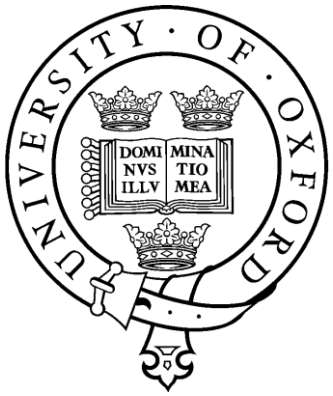
The Role of Fungal-Responsive CD1a-Reactive T cells in the Pathogenesis of Atopic Dermatitis



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Abstract

The Role of Fungal-Responsive CD1a-Reactive T cells in the Pathogenesis of Atopic Dermatitis.

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Atopic dermatitis (AD) is a common chronic inflammatory skin condition characterised by a skin-barrier defect, altered lipid profile and enrichment of CD1a-expressing antigen presenting cells. Although several endogenous epidermal CD1a ligands have been identified, non-endogenous ligands of CD1a are still to be elucidated. There is an association between the presence of fungi such as *C. albicans* on the skin and AD pathogenesis, but no previous studies have investigated the specific role of fungal-responsive CD1a-reactive T cells. We hypothesised that AD is associated with an altered frequency and phenotype of fungal-responsive CD1a-reactive T cells in human blood and skin and that fungal-responsive CD1a-reactive T cells contribute to fungal immune responses, cutaneous immunity and AD. We tested this hypothesis by investigating the frequency and functional profile of fungal-responsive CD1a-reactive T cells in the blood and skin of healthy donors and patients with AD. We have shown that fungi may be sensed through the CD1a pathway through recognition of fungal lipids by CD1a-reactive T cells. We have characterised fungal-responsive CD1a-reactive T cell clones and identified their T cell receptor usage and co-receptor usage. Fungal antigens including unique fungal lipids such ergosterol and ergosterol esters and modified self-lipids such as squalene peroxide were identified to be possible novel CD1a-binding lipids. The fungal-responsive CD1a-reactive T cells may contribute to fungal defence and skin inflammation defining a broader understanding of the mechanisms underlying AD which will likely help in the design of novel therapeutics in the future.

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Abbreviations

AD	Atopic dermatitis
AD-HIES	Autosomal dominant hyper-IgE syndrome
ADP	Adenosine diphosphate
AIRE	Autoimmune regulator
AMPs	Anti-microbial peptides
APCs	Antigen-presenting cells
APS-1	Autoimmune polyglandular syndrome type 1
AR	Autosomal recessive
ARF6	Adenosine diphosphate ribosylation factor 6
BSA	Bovine serum albumin
C. albicans	*Candida albicans*
CARD9	Caspase recruitment domain family member 9
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD1	Cluster of differentiation 1
cDC1	Conventional DCs type 1
cDC2	Conventional DCs type 2
CER	Ceramide
CLA	Cutaneous lymphocyte-associated antigen
CLRs	C-type lectin receptors
CR3	Complement receptor 3
CSF2	Colony stimulating factor 2
DAGs	Di-acyl glycerols
DCs	Dendritic cells
DETCs	Dendritic epidermal T cells
DMSO	Dimethyl sulfoxide
dNKT	Diverse NKT cells
DOK8	Dedicator of cytokinesis 8
EASI	Eczema Area and Severity Index
ELISA	Enzyme-linked immunosorbent assay

EpCAM	Epithelial cell adhesion molecule
ER	Endoplasmic reticulum
FCS	fetal bovine serum
FcεR1s	High-affinity immunoglobulin-ε receptors
FLG	Filaggrin gene
FMOs	Fluorescence minus one controls
GalACer	Galacturonosylceramide
GalADAG	Galacturonosyldiacylglycerol
GalCer	Galactosylceramide
GalDAG	Galactosyldiacylglycerol
GD3	Ganglioside D3
GEM	Germline-encoded mycolyl-specific
GlcCer	Glucosylceramide
GM-SCF	Granulocyte-macrophage colony-stimulating factor
HBD-2	Human beta-defensin 2
HDM	House dust mites
HLA-DR	Human leukocyte antigen-DR
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ICAM-1	Intercellular adhesion molecule-1
IDECs	Inflammatory dendritic epidermal cells
IFN	Interferon
iGb3	Isoglobotrihexosylceramide 3
IGF-1	Insulin-like growth factor 1
IKBG	Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma
ILC1s	Group 1 ILCs
ILC2s	Group 2 ILCs
ILC3s	Group 3 ILCs
ILCs	Innate-lymphoid cells
iNKT	Invariant NKT cells
IPC	Inositolphosphorylceramide
ISAAC	The International Study of Asthma and Allergies in Childhood
LCs	Langerhans cells

Lyso-PC	Lysophophatidylcholine
Lyso-PE	Lysophophatidylethanolamine
Lyso-PG	Lysophophatidylglycerol
Lyso-PL	Lysophospholipase
M(IP)2C	Mannosyldiinositolphosphorylceramide
M1	Type 1 macrophages
M2	Type 2 macrophages
MHC	Major histocompatibility complex
MIPC	Mannosylinositolphosphorylceramide
mLPAs	Methyl-lysophosphatidic acids
M-MLV RT	Moloney murine leukaemia virus reverse transcriptase
MNCs	The mononuclear cells
MO-DCs	Monocyte-derived dendritic cells
MST1	Macrophage stimulating 1
MTP	Microsomal triglyceride transfer protein
NETs	Neutrophil extracellular traps
NK	Natural killer cells
NKT	Natural killer T cells
NLRs	NOD-like receptors
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
N-UVB	Narrowband ultra-violet B
ORAI1	Calcium release-activated calcium channel protein 1
PA	Phosphatidyl acid
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
pDCs	Plasmacytoid DCs
PDE4	Phosphodiesterase 4
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGD2	Prostaglandin D2
PGLs	Phosphoglycerides

PI	Phosphatidylinositol
PL	Phospholipases
PPBF	Phenyl 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonate
PRRs	Pattern-recognition receptors
PS	Phosphatidyl serine
ROS	Reactive oxygen species
RT-qPCR	Quantitative and real-time polymerase chain reaction
SB	Stratum basale
SC	Stratum corneum
SCID	Severe combined immunodeficiency
SCORAD	Scoring atopic dermatitis
SEs	Sterol esters
SG	Stratum granulosum
SLE	Systemic lupus erythematosus
SLs	Sphingolipids
SNP	Single nucleotide polymorphism
SP	Squalene peroxide
SQ	Squalene
SRDs	Sterol-rich domains
SS	Stratum spinosum
STAT	Signal transducer and activator of transcription
STK4	Serine/threonine kinase 4
SYK	Spleen tyrosine kinase
TAGs	Tri-acyl glycerols
TCI	Topical calcineurin inhibitors
TCR	T cell receptor
TCS	Topical corticosteroids
Tem	Effector memory T cells
Th1 cells	Type 1 T-helper cells
Th17	T helper 17 cells
Th2 cells	Type 2 T-helper cells
TLR	Toll-like receptor
Tmm	Migratory memory T cells

TNF	Tumour-necrosis factor
Treg cells	Regulatory T cells
Trm	Resident-memory T cells
TSLP	Thymic stromal lymphopoietin
vNKT	Variant NKT
α-GalCer	Alpha-galactosylceramide

1. Introduction

1.1. Skin immune mechanisms

The skin is composed of four distinct functional barriers: the microbial barrier, the chemical barrier, the physical barrier and the immune barrier that orchestrate together to maintain protection from exogenous challenges (Eyerich, Eyerich et al. 2018). The components of each of these barriers are illustrated in Figure 1.1.

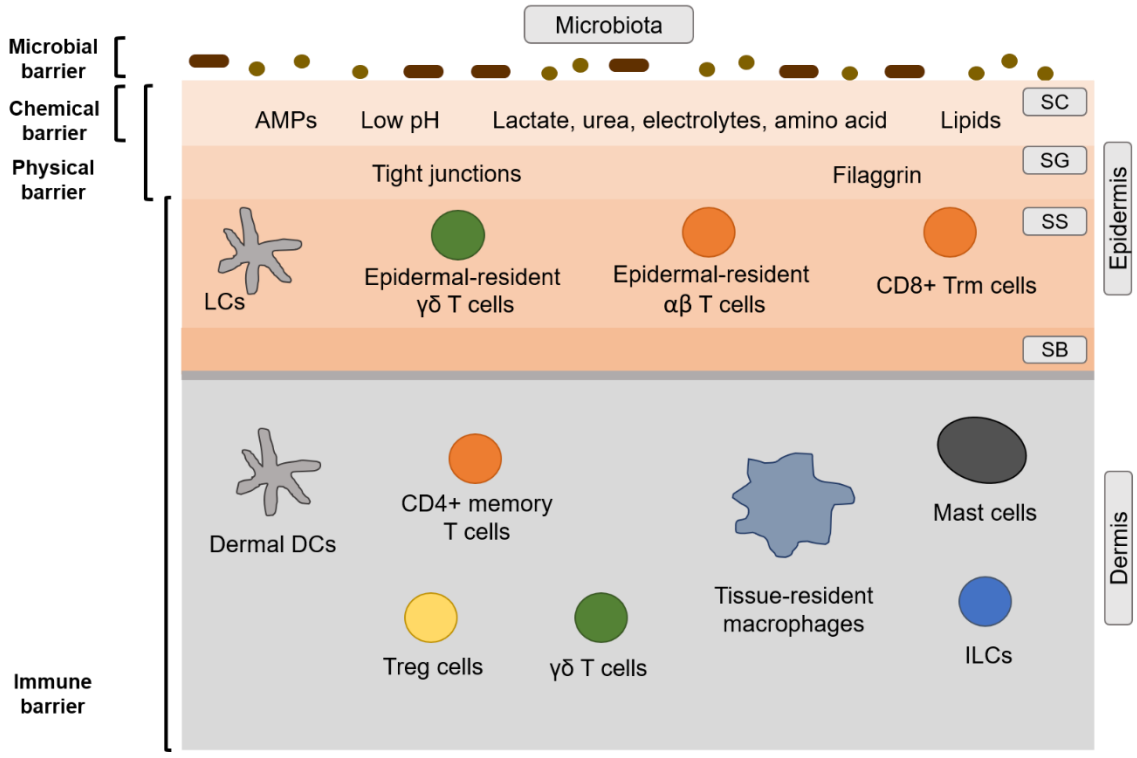


Figure 1.1 The components of the skin immune barriers.

Abbreviation: AMPs, anti-microbial peptides; SC, stratum corneum, SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; LCs, Langerhans cells; Trm, resident-memory T cells; Th cells, T helper cells; DCs, dendritic cells; ILCs, innate-lymphoid cells; Treg cells, regulatory T cells. Modified from (Heath and Carbone 2013, Malissen, Tamoutounour et al. 2014, Pasparakis, Haase et al. 2014, Eyerich, Eyerich et al. 2018).

1.1.1. The microbial barrier

The skin is a complex and dynamic environment that is a home for different types of microorganisms including bacteria, fungi and viruses (Byrd, Belkaid et al. 2018) that have essential roles in educating the immune system and protecting from invading pathogens (Belkaid and Segre 2014).

On the surface of healthy skin, bacteria predominate other types of microbiota across different skin sites. In contrast, fungi are the least abundant (Oh, Byrd et al. 2016). In sequencing surveys of healthy individuals, the composition of the bacterial skin microbiota varied on different skin sites depending on the moisturisation or dryness of the skin site and the presence or the absence of sebaceous glands (Costello, Lauber et al. 2009, Grice, Kong et al. 2009). For instance, sebaceous sites were dominated by lipophilic bacteria (e.g. *Propionibacterium* species), moist skin sites were inhabited by *Staphylococcus* and *Corynebacterium* species and dry skin sites were predominantly occupied by *Proteobacteria* (Grice, Kong et al. 2009, Findley, Oh et al. 2013). Conversely, fungal microbiota composition did not show major differences in term of genus predominance across skin sites as *Malassezia* species predominate throughout. Nevertheless, differential densities of *Malassezia and subtypes* are well known, for example enrichment at sites of sebum production. However, in the foot, diverse species of fungi were present including *Malassezia*, *Aspergillus*, *Candida, Cryptococcus*, *Rhodotorula* and *Epicoccum* species. (Findley, Oh et al. 2013). Changes in the microbiota in AD are discussed in section 1.3.3.4.

In the steady state, there is a mutualistic interaction between the microbiota and the skin (Belkaid and Tamoutounour 2016). Skin microbiota utilise the resources and nutrients that are present in sweat, sebum and the stratum corneum. For

instance *Malassezia* (Wu, Zhao et al. 2015) and *Corynebacterium* (Scharschmidt and Fischbach 2013) species are unable to fully produce their own lipids and therefore rely on the lipids of the sebum for nutrition. Moreover, *Staphylococcus* species have the ability to utilize the urea in the sweat as a nitrogen source (Scharschmidt and Fischbach 2013). In addition, some bacterial and fungal microbiota can produce proteases to hydrolyse skin proteins to utilise the amino acids released and lipases to degrade sebum-derived lipids and release free fatty acids to facilitate their adherence to the skin surface and induce acidification of the skin (Takahashi, Banno et al. 1991, Gribbon, Cunliffe et al. 1993, Fluhr, Kao et al. 2001).

In the same context, the skin and the immune system benefit from the presence of these microbiota by several ways. Firstly, because both the skin microbiota and pathogenic microorganisms compete for the same ecological niche the skin microbiota limit pathogen colonization, growth and survival through the competition for space and nutrition and changing the skin environment, for example affecting the pH (Buffie and Pamer 2013). Secondly, the skin microbiota regulate the innate immune responses in the skin and increase the resistance toward pathogens (Belkaid and Tamoutounour 2016). This is achieved by their contribution to preserve the integrity of the skin and promote tissue repair (Lai, Di Nardo et al. 2009), induce the expression of anti-microbial peptides (AMPs) (Nagy, Pivarcsi et al. 2006) and stimulate the complement-mediated immune pathway (Chehoud, Rafail et al. 2013). Thirdly, cutaneous microbiota modulate the adaptive immune responses in the skin (Belkaid and Tamoutounour 2016). The enhancement of innate immunity promotes adaptive immune responses through the activation of resident T lymphocyte function through IL-1 signalling (Naik, Bouladoux et al.

2012). In addition, skin microbiota shape adaptive immune responses by establishing IL-17A-producing T cells, contributing to effective immune defence against invading pathogens (Eyerich, Eyerich et al. 2018). In addition, the skin microbiota have been shown to establish tolerance by promoting the accumulation of regulatory T cells (Treg cells) in the skin (Volz, Skabytska et al. 2014), stimulating IL-10 production by dendritic cells (DCs) *in vitro* (Laborel-Preneron, Bianchi et al. 2015) and inhibiting the expression of epidermal-derived thymic stromal lymphopoietin (TSLP) (Yockey, Demehri et al. 2013).

1.1.2. The chemical barrier

The chemical barrier of the skin is composed of factors that contribute, for example, to the acidic surface pH which include amino acids and their derivatives, lactates, urea, and electrolytes (Eyerich, Eyerich et al. 2018). The chemical barrier is tightly connected to the physical barrier and any alteration in the chemical barrier influences the stratum corneum (SC) pH, protein content and lipid content (Verdier-Sevrain and Bonte 2007, Ali and Yosipovitch 2013). In addition, AMPs produced by the keratinocytes and cutaneous immune cells represent an integral component of the chemical barrier of the skin that also trigger and synchronise with the innate and adaptive immune cells (Schauber and Gallo 2009).

1.1.3. The physical barrier

The outermost component of the physical barrier is the SC (Eyerich, Eyerich et al. 2018) which is formed by the terminal differentiation of maturing keratinocytes that move up the epidermal layers (starting from stratum basale (SB) moving toward the stratum spinosum (SS) then to stratum granulosum (SG) and finally reach the SC). When the keratinocytes reach the upper-most layer of the epidermis, they

lose their nuclei and form corneocytes in a process of cell death, termed cornification. Those protein-enriched corneocytes are embedded in a lipid matrix to form the SC (Matsui and Amagai 2015, Eyerich, Eyerich et al. 2018).

Keratinocytes of the SG are connected by tight junctions. Tight junctions are cell-cell junctions consisting of transmembrane proteins, junctional adhesion molecules and plaque proteins and are connected with an actin filament cytoskeleton. The main function of skin tight junctions is to form a two-way barrier separating the internal/external environments and influence other skin barrier components like the SC, microbial barrier, chemical barrier and the immune cells (Basler, Bergmann et al. 2016). In addition, the SG contain granules that are composed of important proteins that contribute to the physical barrier such as filaggrin, loricrin, and keratin filaments in addition to lamellar bodies that contain lipids, corneodesmosins and kallikreins (Basler, Bergmann et al. 2016, Eyerich, Eyerich et al. 2018). The role of filaggrin in skin barrier is discussed in detail in section 1.3.3.1.

In addition, cells in the epidermis contribute to the production of important lipids, delivering them into the SC to provide an effective skin barrier and regulate transepidermal water loss. Keratinocytes for example deliver triglycerides and cholesterols and the sebaceous glands secrete triglycerides, wax esters, and squalene (Eyerich, Eyerich et al. 2018).

1.1.4. The immune barrier

The immune barrier of the skin is composed of skin-resident and migratory immune cells which all coordinate to maintain the cutaneous immune function (Heath and Carbone 2013, Eyerich, Eyerich et al. 2018).

1.1.4.1. Immune cells in the epidermis

The epidermis in human skin is rich with several types of immune cells such as Langerhans cells (LCs), epidermal-resident αβ and γδ T cells and resident-memory CD8+ T cells (Trm).

1) LCs

LCs are the main type of professional antigen-presenting cells (APCs) in the epidermis. They express CD45, MHC class II, CD172α, langerin (CD207), CD11b, CD1a, CD1c, E-cadherin and epithelial cell adhesion molecule (EpCAM). (Malissen, Tamoutounour et al. 2014, Kashem, Haniffa et al. 2017). In the steady state, LCs repopulate locally and upon stimulation, LCs have the capacity to protrude their dendrites through the epidermal tight junctions to acquire antigen from the SC (Kubo, Nagao et al. 2009) and then migrate to the skin-draining lymph nodes to prime T cells (Stoitzner, Tripp et al. 2005). LCs were shown to induce the differentiation of type 2 T-helper cells (Th2 cells) and to prime and cross-prime CD8+ T cells to induce inflammatory responses (Klechevsky, Morita et al. 2008). In addition, they can contribute to tissue homeostasis through the activation of Treg cells (Seneschal, Clark et al. 2012).

2) Epidermal-resident αβ T and γδ T cells

In addition to LCs, human epidermis contains resident αβ T cells and γδ T cells ($V\delta1^+$ T cells predominantly) which express high levels of the skin homing marker cutaneous lymphocyte-associated antigen (CLA) and are therefore speculated to have a potential to function in immune surveillance in the skin (Toulon, Breton et al. 2009). Resident epidermal T cells regulate skin homeostasis through the production of insulin-like growth factor 1 (IGF-1) and also contribute to wound

healing (Toulon, Breton et al. 2009). Compared to murine epidermal γδ T cells, γδ T cells in human epidermis represent a very small population (1-2% of total T cells in healthy skin). γδ T cells in the murine epidermis comprise the vast majority of all epidermal T cells and are known as dendritic epidermal T cells (DETCs) which express an invariant T cell receptor (TCR) identified as Vγ3 Vδ1^{+} (Macleod and Havran 2011). Upon activation, DETCs secrete different types of cytokines and chemokines (e.g. CCL3, CCL4, CCL5 and XCL1, IFN-γ, IL-2 , IL-13) which induce inflammatory responses and contribute to cutaneous microbial defence, tumour immunosurveillance, tissue repair and homeostasis (Macleod and Havran 2011).

3) Resident-memory CD8+ T cells (Trm)

In models of herpes simplex virus infection, resident-memory CD103+ CD8^{+} T cells were found to be present in skin are thought to control local reactivations (Wakim, Waithman et al. 2008, Gebhardt, Wakim et al. 2009, Mackay, Rahimpour et al. 2013). These cells were found to have the capacity for self-renewal either through homeostatic or antigen-stimulatory mechanisms (Wakim, Waithman et al. 2008, Gebhardt, Wakim et al. 2009). The entry and local signalling of the CD8+ Trm in the epidermis is related to the presence of specific chemokines (CXCL9 and CXCL10), cytokines (TGF-β and IL-15) and surface markers (CD69 and CD103) (Mackay, Rahimpour et al. 2013).

1.1.4.2. Immune cells in the dermis

Immune cells present in the dermis are predominantly dermal DCs, dermal macrophages, T cell subsets, mast cells and innate-lymphoid cells (ILCs).

1) Dermal DCs

The dermal DCs are phenotypically diverse and highly plastic group of cells. The subtypes of DCs present in the dermis are the conventional DCs type 1 (cDC1) or CD141+ DCs, the conventional DCs type 2 (cDC2) or CD1c+ DCs , the CD14+ DCs and the plasmacytoid DCs (pDCs), although the latter are rare in healthy skin (Segura 2016, Kashem, Haniffa et al. 2017).

Dermal DCs carry antigen acquired in dermis to draining lymph for the priming naive T cells and therefore contribute to skin immunosurveillance and trigger immune responses (Matejuk 2018). For instance, the functional specialization of the two subsets of the conventional DCs has been well established. While cDC1 induce the immunity against intracellular pathogens by the induction of CD8 T cell and type 1 T-helper cells (Th1 cells) (Mashayekhi, Sandau et al. 2011, Martinez-Lopez, Iborra et al. 2015), cDC2 -the most predominant dermal DC population- provide responses toward extracellular pathogens via promoting Th2 and T helper 17 cell (Th17) priming (Gao, Nish et al. 2013, Persson, Uronen-Hansson et al. 2013). The pDCs are a highly specialized type-1 interferon (IFN)-producing DC population that play a major role in the protection against viral infections and potentially contributing to autoimmune diseases (Reizis, Bunin et al. 2011).

2) Dermal macrophages

Skin resident dermal macrophages are integral components of the mononuclear phagocyte system in the skin. They play an essential role in the maintenance of skin homeostasis and integrity as they contribute to wound healing, tissue repair, hair follicle regeneration, and the stress response (Yanez, Lacher et al. 2017). The dermal macrophages can be polarised into two distinct types M1 and M2 which have pro-inflammatory or anti-inflammatory functions, respectively. Inflammatory

stimuli sensed by macrophage receptors, resulting in downstream signalling cascades (Davies and Taylor 2015). M1 cells have been reported to be enriched in psoriasis (Clark and Kupper 2006) and atopic dermatitis (AD) (Kasraie and Werfel 2013).

3) T cell subsets

Clark and colleagues characterised the skin resident T cells phenotype and found that most of these cells expressed high levels of the skin homing markers: CLA, chemokine receptor 4 (CCR4), and chemokine receptor 6 (CCR6). Interestingly, 98% of CLA+ effector memory T cells were found to reside in normal skin in steady state. Moreover, this study found that the skin T cells had diverse αβ TCR repertoire and 80% of these cells were effector memory T cells and were mostly Th1 subtype, consistent with these T cells contributing to cutaneous immunosurveillance. In addition to Th1 cells, other types of T cells were found to be present in the dermis including Th2 and Treg cells (Clark, Chong et al. 2006).

The memory T cells in the dermis are divided broadly into non-recirculating resident memory T cells (Trm) and recirculating memory T cells. Two distinct subsets of the Trm were characterised according to CD103 expression. The CD103+ population resides in the epidermis whereas the CD103- population resides in the dermis (Watanabe, Gehad et al. 2015). Most of the Trm cells in human dermis were reported to be CD4+ (Heath and Carbone 2013, Watanabe, Gehad et al. 2015). The recruited memory T cells can be either CCR7+/L-selectin+ central memory T cells (Tcm), CCR7+/L-selectin- migratory memory T cells (Tmm) or effector memory T cells (Tem) (Watanabe, Gehad et al. 2015).

In addition to the αβ memory T cell subtypes present in the dermis, γδ T cells have been identified in peripheral tissue (Cruz, Diamond et al. 2018). IL-23 responsive IL-17A-secreting γδ T cells were found to reside in the dermis and play an important role in psoriasis pathogenesis (Cai, Shen et al. 2011). Dermal γδ T cells recognise a wide variety of peptide and non-peptide antigens released by stressed, damaged, malignant, or infected cells (Cruz, Diamond et al. 2018).

Moreover, Treg cells are an important subset of $CD4^+$ T cells in dermis that regulate immune responses and maintain tissue homeostasis. An imbalance between pathogenic effector T cells and Treg cells results in chronic tissue inflammation and autoimmunity (Ali and Rosenblum 2017). Unique populations of Treg cells reside or infiltrate peripheral tissues where they mediate specific functions including in the skin (Ali and Rosenblum 2017). In normal human adult skin, approximately 20% of skin-resident CD4+ T cells are Treg cells, compared with only 5% found in peripheral blood (Sanchez Rodriguez, Pauli et al. 2014).

4) Mast cells

Mast cells have multivalent functions in the skin as they contribute to the first-line defence against harmful pathogens by direct phagocytosis and participate in tuning the innate and adaptive immune pathways (Cardamone, Parente et al. 2016). Upon activation, mast cells degranulate and secrete a wide spectrum of mediators (e.g. histamine, proteoglycans, serotonin, tumour-necrosis factor (TNF), lipid mediators) that promote the inflammatory process and recruit innate immune cells to the inflammation site (Cardamone, Parente et al. 2016). Mast cells also trigger adaptive immunity by recruiting T cell subsets through the expression of chemotactic factors: CCL3, CCL4, CXCL9 and CXCL10 for Th1; CCL5 and CCL11

for Th2; CCL2 and CCL20 for Th17 (Marshall 2004). In addition, they secrete different types of cytokines which trigger Th cell polarization and activation such as IL-12 and IFN-γ for Th1, IL-4 for Th2, IL-6 and TGFβ1 for Th17, IL-6 and TNFα for Th22 (Galli, Kalesnikoff et al. 2005). Mast cells also interact with B cells and promote their differentiation and proliferation (Merluzzi, Betto et al. 2015) and modulate Treg cell responses (Lu, Lind et al. 2006).

5) ILCs

In recent years there has been a marked interest in exploring the role of ILCs in inflammatory diseases in general and in cutaneous immune responses in particular (Ebbo, Crinier et al. 2017). ILCs are a family of innate immune cells that are derived from a common lymphoid progenitor (Kim 2015). ILCs are classified into different groups based on their expression of transcription factors and cytokine production and align with analogue Th cell subsets. Group 1 ILCs (ILC1s) express T-bet and produce IFN-γ and TNF. Group 2 ILCs (ILC2s) express GATA3, BCL11B and GFI1 and produce type 2 cytokines and amphiregulin. Group 3 ILCs (ILC3s) express RORγt and produce IL-17A, IL-17F and IL-22. All three types of ILCs were identified in the skin (Ebbo, Crinier et al. 2017). ILC2s and ILC3s were reported to be highly associated with inflammatory skin conditions such as AD (Eyerich, Eyerich et al. 2015) and psoriasis (Villanova, Flutter et al. 2014), respectively.

1.2. CD1 molecules

Since the discovery of the cluster of differentiation 1 (CD1) molecules more than three decades ago (McMichael, Pilch et al. 1979, Bernard and Boumsell 1984), as a complementary antigen-presentation system to major histocompatibility complex (MHC)-peptide presentation, much work has been done to investigate and

understand the role of these molecules in lipid-presentation, in addition to their contribution to the immune pathways in health and disease (Silk, Salio et al. 2008, Van Kaer, Wu et al. 2016). In this section, aspects of CD1 molecules are discussed including: CD1 genes, structure, expression, assembly and trafficking, CD1-binding lipids and CD1-reactive T cells with emphasis on the CD1a molecule and CD1a-reactive T cells.

1.2.1. CD1 genes

The human CD1 gene complex is located outside the MHC region in a cluster on chromosome 1 (1q23.1) which encodes five CD1 genes (CD1a, b, c, d, and e) corresponding to five CD1 proteins (McMichael, Pilch et al. 1979, Calabi and Milstein 1986, Calabi and Milstein 2000). Mice lack CD1 a, b and c genes and express only CD1d genes (CD1d1 and CD1d2) on chromosome 3 (Calabi and Milstein 2000, Brigl and Brenner 2004). CD1 genes are structured with a similar intron/exon arrangement to MHC class I genes with resulting type 1 membrane proteins comprising α1, α2, and α3 domains (Brigl and Brenner 2004). Based on the sequence homology, CD1 molecules were divided into 3 groups: group 1 (CD1a, b and c), group 2 (CD1d) and group 3 (CD1e) (Calabi, Jarvis et al. 1989, Calabi and Milstein 2000, Brigl and Brenner 2004).

In contrast to MHC genes, CD1 genes are relatively non-polymorphic (Han, Hannick et al. 1999), however, some allelic variations of CD1 were reported in the literature. For example two allelic variants were characterised for CD1a (CD1a*01 and CD1a*02) (Han, Hannick et al. 1999, Oteo, Arribas et al. 2001). CD1a alleles have substitutions at amino acid positions 13 and 51. As a consequence, allele 1 contains an isoleucine at position 13 and a tryptophan at position 51 whereas allele 2 has a threonine at position 13 and a cysteine at position 51. These substitutions

might have a functional impact on the CD1a interactions with T cell receptors (Han, Hannick et al. 1999). The percentages of individuals positive for CD1a allele 1 and allele 2 are 23% and 98%, respectively in unrelated individuals representing diverse ethnic backgrounds, but this is likely to vary across different populations. (Han, Hannick et al. 1999).

1.2.2. CD1 structure

The crystal structures of CD1 molecules have shown some similarities and differences between CD1 molecules and MHC class I. Like MHC class I, CD1 heterodimers are composed of two non-covalently bound polypeptide chains, α heavy chain and β2-microglobulin. The α chain is composed of α1, α2, and α3 domains (Van Kaer, Wu et al. 2016).

In contrast to MHC molecules, CD1 have a hydrophobic and deeper antigen-binding groove (Van Kaer, Wu et al. 2016). While MHC class I contains 6 antigen-binding pockets (A-F), CD1a, c and d molecules contain only two (A′ and F′) (Gadola, Zaccai et al. 2002, Zajonc, Elsliger et al. 2003, Koch, Stronge et al. 2005, Scharf, Li et al. 2010). CD1b is an exception, it contains additional C′ and T′ pockets (Moody, Briken et al. 2002). Overall, the A′ pocket is located to one side and F′ pocket to the contralateral side. The surface above the A′ pocket is covered by the A′ roof and a portal over the F′ pocket allows antigens to protrude to the surface (Cotton, Shahine et al. 2018). Among other CD1 molecules, CD1a has the smallest antigen-binding groove (1.35 nm^3) while CD1b has the biggest (2.20 nm^3) (Van Kaer, Wu et al. 2016).

These distinct antigen-binding groove properties of CD1 molecules, determine their unique ligand binding characteristics; distinct CD1 isoforms can bind lipids

containing specific alkyl chains lengths that match the groove size (Silk, Salio et al. 2008). For examples, group 1 CD1 molecules bind lipids with an overall chain lengths of C36-42 (Gadola, Zaccai et al. 2002, Moody, Briken et al. 2002, Zajonc, Elsliger et al. 2003, Koch, Stronge et al. 2005, Scharf, Li et al. 2010). However, CD1 molecules can bind to lipids with shorter alkyl chains lengths because of spacer lipids present in the binding groove as reported for CD1b (Garcia-Alles, Versluis et al. 2006) or accommodate longer tail lipids in the presence of the accessory portals for CD1c and b to allow the lipids to protrude laterally from the cleft (Van Kaer, Wu et al. 2016, Moody and Cotton 2017).

1.2.3. CD1 expression

Group 1 CD1 molecules (CD1a-c) are expressed on cortical thymocytes and professional APCs (e.g. DCs and LCs) (Dougan, Kaser et al. 2007). CD1c molecule is also expressed on subsets of B cells including marginal zone and peripheral blood B cells (Delia, Cattoretti et al. 1988, Brigl and Brenner 2004). Hardman et al found that CD1a molecules were also expressed by epidermal ILC2s under certain circumstances (Hardman, Chen et al. 2017). The group 2 CD1 molecule (CD1d) has a wider tissue distribution and is expressed by hematopoietic, as well as, non-hematopoietic cells including DCs, marginal zone B cells, hepatocytes, intestinal epithelial cells, keratinocytes and activated T cells (Dougan, Kaser et al. 2007, Silk, Salio et al. 2008). The group 3 CD1 molecule CD1e is the only member of the CD1 family that is not expressed on the cell surface; it is localised in the late endosomal compartment. CD1e doesn’t have a direct role in antigen presentation but it is involved in antigen processing (Angenieux, Salamero et al. 2000).

1.2.4. CD1 assembly and trafficking

The CD1 heavy chain is folded and assembled with the β2-microglobulin in the endoplasmic reticulum (ER) and then acquires endogenous lipids or spacer lipids (Moody and Cotton 2017). The assembled CD1 molecules then traffic to the plasma membrane through the secretory pathway. On the cell surface, CD1a (Manolova, Kistowska et al. 2006) and CD1c (Briken, Jackman et al. 2000, Sugita, van Der Wel et al. 2000) have the ability to capture exogenous lipids, however, this is seen in a lesser extent with CD1b (Jackman, Stenger et al. 1998, Moody, Briken et al. 2002) and CD1d (Spada, Koezuka et al. 1998, Sugita, Cao et al. 2002).

All CD1 molecules are internalised through clathrin-coated pits (Brigl and Brenner 2004) except CD1a which is internalised in a clathrin-independent manner (Barral, Cavallari et al. 2008). After internalisation, CD1a is sorted into the early endosomes and then to the recycling endosomes, where it acquires foreign lipids, before trafficking back to the plasma membrane through the adenosine diphosphate (ADP) ribosylation factor 6 (ARF6)-dependent pathway (Sugita, Grant et al. 1999). Whereas, CD1b and CD1d are sorted firstly into the early endosomes, secondly into the late endosomes and finally into the lysosomes before migrating to the cell surface. CD1c can traffic in both early recycling endosomes and late endosomes and lysosomes (Brigl and Brenner 2004, Moody and Cotton 2017).

The differences in the trafficking pathway between CD1 molecules can be explained by the presence of a tyrosine-based sorting motif that binds to the adaptor protein complex for CD1b, c and d which facilitates entry into a variety of endosomal compartments (Jackman, Stenger et al. 1998, Briken, Jackman et al. 2000, Sugita, van Der Wel et al. 2000, Sugita, Cao et al. 2002, Lawton, Prigozy et

al. 2005) and the lack of this sorting motif for CD1a (Barral, Cavallari et al. 2008, Van Kaer, Wu et al. 2016).

1.2.5. CD1-binding lipids

Several endogenous and exogenous lipids have been identified to bind CD1 molecules including glycolipids, phospholipids, lipopeptides and oils. Group 1 CD1 molecules present endogenous, mycobacterial and other bacterial lipids to αβ and/or γδ T cells expressing diverse or semi-invariant TCR repertoire. Group 2 CD1 molecules (CD1d) present endogenous and exogenous lipid antigens to natural killer T (NKT) cells with semi-invariant, biased, or diverse TCR repertoire expression (Van Kaer, Wu et al. 2016).

On one hand, some lipids can bind to several CD1 molecules. Sulfatide for instance promiscuously binds to all CD1 isotypes (CD1a-d) (Shamshiev, Gober et al. 2002). Moreover, Lysophosphatidylcholine (Lyso-PC) binds to CD1a, c and d (Lopez-Sagaseta, Sibener et al. 2012, Birkinshaw, Pellicci et al. 2015, Roy, Ly et al. 2016), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) bind to CD1a and d (Cox, Fox et al. 2009, de Jong, Cheng et al. 2014, Birkinshaw, Pellicci et al. 2015) and mycobacterial-derived lipids bind to CD1a, b and c (Gilleron, Stenger et al. 2004, Moody, Young et al. 2004, Van Rhijn, Zajonc et al. 2005, Roy, Ly et al. 2016). On the other hand, CD1 binding groove can accommodate specific lipids that are unique to each CD1 isomer. The lipid antigens presented by CD1 molecules and their TCR recognition is summarised in Table 1-1.

Table 1-1 lipid antigens presented by CD1 molecules and their TCR recognition.

CD1 isotype	TCR	Antigen-recognised	References
CD1a	αβ	Sulfatide, PE, PI, PC, lyso-PC, fatty acids, squalene,	(Shamshiev, Gober et al. 2002, Van Rhijn,

		triacylglycerol, wax esters, dideoxymycobactin	Zajonc et al. 2005, de Jong, Cheng et al. 2014, Birkinshaw, Pellicci et al. 2015)
	γδ (TCRVδ1^{+})	PE 16:0/16:0	(Agea, Russano et al. 2005)
CD1b	αβ	Sulfatide, mycolic acid, glycerol monomycolate, diacylated sulfoglycolipids, glucose monomycolate, phosphatidylglycerol	(Beckman, Porcelli et al. 1994, Sieling, Chatterjee et al. 1995, Shamshiev, Gober et al. 2002, Gilleron, Stenger et al. 2004, Van Rhijn, Kasmar et al. 2013, Van Rhijn, Gherardin et al. 2014, Van Rhijn, van Berlo et al. 2016)
CD1c	αβ	Lipopeptides and isoprenoid glycolipids	(Moody, Ulrichs et al. 2000, Matsunaga, Bhatt et al. 2004)
	γδ	Sulfatide, lyso-PC, phosphomycoketide, mannosyl-phosphomycoketide	(Shamshiev, Gober et al. 2002, Roy, Ly et al. 2016)
CD1d	αβ (Type I NKT or iNKT cells)	α-GalCer, β-GalCer, α-GlcCer, α-GalACer, α-GalDAG, α-GlcDAG, cholesteryl α-glucoside, asperamide B, GD3, iGb3, PE, PI, PC, lyso-PC, lysopeptidophosphoglycan	(Kawano, Cui et al. 1997, Wu, Segal et al. 2003, Zhou, Mattner et al. 2004, Kinjo, Wu et al. 2005, Mattner, Debord et al. 2005, Sriram, Du et al. 2005, Fox, Cox et al. 2009, Brennan, Tatituri et al. 2011, Kinjo, Illarionov et al. 2011, Lopez-

			Sagaseta, Sibener et al. 2012, Albacker, Chaudhary et al. 2013, Wieland Brown, Penaranda et al. 2013, Brennan, Tatituri et al. 2014, Kain, Webb et al. 2014)
	αβ (Type II NKT, dNKT, or vNKT cells)	Sulfatide, β-GlcCer, β-GalCer, α-GalCer, PG, PI, cardiolipin, lyso-PE non-lipid small molecules (e.g., PPBF), peptides (synthetic, ovalbumin-derived, collagen-derived)	(Shamshiev, Gober et al. 2002, Jahng, Maricic et al. 2004, Van Rhijn, Young et al. 2004, Liu, Teige et al. 2011, Rhost, Lofbom et al. 2012, Tatituri, Watts et al. 2013, Wolf, Tatituri et al. 2015, Girardi, Wang et al. 2016, Le Nours, Praveena et al. 2016)
	γδ ($TCR\gamma\delta^+$ NKT cells)	Cardiolipin	(Dieude, Striegl et al. 2011)
CD1e	-	Sulfatide, PI, dimannosylated PI, diacylated sulfoglycolipids, hemi-bis(monoacylglycero)-phosphate	(Facciotti, Cavallari et al. 2011, Cala-De Paepe, Layre et al. 2012)

Abbreviations: dNKT, diverse NKT; GalCer, galactosylceramide; GalACer, galacturonosylceramide; GalADAG, galacturonosyldiacylglycerol; GalDAG, galactosyldiacylglycerol; GD3, ganglioside D3; GEM, germline-encoded mycolyl-specific; GlcCer, glucosylceramide; iGb3, isoglobotrihexosylceramide; iNKT, invariant NKT; NKT, natural killer T cells; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PPBF, phenyl 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonate; vNKT, variant NKT. Modified from (Van Kaer, Wu et al. 2016).

1.2.5.1. CD1a-binding lipids

CD1a groove has the capacity to bind a large variety of foreign and self-antigens (Van Rhijn, Godfrey et al. 2015). Many endogenous ligands of CD1a have been identified in the literature including permissive stimulatory ligands (phospholipids, Lyso-PC and fatty acids) and non-permissive inhibitory ligands (sphingomyelin and sulfatide) (Birkinshaw, Pellicci et al. 2015, de Jong 2015). CD1a molecules bind to headless self-lipids such as wax esters, fatty acid, methyl esters, triacylglycerides and squalene (Layre, de Jong et al. 2014). These permissive headless antigens sit within the CD1a pocket and change the conformation of CD1a which allows TCR binding without direct contact (de Jong, Cheng et al. 2014).

Few exogenous lipids have been identified in the literature to bind CD1a. The binding of CD1a to the mycobacterial derived lipid dideoxymycobactin is well established (Moody, Young et al. 2004, Van Rhijn, Zajonc et al. 2005) . Another exogenous ligand of CD1a is the urushiol-derived lipid diunsaturated pentadecylcatechol (C15:2), which binds CD1a and mediates the pathogenesis of poison-ivy mediated dermatitis in CD1a-transgenic mice (Kim, Hu et al. 2016). In addition, there is some evidence that CD1a-reactive T cells can be stimulated with pollen-derived phospholipids (Agea, Russano et al. 2005).

It has been also found that some exogenous- or self-enzymes generate self neolipid antigens that bind and activate CD1a in the skin. This include bee-, wasp venom- and house dust mite-derived phospholipases which hydrolyse skin phospholipids and trigger the CD1a immune pathway (Bourgeois, Subramaniam et al. 2015, Jarrett, Salio et al. 2016, Subramaniam, Aslam et al. 2016).

1.2.6. CD1 antigen presentation to T cells

Moody and Cotton hypothesised four different models of CD1 antigen presentation to T cells (Moody and Cotton 2017) illustrated in Figure 1.2 and summarised as follows:

1) Headgroup discrimination model:

In this model, the TCR contacts the lipid head group and the CD1 molecule. The lipid's alkyl chains are inserted within the CD1 binding pocket, while the carbohydrate, phosphate, sulphate or peptide headgroups protrude above the surface and contact with the TCR. This was confirmed for example for the crystal structure of α-galactosylceramide bound to CD1d (Borg, Wun et al. 2007) and glucose monomycolate bound to CD1b (Gras, Van Rhijn et al. 2016).

2) Interference model:

In this model, lipids with large head groups interfere with the CD1a A′ roof and block TCR binding. An example of this model was solved for crystal structure of the autoreactive BK6 TCR which could not interact with sphingomyelin/CD1a (Birkinshaw, Pellicci et al. 2015).

3) Absence of interference model:

In this model, small lipids lacking head groups emerge from right side of the cleft, and the TCR binds to the left side of the CD1 molecule (e.g. CD1a A′ roof) without a direct contact with the lipid; a phenomenon known as the left–right mismatch (Cotton, Shahine et al. 2018). This was shown for the crystal structure of the autoreactive BK6 TCR with lysophosphatidylcholine bound to CD1a (Birkinshaw, Pellicci et al. 2015).

4) CD1 remodelling model:

In this model, loading the CD1 groove with lipids causes remodelling in TCR recognition surfaces of the CD1 molecule. This was demonstrated for CD1c binding to C12-C18 fatty acids or monoacylglycerol. (Mansour, Tocheva et al. 2016, Wun, Reijneveld et al. 2018). The crystal structure of 3C8 clone TCR with CD1c bound to monoacylglycerol and spacer lipids has been solved and showed that the TCRα chain overlies the A′ roof with the TCRβ chain crossing the F′ portal (Wun, Reijneveld et al. 2018).

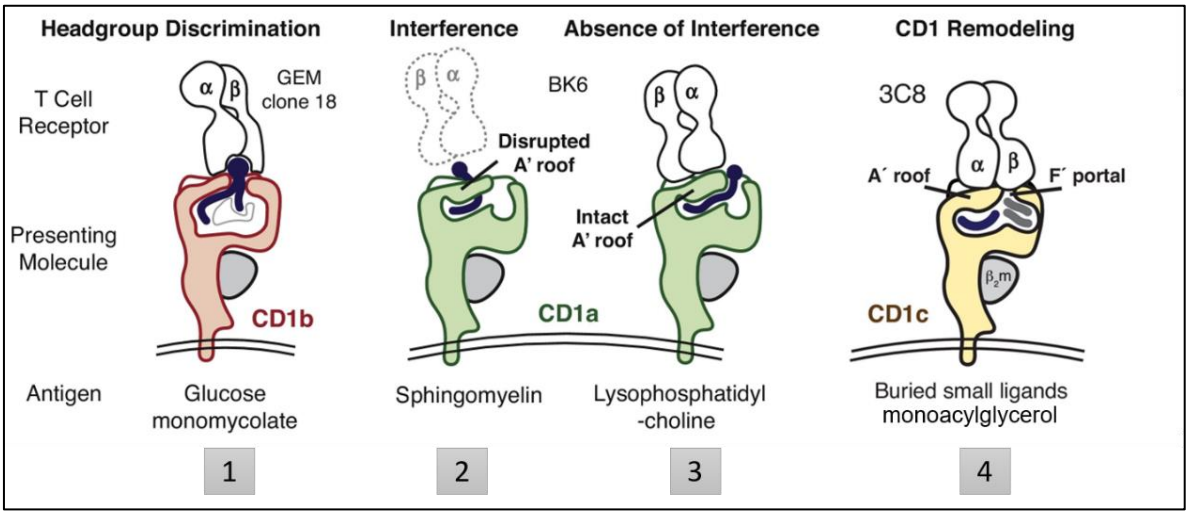


Figure 1.2 Four models for CD1 lipid display to TCR.

Modified from (Moody and Cotton 2017, Cotton, Shahine et al. 2018). Permission to reproduce this figure has been granted by ELSEVIER.

1.2.7. CD1-reactive T cells repertoire

Based on findings from de Lalla and colleagues, high frequencies of autoreactive CD1-reactive T cells were detected in the peripheral blood (1/10-1/300 of circulating T cells) and most predominantly they were CD1a-reactive T cells, followed by CD1c and CD1d-reactive T cells (de Lalla, Lepore et al. 2011). The CD1-reactive T cells were found to be a part of the CD4+, CD8+ or the double

negative T cell population (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Pena-Cruz et al. 2010, de Lalla, Lepore et al. 2011, de Jong, Cheng et al. 2014, Salio, Silk et al. 2014, Birkinshaw, Pellicci et al. 2015). It is known that CD8 plays an important role in facilitating TCR-MHC interaction, peptide antigen recognition and T cell activation. However, it is not known whether CD8 can bind CD1a molecule and consequently the contribution of CD8 in TCR-CD1a interaction is still to be elucidated.

The CD1a-reactive T cells express diverse TCRs and they are normal components of the human αβ T cell repertoire (de Jong, Pena-Cruz et al. 2010). According to Moody and colleagues the frequency of CD1a-autoreactive T cells among memory T cells in the blood was 0.02% to 0.4% with higher frequencies estimated in specific tissues like the skin (de Jong, Pena-Cruz et al. 2010). Most of the CD1a-reactive T cells reported in literature were αβ (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Cheng et al. 2014, Birkinshaw, Pellicci et al. 2015), however one study reported a γδ TCR from one T cell clone (Agea, Russano et al. 2005). Strong activation of CD1a-reactive T cells promote overproduction of IL-22, IL-2, IL-13 and IFN-γ which contribute to disease pathogenesis (de Jong, Pena-Cruz et al. 2010).

The CD1b-reactive T cells express diverse (Beckman, Porcelli et al. 1994, Shamshiev, Gober et al. 2002, Gilleron, Stenger et al. 2004), semi-invariant (Van Rhijn, Kasmar et al. 2013) or biased (Van Rhijn, Gherardin et al. 2014) αβ TCRs. While the CD1c-reactive T cells express diverse αβ (Moody, Ulrichs et al. 2000, Matsunaga, Bhatt et al. 2004) or diverse γδ TCR repertoire (Shamshiev, Gober et al. 2002, Roy, Ly et al. 2016).

CD1d-reactive type I NKT cells express semi-invariant αβ TCR (Kawano, Cui et al. 1997, Wu, Segal et al. 2003, Zhou, Mattner et al. 2004, Kinjo, Wu et al. 2005, Mattner, Debord et al. 2005, Sriram, Du et al. 2005, Fox, Cox et al. 2009, Brennan, Tatituri et al. 2011, Kinjo, Illarionov et al. 2011, Lopez-Sagaseta, Sibener et al. 2012, Albacker, Chaudhary et al. 2013, Wieland Brown, Penaranda et al. 2013, Brennan, Tatituri et al. 2014, Kain, Webb et al. 2014) compared to CD1d reactive type II NKT cells which express variable TCR (Shamshiev, Gober et al. 2002, Jahng, Maricic et al. 2004, Rhost, Lofbom et al. 2012) or diverse αβ TCR (Van Rhijn, Young et al. 2004, Tatituri, Watts et al. 2013, Wolf, Tatituri et al. 2015, Girardi, Wang et al. 2016, Le Nours, Praveena et al. 2016). Some CD1d-reactive NKT cells with biased TCR were γδ (Dieude, Striegl et al. 2011).

1.2.8. CD1-reactive T cells in human disease

1.2.8.1. Role of CD1d-reactive NKT cells in human disease

CD1d-reactive NKT cells contribute to antimicrobial responses, tumour immune surveillance and autoimmunity (Brigl and Brenner 2004). CD1d-reactive NKT cells were found to control growth of microorganisms, influence antibody production and adaptive immune responses, and contribute to immunopathologic tissue injury (Brigl and Brenner 2004). CD1d-reactive NKT cells contribute to antimicrobial immune responses toward different bacteria (Szalay, Ladel et al. 1999, Kumar, Belperron et al. 2000, Nieuwenhuis, Matsumoto et al. 2002, Gansert, Kiessler et al. 2003), parasites (Ishikawa, Hisaeda et al. 2000, Faveeuw, Angeli et al. 2002, Hansen, Siomos et al. 2003), viruses (Baron, Gardiner et al. 2002, Exley, He et al. 2002, Johnson, Hong et al. 2002, Ashkar and Rosenthal 2003, Grubor-Bauk, Simmons et al. 2003) and fungi (Kawakami, Kinjo et al. 2001).

CD1d-reactive NKT cells can have a regulatory function in tumour immunity. CD1d-reactive type I NKT cells induce antitumor immunity and help to eradicate the tumours through the production of IFN-γ, stimulation of DCs to produce IL-12, and activation of CD8+ T cells in the presence of CD1d ligands such as α-galactosylceramide (α-GalCer) (Terabe and Berzofsky 2018). This has been well established for example for multiple myeloma, colorectal cancer and head and neck squamous cell carcinoma. Conversely, CD1d-reactive type II NKT cells can inhibit tumour immunosurveillance (Terabe and Berzofsky 2018).

In addition to this, CD1d-reactive NKT cells were also studied in relation to autoimmune diseases such as type I diabetes mellitus, where they modulate the autoimmune inflammation in the disease (Lehuen, Lantz et al. 1998, Sharif, Arreaza et al. 2001).

1.2.8.2. Role of group 1 CD1-reactive T cells in human disease

The absence of group 1 CD1 molecules in mice has limited the study of the role of group 1 CD1-reactive T cells in disease pathogenesis. However, the development of antigen-loaded CD1 tetramers (Kasmar, van Rhijn et al. 2011, Kasmar, Van Rhijn et al. 2013, Van Rhijn, Kasmar et al. 2013, Mansour, Tocheva et al. 2016, Roy, Ly et al. 2016) and human CD1-transgenic mice and guinea pigs (Hiromatsu, Dascher et al. 2002, Felio, Nguyen et al. 2009, Li, Choi et al. 2011, Kim, Hu et al. 2016) opened the doors for a better understanding of the contribution of these molecules in infections, cancer, autoimmunity and skin diseases (Chancellor, Gadola et al. 2018).

1.2.8.2.1. Infection

The role of group 1 CD1 molecules in *Mycobacterial tuberculosis* immune responses is well established. CD1b-reactive T cell responses toward mycobacterial mycolate have been studied extensively and revealed that mycolic acid-specific CD1b-reactive T cell population contributes to the immune response to human tuberculosis infection indicating a vaccination potential against *Mycobacterium tuberculosis* (Montamat-Sicotte, Millington et al. 2011, Zhao, Siddiqui et al. 2015, Van Rhijn, Iwany et al. 2017). Moreover, CD1c-reactive T cells are enriched in *Mycobacterium tuberculosis*-infected patients and are able to recognise isoprenoid glycolipids (Moody, Ulrichs et al. 2000). Besides this, CD1a binds dideoxymycobactin and presents it to CD1a-reactive T cells (Cheng, Liu et al. 2017). Binding of mycobacterial-derived lipids to Toll-like receptor 2 (TLR2) upregulate the expression of group 1 CD1 molecules on DCs and therefore selectively enhance the presentation of exogenous bacterial lipids by those APCs (Krutzik, Tan et al. 2005, Roura-Mir, Wang et al. 2005). Beside *Mycobacterial tuberculosis*, group 1 CD1 proteins have a potential role in the antimicrobial immunity against *Mycobacterium leprae* in human disease in vivo (Sieling, Jullien et al. 1999). CD1-reactive T cells contribute to the immunity against other important bacteria. For instance, CD1b-reactive T cells target phospholipids present in *Staphylococcus* and *Salmonella* (e.g. phosphatidylglycerol (PG) and PE) (Van Rhijn, van Berlo et al. 2016).

1.2.8.2.2. Cancer

The potential role of CD1b-reactive T cells in lymphoma was studied in a transgenic mouse model expressing human group 1 CD1 molecules and an autoreactive CD1b-reactive T cell receptor. In this study, the autoreactive CD1b-

reactive T cells responded more potently to tumour-derived lipids than normal cell-derived lipid-reactive T cells and gave protection against T cell lymphoma with enhancement of the antitumor effect (Bagchi, Li et al. 2016). High CD1a+ DCs were found to associate with improved survival of patients with papillary thyroid carcinoma (Hilly, Rath-Wolfson et al. 2015) and tongue carcinoma (Goldman, Baker et al. 1998). Autoreactive CD1c-reactive T cells recognise the self-lipid methyl-lysophosphatidic acids (mLPAs) which is enriched in leukaemia cells and it was found that mLPA-specific T cells can efficiently kill CD1c+ acute leukaemia cells demonstrating that lipid-specific T cells contribute to leukaemia immune surveillance and might have a role in immunotherapy (Lepore, de Lalla et al. 2014).

1.2.8.2.3. Autoimmunity

DCs expressing group 1 CD1 molecules infiltrate thyroid glands in Graves' disease and Hashimoto's thyroiditis and CD1-autoreactive T cells present within the thyroid are capable of lysing thyroid target cells in a CD1a- and CD1c-dependent manner *ex vivo* (Roura-Mir, Catalfamo et al. 2005). Moreover, CD1c-reactive T cells from systemic lupus erythematosus (SLE) patients stimulate pathogenic autoantibody responses through the induction of CD1c-expressing B cells for IgG production (Sieling, Porcelli et al. 2000).

1.2.8.2.4. Skin disease

Autoreactive CD1a-reactive T cells migrate to the skin as they express skin-homing markers (de Jong, Pena-Cruz et al. 2010, de Jong, Cheng et al. 2014). CD1a-reactive T cells are enriched in the blood and skin in individuals with psoriasis and AD (Cheung, Jarrett et al. 2016, Jarrett, Salio et al. 2016) and therefore CD1a is thought to contribute to the skin inflammatory pathway in AD (Jarrett, Salio et al. 2016) and psoriasis (Cheung, Jarrett et al. 2016, Kim, Hu et al. 2016). CD1a-

reactive T cells also enhance the pathogenesis of allergic contact dermatitis to common cosmetic compounds (Betts, Perkovic et al. 2017) and poison ivy (Kim, Hu et al. 2016).

1.3. Atopic dermatitis

1.3.1. Epidemiology

AD is one of the most common skin diseases in the world based on global studies conducted by The International Study of Asthma and Allergies in Childhood (ISAAC) on more than a million children and adolescents (Odhiambo, Williams et al. 2009). Overall, AD affects 15-20% of children and 1-3% of adults worldwide with 2- to 3-fold increase in the newly reported cases in the past decades (Odhiambo, Williams et al. 2009, Nutten 2015) with similar trends reported in the UK (Deckers, McLean et al. 2012).

AD can manifest at any age, however, in 80% of patients the age of onset is before 6 years (Perkin, Strachan et al. 2004). AD has a chronic relapsing-remitting clinical course (Weidinger, Beck et al. 2018) and persistent AD was found in 50% of those diagnosed at school age (Mortz, Andersen et al. 2015).

AD has a significant psychosocial burden on the patients and their families as the disease affects their quality of life, social function and mental health (Linnet and Jemec 1999, Lundberg, Johannesson et al. 2000, Lapidus and Kerr 2001, Kiebert, Sorensen et al. 2002, Carroll, Balkrishnan et al. 2005, Chernyshov 2016, Drucker, Wang et al. 2017). In addition to this, AD causes a huge financial burden on the patients or their families (Filanovsky, Pootongkam et al. 2016, Drucker, Wang et al. 2017) and on the health care systems (Drucker, Wang et al. 2017, Shrestha,

Miao et al. 2017). For instance, in the Unites States the total annual costs of AD is more than 4 billion dollars (Bickers, Lim et al. 2006).

1.3.2. Clinical manifestations

The main clinical manifestations of AD -based on the Hanifin and Rajka Criteria (Rothe and Grant-Kels 1996) and the American Academy of Dermatology Consensus Criteria (Eichenfield, Tom et al. 2014)- are intense pruritus, eczematous lesions and chronic or relapsing disease course. The eczematous lesions are shown in Figure 1.3 and they are characterised by erythema, oedema and excoriations. Lesions with superimposed infection manifest with oozing and crusting. AD lesions usually show an age-dependent distribution pattern; in infants and children the lesions are often located on the face, neck and extensor surfaces, however in adults the lesions are most commonly found on the hands, eyelids and flexure surfaces and sometimes they can localise in the upper trunk, shoulders and scalp (Eichenfield, Tom et al. 2014, Weidinger and Novak 2016, Weidinger, Beck et al. 2018).

The severity of AD is measured by using validated scoring systems such as the Eczema Area and Severity Index (EASI) and the Scoring Atopic Dermatitis (SCORAD) (Chopra, Vakharia et al. 2017). While EASI depends on objective physician estimates of disease extent and severity, SCORAD relies on both objective physician estimates of extent and severity and subjective patient assessment of pruritus and sleep pattern (Eichenfield, Tom et al. 2014).



Figure 1.3 Clinical appearance of atopic dermatitis skin lesions.

Typical skin lesions of AD (left and middle) which can be complicated with secondary bacterial infection (right). Adapted from (Weidinger and Novak 2016, Weidinger, Beck et al. 2018). Permission to reproduce this figure has been granted by ELSEVIER and SPRINGER NATURE.

1.3.3. Pathogenesis

AD pathogenesis is a complex interaction between genetic, environmental and immunological factors (Eyerich, Eyerich et al. 2015) that subsequently lead to skin barrier dysfunction, immune dysregulation of innate and adaptive immunity and skin microbial dysbiosis (Eyerich, Eyerich et al. 2015, Weidinger, Beck et al. 2018) – the hallmarks of AD.

1.3.3.1. Genetic susceptibility

Family history of atopy is one of the most important risk factors for the development of AD as there is a strong association between parental history of atopy in general and AD in particular and the development of AD in the offspring (Wadonda-Kabondo, Sterne et al. 2004). The prevalence of AD was twofold higher when the parents reported a history of atopy (Apfelbacher, Diepgen et al. 2011). In a twin study, genetic factors accounted for more than 80% of the individual susceptibility to develop AD, however, this might be an overestimation due to the weakness of the methods used in this study. (Thomsen, Ulrik et al. 2007).

From genome-wide and targeted high-throughput studies, more than 30 different genomic regions have been linked to AD development (Paternoster, Standl et al. 2015, Weidinger, Beck et al. 2018). Those loci are enriched for genes that are involved in cytokine-mediated signalling, cytokine production and leukocyte differentiation especially in relation to innate immune cells and T cells which are important in AD pathogenesis.

Loss-of-function mutations of the *filaggrin* gene (*FLG*) have been studied extensively in AD. A strong association between *FLG* mutation and the development of the disease was discovered in the past decade (Palmer, Irvine et al. 2006). This was followed by many studies that confirmed these findings and included two big meta-analysis studies (Baurecht, Irvine et al. 2007, van den Oord and Sheikh 2009). 30-50% of individuals presenting with moderate to severe AD have loss of function mutations of *FLG* (van den Oord and Sheikh 2009). *FLG* encodes the epidermal protein filaggrin which has a major role in maintaining the skin barrier and haemostasis. Defective filaggrin formation results in epidermal barrier defect (Gruber, Elias et al. 2011, Jakasa, Koster et al. 2011), upregulation of pro-inflammatory cytokines expression (Kezic, O'Regan et al. 2012), skin microbiota dysbiosis with alternation of the epidermal colonisation with *Staphylococcus aureus* (Tollefson, Bruckner et al. 2014) or gram-positive anaerobic cocci (Zeeuwen, Ederveen et al. 2017) and enrichment of the CD1a-reative T cell-mediated inflammatory pathway through phospholipase A2 (PLA2) (Jarrett, Salio et al. 2016). This epidermal barrier defect and inflammation due to filaggrin defect was reported to augment percutaneous allergen priming in a mouse model (Fallon, Sasaki et al. 2009). Collectively, this increases the predisposition for AD. In addition to the genetic defect of filaggrin, secondary filaggrin

downregulation was observed in response to type 2 cytokines, for example through IL-33 induction and IL-4/13 cytokines (Howell, Kim et al. 2007, Seltmann, Roesner et al. 2015).

Cutaneous manifestations are common in primary immune deficiency diseases, affecting 40-70% of patients with primary immunodeficiencies (Berron-Ruiz, Berron-Perez et al. 2000, Moin, Farhoudi et al. 2006) and AD affects 19% of children with primary immunodeficiencies (Al-Herz and Nanda 2011). Primary immunodeficiencies associated with AD includes hyperimmunoglobulin-E syndrome, autosomal dominant STAT3 mutation and autosomal recessive DOK8 mutation. These gene mutations were also linked to increase susceptibility to *C. albicans* infection (refer to section 1.4). In addition, Wiskott–Aldrich syndrome and Omenn syndrome also manifest with eczematous lesions (Lehman 2014).

1.3.3.2. Skin barrier defect

The skin is composed of a layer of a stratified squamous epithelium called the epidermis that is underlined by a vascular connective tissue known as the dermis. The outermost layer of the epidermis -the SC- is composed of a dense protein-lipid matrix that is important in the mechanical and physical function of the skin as It provides a barrier from the outside environment and maintain the pH and the fluid and electrolyte balance of the skin (van Smeden and Bouwstra 2016), as described in section 1.1.3. Disruption of the SC leads to skin barrier breach and predisposes to AD. This disruption is attributed to changes in the lipid content and organisation of the SC, defect in the epidermal structural proteins, compromise in the epidermal tight junctions and the physical trauma due to repeated itching associated with AD.

The major lipid constituents of the SC are ceramides, free fatty acids and cholesterol (van Smeden and Bouwstra 2016). Changes in the lipid content and organisation in the SC, especially in the ceramides' subclasses and chain length, have been attributed to the skin barrier dysfunction and have been correlated with the severity of the disease (Di Nardo, Wertz et al. 1998, Ishikawa, Narita et al. 2010, Janssens, van Smeden et al. 2012, Joo, Hwang et al. 2015, Shen, Zhao et al. 2018). Increased proportion of short-chain ceramides, sphingomyelins and Lyso-PC, and decline in the proportion of corresponding long-chain lipids has been reported in AD skin lesions (Berdyshev, Goleva et al. 2018). Besides this, decline of the lipid/protein ratio and the reduction of SC thickness of AD skin lesions are responsible for this barrier defect (Janssens, van Smeden et al. 2014).

Besides the SC lipid disturbances, structural protein defects, especially in filaggrin, contributes widely to the skin barrier dysfunction in AD and this is discussed in section 1.3.3.1. Moreover, compromise in the epidermal tight junctions can lead to barrier defects in AD and this allows a better exposure of the skin antigen presenting cells to foreign antigens by sending dendrites out through these defective epidermal tight junctions and potentially contributing to the inflammatory responses (Kubo, Nagao et al. 2009, De Benedetto, Rafaels et al. 2011).

1.3.3.3. The role of allergy

The diagnosis of AD in early life increases the risk of the development of other types of atopic diseases such as allergic rhinitis and asthma later in life which is known as the 'atopic march' (Bantz, Zhu et al. 2014). Moreover, a personal or family history of atopy is a major risk factor for developing AD (Naldi, Parazzini et al. 2009, Eichenfield, Tom et al. 2014) demonstrating that atopy and allergy associate strongly with the disease.

Allergens are important in the pathogenesis of AD. AD patients have high serum IgE levels toward various allergens and this is considered a risk factor to develop AD in children (Fukiwake, Furusyo et al. 2008). Similarly, adults with high levels of IgE have more severe and persistent form of AD (Katoh, Hirano et al. 2008). Allergen-specific IgE reactivities to several exogenous, microbial and endogenous allergens were detected in 83% and 92% of patients with moderate and severe AD, respectively (Mittermann, Wikberg et al. 2016). Food and environmental allergens play a major role in AD as around 50% of children and 35% of adults with AD were found to be sensitised to these allergens (Schafer 2008). The relationship between food and AD exacerbation has been studied extensively, but remains controversial (Werfel and Breuer 2004). In contrast, environmental allergens are more widely accepted to trigger AD and include aeroallergens such as house dust mites (HDM) (Nankervis, Pynn et al. 2015) and pollens (Werfel, Heratizadeh et al. 2015) and fungi such as *Malassezia* (Darabi, Hostetler et al. 2009) and *Candida* (Khosravi, Bandghorai et al. 2009).

There are several areas of evidence in the literature about the relevance of *C. albicans* allergic responses in the pathogenesis of AD. 31.6% to 33% of AD patients had positive *C. albicans*-specific IgE in their sera (Back, Scheynius et al. 1995, Lindgren, Wahlgren et al. 1995, Khosravi, Bandghorai et al. 2009). Higher percentages were reported in other studies where *C. albicans*-specific IgE antibodies were reported in 85.6% (Chang, Lee et al. 2011) and 76.1% (Kimura, Tsuruta et al. 2000) of AD patients. The difference among these studies could be attributed to the variations in the *C. albicans* extracts used, the severity of AD, ethnicity and age. Skin sensitization to *C. albicans* is strongly correlated with the development of AD in an Asian population (Chang, Lee et al. 2011). In another

study, positive skin-prick test reactions toward *C. albicans* was observed in 52.6% of AD and only 4.3% of healthy controls (Khosravi, Bandghorai et al. 2009). Besides, *C. albicans*, allergic responses toward *Malassezia* antigen were associated with AD. For instance, more than 60% of the AD patients have atopy patch test positive reaction to at least one of the *Malassezia* allergens (Johansson, Sandstrom et al. 2003). Moreover, elevated serum levels of *Malassezia*-specific IgE were found in 29%-65% of AD patients (Glatz, Buchner et al. 2015) and 30%-80% of adult AD patients have a positive skin prick test for *Malassezia* (Johansson, Eshaghi et al. 2002).

1.3.3.4. The role of skin microbiota dysbiosis

The skin and its appendages are inhabited by diverse microbial species including bacteria, fungi and viruses. In physiologic states, there is usually a mutualistic interaction between the skin and the microbiota as they help to promote the innate and adaptive immunity of the host and limit pathogenic infections as discussed in section 1.1.1. However, microbial factors (virulence factors, localisation and co-infection) or host factors (immune system, genetic predisposition and skin barrier defect) can lead to the transformation of this neutral beneficial state between the microbiota and the skin into a pathogenic state, where microbes penetrate the skin and trigger an inflammatory process (Belkaid and Tamoutounour 2016).

The change in the skin microbiota composition or what is known as ‘dysbiosis’ contributes to the pathogenesis of several skin inflammatory diseases (Byrd, Belkaid et al. 2018). This is well-established for AD where the skin barrier defect, microbial dysbiosis and superimposed infections contribute to this amplification of the inflammatory process (Belkaid and Tamoutounour 2016). In AD, a shift in the microbial composition with less bacterial diversity and *Staphylococcus aureus*

predominant colonization, especially in the severe form of the disease, were reported (Leyden, Marples et al. 1974, Byrd, Deming et al. 2017). *Staphylococcus* species proportion, particularly *Staphylococcus aureus,* increases during AD flares and the presence of these bacteria is associated with AD exacerbation as shown in longitudinal studies of children with AD (Kong, Oh et al. 2012, Chng, Tay et al. 2016, Byrd, Deming et al. 2017). This can be partly explained by that *Staphylococcus aureus* superantigen can promote the capacity of keratinocytes to present allergen to T cells and therefore induce the inflammatory process (Ardern-Jones, Black et al. 2007). Interestingly, colonization of the skin with *Staphylococcus aureus* early in life (before the age of 2 months) is associated with a lower risk of AD development at 1 year (Kennedy, Connolly et al. 2017) proposing that exposure to *Staphylococcus aureus* in early infancy contributes to the education and tuning of the immune system. In addition, *Staphylococcus aureus* dysbiosis was a causative factor of skin inflammation in a mouse model with skin barrier defect and this was reversed with antibiotics administration, proposing a causal link between skin barrier defect, microbiome dysbiosis and dermatitis (Kobayashi, Glatz et al. 2015). In addition to the bacterial dysbiosis, fungal microbiota disturbance has been documented in AD. Compared to healthy skin which is occupied mainly by *Malassezia* species, AD skin exhibits a more diverse array of fungal microbiota including *Candida albicans*, *Wickerhamomyces anomalus*, *Cryptococcus diffluens*, and *Cryptococcus liquefaciens* (Zhang, Tanaka et al. 2011). This difference may be explained by the physiological and environmental conditions of the skin in AD as discussed above. Bacterial and fungal dysbiosis is associated with other inflammatory skin disease as well such

as psoriasis (Fry and Baker 2007, Gao, Tseng et al. 2008, Gaitanis, Magiatis et al. 2012).

1.3.3.5. Immune mechanisms in AD

Innate and adaptive immune cells contribute to the inflammatory mechanisms in AD. The immune pathways of AD are summarised in Figure 1.4.

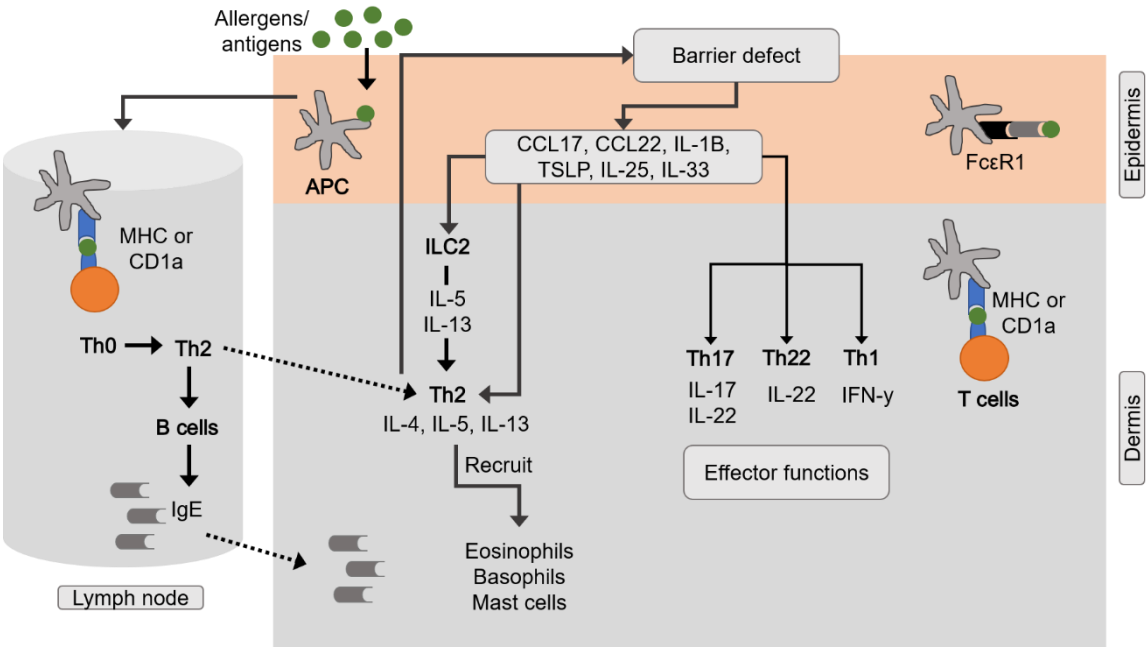


Figure 1.4 Inflammatory pathways of AD.

The keratinocytes are stimulated to express CCL17, CCL22, IL-1β, IL-33 and thymic stromal lymphopoietin (TSLP) primarily by external stimuli due to barrier defect in AD or secondarily by T helper 2 (Th2) cytokines. These mediators activate Th2 cells and group 2 innate lymphoid cells (ILC2s). Th2 cells produce type 2 cytokines including IL-4, IL-5 and IL-13 which induce immunoglobulin E (IgE) class switching, enhance the B cell production of antigen-specific IgE and recruit eosinophils, basophils and mast cells to the dermis. ILC2s produce IL-5 and IL-13 which amplify Th2 immune responses. Antigen-specific IgE antibodies bind to high-affinity immunoglobulin-ε receptors (FcεR1s) expressed on the surface of the skin APCs (inflammatory dendritic epidermal cells (IDECs), Langerhans cells (LCs) or dermal dendritic cells) which facilitates the uptake of allergens and initiate T cell-mediated immune response through MHC or CD1a. Skin APCs take up exogenous antigens or self-antigens released from damages/stressed cells, migrate to regional lymph

node to prime T cells which then upregulate skin homing markers (CLA, CCR4, CCR10) and subsequently migrate to the dermis. Antigen-primed circulating T cells or T resident memory (Trm) cells can response rapidly in the secondary exposure to the antigen. In addition to Th2, Th22 and Th17 cells are important in AD pathogenesis. With disease progression, there is switch toward Th1 immune responses (Gittler, Shemer et al. 2012, Jarrett and Ogg 2016, Lin, Li et al. 2017, Weidinger, Beck et al. 2018).

1.3.3.5.5. Innate Immunity

The innate immune system has a crucial role in AD pathogenesis. The most important components of the innate immunity responses in AD are the epidermal barrier (discussed in section 1.3.3.2), ILC2s, AMPs, TLRs and DCs subsets.

ILC2s are first-line innate sensors that react to AD-associated triggers and functionally contribute to type 2 immune responses and barrier defect (Eyerich, Eyerich et al. 2015). Salimi et al found that the frequencies of skin-resident ILC2s are elevated in AD and they are recruited in an allergen-mediated manner, potentially via IgE-induced mast cell production of prostaglandin D2 (PGD2) which is a chemoattractant for ILC2s (Salimi, Barlow et al. 2013). ILC2s are activated by the keratinocyte alarmins: IL-33, IL-25, and TSLP produced in response to the skin barrier breach to secrete IL-5, IL-4 and IL-13 (Kim, Wojno et al. 2013, Salimi, Barlow et al. 2013, Leyva-Castillo and Geha 2018) and therefore contribute to the Th2-mediated immune pathway in AD. Interestingly, Hardman et al found that human skin-derived ILC2s can present endogenous antigens to T cells through CD1a which can promote the skin inflammation in AD (Hardman, Chen et al. 2017).

The AMPs are important components of the innate immune defence which provide defence against invading micro-organisms (Wollenberg, Rawer et al. 2011). In acute and chronic AD lesions reduced expression of AMPs was observed (e.g.

human cathelicidin and human beta-defensin 2 (HBD-2)) (Ong, Ohtake et al. 2002). This decline in AMPs predisposed AD patients to disseminated infections (Ong, Ohtake et al. 2002) and led to Eczema herpeticum with disseminated herpes simplex virus infection (Howell, Wollenberg et al. 2006, Hata, Kotol et al. 2010). Failure to upregulate cathelicidin in AD is likely to affect the AMPs-mediated protection and tissue repair and therefore exacerbate the disease (Mallbris, Carlen et al. 2010).

TLR2-mediated cytokine production by monocytes is impaired in AD (Hasannejad, Takahashi et al. 2007). TLR2 gene polymorphism was associated with severe AD phenotype (Oh, Schumann et al. 2009, Salpietro, Rigoli et al. 2011) as it increases the predisposition to secondary infections. In addition to TLR2, TLR9 genetic variation increased the susceptibility to AD in a subgroup of patients (Novak, Yu et al. 2007).

DCs subsets contributing to AD pathogenesis include: LCs, inflammatory dendritic epidermal cells (IDECs), and dermal dendritic cells including pDCs (Wollenberg, Rawer et al. 2011). DCs initiate the adaptive immunity in AD by triggering T cell priming and therefore T cell effector responses through antigen presentation by MHC class I, MHC class II and CD1a (Wollenberg, Rawer et al. 2011). This is done more efficiently in the presence of high-affinity immunoglobulin-ε receptors (FcεR1s) which bind to the antigen-specific IgE antibodies, leading to a rapid and more potent activation of LCs in AD (Maurer, Ebner et al. 1995, Bieber 1997) and through IgE-mediated antigen uptake and presentation to T cells (Cooper, Hales et al. 2004).

1.3.3.5.6. Adaptive immunity

An immune dysregulation of the adaptive immunity mediated by T cells plays a critical role in AD development. AD was believed to be driven solely by Th2-mediated immune responses, however, the identification of other CD4+ T cell subsets and their effector cytokines in AD lesions provided a better understanding and interpretation of the diverse immune mechanisms underlying AD pathogenesis. Th cell subsets are critical in the initiation and sustainment of the inflammatory immune pathways in the different stages of AD (Wang and Xu Landen 2015). Effector memory T cells continuously recirculate to the skin through regional lymph nodes or permanently reside within the skin tissue to provides rapid recall responses (Mueller and Mackay 2016).

In the acute phase of AD, Th2 immune responses predominate. This is partly explained by the production of keratinocyte-derived chemokines and alarmins like CCL17, CCL22, IL-1β, IL-33 and TSLP in response to the skin injury and barrier defect (Wang and Xu Landen 2015, Weidinger, Beck et al. 2018). TSLP is highly expressed by keratinocytes in AD skin and associated with LCs migration (Soumelis, Reche et al. 2002). After the activation of the APCs by TSLP, they migrate to regional lymph nodes where they prime the naive T cells into Th2 subtype and facilitate the production of IL-4, IL-5, IL-13 and IL-31 (Soumelis, Reche et al. 2002) through OX40 ligand (Ito, Wang et al. 2005). Overproduction of type 2 cytokines results in an allergic inflammation which induce the morphologic and molecular features of AD (Kamsteeg, Bergers et al. 2011) in addition to the intense pruritus (Oetjen, Mack et al. 2017). Patients with AD exhibit high levels of type 2 cytokines in the serum and skin lesions (Nomura, Goleva et al. 2003, Kamsteeg, Bergers et al. 2011, Szegedi, Kremer et al. 2012) which is correlated

with the serum IgE level and the severity of the disease (Kamsteeg, Bergers et al. 2011, Wang and Xu Landen 2015). The role of type 2 cytokines is well established in AD pathogenesis. IL-4 and IL-13 enhance the barrier defect in AD by altering the SC lipids (Berdyshev, Goleva et al. 2018). IL-4, IL-13 and IL-31 further disrupt the barrier function of the skin through the down-regulation of *filaggrin* (Howell, Kim et al. 2007, Howell, Kim et al. 2009, Sehra, Yao et al. 2010, Cornelissen, Marquardt et al. 2012). Beside this, IL-4 and IL-13 reduce the expression of the AMPs (Nomura, Goleva et al. 2003) or impair the mobilization of the AMPs from the cytoplasm to the bacterial surface (Kisich, Carspecken et al. 2008) increasing the predisposition to superimposed bacterial infections. Moreover, IL-4 and IL-13 mediate IgE class switching (Geha, Jabara et al. 2003). IL-5 is another type 2 cytokine critical for eosinophil development and proliferation (Kouro and Takatsu 2009).

AD is not only mediated by Th2 cells, it is currently considered to be a Th2/Th22-centric disease, particularly during chronic disease (Gittler, Shemer et al. 2012, Suarez-Farinas, Ungar et al. 2015, Sanyal, Pavel et al. 2019). AD skin is enriched with Th22 cells which correlate with AD severity (Nograles, Zaba et al. 2009). IL-22 contributes to AD pathogenesis by downregulating *profilaggrin* or *filaggrin* expression in keratinocytes at both mRNA and protein levels (Gutowska-Owsiak, Schaupp et al. 2011) and by inducing epidermal hyperplasia and stimulating keratinocyte proliferation (Boniface, Bernard et al. 2005).

Th17 cells are also important mediators of the inflammatory pathway of AD in some individuals, for example in Asian ethnicities. Koga et al found that Th17 cells were increased in peripheral AD blood and acute skin lesions and this correlated with the severity of the disease (Koga, Kabashima et al. 2008). This was confirmed by

subsequent studies in Asian and paediatric AD patients where increased Th2/Th17 activation was observed (Noda, Suarez-Farinas et al. 2015, Esaki, Brunner et al. 2016, Brunner, Israel et al. 2018, Nomura, Honda et al. 2018). IL-17 contributes to AD development by downregulating the expression of *filaggrin* and other genes important in cellular tight junctions and adhesion molecules which contribute to the barrier defect (Gutowska-Owsiak, Schaupp et al. 2012). Teunissen et al found that IL-17 with the synergistic effect of IFN-γ can induce the secretion of IL-6 and IL-8 and the expression of the intercellular adhesion molecule (ICAM-1) and the human leukocyte antigen-DR (HLA-DR) (Teunissen, Koomen et al. 1998).

The Th2 immune response in acute AD is converted into a chronic Th1-mediated inflammation through the activation of DCs by TLR2 ligands and IL-4 (Kaesler, Volz et al. 2014). It was reported that with disease progression Th2 and Th17 cells were decreased and Th1 cells were increased (Su, Yang et al. 2017). Another study reported an upregulation of Th1/Th17 cells in chronic AD (Orciani, Campanati et al. 2017). The IFN-γ producing CD1a-reactive T cells were enriched in AD blood and skin in response to HDM challenge (Jarrett, Salio et al. 2016). IFN-γ contributes to AD pathogenesis by enhancing skin hypertrophy in AD (Spergel, Mizoguchi et al. 1999), inducing epithelial ICAM-1 expression and monocyte adhesion (Chang, Holtzman et al. 2002) and inducing the expression of the MHC class II on keratinocytes (Czernielewski and Bagot 1986, Barker, Navsaria et al. 1988).

1.3.4. Treatment

The aim of AD management is to reduce symptoms, accomplish long-term disease control and prevent disease exacerbation and complications (Weidinger and Novak 2016). In brief, this is achieved by several approaches; firstly, to avoid

triggering factors of the disease, secondly, to restore the skin barrier and thirdly, to reduce the skin inflammation and pruritus (Weidinger, Beck et al. 2018). Based on the American Academy of Dermatology guidelines, the current treatment modalities of AD include topical therapy, phototherapy and systemic therapy (Eichenfield, Tom et al. 2014, Sidbury, Davis et al. 2014, Sidbury, Tom et al. 2014). An integral part of the treatment of AD is patients' and caregivers' education which increases their knowledge about the disease mechanism and course and instruction on the correct use of the topical treatments which subsequently leads to a better disease control (Cork, Britton et al. 2003, Staab, Diepgen et al. 2006, Kupfer, Gieler et al. 2010). The choice of the therapy option is based on the severity of the disease, the patient's age and the presence of other comorbidities or complications (Weidinger, Beck et al. 2018).

1.3.4.1. First-line treatment: Topical therapy

Skin hydration is a key component of the management of AD (Eichenfield, Tom et al. 2014, Boguniewicz, Fonacier et al. 2018). Using moisturisers and emollients improves AD severity, reduces the frequency of disease flares (van Zuuren, Fedorowicz et al. 2017) and prevents AD in high risk infants (Simpson, Chalmers et al. 2014). The moisturizers used to manage AD contains occlusive and humectants agents that coat the surface of the skin and attract water from the surrounding environment, respectively (Elias and Sugarman 2018) to create a physical barrier that increases the skin hydration.

If regular use of emollients and moisturisers fail, topical corticosteroids (TCS) are recommended for the treatment of AD (Eichenfield, Tom et al. 2014). TCS have an anti-inflammatory effect that inhibits some immune pathways involved in AD

pathogenesis and broadly affects the barrier responses (Brunner, Khattri et al. 2016).

In some clinical situations, topical calcineurin inhibitors (TCI) are used as an alternative for TCS (Eichenfield, Tom et al. 2014). TCI (e.g. Tacrolimus and Pimecrolimus) are non-steroidal anti-inflammatory agents that decrease the production of proinflammatory cytokines and mediators of AD through inhibiting calcineurin-dependent T-cell activation (Grassberger, Baumruker et al. 1999, Winiski, Wang et al. 2002).

Crisaborole (phosphodiesterase 4 (PDE4) inhibitor), a relatively new non-steroidal anti-inflammatory topical treatment of AD, was licensed in the US in 2016. Controlled randomised double-blind studies and clinical trials demonstrated its safety profile and showed improvement of AD symptoms and severity (Murrell, Gebauer et al. 2015, Paller, Tom et al. 2016).

1.3.4.2. Second-line treatment: Phototherapy

If AD cannot be controlled with topical measures, phototherapy is considered as a second-line treatment option (Sidbury, Davis et al. 2014). Numerous studies investigated the efficacy of phototherapy for AD (Garritsen, Brouwer et al. 2014), from which the most commonly recommended and widely used phototherapy for AD was the narrowband ultra-violet B (N-UVB) because of its high safety profile and good efficacy (Sidbury, Davis et al. 2014). N-UVB has immunomodulatory effects that lead to the improvement of AD symptoms. This is thought to be achieved by the suppression of Th1, Th2 and Th22 cytokine production, the reduction in epidermal proliferation and the suppression of inflammatory-cell infiltrates in the site of active AD inflammation including T cells, DCs, eosinophils

and mast cells (Tintle, Shemer et al. 2011). Interestingly, phototherapy was found also to inhibit the CD1a expression of LCs in the skin (Tintle, Shemer et al. 2011).

1.3.4.3. Third-line treatment: Systemic therapy

When topical treatment and phototherapy fail to control the signs and symptoms of the disease, systemic therapies are considered (Sidbury, Davis et al. 2014). The systemic immune-modulators used for AD includes: ciclosporin (van Joost, Kozel et al. 1992, Berth-Jones, Graham-Brown et al. 1997), azathioprine (Berth-Jones, Takwale et al. 2002, Murphy and Atherton 2002, Schram, Roekevisch et al. 2011), methotrexate (Schram, Roekevisch et al. 2011, Shah, Alhusayen et al. 2018), and mycophenolate mofetil (Prussick, Plotnikova et al. 2016). Systemic corticosteroids are not commonly used for the long-term treatment of AD due to their unfavourable side-effects (Weidinger and Novak 2016).

There is a growing interest in the cytokine-targeted therapies in the past few years. Dupilumab, a fully humanised monoclonal anti-IL-4Rα antibody that inhibits IL-4 and IL-13 signalling (Hamilton, Suarez-Farinas et al. 2014, Guttman-Yassky, Bissonnette et al. 2019) was approved in the UK in 2018 as the first biologic treatment of moderate-to-severe AD. Clinical trials have proven its safety and efficacy in AD treatment (Simpson, Bieber et al. 2016, Thaci, Simpson et al. 2016, Blauvelt, de Bruin-Weller et al. 2017). Besides this, clinical trials on anti-IL-33 antibody (ANB020) are in Phase-2a (Ogg et al. 2018 May; presented at the 2018 European Academy of Allergy and Clinical Immunology (EAACI) Congress). The safety profile and the pharmacologic characteristics of the antibody was confirmed in a phase 1 trial on healthy individuals (Schram, Roekevisch et al. 2011).

1.4. *Candida albicans*

The genus *Candida* consists of more than 300 species. However, only a few members of this genus are relevant to human disease or are part of the human microbiome. This includes *Candida albicans (C. albicans)*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida dubliniensis* (McManus and Coleman 2014).

C. albicans is a diploid polymorphic yeast that can grow in three forms; budding yeast, pseudohyphae, or hyphae *(Staniszewska, Bondaryk et al. 2013).* The virulence of *C. albicans* is attributed to an ability to adhere to epithelial cells, transform from one morphotype to the other, form biofilms, secrete enzymes and adapt to different environmental conditions (e.g. pH or metabolic change) (Khan, Ahmad et al. 2010, Boral, Metin et al. 2018).

C. albicans is a member of the human microbiota and is usually a harmless commensal fungus that is present in the gastrointestinal, respiratory and genitourinary systems. However, it can be pathogenic and causes superficial vaginal or mucosal oral infections or in some circumstances causes systemic and deep-tissue infection particularly in immunocompromised or immunologically deficient individuals (Dadar, Tiwari et al. 2018). Several primary immunodeficiencies predispose to *C. albicans* infection. For instance, chronic mucocutaneous candidiasis -recurrent *C. albicans* infection of the oral mucosa, oesophagus, digestive tract, genital mucosa, nails and skin- is associated with various inherited primary T cell immunodeficiencies summarised in Table 1-2 (Lanternier, Cypowyj et al. 2013).

Table 1-2 Primary immunodeficiencies associated with chronic mucocutaneous candidiasis.

Disease	Immunological phenotype	Gene, transmission
SCID	No T cells, with or without B and/or NK cell lymphopenia	>30 genes: *IL2RG*, XL; *JAK3*, AR; *RAG1*, AR; *RAG2*, AR; *ARTEMIS*, AR; *ADA*, AR; *CD3*, AR.
CD25 deficiency	T cell defect	*IL2RA*, AR
IKBG deficiency	T cell defect	*IKBG* X-linked
DOCK8 deficiency	T cell defect	*DOCK8*, AR
TCR-α deficiency	T cell defect	*TCRA*, AR
ORAI1 deficiency	T cell defect	*ORAI1*, AR
MST1/STK4 deficiency	T cell defect	*MST1/STK4*, AR
MHC class II deficiency	T cell defect	*CIITA, RFXANK, RFXC, RFXAP*, all AR
Idiopathic CD4 lymphopenia	Low CD4 T cells	*MAGT1*, X-linked RAG1, AR
IL-12Rβ 1 and IL-12p40 deficiencies	Deficit of IL-17 producing T cells	*IL12RB1*, AR *IL12B*, AR
STAT3 deficiency (AD-HIES)	Hyper IgE, deficit of IL-17-producing T cells	*STAT3,* AD
APS-1	Neutralizing anti-IL-17A, IL-17F and/or IL-22 autoantibodies	*AIRE*, AR
CARD9 deficiency	Deficit of IL-17-producing T cells	*CARD9*, AR
Complete IL-17RA deficiency	No IL-17 response	*IL17RA,* AR
Partial IL-17F deficiency	Impaired IL-17F, IL-17A/F function	*IL17F,* AD
***STAT1* gain of function mutations**	Low IL-17 producing T cells	*STAT1*, AD

Abbreviations: AD, autosomal dominant; AD-HIES, autosomal dominant hyper-IgE syndrome (Job syndrome); AIRE, autoimmune regulator; APS-1, autoimmune polyglandular syndrome type 1; AR, autosomal recessive; CARD9, caspase recruitment domain family member 9; DOK8, dedicator of cytokinesis 8; IKBG, inhibitor of nuclear factor kappa B kinase regulatory subunit gamma; MST1, macrophage stimulating 1; ORAI1, calcium release-activated calcium channel protein 1; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription; STK4, serine/threonine kinase 4. Modified from (Lanternier, Cypowyj et al. 2013).

1.4.1. Immune defence against *C. albicans* infections

1.4.1.1. Recognition of *C. albicans* by innate immune cells

C. albicans pathogen-associated molecular patterns (PAMPs) are recognised by several types of pattern-recognition receptors (PRRs) (summarised in Figure 1.5). The PRRs includes C-type lectin receptors (CLRs), TLRs and NOD-like receptors (NLRs) which are expressed by innate immune cells (Netea, Joosten et al. 2015).

CLRs are the most important family of innate receptors for the recognition of *C. albicans* PAMPs (Netea, Joosten et al. 2015). β-glucans are recognised by the CLRs: dectin-1 and complement receptor 3 (CR3) (Brown and Gordon 2001, van Bruggen, Drewniak et al. 2009, Lowman, Greene et al. 2014). Mannans and mannoproteins are recognised by other types of CLRs, including mannose receptor, dectin-2 and DC-specific ICAM3-grabbing non-integrin (DC-SIGN) (Cambi, Netea et al. 2008, van de Veerdonk, Marijnissen et al. 2009, Yamasaki, Matsumoto et al. 2009, Saijo, Ikeda et al. 2010). Dectin-1 and dectin-2 induce responses through spleen tyrosine kinase (SYK) or RAF1 kinase signalling pathway (Netea, Joosten et al. 2015).

TLRs also play a major role in candida recognition. This includes the membrane-bound TLRs (TLR2, TLR4 and TLR6) which bind to mannoprotein of the fungal cell wall (Netea, Brown et al. 2008) and the intracellular TLRs (TLR3 and TLR9) that recognise cytoplasmic nucleic acids (Nahum, Dadi et al. 2011, Wagener, Malireddi et al. 2014). In addition, TL9 and NLRs recognise the fungal polysaccharide, chitin, which leads to the production of IL-10 through a nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-dependent mechanism (Wagener, Malireddi et al. 2014).

This interaction between the *Candida* PAMPs and the PRRs induces intracellular signalling pathways and therefore trigger effector immune responses. For instance, signalling through the mannose receptor induces the production of pro-inflammatory cytokines especially IL-17A (van de Veerdonk, Marijnissen et al. 2009) and signalling through DC-SIGN triggers Th cell differentiation (Cambi, Netea et al. 2008).

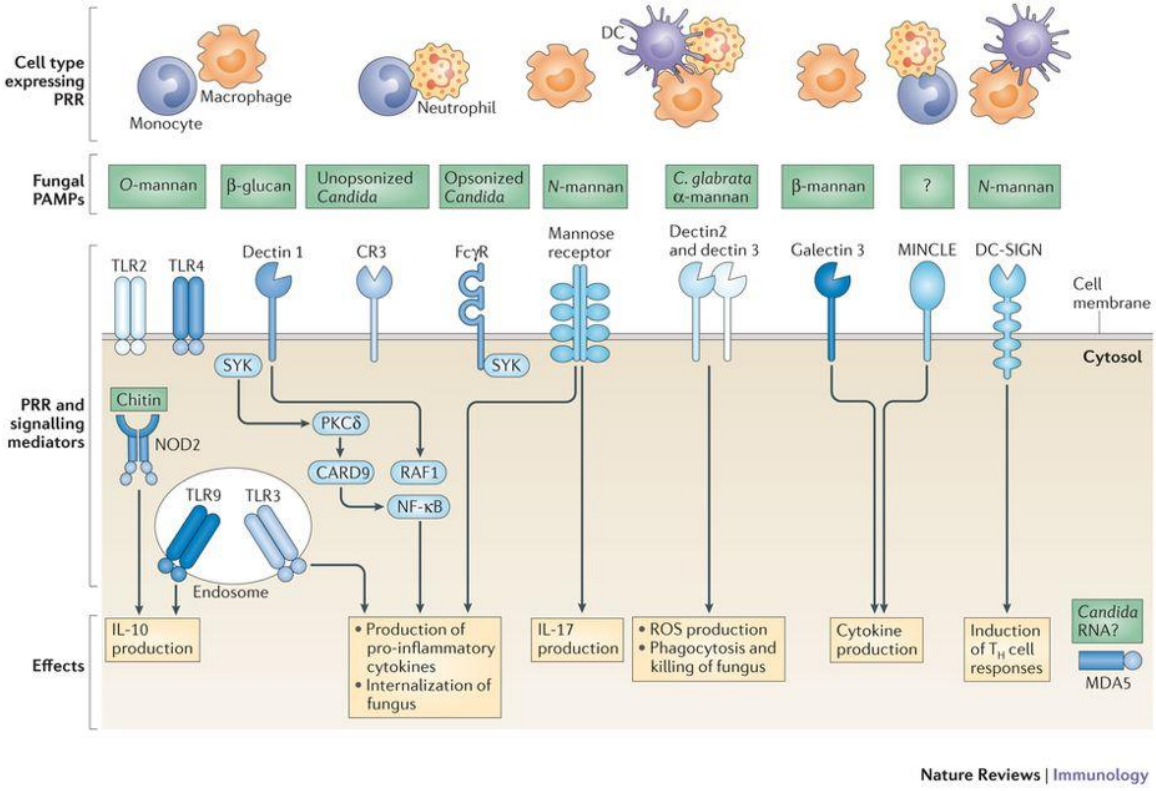


Figure 1.5 Innate sensing of *C. albicans*.

***C. albicans* PAMP are recognised by PRRs expressed on the surface of monocytes, macrophages, neutrophils and DCs (e.g. dectin-1, dectin-3, dectin-3, TLR2, TLR4, CD3 and DC-SIGN) or expressed in the endosomal compartment (e.g. TLR3 and TLR9) which stimulate signalling cascade to induce the secretion of cytokines and chemokines and initiate phagocytosis to clear *Candida* infections. For instance, *C. albicans* β-glucan binds to the PRR dectin-1 which leads to receptor phosphorylation, then recruitment of the spleen tyrosine kinase (SYK) and consequently activation of the CARD complex, which stimulates nuclear translocation of the transcription factor NF-kB. The NF-kB transcription factor induces the expression of pro-inflammatory cytokines and co-stimulatory**

molecules required during antigen presentation (Netea, Joosten et al. 2015). Permission to reproduce this figure has been granted by SPRINGER NATURE.

1.4.1.2. Effector mechanisms for the clearance of *C. albicans*

When the mucosal surface is breached or in microbiota dysbiosis, *C. albicans* can penetrate the epithelial cells by induced endocytosis or active penetration (Netea, Joosten et al. 2015). The first responders that encounter the *C. albicans* antigens are the tissue-resident macrophages which subsequently produce inflammatory cytokines and chemokines that recruit and activate other immune cells at the site of the infection (Netea, Joosten et al. 2015). In addition, blood monocytes (classical $CD14^{hi}CD16^{-}$ and non-classical $CD14^{+}CD16^{low}$ monocytes) are recruited to the infected tissue via CCR2 and its ligand CCL2 where they differentiate into inflammatory macrophages and enhance the effector response of the skin-resident macrophages (Smeekens, van de Veerdonk et al. 2011). The pro-inflammatory cytokines produced by the macrophages and epithelial cells (e.g. IL-1β, TNF and IL-6) recruit neutrophils (Netea, Brown et al. 2008). Neutrophils contribute to fungal killing by several mechanisms including phagocytosis, the production of reactive oxygen species (ROS) and the release neutrophil extracellular traps (NETs) that capture *Candida* conidia and hyphae and contain antimicrobial proteins that inhibit fungal growth (Netea, Joosten et al. 2015). Natural killer (NK) cells also contribute to the rapid innate immune response against invading *C. albicans*. The recognition of *C. albicans* by NK cells results in degranulation and the release of perforin and granzyme B in addition to the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ and TNF-α (Voigt, Hunniger et al. 2014).

Another major contributor to the early immune responses to *C. albicans* infection are the γδ T cells. γδ T cells isolated from human immunodeficiency virus (HIV)-

infected patients and healthy controls have been shown to proliferate and secrete IFN-γ and IL-17A in response to *C. albicans* infection and interestingly they have been shown to express CCR4 and CCR6 which facilitates their skin-homing (Fenoglio, Poggi et al. 2009).

Moreover, ILCs contribute to the anti-fungal immunity through the production of pro-inflammatory cytokines (Netea, Joosten et al. 2015). In a mouse model of oropharyngeal *Candida* infection, the control of the fungal infection was found to be related to IL-17A-secreting ILCs and the depletion of ILCs in mice resulted in a disseminated infection (Gladiator, Wangler et al. 2013). Humoral immunity also contributes to fungal immunity. For example, the activation of the complement system in *C. albicans* infection results in the induction of proinflammatory cytokine production via C5a-C5aR signalling (Cheng, Sprong et al. 2012).

If an infection is not promptly controlled, adaptive immune cells are activated as a second line defence. DCs bind to the antigens and process them before migrating to the regional lymph nodes to present the antigens to naïve Th cells and induce their differentiation into Th17 cells which produce IL-17 that recruits and activates neutrophils, and IL-22 that induces the secretion of β-defensins and Th1 cells which produce IFN-γ which strongly activates phagocytic cells (Liang, Tan et al. 2006, Zielinski, Mele et al. 2012). The role of type 2 cytokines in fungal defence is controversial. In some studies, type 2 cytokines appear to favour fungal infections, allergy and disease relapse (Szymczak and Deepe 2009, Romani 2011). In contrast, IL-4 was shown to be important in the *C. albicans* immune response by paradoxically inducing Th1 cells under some circumstances (Mencacci, Del Sero et al. 1998).

2. Hypothesis and Aims

From what we discussed earlier, it is known that atopic skin lesions are characterised by an altered lipid profile and CD1a-expressing APCs are enriched in AD lesions and it is known that endogenous epidermal lipids can represent CD1a ligands, raising the possibility that CD1a-reactive T cells contribute to sensing of epidermal compromise. In addition, there is a strong association between fungi such as *C. albicans* and AD exacerbation, but no previous studies have investigated the specific role of fungal-responsive CD1a-reactive T cells in the pathogenesis of AD. The contribution of CD1a-reactive T cells to cutaneous immune responses and particularly the ability of fungal antigens to enhance this inflammatory process are not yet elucidated. In order to further understand the fungal-responsive CD1a-reactive T cells, we hypothesised that AD is associated with an altered frequency and phenotype of fungal-responsive CD1a-reactive T cells in human blood and skin and that fungal-responsive CD1a-reactive T cells contribute to fungal immune responses, cutaneous immunity and AD. This hypothesis was investigated by the following aims:

1. Investigation of the frequency and functional profile of polyclonal fungal-responsive CD1a-reactive T cells in the blood and skin of AD and healthy controls (Chapter 4).
2. Isolation, phenotypical and functional analysis of fungal-responsive CD1a-reactive monoclonal T cells (Chapter 5).
3. Understanding the underlying mechanisms of fungal activation of CD1a-reactive T cells and characterisation of novel fungal-derived lipids that bind CD1a (Chapter 6).

3. Materials and Methods

3.1. Fungi and fungal extracts

Table 3-1 Fungi and fungal extracts used in the experiments.

Fungus/extract	Experiments	Source	Identifier
Candida albicans, skin prick test	Used for all experiments, unless stated otherwise.	Inmunotek	P904
Candida albicans, lyophilised (defatted)	Used for pulsing the cells in Table 6-2, Figure 6.5, Figure 6.6, Figure 6.7, and Figure 6.8.	GREER	XPM15D3A2.5 Lot # 346799
Candida albicans, heat-killed	Used for lipid fractionation and then pulsing the cells in section 6.2.2.	Invivogen	tlrl-hkca
Candida albicans, cryopreserved	Used to culture *C. albicans* and generate supernatant to pulse the cells in Figure 6.9, Figure 6.10 and Table 6-3.	BEI resources	NR-29449 *Candida albicans* 19F

3.2. Lipids

Table 3-2 Lipids used in the experiments.

Lipid	Source	Identifier
Ergosterol	SIGMA	CAS # 57-87-4, item 47130-U
Ergosterol	Cayman chemical	CAS # 57-87-4, Item 19850
Ergosterol esters	Professor Gurdyal Besra (University of Birmingham)	-
Squalene peroxide	Professor Gurdyal Besra (University of Birmingham)	-
Squalene	SIGMA	CAS # 111-02-4, item S3626

For long term storage at -20ºC, the lipids above were dissolved in chloroform at 10 mg/ml. To use in cellular assays the chloroform was first evaporated using nitrogen flow and then the lipids were dissolved in phosphate-buffered saline (PBS) 20% methanol (vol/vol, vehicle) and sonicated for 10 minutes. The dissolved fractions were then used in different functional assays at various dilutions with a final methanol concentration of 2% (Lepore, Sansano et al. 2015). In some experiments dimethyl sulfoxide (DMSO) was used to dissolve the lipids with a final concertation of 0.3% when pulsing the cells.

3.3. Flow cytometry

3.3.1. Antibodies and reagents

Table 3-3 Antibodies and reagents used for flow cytometry.

Antibody/reagent	Source	Identifier
Alexa Fluor® 488 anti-human CD4 Antibody	BioLegend	Clone: OKT4 Cat # 317420
Alexa Fluor® 488 anti-human TLR2 Antibody	BioLegend	Clone: TL2.1 Cat # 309712
Alexa Fluor® 700 anti-human CD4 Antibody	BioLegend	Clone: OKT4 Cat # 317426
Alexa Fluor® 700 anti-human TCR Vδ2 Antibody	BioLegend	Clone: B6 Cat # 331416
APC anti-human CD1a Antibody	BD Pharminge™	Clone: HI149 Cat # 559775
APC anti-human CD314 (NKG2D) Antibody	BioLegend	Clone: 1D11 Cat # 320808
APC anti-human Dectin-1 Antibody	BioLegend	Clone: 15E2 Cat # 355406
APC anti-human TCR α/β Antibody	BioLegend	Clone: IP26 Cat # 306718
APC anti-human TCR γ/δ Antibody	BioLegend	Clone: B1 Cat # 331212

APC/Cy7 anti-human TCR α/β Antibody	BioLegend	Clone: IP26 Cat # 306728
Brilliant Violet 421™ anti-human TLR4	BioLegend	Clone: HTA125 Cat # 312811
Brilliant Violet 605™ anti-human CD194 (CCR4) Antibody	BioLegend	Clone: L291H4 Cat # 359418
Brilliant Violet 605™ anti-human CD314 (NKG2D) Antibody	BioLegend	Clone: 1D11 Cat # 320832
Brilliant Violet 605™ anti-human TCR α/β Antibody	BioLegend	Clone: IP26 Cat # 306732
Brilliant Violet 650™ anti-human CD11c Antibody	BioLegend	Clone: 3.9 Cat # 301638
Brilliant Violet 785™ anti-human CD3 Antibody	BioLegend	Clone: OKT3 Cat # 317330
Pacific Blue™ anti-human/mouse Cutaneous Lymphocyte Antigen (CLA) Antibody	BioLegend	Clone: HECA-452 Cat # 321308
PE anti-human Dectin-2 Antibody	R&D Systems	Clone: 545943 Cat # FAB3114P
PE anti-human TCR γ/δ Antibody	BioLegend	Clone: B1 Cat # 331210
PE/Cy7 anti-human TCR γ/δ Antibody	BioLegend	Clone: B1 Cat # 331222
PerCP anti-human CD14	BioLegend	Clone: HCD14 Cat # 325632
PerCP anti-human CD8a Antibody	BioLegend	Clone: RPA-T8 Cat # 301030
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	Invitrogen™	L34957
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit, for 405 nm excitation	Invitrogen™	L34955
CellTrace™ Violet Cell Proliferation Kit, for flow cytometry	Invitrogen™	C34557
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (RUO)	BD Biosciences	552843
ArC™ Amine Reactive Compensation Bead Kit	Invitrogen™	A10346

eBioscience™ Brefeldin A Solution (1000X)	Invitrogen™	00-4506-51

3.3.2. Staining cells for flow cytometry

100,000 – 500,000 cells/well were stained in a round-bottom 96-well plate. 100 µl/well of the cell suspension was aliquoted and the cells were washed twice with 200 µl/well PBS. 40 µl/well of the fluorescent viability dye stock solution (Invitrogen™) was added, mixed well and incubated for 20 minutes at 4°C in the dark. After this, the cells were washed twice with 200 µl/well flow cytometry staining buffer (3% fetal bovine serum (FBS) in PBS) and 40 µl/well of the staining master mix, containing all the conjugated fluorescent antibodies at the recommended dilution, was added, mixed well and incubated for 20 minutes at 4°C in the dark. Then, the cells were washed twice with 200 µl/well flow cytometry staining buffer. The cells were then fixed with 50 µl/well 4% methanol-free formaldehyde (Pierce™ Thermo Scientific™ 28906) for 10 minutes at room temperature protected from light, before they were washed twice with 200 µl/well flow cytometry staining buffer and finally were resuspended in flow cytometry staining buffer and transferred into flow cytometry tubes for flow cytometry analysis using BD LSRFORTESSA or BD LSRFORTESSA X20 (BD Biosciences). Single stained cells or beads controls (BD Biosciences) were used to set-up voltages and determine the levels of compensation. Fluorescence minus one controls (FMOs) were included to set up the positive and negative gates. The flow cytometry data was analysed using FlowJo 10.4.2 (FlowJo, LLC).

3.4. Culturing *Candida albicans*

Candia albicans fungus was cultured in Sabouraud 4% dextrose chloramphenicol agar (100884CF, VWR®). The agar plate was inoculated with approximately 50 µL

of thawed culture and incubated at 25°C to 30°C for 3 days. Figure 3.1 shows the appearance of C. albicans colonies on the agar at day 3. The colonies were then harvested in Sabouraud dextrose broth (AX021154, VWR®) supplemented with 100 mg/litre chloramphenicol (29231-5VL-F, Sigma-Aldrich) and incubated at 25°C to 30°C for 16 hours on an orbital shaker. The cultured fungal cell number was determined using haemocytometer and the optical density (OD) was measure at 600nm using a spectrophotometer. The cells were then span at 3000 rpm for 20 minute and the supernatant was collected and filtered through 0.22 µm filter. The supernatant was used to pulse APCs in functional assays.



Figure 3.1 *C. albicans* on Sabouraud 4% dextrose chloramphenicol agar after 3 days of culture at 25°C to 30°C.

3.5. PBMCs isolation

PBMCs were isolated from whole blood by density gradient centrifugation. Blood received from the dermatology clinic at the Churchill Hospital or from donors at the WIMM was diluted at least 1:1 in RPMI 1640 medium. 15 ml lymphoprep solution was added into a 50ml falcon centrifuge tube, then a maximum of 35 ml of the

diluted blood was layered over the surface of the lymphoprep solution. The tubes were spun at 2000 rpm without brake for 20 minutes. The PBMCs layer was then aspirated using plastic Pasteur pipette and transferred into a new 50 ml falcon tube. The cells were washed with RHEPES media (500 ml RPMI-1640 (SIGMA), 5 ml HEPES buffer solution (Gibco by Life Technologies)) before they were spun at 1800 rpm for 10 minutes (with brake). After that, the cells were washed again with RHEPES and spun at 1500 rpm for 5 minutes (with brake). Finally, cells were resuspended in culture medium before they were frozen for future use or processed using CD3+ or CD14+ MACS bead separation.

3.6. Isolation of mononuclear cells from skin blister fluid.

Skin blister was performed following a well-established protocol in our lab as described in (Jarrett, Salio et al. 2016). In summary, suction was applied to the skin at 200 mmHg for 1 hour to induce a separation between the epidermis and dermis. After 24 hours, the blister fluid was aspirated, and the cells were separated by centrifugation. Blister-derived T cells were sorted using flow cytometry and then expanded using the rapid expansion mix before using them in functional assays.

3.7. MACS® separation using MicroBeads

CD3+ or CD14+ cells were separated from total PBMCs using CD3 or CD14 MicroBeads, respectively (MACS Miltenyi Biotec) following the manufacturer's protocol as follows:

3.7.1. Magnetic labelling

PBMCs were counted and then centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 80 μl cold MACS buffer (PBS, 2% FBS and 2 mM EDTA) and 20 μl MicroBeads per 10^7 total cells before it was mixed well and incubated for

15 minutes at 4-8°C. After this, cells were washed with 1-2 ml of cold MACS buffer, centrifuged at 1500 rpm for 5 minutes and resuspended in 500 μl cold MACS buffer. All the reagent volumes were scaled up when working with more than 10^7 total cells.

3.7.2. Magnetic separation

LS column (MACS Miltenyi Biotec) was placed in the magnetic field of the MACS separator (MidiMACS™) and was rinsed with 3 ml of cold MACS buffer. Then, the cell suspension was applied onto the column and washed 3 times with 3 ml of cold MACS buffer. The total effluent of the unlabelled cell fraction was collected in a 50 ml falcon placed under the column. The column was then removed from the magnetic field of the MACS Separator and the labelled cells were flushed with 5 ml of cold MACS buffer using the plunger supplied with the column. The labelled cell fraction was then washed and resuspended in culture medium and the unlabelled cells were froze or used for further magnetic bead separation.

3.8. Culturing cells

3.8.1. Culturing K562 cells

K562-EV and K562-CD1a cell lines (a gift from Professor Branch Moody, Harvard University, United States) were cultured in K562 media (500 ml RPMI-1640 (SIGMA), 50 ml heat-inactivated FBS (SIGMA, lot # BCBS0536V or BCBS1626V), 5 ml L-glutamine solution (SIGMA), 5 ml HEPES buffer solution (Gibco by Life Technologies), 5 ml penicillin-streptomycin (SIGMA), 5 ml non-essential amino acids solution (Gibco by Life Technologies), 5 ml sodium pyruvate (Gibco by Life Technologies), 500 µl 2-mercaptoethanol (Gibco by Life Technologies) and 200 µg/ml G418 Sulphate (Life Technologies)). The cells were cultured in T75 flasks at

0.4 million cells/ml and they were splitted on alternative days when the density reached approximately 0.8-1 million cells/ml.

3.8.2. Culturing monocyte-derived dendritic cells (MO-DCs)

MO-DCs were differentiated from CD14+ monocytes following a published protocols (Spadaro, Montone et al. 2014) and summarised as follows: CD14+ human cells were isolated from peripheral blood mononuclear cells (PBMCs) using MACS® separation (MACS Miltenyi Biotec). CD14+ cells were cultured in 24-well Nunc™ Non-Treated plates (thermo scientific cat no. 14453024) (0.5-1 × 10^6 cells/ml) in 1 ml of complete media per well (500 ml RPMI-1640 (SIGMA), 50 ml heat-inactivated fetal bovine serum (FBS) (SIGMA, lot # BCBS1626V), 5 ml L-glutamine solution (SIGMA), 5 ml HEPES buffer solution (Gibco by Life Technologies), 5 ml penicillin-streptomycin (SIGMA), 5 ml non-essential amino acids solution (Gibco by Life Technologies), 5 ml sodium pyruvate (Gibco by Life Technologies), 500 µl 2-mercaptoethanol (Gibco by Life Technologies)) supplemented with 10 ng/ml recombinant human IL-4 (BioLegend, 574008) and 100 ng/ml recombinant human GM-CSF (BioLegend, 572905). On day 3 the media was replaced with fresh supplemented culture medium. The differentiated MO-DCs were harvested on day 5 and subsequently stained and checked by flow cytometry for dendritic cells surface markers (CD1a+ CD11c+ CD14-) before the functional assays were carried on.

3.8.3. Culturing T cells

CD3+ T cells were isolated from PBMCs using MACS® separation (MACS Miltenyi Biotec) and cultured in T cells media containing: 500 ml RPMI-1640 (SIGMA), 50 ml heat-inactivated human serum (HS) (SIGMA, lot # SLBK1775V and

SLBN8827V), 5 ml L-glutamine solution (SIGMA), 5 ml HEPES buffer solution (Gibco by Life Technologies), 5 ml penicillin-streptomycin (SIGMA), 5 ml non-essential amino acids solution (Gibco by Life Technologies), 5 ml sodium pyruvate (Gibco by Life Technologies), 500 µl 2-mercaptoethanol (Gibco by Life Technologies) and 250 unit/ml recombinant human IL-2 (BioLegend). T cells were cultured for 3-4 days in T cell media at 1.8-2 million cells/well in a 48-well plate before they were used for functional assays.

3.9. ELISpot

To enumerate the number of cytokine-secreting CD1a-reactive T cells, ELISpot kit was used from MABTECH for IFN-γ (3420-2A), IL-22 (3475-2A) and IL-17A (3520-2A). Each kit contains a matched pair of monoclonal capture antibody and monoclonal biotinylated detection antibody and streptavidin-alkaline phosphatase. The cells were prepared as previously described (Cheung, Jarrett et al. 2016, Jarrett, Salio et al. 2016) and the ELISpot was done following the manufacturer's protocol as follows:

3.9.1. Day 1

The ELISpot plate (MSIPS4510, Merck Millipore) was pre-treated with 25 µl/well 35% ethanol for less than 1 minute, then it was rinsed 5 times with 200 µl/well distilled water. The cytokine-specific monoclonal capture antibody was diluted in PBS at 15 µg/ml for IFN-γ or 10 µg/ml for IL-17A and IL-22 and then 100 µl/well was added to the ELISpot plate and incubated overnight at 4°C. The APCs (K562 or MO-DCs) and the T cells (isolated from PBMCs and cultured for 3-4 days in T cell media at 1.8-2 million cells/well in a 48-well plate) were washed, counted and resuspended in complete media at a concentration of 1 million cells/ml and 2

million cells/ml, respectively. The APCs were then pulsed with the antigen of interest. The cells were incubated for 16-20 hours in a 37°C humidified incubator with 5% CO_2.

3.9.2. Day 2

The pulsed antigen presenting cells were washed twice with RHEPES media, counted and resuspended in complete media at 0.2-0.25 million cells/ml. The concentration of the rested T cells was adjusted to 0.5-0.75 million cells/ml in complete media. The ELISpot plate was washed 5 times with 200μl/well PBS and then blocked for 30-60 minutes with 200 μl/well complete media.

25,000-20,000 APCs/well (100 μl) and 50,000-75,000 T cells/well (100 μl) were added per well in the ELISpot plate. In some experiments 10 μg/ml anti-human CD1a antibody (OKT-6, in house or HI149, BioLegend) or 10 μg/ml purified mouse IgG1 κ isotype control antibody (BioLegend) were added to the antigen presenting cells for 1 hour before co-culturing with T cells. When MO-DCs were used MHC class I and MHC class II were blocked with 10 μg/ml anti-HLA-ABC (clone: W6/32, BioLegend) and anti-HLA-DR (clone: L243, BioLegend), respectively, prior to co-culture with T cells. Wells were set-up in duplicate in the ELISpot plate. Phorbol myristate acetate (PHA) 10 ng/ml and Ionomycin 500 ng/ml were added to T cells in the positive control wells, and T cells alone were included as a negative control. The ELISpot plate was incubated for 16-20 hours in a 37°C humidified incubator with 5% CO_2.

3.9.3. Day 3

The ELISpot plate was washed 5 times with PBS. Cytokine-specific monoclonal biotinylated detection antibody (MABTECH) was diluted at 1 μg/ml for IFN-γ or 0.5

μg/ml for IL-17A and IL-22 in PBS and then 100 μl/well was added and incubated for 2-3 hours at room temperature. After this, the ELISpot plate was washed 5 times with PBS and 100 μl/well of diluted streptavidin-alkaline phosphatase (1:1000 in PBS) (MABTECH) was added and incubated for 1-2 hours at room temperature. Next, the plate was washed 4 times with PBS and 3 times with distilled water. 100 μl/well of developing buffer (alkaline phosphatase conjugate substrate kit, Bio-Rad Laboratories Ltd) was added and incubated for 10-15 min minutes or until the spots in the positive control wells appeared at room temperature. To terminate the colour development reaction, plates were washed 5 times under running distilled water. The plate was kept drying overnight in the dark and then it was read using an automated ELISpot reader (Autimmun Diagnostika ELISpot Reader).

3.10. CD1a plate-bound assay

CD1a plate-bound assay protocol was modified from previously published studies (de Jong, Cheng et al. 2014, Bourgeois, Subramaniam et al. 2015). Streptavidin coated plate (Pierce™ Streptavidin Coated High Capacity Plates, Clear, 8-Well Strip, catalogue number 15501) was washed with washing buffer (0.1% BSA PBS) and then coated with biotinylated CD1a (National institutes of health (NIH) tetramer core facility, Emory University) at 10 μg/ml in 100 μl PBS and then incubated at 4^{o}C overnight. On the next day, the plate was washed 3 times with washing buffer before the antigens of interest were loaded at the desired concentration in 100 μl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, the plate was washed 4 times with washing buffer and 100,000 T cells/well were added in 200 μl of complete media and incubated in a 37°C humidified incubator with 5%

CO_2. In some experiments T cells were stimulated with 500 pg/ml IL-12p70 (BioLegend). After 24 hours, the supernatant was collected, and the cytokines produced by T cells were measured by enzyme-linked immunosorbent assay (ELISA). The recombinant CD1a used in this experiment was validated using western blot (Figure 3.2).

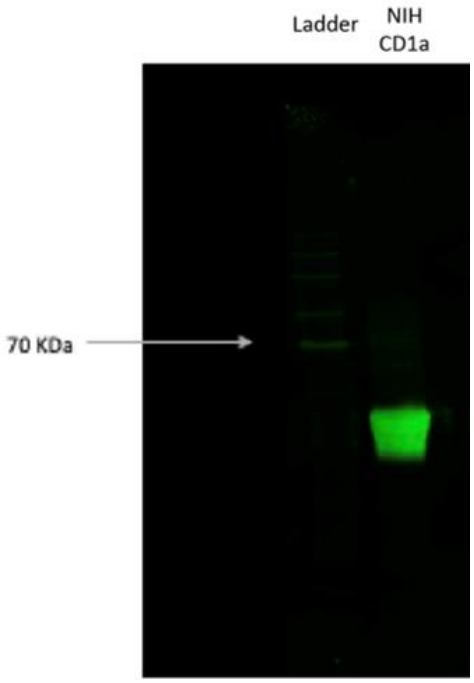


Figure 3.2 Recombinant CD1a validation using western blot showing the molecular size of NIH CD1a at around 49 kDa.

This experiment was done by Yi-Ling Chen and Koshika Yadava (Ogg lab).

3.11. CD1a-beads assay

3.11.1. Loading the CD1a with lipids/antigens

Lipids of interest were dissolved in 2.5% DMSO 0.5% Tyloxapol and were sonicated at 50 degrees for 15-20 minutes. Biotinylated CD1a (2 mg/ml stock, National institutes of health (NIH) tetramer core facility, Emory University) was loaded with the lipids of interest at 30X molar excess or 100X molar excess of

CD1a protein in citrate buffer (pH 6) for 24 hours at room temperature. The recombinant CD1a was validated using western blot

3.11.2. Preparing and adding the beads

Dynabeads™ M-270 Streptavidin (10 mg/ml stock, Invitrogen™) were prepared as per manufacturer's instructions with some modifications. The required volume of the beads was calculated based on their binding capacity. The beads were washed 3 times with equal volume of PBS using DynaMag™ magnet. After the last wash, the supernatant was removed, and the beads were resuspended in 0.1% BSA PBS. 20 µg of the biotinylated CD1a loaded with the lipids was added to 1 mg Dynabeads™ in 300 µl total volume and then they were mixed for 30 minutes using a rotator at room temperature. After this, they were washed 3 times with 300 µl 0.1% bovine serum albumin (BSA) PBS using DynaMag™ magnet. After the last wash, supernatant was removed, resuspend in complete media and incubated overnight at 4^{o}C.

3.11.3. Co-culturing with T cells

T cells were washed twice with RHEPES media, counted, and then resuspend at 1 million/ml in complete media. The beads were vortexed for 30 seconds, counted and resuspended in complete media at 2 million/ml. 100,000 T cells and 200,000 beads were cocultured in 96-well round bottom plate in 200 µl total volume for 24 hours in a 37^{o}C humidified incubator with 5% CO_2. T cells were stimulated with 500 pg/ml IL-12p70 (BioLegend). After 24 hours, the supernatant was collected and analysed by ELISA.

3.12. ELISA

ELISA was done following the manufacturer's protocol of the following kits: human IFN-γ ELISA ready-set-go kit (affymetrix eBioscience), human IFN-γ ELISA MAX™ Deluxe (BioLegend).

3.13. Quantitative and real-time polymerase chain reaction (RT-qPCR)

K562-EV and K562-CD1a cells were washed twice with RHEPES media, counted, resuspended in complete media at 1 million cells/ml and then pulsed with the antigens of interest for 16-20 hours in a 37°C humidified incubator with 5% CO_2. On the next day, the pulsed K562 cells were washed twice with RHEPES media, counted and resuspended in complete media at the 100,000 million cells/ml. T cells were washed, counted and resuspended in complete media at 1 million cells/ml. K562 cells and T cells were co-cultured in a round-bottom 96 well plate at a ratio of 1:10 (10,000 K562s: 100,000 T cells) and were kept in a 37°C humidified incubator with 5% CO_2. After 6 hours, the cells were collected by centrifugation.

mRNA extraction was done using TurboCapture 96 mRNA kit (QIAGEN) following the manufacturer's protocol. The isolated mRNA was then used directly in cDNA synthesis using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (cat. 28025013, Invitrogen™) and then was ran in the PCR machine (T100™ Thermal Cycler, BIO-RAD).

Gene expression of the cDNA samples was measured using the following TaqMan Gene Expression Assays (Applied Biosystems™ TaqMan®): CD2 Hs00233515_m1, IFN-γ Hs00989291_m1, IL-13 Hs00174379_m1, IL-17F

Hs01028648_m1, IL17A Hs0017438, IL-17C Hs00171163_m1, GM-CSF Hs00929873_m1, IL-22 Hs01574154_m1. The samples were then loaded into QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems™) for analysis.

3.14. Secretion assay to generate T cell lines/clones

The cytokines produced by CD1a-reactive T cells were detected and analysed using cytokine-specific secretion assay detection kits from MACS Miltenyi Biotec: IFN-γ-PE (130-054-202), IFN-γ-FITC (130-090-433), IFN-γ-APC (130-090-762). The cytokine secreting cells were sorted to generate T cell lines/clones. This was done following the manufacturer's protocol as follows:

3.14.1. Preparing, pulsing and co-culturing the cells.

The K562 cells and T cells were prepared, pulsed and co-cultured similar to what was done in section 3.9.

3.14.2. Labelling with catch reagent

The cells were collected in 15 ml falcon tubes, washed with 10 ml of cold buffer (0.5% FBS in PBS with 2 mM EDTA) and centrifuged at 1500 rpm for 5 minutes at 4^{o}C. The supernatant was removed completely, and the cells were resuspended in 90 µl of cold complete media and 10 µl of the catch reagent per 10^6 total cells. Then, they were mixed well and incubated for 5 minutes at 4^{o}C. After this, 5-10 ml of warm complete media was added to dilute the cells before they were incubated for 45 minutes at 37^{o}C under slow continuous rotation.

3.14.3. Labelling with the cytokine detection antibody

The cells were washed with cold buffer and centrifuged at 1500 rpm for 5 minutes at 4^{o}C. The supernatant was removed completely, and the cells were resuspended

in 90 µl of cold buffer and 10 µl of the detection antibody per 10^6 total cells which is conjugated to a fluorochrome (e.g. FITC or APC) for detection by flow cytometry. Then, they were mixed well and incubated for 10 minutes at 4°C in the dark. After this, 10 ml of cold buffer was added and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed completely, and additional staining reagents were added, (e.g. CD3, CD4, CD8, TCR αβ, TCR γδ). The stained cells were incubated for 10 minutes at 4°C in the dark, then washed with cold buffer, centrifuged at 1500 rpm for 5 minutes at 4°C and resuspended in cold buffer. Finally, they were analysis by flow cytometry and the autoreactive or antigen-reactive CD1a-reactive T cells were sorted.

3.15. Rapid expansion of T cell lines/clones

Autoreactive or antigen-reactive CD1a-reactive T cells were sorted at 1-50 cells/well in a round-bottom 96-well plate and cultured in 200 µl/well T cell media containing 400,000 cells/ml of irradiated B cell line (9000 RAD), 1.5 million cells/ml of irradiated PBMCs from one donor (3000 RAD), 50 ng/ml of CD3 monoclonal antibody (OKT3, eBioscience™), 500X phytohemagglutinin-L solution (PHA-L) (eBioscience™). For further expansion of the autoreactive or antigen-reactive CD1a-reactive T cell the lines/clones 10,000 - 100,000 T cells were added per well in a 48-well plate and cultured in 1 ml/well T cell media containing the same rapid expansion mix mentioned above. On day 5 post-expansion, the media was changed and then changed every 2-3 days. The cells were splitted as required. 2-4 weeks post-expansion the cells were stained for flow cytometry analysis and they were tested functionally by ELISpot or other functional assays.

3.16. Fungal lipid extraction and fractionation

Fungal lipid extraction and fractionation was done with David Priestman (senior research fellow, Platt laboratory, Department of Pharmacology, University of Oxford). David Priestman's protocol is modified from (Bodennec, Brichon et al. 2000). To summarise, 0.9 ml of the fungal preparation was mixed with 1.5 ml chloroform and 1.5 ml methanol in a 15 ml falcon tube, vortexed and then kept overnight at 4^{o}C. The next day, the tube was centrifuged at 3000 rpm for 15 minutes. Three phases were formed; an upper phase (aqueous), a lower phase (organic) and a middle phase (protein layer). Each of the phases was collected in a separate 15 ml falcon tube and then was dried under nitrogen flow at 42^{o}C using a sample concentrator (TECHNE). The organic phase was dissolved in 1 ml of chloroform and then applied into an aminopropyl column (Supelclean™ LC-NH_2 SPE Tube 504483) and the elute was collected in a 15 ml falcon tube (unbound lipid fraction). Then the aminopropyl column was moved to the next 15 ml falcon tubes one by one and the proper solvent was used for each fraction (lipid fraction I-VI) (Table 3-4). The lipid fractions were dried under nitrogen flow and then dissolved in DMSO and stored at -20^{o}C.

Table 3-4 Solvents used to elute lipid fractions (I-VI).

Modified from (Bodennec, Brichon et al. 2000).

Fraction	Solvent (v/v)
Unbound	Chloroform
I	Diethyl ether
II	Chloroform–methanol (23:1)
III	Diisopropyl ether–acetic acid (98:5)
IV	Acetone–methanol (9:1.2)
V	Chloroform–methanol (2:1)
VI	0.2 M ammonium acetate in methanol followed by DW

3.17. sPLA2 activity

the sPLA2 activity of the *C. albicans* extract was measured using sPLA2 kit (Cayman Chemicals, item no. 765001) and following the manufacturer's protocol. This assay is based on a plate-based colorimetric measurement of sPLA2, where the 1,2-dithio analogue of diheptanoyl phosphatidylcholine is used to serve as a substrate for sPLA2. Upon hydrolysis of the thio ester bond at the sn-2 position by sPLA2, free thiols are detected using DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) which produce a coloured participant. The absorbance was read by a spectrophotometer over time (415nm). The sPLA2 activity was calculated using a formula provided by the manufacturer.

3.18. Ethical approval

All donors and patients signed an informed consent. OUH NHS dermatology clinic: blood obtained from healthy donors or patients; NHS REC approved 14/SC/0106. WIMM phlebotomy room: blood obtained from individuals within the WIMM, consent given and recorded, NHS REC approved 14/SC/0106. OUH NHS dermatology clinic and Manor Hospital, Oxford: skin samples taken of routine skin procedures or abdominoplasty surgeries, NHS REC approved 14/NW/1153 and 09/H0606/71.

3.19. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. When more than two groups were compared ordinary one-way ANOVA test and Tukey's multiple comparison test or Kruskal-Wallis test and Dunn's multiple comparison test were conducted. When two groups were compared student's t-test was used.

4. Investigation of the frequency and functional profile of polyclonal fungal-responsive CD1a-reactive T cells in the blood and skin of healthy controls and AD patients.

4.1. Introduction

The CD1-lipid presentation system is a complementary antigen-presentation system to the MHC-peptide presentation that contributes to the immune pathways in health and disease (Silk, Salio et al. 2008, Van Kaer, Wu et al. 2016). In brief, the CD1a-reactive T cells express diverse TCRs and they are a normal component of the human αβ T cell repertoire (de Jong, Pena-Cruz et al. 2010, de Lalla, Lepore et al. 2011). Activation of CD1a-reactive T cells by lipids presented by CD1a molecules expressed on the surface of professional APCs (DCs and LCs) can promote production of pro-inflammatory cytokines which contribute to inflammatory skin disease pathogenesis (Dougan, Kaser et al. 2007, de Jong, Pena-Cruz et al. 2010, Jarrett, Salio et al. 2016). As discussed in section 1.2.5.1, the CD1a groove has the capacity to bind a large variety of foreign and self-antigens (Van Rhijn, Godfrey et al. 2015). Despite the wide spectrum of endogenous lipid classes that have been identified in the literature to bind CD1a (Layre, de Jong et al. 2014, Birkinshaw, Pellicci et al. 2015, de Jong 2015), few exogenous lipid ligands have been established including the mycobacterial derived lipid dideoxymycobactin (Moody, Young et al. 2004, Van Rhijn, Zajonc et al. 2005), urushiol-derived lipids (Kim, Hu et al. 2016) and pollen-derived phospholipids (Agea, Russano et al. 2005). Autoreactive CD1a-reactive T cells migrate to the skin as they express skin-homing markers such as CLA, CCR4 and CCR10 (de Jong, Pena-Cruz et al. 2010, de Jong, Cheng et al. 2014) and they were found to be enriched in the blood

and skin of individuals with AD; and therefore CD1a-reactive T cells are thought to contribute to the skin inflammatory pathway in AD (Jarrett, Salio et al. 2016).

The skin microbiota dysbiosis contributes to the pathogenesis of AD (Byrd, Belkaid et al. 2018) where the skin barrier defect, microbial dysbiosis and superimposed infections contribute to this amplification of the inflammatory process (Belkaid and Tamoutounour 2016). Compared to healthy skin, AD skin exhibits a more diverse array of fungal microbiota including *C. albicans* and other species which contribute to the disease development. (Zhang, Tanaka et al. 2011).

C. albicans is a diploid polymorphic yeast (Staniszewska, Bondaryk et al. 2013). It is a member of the human microbiota and is usually a harmless commensal fungus. However, it can be pathogenic and cause local or systemic infection (Dadar, Tiwari et al. 2018). After recognition of *C. albicans* peptides by innate immune cells through PRRs, effector immune mechanisms are activated for the clearance of the fungal invasion which include innate and adaptive immune cells that reside in the skin or are recruited after the infection as discussed in detail in section 1.4.1.2. Although 200 molecular lipid species have been identified within *C. albicans* (Singh and Prasad 2011), no previous studies have investigated the role of the CD1a antigen presentation system in this potential anti-fungal immune pathway.

Given that CD1a is highly expressed by LCs, CD1a-reactive T cells are enriched in AD lesions and *C. albicans* contributes to the pathogenesis of AD with no previous studies investigated the role of fungi in the induction of CD1a-reactive responses in AD, we hypothesised that AD is associated with an altered frequency and phenotype of fungal-responsive CD1a-reactive T-cells in human blood and

skin. Therefore, we used *C. albicans* as a model system to investigate novel pathways of fungal responsive CD1a-reactive T cell inflammation in the skin. The key aim of this chapter was to identify polyclonal *C. albicans*-responsive CD1a-reactive T cells in blood and skin of healthy individuals and AD patients and characterise their functional profile based on relevant cytokine production.

4.2. Results

4.2.1. Fungal-responsive CD1a-reactive T cell responses to CD1a in the blood of healthy controls and AD patients.

CD3+ T cells were positively selected from healthy controls (HC) or AD total PBMCs using CD3 microbeads MACS separation technology. The purity of T cells post-MACS bead separation was determined using flow cytometry which showed excellent yield of CD3+ T cells (Figure 4.1).

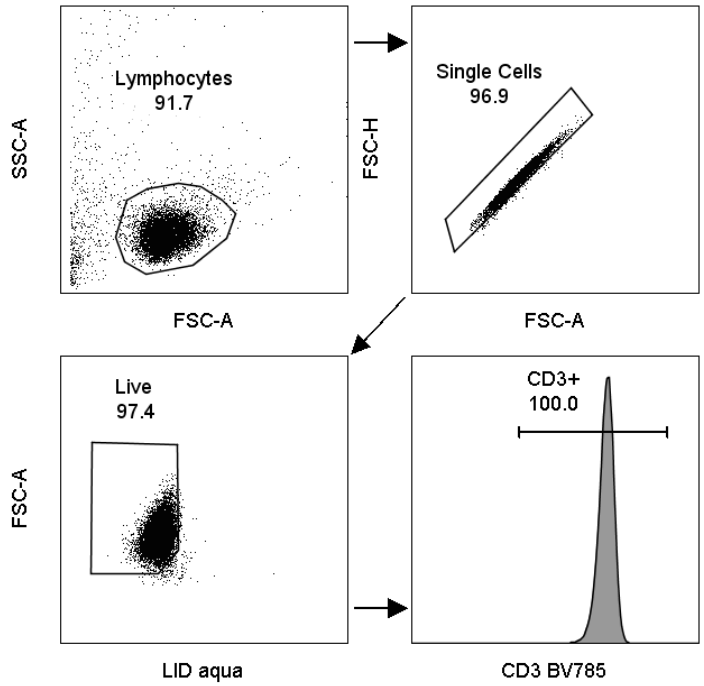


Figure 4.1 Flow cytometry plot of CD3+ T cells post-MACS bead separation.

A representative example of CD3+ T cells flow cytometry. Gating on lymphocytes, singlets, live cells and CD3+ cells. The compensation was based on single-colour stained beads. Gating was based on FMO controls.

To test the CD1a-reactivity of polyclonal CD3+ T cells from unrelated donors under equivalent conditions and bypassing alloreactivity, K562 cells (gift from Branch Moody, Harvard) were used for antigen presentation to T cells; K562 cells were

transfected with CD1a (K562-CD1a) or empty vector (K562-EV) and lacked the expression of MHC class I and II. CD1a expression of the K562-CD1a cells was checked regularly by flow cytometry and was maintained above 85% in the presence of a selection antibiotic (Figure 4.2).

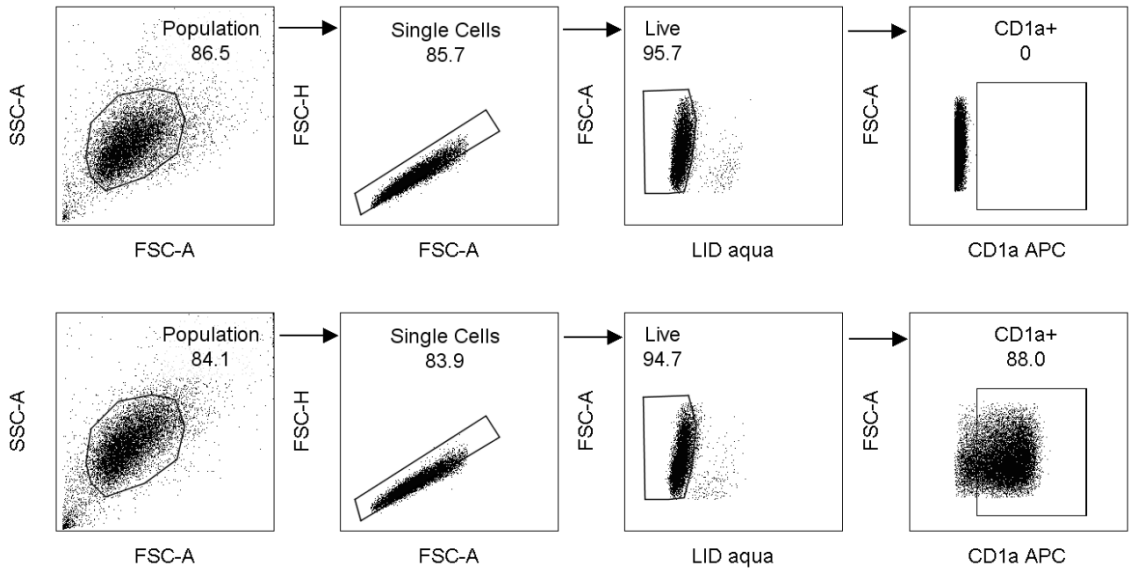


Figure 4.2 Flow cytometry plot of K562 cells.

Flow cytometry plots of K562-EV (upper panel) and K562-CD1a (lower panel). Gating on cell population, singlets, live cells and CD1a+ cells. The compensation was based on single-colour stained beads. Gating was based on FMO controls.

Before using the *C. albicans* extract (skin-prick test solution) to pulse the K562 cells for the functional assay, we tested the effect of the *C. albicans* extract on the viability of K562-CD1a cells to determine the most suitable concentration of the antigen to be used in the functional assays. Some toxicity was noted with the highest concentration (2.5 µg/ml) of the *C. albicans* extract with a reduction of the viability of K562-CD1a cells by approximately 20% compared to unpulsed cells (Table 4-1); as a result the concentration used was 1 µg/ml in the subsequent experiments.

Table 4-1 The viability of K562-CD1a cells after pulsing with *C. albicans* extract.

K562-CD1a cells were pulsed with three different concentrations of *C. albicans* extract (P1: 0.4 µg/ml, P2: 1 µg/ml, P3: 2.5 µg/ml) for 16-20 hours before they were stained with LIVE/DEAD™ Fixable Dead Cell Stain and the viability of cells was determined by flow cytometry.

Antigen	Concentration	% Live cells
Unpulsed		94.9%
Pulsed with *C. albicans* extract	P1	86.3 %
	P2	93.7 %
	P3	75.2 %

After determining the effect of the fungal extract on the viability of K562 cells, we tested whether pulsing the K562 cells with this antigen has any effect on the CD1a expression using flow cytometry. No difference of the CD1a expression was observed after pulsing the K562-CD1a cells with *C. albicans* extract in three different concentrations compared to the unpulsed condition (Figure 4.3).

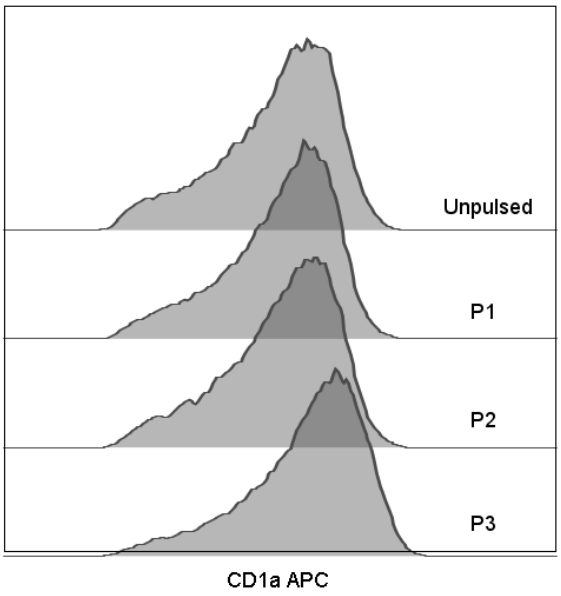


Figure 4.3 Flow cytometry histograms comparing the CD1a expression of the K562-CD1a cells after pulsing with *C. albicans* extract.

K562-CD1a cells were pulsed with three different concentrations of *C. albicans* extract (P1: 0.4 µg/ml, P2: 1 µg/ml, P3: 2.5 µg/ml) for 16-20 hours before the CD1a expression was determined by flow cytometry. Gating on cell population, singlets, live cells and CD1a+ cells. The compensation was based on single-colour stained beads. Gating was based on FMO controls.

In order to assess whether *C. albicans* antigens are presented to T cells by CD1a molecules on the surface of K562s cells, CD3+ T cells were rested for 3-4 days in IL-2 containing media and then co-cultured with K562-CD1a or K562-EV cells that were pulsed or unpulsed with *C. albicans* extract for 16-20 hours. Then, IFN-γ-producing T cells were enumerated using ELISpot (refer to section 3.9 for more details about the ELISpot protocol). PMA/Ionomycin-pulsed T cells were used as a positive control and unpulsed T cells were used as a negative control (Figure 4.4 (B)). A significant induction of IFN-γ production by CD3+ T cells (derived from the PBMCs of an AD patient) was noted when co-cultured with K562-CD1a cells compared to K562-EV in the absence of *C. albicans* antigen, demonstrating the reactivity to endogenous lipids that bind CD1a. However, when T cells were co-cultured with K562-CD1a cells in the presence of 1 µg/ml *C. albicans,* a significant increase in the IFN-γ-producing T cells was observed over K562-EV cells pulsed with *C. albicans* and over the autoreactive responses. This demonstrated that T cells produce IFN-γ in response to *C. albicans* components in a CD1a-dependent manner (Figure 4.4 (A)). The CD1a-reactive T cell response to *C. albicans* extract was shown to be dose dependent as the IFN-γ producing cells were titrated down with lower concentrations of the *C. albicans* extract (Figure 4.4 (A)).

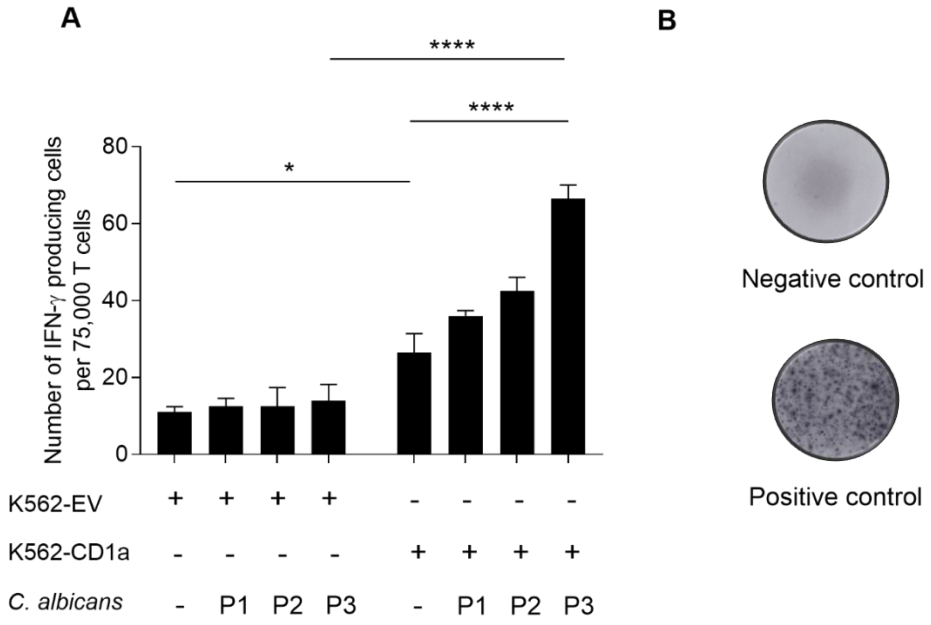


Figure 4.4 *C. albicans* induces IFN-γ production by T cells through CD1a expressed on the surface of K562 cells.

Representative example of IFN-γ ELISpot experiment where AD-derived T cells were co-cultured with K562-CD1a or K562-EV in the presence or absence of 3 different concentrations of *C. albicans* (P1: 0.16 µg/ml, P2: 0.4 µg/ml, P3: 1 µg/ml) (A). Data represent mean and standard deviation (SD) of duplicate measurements. A snapshot of the positive control and negative control wells are shown (B). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

The IFN-γ ELISpot experiment was expanded further in a larger cohort of 15 AD patients and 15 HC donors to compare the frequency of the autoreactive and *C. albicans*-responsive CD1a-reactive T cells in both groups according to number of CD3+ T cells producing IFN-γ (Figure 4.5).

In the HC cohort there was non-significant induction of IFN-γ production by CD3+ T cells when co-cultured with K562-CD1a cells compared to K562-EV in the absence of *C. albicans* antigen demonstrating non-significant reactivity to endogenous lipids. When HC T cells were co-cultured with K562-CD1a cells in the presence of 1 µg/ml *C. albicans* although a significant increment of the IFN-γ-

producing-T cells was observed over K562-EV cells pulsed with *C. albicans*, non-significant induction was observed over K562-CD1a unpulsed. In the AD cohort there was a significant induction of IFN-γ production by CD3+ T cells when co-cultured with K562-CD1a cells compared to K562-EV in the absence of *C. albicans* antigen, demonstrating the reactivity to endogenous lipids. When AD T cells were co-cultured with K562-CD1a cells in the presence of 1 μg/ml *C. albicans* a significant increment of the response by IFN-γ-producing T cells was observed over K562-EV cells pulsed with *C. albicans* and over K562-CD1a unpulsed, demonstrating that the production of IFN-γ in response to *C. albicans* components in the AD cohort is CD1a-dependent. When the autoreactivity and the *C. albicans* reactivity were compared between the two groups, no difference between the autoreactive CD1a-reactive T cell responses of HC and AD was seen, however, the *C. albicans*-responsive CD1a-reactive T cell responses were significantly higher in AD compared to HC. This demonstrated enrichment of the *C. albicans*-responsive CD1a-reactive T cells in AD (Figure 4.5). We did not observe a specific pattern of responses of autoreactive or *C. albicans*-responsive CD1a-reactive T cells based on the disease severity (Figure 4.5).

To examine whether the IFN-γ-producing autoreactive and *C. albicans*-responsive T cells are reactive to other members of group 1 CD1 molecules. CD3+ T cells were co-cultured with K562-EV and K562-CD1a or K562-CD1b or K562-CD1c in the presence or absence of *C. albicans* extract and then IFN-γ production was measured by ELISpot. Overall, co-culturing T cells with K562-CD1a gave the most predominant autoreactive and *C. albicans*-reactive responses compared to CD1b and CD1c (Figure 4.6).

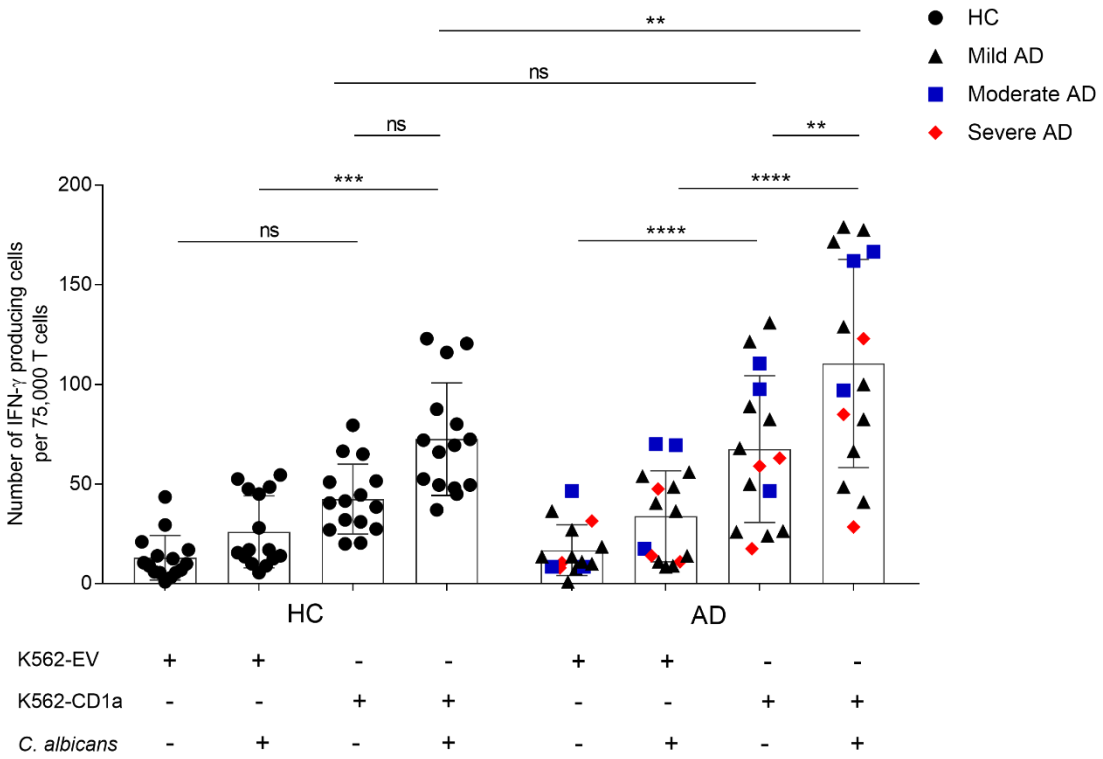


Figure 4.5 Comparing the autoreactive and *C. albicans*-responsive CD1a-reactive T cells between HC and AD patients.

CD3+ T cells from HC (n=15) or AD patients (n=15) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 1 µg/ml *C. albicans* extract (skin prick test solution) and then IFN-γ production was measured by ELISpot. The number of IFN-γ producing T cells was compared. The AD samples were colour-coded based on the severity of the disease. Data represent mean and standard deviation (SD) of biological replicates. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

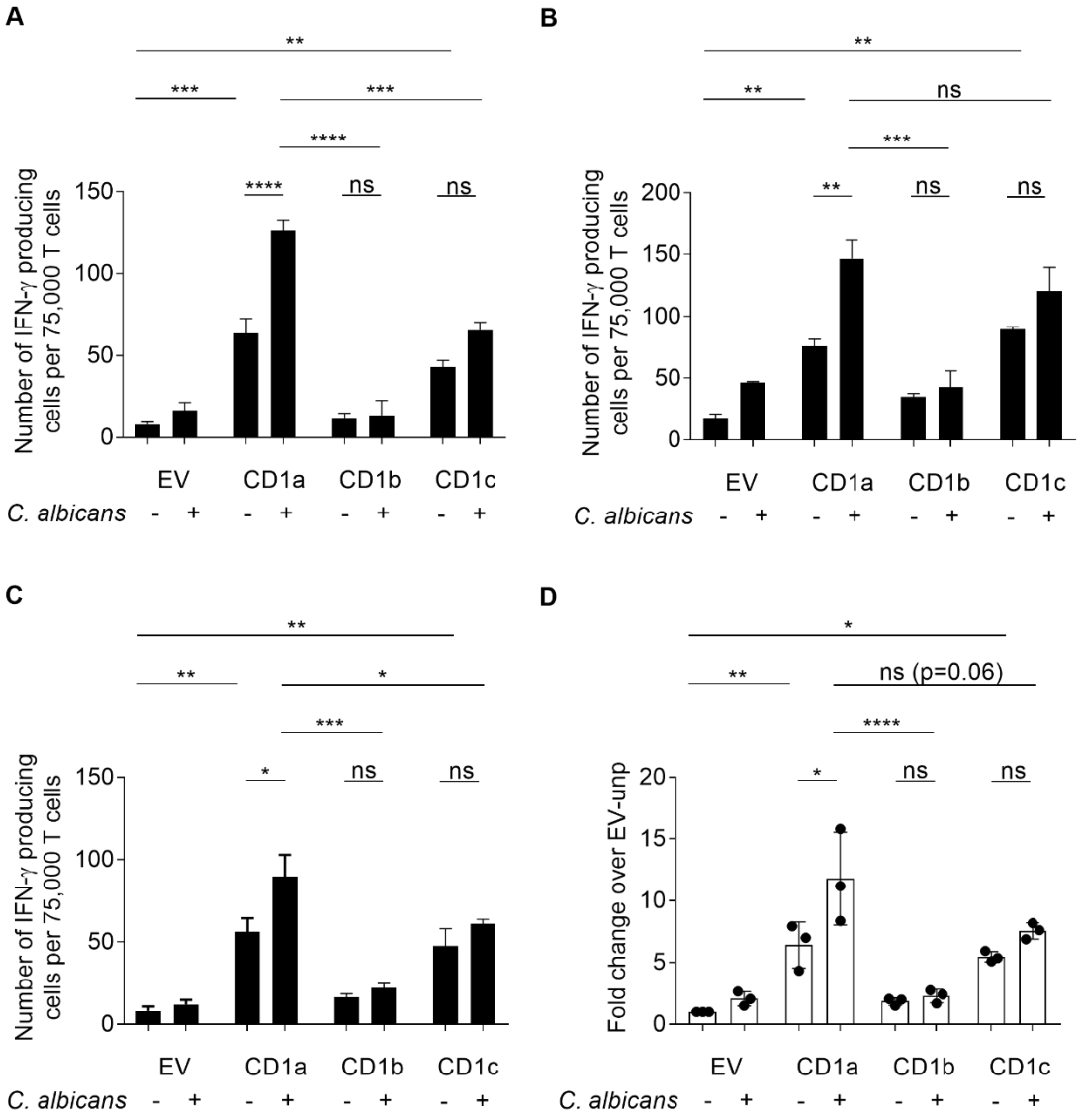


Figure 4.6 Comparing the IFN-γ-producing autoreactive and *C. albicans*-responsive T cells reactivity toward CD1a, CD1b and CD1c.

CD3+ T cells from three HC donors (W015 (A), C2399 (B) and C2402 (C)) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-EV or K562-CD1a or K562-CD1b or K562-CD1c in the presence or absence of 1 µg/ml *C. albicans* extract (skin prick test solution) and then IFN-γ production was measured by ELISpot. The number of IFN-γ producing T cells was plotted in the bar charts. Data represent mean and standard deviation (SD). The mean data from the three donors were plotted and presented as fold change over EV-unpulsed (D). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

To confirm the ELISpot findings, APC-free CD1a-plate bound assay was used. Briefly, streptavidin plate was coated with biotinylated CD1a and incubated at 4ºC overnight. Then the CD1a was loaded with *C. albicans* extract in 100 µl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, 100,000 T cells/well were added and incubated for 24 hours at 37°C. After 24 hours, the concentration IFN-γ in the supernatant was measured by ELISA. The ELISA results of the CD1a-plate bound assay showed an induction of T cells to produce IFN-γ in a CD1a-reactive manner when pulsed with *C. albicans* extract (Figure 4.7), confirming the findings of the cellular assay and demonstrating that the CD1a-reactivity of *C. albicans* was not due to processing of the fungus by the APCs.

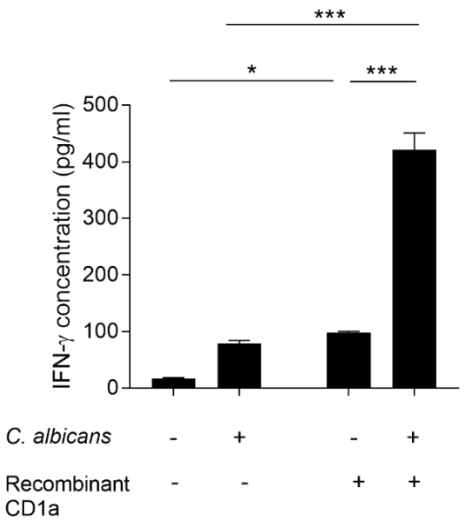
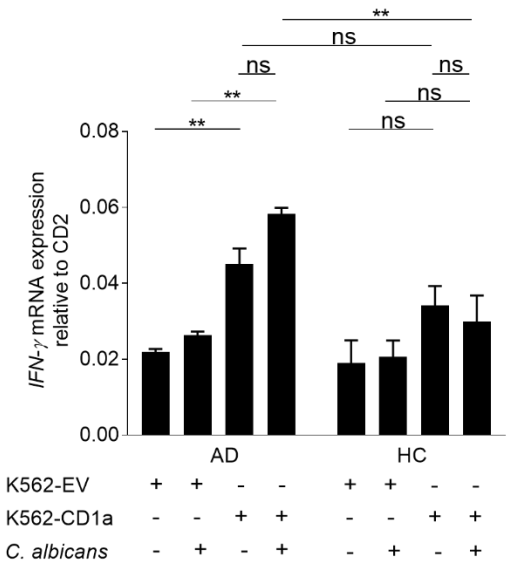


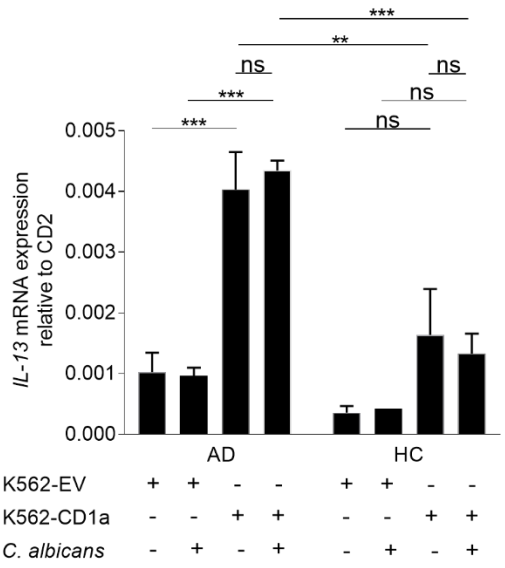
Figure 4.7 CD1a-plate bound assay of AD-derived polyclonal T cells pulsed with *C. albicans*.

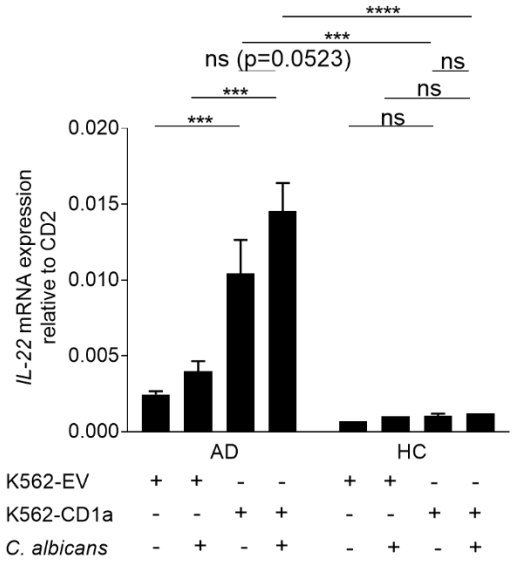
Streptavidin plate was coated with biotinylated CD1a and then incubated at 4ºC overnight. Then, the CD1a was loaded or not loaded with *C. albicans* extract (skin prick test solution) in 100 µl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, 100,000 T cells/well (AD2919) were added and incubated for 24 hours at 37°C. After 24 hours, the concentration IFN-γ in the supernatant was measured by ELISA. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

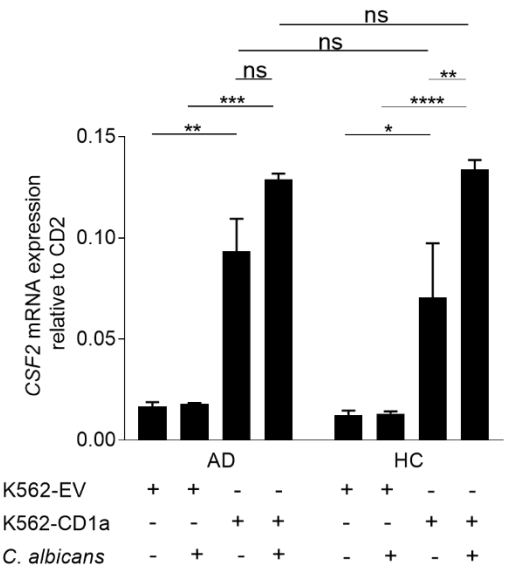
At mRNA level the expression of *IFN-γ* mRNA in addition to other relevant cytokines were measured by RT-qPCR. CD3+ T cells were co-cultured with K562-EV or K562-CD1a in the presence or absence of *C. albicans* extract for 6 hours at a ratio of 10:1. After 6 hours the cells were collected before proceeding with mRNA extraction and cDNA synthesis. Gene expression of the cDNA was measured using the TaqMan Gene Expression Assay (Figure 4.8).

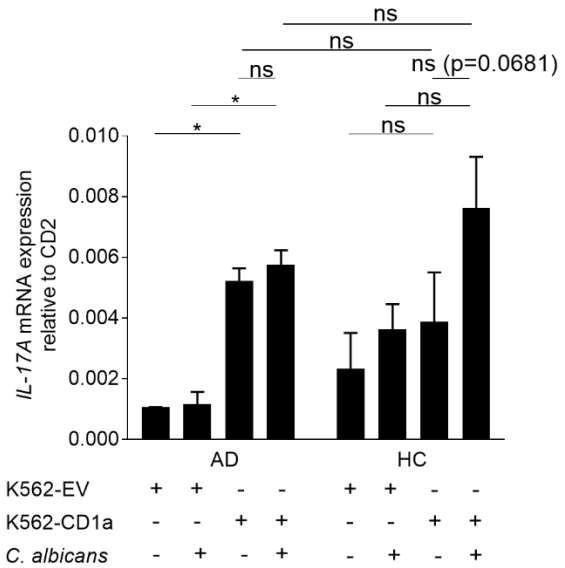
Overall, *CSF2* (the gene for GM-CSF*)* followed by *IFN-γ* were the highest genes expressed by the autoreactive CD1a-reactive T cells and the *C. albicans*-responsive CD1a-reactive T cells in the HC and AD samples. However, the expression of *IFN-γ, IL-13* and *IL-22* genes predominated in the AD samples. In AD, significant autoreactive CD1a-reactive T cell gene expression was observed for all genes tested. However, in the HC the expression of *CSF2* and *IL-17F* only showed significant autoreactive responses. In AD the expression of all genes tested was induced when T cells were co-cultured with K562-CD1a pulsed with *C. albicans* extract compared to K562-EV pulsed with *C. albicans*. However, the induction of the *C. albicans*-responsive CD1a-reactive T cells gene expression over autoreactive responses was observed for some cytokines including *IFN-γ*, *IL-22*, *CSF2* and *IL-17F* with significant results for *IL-22* only. *IL-22* gene expression by *C. albicans*-responsive CD1a-reactive T cells in AD was significantly higher than HC. In HC the expression of *CSF2* and IL-17F genes only were induced when T cells were co-cultured with K562-CD1a pulsed with *C. albicans* extract compared to K562-EV pulsed with *C. albicans*. And when comparing CD1a-unpulsed with CD1a-pulsed in HC, *CSF2* (significant), *IL-17F* (non-significant) and *IL-17A* (non-significance) genes showed a trend of induction (Figure 4.8).


**
ns
ns
ns
**
ns
**
0.08
0.06
0.04
0.02
0.00
IFN-γ mRNA expression relative to CD2
AD
HC
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
C. albicans - + - + - + - +


***
**
ns
ns
***
ns
***
0.005
0.004
0.003
0.002
0.001
0.000
IL-13 mRNA expression relative to CD2
AD
HC
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
C. albicans - + - + - + - +


****
***
ns (p=0.0523)
ns
***
ns
***
ns
0.020
0.015
0.010
0.005
0.000
IL-22 mRNA expression relative to CD2
AD
HC
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
C. albicans - + - + - + - +


ns
ns
ns
**
***
****
**
*
0.15
0.10
0.05
0.00
CSF2 mRNA expression relative to CD2
AD
HC
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
C. albicans - + - + - + - +


ns
ns
ns
ns (p=0.0681)
*
ns
*
ns
0.010
0.008
0.006
0.004
0.002
0.000
IL-17A mRNA expression relative to CD2
AD
HC
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
C. albicans - + - + - + - +

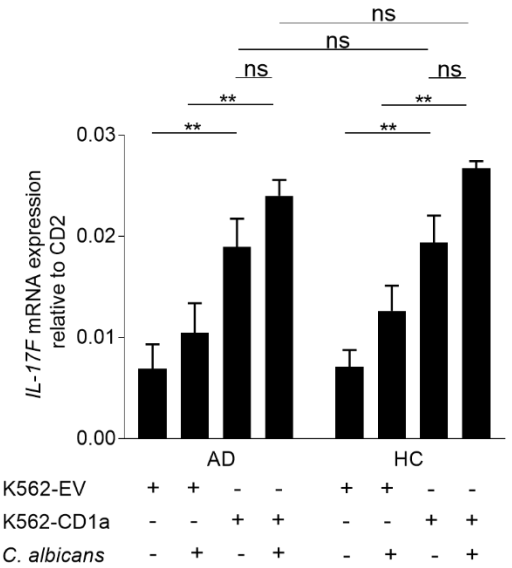

ns
ns
ns
ns
**
**
**
**
0.03
0.02
0.01
0.00
IL-17F mRNA expression relative to CD2
AD
HC
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
C. albicans - + - + - + - +

Figure 4.8 RT-qPCR analysis of *IFN-γ*, *IL-13*, *IL-22*, *CSF2*, *IL-17A* and I*L17F* gene expression by CD3+ T cells in response to K562 cells unpulsed or pulsed with *C. albicans* extract.

RT-qPCR analysis of *IFN-γ*, *IL-13*, *IL-22*, *CSF2*, *IL-17A* and I*L17F* gene expression of T cells (W003 (HC) and AD2520) mRNA level in response to K562-EV and K562-CD1a cells unpulsed or pulsed with *C. albicans* extract after co-culturing for 6 hours at a ratio of 10:1. Results were normalised to CD2. Representative data from paired HC and AD donors are shown. Data represent mean and standard deviation (SD) of duplicate measurements.

4.2.2. Fungal-responsive CD1a-reactive T cell responses to primary autologous CD1a+ MO-DCs in the blood of healthy controls and AD patients.

MO-DCs were differentiated from CD14+ monocytes in conditional media containing 10 ng/ml recombinant human IL-4 and 100 ng/ml recombinant human GM-CSF for 5 days. On day 5 the cells showed dendritic morphology (Figure 4.9) and subsequently the cells were harvested, stained and checked by flow cytometry for dendritic cells surface markers which showed evidence of differentiation (Figure 4.10).

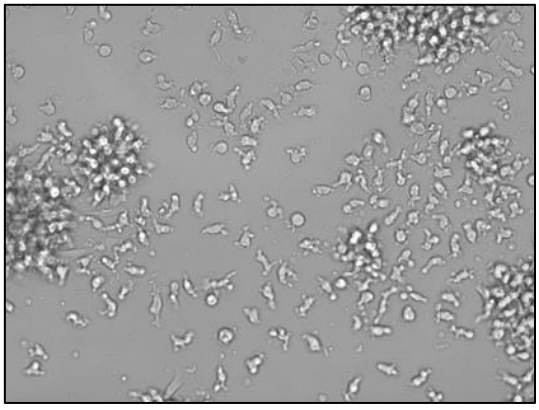


Figure 4.9. Microscopic morphological appearance of MO-DCs.

MO-DCs were examined under the microscope for morphological differentiation on day 5 after culturing in conditional media containing IL-4 and GM-CSF.

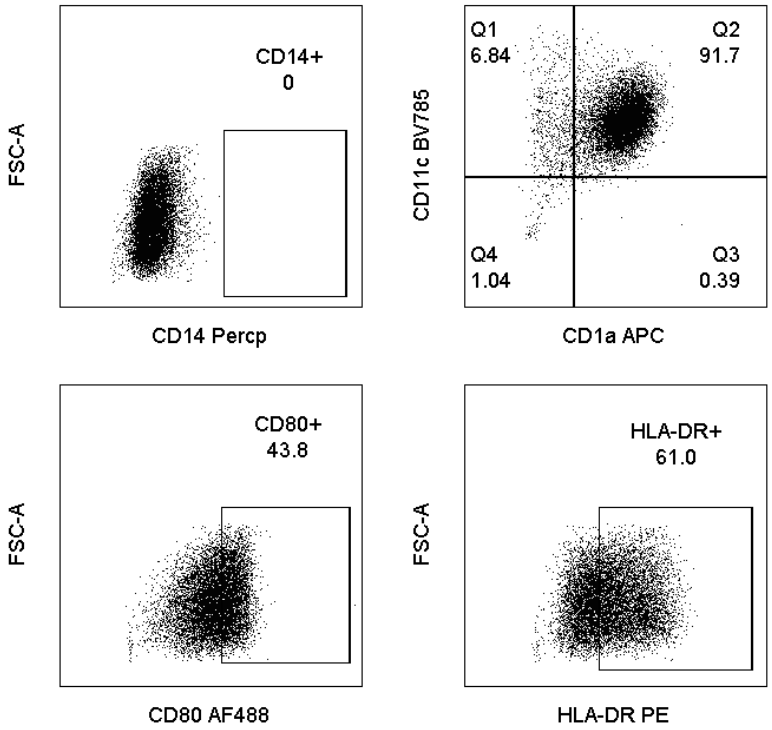


Figure 4.10 Flow cytometry plot of MO-DCs.

Flow cytometry plots of MO-DCs stained on day 5 after differentiation in MO-DCs conditional media containing IL-4 and GM-CSF. Phenotypically MO-DCs were CD14- CD11c+ CD1a+ and upregulated CD80 and HLA-DR. Gating strategy based on cell population, singlets and live cells. The compensation was based on single-colour stained beads. Gating was based on FMO controls.

The effect of the *C. albicans* extract on the viability of MO-DCs was tested in three different concentrations to determine the toxic effect of the antigen on MO-DCs. Some toxicity was noted with the highest concentration (2.5 µg/ml) of the *C. albicans* extract with a reduction of the viability by approximately 41% compared to unpulsed cell (Table 4-2); as a result 1 µg/ml of *C. albicans* extract was used for pulsing the MO-DCs in the subsequent experiments.

Table 4-2 The viability of MO-DCs after pulsing with *C. albicans* extract.

MO-DCs were pulsed with three different concentrations of *C. albicans* extract (P1: 0.4 µg/ml, P2: 1 µg/ml, P3: 2.5 µg/ml) for 16-20 hours before they were stained with

LIVE/DEAD™ Fixable Dead Cell Stain Kit to determine the viability of cells using flow cytometry.

Antigen	Concentration	% Live cells
Unpulsed		94.9%
Pulsed with *C. albicans* extract	P1	86.4%
	P2	84.6%
	P3	54.0%

After determining the effect of the fungal extract on the viability of MO-DCs, we tested whether pulsing the MO-DCs with this antigen has any effect on the CD1a expression and MO-DCs differentiation markers expression using flow cytometry. No substantial differences of the CD1a expression nor other surface markers expression were observed after pulsing the MO-DCs cells with *C. albicans* extract in three different concentrations compared to the unpulsed condition (Figure 4.11).

In order to assess whether *C. albicans* antigens are presented to T cells by CD1a molecules on the surface of MO-DCs or not, CD3+ T cells were rested for 3-4 days in IL-2 containing media and then co-cultured with autologous MO-DCs (as CD1a expressing APCs) that were pulsed or unpulsed with *C. albicans* extract for 16-20 hours in the presence of anti-MHC I and anti-MHC II blocking antibodies with or without anti-CD1a blocking antibody (clone HI149) or isotype control. Then, IFN-γ-, IL-22- and IL-17A-producing T cells were enumerated using ELISpot (Figure 4.12). CD1a autoreactivity was detected in T cells co-cultured with unpulsed MO-DCs, however, there was a significantly higher production of IFN-γ, IL-22 and IL-17A when T cells were co-cultured with *C. albicans*-pulsed MO-DC. The IFN-γ-producing *C. albicans*-responsive CD1a-reactive T cell frequency was higher than the frequency seen with K562 cells. Moreover, In the presence of the anti-CD1a

antibody (HI149), the CD1a reactivity reduced markedly compared to isotype control demonstrating that this response is likely CD1a dependent (Figure 4.12).

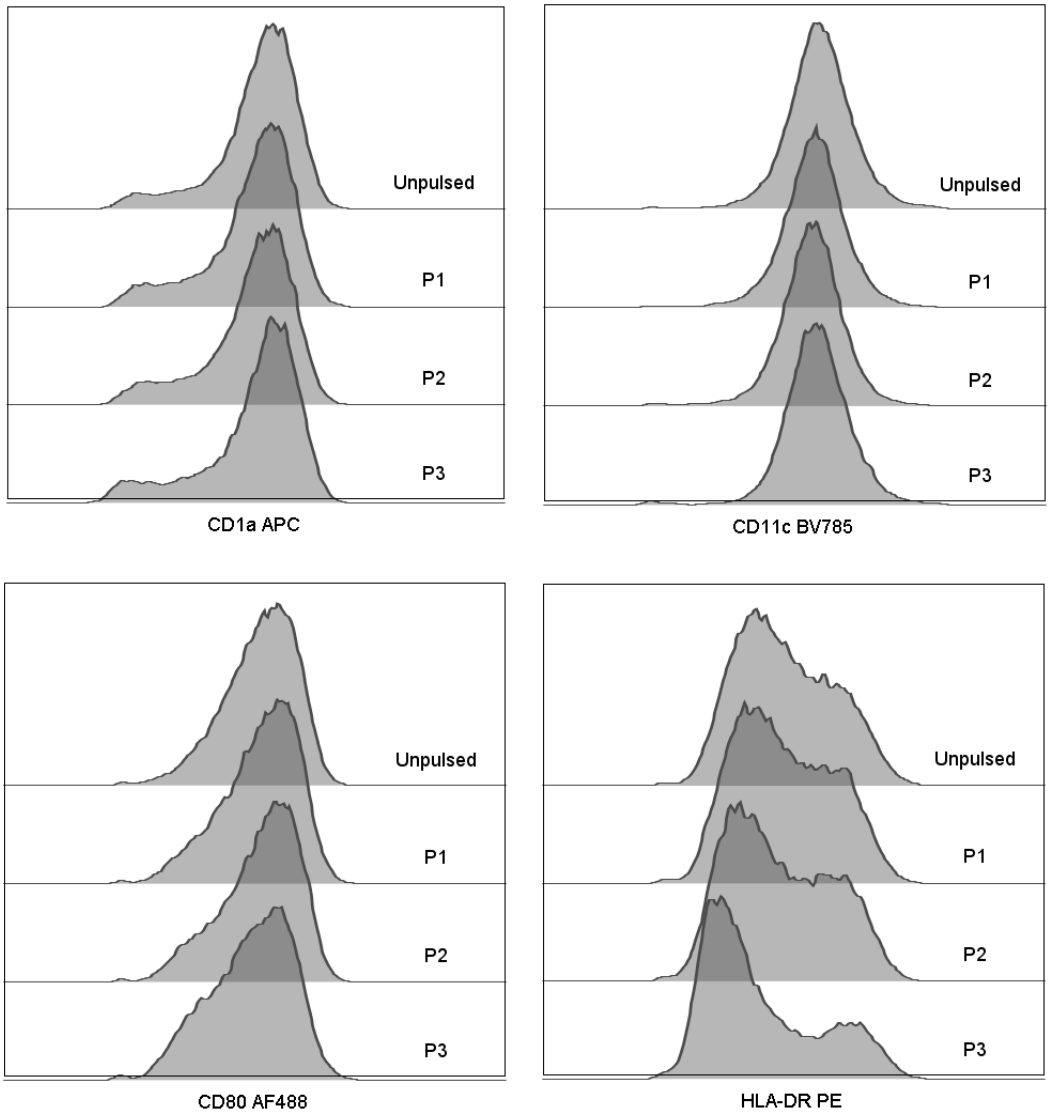


Figure 4.11 Flow cytometry histograms comparing the expression of MO-DCs surface markers after pulsing with *C. albicans* extract.

MO-DCs were pulsed with three different concentrations of *C. albicans* extract (P1: 0.4 µg/ml, P2: 1 µg/ml, P3: 2.5 µg/ml) for 16-20 hours before the CD1a, CD11c, CD80 and HLA-DR surface expression was determined by flow cytometry gating on cell population, singlets and live cells. The compensation was based on single-colour stained beads and the gating was based on FMOs.

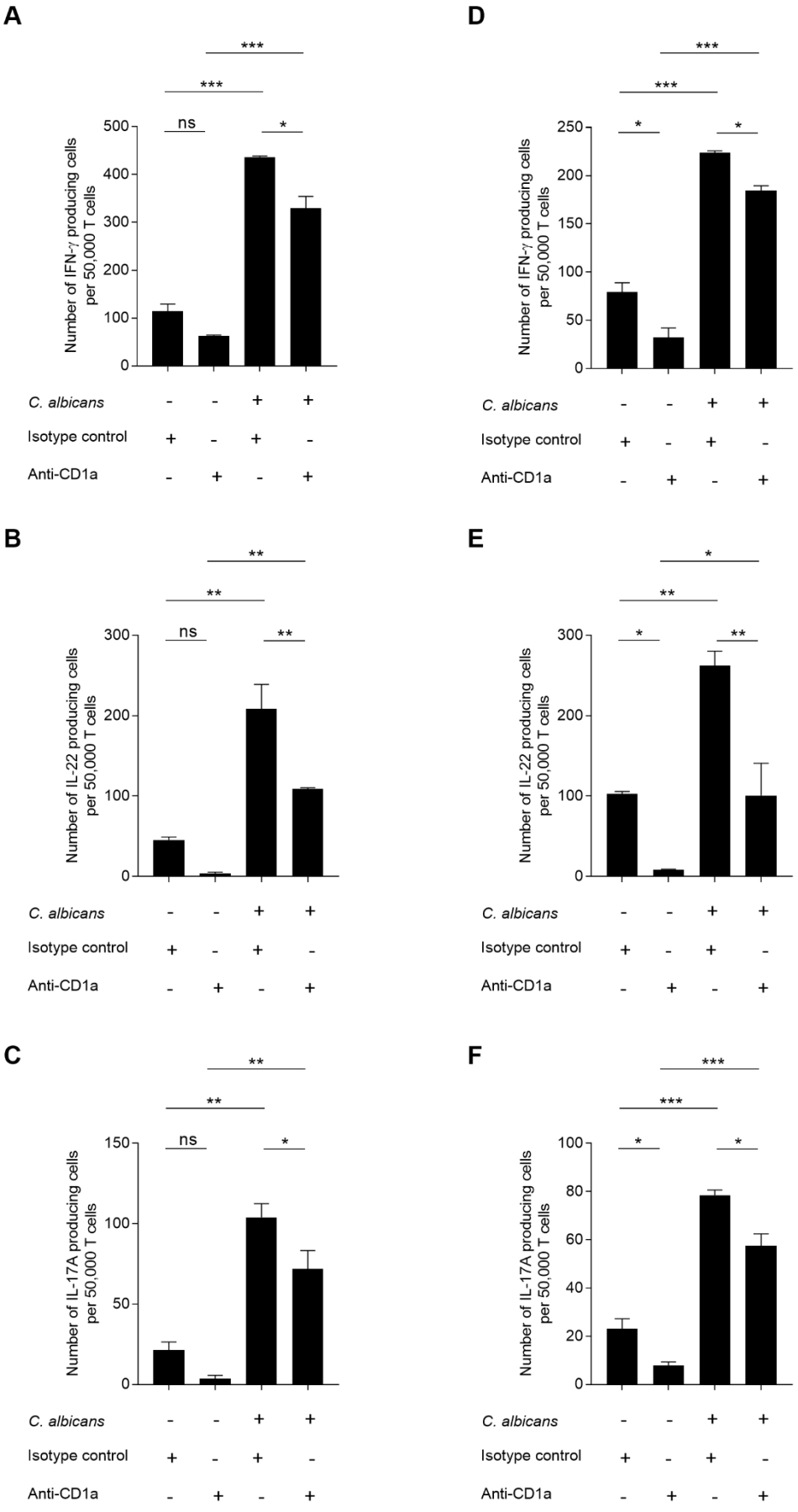

A
***
***
ns
*
Number of IFN-γ producing cells per 50,000 T cells
500
400
300
200
100
0
C. albicans - - + +
Isotype control + - + -
Anti-CD1a - + - +
D
***
***
*
*
Number of IFN-γ producing cells per 50,000 T cells
250
200
150
100
50
0
C. albicans - - + +
Isotype control + - + -
Anti-CD1a - + - +
B
**
**
ns
**
Number of IL-22 producing cells per 50,000 T cells
300
200
100
0
C. albicans - - + +
Isotype control + - + -
Anti-CD1a - + - +
E
*
**
*
**
Number of IL-22 producing cells per 50,000 T cells
300
200
100
0
C. albicans - - + +
Isotype control + - + -
Anti-CD1a - + - +
C
**
**
ns
*
Number of IL-17A producing cells per 50,000 T cells
150
100
50
0
C. albicans - - + +
Isotype control + - + -
Anti-CD1a - + - +
F
***
***
*
*
Number of IL-17A producing cells per 50,000 T cells
100
80
60
40
20
0
C. albicans - - + +
Isotype control + - + -
Anti-CD1a - + - +

Figure 4.12 *C. albicans* induces the production of IFN-γ, IL-22 and IL-17A by T cells through CD1a molecules expressed on the surface of autologous MO-DCs.

CD3+ T cells were isolated by MACS separation technology from PBMCs, and co-cultured with autologous MO-DCs in the presence or absence of 1 µg/ml *C. albicans* extract (skin prick test solution) and then IFN-γ (A,B) , IL-22 (B,E) and IL-17A (C,F) production was measured by ELISpot. The number of cytokine-producing T cells was plotted for two HC donors (W021 (A, B, C) and W060 (D, E, F)). Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

4.2.3. Fungal-responsive CD1a-reactive T cells in the skin

To examine whether *C. albicans*-responsive CD1a-reactive T cells were present in the skin, we used a well-established method in our lab which is the skin suction blister method (refer to section 3.6). T cells were isolated from unchallenged skin and then expanded using rapid expansion mix (refer to section 3.15). T cells were then co-cultured with K562-CD1a or K562-EV in the presence or absence of *C. albicans* extract for 16-20 hours and IFN-γ production was measured by ELISpot (Figure 4.13). Like PBMC-derived T cells, skin-blister derived T cells were able to induce CD1a-autoreactive and *C. albicans* responsive CD1a reactive responses.

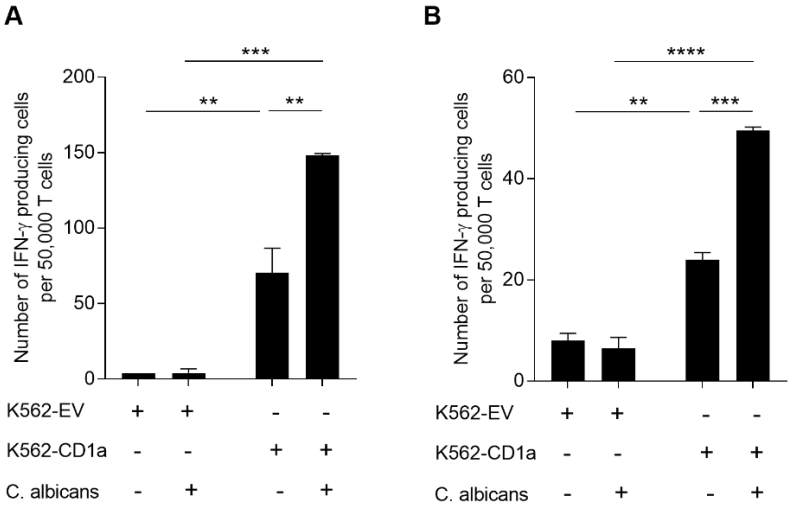


Figure 4.13 *C. albicans*-responsive CD1a-reactive T cells are enriched in the skin.

Skin blister T cells were isolated from unchallenged skin -of an atopic asymptomatic donor with mild asthma (AA9005) (A) and an AD donor (AD229) (B)- using skin blister method. Suction was applied to the skin at 200 mmHg for 1 hour to induce a separation between the epidermis and dermis. After 24 hours, the blister fluid was aspirated, and the cells were separated by centrifugation. Blister-derived T cells were sorted using flow cytometry and then expanded using the rapid expansion mix. Then T cells were co-cultured with K562-CD1a or K562-EV in the presence or absence of *C. albicans* extract (skin prick test solution) for 16-20 hours. IFN-γ production was measured by ELISpot and expressed as number of cells. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

To examine whether the *C. albicans*-responsive CD1a-reactive T cell response is enriched in the skin homing fraction of T cells, PBMC-derived polyclonal T cells were isolated by MACS bead separation and then further separated into CLA+ and CLA- fraction by MACS bead separation as well. The CLA+ and CLA- cells were stained for CLA expression by flow cytometry prior to proceeding with functional assays. The CLA- fraction was contaminated with some CLA+ cells (7.05%). However, the CLA+ fraction expressed higher percentage of CLA (40.2%) (Figure 4.14).

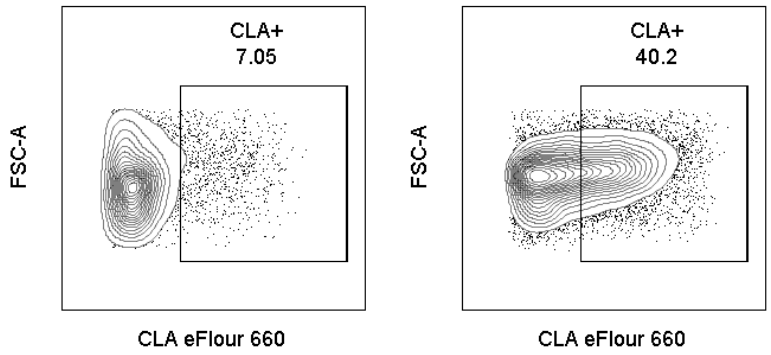


Figure 4.14 Flow cytometry plot of CLA-stained T cells.

Flow cytometry plots of T cells CLA- (left) and CLA+ (right) post-MACS separation. Gating strategy based on lymphocytes, singlets, live cells and CD3+ cells. The

compensation was based on single-colour stained beads. Gating was based on FMO controls.

CLA+ and CLA- T cells from three HC donors were co-cultured with K562-EV or K562-CD1a cells that were unpulsed or pulsed with *C. albicans* extract for 16-20 hours. IFN-γ production by T cells was measured by ELISpot (Figure 4.15). It was observed that the number of spots in this experiment was higher than previous experiments where CD3+ T cells were used, although the negative control which contain T cells only did not show T cells stimulation. When CLA+ T cells were co-cultured with K562-CD1a in the absence of *C. albicans* antigens, a significant autoreactivity was induced and when they were co-cultured with K562-CD1a cells in the presence of 1 µg/ml *C. albicans* extract, a significant increment of the response by IFN-γ-producing T cells was observed over K562-CD1a-unpulsed. Similar pattern of responses was seen with the CLA- T cell fraction. However, the autoreactive CD1a-reactive T cells and the *C. albicans* responsive CD1a-reactive T cells were enriched in the CLA+ fraction compared to the CLA- fraction (Figure 4.15).

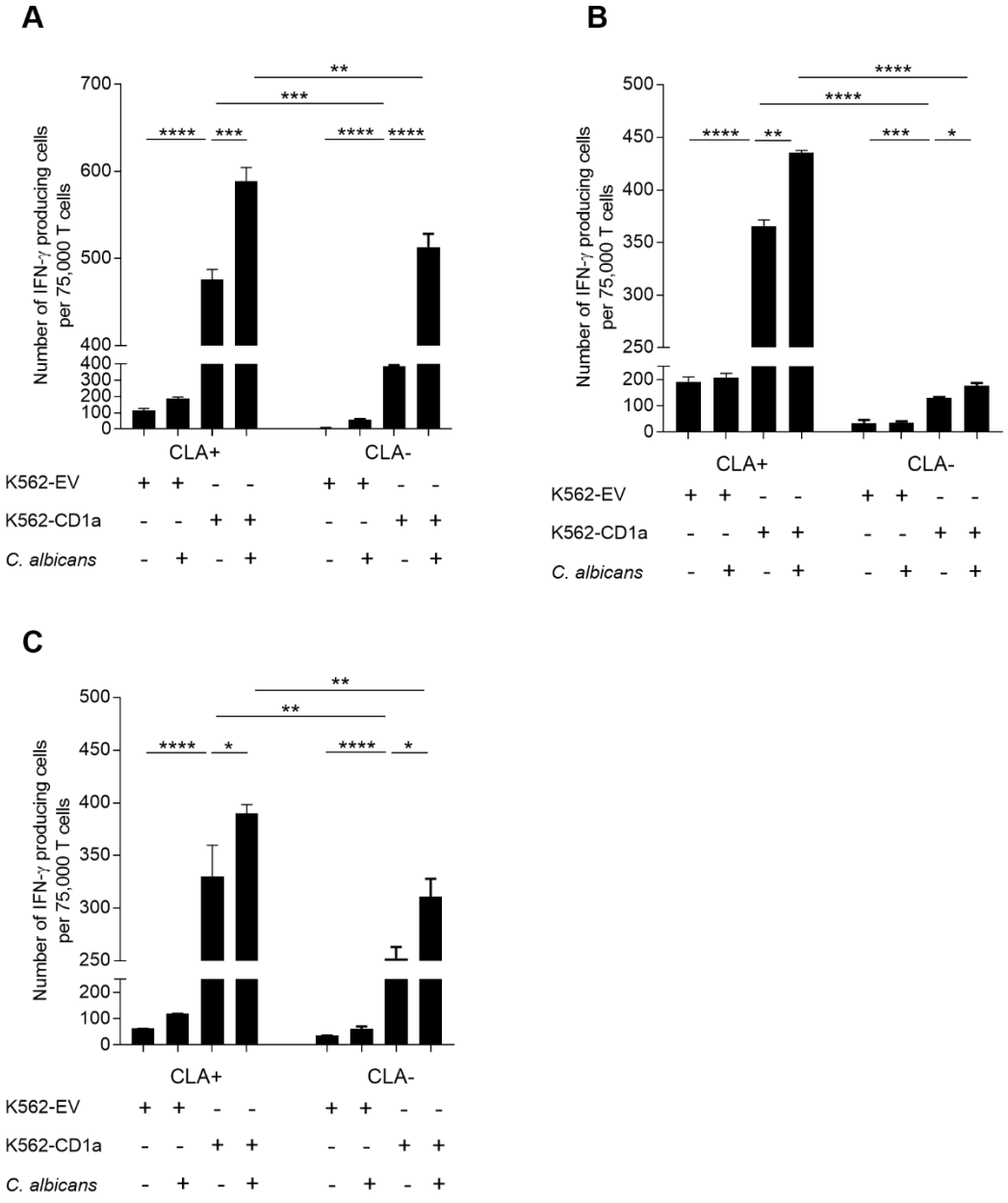


Figure 4.15 IFN-γ ELISpot comparing the CD1a-reactivity of CLA+ and CLA- T cells toward *C. albicans*.

Pan T Cell isolation kit (Miltenyi Biotec) was used to isolate untouched T cells of three HC donors ((A) W079, (B) W020, (C) W023) and then CLA+ and CLA- T cells were separated by MACS bead technology (positive selection). CLA+ or CLA- T cells were rested in IL-2 containing media for 3-4 days before they were co-cultured with K562-EV or K562-CD1a cells in the presence or absence of *C. albicans* extract for 16-20 hours. IFN-γ production of T cells was measured by ELISpot. The data are expressed as number of T cells producing IFN-γ. Data represent mean and standard deviation (SD) of duplicate measurements.

4.3. Summary and discussion

To study the polyclonal fungal-responsive CD1a-reactive T cells, K562 cells (gift from Branch Moody, Harvard) were used for antigen presentation to T cells. K562 cells are engineered APCs derived from human myelogenous leukaemia cells that were transfected with CD1a (K562-CD1a) or empty vector (K562-EV) and lacked the expression of MHC class I and II (de Jong, Pena-Cruz et al. 2010, Bourgeois, Subramaniam et al. 2015). As a result, K562 cells were the most suitable cells to be used in our model to bypass alloreactivity and to compare and quantify responses of different donors under standardised conditions. Although, the K562 CD1a-reactive T cell responses might not necessarily reflect the actual physiologic responses because K562s express different costimulatory molecules and have different lipid-processing mechanisms compared to primary APCs (Bourgeois, Subramaniam et al. 2015), in previous studies, K562-CD1a responses have been shown to accurately reflect responses observed to primary CD1a-expressing cells (Bourgeois, Subramaniam et al. 2015, Jarrett, Salio et al. 2016).

We used the MACS bead method to positively select CD3+ T cells from PBMCs and this method was very reliable in obtaining high yields of CD3+ T cell populations. We studied the CD3+ T cells in order to include all CD1a-reactive T cell subtypes in our functional assays as the CD1-reactive T cells were found to be a part of the CD4+, CD8+ or the double negative T cell population (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Pena-Cruz et al. 2010, de Lalla, Lepore et al. 2011, de Jong, Cheng et al. 2014, Salio, Silk et al. 2014, Birkinshaw, Pellicci et al. 2015).

After pulsing the K562s-EV or K562-CD1a cells with *C. albicans* extract (skin prick test solution) for 16-20 hours, they were cocultured with CD3+ T cells for 16-20 hours before the IFN-γ producing T cells were quantified using ELISpot. We observed that *C. albicans* presented on the surface of K562-CD1a induced T cells to produce IFN-γ in a CD1a-reactive dose-dependent manner. We have demonstrated that the concentration of the *C. albicans* used in the subsequent experiment was not toxic to the K562s cells or MO-DCs and did not alter the expression of CD1a on their surface. This was essential in order to eliminate the effect of cellular toxicity and CD1a expression downregulation or upregulation on the functional assays results.

In our cohort of AD and HC blood-derived T cells, we observed an enrichment of the IFN-γ-producing *C. albicans*-responsive CD1a-reactive T cells in AD blood compared to HC with a mean frequency 0.15% in AD, which was a 1.5-fold higher that what was documented with HC samples (p-value= 0.008). This suggested that *C. albicans*-responsive CD1a-reactive T cells might contribute to some extent to the pathogenesis of AD. Disease severity did not correlate with the frequency of IFN-γ-producing CD1a-reactive *C. albicans*-responsive T cells, but it will be important to investigate other cytokines and to examine skin T cell responses.

Then we observed that *C. albicans*-responsive CD1a-reactive T cell responses predominated other group 1 CD1 molecules including CD1b and CD1c demonstrating CD1a relevance. This finding added strength to our hypothesis as CD1a is the most abundantly expressed isoform in the skin and this was consistent with previously published studies (de Jong, Pena-Cruz et al. 2010).

At mRNA level, we saw an induction of the expression of some relevant cytokine genes when K562-CD1a cells were pulsed with *C. albicans*. Overall, the expression of *CSF2*, *IL-17A* and *IL-17F* was induced in both HC and AD samples in a CD1a-reactive pattern. However, some genes predominated in AD such as *IL-22* and *IFN-γ*. Although *IL-13* gene expression was higher in AD as expected, there was no difference between *IL-13* expression comparing K562-CD1a-unpulsed with K562-CD1a-pulsed with *C. albicans*. This is perhaps not surprising as the role of type 2 cytokines in the fungal immune responses in not well established and remains controversial.

The K562s-CD1a cell results were confirmed by autologous MO-DCs which gave a comparable pattern of response for IFN-γ production after blocking the MHC I and II. However, there was 1.4-fold and 2-fold higher frequencies of autoreactive and *C. albicans*-responsive CD1a-reactive T cells, respectively, compared to K562 cells responses. We were able also to measure other relevant cytokines when ELISpot was conducted with MO-DCs including IL-17A and IL-22 which are hallmarks in fungal immune responses and AD. When CD1a was blocked there was a significant inhibition of the autoreactivity and *C. albicans* reactivity, demonstrating that CD1a contribute to the reactivity toward *C. albicans* through the production of IFN-γ, IL-22 and IL-17A. The partial blockade of the CD1a-reactive response with anti-CD1a antibody raised the question whether other antigen-presenting molecules also contribute to this response.

Our finding with circulatory CD1a-reactive T cells was confirmed in the skin, using skin-blister-derived T cells method, published in previous work done in our lab (Jarrett, Salio et al. 2016). We found that the skin-blister derived T cells were able to induce CD1a-autoreactive and *C. albicans*-responsive CD1a-reactive T cell

responses comparable to those seen with PBMC-derived T cells. This confirmed the importance of these cells in the immune pathways in the skin. The limited number of blister-derived T cells available for our study limited the number of functional assays we were able to undertake.

Enrichment of the CD1a-reactive T cells was documented in the CLA+ circulatory T cells, demonstrating that T cells expressing skin homing markers can contribute to CD1a-reactive fungal responsive immune pathway in the skin.

We chose IFN-γ as a readout in the K562 ELISpot experiments because of the evidence in the literature that supported the role of Th1 cytokines in the pathogenesis of AD as discussed in section1.3.3.5. IFN-γ contributes to AD pathogenesis by enhancing skin hypertrophy in AD (Spergel, Mizoguchi et al. 1999), inducing epithelial ICAM-1 expression and monocyte adhesion (Chang, Holtzman et al. 2002) and inducing the expression of MHC class II on the keratinocytes (Czernielewski and Bagot 1986, Barker, Navsaria et al. 1988). In addition to its essential contribution in the development of AD, IFN-γ plays a major role in the adaptive immunity against *C. albicans* where naïve Th cells are differentiation into Th1 cells through APCs. Th1 cells produce IFN-γ which strongly activates phagocytic cells and therefore fungal clearance (Liang, Tan et al. 2006, Zielinski, Mele et al. 2012). In addition, IFN-γ has been used as a robust readout of CD1a-reactive T cell responses previously (Bourgeois, Subramaniam et al. 2015, Jarrett, Salio et al. 2016). However, it is recognised that other cytokines have been contributed to AD and *C. albicans* immune responses and it would be important to further investigate them in future experiments.

For instance, IL-22 and IL-17A are important cytokines in AD and fungal immunity. IL-22 contributes to AD pathogenesis by downregulating *profilaggrin* or *filaggrin* expression in keratinocytes at both mRNA and protein levels (Gutowska-Owsiak, Schaupp et al. 2011) and by inducing epidermal hyperplasia and stimulating keratinocyte proliferation (Boniface, Bernard et al. 2005). IL-17A contributes to AD development by downregulating the expression of *filaggrin* and other genes important in cellular tight junctions and adhesion molecules which contribute to the barrier defect (Gutowska-Owsiak, Schaupp et al. 2012). Teunissen et al found that IL-17A with the synergistic effect of IFN-γ can induce the secretion of IL-6 and IL-8 and the expression of the intercellular adhesion molecule (ICAM-1) and the human leukocyte antigen-DR (HLA-DR) (Teunissen, Koomen et al. 1998). In fungal immunity, IL-17 was shown to recruit and activate neutrophils, and IL-22 induced the production of β-defensins (Liang, Tan et al. 2006, Zielinski, Mele et al. 2012).

To summarise we found that autoreactive and *C. albicans*-responsive CD1a-reactive T cells present in the blood and skin of HC and are enriched in AD samples and secrete relevant cytokines to AD pathogenesis and fungal immunity. The cells should be further characterised phenotypically and functionally through studying T cell clones or lines that are specific to this fungus and these will be addressed in the next chapter.

5. Isolation, phenotypical and functional analysis of fungal-responsive CD1a-reactive monoclonal T cells.

5.1. Introduction

The CD1a-reactive T cells express diverse TCRs and they are normal components of the human αβ T cell repertoire (de Jong, Pena-Cruz et al. 2010). According to Moody and colleagues the frequency of CD1a-autoreactive T cells among memory T cells in the blood was 0.02% to 0.4% with higher frequencies estimated in specific tissues like the skin (de Jong, Pena-Cruz et al. 2010). Most of the CD1a-reactive T cells reported in literature from solved crystal structures were αβ (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Cheng et al. 2014, Birkinshaw, Pellicci et al. 2015), however one study reported a γδ TCR from one T cell clone (Agea, Russano et al. 2005). The role of γδ T cells in triggering CD1a-reactive responses should be further elucidated, especially that CD1d- and CD1c-reactive γδ T cells have been characterised (Bai, Picard et al. 2012, Uldrich, Le Nours et al. 2013, Roy, Ly et al. 2016).

γδ T cells are heterogeneous group of cells that link the innate and adaptive immunity. γδ TCR is formed by a combination of V (variable), D (diversity, for the delta chain), and J (joining) gene segments. The germline genes used to rearrange γδ TCR in human are Vγ (Vγ 2,3,4,5,8,9) and Vδ (Vδ 1,2,3,4,6,7,8) (Blazquez, Benyamine et al. 2018). Certain Vδ chains are strictly paired with specific Vγ chains, for example Vγ9 is typically paired with Vδ2 in humans (Blazquez, Benyamine et al. 2018).

γδ T cells represent 1–5% of the total circulating T cells, however, higher proportion (10-100%) of specific γδ T cells subsets are present in murine epithelial tissue as

a part of the tissue-resident intraepithelial lymphocytes which contribute largely to the epithelial immune defence (Nielsen, Witherden et al. 2017). In mice, γδ T cells are found in the epidermis and known as dendritic epidermal T cells (DETCs) which are a highly specialised and well characterised subset of γδ T cells (Havran and Jameson 2010). Human epidermis and dermis also contain γδ T cells which appear to have overlapping effector functions to DETCs (Toulon, Breton et al. 2009). Compared to murine epidermal γδ T cells where γδ T cells represent the vast majority of all epidermal T cells, γδ T cells in human epidermis is a very small population (1-2% of total T cells in healthy skin) (Macleod and Havran 2011). Dermal γδ T cells in healthy human skin largely express Vδ1 TCR with an oligoclonal repertoire that is distinct from circulating γδ T cells (Holtmeier, Pfander et al. 2001). 50 to 95% of γδ T cells subsets in human peripheral blood are γ9δ2 T cells (Blazquez, Benyamine et al. 2018).

Upon activation, DETCs secrete different cytokines and chemokines (e.g. CCL3, CCL4, CCL5 and XCL1, IFN-γ, IL-2 , IL-13) which induce inflammatory responses and contribute to cutaneous microbial defence, tumour immunosurveillance, tissue repair and homeostasis (Macleod and Havran 2011, Nielsen, Witherden et al. 2017). Human epidermal γδ T cells express high levels of the skin homing marker cutaneous lymphocyte-associated antigen (CLA) (Toulon, Breton et al. 2009). In addition, epidermal γδ T cells were found to aid wound healing by producing epithelial growth factors (Toulon, Breton et al. 2009). Moreover, epidermal γδ T cells have an anti-bacterial effect by promoting the recruitment of neutrophils to the skin upon *Staphylococcus aureus* infection through IL-17A production (Cho, Pietras et al. 2010). In addition to this, they exert an anti-tumour effect (Gao, Yang et al. 2003) through a TCR-independent pathway and mediated potentially through

NKG2D (Ibusuki, Kawai et al. 2014). Several ligands of γδ T cells have been identified in the literature which exhibit diverse homology, biology, and structure (Cruz, Diamond et al. 2018, Willcox and Willcox 2019). Examples of some of these identified ligands for each γδ T cell subset are summarised in Table 5-1.

Table 5-1 Examples of identified ligands to Vδ1 and Vδ2 γδ T cells.

γδ T cell subset	Location of highest prevalence	Identified ligands	Reference
Vδ1	Epithelial tissues	MICA and MICB, CD1d-sulfatides, CD1d–α-GalCer, CD1c-sulfatides, CD1c-*Mycobacterium tuberculosis* phosphomycoketide, CD1a-phosphoethanolamine.	(Wu, Groh et al. 2002, Agea, Russano et al. 2005, Xu, Pizarro et al. 2011, Bai, Picard et al. 2012, Uldrich, Le Nours et al. 2013, Roy, Ly et al. 2016).
Vδ2	Peripheral blood	Phosphoantigens, ULBP4.	(Constant, Davodeau et al. 1994, Tanaka, Morita et al. 1995, Kong, Cao et al. 2009).

Recently, a lot of research has been done on the contribution of butyrophilins (BTN) and butyrophilins-like (BTNL) family of proteins in humans in the modulation of the γδ T cells (Kabelitz, Lettau et al. 2017, Blazquez, Benyamine et al. 2018, Melandri, Zlatareva et al. 2018). Importantly, BTN3A proteins were identified as the key mediators of phosphoantigen sensing by human γ9δ2 T cells (Harly, Guillaume et al. 2012) which react to intracellular phosphoantigens accumulated in infected cells (e.g. hydroxy-methyl-butyl-pyrophosphate (HMB-PP)) or in tumour cells (e.g. isopentenyl pyrophosphate (IPP)) (Bukowski, Morita et al. 1995, Gober, Kistowska et al. 2003).

γδ T cells are also activated in a TCR-independent manner via NKG2D. NKG2D is a cell surface receptor that is expressed on cytotoxic immune cells including NK cells, NKT cells, and CD8+ γδ T cells (Wensveen, Jelenčić et al. 2018). The ligands of NKG2D that have been identified in humans are MICA, MICB, and six members of the ULBP family (Wensveen, Jelenčić et al. 2018), which are induced in response to infection or malignancy (Raulet, Gasser et al. 2013). The NKG2D receptor is a homodimer of two disulfide-linked transmembrane proteins, with short intracellular domains and it signals through the adaptor molecule, DAP10 (Zafirova, Wensveen et al. 2011). NKG2D mediates immune responses under cellular stress against tumours, virally infected cells, and organ transplants (Wensveen, Jelenčić et al. 2018). NKG2D is considered a co-stimulatory molecule for T cells where activation of NKG2D by MICA, for instance, augments human CD8 T cell activity and production of cytokines like IFN-γ, TNF, and IL-2 (Groh, Rhinehart et al. 2001).

There are two crystal structures identified to date for TCR-CD1a complex. The first is for the CD1a ternary complex with lysophosphatidylcholine and BK6 αβ TCR (Birkinshaw, Pellicci et al. 2015), the second is for the CD1a ternary complex with oleic acid and BK6 TCR (Birkinshaw, Pellicci et al. 2015). BK6 TCR has been sequenced and shown to be TRAV12-3*01 TRBV6-2*01. However, there are few other CD1a-reactive T cell clones that have been well defined in the literature.

The key aim of this chapter was to identify *C. albicans*-responsive CD1a-reactive monoclonal T cells in blood and characterise them phenotypically and functionally.

5.2. Results

5.2.1. Generating fungal-responsive CD1a-reactive monoclonal T cells.

In order to characterise the fungal-responsive CD1a-reactive T cells phenotypically and functionally, fungal-responsive CD1a-reactive monoclonal T cells were generated using IFN-γ secretion assay. After pulsing the K562-CD1a cells with *C. albicans* extract for 16-20 hours, they were cocultured with CD3+ T cells for 6 hours at 1:2 ratio before the secretion assay was conducted. The secretion assay protocol is detailed in section 3.14. Briefly, T cells were labelled with an IFN-γ catch reagent which binds to the surface of the cells to capture the cytokine secreted by the T cells. This was detected by adding IFN-γ detection antibody which is conjugated to a fluorochrome (e.g. PE, APC or FITC) for detection by flow cytometry (Figure 5.1). The *C. albicans*-responsive CD1a-reactive T cells were then sorted at one cell per well and expanded in rapid expansion mix (refer to section 3.15). When an adequate number of T cells was reached, the cells were tested functionally by ELISpot and were stained for flow cytometry analysis.

5 clones were successfully generated. 3 out of 5 clones (6C6, 1D6 and 7D8) had significant IFN-γ production with CD1a-unpulsed K562 cells demonstrating reactivity to endogenous lipids (frequency of autoreactivity ranges between 0.5% and 1.5%), however a significant induction of CD1a-reactive T cells was observed when pulsed with *C. albicans* extract (frequency of *C. albicans*-reactivity ranges between 1.1% and 3.4%) (Figure 5.2). The other 2 clones (2D8 and 3E8) were *C. albicans*-responsive CD1a-reactive with a percentage of 0.9-0.6% and did not show a significant autoreactivity (Figure 5.3). We noted that *C. albicans* could

sometimes induce a functional response even when pulsed onto EV-K562, which likely represents non-specific mechanisms such as stress-receptor recognition.

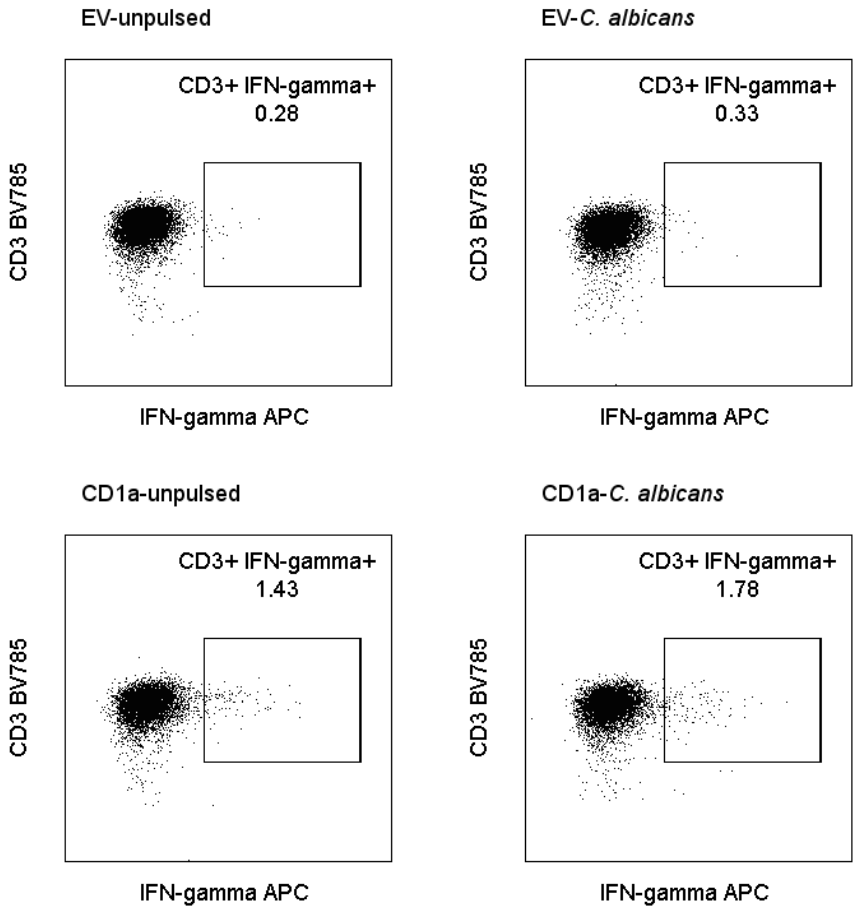


Figure 5.1 Flow cytometry plot of IFN-γ secretion assay to generate *C. albicans*-responsive CD1a-reactive T cell clones.

Flow cytometry plots of IFN-γ secretion assay of T cells from a HC donor (W016) co-cultured with K562-EV or K562-CD1a unpulsed or pulsed with 1 µg/ml *C. albicans* extract (skin prick test solution). Gating on lymphocytes, singlets, live cells, CD3+ and IFN-γ+ cells. The compensation was based on single-colour stained cells.

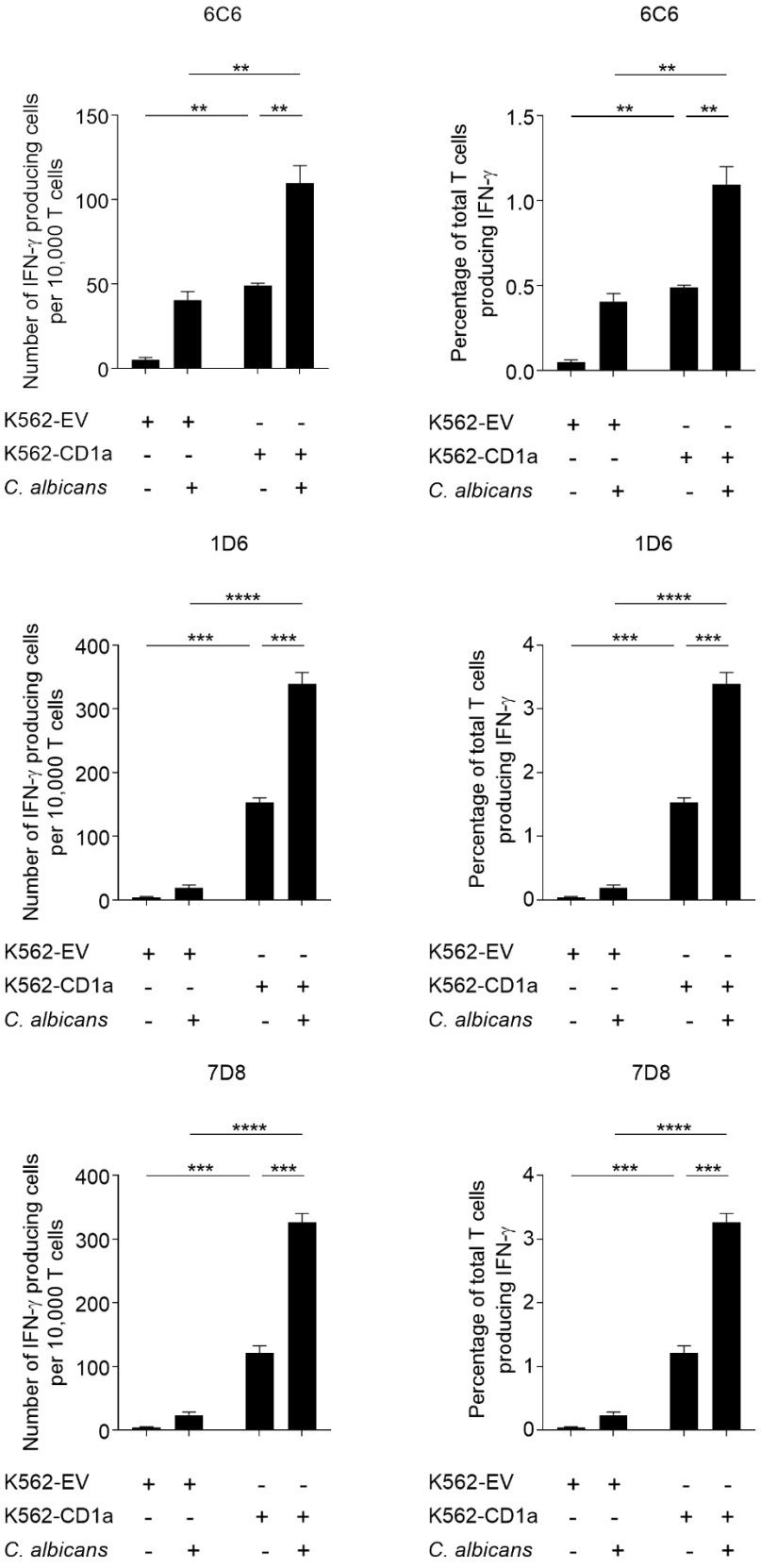


Figure 5.2 IFN-γ ELISpot of autoreactive and *C. albicans*-responsive CD1a-reactive T cell clones.

K562-CD1a or K562-EV cells were pulsed with 1 µg/ml *C. albicans* extract (skin prick test solution) for 16-20 hours and then co-cultured with T cell clones 6C6, 1D6 and 7D8. The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD) of duplicate measurements. The number of CD3+ T cells producing IFN-γ (left panel) and the percentage of total T cells producing IFN-γ (right panel) are illustrated. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

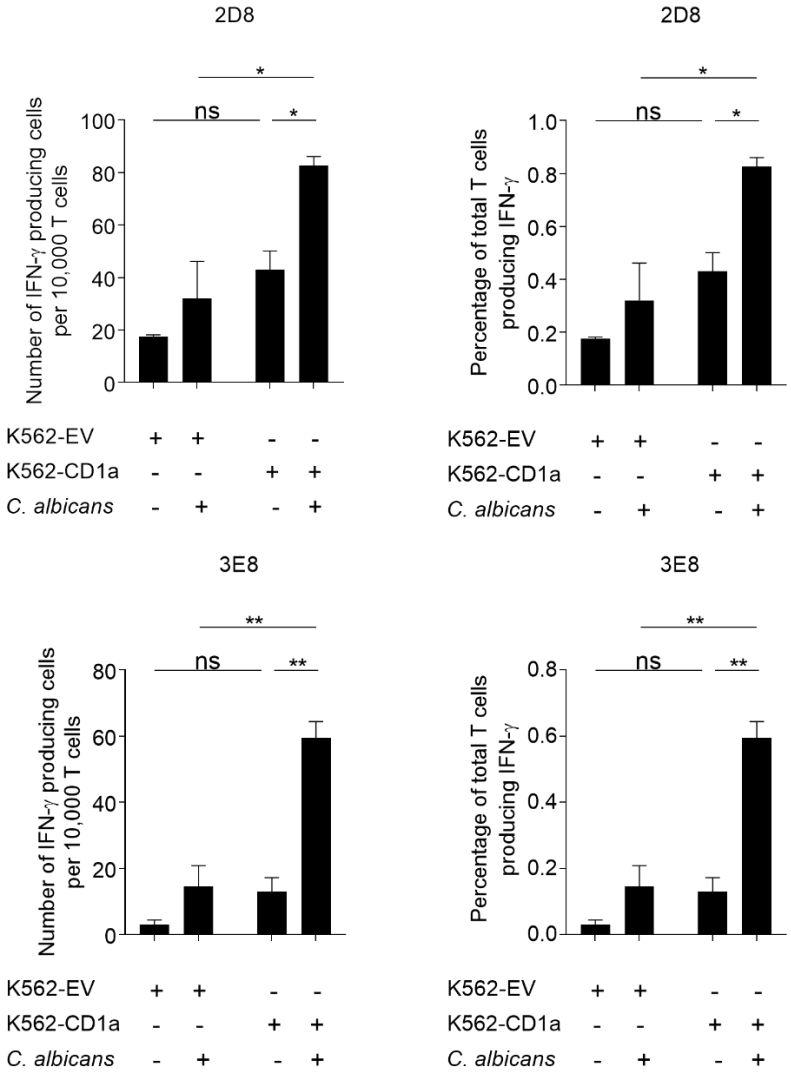


Figure 5.3 IFN-γ ELISpot of *C. albicans*-responsive CD1a-reactive T cell clones.

K562-CD1a or K562-EV cells were pulsed with 1 µg/ml *C. albicans* extract (skin prick test solution) for 16-20 hours and then co-cultured with T cell clones 2D8 and 3E8. The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD) of duplicate measurements. The number of CD3+ T cells producing

IFN-γ (left panel) and the percentage of total T cells producing IFN-γ (right panel) are illustrated. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

The clones were characterised phenotypically by staining for surface markers. Clones 2D8, 3E8 and 6C6 express γδ TCR. Clone 2D8 was CD8+ and clones 3E8 and 6C6 were CD8/CD4-double negative (Figure 5.4). On the other hand, the T cell clones 1D6 and 7D8 were TCR αβ+ and both were CD8+ (Figure 5.5). CD8+, CD4+ and double negative CD1a-reactive T cells have been described previously (de Jong, Pena-Cruz et al. 2010). Occasionally, minor populations would be present, but these were noted to be variable. These could have indicated non-clonality or perhaps may have been related to feeder cells before their final irradiation-induced death. Therefore, we proceeded to test for clonality using TCR sequencing in collaboration with David Price (Cardiff University).

The TCRs of these T cell clones were sequenced, confirming their monoclonality (Table 5-2). The αβ clones showed identical TCR sequence (TRAV30-1 TRBV 4-1) with the same DNA sequence demonstrating that they originated from the same original parent clone. The three γδ clones were γ9δ2 subset.

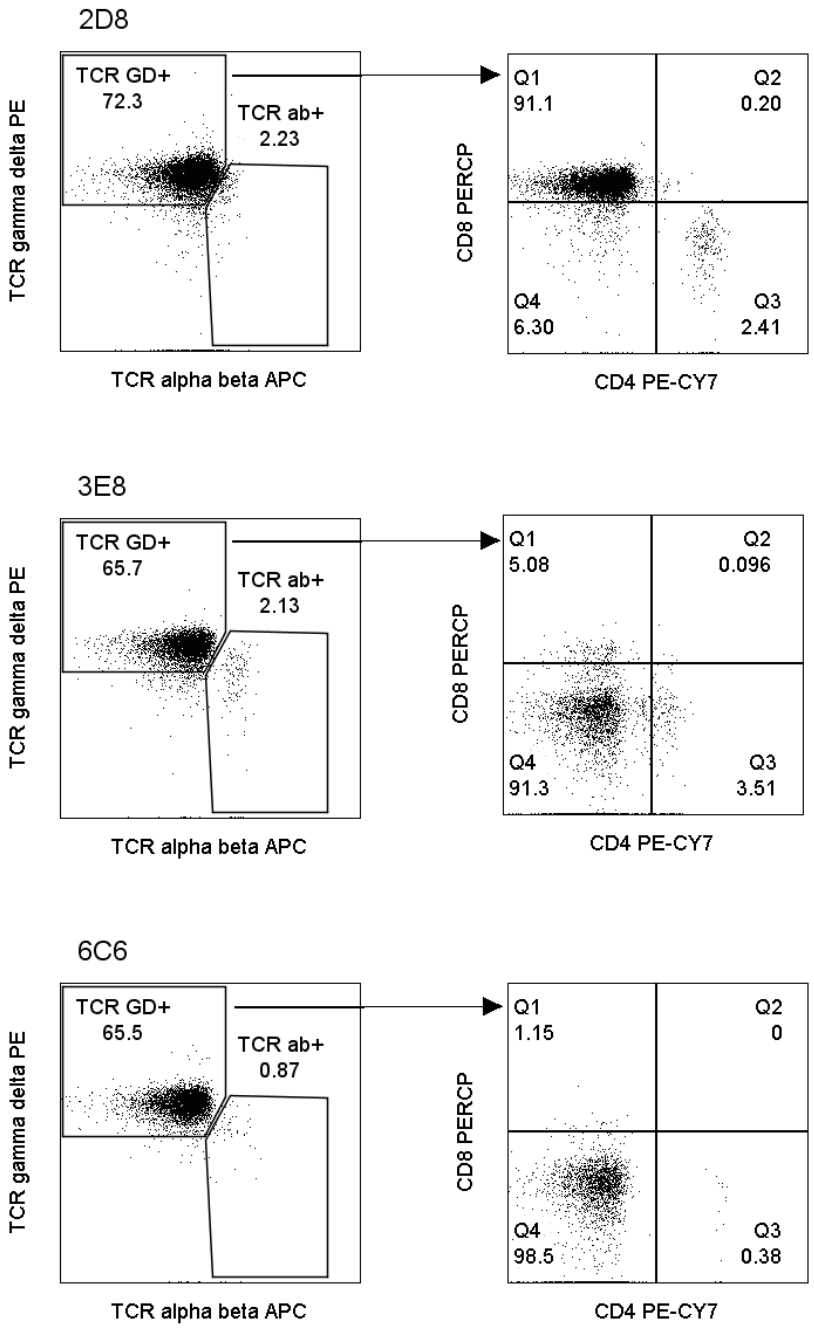


Figure 5.4 Flow cytometry plots of the *C. albicans*-responsive CD1a-reactive γδ T cell clones.

The *C. albicans*-responsive CD1a-reactive T cell clones were stained for surface markers (CD3, TCR αβ, TCR γδ, CD4 and CD8). Gating on population, singlets, live, CD3+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

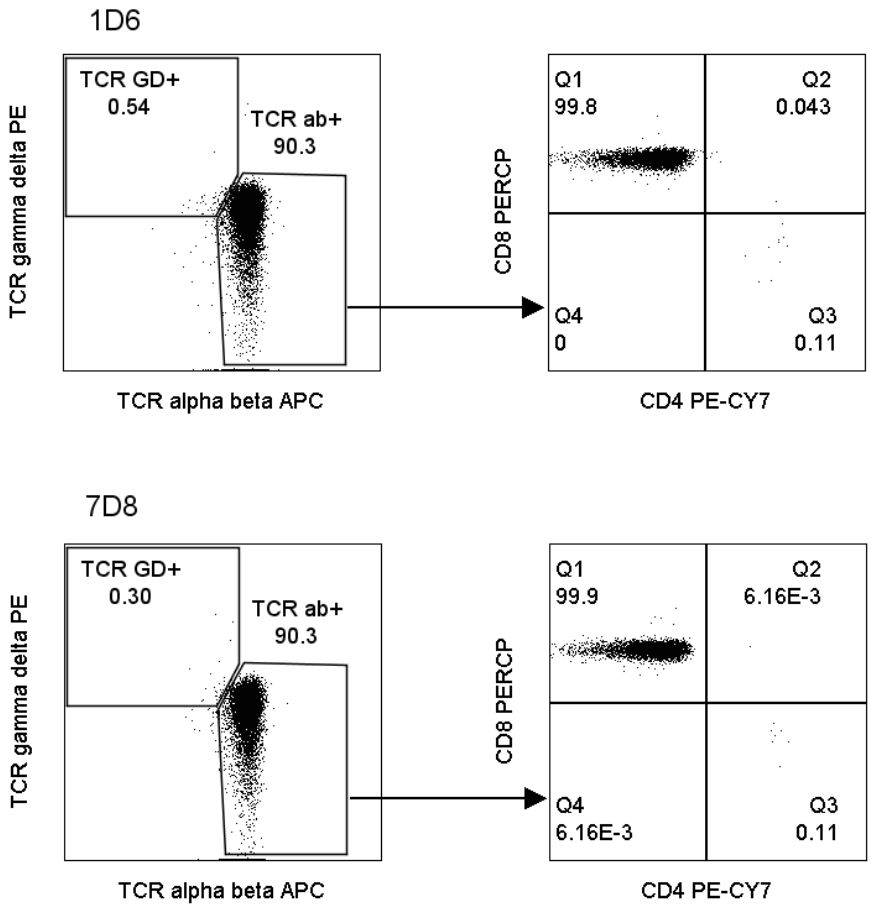


Figure 5.5 Flow cytometry plots of the *C. albicans*-responsive CD1a-reactive αβ T cell clones.

The *C. albicans*-responsive CD1a-reactive T cell clones were stained for surface markers (CD3, TCR αβ, TCR γδ, CD4 and CD8). Gating on population, singlets, live, CD3+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

Table 5-2. *C. albicans*-responsive CD1a-reactive T cell clones TCR sequence.

Clone 2D8

TRGV	CDR3	TRGJ
9	CALWEVLELGKKIKVF	P

TRDV	CDR3	TRDJ
2	CACDTVVGDTSYTDKLIF	1

Clone 3E8

TRGV	CDR3	TRGJ
9	CALWEVQELGKKIKVF	P

TRDV	CDR3	TRDJ
2	CACDTVINPVLGDTHKYTDKLIF	1

Clone 6C6

TRGV	CDR3	TRGJ
9	CALWEATQLGKKIKVF	P

TRDV	CDR3	TRDJ
2	CACDTLGGHTDKLIF	1

Clone 1D6

TRAV	CDR3	TRAJ	DNA
30-1	CGTSLGNEKLT	48	tgtggcacatcacttggaaatgagaaattaacc

TRBV	CDR3	TRBJ	DNA
4-1	CASSQDGGRAYNEQF	2-1	tgcgccagcagccaagatggcgggagggcctacaatgagcagttc

Clone 7D8

TRAV	CDR3	TRAJ	DNA
30-1	CGTSLGNEKLT	48	tgtggcacatcacttggaaatgagaaattaacc

TRBV	CDR3	TRBJ	DNA
4-1	CASSQDGGRAYNEQF	2-1	tgcgccagcagccaagatggcgggagggcctacaatgagcagttc

Then, the T cell clones were stained with NIH CD1a “endo”-tetramer (termed “endo” as it has been shown to contain multiple endogenous lipids derived from the cells of production – (Birkinshaw, Pellicci et al. 2015)) and this showed that all

γδ and αβ *C. albicans*-responsive CD1a-reactive T cell clones were tetramer negative (Figure 5.6 and Figure 5.7).

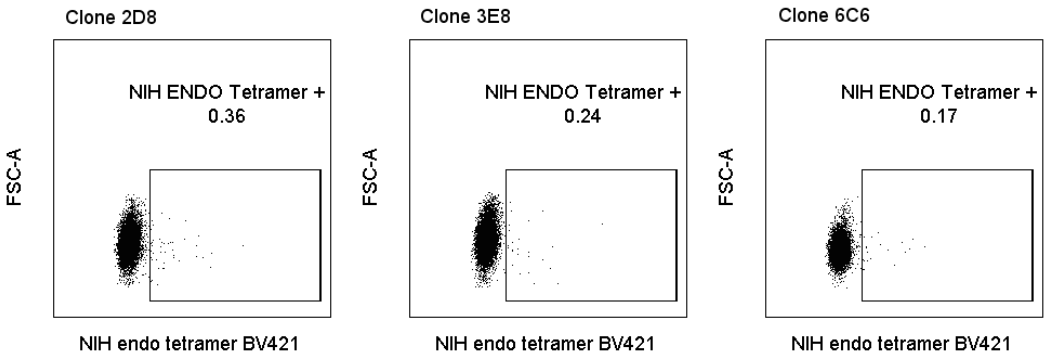


Figure 5.6 CD1a-endo tetramer staining of the *C. albicans*-responsive CD1a reactive γδ T cell clones.

The *C. albicans*-responsive CD1a-reactive γδ T cell clones (2D8, 3E8 and 6C6) were stained with NIH CD1a endo-tetramer. Gating on population, singlets, live, CD3+, endo-tetramer+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

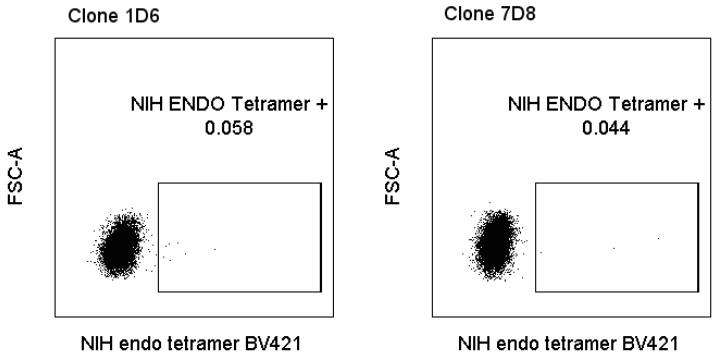


Figure 5.7 CD1a-endo tetramer staining of the *C. albicans*-responsive CD1a reactive αβ T cell clones.

The *C. albicans*-responsive CD1a-reactive αβ T cell clones (1D6 and 7D8) were stained with NIH CD1a endo-tetramer. Gating on population, singlets, live, CD3+, endo-tetramer+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

5.2.2. Fungal-responsive CD1a-reactive monoclonal T cell responses to primary autologous CD1a+ MO-DCs.

In order to confirm the *C. albicans*-reactivity of the T cell clones, MO-DCs unpulsed or pulsed with *C. albicans* extract were used as APCs. T cell clones were co-cultured with autologous MO-DCs for 16-20 hours in the presence of anti-MHC I and anti-MHC II blocking antibodies with or without anti-CD1a blocking antibody (clone HI149) or isotype control. Then, IFN-γ-producing T cells were enumerated using ELISpot. A representative example of clone 6C6 is illustrated in Figure 5.8. A significant induction of IFN-γ production by 6C6 T cell clone was observed when it was co-cultured with *C. albicans*-pulsed MO-DCs. The IFN-γ-producing *C. albicans*-responsive CD1a-reactive T cell frequency was 4.78% which is 4.4-fold higher than the frequency seen with K562-CD1a cells. Moreover, in the presence of the anti-CD1a antibody (HI149), the CD1a reactivity was reduced by 36% compared to isotype control demonstrating that this response is partially dependent on CD1a lipid presentation (Figure 5.8).

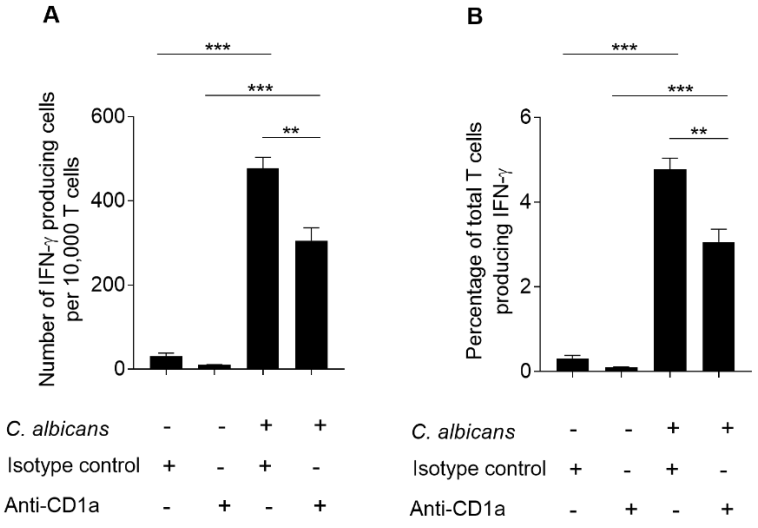


Figure 5.8. *C. albicans* induces the production of IFN-γ by T cell clone through CD1a molecules expressed on the surface of MO-DCs.

Monoclonal T cells (clone 6C6) were co-cultured with autologous MO-DCs pulsed or unpulsed with 1 µg/ml *C. albicans* extract (skin prick test solution) in the presence of 10 µg/ml anti-MHC I and anti-MHC II blocking antibodies with or without 10 µg/ml anti-CD1a blocking antibody (clone HI149) or isotype control then IFN-γ was measured by ELISpot. The number of IFN-γ-producing T cells (A) and the percentage of IFN-γ-producing T cells (B) are plotted. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

5.2.3. Activation of fungal-responsive CD1a-reactive T cell clones by recombinant plate-bound CD1a protein.

The reactivity toward *C. albicans* was confirmed for the T cell clones by CD1a-plate bound assay and CD1a-beads based assay (Figure 5.9), in order to eliminate the effect of the APCs processing of the *C. albicans* antigens. Streptavidin plate was coated with biotinylated CD1a and then incubated overnight. Then, the CD1a was loaded or not loaded with *C. albicans* extract and incubated for 24 hours. After this, T cell clones were added and incubated for 24 hours. After 24 hours, the concentration of IFN-γ in the supernatant was measured by ELISA. Using this method, although we observed some induction of IFN-γ when T cells where co-cultured with CD1a loaded with *C. albicans*, no significant difference was seen over autoreactivity (Figure 5.9 (A and C)).

As the CD1a-plate bound assay was not sensitive enough to detect the difference between autoreactivity and *C. albicans*-responsive CD1a-reactivity, we conducted another APC-free method, namely CD1a beads-based assay. Biotinylated-CD1a was loaded with *C. albicans* extract and then incubated with streptavidin-dyna beads before co-culturing with monoclonal T cells (clone 7D8). This was followed by measurement of IFN-γ concentration in the supernatant using ELISA. We found

that this method detected the CD1a-reactive T cell responses toward *C. albicans* over autoreactive responses compared to CD1a plate-bound assay (Figure 5.9 (B)).

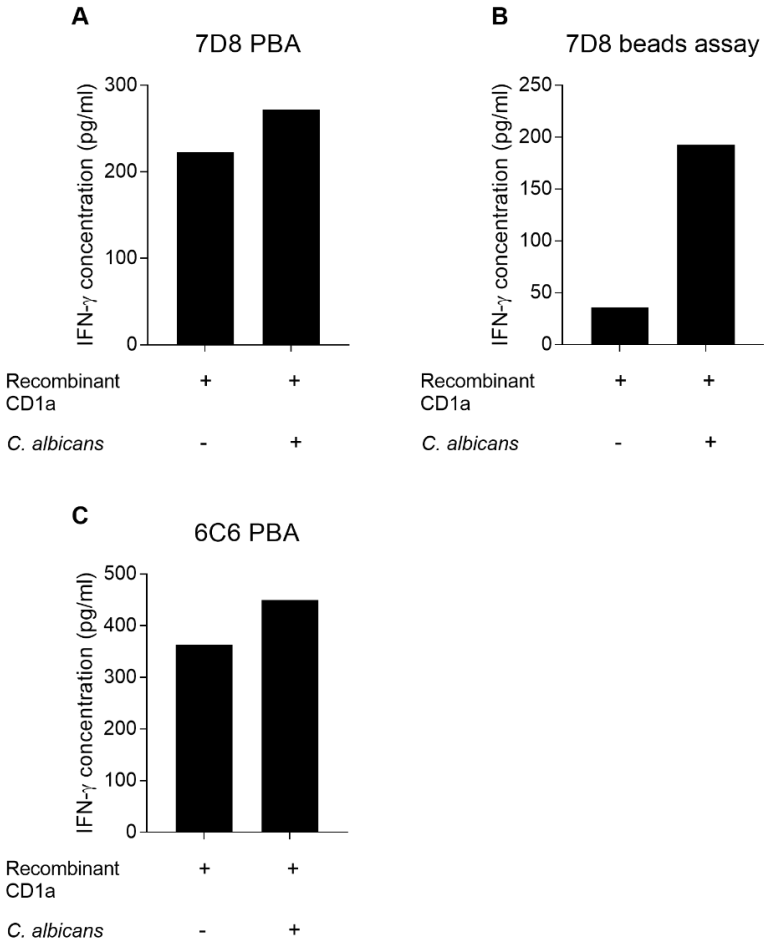


Figure 5.9 CD1a-plate bound assay of *C. albicans*-responsive CD1a-reactive T cells pulsed with *C. albicans*.

Streptavidin plate was coated with biotinylated CD1a and then incubated at 4ºC overnight. Then, the CD1a was loaded or not loaded with *C. albicans* extract (1 µg/ml) in 100 µl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, 100,000 T cells/well (clone 7D8 or clone 6C6) were added and incubated for 24 hours at 37°C. After 24 hours, the concentration of IFN-γ in the supernatant was measured by ELISA (A and C). Biotinylated-CD1a was loaded *C. albicans* extract and then incubated with streptavidin-dyna beads before co-culturing with T cell clone 7D8. the concentration IFN-γ in the supernatant was measured by ELISA (B).

5.2.4. NKG2D receptor expression and contribution to the CD1a-reactive T cell responses toward *C. albicans.*

Before testing the role of NKG2D receptor of the CD1a-reactive T cell responses toward *C. albicans*, we checked the K562 cells expression of the NKG2D ligands, MICA and MICB by flow cytometry. This showed that the K562 cells largely express MICA and MICB (Figure 5.10).

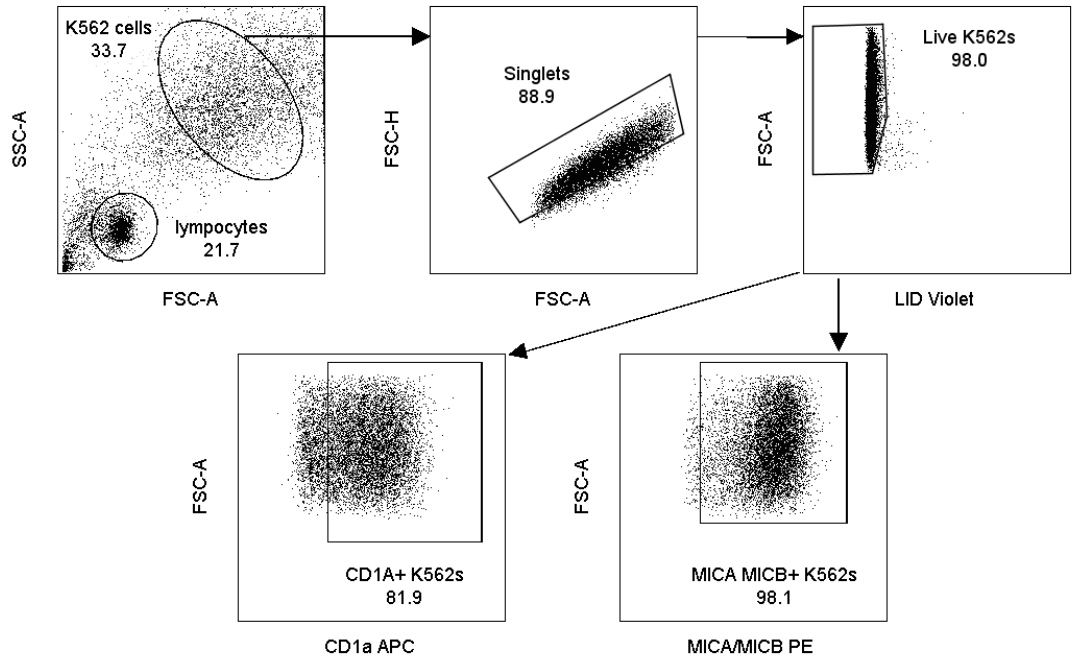


Figure 5.10 Flow cytometry plots of K562-CD1a cells stained for MICA/MICB.

The K562-CD1a cells were stained with CD1a and MICA/MICB expression. Gating on population, singlets, live, CD1a+ and MICA/MICB. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

Then the γδ T cell clones were stained for NKG2D expression. The γδ T cell clones 2D8 and 6C6 were NKG2D+, whereas clone 3E8 was NKG2D- (Figure 5.11).

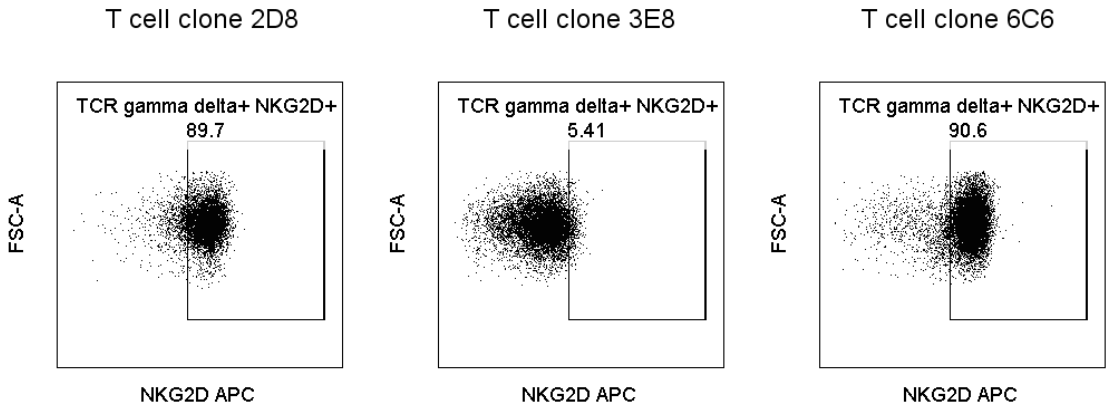


Figure 5.11 Flow cytometry plots of *C. albicans*-responsive CD1a-reactive γδ T cell clones stained for NKG2D.

The *C. albicans*-responsive CD1a-reactive γδ T cell clones were stained for NKG2D expression. Gating on population, singlets, live, CD3+, TCR γδ+ and NKG2D+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

The γδ T cell receptor NKG2D has been shown to play an important role in T cell activation and function, as discussed in the introduction. We thus investigated the role of NKG2D in T cell activation upon co-culturing with K562-CD1a cells unpulsed or pulsed with *C. albicans* with or without anti-NKG2D blocking antibody or isotype control. Then the expression of the activation marker CD69 was compared as a readout. The percentage of CD69 expression by autoreactive CD1a-reactive T cells and the percentage of *C. albicans*-responsive CD1a-reactive T cells were blocked partially with anti-NKG2D antibody compared to isotype control (Figure 5.12). The NKG2D partial blockade affected the K562-EV, as well as the CD1a-dependent autoreactive and fungal-reactive responses. However, it was noted that the reduction in the K562-CD1a-induced T cell response did not bring the activity down to that detected with K562-EV.

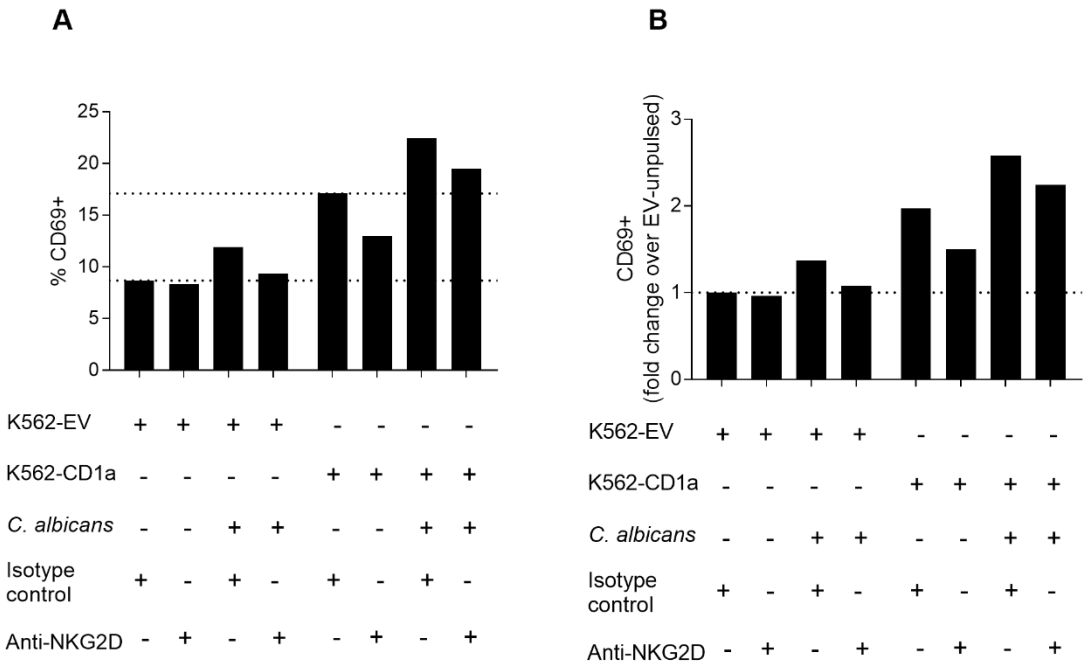


Figure 5.12 The effect of NKG2D blocking on the *C. albicans*-responsive CD1a-reactive T cell responses.

6C6 T cell clone was co-cultured with K562-CD1a cells unpulsed or pulsed with *C. albicans* in the presence or absence of anti-NKG2D blocking antibody (10 μg/ml) or isotype control (10 μg/ml) and then the expression of the activation marker CD69 was compared by flow cytometry. The percentage of CD69+ cells (A) and the fold change over EV-unpulsed (B) are illustrated. Gating on lymphocytes, singlets, live cells, CD3+ cells, γδ+ T cells and CD69+ cells. The compensation was based on single-colour beads and the gating was based on FMO controls.

5.3. Summary and discussion

To summarise, IFN-γ secretion assay was used to generate *C. albicans*-responsive CD1a-reactive T cell clones, as published previously (Jarrett, Salio et al. 2016), for further characterisation phenotypically and functionally in order to broaden our knowledge of their contribution to health and disease. We generated five *C. albicans*-responsive CD1a-reactive T cell clones using IFN-γ secretion assay. Some of the clones express αβ TCR and others express γδ TCR which were sequenced accordingly. Most of the clones generated were CD8+ and some where CD4 CD8 double negative in term of co-receptor expression. The reactivity of the T cell clones was examined by ELISpot using K562-CD1a cells or autologous MO-DCs and then confirmed using CD1a-plate bound assay or CD1a beads-based assay. We then found that the NKG2D receptor expressed on the surface of 6C6 γδ T cell clone contributed partially to the responses, demonstrating that in these two cases CD1a-reactive response was a combination of TCR-dependent and TCR-independent recognition.

It is of interest that 3 out of 5 clones generated were γδ T cells. Dermal γδ T cells in human skin largely express Vδ1 TCR with an oligoclonal repertoire that is distinct from circulating γδ T cells which largely express Vδ2 TCR (Holtmeier, Pfander et al. 2001). Interestingly, to date, virtually all reported CD1a-reactive T cell clones were αβ (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Cheng et al. 2014, Birkinshaw, Pellicci et al. 2015), however one study reported a γδ1+ TCR from one T cell clone (Agea, Russano et al. 2005). All the γδ T cells we generated were γ9δ2+. This was perhaps not surprising as these clones were generated from PBMC-derived T cells. It is of interest that some studies have suggested these cells are enriched in psoriatic skin lesions (Laggner, Di Meglio et

al. 2011). We have confirmed that the CD1-reactive T cells were found to be a part of the CD4+, CD8+ or the double negative T cell population (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Pena-Cruz et al. 2010, de Lalla, Lepore et al. 2011, de Jong, Cheng et al. 2014, Salio, Silk et al. 2014, Birkinshaw, Pellicci et al. 2015). The fact that most of the clones we generated were CD8+ and not CD4+ raised the question of whether these clones are of cytotoxic phenotype. However, an explanation might be the selection bias due to the method used to generate and expand the clones.

The sequence of the γδ TCR of the clones we generated was compared to sequences of the γδ TCRs from healthy control donors and cord blood samples generated by Martin Davey (Jamie Rossjohn lab, Monash University). The TCRγ sequences for clones 2D8 and 3E8 were found to be well represented in the population. Clone 3E8 has the most prevalent Vγ sequence in the circulation in adults and neonates (10-15% of all sequences). However, clone 6C6 is a remarkably uncommon sequence, found in one adult donor in their cohort and not in the cord blood samples. The TCRδ sequences were diverse and this is similar to what Davey et al found in their recent study (Davey, Willcox et al. 2018). Interestingly, each TCRδ was found to contain a hydrophobic residue at position 6, and this residue was essential for phosphoantigen (HMB-PP) activation by Vδ2+ T cells. Davey et al also found in their study clonal expansion of uncommon TCRγ (and TCRδ) in some donors (Davey, Willcox et al. 2018). It would be of interest to investigate if 6C6 is a dual reactive γδTCR for both CD1a and HMB-PP and particularly in the context of modulation by fungal lipids. The next step would be to generate Jurkat cell lines expressing this TCR and then testing their reactivity against phosphoantigens. The possibility that fungal-derived non-

phosphoantigens might act as potential ligands for γδ T cells should be investigated further in future studies.

As CD8+ γδ T cells express NKG2D surface receptor (Wensveen, Jelenčić et al. 2018), we sought to investigate the role of this receptor in contributing to the CD1a-reactive T cell responses toward *C. albicans* of our T cell clones. We found that a partial inhibition of the expression of CD69 by the T cell clone 6C6 in the presence of anti-NKG2D blocking antibody. This demonstrated that NKG2D might bind to MICA and MICB expressed on the surface of K562 cells and contribute partially to the induction of T cells to produce IFN-γ. However, it would be essential to undertake more experiments to confirm this finding. We speculated that NKG2D enhances the CD1a-reactive TCR-dependent responses seen with *C. albicans* especially during inflammation for example in AD. However, one of the γδ T cell clones was NKG2D-negative suggesting that NKG2D cannot contribute in all cases.

It would be very interesting to generate *C. albicans*-responsive CD1a-reactive T cell clones that secrete other relevant cytokines such as IL-22 or IL-17A which were shown to be induced with polyclonal T cells as seen in the previous chapter. Additionally, extending this work to generate T cell clones from the skin would be important in order to understand the role of *C. albicans*-responsive CD1a-reactive T cell in the skin especially in the context of AD and other inflammatory skin diseases. It is important to investigate the CD1a-binding fraction of *C. albicans* further and determine *C. albicans*-derived CD1a ligands, and this will be examined in the next chapter. However, based on the findings in this and the next chapter, co-crystal structures of 6C6/CD1a and 7D8/CD1a are underway at Monash University (Jamie Rossjohn).

6. Understanding the underlying mechanism of fungal activation of CD1a-reactive T cells.

6.1. Introduction

C. albicans is structurally rich with lipids; we hypothesized that the CD1a-reactive responses toward *C. albicans* are driven by *C. albicans*-derived lipids. Over 200 molecular lipid species were identified within *C. albicans* using high throughput mass spectrometry based lipidome analysis (Singh and Prasad 2011). These were divided into six major groups of lipids as follows:

i. Phosphoglycerides (PGLs): phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl glycerol (PG) and phosphatidyl acid (PA)
ii. Sphingolipids (SLs): ceramide (CER), inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC), mannosyldiinositolphosphorylceramide ($M(IP)_2C$)
iii. Sterols and sterol esters (SEs),
iv. Di-acyl glycerols (DAGs),
v. Tri-acyl glycerols (TAGs),
vi. Lyso-lipids: lysophophatidylcholine (Lyso-PC), lysophophatidylethanolamine (Lyso-PE) and lysophophatidylglycerol (Lyso-PG).

One of the major components of the fungal lipid pool are the sterols. The sterols found in mammalian cells and lower eukaryotes such as fungi respectively are cholesterol and ergosterol (Sood and Kinnunen 2008). These sterols share similar overall chemical structure with hydrophobic and planar fused tetracyclic rings, a six-carbon atom side chain, and an OH-group at position 3. However, they differ in

the number of double bonds present. While cholesterol has only one double bond, ergosterol has three (Sood and Kinnunen 2008). Fungal sterols include ergosterol and its metabolites (zymosterol, ergostatetraenol, episterol, fecosterol and lanosterol) which can be present either in the free form or the esterified form (Singh and Prasad 2011). The composition of sterol esters of *C. albicans* is detailed in Table 6-1.

Table 6-1 Composition of sterol esters of *C. albicans*.

The average percentage of total sterol ester mass spectral signal of 16 different strains of *C. albicans* is detailed in the tables. Modified from (Singh and Prasad 2011).

Sterol esters 16:1	**%**
Zymosterol	0.6
Ergostatetraenol	0.1
Ergosterol	2.2
Episterol + Fecosterol	1.1
Lanosterol	1.1
Total 16:1	5.0

Sterol esters 16:0	**%**
Zymosterol	0.4
Ergostatetraenol	0.1
Ergosterol	1.4
Episterol + Fecosterol	1.0
Lanosterol	1.3
Total 16:0	4.2

Sterol esters 18:3	**%**
Zymosterol	0.7
Ergostatetraenol	0.6
Ergosterol	5.8
Episterol + Fecosterol	1.5
Lanosterol	2.7
Total 18:3	11.3

Sterol esters 18:2	**%**
Zymosterol	2.3
Ergostatetraenol	0.6
Ergosterol	12.4
Episterol + Fecosterol	4.1
Lanosterol	4.0
Total 18:2	23.3

Sterol esters 18:1	**%**
Zymosterol	5.6
Ergostatetraenol	1.7
Ergosterol	24.5
Episterol + Fecosterol	9.2
Lanosterol	10.0
Total 18:1	51.0

Sterol esters 18:0	**%**
Zymosterol	0.6
Ergostatetraenol	0.2
Ergosterol	2.1
Episterol + Fecosterol	1.1
Lanosterol	1.1
Total 18:0	5.1

Total sterol esters	**%**
Lanosterol esters	20.2
Zymosterol esters	10.1
Episterol + Fecosterol esters	18.0
Ergostatetraenol sterol esters	3.3

Ergosterol esters	48.3
Total sterol esters	100.0

On a molar basis cholesterol represents 30–50 mol % of the total lipid fraction of the mammalian plasma membrane (Miao, Nielsen et al. 2002). The fungal plasma membrane lipid content is like that of mammalian membrane with 30-40 mol % sterols. In addition to the sterols, fungal plasma membrane contains 10-20 mol % sphingomyelin and a small fraction of glycosphingolipids (Alvarez, Douglas et al. 2007). In addition to the plasma membrane, significant amounts of sterols are present in the membranes of the intracellular organelles such as the peroxisomes, mitochondria, vacuoles and the ER (Schneiter, Brugger et al. 1999).

Fungal sterols are tightly packed with sphingolipids forming lipid rafts. The lipid rafts cluster to form large sterol-rich domains (SRDs) which are unique to fungi and not present in mammalian cells (Alvarez, Douglas et al. 2007). The SRDs are found in different types of fungi such as *Saccharomyces cerevisiae*, *Cryptococcus neoformans* as well as *C. albicans* (Alvarez, Douglas et al. 2007). They have potential roles in membrane organization and they may therefore contribute to fungal virulence (Alvarez, Douglas et al. 2007). In addition, sterols (and ergosterol particularly) have a major role in cell permeability (Young, Hull et al. 2003, Mishra, Prasad et al. 2008) by maintaining the fluidity of the fungus plasma membranes and architecture (Kodedova and Sychrova 2015). Moreover, ergosterol contributes to protein sorting, cytoskeleton organisation and endocytosis (Kodedova and Sychrova 2015). Alteration in ergosterol levels causes variation in the cellular osmotic pressure and therefore can be lethal to the cells (Zavrel, Hoot et al. 2013). As a result, ergosterol biosynthesis pathway served as a good target for antifungal drugs (Young, Hull et al. 2003). In *C. albicans* for instance, the use of amphotericin

B (polyene class of antifungal drugs) impairs fungal cell membrane integrity (Young, Hull et al. 2003) and the use of the anti-fungal drug ketoconazole (ergosterol inhibitor) or myriocin (sphingolipid inhibitor) affects the formation of the SRDs and inhibits the yeast to hyphal transition resulting in decreased virulence (Martin and Konopka 2004). In addition to the importance of fungal sterols as major components in the structural and metabolism of the fungus, they also play an important role in multi-drug resistance toward anti-fungal drugs as upregulation of the sterol biosynthetic pathway was reported in the resistant strains of *C. albicans* (Singh, Yadav et al. 2012).

The sterol biosynthetic pathway in eukaryotes is conserved. Cholesterol and ergosterol are synthesized from the same intermediate, namely lanosterol. Different enzymes are used for each biosynthesis pathway. The two rate-limiting enzymes in this pathway are the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is necessary in regulation of cholesterol synthesis in mammals and the lanosterol 14-alpha-demethylase, which is essential in the ergosterol synthesis in fungi (Lv, Yan et al. 2016). The biosynthesis pathway of ergosterol has been characterised for *C. albicans* and is illustrated in Figure 6.1.

Several genes have been identified to be involved in the regulation of the ergosterol biosynthesis pathway including essential genes (ERG1, ERG7, ERG9, ERG25, ERG26, and ERG27) and non-essential genes (ERG2, ERG3, ERG5, ERG6, and ERG24) (Jensen-Pergakes, Kennedy et al. 1998, Kennedy, Johnson et al. 2000, Aaron, Pierson et al. 2001, Jia, Arthington-Skaggs et al. 2002, Pierson, Jia et al. 2004, Pasrija, Krishnamurthy et al. 2005, Martel, Parker et al. 2010). Different mutations in the ergosterol genes have been reported in the literature and believed to cause alterations in enzyme activity, potentially affecting drug binding

sites leading to resistance to anti-fungal drugs especially the azoles (Wang, Kong et al. 2009, Martel, Parker et al. 2010, Golabek, Strzelczyk et al. 2015). The antifungals inhibit the activities of the enzymes involved in the ergosterol synthesis. In addition to this, they contribute to the accumulation of altered forms of sterols with a C14-methyl group which leads to changing the properties of the plasma membrane (Abe, Usui et al. 2009, Lv, Yan et al. 2016).

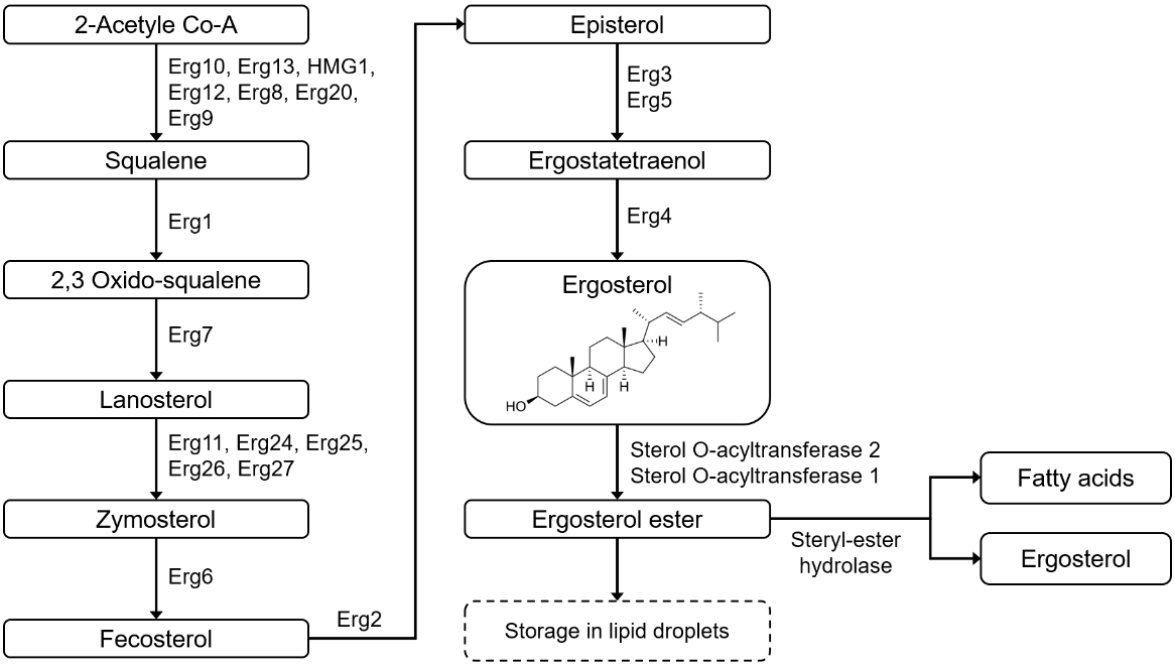


Figure 6.1 Schematic representation of the ergosterol biosynthetic pathway in *C. albicans* including the main enzymes and metabolites.

Enzymes abbreviations: Erg10, Acetyl-CoA C-acetyltransferase; Erg13, Hydroxymethylglutaryl-CoA synthase; HMG1, HMG-CoA reductase; Erg12, Mevalonate kinase; Erg8, Phosphomevalonate kinase; Erg20, Farnesyl pyrophosphate synthetase; Erg9, Squalene synthase; Erg1, Squalene monooxygenase/epoxidase; Erg7, Lanosterol synthase; Erg11, Lanosterol 14-alpha-demethylase; Erg24, C-14 sterol reductase; Erg25, C-4 methyl sterol oxidase; Erg26, C-3 sterol dehydrogenase; Erg27, 3-keto sterol reductase; Erg6, Delta(24)-sterol C-methyltransferase; Erg2, C-8 sterol isomerase; Erg3, C-5 sterol desaturase; Erg5, C-22 sterol desaturase; Erg4, C-24(28) sterol reductase. Modified from (Sanglard, Ischer et al. 2003, Lv, Yan et al. 2016).

Although the synthesis of sterols in *C. albicans* is well established, the storage of sterols, the transformation between free sterol and sterol esters and the enzymes involved in this process have not been extensively investigated. However, this is well established for *Saccharomyces cerevisiae* and because they have homologue genes, it is speculated that they have the same regulatory pathways (Lv, Yan et al. 2016). These regulatory genes control the storage of the ergosterol in its esterified form in specialised lipid droplets and then the decomposition of it when needed. These lipid droplets contain a neutral lipid core consisting of triacylglycerols, sterols and sterol esters surrounded by protein-bearing phospholipid membrane (Beller, Thiel et al. 2010).

In addition to the lipid composition, fungi secrete enzymes which contribute to their pathogenesis and virulence. Fungi in general and *C. albicans* in particular secrete two main types of enzymes, proteinases (Naglik, Challacombe et al. 2003, Rapala-Kozik, Bochenska et al. 2018) and phospholipases (Ghannoum 2000). For the relevance of the thesis, I will be focusing on the fungal phospholipases (PL). PL are a heterogeneous group of enzymes that hydrolyse one or more ester bonds in glycerophospholipids. They have been divided into 5 types (PLA1, PLA2, PLB, PLC and PLD) according to the cleavage position of the specific ester bond (Figure 6.2).

The most abundant type of PL secreted by *C. albicans* is PLB (Banno, Yamada et al. 1985, Leidich, Ibrahim et al. 1998, Ghannoum 2000). PLB cleaves the acyl chains at the sn1 and sn2 positions (PBL activity). In addition to the PLB activity, PLB has a lysophospholipase (Lyso-PL) activity by removing the single fatty acyl chain on the lysophospholipid to produce glycerophosphocholine. PLB exert also a Lyso-PL trans-acylase activity by the re-esterification of the acyl chain onto a

lysophospholipid, to regenerate a glycerophospholipid (Takahashi, Banno et al. 1991, Mirbod, Banno et al. 1995, Leidich, Ibrahim et al. 1998). Besides PLB, *C. albicans* express a membrane-associated PLD enzyme which was reported to be an important regulator of yeast-hyphae transition of the fungus (McLain and Dolan 1997, Ghannoum 2000).

C. albicans phospholipase genes identified by gene cloning are two genes coding for PLB (*caPLB1* (Leidich, Ibrahim et al. 1998), *caPLB2* (Sugiyama, Nakashima et al. 1999)) and one gene coding for PLD (*caPLD* (Kanoh, Nakashima et al. 1998)). The expression of *caPLB1* by *C. albicans* was found to be regulated by different environmental and physiologic factors (pH, temperature and nutrient supplementation) (Mukherjee, Chandra et al. 2003) and the deletion of this gene was shown to decrease the virulence of *C. albicans* in a murine model of haematogenous disseminated candidiasis by reducing the ability for host cell penetration (Leidich, Ibrahim et al. 1998).

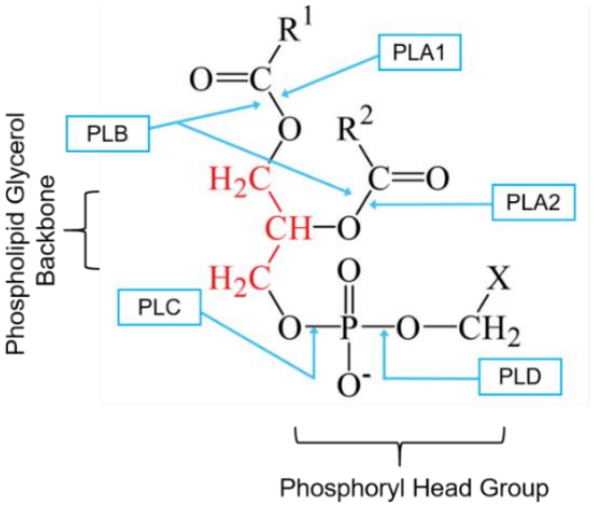


Figure 6.2 The phospholipid cleavage sites of phospholipase enzymes.

PLA1 hydrolyses the fatty acyl ester at the sn1 position to yield free fatty acids and 2-acyl lysophospholipids, PLA2 cleaves at sn2 position to yield free fatty acids and 1-acyl lysophospholipids, PLB cleaves acyl chains at the sn1 and sn2 positions,

PLC cleaves the phosphodiester bond in the head group proximal to the glycerol backbone to yield 1,2-diacylglycerol and the phosphoryl head group depending on the specific phospholipid species involved (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol), PLD cleaves at the phosphodiester bond proximal to X after the phosphate group, yields phosphatidic acid and X, which can be either choline, ethanolamine, serine, inositol). Modified from (Djordjevic 2010). Permission to reproduce this figure has been granted by FRONTIERS.

In addition to PL, fungi can process endogenous lipids such as squalene to produce squalene peroxide. Squalene which is part of the ergosterol synthetic pathway, is a known CD1a ligand (Layre, de Jong et al. 2014). Squalene is a major human sebaceous lipid (Niki 2015) and due to its unique structure of six double bonds, squalene is oxidised easily in the presence of UV light or lipoxygenases enzymes giving rise to squalene peroxide (Niki 2015). Lipoperoxide and their metabolic products like leukotrienes are thought to induce keratinocyte hyperproliferation and therefore contribute to the development of inflammatory skin lesions. In addition, squalene peroxide contributes to the inflammatory process in acne vulgaris through the induction of keratinocytes proliferation, lipoxygenase activity, and NF-κB activation with the consequent secretion of IL-6 (Ottaviani, Alestas et al. 2006). Squalene peroxides play a major role in the development of dandruff which could be mediated by *Malassezia* which induce squalene peroxidation in the scalp (Jourdain, Moga et al. 2016). In this study it was speculated that *Malassezia* induces squalene peroxidation and therefore the squalene peroxide formed penetrates the skin and stimulates inflammation and keratinocyte proliferation which result in the formation dandruff with typical clinical signs such as skin barrier impairment and itching. We speculated that a similar model might be applied to AD and *C. albicans*.

6.2. Results

6.2.1. The role of fungal-derived proteins in the CD1a-driven T cell responses.

PLA2 derived from HDM and bee venom was shown to induce CD1a-reactive T cell responses through the production of neolipid antigens that bind CD1a (Bourgeois, Subramaniam et al. 2015, Jarrett, Salio et al. 2016); therefore we sought to investigate the role of fungal-derived PLA2 in this pathway. We first measured the activity of *C. albicans'* secretory PLA2 (sPLA2) and compared it to wasp venom sPLA2 as a positive control. We found that *C. albicans* secretes very small amount of sPLA2 compared to wasp venom (Figure 6.3). As a result, we thought it unlikely that *C. albicans* derived sPLA2 explained CD1a-reactive T cell responses in AD.

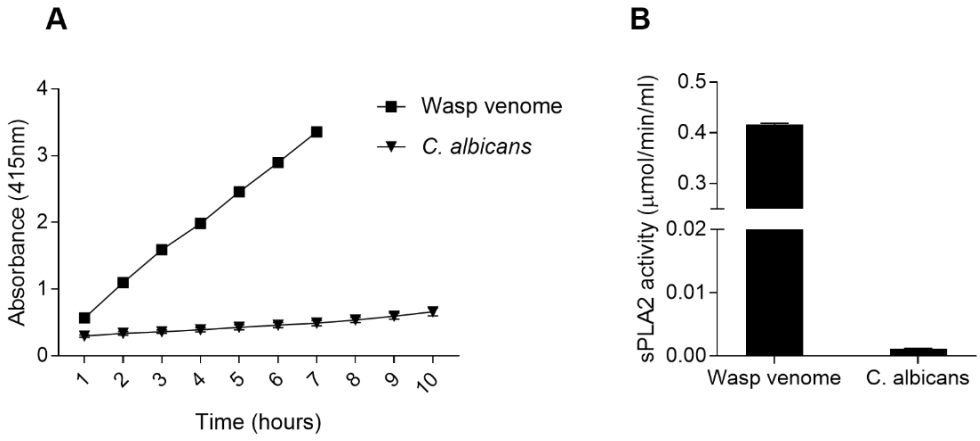


Figure 6.3 sPLA2 activity of *C. albicans* extract.

The sPLA2 activity of the *C. albicans* extract was measured using a plate-based colorimetric measurement (sPLA2 kit). In this assay the 1,2-dithio analogue of diheptanoyl phosphatidylcholine is used as a substrate for sPLA2. Upon hydrolysis of the thio ester bond at the sn-2 position by sPLA2, free thiols are detected using DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) which produce a coloured participant.

The absorbance was measured by a spectrophotometer hourly over 10 hours (415nm) (A) and then the sPLA2 activity was calculated and compared to wasp venom (B).

As discussed in the introduction, the most abundant type of phospholipase produced by *C. albicans* is PLB. In order to further test for potential roles for phospholipase, we next investigated the induction of *C. albicans*-responsive CD1a-reactive T cell responses. This was done using by a PLB inhibitor compound known as alexidine dihydrochloride. Alexidine dihydrochloride was added to the *C. albicans* extract for 1-2 hours before pulsing the K562-CD1a cells for 16-20 hours. Then the K562-cells were co-cultured with T cells for another 16-20 hours and IFN-γ ELISpot was conducted. Compared to K562-CD1a cells that were pulsed with *C. albicans* extract without PLB inhibition but with the vehicle control, no difference was seen when PLB inhibitor was added in 3 different concentrations in two HC donors (Figure 6.4). This demonstrated that PLB was not contributing to the *C. albicans*-responsive CD1a-reactive T cell activation.

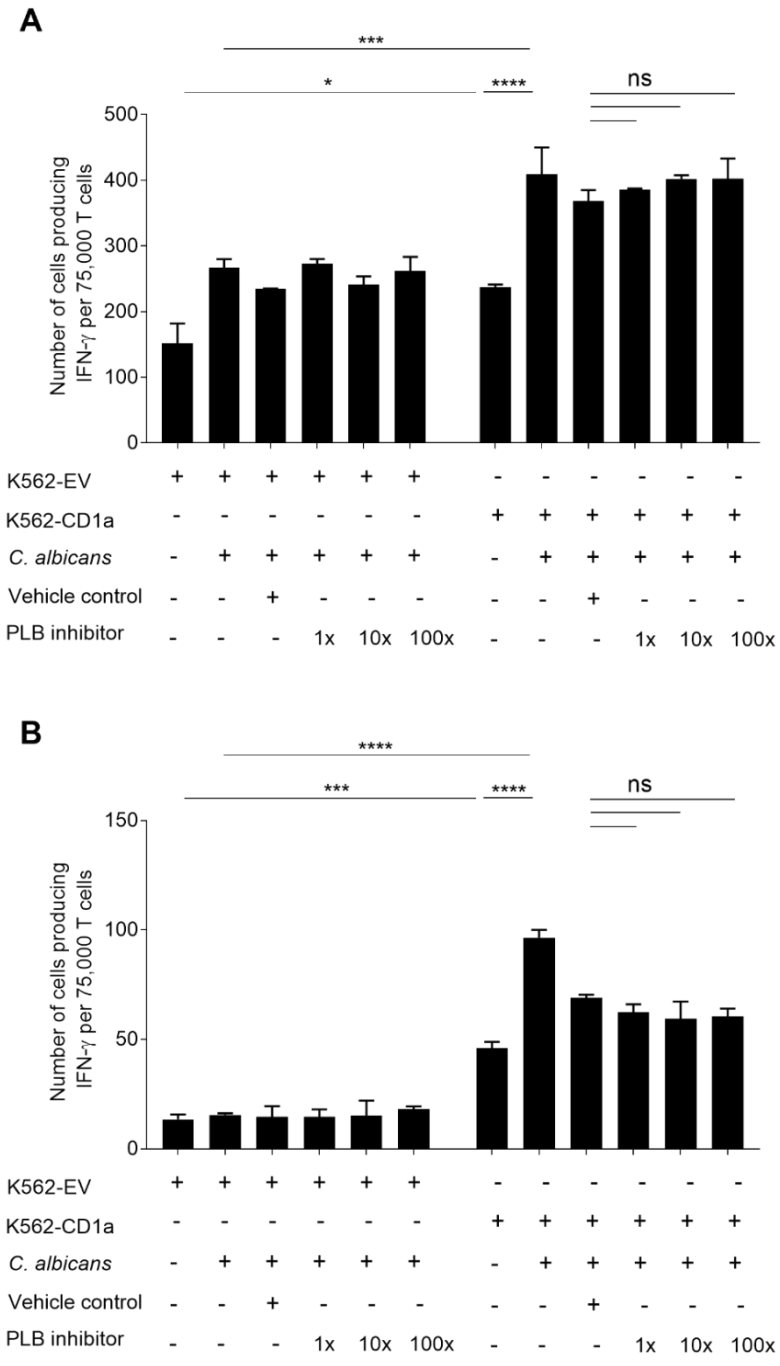


Figure 6.4 The effect of PLB inhibition on the *C. albicans*-responsive CD1a-reactive T cell responses.

PBMCs-derived CD3+ T cells from HC donors (W021 (A) and W071 (B)) were co-cultured with K562-CD1a or K562-EV in the presence or absence of *C. albicans* extract (skin prick test solution) with or without the PLB inhibitor alexidine dihydrochloride in 3 different concentrations (1x: 0.15 µg/ml, 10x: 1.5 µg/ml, 100x: 15 µg/ml). Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

To confirm that *C. albicans*-derived enzymes were not contributing to the CD1a-driven responses, we tested the CD1a-reactive T cell responses of a commercially available defatted-*C. albicans* preparation. Firstly, we demonstrated that this defatted-*C. albicans* preparation had no toxic effect on the K562-CD1a cells (Table 6-2). Secondly, and as expected, we didn't find any altered CD1a expression on the surface of the K562 cells when pulsing with the defatted-*C. albicans* preparation (Figure 6.5).

Table 6-2 The viability of K562-CD1a cells after pulsing with defatted-*C. albicans* preparation.

K562-CD1a cells were pulsed with three different concentrations of defatted-*C. albicans* preparation (P1: 0.1 µg/ml, P2: 1 µg/ml, P3: 10 µg/ml) for 16-20 hours before they were stained with LIVE/DEAD™ Fixable Dead Cell Stain and the viability of cells was determined by flow cytometry.

Antigens	Concentration	% Live cells
Defatted *C. albicans*	P3	96.3 %
	P2	95.1 %
	P1	95.8 %
Unpulsed		94.9 %

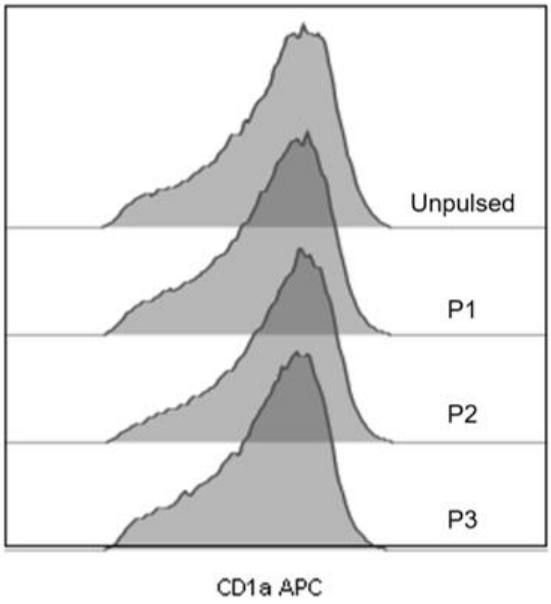


Figure 6.5 Flow cytometry histograms comparing the CD1a expression of the K562-CD1a cells after pulsing with defatted-*C. albicans* preparation.

K562-CD1a cells were pulsed with three different concentrations of defatted-C. albicans preparation (P1: 0.1 µg/ml, P2: 1 µg/ml, P3: 10 µg/ml) for 16-20 hours before the CD1a expression was determined by flow cytometry. Gating on cell population, singlets, live cells and CD1a+ cells. The compensation was based on single-colour stained beads. Gating was based on FMO controls.

Then we tested the effect of this preparation functionality using IFN-γ ELISpot on three HC donors. We observed a significant induction of IFN-γ production by CD3+ T cells when co-cultured with K562-CD1a and K562-CD1c cells compared to K562-EV in the absence of *C. albicans* antigen, demonstrating the reactivity to endogenous lipids. When T cells were co-cultured with K562-CD1a cells in the presence of 1 µg/ml defatted *C. albicans* preparation a non-significant difference of the IFN-γ-producing T cells was observed over unpulsed conditions. The same observation was seen with other CD1 isoforms tested (Figure 6.6).

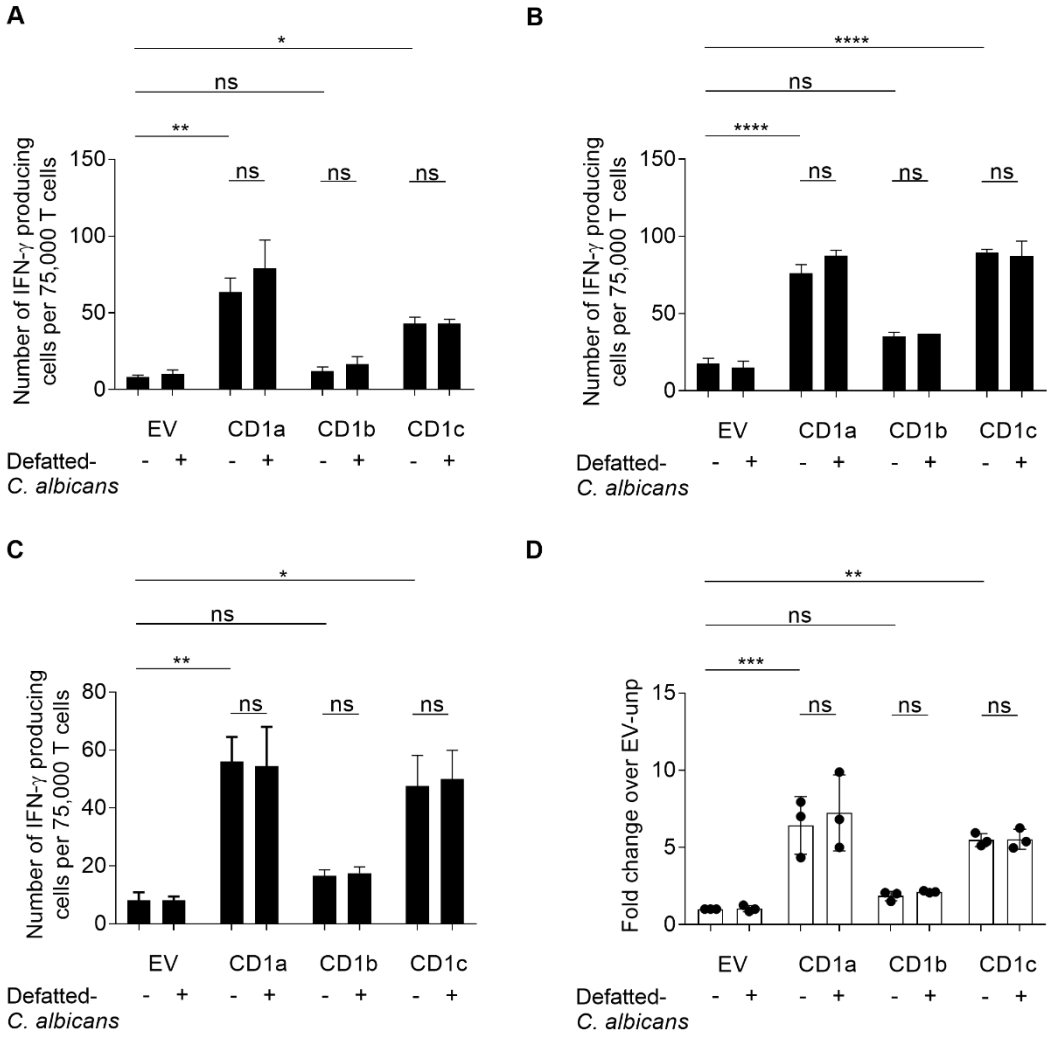


Figure 6.6 Comparing the IFN-γ-producing autoreactive and defatted-*C. albicans*-responsive T cells reactivity toward CD1a, CD1b and CD1c.

CD3+ T cells from three HC donors (W015 (A), C2399 (B) and C2402 (C)) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-EV or K562-CD1a or K562-CD1b or K562-CD1c in the presence or absence of 1 µg/ml defatted-C. albicans preparation and then IFN-γ production was measured by ELISpot. The number of IFN-γ producing T cells is plotted in the bar chart. Data represent mean and standard deviation (SD) of duplicate measurements. The mean data from the three donors were plotted and presented as fold change over EV-unpulsed (D). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

In a cohort of 7 HC donors we observed a significant induction of autoreactive T cells, however, when T cells were co-cultured with 1 µg/ml defatted-*C. albicans*

preparation a non-significant difference in the IFN-γ-producing T cells was observed over K562-CD1a unpulsed (Figure 6.7), demonstrating that defatted-*C. albicans* preparation did not induce T cell production of IFN-γ through the CD1a-presentation pathway.

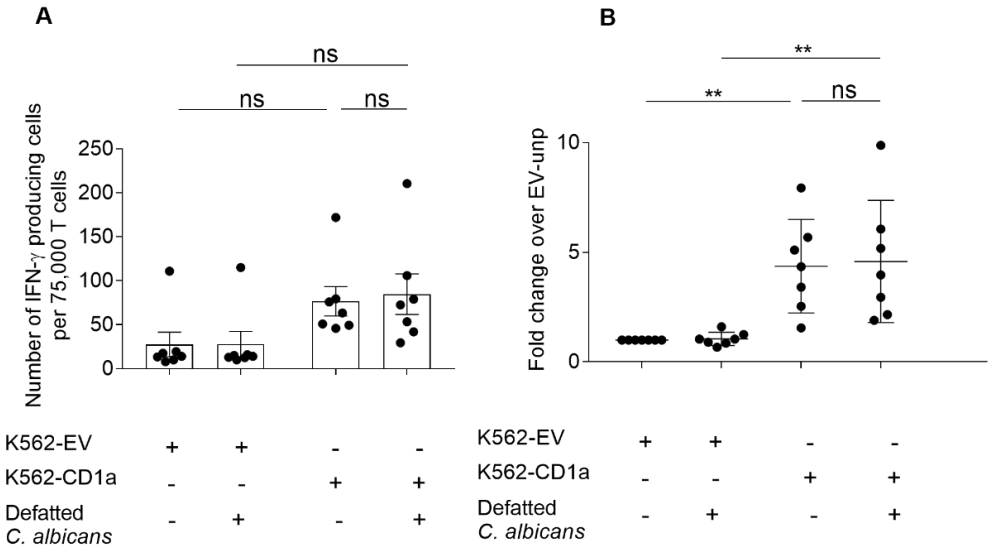


Figure 6.7 The autoreactive and defatted *C. albicans*-responsive CD1a-reactive T cells cohort.

CD3+ T cells from HC donors (n=7) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 1 µg/ml defatted C. albicans preparation and then IFN-γ production was measured by ELISpot. The number of cells producing IFN-γ (A) and the fold change over EV-unpulsed (B) are illustrated. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

We then compared the CD1a-reactive T cell responses of K562-cells pulsed with defatted *C. albicans* preparation or *C. albicans* extract (non-defatted) in a cohort of 3 health donor PBMC-derived T cells. While, pulsing with the *C. albicans* extract gave a significant induction of IFN-γ in a CD1a-reactive manner, pulsing with the defatted preparation was not significant over the autoreactive responses.

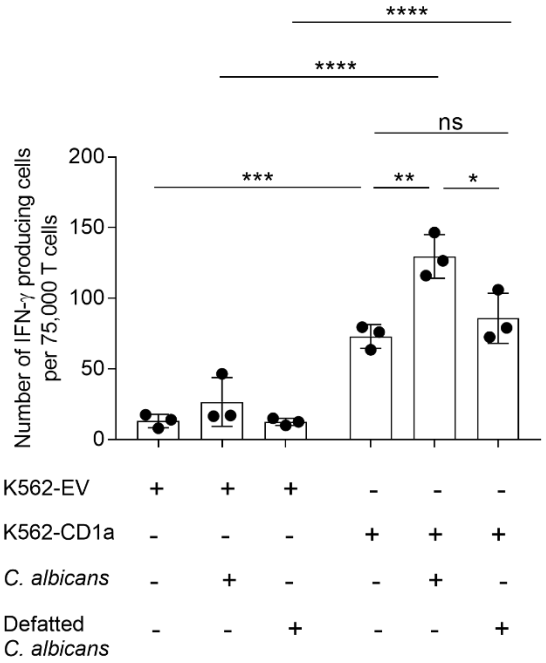


Figure 6.8 Comparing the IFN-γ-producing CD1a-reactive T cells responses of defatted-*C. albicans* and non-defatted *C. albicans* extract.

CD3+ T cells from HC donors (n=3) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 1 µg/ml *C. albicans* extract or 1 µg/ml defatted-*C. albicans* preparation and then IFN-γ production was measured by ELISpot. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

Our findings were confirmed when we did not observe any effect of the *C. albicans* supernatant, which contains different types of secretory enzymes, on inducing CD1a-reative T cells to produce IFN-γ in a cohort of 4 healthy donor PBMC-derived T cells (Figure 6.9). Like other *C. albicans* preparations used in previous experiment, *C. albicans* supernatant did not alter the CD1a-expression on the surface of K562-CD1a (Figure 6.10) nor affect their viability in three different concentrations (Table 6-3)

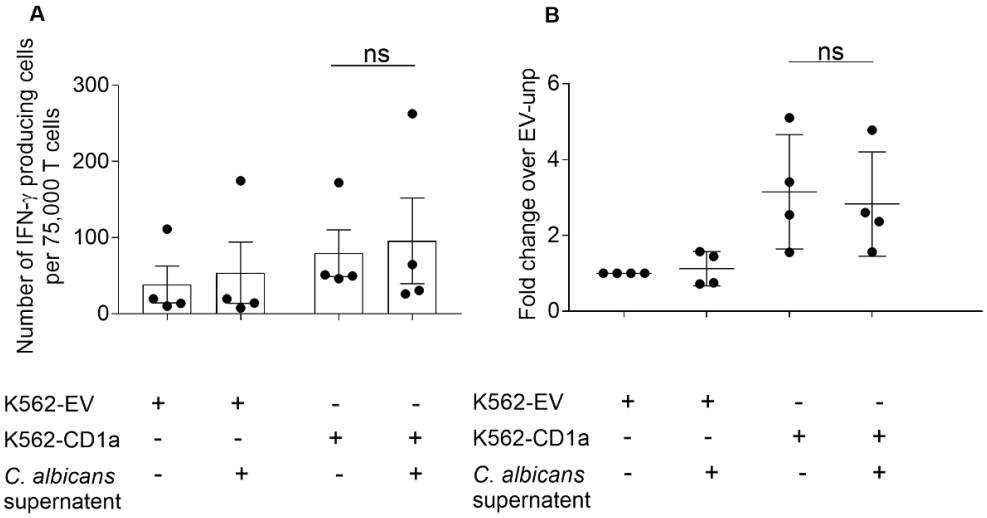


Figure 6.9 The autoreactive and *C. albicans* supernatant-responsive CD1a-reactive T cells cohort.

CD3+ T cells from HC donors (n=4) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 100 µl *C. albicans* supernatant and then IFN-γ production was measured by ELISpot. The number of cells producing IFN-γ (A) and the fold change over EV-unpulsed (B) are illustrated. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

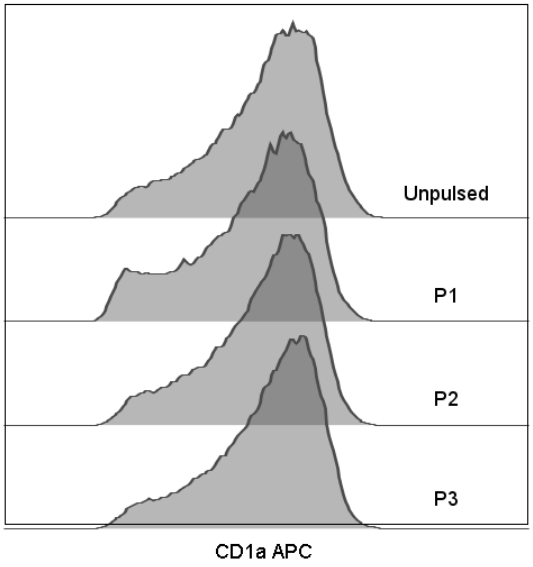


Figure 6.10 Flow cytometry histograms comparing the CD1a expression of the K562-CD1a cells after pulsing with *C. albicans* supernatant.

K562-CD1a cells were pulsed with three different concentrations of *C. albicans* supernatant (P1: 1 µl/ml, P2: 10 µl/ml, P3: 100 µl/ml) for 16-20 hours before the CD1a expression was determined by flow cytometry. Gating on cell population, singlets, live cells and CD1a+ cells. The compensation was based on single-colour stained beads. Gating was based on FMO controls.

Table 6-3 The viability of K562-CD1a cells after pulsing with *C. albicans* supernatant.

K562-CD1a cells were pulsed with three different concentrations of *C. albicans* supernatant (P1: 1 µl/ml, P2: 10 µl/ml, P3: 100 µl/ml) for 16-20 hours before they were stained with LIVE/DEAD™ Fixable Dead Cell Stain and the viability of cells was determined by flow cytometry.

Antigens	Concentration	% Live cells
C. albicans supp.	P3	93.8 %
	P2	95.3 %
	P1	96.1 %
Unpulsed		94.9 %

Taken together, the above findings demonstrated that *C. albicans* derived enzymes were not contributing to induce the CD1a-reactive T cell response, hence other components of the *C. albicans* extract were likely responsible for deriving this response and this is most probably the lipid content of the fungus.

6.2.2. The CD1a-reactive T cell responses toward fungal-derived lipid fractions.

In order to study the role of *C. albicans*-derived lipids of the CD1a-drived responses of T cells, we extracted the lipid mass from heat-killed *C. albicans* and then undertook lipid fractionation and tested the role of each of the lipid fractions separately (refer to section 3.16 for details about the lipid fractionation protocol).

The K562-CD1a or K562-EV cells were pulsed with 3 different concentrations of each of the lipid fractions for 16-20 hours and then co-cultured with PBMC-derived T cells from HC donors. The CD1a-reactivity was tested by IFN-γ ELISpot. Lipid fractions I, IV and V did not induce any CD1a-reactive responses over autoreactivity (Figure 6.11 (A), (D) and (E)). However, when pulsing with lipid fraction III, we observed a significant inhibition of the CD1a-reactive T cell responses below autoreactivity and the vehicle control in a dose-dependent manner, demonstrating that this lipid fraction contained CD1a-inhibitory ligands (Figure 6.11 (C)). Similarly, pulsing with lipid fraction II inhibited the CD1a-reactive T cell with the middle concentration of the lipid but did not show a dose dependent response (Figure 6.11 (B)). Conversely, pulsing with the unbound lipid fraction induces the production of IFN-γ by CD1a-reactive T cells above autoreactivity and vehicle control, demonstrating that this lipid fraction contained CD1a-stimulatory ligands (Figure 6.11 (F)).

From mass spectrometry analysis (kindly done by David Priestman), it was noted that the unbound lipid fraction is rich with sterols and their ester derivatives. Because ergosterol and its metabolites are unique to fungi and not present in human tissue, we went to further investigate their role in the CD1a-reactivity of T cells as exogenous CD1a-ligands candidates.

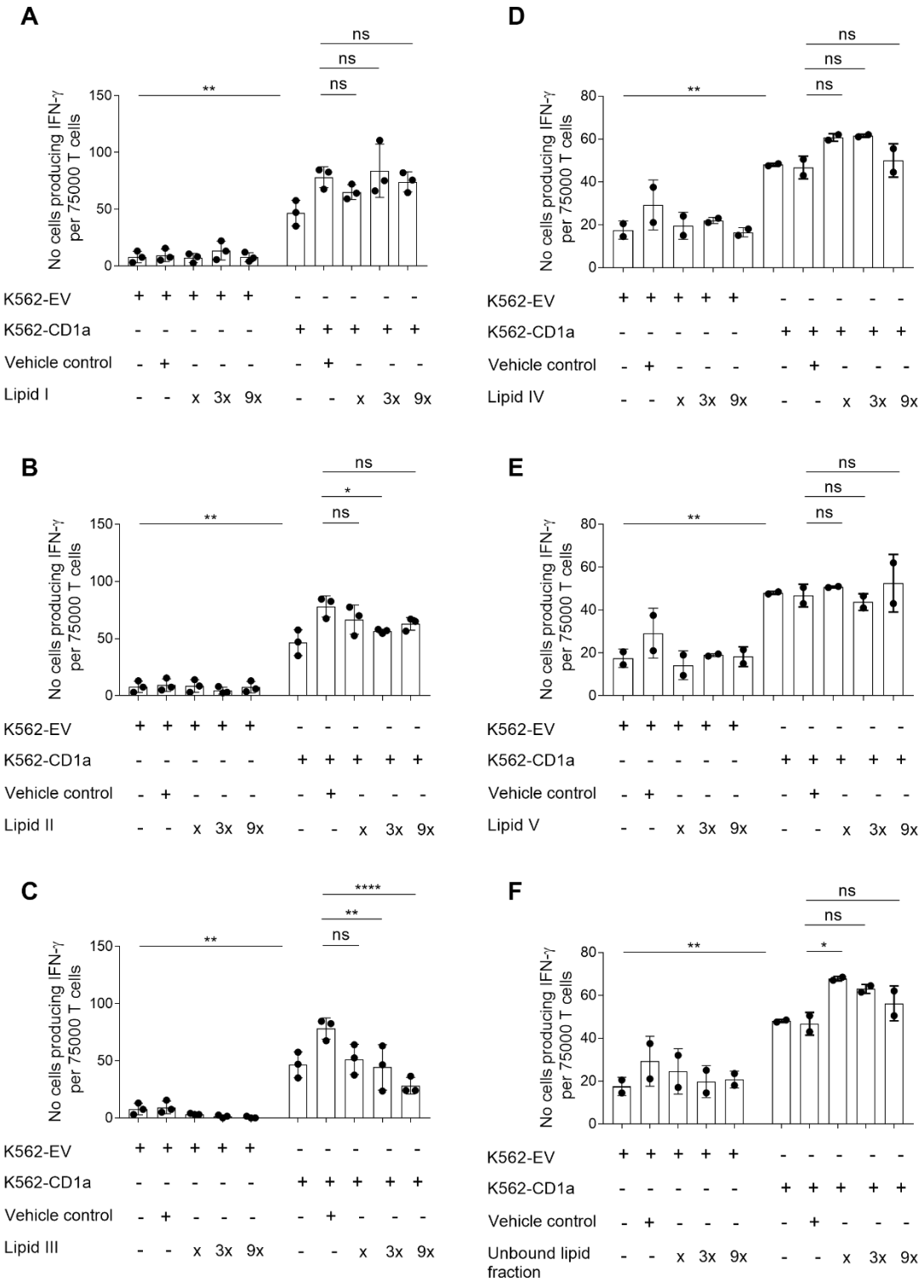


Figure 6.11 Testing the CD1a-reactive T cell responses toward *C. albicans*-derived lipid fraction.

K562-CD1a or K562-EV cells were pulsed with x, 3x and 9x of the lipid fractions I, II, III IV, V and the unbound lipid fraction for 16-20 hours and then co-cultured with T cell from HC donors. The CD1a-reactivity was tested by IFN-γ ELISpot. Data

represent mean and standard deviation (SD). The T cell samples used in A, B and C were W015, W003 and W030. The T cell samples used in D, E and F were C2535 and C2536. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

6.2.3. The CD1a-reactive T cell responses toward ergosterol.

The CD1a-reactive T cell responses to ergosterol was tested using IFN-γ ELISpot in a cohort of 11 HC and 11 AD samples. In the AD cohort there was a significant induction of IFN-γ production by CD3+ T cells when co-cultured with K562-CD1a cells compared to K562-EV in the absence of ergosterol, demonstrating the reactivity to endogenous lipids. When AD T cells were co-cultured with K562-CD1a cells in the presence of 2 µg/ml ergosterol a significant increment of the IFN-γ-producing T cells was observed over K562-EV cells pulsed with ergosterol and over K562-CD1a unpulsed, demonstrating that the production of IFN-γ in response to ergosterol in the AD cohort is CD1a-dependent (Figure 6.12 (A)). In the HC cohort, similar responses were seen (Figure 6.12 (A)). As a further test of functional relevance, when the fold change of ergosterol-reactive response over autoreactivity was calculated for both groups, a significant induction of ergosterol-responsive CD1a-reactive T cells over autoreactivity was seen in the AD cohort and the HC group with no difference between the ergosterol-reactivity of both groups (Figure 6.12 (B)).

When the severity of AD was compared in the same cohort we did not observe a specific pattern of responses of autoreactive or ergosterol-responsive CD1a-reactive T cells when the number of IFN-γ-producing T cells was plotted (Figure 6.12 (A)), however, when the fold change of ergosterol-reactive response over autoreactivity was plotted, two out of three severe AD samples were shown to have

the highest ergosterol-responsive CD1a-reactive T cell responses (Figure 6.12 (B)).

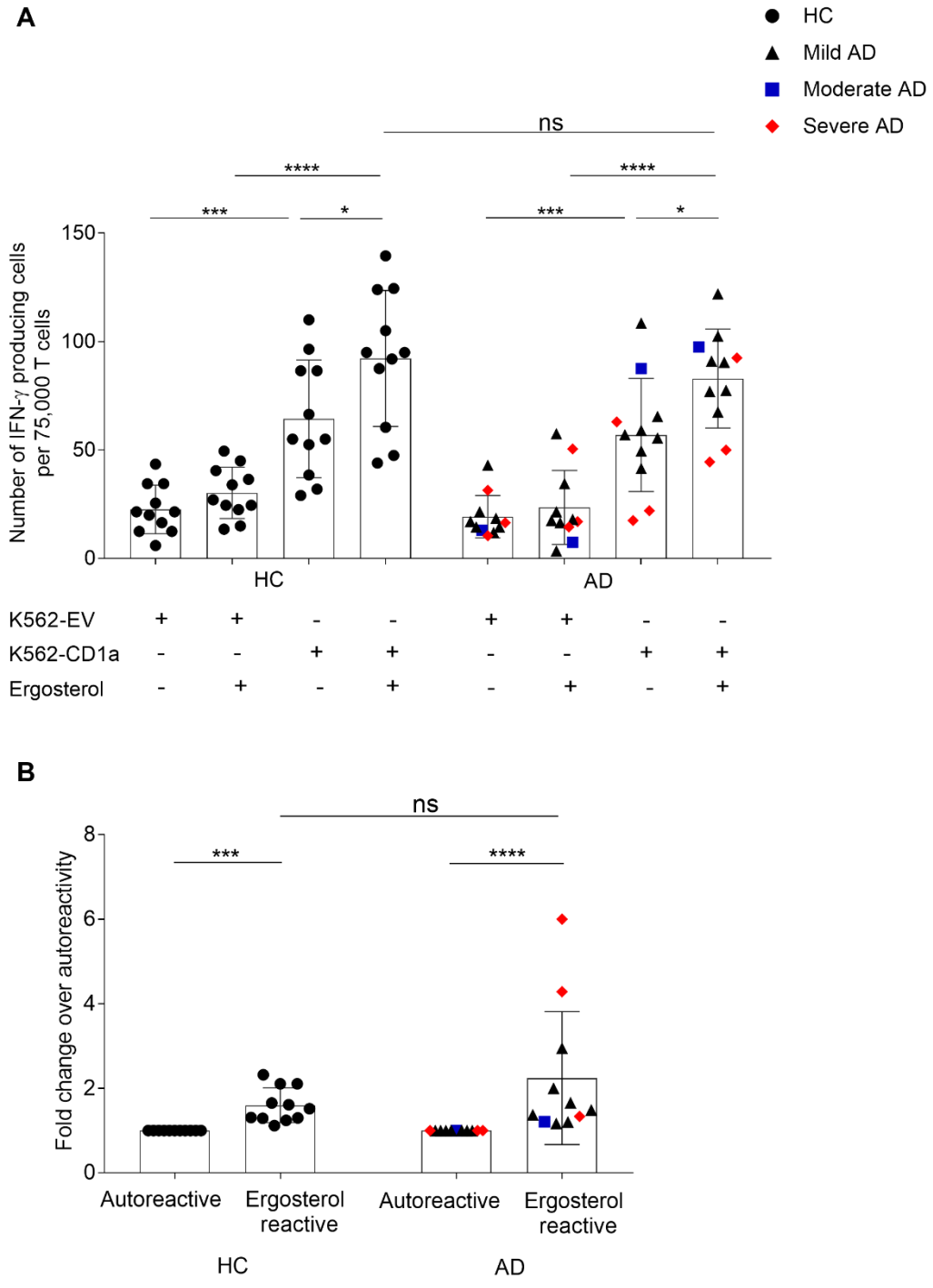


Figure 6.12 Comparing the ergosterol-responsive CD1a-reactive T cells between HC and AD.

CD3+ T cells from HC (n=11) or AD patients (n=11) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 2 µg/ml ergosterol and then IFN-γ production was measured by ELISpot. The number of IFN-γ producing T cells was compared (A) and the fold change over autoreactivity was plotted for the same cohort (B). The AD samples were colour-coded based on the severity of the disease. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

To confirm the ELISpot findings, APC-free CD1a-plate bound assay was used. Briefly, streptavidin plate was coated with biotinylated CD1a incubated at 4^{o}C overnight. Then the CD1a was loaded with ergosterol at 3 different concentrations (0.2, 2 and 20 µg/ml) in 100 µl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, 100,000 T cells/well were added and incubated for 24 hours at 37°C. After 24 hours, the concentration of IFN-γ in the supernatant was measured by ELISA. The ELISA results of the CD1a-plate bound assay showed a dose-dependent induction of T cells to produce IFN-γ in a CD1a-reactive manner when pulsed with ergosterol (Figure 6.13), confirming the findings of the cellular assay and demonstrating that the CD1a-reactivity of ergosterol detected was not due to processing of ergosterol by the APCs.

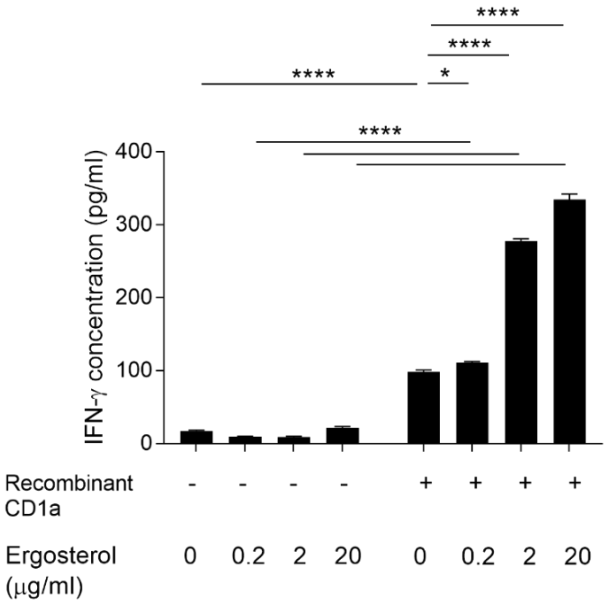


Figure 6.13 CD1a-plate bound assay of AD-derived polyclonal T cells pulsed with ergosterol.

Streptavidin plate was coated with biotinylated CD1a and then incubated at 4ºC overnight. Then, the CD1a was loaded or not loaded with ergosterol at 3 different concentrations (0.2, 2 and 20 µg/ml) in 100 µl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, 100,000 T cells/well (AD2919) were added and incubated for 24 hours at 37°C. After 24 hours, the concentration of IFN-γ in the supernatant was measured by ELISA. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

To study the ergosterol-responsive CD1a-reactive T cells further, IFN-γ secretion assay was used to generated CD1a-reactive T cell clones responsive to ergosterol. After pulsing the K562-CD1a cells with ergosterol for 16-20 hours, they were co-cultured with CD3+ T cells for 6 hours at 1:2 ration before the secretion assay was conducted. The secretion assay protocol is detailed in section 3.14. Briefly, T cells were labelled with an IFN-γ catch reagent which bind to the surface of the cells to capture the cytokine secreted by the T cells. This was detected by adding IFN-γ detection antibody which is conjugated to a fluorochrome (PE) for detection by flow

cytometry (Figure 6.14). The ergosterol-responsive CD1a-reactive T cells were then sorted at one cell per well and expanded in rapid expansion mix (refer to section 3.15). When adequate number of T cells was reached the cells were tested functionally by ELISpot (Figure 6.15).

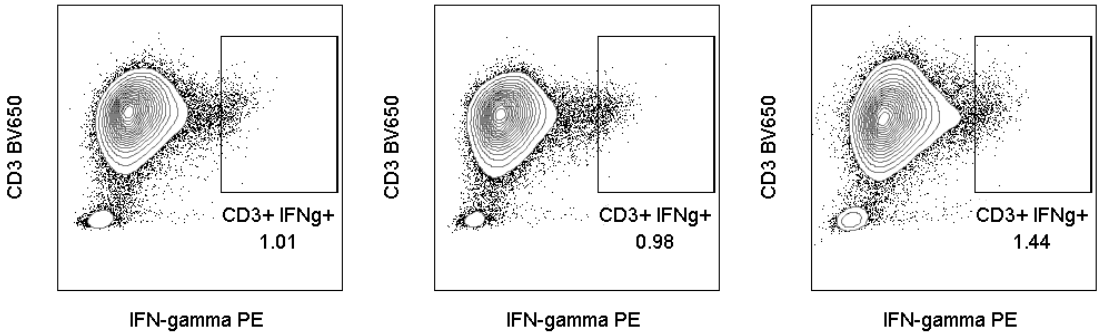


Figure 6.14 Flow cytometry plots of IFN-γ secretion assay to generate ergosterol-responsive CD1a-reactive T cell clones.

Flow cytometry plots of IFN-γ secretion assay using HC T cells (W021) comparing CD1a-unpulsed (left), vehicle control (middle) and CD1a- pulsed with 2 µg/ml ergosterol (right). Gating on cell population, singlets, live cells, CD3+ and IFN-γ+ cells. The compensation was based on single-colour stained cells.

We successfully generated the 3 ergosterol-responsive CD1a-reactive T cell clones; AB3, AB5 and AB12 illustrated in (Figure 6.15). In addition to the significant autoreactivity that was seen with all the clones, a significant ergosterol reactivity over autoreactivity was induced (Figure 6.15).

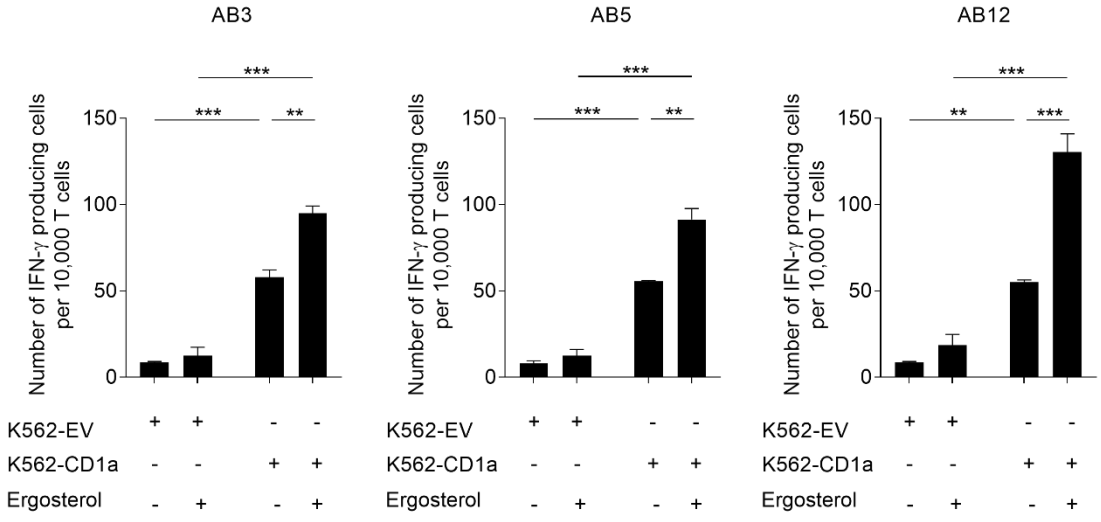


Figure 6.15 IFN-γ ELISpot of the ergosterol-responsive CD1a-reactive T cell clones.

K562-CD1a or K562-EV cells were pulsed with 2 μg/ml ergosterol for 16-20 hours and then co-cultured with T cell clones AB3, AB5 and AB12. The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

The ergosterol-responsive CD1a-reactive T cell clones were characterised phenotypically by flow cytometry (Figure 6.16). All the clones express αβ TCR and they were CD8+ and CLA+. The TCR sequence of the clones showed exactly the same α and β chains sequence with identical DNA sequence of three clones (Table 6-4), demonstrating that they were originated from the same parent clone.

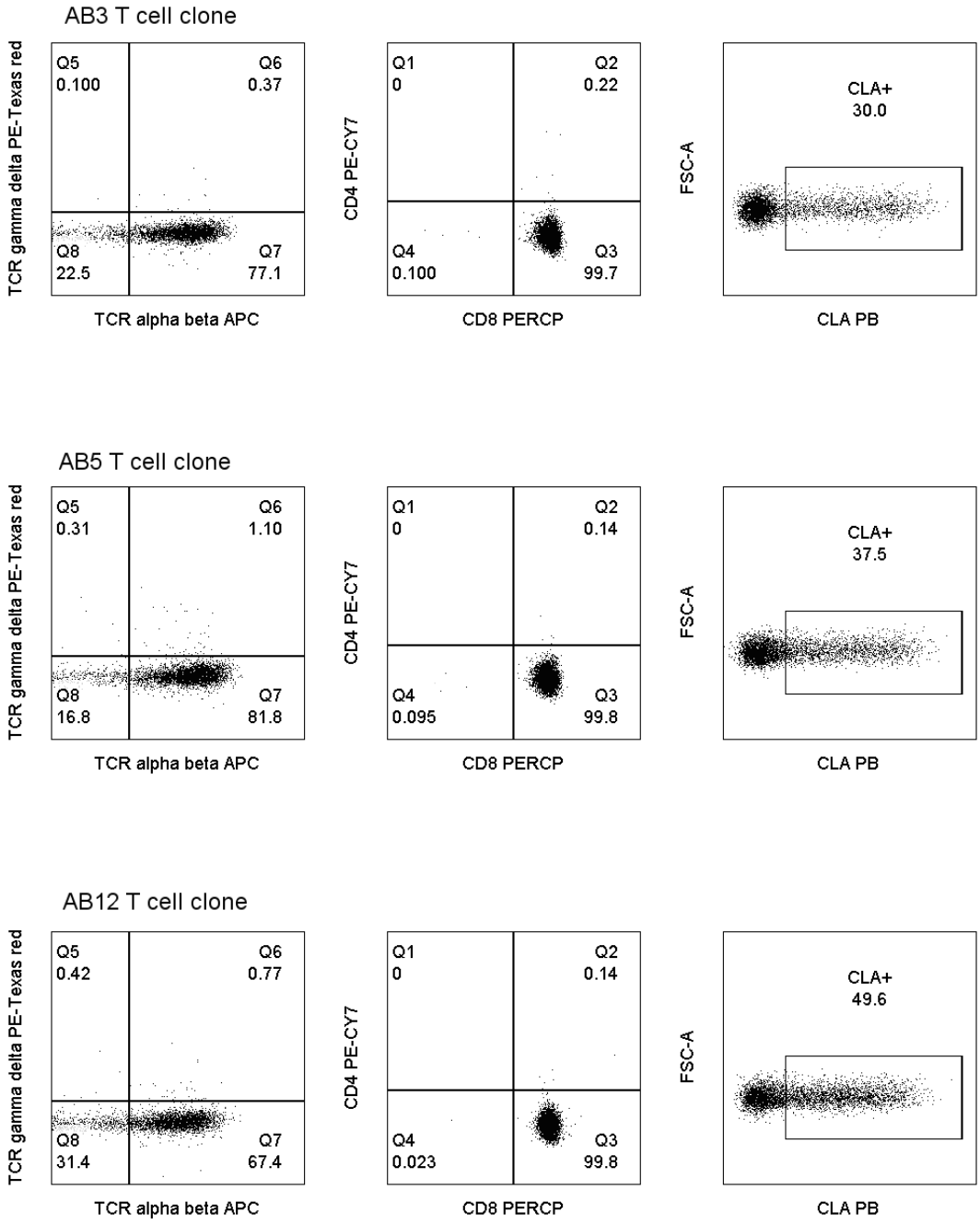


Figure 6.16 Flow cytometry plots of the ergosterol-responsive CD1a-reactive T cell clones.

The ergosterol-responsive CD1a-reactive clones were stained for surface markers (CD3, TCR αβ, TCR γδ, CD4, CD8 and CLA). Gating on population, singlets, live, CD3+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

Table 6-4. TCR sequence of the ergosterol-responsive CD1a-reactive T cell clones.

	Alpha chain			
Clone	**CDR3**	**TRAV**	**TRAJ**	**CDR3 DNA**
AB3	CASRKGSTLGRLYF	TRAV14DV4	TRAJ18	TGCGCAAGTAGGAAAGGCTCAACCCTGGGGAGGCTATACTTT
AB5	CASRKGSTLGRLYF	TRAV14DV4	TRAJ18	TGCGCAAGTAGGAAAGGCTCAACCCTGGGGAGGCTATACTTT
AB12	CASRKGSTLGRLYF	TRAV14DV4	TRAJ18	TGCGCAAGTAGGAAAGGCTCAACCCTGGGGAGGCTATACTTT

	Beta chain			
Clone	**CDR3**	**TRVB**	**TRBJ**	**CDR3 DNA**
AB3	CATTSEGVTDTQYF	TRBV13	TRBJ2-3	TGTGCCACCACAAGCGAGGGAGTGACAGATACGCAGTATTTT
AB5	CATTSEGVTDTQYF	TRBV13	TRBJ2-3	TGTGCCACCACAAGCGAGGGAGTGACAGATACGCAGTATTTT
AB12	CATTSEGVTDTQYF	TRBV13	TRBJ2-3	TGTGCCACCACAAGCGAGGGAGTGACAGATACGCAGTATTTT

As all the T cell clones had the same TCR sequence, in subsequent experiments we considered the three clones were derived from the same cell and treated them as one clone (AB clone).

Then, the AB clone was stained with NIH CD1a “endo”-tetramer (termed “endo” as it has been shown to contain multiple endogenous lipids derived from the cells of production – (Birkinshaw, Pellicci et al. 2015)) and this showed it was tetramer negative (Figure 6.17).

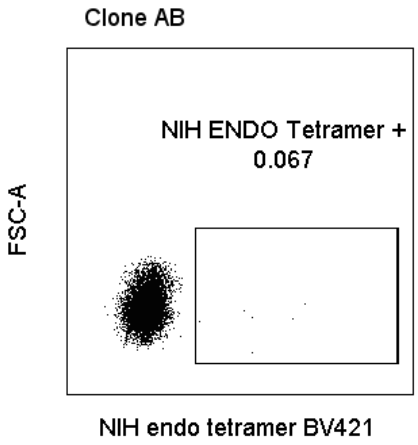


Figure 6.17 CD1a-endo tetramer staining of the AB ergosterol-responsive CD1a reactive T cell clones.

The ergosterol-responsive CD1a-reactive clone (AB) was stained with NIH CD1a endo-tetramer. Gating on population, singlets, live, CD3+, endo-tetramer+. Gating was based on FMO controls and the compensation was based on single-colour stained beads.

The CD1a-reactivity of the AB clone was tested toward *C. albicans* extract and this showed that this ergosterol-responsive clone was also reactive to *C. albicans* extracts (Figure 6.18). In contrast, we have also tested the CD1a-reactivity of the *C. albicans*-responsive CD1a-reactive T cell clones (1D6, 2D8, 6C6 and 7D8) toward ergosterol. We found that the *C. albicans*-responsive CD1a-reactive T cell clones; 2D8 and 7D8 were also reactive to ergosterol (Figure 6.19 C and D). However, clones 1D6 and 6C6 didn't show a significant reactivity to ergosterol. (Figure 6.19 A and B).

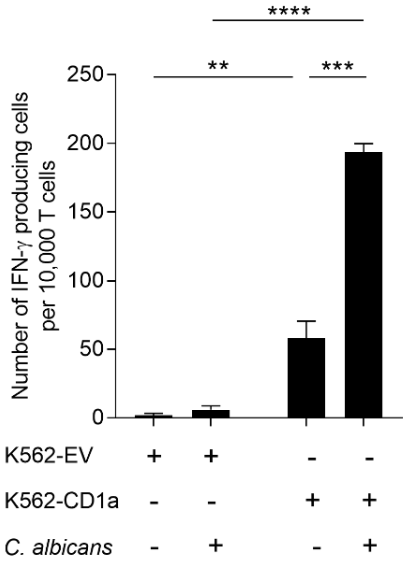


Figure 6.18 The ergosterol-responsive CD1a-reactive T cell clone reactivity toward *C. albicans* extract.

K562-CD1a or K562-EV cells were pulsed with *C. albicans* extract (skin prick test solution) for 16-20 hours and then co-cultured with AB T cell clone. The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

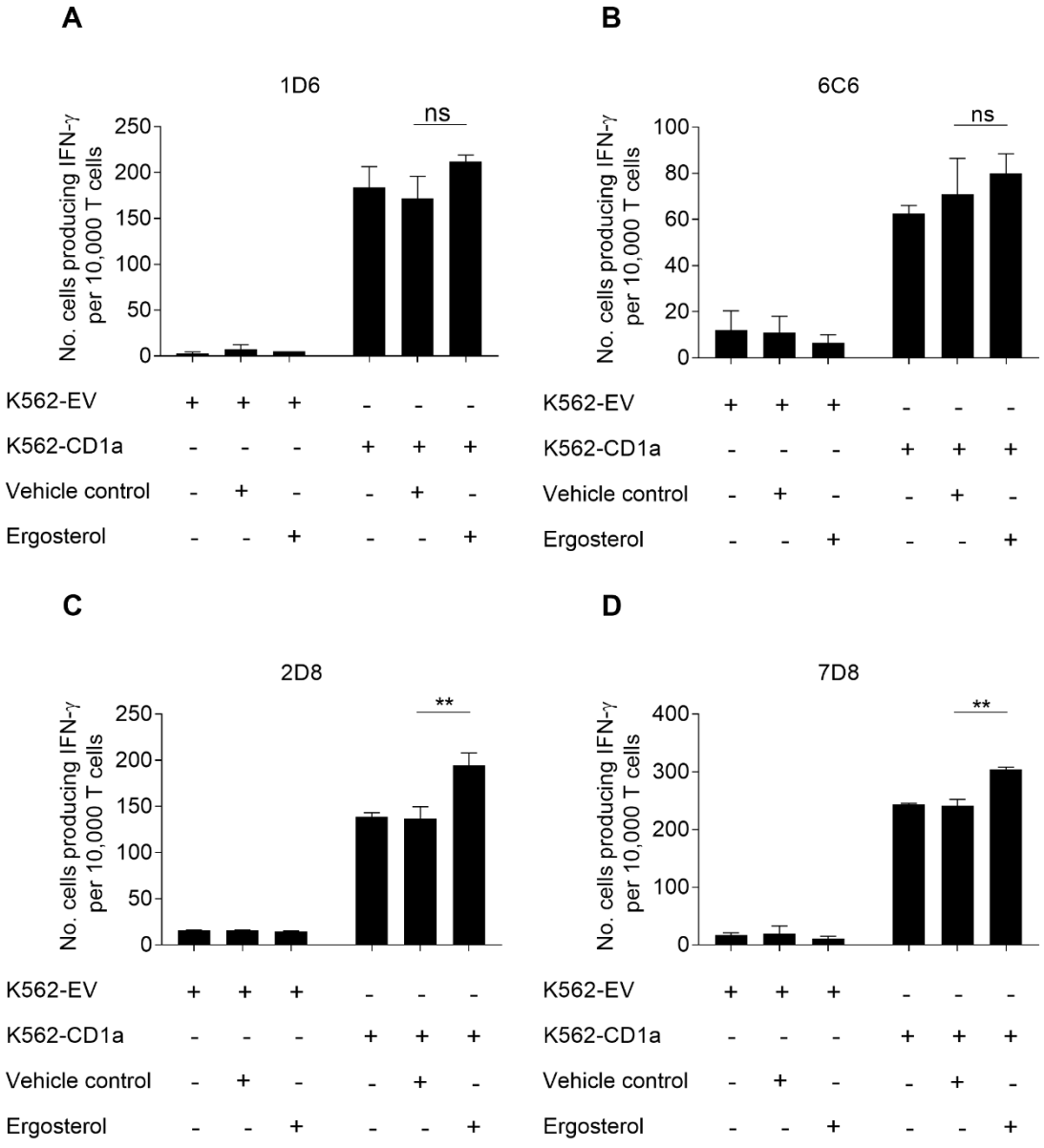


Figure 6.19 The *C. albicans*-responsive CD1a-reactive T cell clones reactivity toward ergosterol.

K562-CD1a or K562-EV cells were pulsed with 2 μg/ml ergosterol for 16-20 hours and then co-cultured with (A) 1D6, (B) 6C6, (C) 2D8 and (D) 7D8 T cell clones. The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

The ergosterol-CD1a reactivity of the AB clone was confirmed with CD1a-plate bound assay, which showed a 6-folds induction of IFN-γ when the CD1a was loaded with ergosterol compared to unloaded CD1a (Figure 6.20).

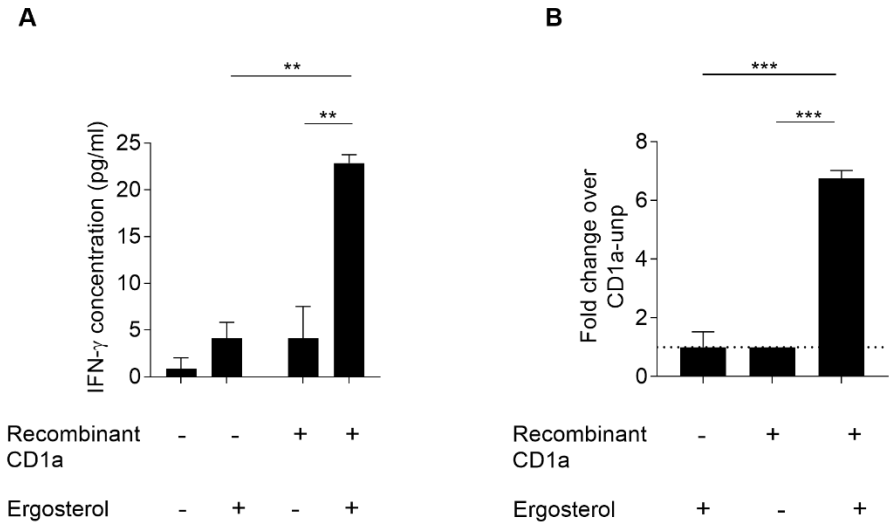


Figure 6.20. CD1a-plate bound assay of the ergosterol-responsive CD1a-reactive T cell clone.

Streptavidin plate was coated with biotinylated CD1a and then incubated at 4ºC overnight. Then, the CD1a was loaded or not with 20 µg/ml ergosterol in 100 µl citrate buffer (pH 6) and incubated for 24 hours at room temperature. After this, 100,000 T cells/well were added and incubated for 24 hours at 37°C. After 24 hours, the concentration IFN-γ in the supernatant was measured by ELISA. The IFN-γ concentration is plotted (A) and the IFN-γ fold-change over CD1a-unpulsed after subtracting the effect of T cells only (B) are illustrated. Data represent mean and standard deviation (SD) of duplicate measurements. *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

6.2.4. The CD1a-reactive T cell responses toward ergosterol esters.

As ergosterol can present in the esterified form in fungus, we then examined the role of ergosterol esters with 16 and 18 alkyl chains lengths with or without double

bonds in inducing CD1a-reactive T cells responses. Like that done in previous experiments, we pulsed K562-CD1a or K562-EV cells with different types of ergosterol esters for 16-20 hours and they were co-cultured with PBMC-derived polyclonal T cells. Then the IFN-γ production was measured by ELISpot. When comparing the total number of cells producing IFN-γ, no difference between autoreactivity and pulsing with any of these ergosterol esters was observed due to large variations between the samples (Figure 6.21 (A)). However, when the results were normalised to EV-unpulsed a clearer difference emerged. Compared to CD1a-pulsed with vehicle control, pulsing with ergosterol 16:1 and ergosterol 18:1 significantly downregulated CD1a-reactive T cell responses, with near significant results for ergosterol 18:2 (Figure 6.21 (B)).

Then we went to examine whether ergosterol esters were able to induce any CD1a-reactivity of the *C. albicans*-responsive T cell clones (1D6, 2D8, 6C6 and 7D8) or the ergosterol-responsive clone (AB). We found that ergosterol 18:1 had some inhibitory effect on the CD1a-reactvity of the T cell clones 1D6, 7D8 and AB (Figure 6.22). On the other hand, no significant difference was seen between autoreactivity and pulsing with ergosterol 18:1 for the clones 2D8 and 6C6 (Figure 6.23). Pulsing with the other types of ergosterol esters did not induce nor inhibit the CD1a-reactive T cells production of IFN-γ.

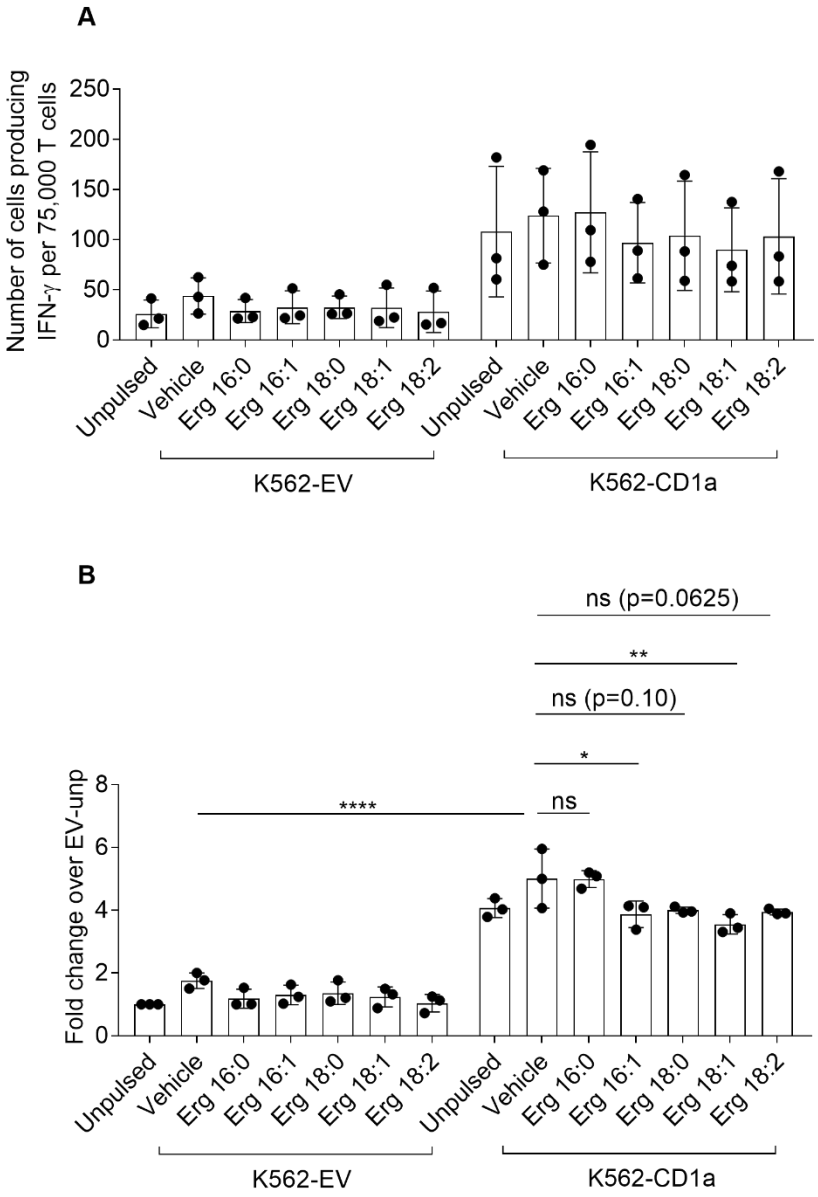


Figure 6.21 The polyclonal CD1a-reactive T cell responses toward ergosterol esters.

K562-CD1a or K562-EV cells were pulsed with 2 μg/ml ergosterol esters for 16-20 hours and then co-cultured with polyclonal PBMC-derived T cells from HC donors (n=3). The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance. Abbreviations: Erg 16:0, ergosterol palmitate; Erg 16:1, ergosterol palmatoleate; Erg 18:0, ergosterol stearate; Erg 18:1, ergosterol oleate; Erg 18:2, ergosterol linoleate.

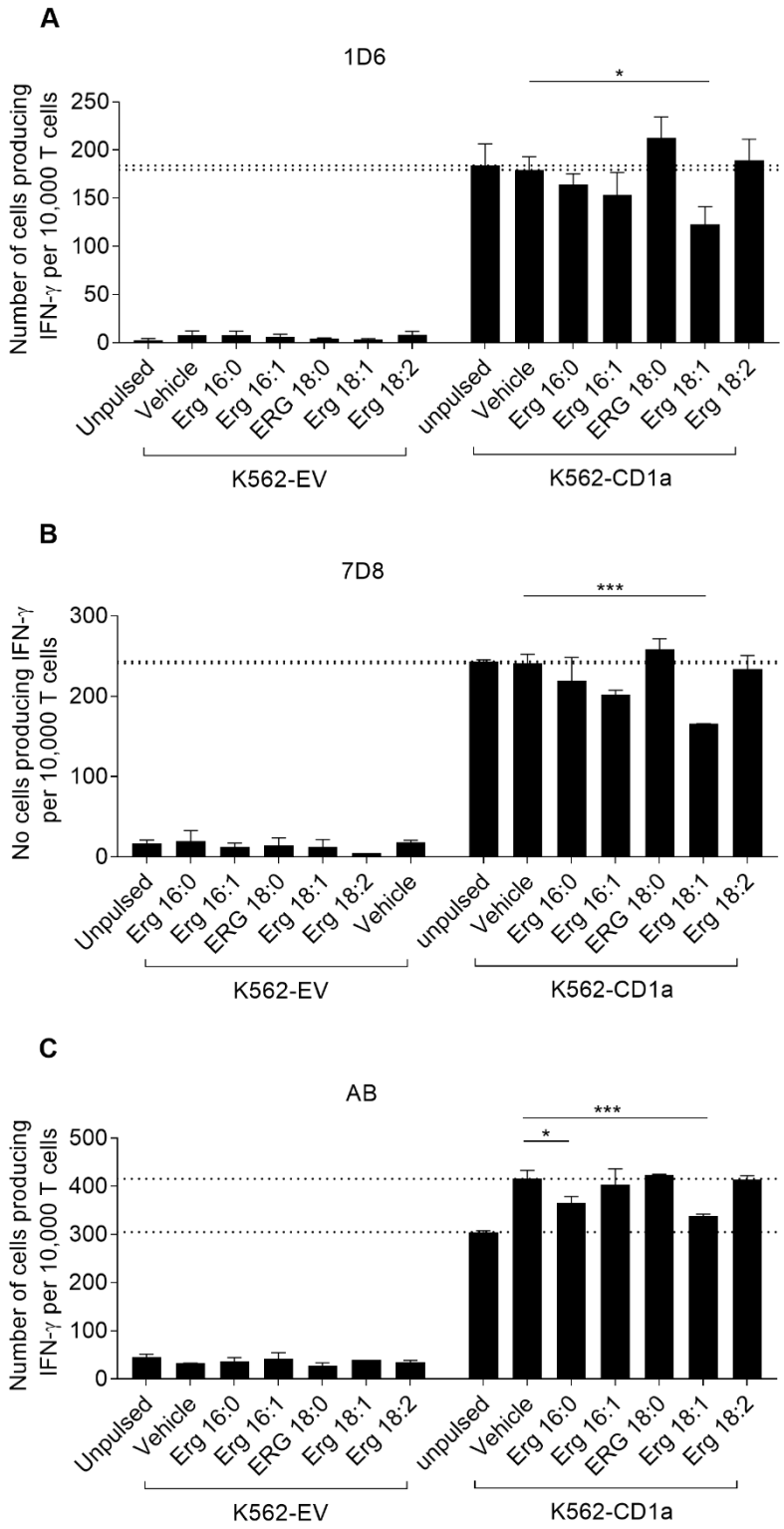


Figure 6.22 The monoclonal CD1a-reactive T cell responses toward ergosterol esters (1D6, 7D8 and AB).

K562-CD1a or K562-EV cells were pulsed with 2 μg/ml ergosterol esters for 16-20 hours and then co-cultured with T cell clones 1D6 (A), 7D8 (B) and AB (C). The CD1a-

reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance. Abbreviations: Erg 16:0, ergosterol palmitate; Erg 16:1, ergosterol palmatoleate; Erg 18:0, ergosterol stearate; Erg 18:1, ergosterol oleate; Erg 18:2, ergosterol linoleate.

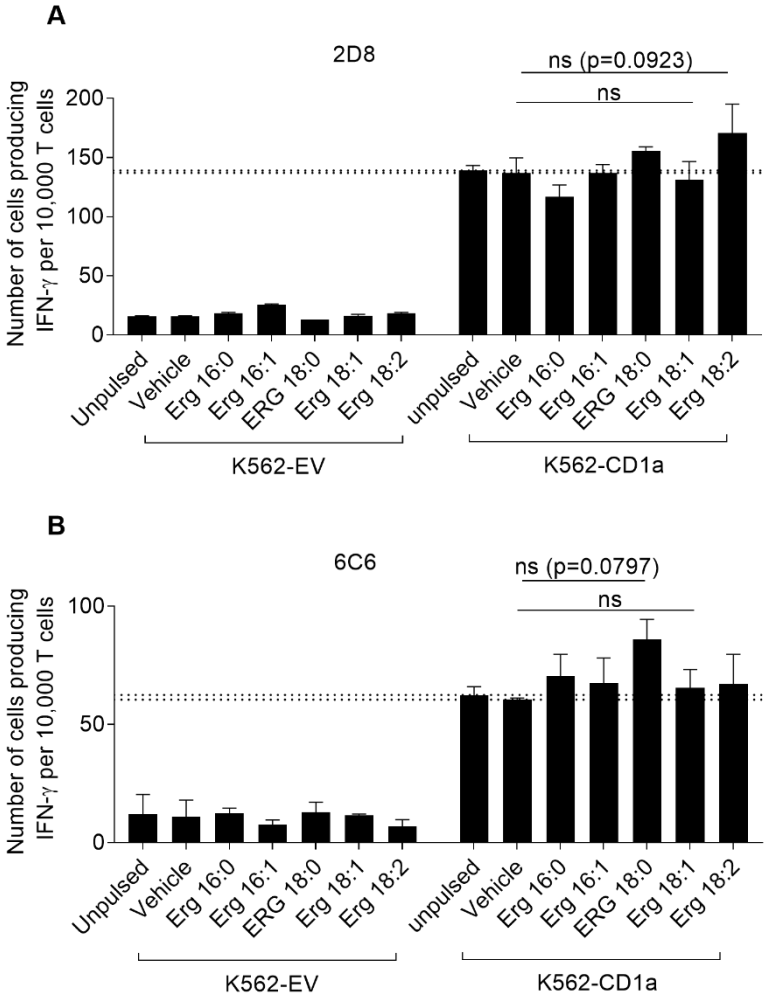


Figure 6.23 The monoclonal CD1a-reactive T cell responses toward ergosterol esters (2D8 and 6C6).

K562-CD1a or K562-EV cells were pulsed with 2 μg/ml ergosterol esters for 16-20 hours and then co-cultured with T cell clones 2D8 (A) and 6C6 (B). The CD1a-reactivity was tested by IFN-γ ELISpot. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance. Abbreviations: Erg 16:0, ergosterol palmitate; Erg 16:1, ergosterol palmatoleate; Erg 18:0, ergosterol stearate; Erg 18:1, ergosterol oleate; Erg 18:2, ergosterol linoleate.

6.2.5. The CD1a-reactive T cell responses toward squalene peroxide

Because fungi have the ability to induce squalene peroxidation, we sought to investigate whether squalene peroxide can induce CD1a-reactivity of T cell responses. K562-EV or K562-CD1a cells were pulsed with 20 μg/ml of squalene peroxide and then co-cultured with T cells for 16-20 hours before IFN-γ produced by T cells was measured by ELISpot. In a cohort of 4 healthy donor PBMC-derived T cells, no difference between autoreactivity and squalene peroxide-reactivity was noted (Figure 6.24). However, a significant stimulation of CD1a-reactive T cells when K562-CD1a cells were pulsed with squalene peroxide was observed over autoreactivity and vehicle control with AD-derived T cells (Figure 6.25).

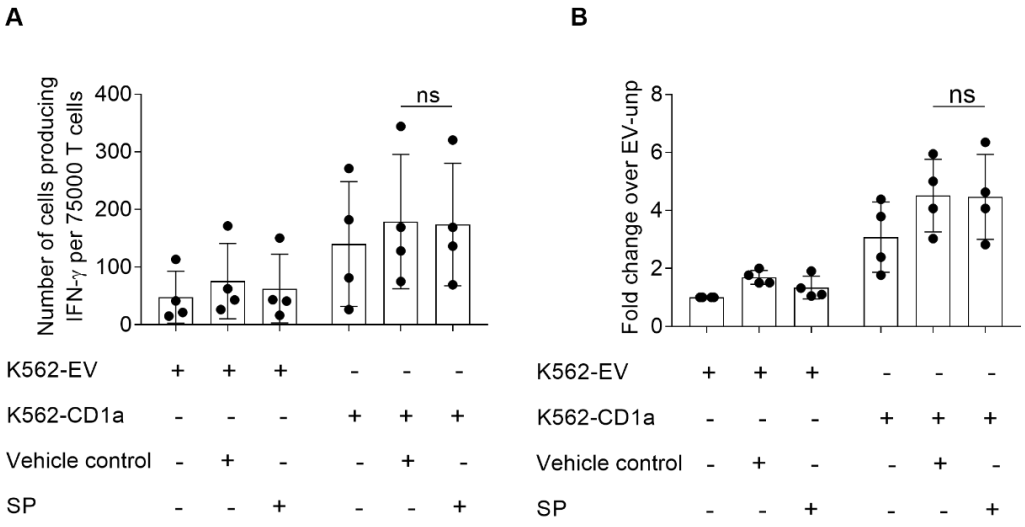


Figure 6.24 The effect of squalene peroxide on the HC polyclonal T cell CD1a-reactivity.

CD3+ T cells from HC donors (n=4) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 20 μg/ml squalene peroxide (SP) and then IFN-γ production was measured by ELISpot. The number of cells producing IFN-γ (A) and the fold change

over EV-unpulsed (B) are illustrated. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

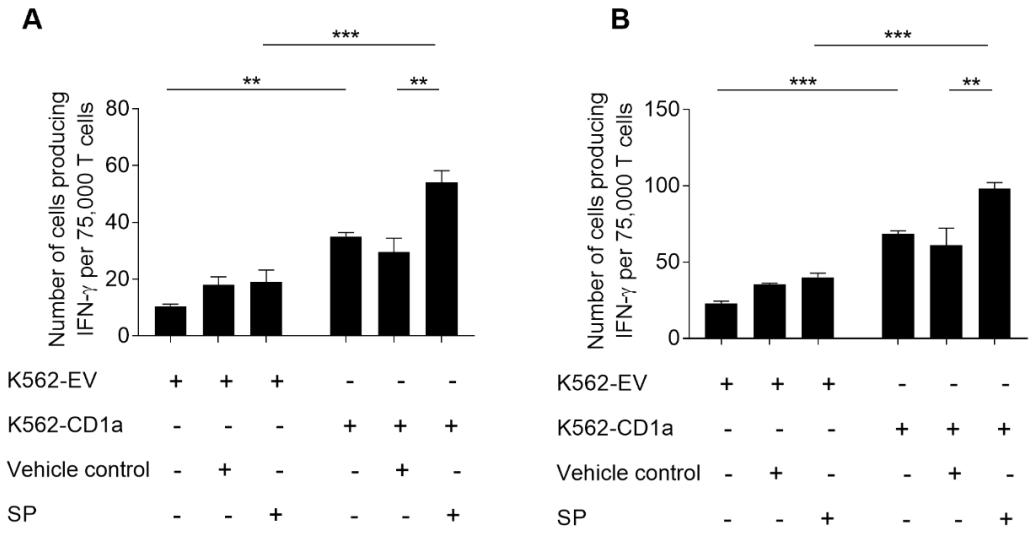


Figure 6.25 The effect of squalene peroxide on the AD polyclonal T cell CD1a-reactivity.

CD3+ T cells from AD pateints ((A) AD2009, (B) AD2013) were isolated by MACS separation technology from PBMCs, and co-cultured with K562-CD1a or K562-EV in the presence or absence of 20 µg/ml squalene peroxide (SP) and then IFN-γ production was measured by ELISpot. The number of cells producing IFN-γ is illustrated. Data represent mean and standard deviation (SD). *p<0.05; **p < 0.01; ***p<0.001; **** p < 0.0001; ns non-significance.

To confirm the ELISpot findings, we conducted an APC-free beads-based CD1a assay where biotinylated-CD1a was loaded with squlane peroxide, squalene or vehicle control and then incubated with streptavidin-dyna beads before co-culturing with PBMC-derived T cells from a HC and an AD donors. This was followed by intracellular cytokine staining for IFN-γ. The flow cytometry plots are shown in Figure 6.26 and the the data are summarised in bar charts in Figure 6.27. We found the percentage of CD3+ T cells producing IFN-γ when CD1a was loaded with squalene peroxide was higher than unloaded-CD1a (beads endo), CD1a-

loaded with vehicle control (beads mock) and CD1a-loaded with squalene in both samples.

In collaboration with Koshika Yadava (Ogg group) and Jamie Rossjohn group at Monash University, whether squalene peroxide and ergosterol esters have the tendency to displace endogenous ligands bound to CD1a was tested using isoelectric focusing (IEF) which separates molecules by differences in their isoelectric point. Compared to CD1a-endo, a shift of some of the bands was observed with lipids tested. The most striking shift was seen with squalene peroxide. The other lipids tested also induced some shift in the band with variable degrees (Figure 6.28 (A)). The crystals of CD1a-squalene peroxide were formed and mounted (Figure 6.28 (B, C)).

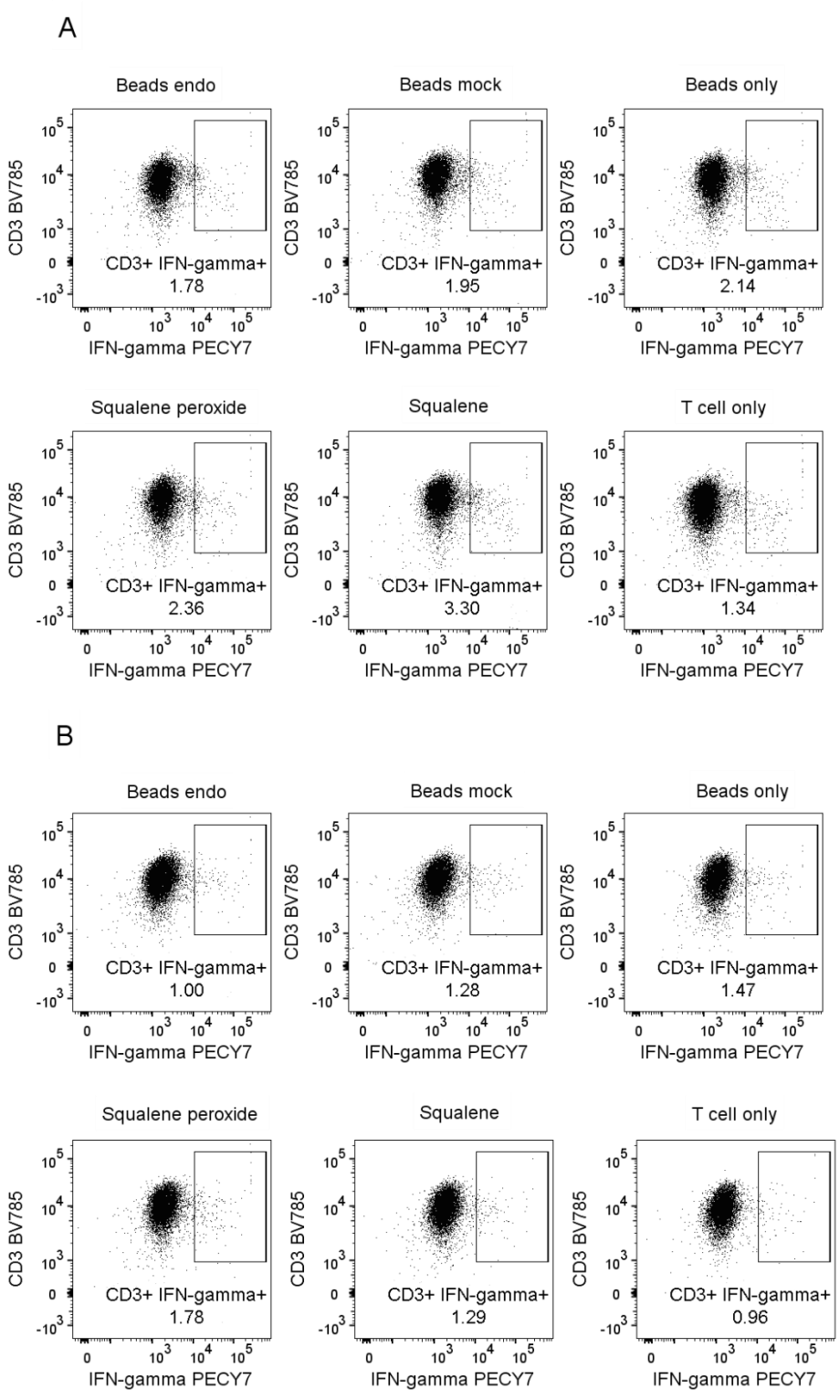


Figure 6.26 Flow cytometry plots of IFN-γ intracellular cytokine staining of polyclonal T cells pulsed with squalne peroxide (CD1a-beads based assay).

Biotinylated-CD1a was loaded with squalene peroxide (SP), squalene (SQ) or vehicle control and then incubated with streptavidin-dyna beads before co-culturing with PBMC-derived T cells from a HC donor (C2532) (A) and an AD donor (AD2531)

(B). This was followed by intracellular cytokine staining for IFN-γ. The percentage of CD3+ T cells producing IFN-γ is illustrated.

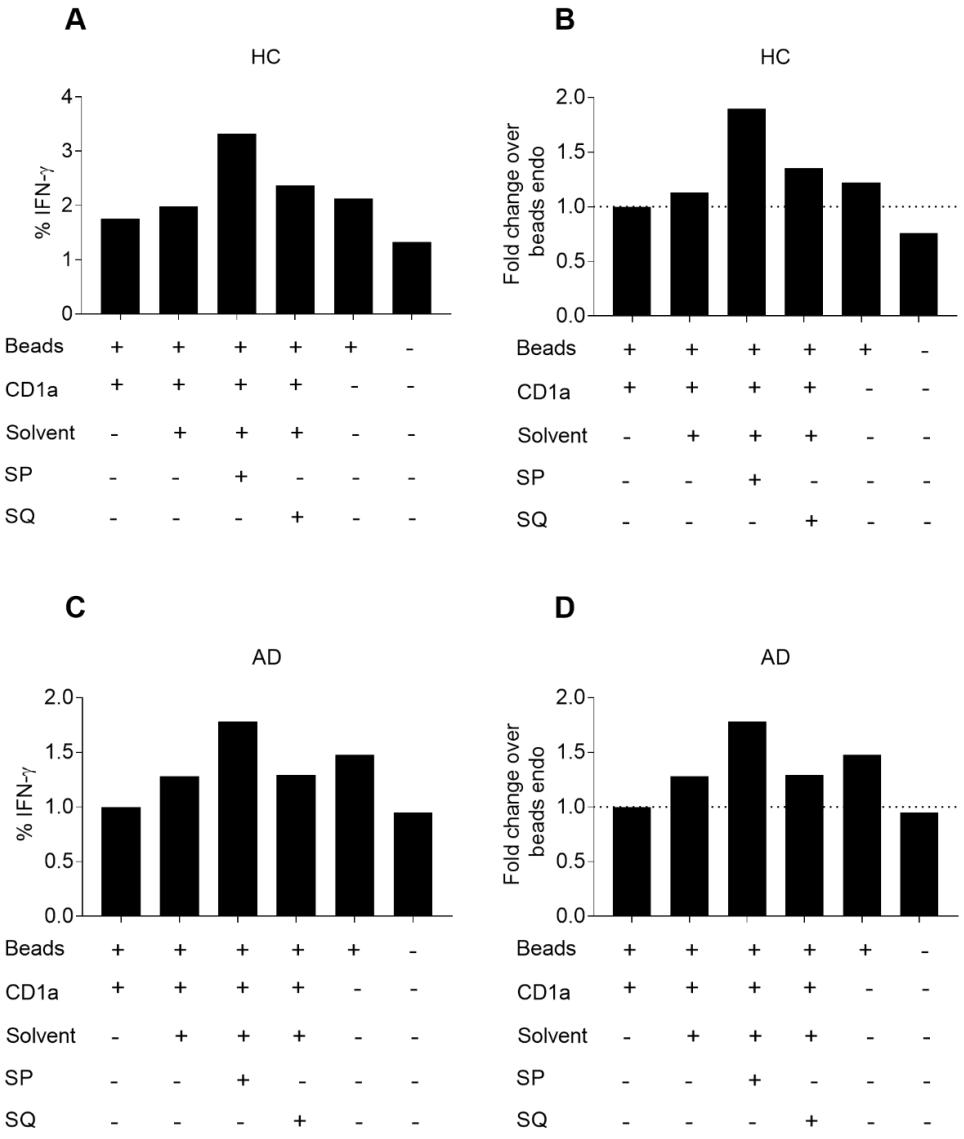


Figure 6.27 CD1a-beads based assay and intracellular cytokine staining of polyclonal T cells pulsed with squalne peroxide.

Biotinylated-CD1a was loaded with squalene peroxide (SP), squalene (SQ) or vehicle control and then incubated with streptavidin-dyna beads before co-culturing with PBMC-derived T cells from HC donor (C2532) (A, B) and an AD donor (AD2531) (C, D). This was followed by intracellular cytokine staining for IFN-γ. The percentage of CD3+ T cells producing IFN-γ (A, C) and the fold change over unloaded-CD1a (B, D) are illustrated.

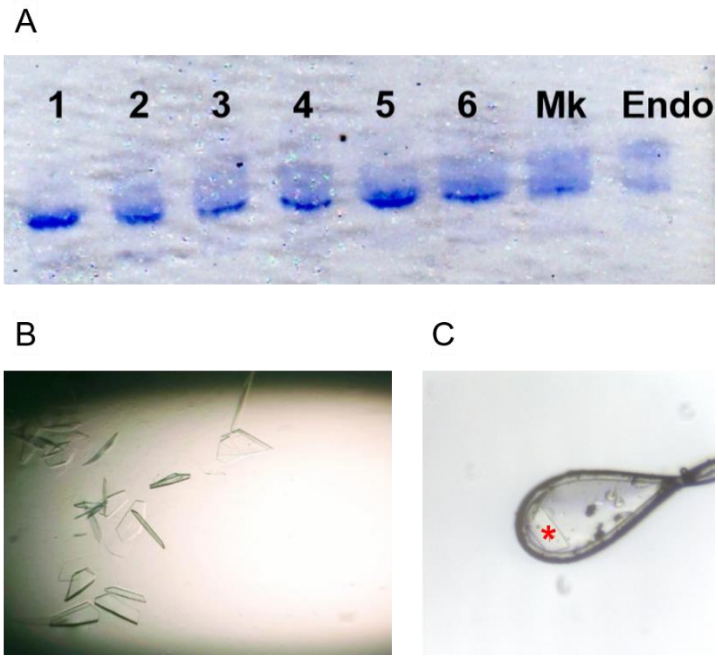


Figure 6.28 Squalene peroxide IEF and crystals

A, IEF gel. 1. squalene peroxide; 2. beta cholesteryl glucoside; 3. ergosteryl palmitate (16:0); 4. ergosteryl palmitoleate (16:1); 5. ergosteryl stearate (18:0); 6. ergosteryl oleate (18:1); Mk: mock control; Endo: CD1a loaded with endogenous lipids. B, CD1a-squalene peroxide crystals. C, mounted CD1a-squalene peroxide crystals (red star). This work was done by Koshika Yadava (Ogg group) in collaboration with Jamie Rossjohn group at Monash University, Australia.

6.3. Summary and discussion

We found that *C. albicans*-responsive CD1a-reactive T cell responses were not significantly induced by the protein components of the fungus as we were not able to see any induction of CD1a-reactive T cells when pulsing with defatted preparation of *C. albicans*. In addition, inhibiting PLB in the fungal extract did not have any inhibitory effect on the *C. albicans*-CD1a reactivity.

There are several studies in literature about the contribution of PL, especially PLA2 in the CD1a immune pathway. From previous work done on HDM responsive CD1a-reactive T cells, HDM-derived PLA2 generated neolipid antigens that activated CD1a-reactive T cell responses in the blood and skin. (Jarrett, Salio et al. 2016). Moreover, wasp and bee venom-derived PLA2 also generated endogenous neolipid antigens through cleaving cell membrane phosphodiacylglycerides into free fatty acids and lysophospholipids that activate T cells through CD1a-protein (Bourgeois, Subramaniam et al. 2015). In addition, secreted endogenous PLA2 enzyme was found to be important for autoantigenic stimulation of NKT cells in a CD1d-reactive manner (Fox, Cox et al. 2009). However, as we found that *C. albicans* extract was not rich with sPLA2, it was speculated that this enzyme was not contributing to the induction of *C. albicans*-responsive CD1a-reactive T cells; this was further consistent with the *C. albicans* genomic repertoire lacking sequence homologs of mammalian PLA1 and PLA2 genes (Theiss, Ishdorj et al. 2006). However, it is known that PLB is the most abundant PL produced by *C. albicans* as discussed in the introduction. Therefore, to study the role of this enzyme we used the PLB inhibitor: alexidine dihydrochloride. Alexidine dihydrochloride is a selective inhibitor of fungal secreted and cell-associated PLB and shown to be non-toxic to mammalian cells

(Ganendren, Widmer et al. 2004). The concentration of alexidine dihydrochloride used in our experiment was calculated according to a published study (Ganendren, Widmer et al. 2004). PLB inhibition did not alter the CD1a-reactivity toward the whole fungal extract, demonstrating that this enzyme was unlikely to be contributing to this response.

From all the trials we undertook in order to examine the role of fungal derived proteins, we found that that the lipid fraction of the fungus is more effective in inducing the T cells' CD1a-reactivity toward *C. albicans* than the fungal derived proteins and enzymes. To investigate this further, we fractionated different classes of lipids from the whole fungal extract and tested them functionally. While some of the lipids did not induce any CD1a-reactive T cells over autoreactivity, one fraction (lipid fraction III) was inhibitory and another fraction (unbound fraction) was stimulatory. This indicated that *C. albicans* lipid mass contains a mix of candidate lipid ligands that inhibit or stimulate CD1a immune reaction. However, because the cumulative effect of *C. albicans* extract induced CD1a-reactive T cell responses, as we discussed in chapter 4, we speculated that the stimulatory effects of the lipid fractions are more potent than the inhibitor ligands.

From work done by David Priestman (Platt laboratory, Department of Pharmacology, University of Oxford), mass spectrometry analysis revealed that the sterols and their ester derivatives were abundant in the unbound lipid fraction. The unbound lipid fraction is the total lipids that were dissolved in chloroform and did not bind to the aminopropyl column. Lipid fraction II and lipid fraction III which gave an inhibitory effect on the CD1a-reactive T cells should be investigated further in future experiments.

As discussed in the introduction, ergosterol and its metabolites are major components of the fungal lipid pool and they are unique to fungi and not present in human tissue, therefore we went on to further investigate their role in the CD1a-reactivity of T cells as exogenous CD1a-ligand candidates. We found that ergosterol induces CD1a-reactive polyclonal T cell responses in the AD and the HC cohort. When disease severity was compared, two out of three severe AD samples were observed to induce the highest CD1a-reactive T cell responses toward ergosterol, predicting that there might be a correlation between the disease severity and the proportion of IFN-γ-producing ergosterol-responsive CD1a-reactive T cells. A bigger cohort of AD samples should be investigated in future experiments in order to draw a conclusion about this prediction.

This was confirmed with CD1a-plate bound assay that demonstrated that the CD1a-reactivity of ergosterol was not due to processing of ergosterol by the APCs. We then generated ergosterol-responsive CD1a-reactive monoclonal T cells and confirmed their functionality after expansion. Phenotypically, all the three clones were CD8+ and TCR αβ+. They also have the ability to home to the skin as they are capable of expressing the skin homing marker CLA. It was noted that not all the cells were CLA-positive; CLA expression is known to vary on individual T cell populations (Seneviratne, Jones et al. 2005). The TCR sequence of the clones revealed that all the clones are generated from the same parent T cell. CD1a ternary complex with lysophosphatidylcholine or endogenous lipids and BK6 T cell clone, BKT6 TCR sequence was TRAV12-3*01 TRBV6-2*01 (Birkinshaw, Pellicci et al. 2015). Although our ergosterol-responsive CD1a-reactive T cell clone also expressed TCR αβ, it has a different variable chain usage (TRAV14-4 TRBV13). Interestingly, the ergosterol-responsive CD1a-reactive T cell clone was negative

for CD1a-endo-tetramer staining. This might be explained by the specificity of this clone to exogenous fungal derived lipid and not to endogenous lipids presented by the endo-CD1a tetramer. As ergosterol is a major component of the fungal lipid mass, it was not surprising that the ergosterol-responsive clone was also responsive to *C. albicans* extract in a CD1a-dependent manner. However, only two out of four *C. albicans*-responsive CD1a-reactive clones tested were reactive to ergosterol. One potential explanation of this is that these clones might be specific to other *C. albicans*-derived lipids, not necessarily the ergosterol. The difference between the reactivity toward ergosterol of the two *C. albicans*-responsive CD1a-reactive αβ T cell clones (1D6 and 7D8) -which have identical TCR sequence- might be attributed to differences in the stage of activation when these clones were sampled, leading to different responses in the experiment.

Since ergosterol is also present in the esterified form within *C. albicans*, we investigated the role of some relevant ergosterol esters in the CD1a-reactvity of T cells. We chose ergosterol esters with C16 and C18 alkyl chain length because the CD1a binding groove select lipids with alkyl chain length (C16-C23) to match the volume of the pocket (Silk, Salio et al. 2008). Interestingly, related cholesterol esters have been shown to be ligands for CD1c (Mansour, Tocheva et al. 2016). This includes β-cholesteryl glucoside (C14), cholesteryl linoleate (C18:2) and cholesteryl oleate (C18:1). While ergosterol stimulated CD1a-reactive T cells, we found that ergosteryl oleate (C18:1) and ergosteryl palmitoleate (C16:1) significantly inhibited polyclonal CD1a-reactive T cells below autoreactivity with near significant inhibition with ergosteryl linoleate (C18:2). Similar results were seen with the some of our *C. albicans*-responsive CD1a-reactive clones (1D6 and 7D8) and the ergosterol responsive CD1a-reactive clone (AB) where we found

some degree of inhibition of below autoreactivity when the K562-CD1a cells were pulsed with ergosteryl oleate (C18:1). It is possible that fungal generation of esterified forms of ergosterol may provide some capacity to escape from CD1a pathway recognition. Beside ergosterol esters, it is of interest to investigate the role of other forms of ergosterol such as the oxidative product of ergosterol, ergosterol peroxide in this CD1a-reactive response. Ergosterol peroxide was studied widely in the field of cancer and showed to exhibit an anti-tumour effects (Russo, Cardile et al. 2010, Han, Sohn et al. 2014, Tan, Pan et al. 2017).

After investigating the role of fungal-derived lipids, we sought to investigate the role of endogenous lipids processed by fungal enzymes such as squalene peroxide. We found that squalene peroxide induces CD1a-reactive T cell responses using ELISpot and CD1a-beads based assay. From work done in collaboration with Jamie Rossjohn group at Monash University, we found that squalene peroxide can displace endogenous ligands bound to CD1a, however the actual fitting of squalene peroxide into the CD1a groove is yet to be confirmed. The crystals of CD1a-squalene peroxide were formed and mounted, and the data are pending. Preliminary analyses show density compatible with squalene peroxide within the CD1a hydrophobic pocket.

We speculated that ergosterol and squalene peroxide might be presented to T cells through the absence of interference model where headless lipids are embedded within the CD1a molecule, and the TCR binds to the left side of the CD1 molecule (e.g. CD1a A′ roof) without a direct contact with the lipid; a phenomenon known as the left–right mismatch (Cotton, Shahine et al. 2018). Whereas ergosterol esters might be presented through the interference model where the head group interferes with the CD1a A′ roof and TCR binding (Cotton, Shahine et al. 2018).

Overall these data showed that fungal-derived lipids like ergosterol and its derivatives or endogenous lipids processed by fungal enzymes like squalene peroxide, were contributing to the CD1a-reactive T cell responses induced by *C. albicans*. Preliminary data suggested that those lipids might serve as neolipid antigens that bind CD1a.

7. General discussion

Atopic skin lesions are characterised by an altered lipid profile and CD1a-expressing APCs are enriched in AD lesions, and there is an association between *C. albicans* and disease, but no previous studies have investigated the specific role of fungal-responsive CD1a-reactive T cells in the pathogenesis of AD. Understanding the pathogenesis of AD is crucial for broader understanding of the underlying mechanism of the disease especially that it is one of the commonest inflammatory skin diseases with a huge burden on the patients' quality of life and health-care resources.

The contribution of CD1a-reactive T cells to cutaneous immune responses and particularly the ability of fungal antigens to enhance this inflammatory process are not yet elucidated. In order to further understand the fungal-responsive CD1a-reactive T cells, this thesis aimed to address several questions about the frequency and phenotype of these cells and their role in fungal immune responses, cutaneous immunity and atopic disease. Polyclonal and monoclonal CD1a-reactive T cells were studied in blood and skin of healthy control donors and patients with AD upon pulsing with *C. albicans* extract or fungal-lipids. This led to the discovery of a novel pathway of inflammation through the induction of CD1a-reactive T cells in response to fungal antigens. Two approaches were followed in this project. The first was based on testing broadly the CD1a-reactive T cell responses of whole fungal extracts. The second approach was based on testing the CD1a-reactivity of fungal-derived lipids through lipid fractionation or testing specific exogenous fungal-lipids (ergosterol and its derivatives) and endogenous lipid modified by fungal enzymes (squalene peroxide).

In the first results chapter (chapter 4), we found that autoreactive and *C. albicans*-responsive CD1a-reactive T cells present in the blood and skin of healthy controls and are enriched in AD samples and secrete relevant cytokines to AD pathogenesis and fungal immunity such as IFN-γ, IL-17A and IL-22. In addition to the important role of these cytokines in AD (as described in chapter 4), type 2 cytokines including IL-4, IL-5 and IL-13 contribute significantly to AD pathogenesis by inducing IgE class switching, enhancing the B cell production of antigen-specific IgE and recruiting eosinophils, basophils and mast cells to the dermis. Antigen-specific IgE antibodies bind to high-affinity immunoglobulin-ε receptors (FcεR1) expressed on the surface of the skin APCs which facilitates the uptake of allergens and initiate T cell-mediated immune response leading to a rapid and more potent activation of LCs in AD (Maurer, Ebner et al. 1995, Bieber 1997) and through IgE-mediated antigen uptake and presentation to T cells (Cooper, Hales et al. 2004). The role of IgE in fungal immune responses in unclear and not fully characterised (Verma, Wuthrich et al. 2014) - type 2 cytokines are not thought to be the key cytokines relevant to the fungal defence immune responses (Szymczak and Deepe 2009, Romani 2011). However, AD patients were found to be sensitised to fungal antigens and around 30% of them were positive for *C. albicans*-specific IgE (Back, Scheynius et al. 1995, Lindgren, Wahlgren et al. 1995, Khosravi, Bandghorai et al. 2009). The role of fungi in triggering the development of the allergic response in patients with AD through fungal-specific IgE is still to be elucidated.

In order to extend our understanding of the role of fungal-responsive CD1a-reactive T cells in health and disease, in the second results chapter (chapter 5) we generated αβ and γδ *C. albicans*-responsive CD1a-reactive T cell clones with diverse TCR and characterized them phenotypically and functionally. It is of

interest that some of the *C. albicans*-responsive CD1a-reactive T cell clones expressed γδ TCR, as to date, virtually all reported CD1a-reactive T cell clones were αβ (Shamshiev, Gober et al. 2002, Van Rhijn, Zajonc et al. 2005, de Jong, Cheng et al. 2014, Birkinshaw, Pellicci et al. 2015), except one study reported a γδ1+ TCR from one T cell clone (Agea, Russano et al. 2005). A further study suggested that γ9δ2+ TCR are enriched in psoriatic skin lesions but antigen specificity was not examined (Laggner, Di Meglio et al. 2011). This raised the question of whether fungal derived phosphoantigens and/or non-phosphoantigens might act as potential ligands for γδ T cells. We next investigated the involvement of T cell co-receptors in the fungal immune response. We found that the NKG2D receptor expressed on the surface 6C6 γδ T cell clone contributed partially to the responses, demonstrating that NKG2D enhances the CD1a-reactive TCR-dependent responses seen with *C. albicans* especially during inflammation, for example in AD. However, one of the γδ T cell clones was NKG2D-negative suggesting that NKG2D cannot contribute in all cases.

Hence *C. albicans* pathogen-associated molecular patterns (PAMPs) are recognised by several types of pattern-recognition receptors (PRRs) expressed on the surface of APCs (Netea, Joosten et al. 2015), it is of interest to discuss how these PRRs such as CLRs contribute to the fungal-sensing system. The CLRs dectin 1, CR3, mannose receptor, dectin 2 and DC-SIGN are the most important family of innate receptors for the recognition of *C. albicans* PAMPs such as β-glucans, mannans and mannoproteins (Brown and Gordon 2001, Cambi, Netea et al. 2008, van Bruggen, Drewniak et al. 2009, van de Veerdonk, Marijnissen et al. 2009, Yamasaki, Matsumoto et al. 2009, Saijo, Ikeda et al. 2010, Lowman, Greene et al. 2014, Netea, Joosten et al. 2015). APCs recognise and take up

microbial pathogens through PRRs and then distribute them to the endocytic compartments (Salio and Cerundolo 2015). CD1a may then acquire these foreign lipids, before trafficking back to the plasma membrane through the adenosine diphosphate (ADP) ribosylation factor 6 (ARF6)-dependent pathway (Sugita, Grant et al. 1999). In addition, CD1a can capture exogenous lipids directly on the cell surface (Manolova, Kistowska et al. 2006). It is of interest that some subtypes of T cells express PRRs (Kabelitz 2007, Dar, Patil et al. 2014). This raised the question whether the response we saw in our system is due to CD1a-TCR interaction or direct activation of T cells through PRRs presented on the surface of T cells that recognise fungal-derived PAMPs. However, we found that all the *C. albicans*-responsive CD1a-reactive T cell clones tested didn't express PRRs such as TLR2, TLR4, dectin-1 and dectin-2 (Appendix, Figure 8.1).

In the third results chapter (chapter 6), we found that *C. albicans*-responsive CD1a-reactive T cell responses were not mediated by the fungal-derived phospholipases in contrast to that published before for HDM and bee/wasp venom (Bourgeois, Subramaniam et al. 2015, Jarrett, Salio et al. 2016) . Instead, we found that fungal-derived lipids such as ergosterol and its derivatives or endogenous lipids processed by fungal enzymes (e.g. squalene peroxide), were contributing to the CD1a-reactive T cell responses induced by *C. albicans*. Preliminary data suggested that those lipids might serve as novel lipid antigens that bind CD1a and this should be confirmed by crystal structure.

Squalene is part of the ergosterol synthetic pathway, is a known CD1a ligand (Layre, de Jong et al. 2014) and is a major human sebaceous lipid (Niki 2015). Due to its unique structure of six double bonds, squalene is oxidised easily into squalene monohydroperoxide or what is known as squalene peroxide and other

oxidative degradation products such as epoxides, and aldehydes (Kostyuk, Potapovich et al. 2012). In general, lipids are oxidized by different mechanisms including enzymatic oxidation, free radical chain oxidation, and non-radical non-enzymatic oxidation (Niki 2015). In the skin, environmental oxidants (e.g. photo-oxidation) and microbial-derived enzymes (e.g. lipoxygenases) play a major role in the formation of lipid oxidation and therefore formation of squalene peroxide (De Luca and Valacchi 2010, Kostyuk, Potapovich et al. 2012, Niki 2015, Pham, Boussouira et al. 2015). Squalene peroxides have been implicated in the pathogenesis of acne (Ottaviani, Alestas et al. 2006) and dandruff (Jourdain, Moga et al. 2016). From our findings we speculated that squalene peroxide might contribute to skin inflammation through the induction of CD1a-reactive T cells in the skin.

It is possible that the CD1a-reactive T cell responses are part of a barrier sensing system that if activated, can induce other adaptive immune responses and cutaneous inflammation. In steady state, there is a physical separation between the fungal antigens on the surface of the skin and the APCs present in the epidermis. In addition, there is a balance between the stimulatory and the inhibitory endogenous CD1a-ligands. These two immune mechanisms may lead to maintaining the control of CD1a-reactive T cell responses. However, we speculated that in the case of skin barrier defects and microbial dysbiosis in the context of AD, fungal-derived antigens penetrate the skin and contact with APCs and therefore amplify the inflammatory pathway. Fungal antigens are taken up and processed by LCs in the epidermis before migrating to local lymph nodes for priming naive T cells through CD1a-lipid presentation. Then T cells upregulate skin homing markers and migrate to the skin where they contact antigen-exposed

DCs/LCs and secrete different cytokines that exacerbate the inflammatory process of AD (including IFN-γ, IL-17 and IL-22). We speculate that migratory LCs or dermal DCs present fungal-derived lipids via CD1a to resident memory T cells or T cells recruited from the circulation which amplifies the cutaneous inflammation in AD. The proposed mechanism of *C. albicans*-responsive CD1a-reactive T cell stimulation is illustrated in Figure 7.1.

The clinical consequences of CD1a deficiency is not elucidated, however lack of CD1a expression was associated with defect in mycobacterial lipid antigens presentation to T cells (Seshadri, Shenoy et al. 2013) and with an increase in susceptibility to *Mycobacterial tuberculosis* infection (Seshadri, Thuong et al. 2014). Reduction in CD1a expression on cutaneous APCs was reported in two Vietnamese healthy individuals and was attributed to a single nucleotide polymorphism (SNP) (Cerny, Thi Le et al. 2016). Another study on a Caucasian population identified another SNP in the 5′ untranslated region that was common and strongly associated with low CD1a expression on the surface of MO-DCs at protein and mRNA levels (Seshadri, Shenoy et al. 2013). This demonstrated that CD1a expression is regulated at the genetic level and the varying expression of CD1a on human DCs may contribute to differences in the autoreactive and antigen-responsive CD1a-reactive T cell responses within the population.

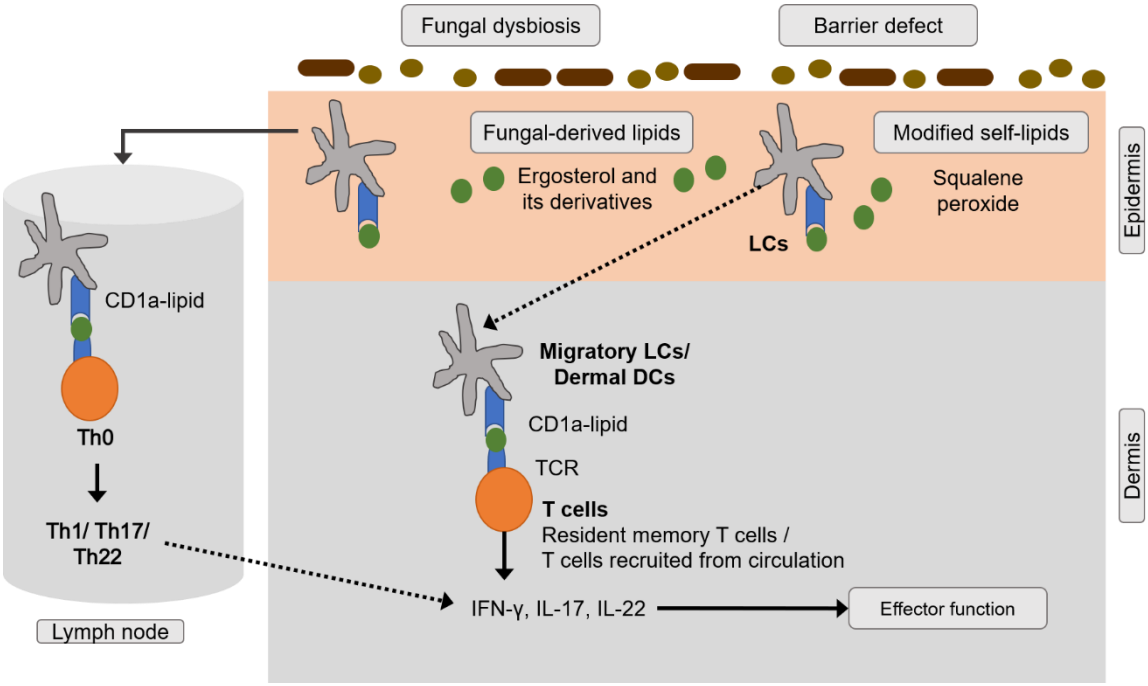


Figure 7.1 Proposed mechanism of *C. albicans*-responsive CD1a-reactive T cell stimulation.

The fungal dysbiosis and the barrier defect in AD allows fungal-derived antigens to penetrate the skin. Skin APCs take up these exogenous antigens or self-antigens modified by fungal enzymes, migrate to regional lymph node to primes T cells through presenting those lipid antigens via CD1a molecule. T cells then upregulate skin homing markers (CLA, CCR4, CCR10) and subsequently migrate to the dermis and release effector cytokines including IFN-γ, IL-17A and IL-22. Alongside this, migratory LCs or dermal DCs present fungal-derived lipids to resident memory T cells or T cells recruited from the circulation via CD1a molecule to promote effector T cell function which may amplify the cutaneous inflammation in AD.

In addition to the genetic regulation of CD1a expression, there is also regulation by other mediators, such as serum lipids. Leslie et al found that serum lipids inhibited the expression of CD1a on the surface of DCs which therefore inhibited DCs maturation and function, and subsequently affected the activation of CD1a-reactive T cells. This alteration of CD1a expression was explained by changes at the level of CD1a gene transcription (Leslie, Dascher et al. 2008). The serum lipids responsible for this inhibition were characterised to be phospholipids presented in

human serum, specifically lysophosphatidic acid and cardiolipin. This was found to be dependent on the activation of the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) that regulates the expression of genes related to lipid metabolism and uptake (Leslie, Dascher et al. 2008). Besides serum lipids, cytokines contribute to the regulation of CD1a expression on the surface of APCs. The differentiation of monocytes into CD1a+ MO-DCs is induced by the presence of IL-4 and GM-CSF together in the culture (Holla, Sharma et al. 2014). IL-4 alone or GM-CSF alone does not have the ability to upregulate CD1a expression (Roy, Bandyopadhyay et al. 2004, Holla, Sharma et al. 2014). The tight regulation of CD1a expression is also accompanied by regulation of loading of self-lipids into CD1a molecules which was found to be maintained by the lipid transfer protein, microsomal triglyceride transfer protein (MTP) (Kaser, Hava et al. 2008). Inhibition of MTP results in decreased activation of CD1a-reactive T cells that are reactive to self-lipids (Kaser, Hava et al. 2008). This raises the question whether fungi contribute to the regulation of CD1a expression by APCs and how does this affect the responses of CD1a-reactive T cells – this will be the focus of future studies.

We do not believe that AD is a fungal infection, however, we speculated that the skin barrier defect (e.g. FLG null mutation), microbial dysbiosis and superimposed infections in AD amplify the inflammatory process and therefore exacerbate the disease. The use of anti-fungal drugs in *Malassezia*- and/or *C. albicans*-IgE-positive AD patients decreased the levels of total serum IgE as well as fungal-specific IgE antibodies. In addition, such treatment has showed some clinical improvement, but without full resolution of the disease, in subgroups of patients with refractory/severe AD or patients with head and neck AD (Back, Scheynius et al. 1995, Kolmer, Taketomi et al. 1996, Adachi, Horikawa et al. 1999, Bäck and

Bartosik 2001, Lintu, Savolainen et al. 2001, Nikkels and Pierard 2003, Ikezawa, Kondo et al. 2004). However, this clinical improvement of AD might not be only explained by the antimycotic effect of the drugs. For instance Ketoconazole in addition to its anti-fungal mechanism of action through inducing cell membrane damage, disruption of microtubule function and inhibition of ergosterol synthesis, it exerts anti-inflammatory effects by the inhibition of the 5-lipoxygenase pathway, inhibition of IL-2-dependent T-cell proliferation, suppression of IL-4 and IL-5 production and interference with allergen-presenting cell function (Nikkels and Pierard 2003).

These are the first demonstrations that fungi can be recognised through the CD1a-lipid presentation pathway and so have implications for fungal defence and skin inflammation; and subsequently would have relevant therapeutic implications. This might be approached through blocking downstream effects of the pro-inflammatory cytokines such as IFN-γ or blocking CD1a on the surface of APCs in the skin directly in order to abolish the inflammation driven by CD1a-reactive T cells. As we found that squalene peroxide is a potential stimulatory ligand for CD1a-reactive T cells, inhibiting fungal-induced peroxidation of squalene would be another treatment modality. Inhibiting ergosterol or ergosterol esters also should be considered. Moreover, some potential inhibitory fungal-derived lipids might be used as topical treatments for AD flares.

In future experiments, the role of fungal-responsive CD1a-reactive T cells in skin inflammation should be studied further using mice with transgenic expression of human CD1a to prove causality and disease association *in vivo* as published in other settings previously (Kim, Hu et al. 2016). One of the limitations of this study was that it focused on IFN-γ to study CD1a-reactive T cells with some data on IL-

22 and IL-17A. Other relevant cytokines should be studied in the future. This includes generating fungal-responsive CD1a-reactive T cells which produce IL-22, IL-17A and type 2 cytokines. In addition, the data on skin-derived T cells was limited in our study; therefore, expanding this further is essential to characterise fungal-responsive CD1a-reactive T cells in the skin. In addition, in our study we focused on *C. albicans* as one of the important clinically relevant fungi in skin inflammation that induced CD1a-reactive T cell responses, however, other relevant fungal pathogens that are important in skin inflammation should be examined in the APC-CD1a-T cells immune pathway such as *Malassezia. Malassezia* yeasts are commensal microorganisms found on normal human skin constituting up to 80% of the total skin fungal population (Gao, Perez-Perez et al. 2010). They produce a wide range of enzymes such as lipases, phospholipases and hydrolases (Ashbee 2006, Pini and Faggi 2011). Many species of *Malassezia* have been identified; the most clinically significant species are *M. furfur, M. globosa, M. sympodialis* and *M. restricta* (Difonzo, Faggi et al. 2013). They are associated with a wide range of skin disease including AD and psoriasis (Difonzo, Faggi et al. 2013). There are several studies in the literature about the importance of Malassezia in AD pathogenesis; for example, a high proportion of AD patients are sensitized to this yeast (Johansson, Sandstrom et al. 2003). More than 60% of the AD patients have atopy patch test positive reactions to at least one of the Malassezia allergens (Johansson, Sandstrom et al. 2003); and elevated serum levels of Malassezia-specific IgE were found in 29%-65% of AD patients (Glatz, Buchner et al. 2015). In addition, 30%–80% of adult AD patients have a positive skin prick test with *Malassezia* (Johansson, Eshaghi et al. 2002). In addition to expanding the fungal species tested, further expansion of the range of fungal-

derived lipids or self-lipids processed by fungal enzymes should be investigated in future research. The possibility that fungal-derived antigens might act as potential ligands for γδ T cells should be investigated further in future studies, as well. This can be done by transducing Jurkat cell lines with TCRs of clones generated and then testing their reactivity against phosphoantigens and non-phosphoantigens and testing the involvement of butyrophilins.

In conclusion, the study has shown that fungi may be sensed through the CD1a pathway and recognised by CD1a-reactive T cells. Fungal antigens include unique fungal lipids, but also modified self-lipids. The fungal-responsive CD1a-reactive T cells may contribute to fungal defence and skin inflammation. Defining a broader understanding of the mechanisms underlying AD will likely help in the design of novel therapeutics.

8. Appendix

Table 8-1 Atopic dermatitis patients and healthy control donors list.

Sample code	Sex	Age	*C. albicans*-specific IgE (KUA/L)	Clinical status
AD1795	F	52	N/A	Mild AD
AD1834	F	37	N/A	Mild AD
AD1855	M	61	N/A	Mild AD
AD1869	F	68	N/A	Mild AD
AD1871	M	24	N/A	Moderate AD
AD1872	F	28	N/A	Severe AD
AD1873	F	24	N/A	Mild AD
AD2009	M	25	N/A	Moderate AD
AD2013	F	69	N/A	Mild AD
AD2101	F	51	N/A	Severe AD
AD2102	M	37	N/A	Moderate AD
AD2103	F	77	N/A	Mild AD
AD2104	F	24	N/A	Severe AD
AD229	F	29	N/A	Severe AD
AD2377	F	30	N/A	Mild AD
AD2467	M	25	0.04	Moderate AD
AD2468	M	32	0.06	Mild AD
AD2469	F	85	0.01	Mild AD
AD2477	F	42	0.01	Mild AD
AD2478	M	40	0.02	Mild AD
AD2531	F	60	0.03	Mild AD
AA9005	M	38	N/A	Mild asthma
C1848	F	36	N/A	HC
C1889	F	51	N/A	HC
C1965	F	69	N/A	HC
C1966	F	77	N/A	HC
C1967	F	40	N/A	HC
C1972	F	33	N/A	HC
C2034	M	60	N/A	HC
C2076	M	23	N/A	HC
C2077	F	30	N/A	HC
C2124	F	93	N/A	HC
C2199	M	37	N/A	HC
C2399	F	73	N/A	HC
C2402	M	23	N/A	HC
C2404	F	24	N/A	HC
C2423	F	41	N/A	HC
C2424	M	29	N/A	HC
C2425	M	35	N/A	HC
C2466	F	36	0.01	HC

C2474	F	29	0.01	HC
C2498	M	28	0.02	HC
C2532	F	74	0.08	HC
C2535	F	69	0.04	HC
C2536	M	60	0.13	HC
C7002	M	28	N/A	HC
C7003	M	29	N/A	HC
C7004	F	26	N/A	HC
W003	F	32	0.01	HC
W007	F	30	0.02	HC
W013	F	49	N/A	HC
W015	M	35	0.01	HC
W016	F	33	N/A	HC
W020	F	30	N/A	HC
W021	M	29	0.09	HC
W023	M	38	N/A	HC
W030	F	27	0.01	HC
W059	F	38	N/A	HC
W060	F	38	0.02	HC
W063	M	37	0.1	HC
W071	M	33	0.06	HC
W079	F	27	N/A	HC

Abbreviations: AD, atopic dermatitis; HC, healthy control; F, female; M, male; N/A, not available. *C. albicans*-specific IgE: 0-0.35, negative; 0.35-0.7, weak positive, >0.7, positive.

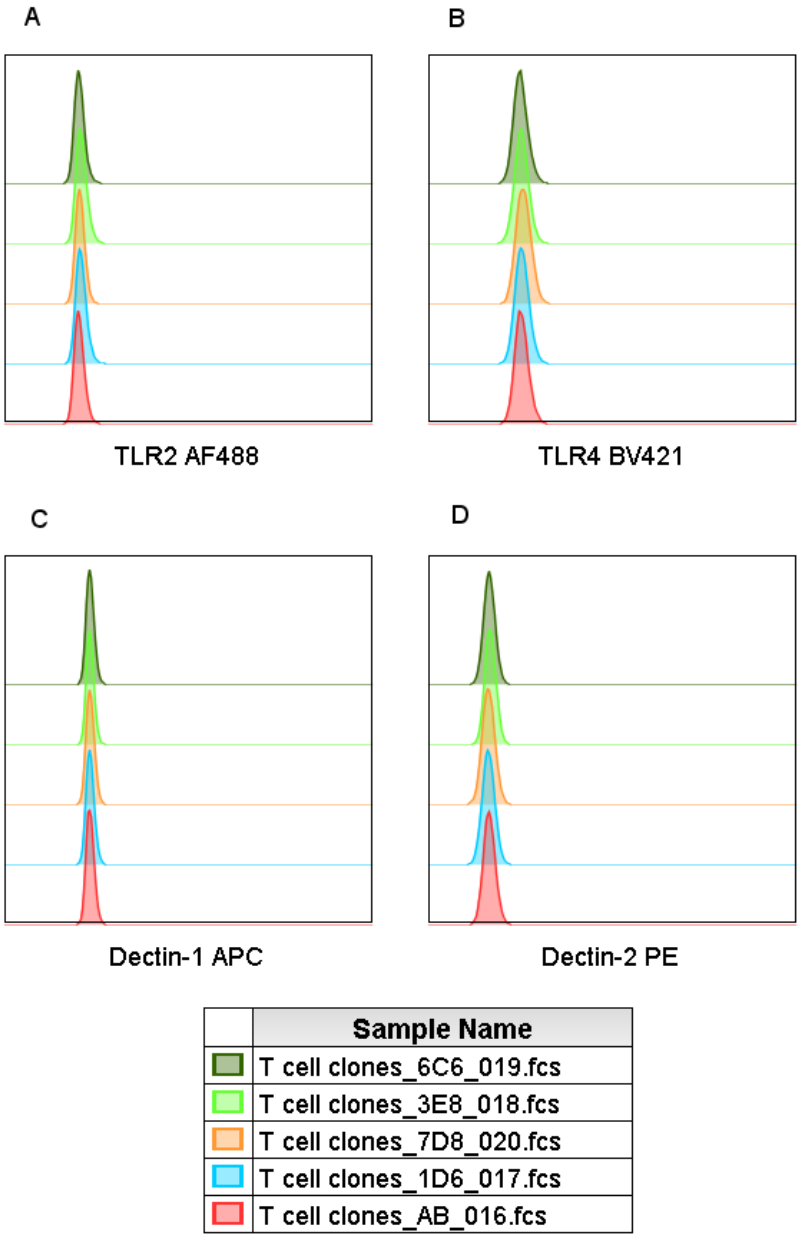


Figure 8.1 Flow cytometry histograms of T cell clones stained for PRRs.

Flow cytometry plots of T cells clones stained for TLR2 (A), TLR4 (B), dectin-1 (C) and dectin-2 (D). Gating on lymphocytes, singlets, live cells, CD3+. Gating based FMO controls. The compensation was based on single-colour stained beads.

9. References

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