coated onto Nunc maxisorp plates. After washing HDM was added for 48 hours, plates were washed again, and AH5(1) (A, left) and H+8 (B, right) T cell lines were added in the presence or absence of anti-CD1a antibody and isotype control. Supernatants were collected after 24 hours and analysed using IFNγ ELISA. Data are mean of duplicate results and SEM. ** P<0.01; ***P<0.001.

A CD1a restricted HDM response could also be seen in the CD1a plate bound assay in polyclonal T cells derived from blood (Figure 6.10A) and skin (Figure 6.10B).

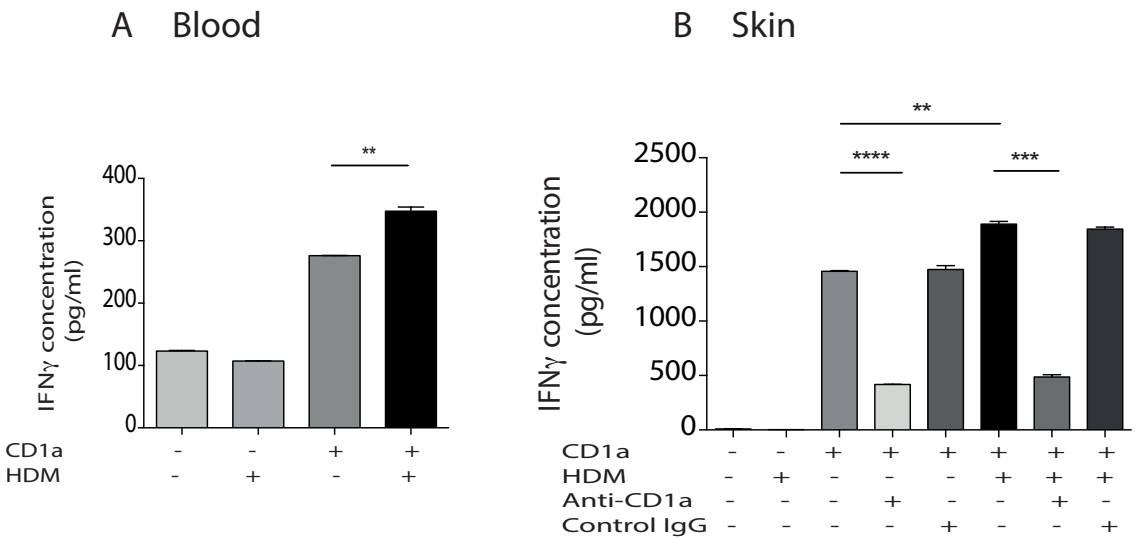


Figure 6.10. HDM stimulates polyclonal HDM responsive CD1a-reactive T cells in the CD1a plate bound assay. CD1a protein was coated onto Nunc maxisorp plates. After washing HDM was added for 48 hours, plates were washed again, and polyclonal T cells derived from blood (A, left) and skin (B, right) were added. Supernatants were collected after 24 hours and analysed using IFNγ ELISA. Data are representative of three separate experiments. Bars represent standard error. ** P<0.01; ***P<0.001, ****P<0.0001.

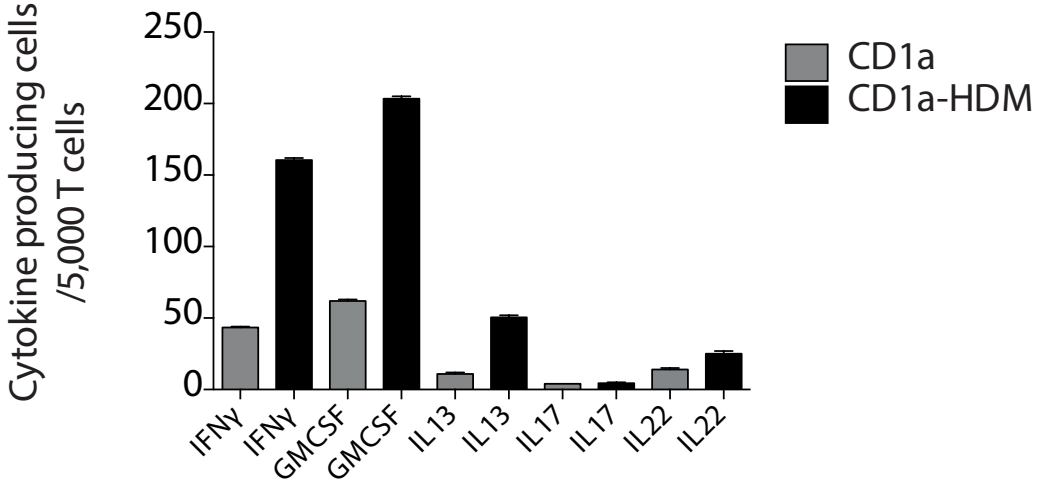
6.4 Cytokine production of HDM responsive CD1a-reactive T cell lines

As shown above, IFNγ and IL-13 have been detected as being produced by both polyclonal HDM responsive CD1a reactive T cells derived from blood (Chapter 4) and skin (Chapter 5), and by HDM responsive CD1a reactive T cell lines (Chapter 6) by ELISpot analysis and by the CD1a plate bound assay. These results indicate broad type 1 and type 2 cytokine production by these cells.

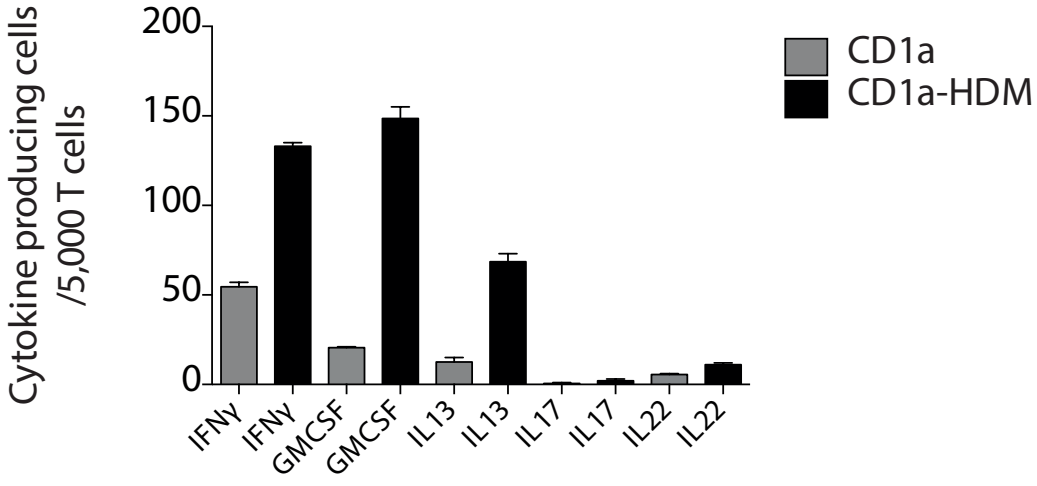
However, it is also important to more broadly characterise their functional profile to gain an insight into their effector function during an immune response.

In order to broadly characterise the functional profile of selected HDM responsive CD1a reactive T cell lines, GM-CSF, IL-17 and IL-22 production were therefore also examined by ELISpot (Figure 6.11 A, B, C), and IFNγ, IL-13, IL-17, IL-22 and IL-10 production were investigated using RT-qPCR (Figure 6.12A, B). In particular, IL-22 production was investigated because, as described, autoreactive CD1a-restricted T cells have been recently shown to produce IL-22 (119), and this cytokine is believed to have an important role in cutaneous immunity in the context of AD (327).

A: AH5(1)



B: AH5(2)



C: H+8

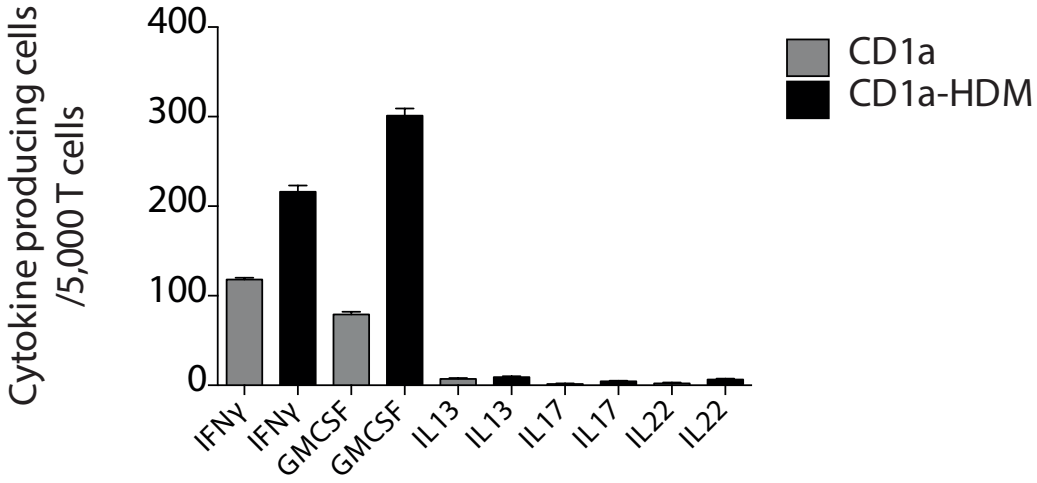


Figure 6.11. Cytokine production of HDM responsive CD1a-reactive T cell lines by ELISpot analysis. CD1a dependent production of IFNγ, GM-CSF, IL-13, IL-17 and IL-22 was determined by cytokine ELISpot analysis with K562 or K562-CD1a in the presence or absence of HDM for the T cell lines AH5(1) (A), AH5(2) (B) and H+8 (C). Data are mean of duplicate experiments. Bars represent standard error.

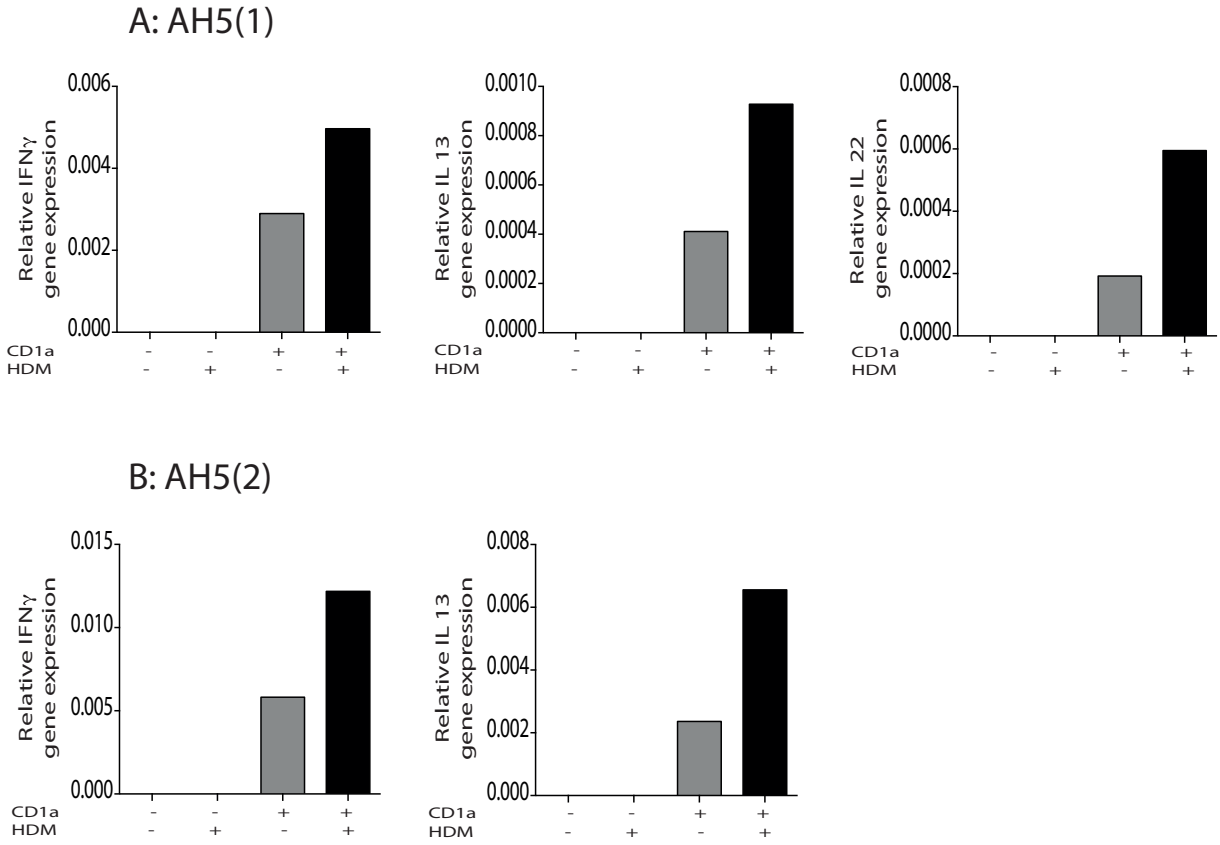


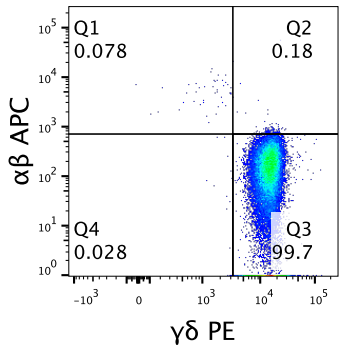
Figure 6.12. IFNγ, IL-13 and IL-22 production by RT-qPCR by CD1a-reactive T cell lines in response to HDM. Real-time PCR analysis of IFNγ, IL-13 and IL-22 message in HDM responsive CD1a-reactive T cell lines AH5(1) (A) and AH5(2) (B) following co-culture with plate bound CD1a protein in the presence or absence of HDM extract. Results are normalised to GAPDH. Data are mean of duplicate experiments.

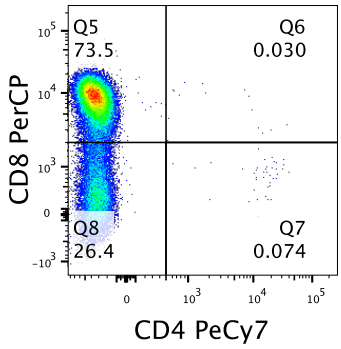
These experiments largely confirmed previous findings, with the HDM responsive CD1a-reactive cell lines investigated producing cytokines classically associated with a type 1 (IFNγ), and type 2 (GM-CSF and IL-13) response (Figure 6.11, Figure 6.12). IL-22 production was also observed by RT-qPCR in AH5(1), one of the investigated T cell lines (Figure 6.12), however it would be interesting to extend these investigations to all of the T cell lines generated, particularly to AA9005 which was derived from the skin. No IL-17 or IL-10 production was detected by ELISpot or RT-qPCR (Figure 6.11, Figure 6.12).

6.5 Cell surface marker expression

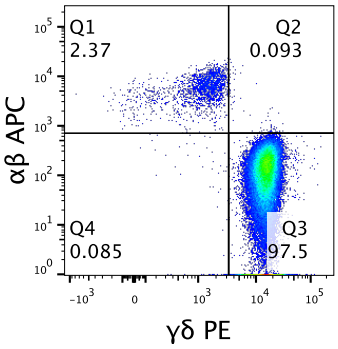
In order to better understand the phenotype of the HDM-responsive CD1a-reactive T cell lines generated, cell surface marker expression of key cell surface molecules was also determined by Flow Cytometry (Figures 6.13, 6.14).

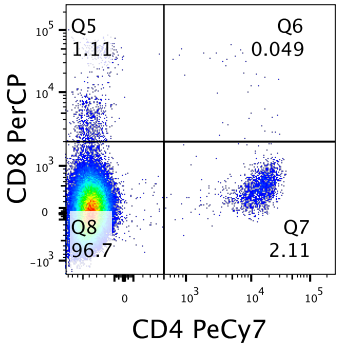
AH5(1)



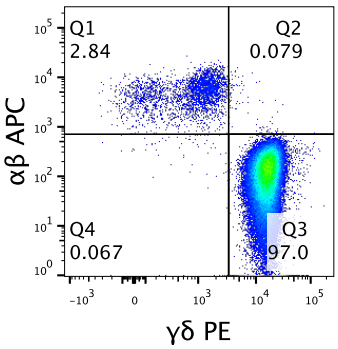


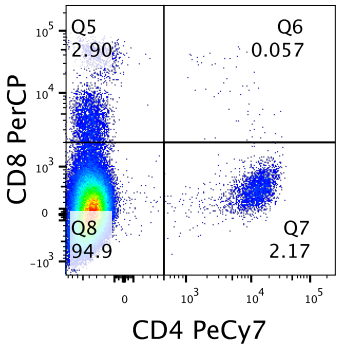
HH11



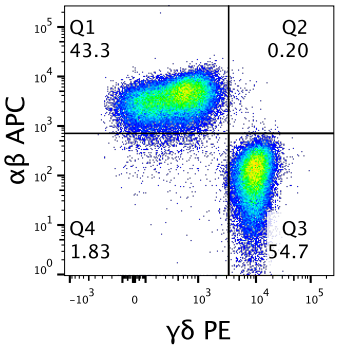


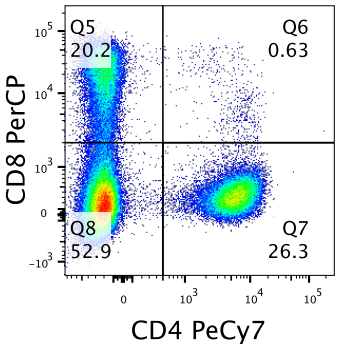
H+8



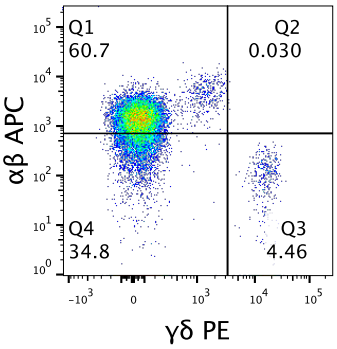


AH5(2)





AA9005



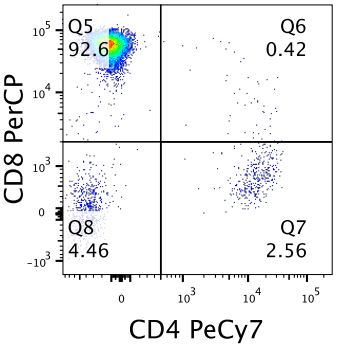


Figure 6.13. Surface expression of cell surface markers on AH5(1), HH11, H+8, AH5(2) and AA9005 HDM responsive CD1a-reactive T cell lines. Flow cytometric analysis of γδ TCR, αβ TCR, CD4 and CD8 on HDM responsive CD1a-reactive T cell lines. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using isotype controls. Left plot: γδ TCR PE (x axis), αβ TCR APC (y axis). Right plot: CD4 PeCy7 (x axis), CD8 PerCP (y axis).

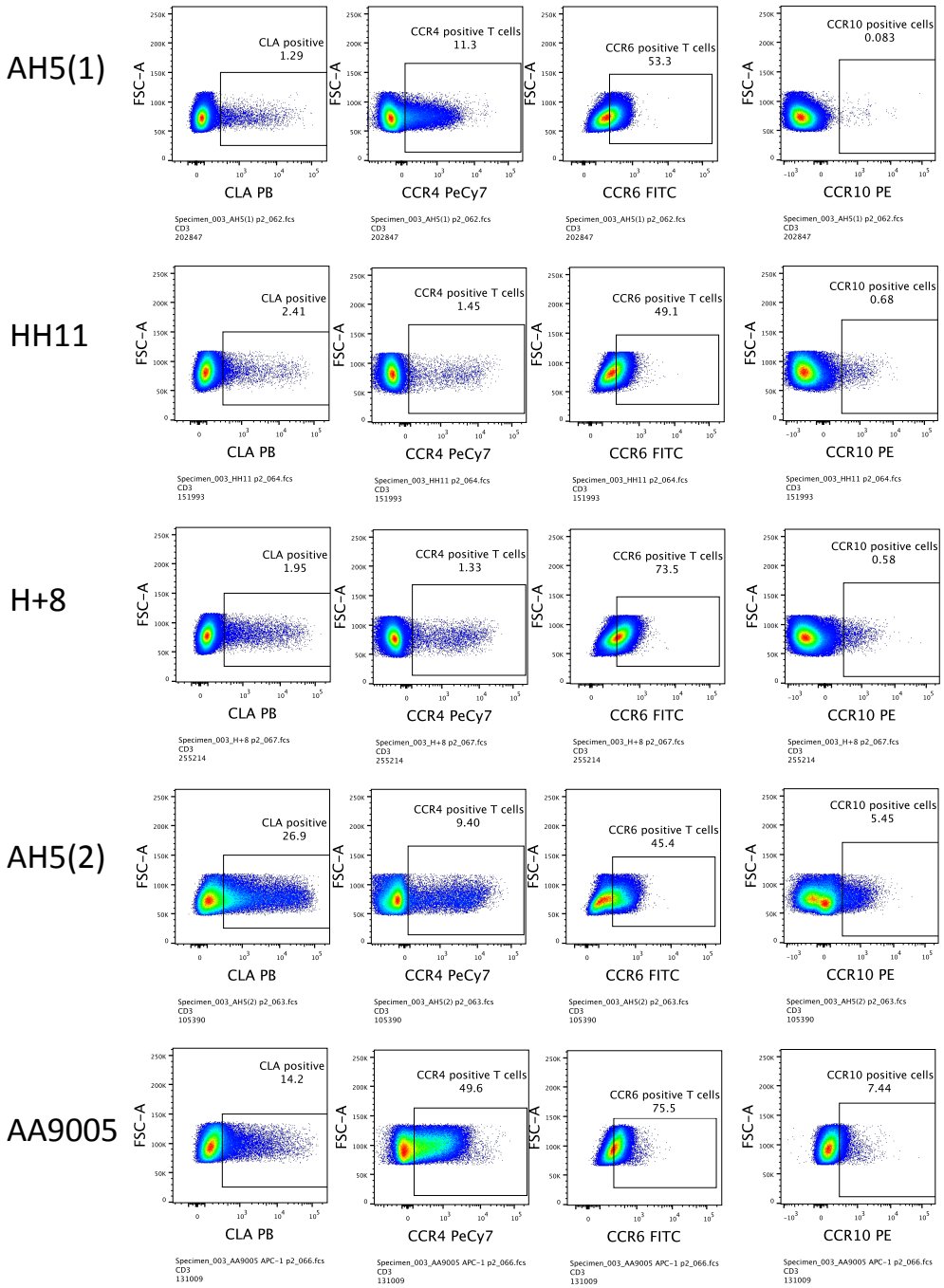


Figure 6.14. Surface expression of skin homing markers on AH5(1), HH11, H+8, AH5(2) and AA9005 HDM responsive CD1a-reactive T cell lines. Flow cytometric analysis of the skin homing markers (left to right) CLA, CCR4, CCR6 and CCR10 on HDM responsive CD1a-reactive T cell lines. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using isotype controls.

Fascinatingly, three of the five T cell lines (AH5(1), HH11 and H+8) generated were composed of predominantly γδ T cells (Figure 6.13), the forth (AH5(2)) consisted of a mixed population of γδ and αβ T cells and the fifth (AA9005) was predominantly αβ T cells. It will obviously be important to attempt to define the responding T cell subset but of note the poorly understood class of γδ T cells have been postulated to have a role in cutaneous immunity (369), and γδ TCRs have been reported to bind CD1 molecules (1). If proven to have a role in the HDM responsive CD1a-reactive T cell response, the involvement of γδ T cells is potentially a new and exciting finding of CD1a-restricted T cells.

In keeping with a γδ T cell predominance, the majority of the HDM responsive CD1a-reactive T cell lines were found to be composed of CD8+ or double negative T cells, although approximately one quarter of the T cells in the T cell line AH5(2) stained positive for CD4 (Figure 6.13).

Supporting a role for these cells in cutaneous immunity, expression of the skin homing markers CLA, CCR4, CCR6 and CCR10 was present but varied throughout the T cell population and between T cell lines (Figure 6.14).

Given the unexpected finding of γδ T cell enriched HDM responsive CD1a-reactive T cell lines, preliminary experiments were conducted to investigate this further. Polyclonal T cells derived from the blood from two donors and skin blister fluid from one donor were sorted into γδ and αβ subsets prior to IFNγ ELISpot to examine

CD1a reactivity. Interestingly, HDM responsive CD1a-reactive T cell responses were enriched in the γδ T cell fraction in 2 out of 3 donors (Figure 6.15).

Furthermore in the IFN-γ secretion assay, a population of αβ TCR negative CD3 positive T cells can be observed within the K562-CD1a HDM reactive population (Figure 6.16).

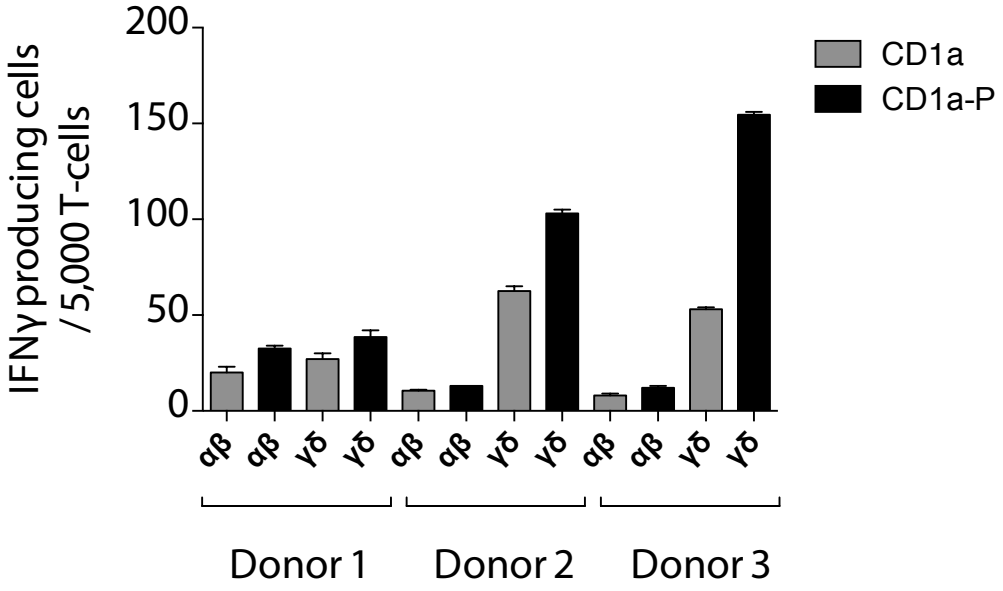


Figure 6.15. HDM responsive CD1a-reactive T cell responses are enriched in the γδ T cell fraction of T cells derived from the blood and skin. *Ex-vivo* PBMCS (donor 1 AD9501A, donor 2 AD9500) and T cells expanded from skin blister fluid (donor 3 AA9005) were FACs sorted into CD3 positive γδ positive and CD3 positive αβ positive subsets prior to IFNγ ELISpot to examine CD1a reactivity. Responses to K562-EV and K562-EV pulsed with HDM were consistently low and have therefore been omitted for graph simplicity. Data representative of three donors.

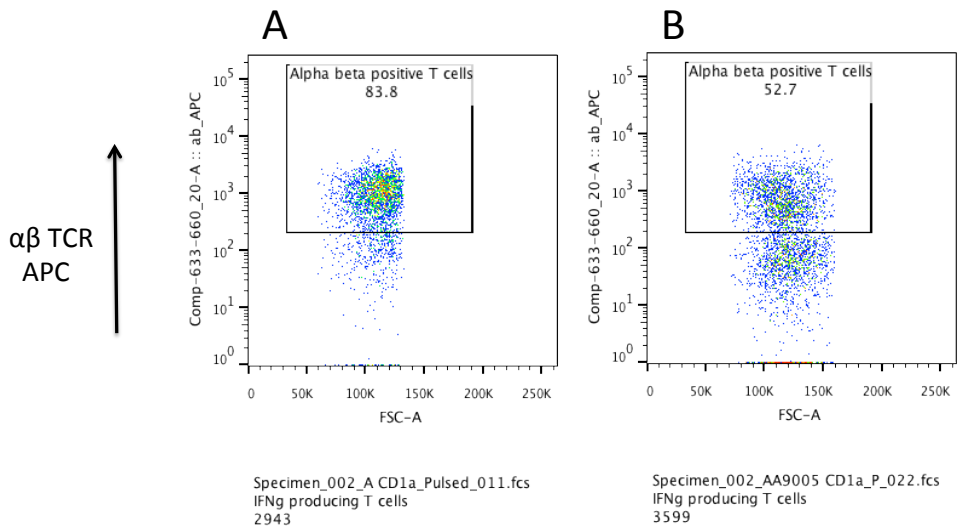


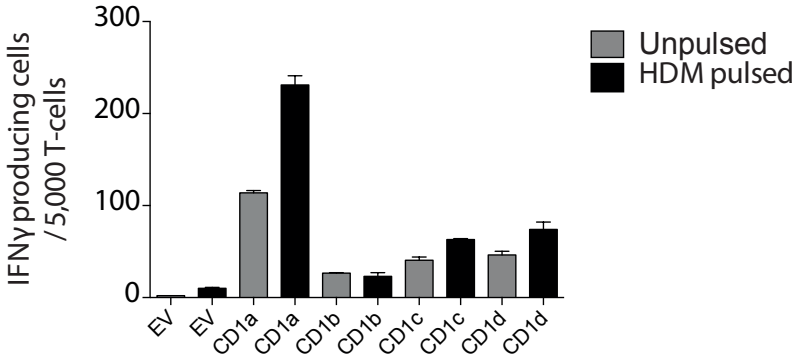
Figure 6.16. Flow cytometric analysis of αβ TCR staining of IFN-γ producing CD3+ cells on stimulation of K562-CD1a pulsed with HDM in the IFN-γ secretion assay. Plots gated on singlets, live cells, and IFN-γ producing CD3+ T cells. A – donor AD9501A (blood-derived T cells), B – donor AA9005 (skin blister-derived T cells).

These experiments constitute preliminary work which needs to be expanded and validated but the involvement of γδ T cells in the HDM responsive CD1a-reactive T cell response remains a fascinating possibility to bear in mind.

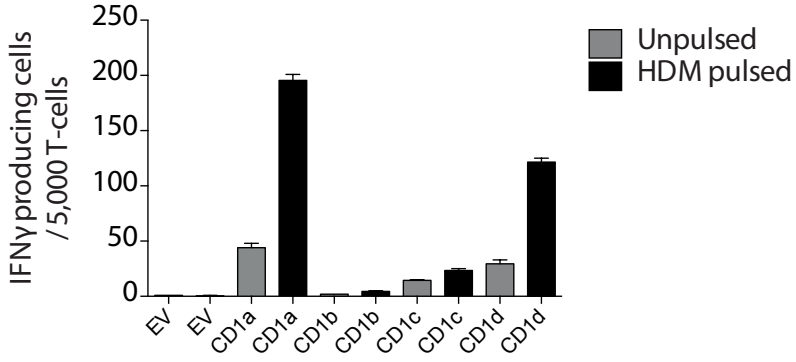
6.6 T cell line reactivity to different CD1 isoforms

HDM responsive CD1a-reactive T cell lines generated were also examined for reactivity to the other CD1 isoforms, by performing IFNγ ELISpot against K562-CD1a, K562-CD1b, K562-CD1c and K562-CD1d in the presence and absence of HDM extract (Figure 6.17).

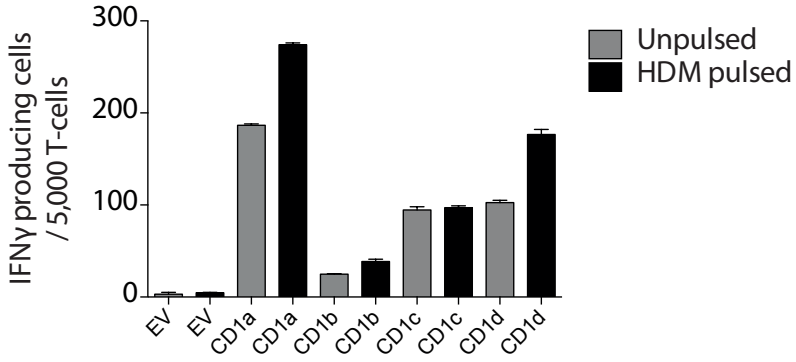
A : AH5(1)



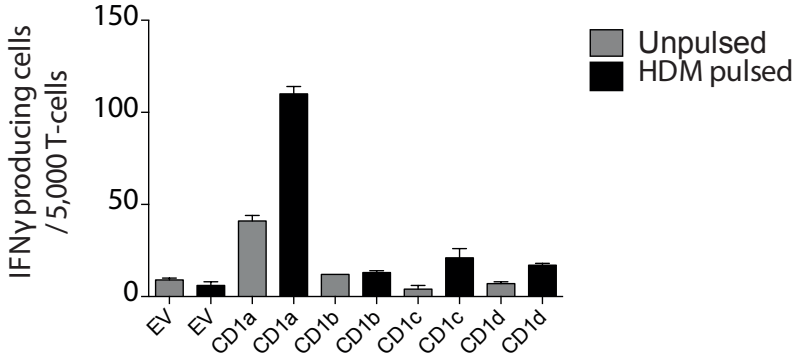
B: HH11



C: H+8



D: AH5(2)



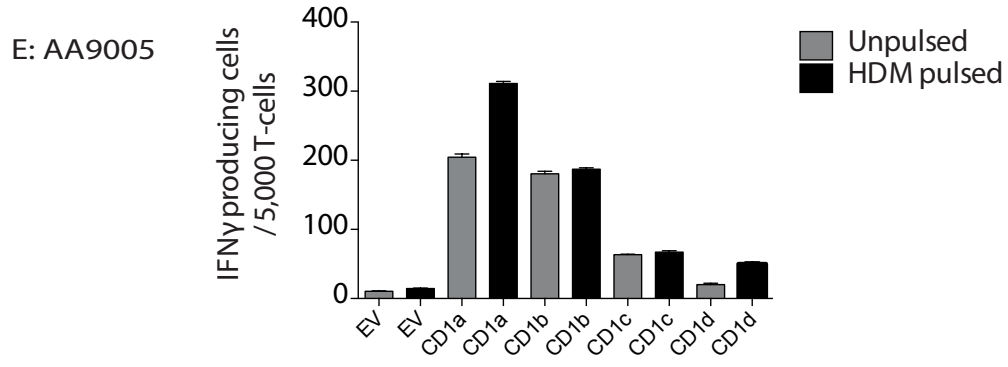


Figure 6.17. HDM responsive CD1a-reactive T cell lines react to more than one CD1 isoform. HDM responsive CD1a-reactive T cell line responses to untransfected or CD1a, CD1b, CD1c or CD1d transfected K562 cells in the presence or absence of HDM were analysed by IFNγ ELISpot. One of two separate experiments shown.

Having previously observed that anti-CD1a antibody abrogated the response of each HDM-responsive CD1a-reactive T cell line, it was interesting to see that each line was able to respond to varying degrees to the other group 1 (K562-CD1a, -b, -c) and group 2 (K562-CD1d) CD1 isoforms (Figure 6.17).

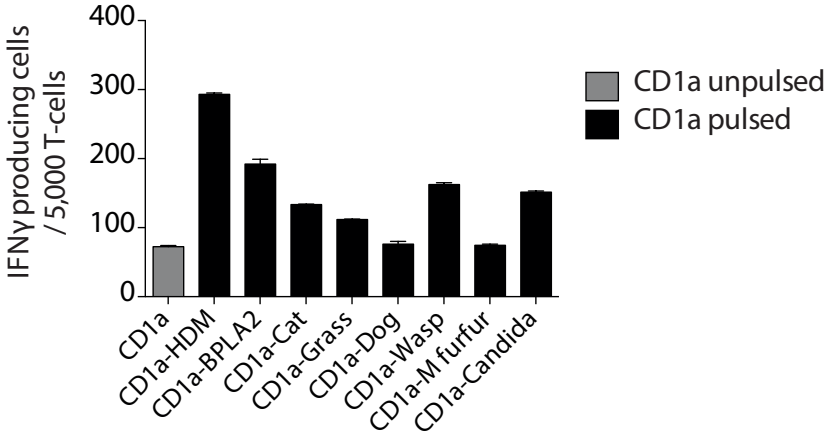
Cross-contamination of the transfected K562-CD1a cells into the flasks of other K562-CD1 isoforms was excluded as a possible explanation for this by flow cytometry (Figure 4.4). A further possible explanation which will be important to explore further as a part of future studies is that the HDM responsive CD1a-reactive T cell lines might contain oligoclonal T cell populations possessing multiple TCR specificities. A degree of antigen promiscuity has been described amongst CD1 molecules (98), however T cell reactivity to multiple CD1 isoforms is yet to be described in the literature. Therefore recognition of multiple CD1 isoforms by a presumed oligoclonal T cell population is an interesting finding which is potentially a novel finding of CD1a-reactive T cells.

6.7 Antigen specificity of HDM responsive CD1a-reactive T cell lines

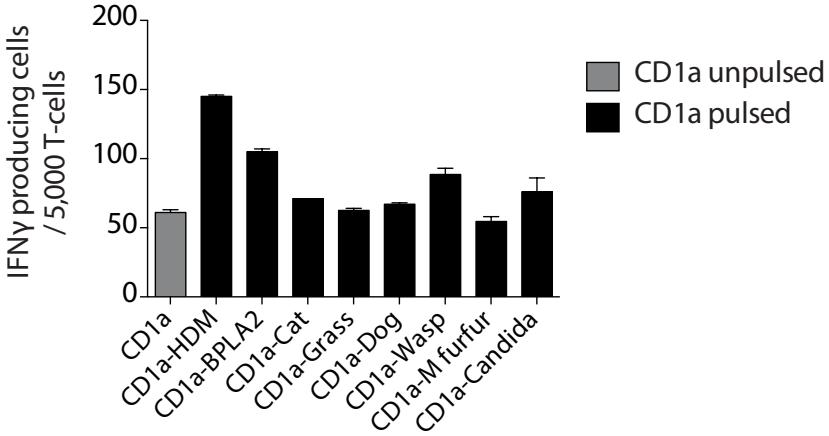
Antigen specificity of the HDM responsive CD1a-reactive T cell lines was next investigated by examining CD1a dependent antigen reactivity to a panel of antigens known to be clinically relevant to AD (524) using IFNγ ELISpot (Figure 6.18).

These included grass pollens, animal danders (dog, cat), and moulds including candida albicans and Malassezia furfur in addition to bee phospholipase A2 and wasp venom, which were investigated because of recently reported findings that insect venoms are capable of eliciting CD1a-dependent T cell responses (469). Responses to K562-EV, either unpulsed or pulsed with antigen were consistently absent and thus have been omitted from Figure 6.18 for simplicity.

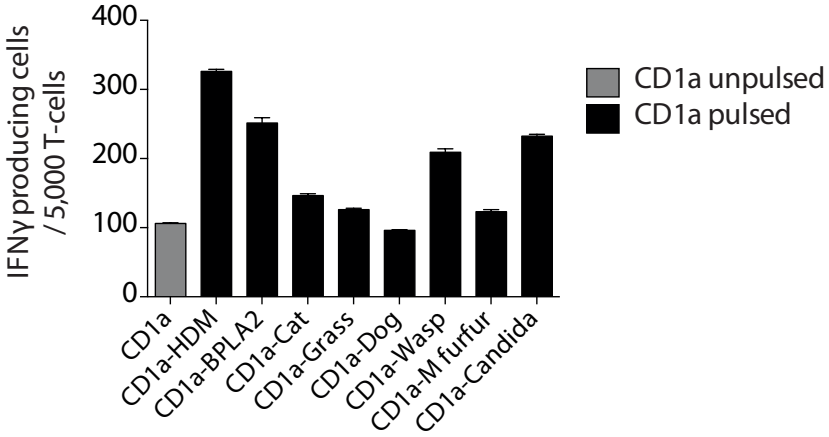
A: AH5(1)



B: HH11



C: H+8



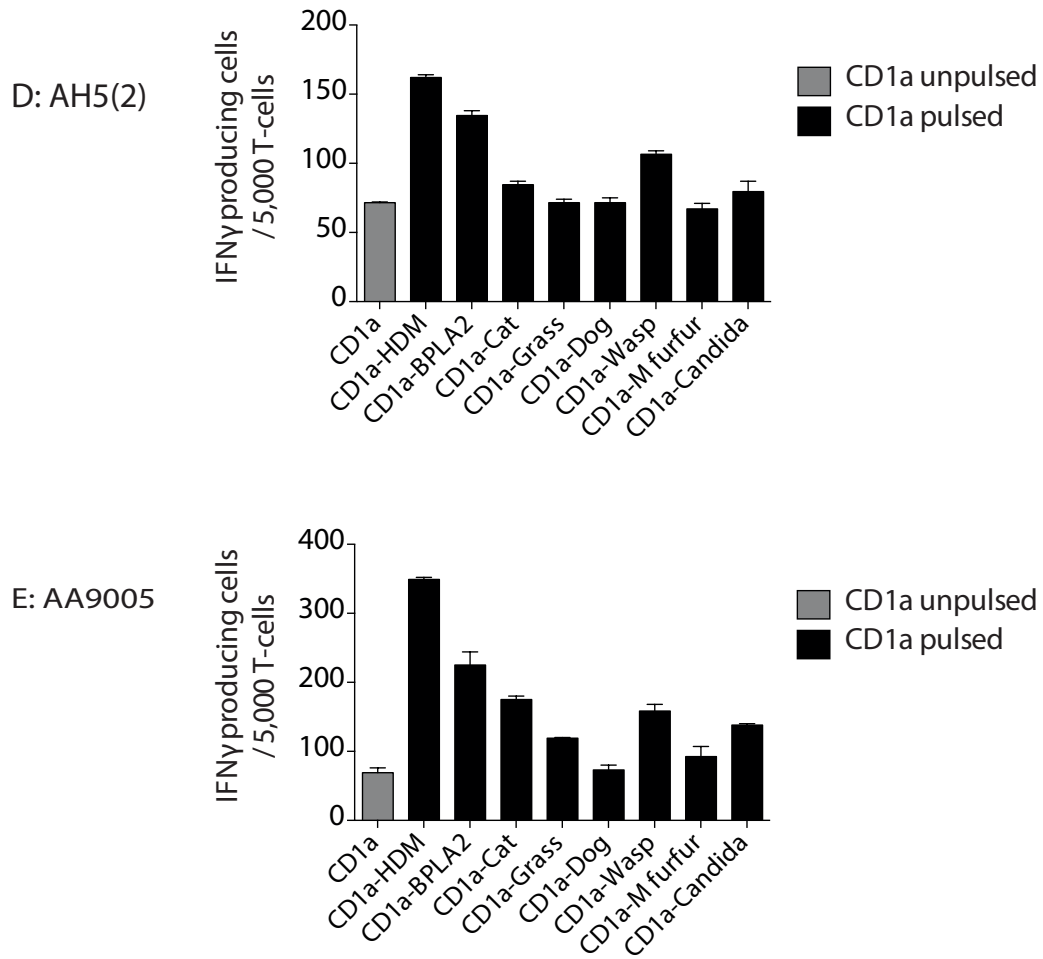


Figure 6.18. HDM responsive CD1a-reactive T cell lines react in a CD1a-restricted manner to numerous allergens. HDM responsive CD1a-reactive T cell line responses to untransfected or CD1a transfected K562 cells in the presence or absence of multiple allergen preparations were analysed by IFNγ ELISpot. Absent untransfected K562 results not shown for graph simplicity. One of two separate experiments shown.

Of interest, although the response to HDM was always the highest at the concentrations used (Figure 6.18), multiple allergens were capable of stimulating CD1a-restricted IFNγ production from HDM-responsive CD1a-reactive T cell lines,

including BPLA2, Wasp venom, and Cat and Candida albicans to varying extents (Figure 6.18), indicating considerable antigen cross reactivity.

There has recently been exciting progress regarding the mechanism by which the CD1a-restricted TCR interacts with the CD1a-lipid complex, with the reported findings that CD1a is capable of presenting headless lipid antigens (55) and the notion of *"permissive"* and *"non permissive"* lipid ligands (525). It has been reported that direct contact between the CD1a molecule and TCR is important for TCR interaction (525) and is thought that bound *"headless"*, or *"permissive"*, lipid antigens stabilise CD1a in the correct conformation for TCR binding by sequestering deep within the CD1a antigen binding groove. In this way, CD1a-reactive T cells may be stimulated by a number of highly hydrophobic substances that fit in the CD1a groove rather than recognising single compounds with high specificity (57).

This proposed model may form the basis for the antigen cross-reactivity observed in the HDM responsive CD1a-reactive T cell lines, or as with the cross-reactivity to other CD1 isoforms it may be that HDM responsive CD1a-reactive T cell lines contain oligoclonal T cell populations with differing TCR specificity. Furthermore a common lipid antigen, or antigen generation pathway may be shared by the IFNγ eliciting antigens.

It is clearly important to investigate this further, particularly with regard to what is the CD1a reactive fraction of HDM. This will be examined in the next chapter: investigation of phospholipase in the generation of CD1a ligands.

Chapter 6 summary

Despite the recent advent of CD1a tetramers, the lack of a readily available surface marker for CD1a-restricted T cells presents a challenge for the examination of the phenotype of HDM responsive CD1a-reactive T cells. Therefore, the IFN-γ secretion assay was utilised as a tool to generate HDM responsive CD1a-reactive T cell clones for further characterisation of their phenotype to increase our understanding of their patho-physiological function.

HDM was found to stimulate HDM responsive CD1a-reactive T cell lines in a CD1a-dependent manner, and this response could be abrogated by anti-CD1a antibody, demonstrating CD1a specificity. HDM responsive CD1a-reactive T cell lines were also found to respond to primary $CD1a^+$ APCs in a CD1a-dependent manner. Dual-colour Fluorospot demonstrated that the majority of type 1 and type 2 cytokine producing cells were unique subsets, and IL-22 was also detected as being produced by these cells in a CD1a-restricted manner on ELISpot and on RT-qPCR. No IL-17 or IL-10 production was detected.

The CD1a-restricted response to HDM seen with the CD1a plate bound assay argues against an altered endogenous lipid profile inducing the response seen on HDM treatment of K562-CD1a cells or other primary $CD1a^+$ APCs. Interestingly, three of the five T cell lines (AH5(1), HH11 and H+8) generated were composed of predominantly γδ T cells, the fourth (AH5(2) consisted of a mixed population of γδ and αβ T cells and the fifth (AA9005) was predominantly αβ T cells. Of note and in keeping with a γδ T cell predominance, the majority of the HDM responsive CD1a-reactive T cell lines were found to be mainly composed of $CD8^+$ or double negative T cells in terms of co-receptor expression. Skin homing marker expression was present but varied throughout the T cell population and between the T cell lines, with the highest expression seen in the skin derived T cell line AA9005.

HDM responsive CD1a-reactive T cell line reactivity to additional CD1a isoforms was observed and considerable antigen cross-reactivity was also seen. This will be explored in future studies in the laboratory.

Cytokine production by the HDM responsive CD1a-reactive T cell lines was essentially the same as the polyclonal HDM responsive CD1a-reactive T cell responses observed in blood (Chapter 4) and skin (Chapter 5), in addition to the autoreactive, and pollen- and venom-reactive CD1a-restricted T cell responses reported previously (109, 119, 469). Activation of HDM responsive CD1a-reactive T cells induced IFN-γ, GM-CSF and IL-13 production. As previously discussed, these cytokines are known to exert multiple effects on keratinocytes and the adaptive and innate immune systems, which would contribute to cutaneous inflammation and the pathogenesis of AD. A heterogeneous role in the immune response is suggested by the production of both type 1 and type 2 cytokines from a presumed oligoclonal population of T cells in a HDM responsive, CD1a-reactive manner. Dual-colour Fluorospot demonstrated the majority of type 1 and type 2 cytokine producing cells to be unique subsets, and this could be further examined using ICS. These findings are in contrast to the well described group 2 CD1d-restricted iNKT cells which are known have an *"innate-like"* function responding rapidly to stimuli with the secretion of both type 1 and type 2 cytokines (66), and in addition to their reported more adaptive-like characteristics (105, 512), may be another distinguishing feature of group 1 CD1-restricted T cells.

IL-22 production was also detected on ELISpot and RT-qPCR further implicating these cells in cutaneous immunity, with IL-22 producing cells having been found to be increased in AD (329) and to exert a number of cutaneous effects including upregulation of AMP production (331) and downregulation of filaggrin expression and epidermal differentiation (332-334). IL-22 producing cells may therefore exaggerate the barrier dysfunction and contribute to the epidermal hyperplasia seen

in AD. No IL-17 secretion was detected, in agreement with previous reports (55, 119). It would be very interesting to extend these investigations to all of the T cell lines generated, particularly AA9005 which was derived from the skin, and indeed determining the existence and role of IL-22 producing HDM responsive CD1a-reactive T cells would be important in advancing our understanding of AD disease pathogenesis. No IL-10 production was observed, arguing against a regulatory function.

The fact that three of the HDM responsive CD1a-reactive T cell lines were predominantly γδ T cells is also a very interesting point for discussion. γδ T cells are present in high numbers and are known to participate in cutaneous immunity in mice, but this class of T cells is poorly understood in human cutaneous T cell responses. Elevated numbers of γδ T cells have been reported in a wide range of dermatological conditions including AD (375) suggesting an involvement in cutaneous pathology. Furthermore γδ TCRs have been reported to bind CD1 molecules (1), and, although the vast majority of CD1a-restricted T cells investigated to date have expressed αβ TCRs, reported studies have identified CD1a-restricted T cells in both the αβ and γδ T cell repertoires (79, 119). It is of interest that, to date, all reported γδ CD1a-restricted T cell clones have consisted of $V\delta1^+$ T cells (109). In contrast to $V\delta2^+$ γδ T cells which constitute the majority of circulating peripheral γδ T cells and mainly associate with the Vγ9 chain (375), $V\delta1^+$ γδ T cells are enriched in tissues, especially mucosa and epithelia, and have been demonstrated to recognise CD1c molecules with resultant production of type 1 cytokines, proliferation and cytotoxic function (526). It will be interesting to investigate whether the HDM responsive CD1a-reactive T cell lines are $V\delta1^+$ or $V\delta2^+$, and although it will be important to determine the responding αβ/γδ T cell subset in these lines, for example with ICS or the IFN-γ secretion assay, the involvement of γδ T cells in the HDM responsive CD1a-reactive T cell response remains an interesting possibility, and is potentially a novel finding of

CD1a-restricted T cells. In addition, although this would need to be examined *ex vivo* as cell surface markers are known to change during *in vitro* culture, it would also be interesting to determine CD45RO, CCR7, CD103 and CD62L expression indicating previous antigen encounter to see if, as expected from the cord blood results and previous reports, they demonstrate adaptive central or tissue resident memory type characteristics (105, 512).

Interestingly, the majority of the HDM responsive CD1a-reactive T cell lines generated were $CD8^+$ or double negative. This is in keeping with a γδ T cell predominance, and suggests a Tc1 or cytotoxic phenotype, although this has yet to be directly investigated. This preponderance of $CD8^+$ T cells amongst HDM responsive CD1a-reactive T cell lines might be explained by the structural analogy of CD1 molecules to MHC Class I molecules. Studies based on large T cell clone libraries have identified CD1a-restricted T cells in the CD4, CD8 and DN T cell repertoire, with the demonstration of both Th1 and Th2 functional phenotypes (97, 98, 102, 119, 512), however previous reports have documented the majority of CD1a-restricted T cells to be CD4+ or double negative (119, 512). $CD8^+$ CD1a-restricted T cells may have been positively selected by using the IFN-γ secretion assay to generate HDM responsive CD1a-reactive T cell lines in these experiments. Such a selection bias might be avoided by using limiting dilution assay to generate T cell lines, with the caveat that a low percentage of HDM-responsive CD1a-reactive T cell lines might be expected to be generated in this way. Expression of the skin homing markers CLA, CCR4, CCR6 and CCR10 was present but varied throughout the HDM responsive CD1a-reactive T cell lines generated, further implicating them in cutaneous inflammation and the pathogenesis of AD. Highest expression of skin homing markers was seen in the skin derived HDM responsive CD1a-reactive T cell line AA9005.

As discussed, it was also an interesting finding that each HDM responsive CD1a-reactive T cell line was capable of being stimulated by the other group 1 (K562-CD1a, -b, -c) and group 2 (K562-CD1d) CD1 isoforms to varying degrees. K562-CD1a cross-contamination of the other transfected K562 cell lines, a possible explanation for this, was excluded by flow cytometry. A further possible explanation is that the HDM responsive CD1a-reactive T cell lines are composed of oligoclonal T cell populations with differing TCR specificity, and it will be important to explore this further with TCR sequencing as a part of future studies. Indeed, amongst CD1 molecules, a degree of antigen promiscuity has been described (98), however T cell reactivity to multiple CD1 isoforms is yet to be reported in the literature. It is also of interest that TCR promiscuity has also previously been reported in the form of dual expression of α- and β-chain TCRs, which might provide a further explanation for these findings (527-529).

In summary, recognition of multiple CD1 isoforms by a presumed oligoclonal T cell population is an interesting finding, which is potentially a novel observation of CD1a-reactive T cells and might have significant functional implications for these cells.

Finally, although the response to HDM was consistently the highest at the concentrations used, it was interesting to see considerable antigen cross-reactivity was observed and that multiple allergens were capable of stimulating IFNγ secretion from HDM-responsive CD1a-reactive T cell lines in a CD1a-restricted manner, including BPLA2, Wasp venom, and Cat and Candida albicans to varying degrees. As discussed, the proposed model of direct CD1a molecule-TCR binding on occupation of the CD1a binding groove with *"permissive"* lipid ligands (55, 57, 64) might form the basis for this antigen cross-reactivity. It is also possible that, as with the cross-reactivity observed to other CD1 isoforms, the HDM responsive CD1a-reactive T cell lines are composed of oligoclonal T cell populations possessing differing TCR specificities. Furthermore a common lipid antigen, or antigen

generation pathway may be shared by the IFNγ eliciting antigens. It is clearly important to investigate the CD1a binding fraction of HDM further, and this will be examined in the next chapter: Investigation of phospholipase in the generation of CD1a ligands.

7 Investigation of the role of phospholipase in the generation of CD1a ligands

Introduction and aims

Having demonstrated HDM recognition by CD1a-reactive T cells in the blood (Chapter 4) and skin (Chapter 5), which were enriched within the blood and skin of AD patients, and successfully generated HDM responsive CD1a-restricted T cell lines from the blood and skin of multiple unrelated donors (Chapter 6), we next sought to determine the CD1a binding fraction of HDM.

As discussed in the introduction, multiple allergens have been identified within HDM. To date, these are all proteins, with 31 groups of protein allergens having been identified (167). Many of these are proteolytic enzymes which facilitate digestion of human skin substrate, originating in the mite's digestive tract and being excreted in the faeces at high concentrations where they become airborne (168, 169). Examples include Der p1, a group 1 allergen, which is a cysteine protease and has been found to cleave occludin, a protein of intercellular tight junctions and other cell surface molecules (170); α amylase and serine protease activities have also been reported (167). It is also interesting to note that some of the described HDM allergens are lipid binding proteins (167), furthermore lipase (530) and phospholipase (531) activities have been reported within HDM extract which are of interest whilst considering possible CD1a ligands in the context of CD1a-restricted T cells.

This aim of this chapter was to investigate the CD1a-reactive constituent of HDM extract.

Considerable antigen cross-reactivity of the HDM responsive CD1a-reactive T cell lines generated in Chapter 6 was additionally observed. As discussed, possible explanations for this include the proposed model of direct CD1a molecule-TCR binding on occupation of the CD1a binding groove with *"permissive"* lipid ligands (55, 57, 64) or that a common lipid antigen, or antigen generation pathway might be shared by the IFNγ eliciting antigens.

By definition and as per all literature on known CD1a ligands to date (11, 53-55, 64, 79), CD1a antigens would be expected to be lipid in biochemical nature, and correspondence with the pharmaceutical manufacturer of the HDM extract (ALK-Abello) suggested that lipids would be expected to be preserved within the HDM extract on allergen preparation. We therefore used polyclonal T cells derived from the blood and the skin, and HDM responsive CD1a-reactive T cell clones generated in Chapter 6 to investigate the CD1a binding fraction of HDM by fractionating total HDM extract into lipid enriched organic, and protein enriched aqueous fractions, to examine the antigenic substance in HDM extract.

These studies revealed that unexpectedly, CD1a reactivity was found to reside within the protein-enriched fractions, and could be explained by sPLA2 activity, most likely by the generation of neolipid CD1a antigens. sPLA2 has long been implicated as having a role in atopic disease (461, 462, 532-537), and this model is consistent with recently reported findings of our laboratory which demonstrated that sPLA2 within insect venoms process human skin lipids for presentation via CD1a (469), bringing the number of allergens in which PLA2 generates CD1a ligands up to three: wasp venom, bee venom, and HDM. The aim of this chapter therefore evolved into the investigation of the role of phospholipase in the generation of CD1a ligands in the context of the HDM responsive CD1a-reactive T cell response. Given the importance of filaggrin in the field of AD and the observation that HDM responsive CD1a-reactive T cell frequencies were found to be elevated in individuals with

filaggrin mutations in Chapter 4, we also investigated the effect of filaggrin on this system.

7.1 HDM responsive CD1a-reactive T cell responses can be explained by sPLA2 activity

7.1(i) CD1a reactivity lies within the protein, not the lipid fraction of HDM extract

To identify the antigenic substance in HDM, chloroform and methanol were used to isolate protein-enriched aqueous and lipid-enriched organic fractions, which were then used to examine HDM responsive CD1a-reactive T cell recognition (Figure 7.1A, left panel). The treatment of HDM extract by heating at 95°C for 4 hours would be expected to denature proteins but not broadly cleave other biochemicals, and this inactivated HDM effects (figure 7.1A, right panel).

These experiments were additionally performed using T cells derived from the skin (Figure 7.1B) and HDM responsive CD1a-reactive T cell lines generated in chapter 6 (Figure 7.1C).

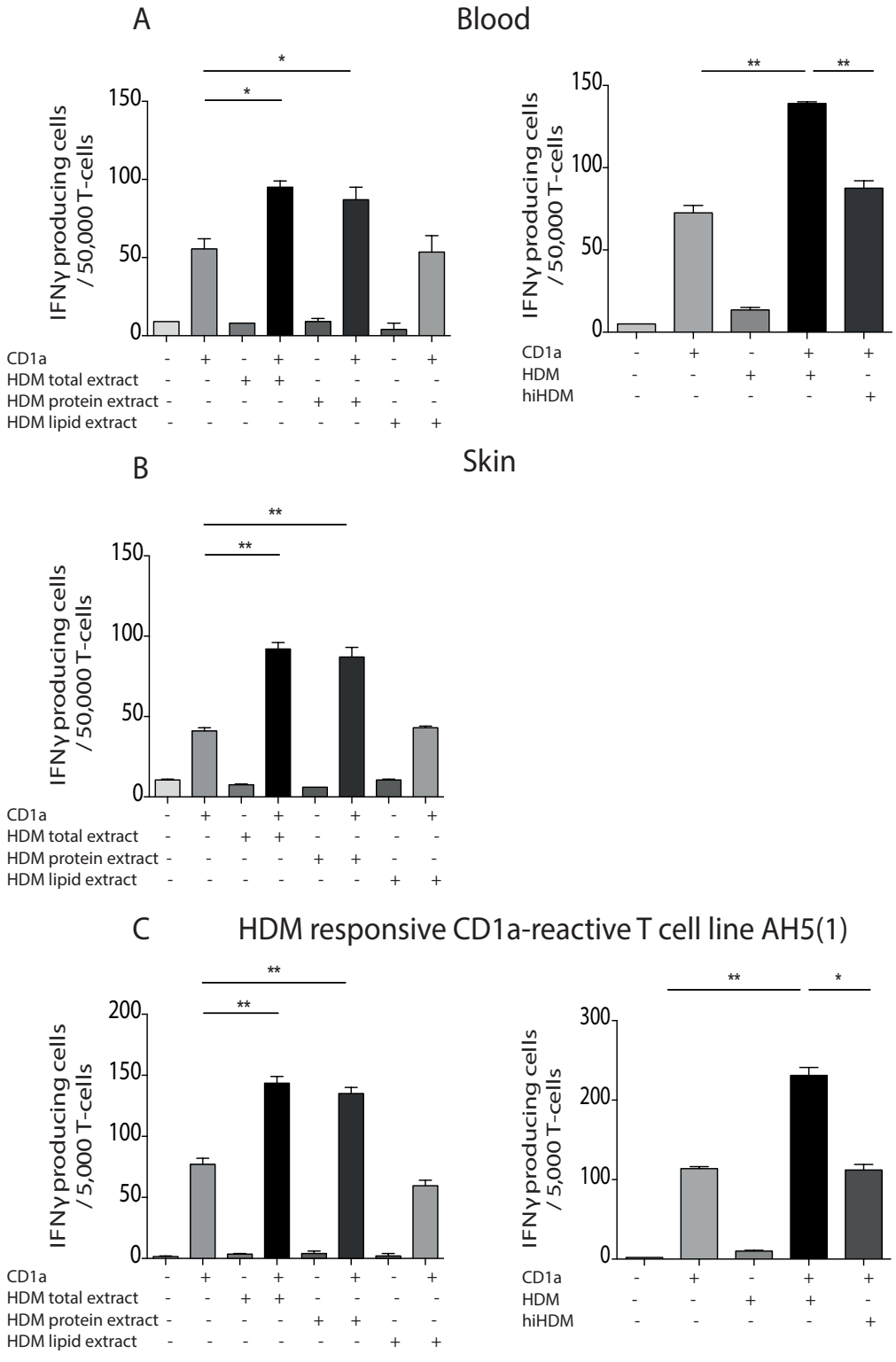


Figure 7.1. CD1a reactivity lies within the protein, not lipid, fraction of fractionated HDM. Blood- (A – AD9500) and skin blister- (B – AD4) derived T cells cells, and HDM responsive CD1a-reactive T cell lines (C – AH5(1)) were incubated

overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM total extract, aqueous protein phase or lipid phase. IFN-γ production was measured by ELISpot. A, C (left) IFN-γ production before, and A,C (right) after HDM heat inactivation (hiHDM). Data are representative of at least three donors. Bars represent standard error. * P<0.05; ** P<0.01.

7.1(ii) HDM extract contains sPLA2 activity *in vivo* and *in vitro*

It was consistently observed that HDM-derived proteins mediate lipid recognition by CD1a-reactive T cells, i.e. that unexpectedly, HDM CD1a reactivity lies within the protein, not lipid, fraction of HDM.

This counterintuitive result could be explained by experiments that were recently reported by our laboratory (538), showing that bee venom CD1a mediated T cell responses were also stimulated by the protein fraction of bee venom. In this model it was reported that bee venom phospholipase generate antigenic free fatty acids and lysophospholipids from intact phospholipids to drive the CD1a mediated response to bee venom.

It was postulated that a parallel mechanism existed here which was tested by examining sPLA2 biochemical activity *in vivo* and *in vitro using* colorimetric thiol detection after release from diheptanoyl thio-PC substrate. Interestingly there was sPLA2 activity measured in all skin blister samples, which significantly increased after HDM challenge (Figure 7.2A). Furthermore, HDM-induced sPLA2 activity within skin blister fluid was significantly inhibited in the presence of the known sPLA2 inhibitor manoalide (Figure 7.2B).

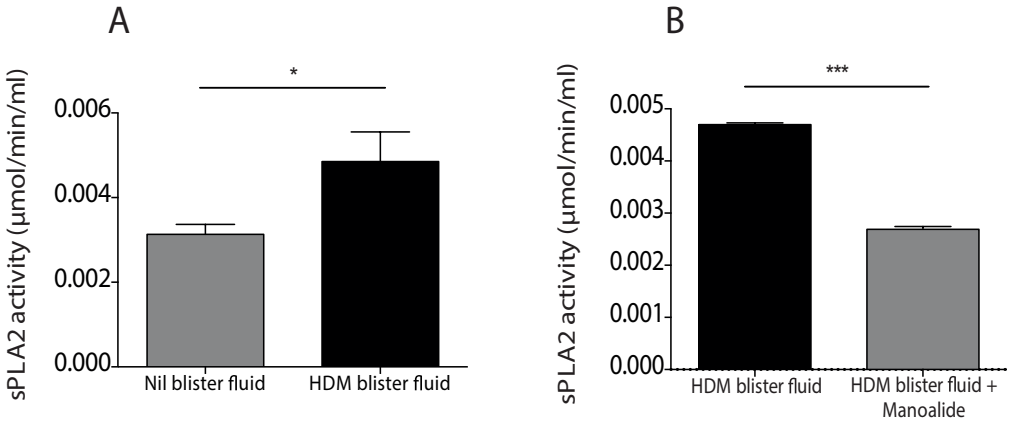


Figure 7.2. HDM extract contains sPLA2 activity *in vivo*. sPLA2 activity in saline or HDM challenged skin blister fluid was detected by measuring free thiol release in the presence of the diheptanoyl thio-PC substrate in the absence (A, n=10) or presence (B) of 10µM of the sPLA2 inhibitor manoalide. Data are representative of three separate experiments. Bars represent standard error. * P<0.05; ***P<0.001.

It was next demonstrated that HDM extract contains sPLA2 activity *in vitro* which could also be inhibited by manoalide (Figure 7.3). This is consistent with previously reported findings of phospholipase activity in HDM (531).

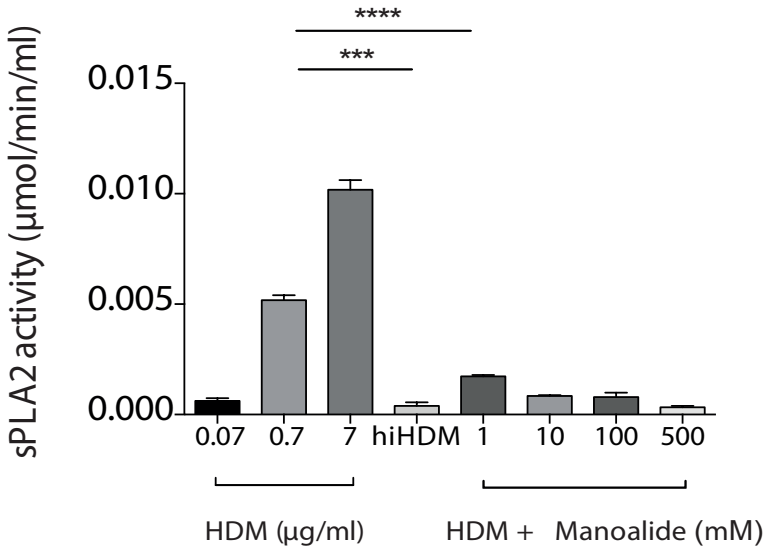


Figure 7.3. HDM extract contains sPLA2 activity *in vitro*. sPLA2 activity in HDM extract *in vitro* was detected by measuring free thiol release in the presence of the diheptanoyl thio-PC substrate and manoalide and after heat inactivation (hiHDM). Data are representative of three separate experiments. Bars represent standard error. ***P<0.001;****P<0.0001.

7.1(iii) HDM-derived sPLA2 generates neolipid antigens for presentation by CD1a to blood and skin T cells

Heat inactivation of house dust mite extract or treatment with the sPLA2 inhibitor manoalide, abrogated the CD1a-dependent recognition of HDM-pulsed K562-CD1a cells, but did not affect the autoreactive T cell response (Figure 7.4). However, manoalide did not affect viral-specific T cell line responses, illustrating the inhibitory specificity of manoalide (Figure 7.5). This suggests that the CD1a-reactive HDM responses are secondary to sPLA2, most likely through the generation of neolipid antigens (538).

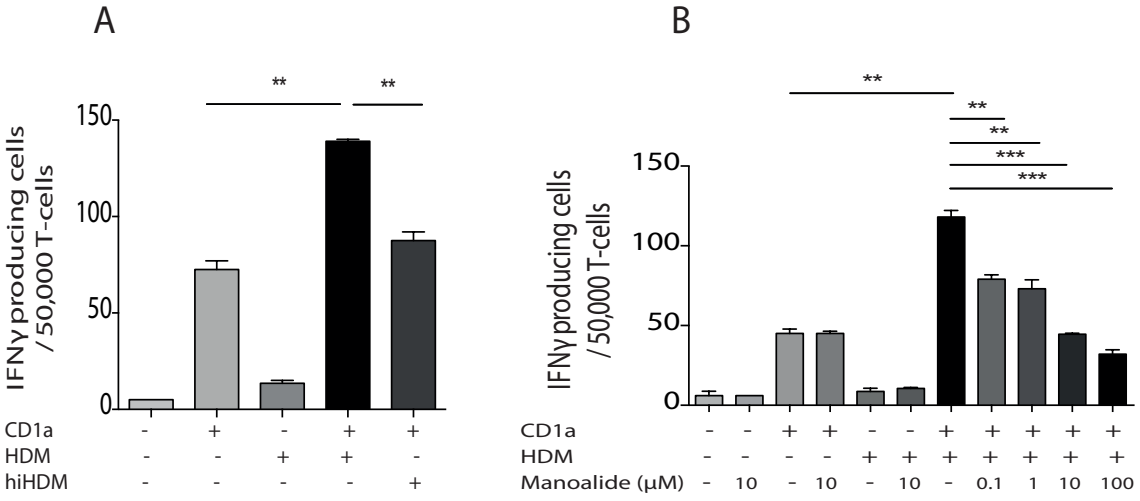


Figure 7.4. HDM-derived sPLA2 generates neolipid antigens for presentation by CD1a to blood T cells. T cells were isolated by CD3 MACS beads from donor PBMCs and incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM total extract. IFN-γ production was measured by ELISpot after HDM heat inactivation (hiHDM) (A) or overnight incubation with a dose titration of the sPLA2 inhibitor manoalide (B). Data are representative of at least three separate experiments. Bars represent standard error. ** P<0.01; ***P<0.001.

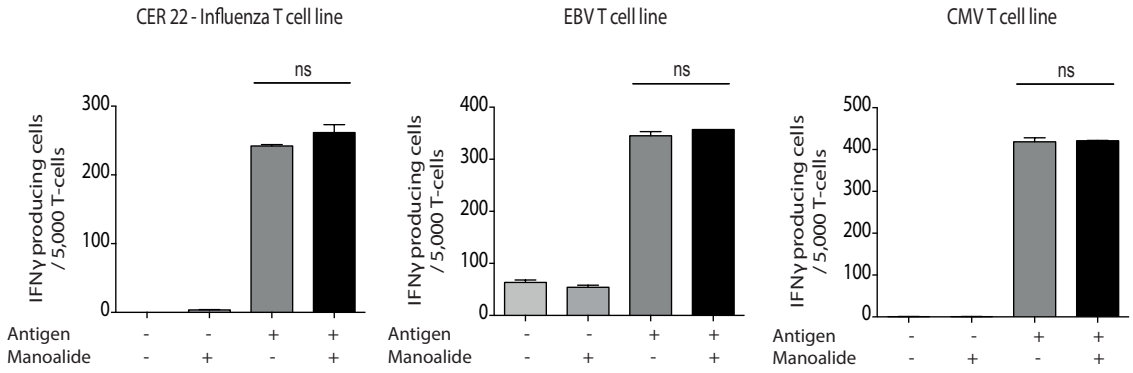


Figure 7.5. Viral-specific T cell responses in the presence of manoalide. Viral specific T cells were incubated with relevant antigen in the presence or absence of 10μM Manoalide. IFNγ production was measured by ELISpot. Data representative of two separate experiments. I am grateful to Mariolina Salio for provision of the viral-specific T cell lines.

Whether skin-derived T cells and HDM-responsive CD1a-reactive T cell lines were also dependent on sPLA2 activity was next investigated (Figure 7.6), and it was found that cytokine production by skin T cells derived *ex vivo* following intra-epidermal HDM challenge to human skin (Figure 7.6 A), and by HDM responsive CD1a-reactive T cell lines (Figure 7.6 B) were also inhibited by heat inactivation of the HDM or by treatment with manoalide.

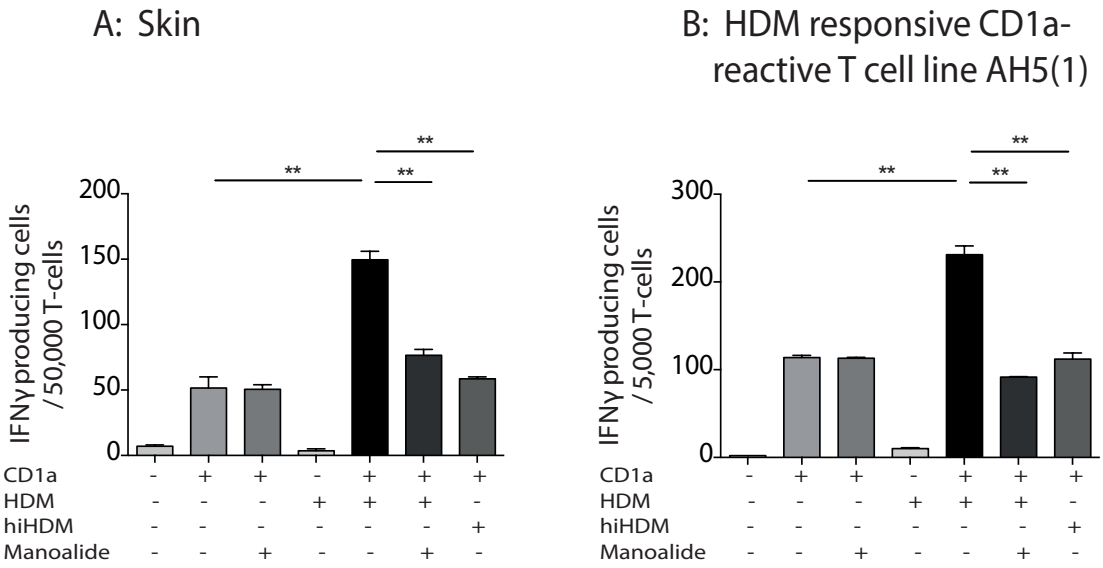


Figure 7.6. HDM-derived sPLA2 generates neolipid antigens for presentation by CD1a to skin T cells and HDM responsive CD1a-reactive T cell lines. A - Skin blister T cells were isolated 24 hours after HDM skin challenge, expanded and incubated with CD1a-transfected or untransfected K562 cells in the presence or absence of HDM extract that had been heat inactivated or incubated with manoalide. IFNγ production was measured by ELISpot. B - the HDM responsive CD1a-reactive T cell line AH5(1) was incubated with CD1a-transfected or untransfected K562 cells in the presence or absence of HDM extract that had been heat inactivated or incubated with manoalide. IFNγ production was measured by ELISpot. Data are representative of at least three separate experiments. Bars represent standard error. ** $P<0.01$.

7.1(iv) Preliminary identification of neolipid antigens generated by HDM sPLA2

Our knowledge of the range of lipid ligands that are able to bind to CD1a molecules would be furthered by characterisation of the neolipid antigens generated by HDM sPLA2.

To date, a limited number of CD1a ligands have been identified. These include the self lipid sulfatide (11), and a mycobacterial lipid, dideoxymycobactin (DDM) (54). More recently, CD1a has been shown to bind and present headless hydrophobic lipid antigens, including fatty acids, squalene and triacylglyceride (55) and a recent study showed that lysophosphatidylcholine and oleic acid, products of phospholipase activity, are also ligands for CD1a (64).

Therefore, in order to begin to investigate the underlying mechanism of neolipid antigen generation further, polyclonal blood and blister T cells and HDM responsive CD1a-reactive T cell line responses to a panel of candidate lipids were examined using IFNγ ELISpot. These included whole phosphatidylcholine substrate and products of cleavage by sPLA2 including purified free fatty acids and lysophospholipids (Figure 7.7).

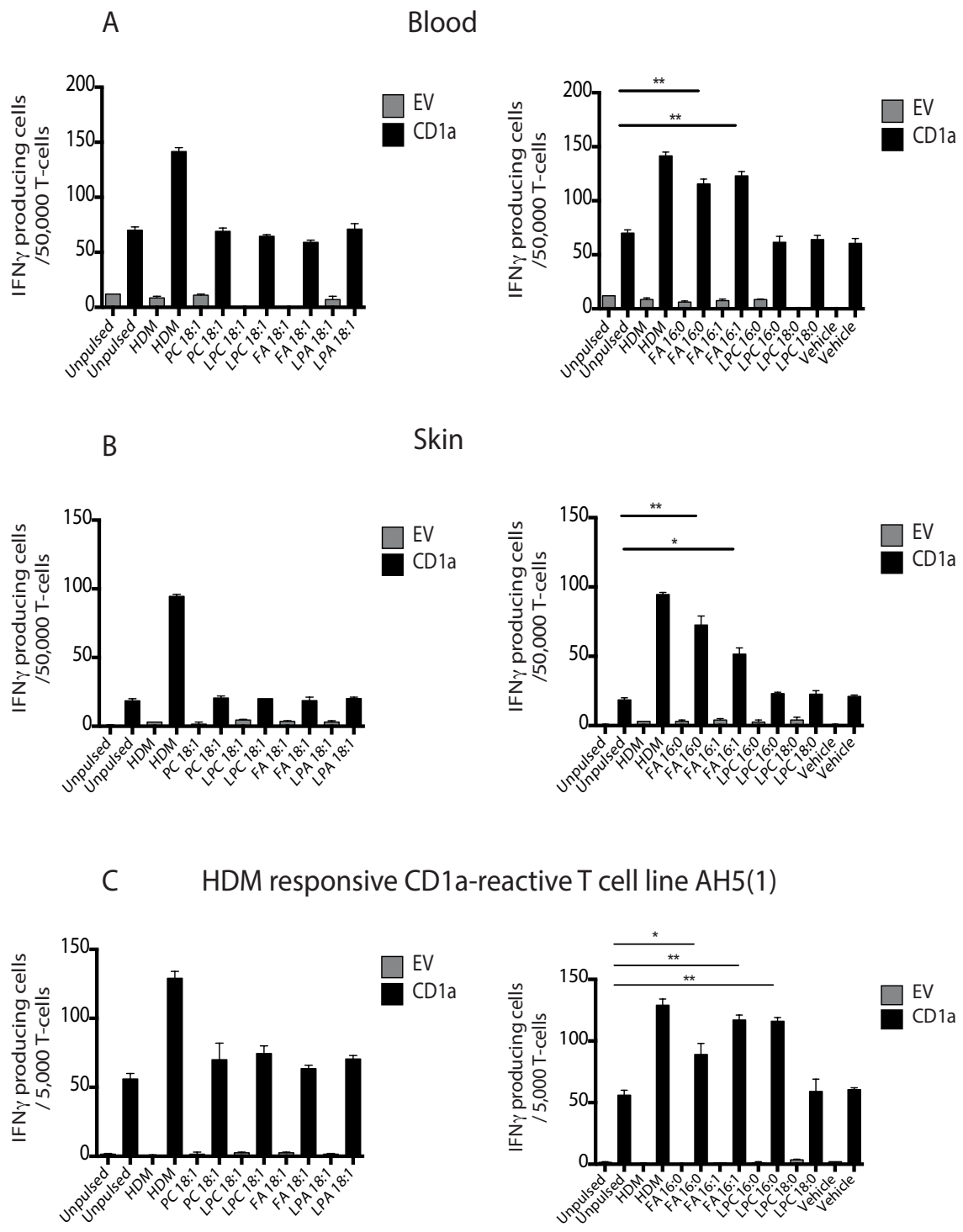


Figure 7.7. Polyclonal blood- and skin- derived T cells, and HDM responsive CD1a-reactive T cell lines respond to free fatty acids. A – polyclonal blood-derived T cells, B – polyclonal skin blister-derived T cells, and C – the HDM responsive CD1a-reactive T cell line AH5(1) were incubated with CD1a-transfected

or untransfected K562 cells in the presence or absence of HDM extract or the presence or absence of a panel of candidate lipids. IFNγ production was measured by ELISpot. Data are representative of at least three separate experiments. Bars represent standard error. ** P<0.01.

As expected, low responses were observed to untransfected K562 cells and an autoreactive response was detected to unpulsed K562-CD1a cells (Figure 7.7). This was further elevated by pulsing K562-CD1a with HDM (Figure 7.7). No increase above autoreactivity was detected to K562-CD1a pulsed with phosphatidylcholine substrate, but products of sPLA2 activity including lysophoshatidylcholine and free fatty acids with a 16 carbon chain length were able to mimic the HDM induced response (Figure 7.7). It is noteworthy that changing the alkyl chain length abrogated the response, suggesting that in agreement with previously reported findings, TCR interaction is determined by the carbon chain length (64).

Therefore these preliminary results suggest that the HDM responsive CD1a-reactive T cell response might be a free fatty acid response, with the most consistent response seen to the free fatty acids palmitic acid and palmitoleic acid with C16:0 and C16:1 acyl chains respectively, which generally caused a greater response than intact phospholipids and lysophospholipids (Figure 7.7). Unsaturated lysophosphatidylcholine with a 16 carbon chain length and one double bond was also able to stimulate AH5(1), a HDM responsive CD1a-reactive T cell line (Figure 7.7).

In agreement with previously published data (64), as both lysophosphatidylcholine C16:1 and palmitic (C16:0) and palmitoleic acids (C16:1) were capable of stimulating AH5(1) these studies potentially suggest that the lipid polar head group is less significant, and that it is the interaction of the lipid's hydrophobic alkyl chain with the CD1a molecule's binding groove that determines TCR interaction with the CD1a molecule and thus T cell stimulation.

7.1(v) HDM responsive CD1a-reactive T cells respond to multiple phospholipase containing allergens

As HDM responsive CD1a-reactive T cell responses can be explained by sPLA2 activity, and sPLA2 has been detected in other allergens (469, 539, 540), CD1a-reactive responses to purified bee venom sPLA2, another recognised sPLA2 containing allergen was also investigated. It can be seen that blood and skin HDM responsive CD1a-reactive T cells are also able to be stimulated by purified bee venom sPLA2 (Figure 7.8). This suggests shared pathways of skin inflammation despite different depths of natural antigen delivery. Furthermore, because the antigen(s) recognised by HDM responsive CD1a-reactive T cells are neolipid antigens, this suggests that the phospholipase activities of two distinct antigens generate the same, or similar neolipid antigens.

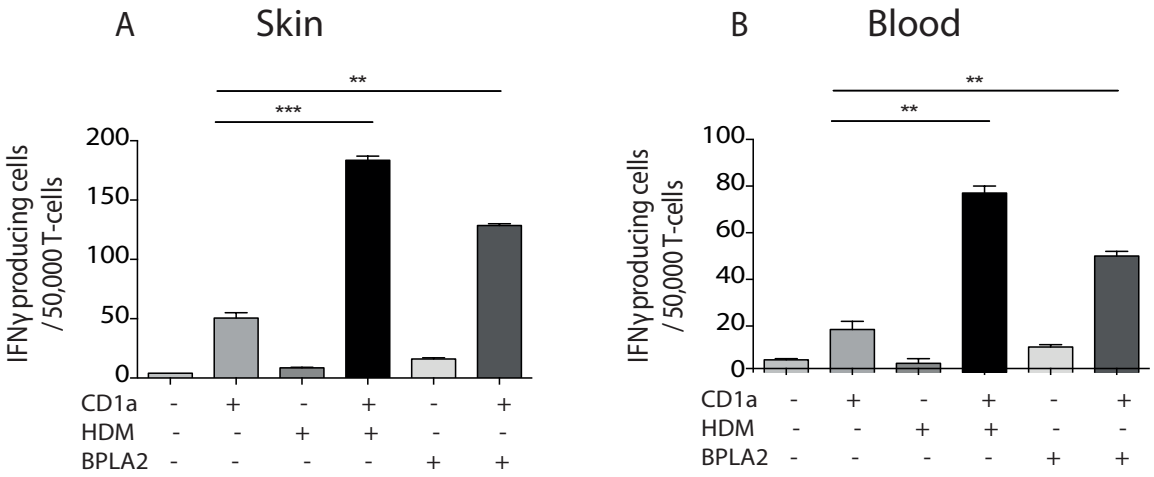


Figure 7.8 Polyclonal T cells from the skin and blood respond to purified bee venom sPLA2 in a CD1a-dependent manner. (A) Skin blister T cells were isolated 24 hours after HDM skin challenge, expanded and incubated with CD1a-transfected

or untransfected K562 cells in the presence or absence of 7μg/ml HDM extract or 1μg/ml purified bee venom PLA2. IFNγ production was measured by ELISpot. (B) Polyclonal T cells derived from the blood were incubated with CD1a-transfected or untransfected K562 cells in the presence or absence of 7μg/ml HDM extract or 1μg/ml purified bee venom PLA2. IFNγ production was measured by ELISpot. Data are representative of at least three separate experiments. Bars represent standard error. ** P<0.01; ***P<0.001.

In addition, sPLA2 activity was found to varying degrees within the panel of allergens able to stimulate HDM responsive CD1a-reactive T cell lines in chapter 6 (Figure 6.18) using the sPLA2 assay (Figure 7.9). Interestingly, there was no correlation between the strength of the CD1a-reactive T cell response seen to a specific allergen and the measured level of sPLA2 activity of that allergen, for example in the sPLA2 assay 7μg HDM had approximately 10% the sPLA2 activity as 10μg BPLA2, whereas the CD1a-restricted T cell response on ELISpot (performed using 7μg/ml HDM and 1μg/ml BPLA2) was around 20-50% greater (Figure 6.18). This difference might possibly be explained by the different allergen specific sPLA2s having differing phospholipid substrate preferences, with consequential differing CD1a ligand generation. Nevertheless, this further supports the suggestion of a common pathway of antigen generation and skin inflammation.

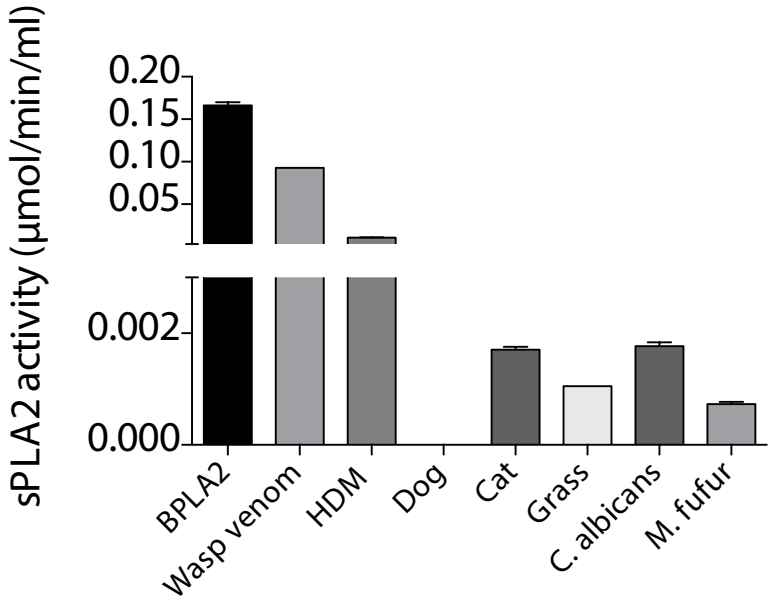


Figure 7.9 sPLA2 activity can be detected in multiple HDM responsive CD1a-reactive T cell line stimulating allergens of interest. Using a phospholipid substrate with a thiol group at the sn-2 position, sPLA2 activity in multiple allergens was detected using DNTB/EGTA which produces a coloured precipitate and measured at 415nm. Data were mean of duplicate measurements ± SEM.

Further supporting this, the sPLA2 responsive CD1a-restricted T cell clone BC2 (a gift from Professor Branch Moody) (469) was additionally found to respond to HDM in a CD1a-restricted manner (Figure 7.10A, B).

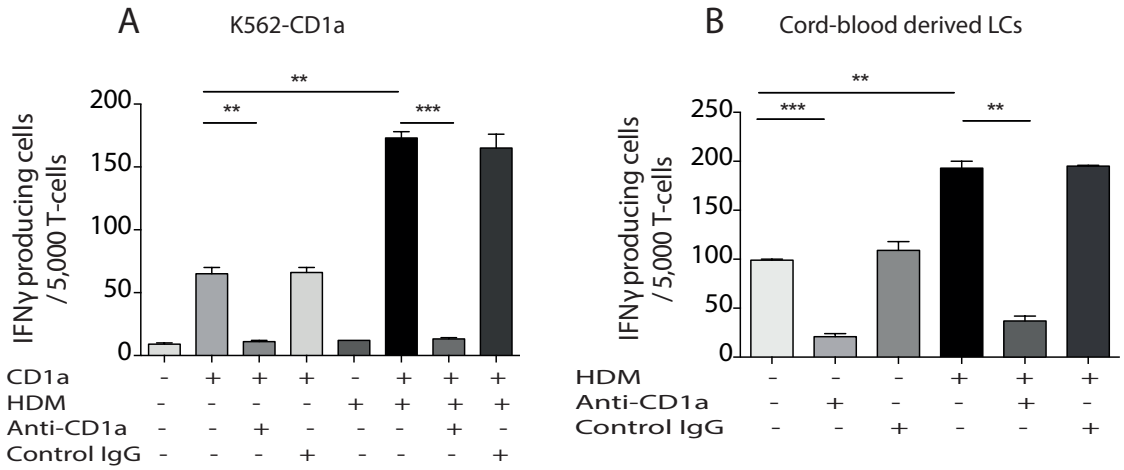


Figure 7.10. The sPLA2 responsive CD1a-restricted T cell clone BC2 additionally responds to HDM. A - BC2 cells were incubated overnight with CD1a-transfected K562 (CD1a) or untransfected K562 (EV) cells pulsed with HDM extract in the presence or absence of anti-CD1a antibody and isotype control. IFN-γ production was measured by ELISpot. B - *In vitro* cord blood derived LCs were pulsed with HDM extract overnight and incubated with BC2 cells in the presence of HLA Class I and II blocking antibodies. IFNγ production was measured by ELISpot in the presence or absence of anti-CD1a antibody. I am grateful to Professor Branch Moody for provison of the PLA2 responsive CD1a-restricted T cell clone BC2.

7.2 Filaggrin inhibits HDM sPLA2 and CD1a-reactive T cell responses

Filaggrin insufficiency is associated with moderate-severe atopic dermatitis and because we have shown an association between CD1a-reactive T cell responses to HDM and filaggrin null mutations, experiments were designed to test the hypothesis that filaggrin directly contributes to the CD1a-reactive T cell response.

The possibility that filaggrin itself might directly inhibit sPLA2 activity was investigated, and it was observed that filaggrin monomers efficiently inhibited sPLA2 biochemical activity at levels equivalent to those present in the stratum corneum *in vivo* (541) (Figure 7.11). The control SUMO protein did not affect sPLA2 activity (Figure 7.11). SUMO protein was used as a control as during filaggrin synthesis, the SUMO protein sequence is added between the His-tag and the target filaggrin monomer allowing further proteolysis using SUMO protease. However, it is

sometimes difficult to completely eliminate SUMO protein from the sample, hence SUMO protein was used as an additional control.

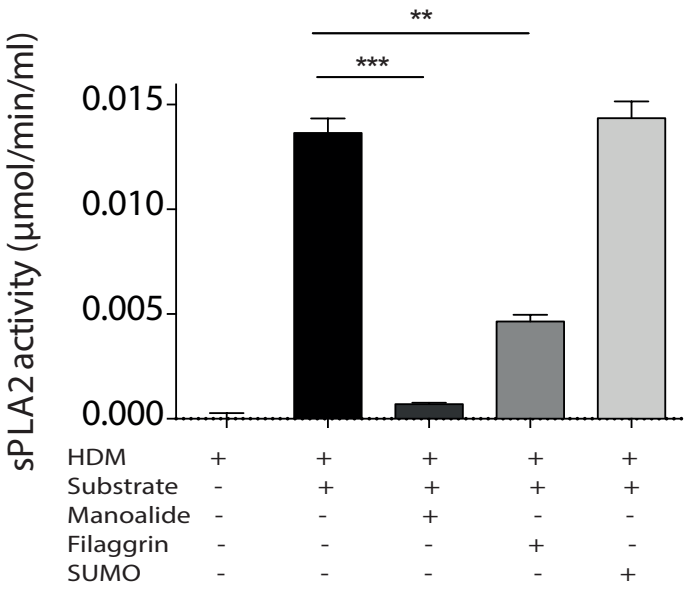


Figure 7.11. Filaggrin inhibits HDM sPLA2 activity. sPLA2 activity in HDM extract *in vitro* was detected by measuring free thiol release in the presence of the diheptanoyl thio-PC substrate, 10µM of the sPLA2 inhibitor manoalide and 1µg/ml human recombinant filaggrin or SUMO protein. Data are representative of at least three separate experiments. Bars represent standard error. ** P<0.01; ***P<0.001. I am grateful to Ewa Podobas for provision of the filaggrin monomers and SUMO protein.

Furthermore recombinant filaggrin monomers, but not SUMO protein, inhibited the sPLA2 activity observed within skin blister fluid after intra-epidermal challenge with HDM (Figure 7.12).

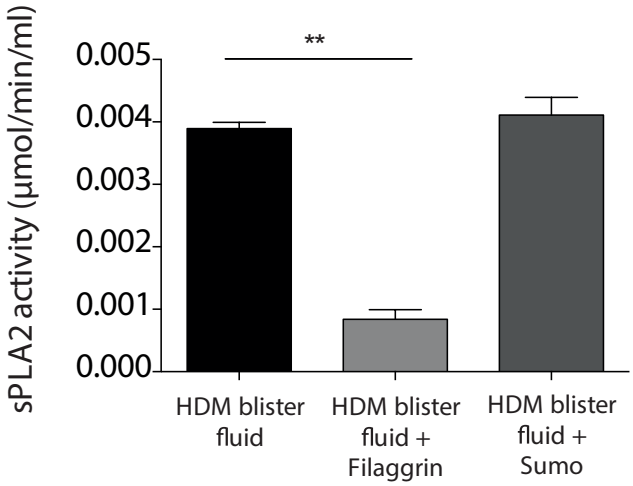


Figure 7.12. Filaggrin inhibits sPLA2 activity within HDM challenged skin blister fluid. sPLA2 activity in HDM challenged skin blister fluid was detected by measuring free thiol release diheptanoyl thio-PC substrate in the absence or presence of filaggrin and SUMO protein. Data are representative of at least three separate experiments. Bars represent standard error. ** P<0.01.

Whether filaggrin could inhibit cytokine production by HDM-responsive CD1a-reactive T cells was next investigated and it was observed that recombinant filaggrin monomers significantly inhibited IFNγ, IL-13 and GM-CSF production by T cells derived from blood *ex vivo* (Figure 7.13A). However there was no filaggrin-inhibition of autoreactive T cells, ruling out a non-specific toxic effect of the filaggrin on T cells. In addition, the control SUMO protein did not affect the HDM responsive CD1a-reactive T cell response (Figure 7.13A), and neither filaggrin, nor SUMO protein affected viral-specific T cell line responses, illustrating the inhibitory specificity of filaggrin (Figure 7.13B).

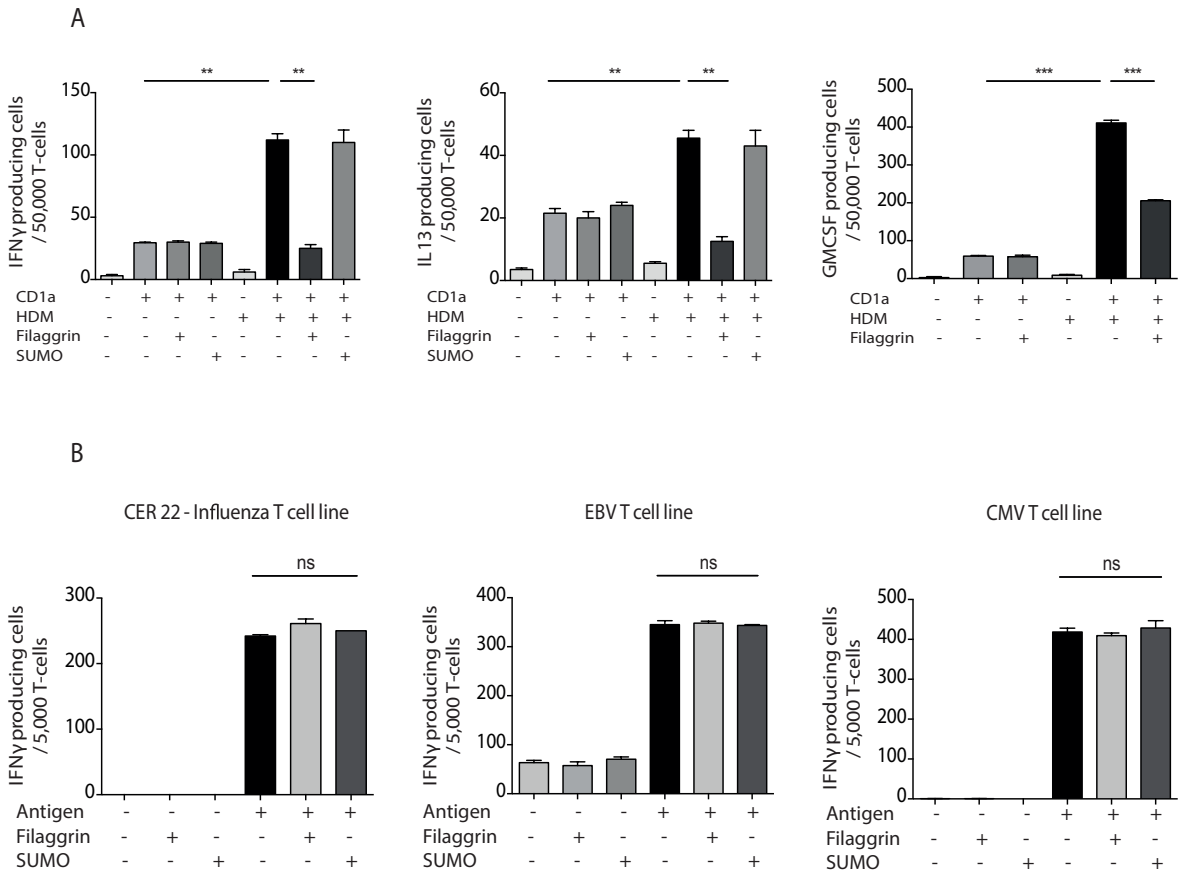


Figure 7.13. Filaggrin inhibits HDM-responsive CD1a-reactive T cell responses of polyclonal T cells derived from blood. (A) T cells were isolated by CD3 MACS beads from donor PBMC and incubated overnight with CD1a-transfected K562 or untransfected K562 cells pulsed with HDM total extract. GM-CSF, IL-13 and IFNγ production was measured by ELISpot in the presence or absence of filaggrin and SUMO protein. (B) Viral specific T cells were incubated with relevant antigen in the presence or absence of filaggrin and SUMO protein. IFNγ production was measured by ELISpot. Data are representative of at least two separate experiments. Bars represent standard error. ** P<0.01; ***P<0.001. I am grateful to Mariolina Salio for provision of the viral-specific T cell lines.

Lastly it was observed that the filaggrin monomers inhibited GM-CSF production by T cells derived *ex vivo* from skin after HDM skin challenge (Figure 7.14A) and HDM responsive CD1a-reactive T cell responses of the HDM responsive CD1a-reactive T cell line AH5(1) (Figure 7.14B). These data suggest that filaggrin may provide barrier function status signals to the innate and adaptive immune responses through effects on PLA2.

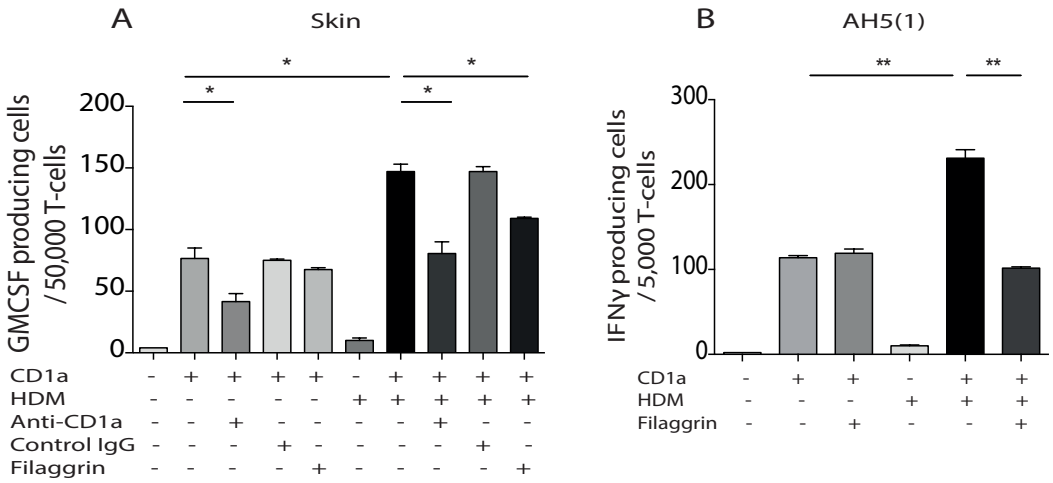


Figure 7.14. Filaggrin inhibits HDM PLA2 activity and inhibits responses of HDM-responsive CD1a-reactive T cells isolated from skin (A) and HDM responsive CD1a-reactive T cell lines (B). Skin blister T cells isolated 24 hours after HDM skin challenge T cells and expanded (A), or HDM responsive CD1a-reactive T cell lines were generated using the IFNγ secretion assay (B), were incubated overnight with CD1a-transfected K562 or untransfected K562 cells pulsed with HDM total extract. GM-CSF (A) and IFNγ production (B) was measured by ELISpot in the presence or absence of filaggrin monomers. Data are representative of at least three separate experiments. Bars represent standard error. * $P<0.05$; ** $P<0.01$.

Chapter 7 Summary

In summary, HDM responsive CD1a-reactive T cell responses were explained by sPLA2 activity, and were found to reside within the protein, not lipid, fraction of fractionated HDM. It was demonstrated that HDM extract contains sPLA2 activity *in vitro* and *in vivo*, and that this could be abrogated by heat inactivation of HDM or treatment with the sPLA2 inhibitor manoalide. The specific actions of the sPLA2 inhibitor manoalide suggest that the CD1a-reactive HDM responses of blood and skin T cells and HDM responsive CD1a-reactive T cell lines are secondary to sPLA2, most likely through the generation of neolipid antigens for presentation via CD1a. Preliminary experiments identified the free fatty acids palmitic acid (C16:0) and palmitoleic acid (C16:1), in addition to lysophosphatidylcholine (C16:1) as potential neolipid antigens generated by HDM sPLA2. It was observed that HDM responsive CD1a-reactive T cell lines respond to multiple phospholipase containing allergens, and recombinant filaggrin monomers were found to inhibit HDM sPLA2 and CD1a-reactive T cell responses.

Overall, these data identify a novel pathway of AD skin inflammation, whereby HDM-derived sPLA2 has the capacity to generate neolipid antigens for recognition by CD1a-reactive T cells, and identify sPLA2 inhibition as a hitherto unappreciated function of filaggrin, suggesting that filaggrin may provide barrier function status signals to the innate and adaptive immune responses through effects on PLA2.

Our data identify a novel mechanism of HDM antigenicity, in which HDM-derived sPLA2 catalyses the hydrolysis of non-antigenic phospholipid substrates into antigenic free fatty acids and lysophospholipids, which then act as CD1a lipid ligands. It is postulated that HDM-derived sPLA2 cleaves keratinocyte cell membrane phospholipids to release these lipid antigens, and indeed CD1a was recently

crystallised with oleic acid and lysophosphatidylcholine, which are both products of PLA2 activity (64).

As shown in Chapter 4 by the findings of increased frequencies in adults compared to cord blood samples, HDM responsive CD1a-reactive T cells are present in low frequencies at birth and likely undergo acquired antigen driven expansion with age. As HDM responsive CD1a-reactive T cell responses are dependent on sPLA2 enzymatic activity, it therefore follows that the responding polyclonal blood/skin/HDM responsive CD1a-reactive T cells have previously encountered products of the HDM sPLA2 activity. HDM responsive CD1a-reactive T cell responses are seen in polyclonal T cells and T cell lines derived from many unrelated donors, implicating HDM-derived sPLA2 generated neolipids as important antigens in CD1a immunobiology and suggesting that this system may be important for sensing epidermal barrier compromise. This model also provides a potential mechanistic pathway for the generation of free fatty acid antigens, which are known to stimulate CD1a-restricted T cell responses (55).

As discussed in the introduction, PLA2s exist as cytosolic, secretory, and inducible forms and sPLA2 activity was detected *in vitro* and *in vivo* in HDM extract, in keeping with other reported enzymatic activities of this allergen (167, 170). To date, the secretory PLA2 family is subdivided into 16 groups, with roles including involvement in inflammation, lipid metabolism, and cell membrane maintenance (543). Indeed phospholipases are known to be present in many other allergens including pollens and fungal allergens (539, 540), and in these studies HDM responsive CD1a-reactive T cell lines responded to multiple phospholipase containing allergens, as validated by the sPLA2 assay. This implies a degree of sPLA2 homology and overlapping cross-reactivity of phospholipid substrates, and suggests that lipid antigen generation may be a feature common to many allergens. However the strength of the observed CD1a-reactive T cell response did not correlate with the detected level of sPLA2

activity. A possible explanation for this is that, as reported for HDM (530), some of the allergens may contain additional phospholipase or lipase groups that in turn have differential phospholipid substrate preferences or hydrolyse the production of CD1a lipid ligands of greater potency. It is also possible that the CD1a-restricted T cell responses seen may have been contributed to by lipid ligands contained within some of the complete allergen preparations.

Given that sPLA2 generation of CD1a ligands has now been shown to be present in three allergens of relevance to humans (469, 544), namely bee venom, wasp venom, and HDM the data suggest that this is part of a broader hypersensitivity system. Human group 3 sPLA2 is known to have the closest sequence homology to bee venom sPLA2 (545), and it might be postulated that a breach in epidermal integrity as seen in AD or with insect venom injection might *"co-opt, via overstimulation, the natural effects of endogenous PLA2s in barrier sensing and the immune response"* (469). This is particularly convincing given the recently reported findings that headless skin antigens are capable of stimulating CD1a-restricted T cells (55), and the notion of *"permissive"* and *"non-permissive"* lipid ligands for CD1a (64), so that HDM-derived PLA2 in the presence of a compromised epidermal barrier in the context of AD may tip this delicate balance into the production of permissive, or stimulatory CD1a lipid antigens which might contribute to cutaneous inflammation seen in this condition. Furthermore, a *"near-neighbour"* model has been proposed in which $CD1a^{+}$ Langerhans cells are predominantly located suprabasally, and antigenic skin lipids, for example wax esters and free fatty acids are found in the stratum corneum and sebum, so that in uncompromised skin with an intact epidermal barrier CD1a molecules and CD1a lipid ligand antigens do not co-localise (55, 523). Skin barrier breaches such as AD, might therefore facilitate delivery of surface antigenic lipid ligands to deeper epidermal layers where Langerhans cells reside, promoting activation of CD1a-restricted T cells (55, 523) with ensuing inflammation.

Indeed, generation of antigenic neolipids by HDM-derived sPLA2 might exacerbate and perpetuate this model. Furthermore, a role of sPLA2s in the CD1 T cell response in many organs might be speculated, with recent studies having demonstrated an important role for PLA2s in CD1d-mediated activation of iNKT cells (81, 466) and in selection of iNKT cells in the thymus (546).

Preliminary experiments identified the free fatty acids palmitic acid (C16:0) and palmitoleic acid (C16:1), in addition to lysophosphatidylcholine (C16:1) as potential neolipid antigens generated by HDM sPLA2. As discussed, recent studies have demonstrated that both free fatty acids and lysophosphatidylcholine, products of sPLA2 activity, stimulate autoreactive CD1a-restricted T cells (55, 64), which is consistent with our data. The finding that both lysophosphatidylcholine C16:1 and palmitic (C16:0) and palmitoleic acids (C16:1) were capable of stimulating the HDM responsive CD1a-reactive T cell line AH5(1) suggest that the lipid's polar head group is of less importance, and that it is the interaction of the lipid's hydrophobic alkyl chain with the CD1a molecule's antigen binding groove that is important for TCR interaction with the CD1a molecule and for CD1a-restricted T cell stimulation. This mechanism has recently been reported for CD1a autoreactive T cells, and will be described in detail below (64). It is also of interest that changing the length of the carbon chain abrogated the HDM responsive CD1a-reactive T cell response, again in agreement with previous published data (64).

These findings are consistent with recently reported studies, which used CD1a tetramers using a TCR from an autoreactive CD1a-restricted T cell clone (64). High performance liquid chromatography followed by mass spectrometry demonstrated that the CD1a molecule was capable of binding and presenting multiple self lipids to the CD1a-restricted TCR, illustrating that many lipids could be eluted from CD1a-lipid-TCR complexes, and lipid ligands eluted from both CD1a-lipid and CD1a-lipid-TCR complexes were designated *"permissive"* ligands, whilst those isolated to the

CD1a-ligand eluent, i.e. that did not bind to the autoreactive TCR were termed *"non-permissive"* ligands (64). Indicating a common TCR docking mode, three common membrane lipids (phosphatidylglycerol, phosphatidylcholine and phosphatidylinositol) were identified as permissive candidate CD1a ligands for the autoreactive TCR in this way (64), in addition to the PLA2 products oleic acid (a headless fatty acid) and lysophosphatidylcholine, (containing a polar head group) which were crystallised with CD1a-TCR (64).

With respect to MHC-peptide-TCR interaction, a diagonal binding mode of the TCR over the MHC peptide antigen binding groove has been reported, with MHC α helices contacting the TCR complementarity determining regions (CDR) 1 and 2, and the peptide antigen interacting with the TCR CDR3 (494). Distinct to this model, the α-GalCer-CD1d-TCR is known to bind in a parallel orientation to the CD1d antigen binding groove, with TCR–CD1d interaction being predominantly mediated by CDR2β and CDR3α and the TCR CDR1α and CDR3α regions facilitating lipid antigen interaction (494). In an analagous manner, the TCR was found to bind to CD1a-lipid antigen in a parallel alignment in a model of the TCR-CD1a-DDM complex (51). In this model, the TCR predominantly contacts the CD1a molecule superior to the CD1a molecule's A' pocket, where CDRs of the TCR α chain interact with the CD1a molecule's α1 and α 2 helices (51). The peptidic head group of DDM protrudes from the CD1a F' pocket, and interacts with CDRs of the TCR β chain (51).

In contrast to the previously reported parallel orientated CD1-lipid-TCR models, CD1a autoreactive TCR engagement of CD1a bound to oleic acid and lysophosphatidylcholine were fundamentally identical, with the TCR docking orthogonally across the CD1a binding groove's long axis (64). The TCR was found to bind over the A' roof of CD1a-antigen, with the TCR α and β chains being situated over the α 1 and α 2 helices of CD1a, contact predominantly being facilitated by the TCR CDR3α and CDR3β loops (64). By virtue of this TCR footprint, the TCR directly

interacted with the CD1a protein and did not contact the lipid antigen, with the A' roof of CD1a uniquely providing an *"antigen free landing platform"* that was opposite the solvent exposed F' portal (64), so that the TCR was distant from and did not interact with the polar head group of lysophosphatidylcholine (C18). A second lipid, sphingomyelin (C24) which was not permissive to TCR binding was found to disrupt this TCR-CD1a contact zone by disrupting a triad of amino acids in the CD1a A' roof (64). Both lysophosphatidylcholine and sphingomyelin have the same polar head group, but the longer C24 acyl chain of sphingomyelin caused the polar head group to protrude further from the F' portal of CD1a, which precluded TCR binding by affecting the CD1a A' roof's architecture and electrostatic properties (64). It was concluded that *"CD1a reactivity can result from direct contact of the TCR with CD1a itself, but only when the surface of CD1a is not disrupted by non permissive ligands"* (64), and this study illustrated how HDM sPLA2 generated neolipid antigens presented by CD1a might stimulate CD1a-restricted T cells. Therefore, whilst previous models of TCR interaction with CD1a-lipid complexes have been centered around protrusion of a polar head group from the CD1a F' portal for TCR recognition (11, 51), recognition of both fatty acids and lysophosphatidycholine by HDM responsive CD1a-reactive T cells suggest that HDM responsive CD1a-reactive T cell stimulation does not require the presence of a lipid ligand polar head group and therefore this novel form of antigen detection (64) may prove to be of relevance to the HDM-responsive CD1a-reactive T cell response. Furthermore it can be speculated that, as part of a barrier sensing mechanism, HDM-derived sPLA2 generates permissive or stimulatory antigenic free fatty acids and lysophosphatidylcholine, tipping the balance away from inhibitory or non permissive ligands, with ensuing cutaneous inflammation in AD.

It is also interesting to note that whilst filaggrin is known to have many roles in the skin which have been discussed in detail in the introduction (224, 226-228, 230),

sPLA2 inhibition is identified as a novel function of filaggrin. These data suggest that filaggrin may provide skin barrier function signals to the innate and adaptive immune responses through effects on PLA2, with loss of filaggrin inhibition of HDM PLA2 providing neolipid signals to CD1a-reactive T cells that barrier compromise has occurred, and downstream inflammatory sequelae. This process would be expected to be compounded by genetically determined or acquired filaggrin insufficiency in the context of AD, and might have potential therapeutic implications in this condition in the form of sPLA2 inhibitors. Interestingly, amelioration of contact dermatitis in patients treated with a topical sPLA2 inhibitor has previously been described (474), supporting the involvement of sPLA2 activity in cutaneous inflammation and a possible therapeutic impact of its inhibition. Furthermore, having identified a novel pathway of cutaneous inflammation in AD, the CD1a and HDM derived sPLA2 pathways may be utilised as potential targets for therapeutic manipulation in AD.

8 Discussion

As discussed, CD1a is a MHC Class I-like antigen presenting molecule that is expressed at high levels by Langerhans cells of the epidermis in addition to a subset of dermal DCs and DC populations at other surface sites (110-114), and the recent identification of CD1a-restricted T cells as a normal component of the human T cell repertoire (118, 119) has sparked interest into the patho-physiological function of CD1a-restricted T cells. CD1a is known to present self (54, 97), and foreign lipid antigens to T cells, so that Langerhans cells of the epidermis, which express CD1a protein at high levels, are well located to bind and present CD1a lipid ligands to CD1a-reactive T cells as a mechanism of detecting skin barrier compromise. Indeed lesional cutaneous atopic tissue carries an altered lipid profile that might influence CD1a-restricted T cell activation (495-499), and furthermore $CD1a^{+}$ cells are enriched within atopic dermatitis lesions (386). Recent studies have reported that CD1a-reactive T cells circulate at higher frequencies than previously considered and can infiltrate normal human skin and recognise natural skin lipids (501-503). These cells produce cytokines that have been associated with skin disease, such as IFN-γ, IL-13 and IL-22 (501), but a defined role for CD1a-restricted T cells in human skin disease is lacking.

Several CD1a-lipid complexes have been crystallised and the floor of the F′ pocket of CD1a is elevated by Val98, Leu116 and Phe144, so that in comparison with other CD1 molecules the F′ pocket is shallow and laterally orientated (517, 547-549). In the structure of CD1a with sulfatide, the A' pocket can contain one ligand acyl chain and has been suggested to act as a 'molecular ruler' to determine lipid moiety binding length as it has a well-defined terminus (11). Unlike other CD1 proteins, CD1a lacks endosomal localisation motifs in its cytoplasmic tail and therefore has a distinct subcellular localisation - mostly on the cell surface, but some CD1a is also

present within an early endosomal compartment suggesting specialised lipid sampling and loading requirements (36, 550). CD1a autoreactive T cells express skin addressins (such as CLA, CCR4, CCR6 and CCR10) and accumulate in human skin (119). *"Headless"* self-lipids which naturally accumulate in the skin and sebum including squalene, wax esters and fatty acids, have recently been shown to be presented by CD1a for recognition by T cells (503) and our laboratory has identified that fatty acids generated by phospholipase activity in wasp and bee venom can be recognized by CD1a-reactive T cells (516). T cell receptor recognition of such headless lipids can occur without direct TCR-lipid contact, and is thought to be mediated by permissive structural changes in the CD1a molecule on lipid binding (517). Together, the natural accumulation of autoantigens, CD1a proteins and CD1a-restricted T cells point to a natural organ-specific function in skin. Further supporting a potential role for phospholipids and their downstream products, pollen-derived phospholipids have been implicated as targets for lipid-specific T cells, including CD1d and CD1a reactive T cell clones (551). Based on these findings, we considered that CD1a might play a role in atopic dermatitis. Specifically, given the enrichment of CD1a-expressing cells and altered lipids in lesional atopic dermatitis skin, we sought to test the hypothesis that CD1a-reactive lipid-specific T cells contribute to the human response to HDM in AD.

Pathogenesis of AD is complex and has been discussed in detail in the introduction, with convincing evidence of skin barrier insufficiency (the "outside-in' hypothesis: that barrier dysfunction through filaggrin null mutations enables exogenous immune stimuli to enter the skin and activate immune responses (208)) and immune dysregulation (the "inside-out' hypothesis: that immune dysregulation causes cutaneous inflammation, with a deleterious effect on skin barrier function) contributing to the cutaneous inflammation seen in this condition. There remains considerable debate about the relative contributions of each model, and this is an

important question to address in order to define how best to target future therapeutic strategies.

The aim of this thesis was to address several research questions regarding the frequency and phenotype of CD1a-restricted T cells and their role in the pathogenesis of AD, to further our understanding of these cells. In these studies, we have shown that HDM generates CD1a ligands for recognition by T cells. Such HDM-responsive CD1a-reactive T cells are enriched in the blood and skin of individuals with atopic dermatitis and correlate with IgE and disease severity, and are significantly elevated in the presence of filaggrin null mutations. HDM responsive CD1a-reactive T cells were detected in higher frequencies in the blood and skin of patients with AD compared to healthy controls, in keeping with reported findings of increased frequencies of pollen-responsive CD1a-restricted T cells in pollen-allergic individuals (79), and venom responsive CD1a-restricted T cells in venom-allergic individuals (79), cumulatively suggesting that these cells may play a role in atopic diseases. Upon activation, HDM responsive CD1a-reactive T cells produced type 1 and type 2 cytokines which exert numerous effects on the innate and adaptive immune systems and keratinocytes (122), contributing to the cutaneous inflammation seen in AD. Of possible relevance, it has recently been reported that regulation of the CD1a gene at the transcriptional level by single nucleotide polymorphisms in the 5' untranslated region causes an inter-donor variation in inducible DC CD1a expression (23). It is unknown whether this in turn affects the frequency of an individual's CD1a-restricted T cells and this phenomenon may therefore have contributed to the results presented in this thesis, and this would be interesting to examine as a part of future work.

We observed that CD1a-reactive T cells infiltrated the skin 24 hours after HDM challenge, which associated with type 2 cytokine production and phospholipase (PLA2) activity *in vivo*. HDM responsive CD1a-reactive T cell lines generated

expressed skin homing markers, predominantly expressed the CD8 co-receptor or were double negative, and interestingly several of the lines generated consisted of predominantly γδ T cells. T cell line reactivity to additional CD1 isoforms and antigen cross reactivity was observed. We showed that the HDM responsiveness of CD1a-reactive T cells was explained by the presence of PLA2 activity within HDM, identifying a potential novel pathway of AD skin inflammation in which HDM-derived PLA2 generates neolipid antigens for CD1a presentation to T cells. Finally HDM-derived PLA2 activity was shown to be inhibited by recombinant filaggrin monomers. Therefore it can be seen that acquired or genetically determined filaggrin insufficiency in AD might have a dual role in compounding ensuing cutaneous inflammation within this system – firstly the resulting impaired skin barrier might allow increased penetration of HDM, with enhanced cleavage of keratinocyte phospholipids and CD1a-restricted T cell activation, compared to healthy skin, with a consequential further degradation of the skin barrier. Secondly, loss of filaggrin inhibition of HDM PLA2 may provide increased neolipid signals to CD1a-reactive T cells that barrier compromise has occurred, with downstream inflammatory sequelae.

Historically, the study of antigen specific T cells has centred on HLA class I and class II restricted peptide-specific responses (175, 176). The data from the current work suggest that HDM PLA2 processed skin lipids should be added as an antigen class amongst T cell antigens potentially relevant to AD. As described, Langerhans cells are enriched in AD lesions (500) where an altered lipid profile has additionally been reported (495-499). This is consistent with a possible role of these cells in CD1a-restricted T cell presentation of lipid ligands. Many allergens including some of those within HDM are known to be lipid binding proteins, which, on binding to their lipid cargo, might become peptide-specific T cell targets. A large literature demonstrating that, despite the fact that frequently the immunogens are not typical T cell peptide antigens, T cells play a role in Type IV hypersensitivity reactions is summarised by

the widely taught Gell and Coombs classification (552). Protein haptenization has been reported as a mechanism by which non-peptide antigens may stimulate T cell-mediated responses (553), but the general applicability of this theory is unknown, and the mechanism(s) of non-peptide and small molecule stimulation of T cells is uncertain in many cases. An appealing contender for the mediation of such T cell responses, the CD1 system has evolved for T cell presentation of lipids (554, 555). Through its expression on Langerhans cells and a subset of dermal dendritic cells, CD1a protein is ideally located to sample non-peptide antigens that are exposed to the skin. The results presented here suggest a broader model of Gell and Coombs hypersensitivity where neolipid recognition by CD1a-reactive T cells is associated with clinical atopic disease. There may therefore exist a multi-hit process where, if activated in it's role as a skin barrier sensing system, the HDM responsive CD1a-reactive T cell response may induce other adaptive immunity, with resultant cutaneous inflammation. This is germane to animal models, in which cutaneous stress responses enhance sensitization (556). The data may also provide insights to why only certain environmental challenges are particularly allergenic. Such pro-allergenic sources, including house dust mite, may preferentially activate a barrier distress system through generation of inflammatory lipids, leading to immune responses to co-existent proteins and subsequent clinical disease. These processes may be compounded by genetically-determined or acquired filaggrin insufficiency.

CD1a protein is a member of the group 1 CD1 family, along with CD1b, and CD1c. The group 1 family is not present in mice. Each member has a distinct cytoplasmic domain, intracellular trafficking and tissue distribution which suggests functional specialisation (554). CD1a-autoreactive T cells were found to infiltrate human skin where they recognise skin derived self-lipids, including fatty acids, presented by primary CD1a-expressing antigen presenting cells (503). Products of phospholipase activity, namely a free fatty acid (oleic acid) and lysophosphatidylcholine were

recently shown to stimulate a CD1a-restricted autoreactive T cell clone (64), and the laboratory has also recently shown that wasp and bee venom derived phospholipase can generate self-lipids for presentation by CD1a protein suggesting shared pathways of skin inflammation, albeit with differing clinical phenotypes dependent on depth of antigen delivery (516). Furthermore, there is evidence that phospholipase activity is required for generation of CD1d-binding NKT cell ligands in a model of hepatitis virus infection (557, 558), and that thymic PLA contributes to the selection of NKT cells (559). However none of these studies have linked CD1 molecules to biological processes relevant in human inflammatory skin disease.

Secretory phospholipase A2 cleaves phospholipid to lysophospholipid and the sn-2 acyl chain, and while mechanisms have not been clear, it has long been implicated as having a role in atopic disease (560-567). Although sPLA2s are likely to have many roles, here we show that HDM-derived PLA-derived lipid products are presented by CD1a for recognition by T cells in the skin. The phospholipid substrate required for this model may be derived from the serum, skin, or HDM extract itself, although the CD1a plate bound assay experiments suggest that adequate quantities of phospholipid substrate for CD1a ligand generation and the stimulation of CD1a-restricted T cells may be supplied by cell membrane phospholipids. Therefore, similar to the model proposed for insect venoms (Figure 8.1), the presence of HDM sPLA2 in the epidermis may cleave keratinocyte-derived phospholipids, generating CD1a neo-lipid antigens including free fatty acids and lysophospholipids, which are presented to CD1a-restricted T cells by CD1a expressing LCs or dermal DCs.

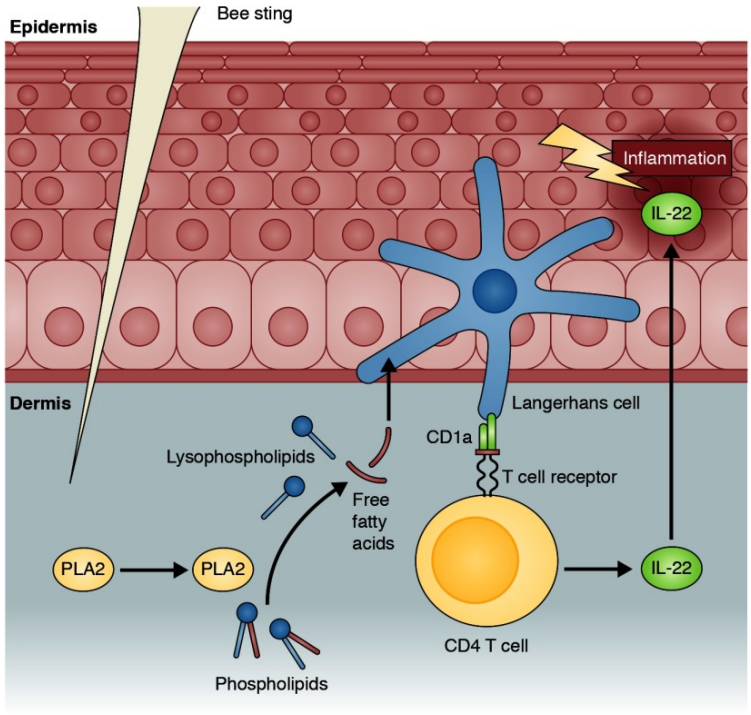


Figure 8.1. Mechanism of CD1a-restricted T cell activation by venom-derived phospholipase antigens. From (568). Bee venom is injected into the skin and hydrolyses cellular phospholipids into antigenic free fatty acids and lysophospholipids. These then act as neolipid antigens which are presented to CD1a-restricted T cells within the skin by CD1a molecules expressed by epidermal LCs and dermal DCs. These CD1a-restricted T cells then produce cytokines such as IL-22 which in turn contribute to cutaneous inflammation (568).

Until very recently, CD1-lipid recognition by CD1-restricted TCRs was thought to be mediated by the lipid's polar head group. However, in 2015 a model of direct CD1a-restricted TCR interaction with the CD1a molecule was suggested, when a CD1a-restricted autoreactive T cell clone stimulated by small apolar *"headless"* lipid ligands including free fatty acids was described, the stimulation of which was found to be abrogated by lipid ligands containing polar head groups (55). Indeed, it has been reported that the outer surface of the CD1a molecule, in addition to other CD1 isoforms, may be modified by lipid ligand binding (45, 58). This was corroborated by a more recent study which solved the crystal structure of the CD1a-lipid-TCR

interaction in complex with an apolar fatty acid, which illustrated that the alkyl chain sequestered deep within the CD1a A' pocket, access to which was blocked by the CD1a A' roof-like structure so that TCR interaction was precluded (64). This A' roof-like structure, which is common to all CD1 isoforms but is largest for the CD1a molecule, formed the TCR docking site. In the same paper the authors additionally solved the CD1a-TCR in complex with lysophosphatidylcholine (a sPLA2 product comprised of a fatty acid chain bound to a polar head group) and showed that lysophosphatidylcholine's fatty acid chain bound in an anlagous orientation to the apolar free fatty acid in the CD1a A' pocket. Lysophosphatidycholine's polar head group was exposed at the F' pocket of CD1a, but this exposure was at the opposite side of the CD1a molecule to the TCR docking site, and hence the polar head group was distant from, and did not interact with, the TCR (64). In chapter 6, functional assays of HDM responsive CD1a-reactive T cell lines demonstrated that both free fatty acids and lysophosphatidylcholine could stimulate HDM responsive CD1a-reactive T cells, suggesting that a similar mechanism may apply here and that the lipid polar head group is less important, and that rather it is the interaction of the TCR with the CD1a molecule containing the bound alkyl chain which is important for T cell stimulation. HDM responsive CD1a-reactive T cell responses were abrogated by changing the carbon chain length of the alkyl chain, again in agreement with previous published data (64) and suggesting that the alkyl chain carbon chain length and saturation (11) determine the HDM responsive CD1a-restricted TCR interaction. Maximal T cell stimulation was elicited by a fatty acid or lysophosphatidylcholine carbon chain length of 16 carbon atoms. Lipids with this carbon chain length are known to be prevalent in human skin (569, 570), and so, following epidermal barrier compromise, or generation by HDM derived sPLA2, might be presented to HDM responsive CD1a-restricted T cells by resident CD1a positive LCs and dermal DCs.

Indeed, fatty acids and long-chain ceramides are the two of the three dominant lipid constituents of the stratum corneum of the epidermis (202), functioning as an key components of the epidermal permeability barrier. Fatty acids additionally play a role in acidic pH maintenance of the stratum corneum which in turn modulates the activity of proteases that have a role in the degradation of keratinocyte intercellular junctions (471). Several endogenous cutaneous sPLA2s have been documented, which are secreted by corneocytes predominantly within the superficial layers of the epidermis (571). These sPLA2s break down phospholipids derived from cellular membranes with the resultant generation of fatty acids, and these lipids are known to be CD1a ligands (55, 469) so that these enzymes might be involved in the production of CD1a-restricted T cell neolipid antigens.

The different epidermal layers are known to contain differential lipid profiles. For example, ceramides with medium-long carbon chain lengths, which as discussed in the introduction have an important role in the maintenance of skin barrier integrity, are enriched within the stratum corneum (572). These ceramides lie within the array of carbon chain lengths capable of binding to the CD1a molecule and activating CD1a-restricted T cells, being predominantly 14-20 carbon atoms in alkyl chain length (572). Therefore, there is enrichment of the epidermis with CD1a ligands, which co-localise with CD1a molecules in the form of LCs which express CD1a at high levels and are the dominant APC of the epidermis. Autoreactive CD1a-restricted T cells have been demonstrated to be a significant component of the T cell repertoire (119) and are predominantly dermal in location. Such physical separation minimises co-localisation of CD1a-restricted T cells with CD1a molecules presenting lipid antigens in the steady state, and indeed this separation of the CD1a-restricted TCR and CD1a-lipid complex might be important in preventing constitutive stimulation of CD1a-restricted T cells (523). Furthermore, there is tight regulation of CD1a protein expression systemically (523), which is highly restricted to thymocytes,

Langerhans cells, and DCs of myeloid origin. Peripheral blood mononuclear cells only express CD1d molecules (failing to express group 1 CD1 molecules), and mDCs have been shown to exhibit an analagous expression profile when cultured in human serum instead of foetal calf serum (573). Indeed, the regulation of CD1 expression at the transcriptional level by human serum components, such as immunoglobulins, has been reported (573, 574). Therefore, restricted expression of CD1a, in combination with tight regulation of this protein might prevent constant stimulation of autoreactive CD1a-restricted T cells (523).

However, a breach in epidermal integrity in the context of AD might result in antigenic epidermal lipids infiltrating the dermis and dermal LC migration, and contact with, and activation of HDM responsive CD1a-reactive T cells. Indeed a dysregulated lipid profile associates with cutaneous inflammation and a compromised epidermal barrier (244, 569, 575, 576). Increased PLA2 activity and levels and altered PLA2 cutaneous locations have additionally been reported in inflammatory dermatoses (571) and in the context of filaggrin insufficiency (576).

Therefore, a breach in skin barrier integrity in the context of AD, further compounded by the presence of HDM-derived sPLA2 might lead to an elevated HDM-derived sPLA2 activity and increased production of sPLA2-generated neolipid antigens for presentation via CD1a (Figure 8.2). CD1a mediated neolipid antigen presentation may then occur via dermal DCs or epidermal LCs which exhibit increased dermal migration in the inflammatory setting (210), where they may encounter HDM responsive CD1a-reactive T cells. In addition, despite the fact that T cells are predominantly dermal in location, they can also traffic to the epidermis in the inflammatory setting so that epidermal LCs might also present HDM-derived neolipid antigens to epidermal CD1a-reactive T cells in addition to dermal CD1a-reactive T cells in inflammatory skin disorders including AD. The latter mechanism of HDM responsive CD1a-reactive T cell stimulation may occur through CD1a presentation

by dermal DCs or by LCs as they transit through the dermis. Hence HDM responsive CD1a-reactive T cells that are stimulated by products of HDM sPLA2 activity might function to sense a compromise in the epidermal barrier (Figure 8.2). Possible future therapeutic implications include the development of PLA2 inhibitors, and it is of interest that one action of corticosteroids, which remain the mainstay of treatment for AD and are commonly used to treat other inflammatory dermatoses, is PLA2 inhibition (431). It may therefore be postulated that inhibition of this HDM-derived sPLA2 CD1a-mediated inflammatory pathway might play a role in corticosteroid-mediated immunosuppression, precise mechanisms of which are poorly understood. Furthermore, amelioration of contact dermatitis in patients treated with a topical sPLA2 inhibitor has also been described (474), further supporting the involvement of PLA2 activity in skin inflammation and a possible therapeutic impact of its inhibition.

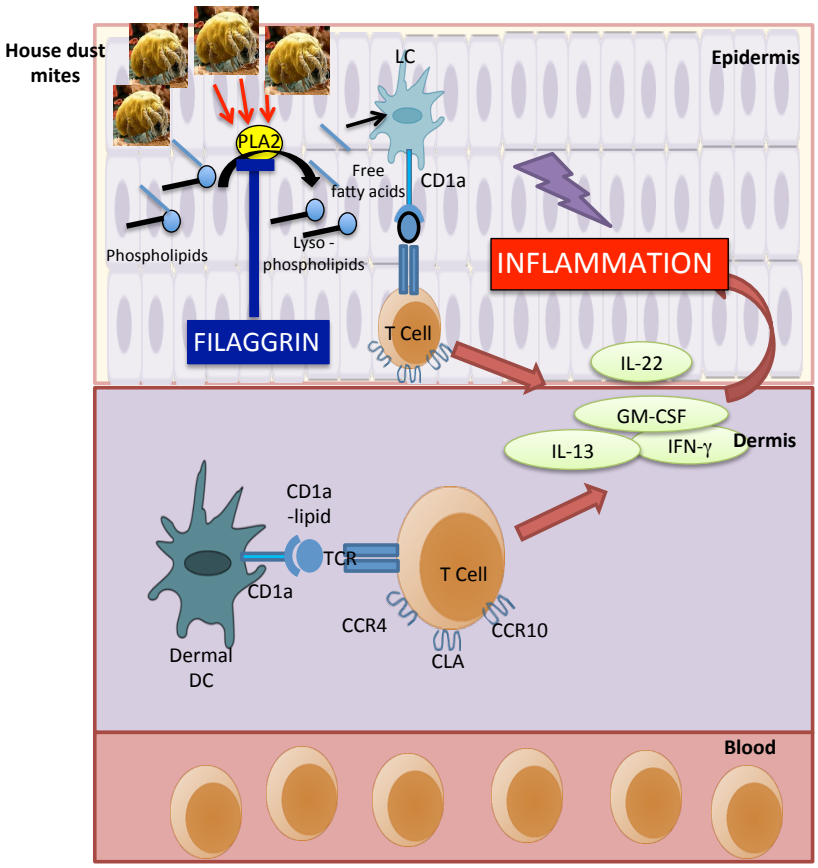


Figure 8.2. Proposed mechanism of HDM responsive CD1a-reactive T cell stimulation. Adapted from (577) and (568). A genetically or acquired impaired epidermal skin barrier in AD results in increased exposure to HDM-derived sPLA2 leading to increased phospholipid hydrolysis into antigenic free fatty acids and lysophospholipids for presentation via CD1a. These CD1a ligands are then presented by cutaneous antigen presenting cells including Langerhans cells which migrate to the dermis and dermal DCs to HDM responsive CD1a-reactive T cells in the dermis and/or epidermis in AD. Barrier insufficiency additionally results in many cutaneous changes that may amplify this process including a dysregulated lipid profile and altered cutaneous pH, reduced protease inhibitor function and elevated sPLA2 activity, and it is possible that increased endogenous, in addition to exogenous (HDM-derived) sPLA2 contribute to CD1a ligand generation, augmenting this process to further signal barrier compromise. Activated HDM responsive CD1a-reactive T cells then secrete a number of cytokines including IFN-γ, IL-13, GM-CSF and IL-22 and signal the breach in the epidermal barrier to initiate a cutaneous immune response. Genetic or acquired filaggrin insufficiency in the context of AD may amplify this model of cutaneous inflammation twofold, firstly by contributing to skin barrier dysfunction facilitating HDM-derived sPLA2 penetration into the epidermis, and secondly through the loss of filaggrin inhibition of HDM sPLA2, which may result in increased neolipid signals to CD1a-reactive T cells that barrier compromise has occurred, with downstream inflammatory sequelae.

Epidermal barrier sensing might be the main role of this inflammatory pathway as surmised above, however insect venom-derived phospholipases have also been reported to utilise this system to generate a CD1a-restricted immune response (469). Within insect venoms, phospholipases are believed to play a role in capturing prey and escaping predators via their capacity to induce pain and toxicity through neurotoxic sPLA2 N-type receptors (523). In addition, phospholipase enzymes

function to in degrade host tissue to facilitate the laying of eggs within this tissue in certain species of parasitic wasp (568, 578). Therefore, whilst the sPLA2 driven CD1a-restricted T cell response might have a major role in skin barrier sensing, it remains a possibility that this system might have evolved for the recognition of parasites in addition to other pathogens, which are also known to contain sPLA2s (434).

The use of HDM responsive CD1a-reactive T cell lines to further characterise these cells highlighted several interesting points for discussion. As previously described, lines expressed cutaneous addressins and produced type 1 and type 2 cytokines, suggesting a heterogeneous role in the immune response. HDM responsive CD1a-reactive T cell lines were predominantly CD8+ or DN, suggesting a cytotoxic phenotype and one of the lines investigated produced IL-22, indicative of a role in cutaneous immunity. No IL-17 or IL-10 production was detected, suggesting an absence of a Th17 or regulatory role. Interestingly, several of the HDM responsive CD1a-reactive T cell lines generated were composed of predominantly γδ T cells, indicating a role for this poorly defined class of T cells in the HDM responsive CD1a-reactive T cell response. T cell lines also exhibited considerable antigen cross-reactivity to phospholipase containing allergens which suggests a shared pathway of CD1a ligand generation and might be partially explained by recently reported experiments that CD1a is able to present multiple headless skin lipids (55) to CD1a-autoreactive T cells, together with the crystal structure of TCR-CD1a-self lipid ligand complexes (64). Of potential relevance, T cells with more than one cell surface T cell receptor permitting specificity to multiple different antigens have been reported (527, 528), or it is possible that the HDM responsive CD1a-reactive T cell lines generated contained oligoclonal T cell populations with multiple TCR specificities. HDM responsive CD1a-reactive T cell lines could additionally be stimulated by the different group 1 and group 2 CD1 isoforms to differing degrees. This finding has not been

previously reported, and as with the antigen cross-reactivity seen, it may be accounted for by the presence of oligoclonal T cell populations with varying TCR specificities, or by the HDM responsive CD1a-reactive T cell lines expressing more than one T cell receptor. It will be important to explore both of these findings further with TCR sequencing as a part of future studies. Nevertheless, recognition of multiple CD1 isoforms by a presumed oligoclonal T cell population is a fascinating observation which is potentially a novel finding of CD1a-reactive T cells.

We also show that recombinant filaggrin can inhibit the PLA2 activity of HDM and the subsequent generation of CD1a neolipid ligands for presentation to T cells. Whilst increased sPLA2 IIA activity in the context of filaggrin insufficiency has been reported (576), this is a hitherto unappreciated function of filaggrin which may help link the presence of filaggrin insufficiency to cutaneous inflammation. The data also provide a potential resolution to the seemingly contrasting "inside-out" and "outside-in" hypotheses of atopic dermatitis, where instead of two independent possibilities, filaggrin can act in both a barrier function/hydration capacity and also as a direct inhibitor of cutaneous lipid-specific immune responses. By inhibiting down-stream T cell effector functions, for example through anti-IL-4Rα, acquired down-regulation of filaggrin may be reversed, leading to enhanced filaggrin inhibition of PLA2, and less barrier distress signals to CD1a-reactive T cells. 20-40% of individuals with moderate-severe atopic dermatitis have filaggrin null mutations, yet acquired filaggrin insufficiency and barrier impairment are common (208, 579). This supports the findings presented herein in which specific aspects related to the downstream immunological events are also important in contributing to the filaggrin insufficiency and compounded atopic cutaneous inflammation (579, 580). The data further substantiate the pursuit of therapeutic strategies that modulate relevant immune responses, perhaps in conjunction with approaches which modulate filaggrin.

Given that PLA2 generation of CD1a ligands has now been shown to be present in three allergens of relevance to humans (516, 523, 544), namely wasp venom, bee venom and HDM, the data suggest that this is part of a broader hypersensitivity system, and the identified roles of CD1a and PLA2 in allergic diseases might facilitate novel approaches for the diagnosis and management of these conditions. Indeed phospholipases are known to be present in many other allergens including pollens and fungal allergens such as *Aspergillus fumigatus* (581-583) and therefore activation of this pathway may promote the allergenic process in allergic rhinitis and allergic bronchopulmonary aspergillosis respectively. By producing type 2 cytokines, CD1a-reactive lipid-specific T cells may lead to down-regulation of filaggrin and anti-microbial peptide expression and thus compound physical and antimicrobial barrier dysfunction. Furthermore, through cytokine secretion, activated T cells may license skin dendritic cells to amplify peptide-specific Th2 responses and subsequent IgE generation. In this respect, it is noteworthy that, as performed in these studies, GM-CSF and IL-4 are routinely used to differentiate monocytes towards mDC and *in vitro* LC-like cells with CD1a and class I/II antigen presenting capacity. If CD1a-reactive responses do contribute to initiation of the allergic process, then we might predict that allergens delivered to anatomical sites that do not contain antigen presenting cells with high levels of CD1a protein would lead to differing systemic responses. This is indeed the case as wasp venom, bee venom and grass pollen subcutaneous immunotherapy are all known to be highly effective (584, 585). In contrast to the epidermis and dermis, subcutaneous tissue has few CD1a-expressing cells. These findings therefore have potential therapeutic implications. As described, it is of interest that corticosteroids are known to potentially inhibit PLA (431), and it may be that in the skin, this is an important mechanism for controlling CD1a-reactive T cell activity. The development of PLA inhibitors that target individual relevant allergenic PLAs may enhance treatment efficacy whilst reducing side effects of broad host PLA inhibition seen with current corticosteroids.

In conclusion, we have identified polyclonal HDM responsive CD1a-reactive T cells, which are enriched in the blood and skin of patients with AD and produce a number of cytokines pertinent to cutaneous immunity and the pathogenesis of AD. HDM-derived sPLA2 was identified as the CD1a reactive fraction of HDM, and generated CD1a neolipid antigens for presentation to CD1a-reactive T cells, thereby identifying a novel pathway of AD cutaneous inflammation. We demonstrated that HDM responsive CD1a-reactive T cells infiltrated the skin after HDM allergen challenge in AD patients, and expressed skin addressins, further implying a function in skin inflammation, and we conclude that these cells may play a role in the detection of epidermal barrier impairment. By additionally defining a novel function of filaggrin in inhibiting PLA2, we are also able to potentially unify the conflicting “outside-in” and “inside-out” hypotheses of atopic dermatitis. As keratinocytes proceed through cornification, profillagrin is cleaved into filaggrin monomers which are incorporated into the cornified cell envelope and exposed to the extracellular space and therefore have the potential to come into contact with HDM-derived sPLA2. The data would support therapeutic approaches to inhibit allergen-derived PLA2 activity, together with treatments that target the downstream immunological effector pathways.

8.1 Future work

Further work is needed to answer questions that some of the results presented in this thesis have raised.

Firstly, the current studies concentrated on IFN-γ, GM-CSF and IL-13 secretion by HDM responsive CD1a-reactive T cells as a broad investigation into type 1 and type 2 cytokine production and to facilitate the quantification of *ex vivo* frequencies of HDM responsive CD1a-reactive T cells in patients with AD and healthy controls. This initial analysis should be expanded to investigate other cytokines, particularly IL-22, which was produced by one HDM responsive CD1a-reactive T cell line

investigated. IL-22 is known to be produced by autoreactive CD1a-restricted T cells and to exert numerous effects in the skin relevant to AD including upregulation of AMP production (331) and downregulation of filaggrin expression and epidermal differentiation (332-334), thereby exaggerating the skin barrier dysfunction and contributing to the epidermal hyperplasia seen in this condition. It would be very important to extend the investigation of this cytokine to all of the T cell lines generated, particularly AA9005, which was derived from the skin, and to polyclonal HDM responsive CD1a-reactive T cell responses which were not studied in this thesis. Examination of the patho-physiological role of IL-22 producing HDM responsive CD1a-reactive T cells in AD would advance our comprehension of disease pathogenesis and reveal potential targets for therapy.

In addition, having identified HDM responsive CD1a-reactive T cells within the blood and skin, their role *in vivo* should be examined in an attempt to establish a causal link to AD. This might be done by using transgenic mice engineered to express human group 1 CD1 molecules (105) and examining T cell responses to challenge with HDM. This would be informative in determining whether HDM responsive CD1a-reactive T cells have a direct role in the pathogenesis of AD, and it would also be informative to examine cytokine secretion by these cells to investigate whether there is the same heterogeneous range of cytokine production, or if there is a predominance of type 1 or type 2 cytokine production *in vivo*. This novel inflammatory pathway additionally highlights novel therapeutic targets for manipulation in AD in the form of PLA2 inhibitors and the use of such animal disease models of AD to examine the effect of PLA2 inhibition might enable the development of innovative treatments for this condition.

Further experiments are also needed to more extensively characterise the HDM responsive CD1a-reactive T cell lines. These include TCR sequencing for examination of T cell clonality and target cell lysis assays to investigate their

suggested cytotoxic phenotype. Furthermore, in addition to the dual colour Fluorospot utilised, intra-cellular cytokine staining (ICS) would be helpful to confirm if, as observed with Fluorospot, the majority of type 1 and type 2 cytokine producing cells are unique subsets, or whether HDM responsive CD1a-reactive T cells are capable of secreting both type 1 and type 2 cytokines. ICS or the IFN-γ secretion assay could also be utilised to determine the responding T cell subset in the HDM responsive CD1a-reactive T cell lines composed of predominantly γδ T cells in order to further define the role of the γδ T cell receptor in the HDM responsive CD1a-reactive T cell response. In addition, as discussed it would also be interesting to determine CD45RO, CCR7, CD103 and CD62L expression suggestive of previous antigen encounter to examine whether, as expected from the cord blood results and previous reports, adaptive central or tissue resident memory type characteristics are seen.

Having demonstrated that filaggrin monomers inhibit HDM-derived sPLA2, further work should examine the mechanism of such inhibition, and indeed it is very interesting to note that increased sPLA2 activity has been reported in filaggrin deficiency in a 3D skin construct (576). Proposed mechansims of filaggrin inhibition of sPLA2 activity include direct binding of filaggrin monomers to PLA2, which might be investigated by a direct ELISA for filaggrin-PLA2 binding or a pull down experiment, or inhibition of sPLA2 via a filaggrin break down product such as urocanic acid. Furthermore, it is possible that an altered epidermal pH in the context of filaggrin insufficiency or that filaggrin chelation of Ca^{2+} ions may impact on sPLA2 enzymatic activity.

Finally, Langerin has been reported to be necessary for CD1a mediated lipid ligand presentation by Langerhans cells (405), and co-localisation of CD1a protein with Langerin in Birbeck granules of these cells has been demonstrated (36). Future work in the laboratory will therefore be to study this association further with respect to

HDM-derived sPLA2 generated neolipid antigens, in addition to other lipid loading pathways.

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