Role of CD1a-restricted T Cells in the Immune Responses to *Staphylococcus aureus*

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Content

Abstract

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CD1a has been demonstrated to contribute to anti-bacterial immune responses by presenting lipid antigens to T cells. Skin *Staphylococcus aureus* (*S. aureus*) colonization has been documented to be strongly associated with the severity of skin inflammatory diseases including atopic dermatitis (AD). However, there have been limited studies of how the bacterial colonization contributes to pathology and exacerbation of disease.

Here, we investigate the involvement of a *S. aureus* exotoxin, sphingomyelinase (SMase), in regulating T cell immune responses via CD1a. We show that SMase enzymatic activity plays an essential role in CD1a-reactive T cell activation in both peripheral blood and skin, leading to production of inflammatory cytokines, including IFNγ, IL-17A, and IL-22. These findings support the possibility that the ceramide product of SMase may serve as a permissive lipid neoantigen and that SMase may reduce the non-permissive effects of sphingomyelin. Patients with

atopic dermatitis had reduced CD1a-dependent IFNγ responses to SMase compared to healthy controls.

The generation of CD1a-reactive SMase-specific T cell lines and clones facilitated the further study of mechanisms underlying CD1a antigen presentation, including the novel identification of a Vγ9/Vδ2 CD1a-autoreactive T cell clone which expanded our understanding of CD1a-reactive T cell repertoire and potential pathways of gamma-delta T cell activation. The work also identified that CD1a may be a marker of activation of peripheral T cells.

In conclusion, we have discovered a previously unrecognized pathway of inflammation where SMase derived from *S. aureus* can modulate CD1a ligands which are presented to lipid-specific T cells.

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List of publications

Oral/Poster presentations:

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Poster presentation at 11th International CD1-MR1 Workshop 2019

The Role of CD1a-reactive T Cells in the Immune Response to *Staphylococcus aureus*. *Poster presentation at WIMM Day 2018*

Bacterial Sensing Through Sphingomyelinase Modulation of CD1a Ligands.

Oral presentation at WIMM Student Presentation 2019

Bacterial Sensing Through Sphingomyelinase Modulation of CD1a Ligands.

Oral presentation at MSD DPhil Day 2018

Abbreviations

α-GalCer: α-alactosylceramide

AD: atopic dermatitis

APC: antigen-presenting cell

aSMase: lysosomal acid sphingomyelinase

CCR: CC chemokine receptor

CD: cluster of differentiation

CLA: cutaneous lymphocyte antigen

CTL: cytolytic T lymphocyte

DC: dendritic cell

DDM: dideoxymycobactin

DN: CD4/CD8-double negative

EDTA: Ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ELISpot: enzyme-linked immunospot

ER: endoplasmic reticulum

FACS: fluorescence-activated cell sorter

FCS: fetal calf serum

FMO: Fluorescence Minus One

GM-CSF: granulocyte-macrophage colony-stimulating factor

HC: healthy control

HLA: human leukocyte antigen

HMB-PP: (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate

IFNγ: gamma-interferon

Ig: immunoglobin

IL: interleukin

ILC2: group 2 innate lymphoid cells

IPP: isopentenyl pyrophosphate

LC: Langerhans cell

LPC: lysophosphatidylcholine

MACS: magnetic cell separation (Miltenyi Biotec)

MAIT cell: mucosal associated invariant T cell

mDC: myeloid dendritic cell

MHC: Major Histocompatibility Complex

mLPAs: methyl-lysophosphatidic acids

NKT: natural killer T cell

nSMase: neutral sphingomyelinase

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: phosphatidyl-ethanolamin

PLA2: phospholipase A2

PMA: phorbol myristate acetate

PS: psoriasis

PsA: psoriasis arthritis

REM: rapid expansion mix

RHepes: RPMI, glutamine, penicillin, streptomycin and Hepes

RPMI-1640: Roswell Park Memorial Institute -1640 (media formulation)

S. aureus: Staphylococcus aureus

SAg: superantigen

SC: stratum corneum

SEB: Staphylococcal enterotoxin B

SEM: standard error of the mean

SM: sphingomyelin

SMase: sphingomyelinase

sSMase: secretory acid sphingomyelinase

TAG: triacylglyceride

TCR: T cell receptor

TCM: T cell media

Th: T helper cell

TNFα: tumor necrosis factor alpha

1. Introduction

1.1 CD1a protein

1.1.1 The CD1 protein family

Peptides are well known to serve as antigens to stimulate T cell receptor (TCR) and trigger downstream activation. The peptide-MHC complex is known as one of the most important discoveries in Immunology, and has large applications such as peptide-based synthetic vaccines or therapeutics (Lau & Dunn, 2018; Skwarczynski & Toth, 2016). However, recent studies show that peptides are not the only molecular type that can act as antigen. Lipids, glycolipids and lipopeptides can also be presented to T cells but instead are presented by the MHC-like family of CD1 proteins (Jong et al., 2014; Moody et al., 2004).

CD1 proteins were first identified in a paper published in 1979 (McMichael et al., 1979). The *CD1* genes were cloned later (F Calabi & Milstein, 1986). Human *CD1* genes are encoded on chromosome 1, outside the MHC locus, which lies on chromosome 6. *CD1* genes are widely conserved in mammals, but the number of genes may be different. Horses, dogs and cattle have many *CD1* genes, while mice only have 1 *CD1* gene, which brings difficulties to the *in vivo* model experimental study of CD1 proteins. (Reinink & Van Rhijn, 2016; Van Rhijn, Godfrey, Rossjohn, & Moody, 2015)

Human *CD1* genes have five isoforms. Based on sequence homology, the CD1 family can be classified into group 1 (CD1a, CD1b, CD1c), group 2 (CD1d) and group 3 (CD1e). All of the five isoforms have different trafficking and tissue distribution, indicating their different physiological roles. (Angénieux et al., 2000; Franco Calabi, Jarvis, Martin, & Milstein, 1989)

The group 2 CD1 protein, CD1d, is the only isoform expressed in commonly used mouse models and has accordingly been studied most. In humans, it is mainly expressed on myeloid lineage cells, independently from group 1 CD1 proteins, and on dermal dendritic cells (DCs), B cells, cortical thymocytes, other lymphocytes and epithelial cells. The group 1 CD1 proteins are mainly expressed on professional antigen-presenting cells (APCs) and thymocytes, and are widely used as DC markers in humans. CD1b is restricted mainly to DCs in lymph nodes, while CD1c is expressed on DCs and subsets of B cells. The group 3 CD1 protein, CD1e, resides in the endocytic compartment, where it provides assistance for lipid editing and their loading onto the other CD1 molecules. (Brigl & Brenner, 2004; Dougan, Kaser, & Blumberg, 2007; Martin, Calabi, Lefebvre, Bilsland, & Milstein, 1987; Van Rhijn et al., 2015)

CD1a was the first identified CD1 protein by monoclonal antibody NA1/34 on human thymocytes (McMichael et al., 1979). CD1a was proved to be expressed on mature human thymocytes but not peripheral T cells, which could serve as

post-selection marker in the life-history of T cells (Sotzik, Boyd, & Shortman, 1993). CD1a belongs to group 1 CD1 proteins, and is expressed on APCs in epithelia of various tissues. It is known as a phenotype-specific marker of human epidermal Langerhans cells (LCs), and is the CD1 isoform that highly and specifically expressed in skin. (Brigl & Brenner, 2004; Dougan et al., 2007; Sotzik et al., 1993) The CD1a expression on dendritic cells, as well as CD1b and CD1c, could be down-regulated by lipids in human serum. (Leslie et al., 2008) CD1a can be induced on human group 2 innate lymphoid cells (ILC2), which are capable of activating T cells via CD1a. (Hardman et al., 2017)

CD1a, CD1b and CD1c expression on APCs has also been observed in bacterial infections and chronic inflammatory conditions, indicating the participation of group 1 CD1 proteins in infections and autoimmune diseases. Group 1 CD1 protein expressions on APCs are selectively up-regulated by bacterial stimuli (Roura-Mir et al., 2005; Yakimchuk et al., 2011). CD1a and CD1b are expressed by macrophages in inflammatory neuropathy and by macrophage-derived foam cells in atherosclerotic, and associate with local T cell activation. (Khalili-Shirazi, Gregson, Londei, Summers, & Hughes, 1998; Melián, Geng, Sukhova, Libby, & Porcelli, 1999; Van Rhijn, Van den Berg, Bosboom, Otten, & Logtenberg, 2000) The CD1a-expressing DCs were also observed in several autoimmune diseases, including rheumatoid synovium and Sjögrens syndrome (Page, Lebecque, & Miossec, 2002; van Blokland et al., 2000) The tumor-infiltrating dendritic cells that

express CD1a on their surface are found in human breast cancer, suggesting the role of CD1a+ DCs in anti-tumor immunity. (Coventry, Austyn, Chryssidis, Hankins, & Harris, 1997; M. Iwamoto et al., 2003)

Although CD1 proteins have a low rate of polymorphism, which is generally not associated with protein function, an intronic polymorphism in *CD1A* is found to specifically influence the expression and ability of CD1a-expressing DCs to present mycobacterial lipopeptide antigen. It further emphasizes the role of CD1a+ DCs in controlling *M. tuberculosis*. (C Seshadri et al., 2014; Chetan Seshadri et al., 2013)

1.1.2 Structure of CD1a protein

CD1 proteins are transmembrane glycoproteins structurally similar to the MHC class I, which are expressed as heterodimers with CD1 heavy chains (domain α1, α2 and α3) and a non-covalently associated β_2-microglobulin. CD1 glycoproteins are assembled in the endoplasmic reticulum (ER), associated with β_2-microglobulin, and loaded with endogenous lipids (Hüttinger, Staffler, Majdic, & Stockinger, 1999; Sugita, Porcelli, & Brenner, 1997). Matured CD1 proteins are trafficked to the cell membrane. The association with β_2-microglobulin is necessary for surface expression, proved by studies in β_2-microglobulin-deficient cell lines. (Bauer et al., 1997; Brigl & Brenner, 2004)

Determined crystal structures of CD1 proteins provide detailed information about CD1 structures and ligand-binding curves. Generally, the CD1 antigen binding cleft is located at α1 and α2 domain and formed by non-polar amino acids, which construct a hydrophobic domain to capture lipids. The binding groove has a narrow opening and larger internal volume, forming a pocket-like structure but not a plate presenting platform. It allows the long and flexible alkyl chains of lipids to fold and insert into the protein. (Moody, Zajonc, & Wilson, 2005; Van Rhijn et al., 2015)

CD1 protein usually has multiple pockets, named as A', C', F' and T' pockets. Each CD1 protein has unique antigen binding cleft structure with different volumes. To bind lipids with varied chain lengths, the scaffold lipids, spacer lipids and accessory portals are used to modify the space of the antigen binding groove. (Moody et al., 2005)

CD1a has the smallest binding groove (~1,350 Å3) in the CD1 protein family, which is mainly composed of two pockets A' and F' in α1 and α2 domains, while α3 domain and β_2-microglobin fold as supporting structure. The crystal structure of CD1a-sulfatide complex offered detailed information of how CD1a binds with antigen. The A' pocket is a semi-circular curve that deeply buried inside the protein, which perfectly fitted 18 carbons of the sphingosine base of sulfatide antigen. The F' pocket is closer to the CD1a surface, and is long and straight. It is

also wider, which can accommodate lipids with various length and headgroup. (Zajonc, Elsliger, Teyton, & Wilson, 2003a)

1.1.3 CD1a presents endogenous and exogenous lipids

The first CD1 lipid antigen found was mycolic acid, which is a family of α-branched, β-hydroxy, long-chain fatty acids found in mycobacteria that could be presented by CD1b to αβ T cells. (Beckman et al., 1994) Later, CD1-reactive T cell clones were found to be stimulated by CD1-expressing APCs without additional foreign antigens, indicating the presence of self-lipid antigens (Porcelli et al., 1989).

General antigens of CD1 proteins have one, two or three long and flexible hydrocarbon chains and a hydrophilic head group. The hydrocarbon chains fit inside binding curve, while polar or charged head groups protrude out and form the TCR binding platform together with CD1a. The orientation of the head group may affect the recognition of T cells. (Van Rhijn et al., 2015; Wang et al., 2010) CD1a, CD1b and CD1c proteins are found to load the same sulfatide on their cell surface and trigger Th1 and Th2 responses, indicating that the same self-antigen may be presented by different CD1 isoforms in some cases. (Shamshiev et al., 2002)

Like other CD1 isoforms, CD1a is able to present self-lipids as well as exogenous lipids. CD1a could bind to endogenous ligands like sphingolipids, phospholipids,

sulfatide, and headless skin oils (Jong et al., 2014; Shamshiev et al., 2002; Zajonc et al., 2003a). It is also demonstrated to bind dideoxymycobactin (DDM) from *Mycobacterium tuberculosis*, and urushiol from plants (J. H. Kim et al., 2016; Moody et al., 2004).

However, the ability of CD1a to present headless skin oils as ligands appears to make CD1a different in some ways from other CD1 isoforms, suggesting other mechanisms of CD1a lipid presentation. Skin oils, such as wax esters, squalene and triacylglycerides (TAG), are extremely hydrophobic and lack the polar or charged head group which is expected for TCR interaction. It suggests that CD1a may not need the direct interaction between TCR and lipid when presenting antigens. Further supporting this hypothesis, CD1a protein can also acquire antigens over time, and carry endogenous ligands with large hydrophilic head groups that block CD1a autoreactivity, when emerging at the cell surface. (Jong et al., 2014)

Based on the crystal structures of CD1a loaded with LPC, oleic acid, sphingomyelin (SM), sulfatide and mycobactin-like lipopeptide, CD1a ligands are divided into the permissive ligands and non-permissive ligands. SM, for example, is a non-permissive ligand of BK6, which interfered with TCR contact by disrupting the CD1a roof and the binding sites of TCR and CD1a. The blocking effect of SM is also proved by CD1a-autoreactive T cell line BC2 by plate-bound assay. While

BK6 permissive ligands, such as LPC and oleic acid, permit TCR binding with CD1a in the absence of interference. (Birkinshaw et al., 2015; Jong et al., 2014; Van Rhijn et al., 2015; Zajonc et al., 2005, 2003a)

Although the crystal structure suggests the disruption by several non-permissive ligands to the interaction of BK6 TCR and CD1a, we should consider that it may be an exclusive mechanism for individual TCRs. In the broader CD1a-reactive repertoire, T cell recognition of CD1a-ligand complex may use the mechanisms of specific antigen recognition. For example, loading of DDM is proved to be necessary for TCR binding, and can induce a T cell response, demonstrating its ability to be recognized by the TCR in a specific manner. (Van Rhijn et al., 2015; D. C. Young et al., 2009)

1.1.4 Antigen-presentation by CD1a

CD1 proteins are assembled in the ER and initially capture self-ligands. The proteins are associated with β_2-microglobin before trafficking to cell membrane. Surface CD1 proteins are internalized again and traffic to different endosomal compartments based on the sorting motifs on their cytoplasmic tails.

CD1b traffics to late endosomes and lysosomes, similar to MHC-II. CD1a, which has a short cytoplasmic tail that lacks tyrosine-based internalization motifs, is internalized through a different pathway that is localized at early endosomes.

(Barral et al., 2008) While CD1c broadly surveys throughout the endocytic system and locates overlapping with CD1a and CD1b. (Sugita, van der Wel, Rogers, Peters, & Brenner, 2000) CD1d also traffics to late endosomes, but its trafficking is regulated by encoded sorting motifs as well as its invariant chains. (Jayawardena-Wolf, Benlagha, Chiu, Mehr, & Bendelac, 2001) The different trafficking pathways of CD1a, CD1b and CD1c allow the proteins to sample lipid contents from different cellular compartments. (Ly & Moody, 2014; Odyniec et al., 2010)

CD1 proteins trafficking through different cellular compartments have gone through microenvironments with different pHs, from mildly acidic pH in early endosomes (pH 6.0), highly acidic pH in lysosomes (pH 5.0), to neutral pH at cell surface (pH 7.4). The stabilized stage and loading requirement of each CD1 isoforms are varied. Among them, CD1a is stable at cell surface with a relatively robust binding curve. Its lipid exchange is not dependent on cellular internalization. As CD1a is not found in late endosomes or lysosomes, the lipid loading of CD1a may not require low pH, which is different from other group 1 CD1 proteins. This is further proved by the ability of CD1a to exchange lipids at cell surface, which is not common to CD1b or other antigen-presenting molecules. (Cernadas et al., 2010; Garzón, Anselmi, Bond, & Faraldo-Gómez, 2013; Manolova et al., 2006)

1.2 CD1a-reactive T cells

1.2.1 Introduction to CD1-reactive T cells

CD1-reactive T cells that recognize lipid antigens presented by CD1 belong to the family of unconventional T cells, which also include MR1-restricted mucosal associated invariant T cells (MAIT cells), and γδ T cells. (Godfrey, Uldrich, Mccluskey, Rossjohn, & Moody, 2015) The presence of CD1-reactive T cells was initially proved by the successful isolation of two CD1-reactive T cell lines in human PBMCs. These T cells are an abundant population in the peripheral blood, and are able to respond rapidly within hours or days of stimulation. Unlike MHC-reactive T cells, the TCR diversity of unconventional T cells is more limited in some cases. (Godfrey et al., 2015; Porcelli et al., 1989)

Among all CD1-reactive T cells, the subset that recognizes CD1d is the most studied, due to the available experimental mouse models. The CD1d-reactive T cells are termed natural killer T cells (NKT), as some co-express NK markers. (Godfrey, MacDonald, Kronenberg, Smyth, & Kaer, 2004) The NKT cells are divided into two groups: type I NKT cells or iNKT cells that express an invariant TCRα chain and a limited range of TCRβ chains, and type II NKT cells that have more diverse αβ TCRs.

Type I NKT cells consist of about 0.1% of T cells in human peripheral blood, and are more frequent in the mouse. One of the best known lipid antigens for these cells is α-galactosylceramide (α-GalCer). The α-GalCer-loaded CD1d-tetramer is

the most sensitive way to detect type I NKT cells. These cells are further proved to participate in autoimmunity, infection, tumor growth and other diseases (Godfrey & Kronenberg, 2004). Type II NKT cells do not have defining markers. The research about them is relatively less, and mainly based on genetic knock-out mouse models. (Godfrey et al., 2015; Kawano et al., 1997)

Compared to well-studied NKT cells, the understanding of group 1 CD1-reactive T cells is limited, partly because the proteins are not expressed in mouse and the lack of defining surface markers. But the development of CD1-tetramers allows the study of these T cells in human. (Kasmar et al., 2014, 2011; Ly et al., 2013) Group 1 CD1-reactive T cells are found to recognize microbial lipids as well as self-antigens. They are found to have diverse TCRs. (Godfrey et al., 2015) The frequency of CD1-autoreactive T cells is 0.3-10% in peripheral blood, tested by IFNγ production, with a predominance of T cells recognizing CD1a, followed by CD1c and CD1d, and seldom CD1b-autoreactive T cells. (De Lalla et al., 2011) Human CD1c-reactive T cells are also found to recognize methyl-lysophosphatidic acids (mLPAs), the self-lipid accumulated in leukemia cells, which indicate its potential role in anti-tumor immunity. (Lepore et al., 2014)

1.2.2 The CD1a-reactive αβ T cells

BK6 was the first T cell line proved to be CD1a-reactive, derived from the peripheral blood of a patient with systemic lupus erythematosus in 1989. It is a

CD4/CD8-double negative (DN), αβ TCR expressing cytolytic T lymphocyte (CTL) line, which could direct the lysis of CD1a-expressing MOLT-4 cells without additional foreign antigens, indicating its CD1a-autoreactivity. It is broadly used for studying CD1a-autoreactive T cell response. (Porcelli et al., 1989)

Later, CD4+ T cells and CD8+ T cells were found to be CD1a-reactive and able to recognize microbial lipids, which extended the CD1a-reactive repertoire. (Rosat et al., 1999; Sieling et al., 2000) These CD1a-reactive αβ T cell lines are mainly Th1 subsets that produce IFNγ and TNFα, and participate in anti-bacterial immunity by recognizing microbial antigens.

Most of the studies of CD1a-reactive T cells relied on the generated long-term T cell lines and clones, until de Jong in 2010 published the research of CD1a-reactive T cells at the polyclonal level by taking advantage of K562 cells transfected with human CD1 genes. The K562 cell line has low levels of MHC expression and is transfected with high CD1 levels on the surface, which avoids alloreactive responses to MHC. It also provides an efficient tool to study CD1a-autoreactivity without requiring prior knowledge of antigens. The research proved that CD1a-reactive T cells are a normal component of the human αβ T cell repertoire, and commonly present in human blood and skin. Besides IFNγ, the cells are also able to significantly produce IL-22, which induces the proliferation of keratinocytes amongst other effects. The CD1a-autoreactive T cells express

skin-homing markers CLA, and could be activated by epithelial CD1a-expressing LCs. (De Jong et al., 2010) Importantly, the localization of CD1a-expressing LCs at skin, the skin-homing character of CD1a-reactive T cells, and the potential of skin oils as CD1a ligands, indicate the participation of CD1a in skin immunity.

1.2.3 The CD1a-reactive γδ T cells

The percentage of γδ T cells is 0.5–16% of circulating T cells in humans, with a mean of 4%. Similar frequencies are found in lymphoid tissues, gut and skin. (Groh et al., 1989) The MHC presentation of peptide is not required for γδ T cell activation, which separates them from the majority of αβ T cells. (Vantourout & Hayday, 2013) They often also lack the high expression of co-receptor CD4 and CD8. (Godfrey et al., 2015) Subsets of γδ T cells have been proved to be CD1-reactive.

Unlike αβ T cells, the numbers of V gene segments of TCRγ and TCRδ are limited. In human there are mainly two types of γδ T cells, named based on their TCR variable segments. The Vδ1 γδ T cells are abundant at skin and large intestine, while Vδ2 γδ T cells are mainly resident at blood. (Chien, Meyer, & Bonneville, 2014; Deusch et al., 1991; Ebert, Meuter, & Moser, 2006; Toulon et al., 2009)

Some human Vδ1 γδ T cells were found to recognize human CD1c, proved by the isolation of CD4- $CD8^{low}$ γδ cytolytic T cell lines. These T cells can induce the lysis

of CD1c-expressiong cells without exogenous ligands, and produce Th1 cytokines as well as antimicrobial proteins. (Porcelli et al., 1989; Spada et al., 2000) Some human Vδ1 γδ T cell lines recognize lipids presented by CD1d, including sulfatide, α-GalCer and pollen-derived phosphatidylethanolamine. (Bai et al., 2012; Luoma et al., 2013; Russano et al., 2006; Uldrich et al., 2013)

Human Vδ2 cells usually express Vγ9 chain to form Vγ9Vδ2 TCR. These T cells are the most abundant γδ T cells population in human blood (Karunakaran, Göbel, Starick, Walter, & Herrmann, 2014) and can be activated by phosphoantigens, including isopentenyl pyrophosphate (IPP) from mammalian cells and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) from bacteria. (Hintz et al., 2001). Phosphoantigens may activate γδ T cells through binding to butyrophilin 3A1 on cell surface, but the mechanism is still unclear. So far it is unknown whether Vγ9Vδ2 T cells recognize any other antigen-presenting molecules. (Godfrey et al., 2015)

The human Vδ3 γδ T cells, a small population of γδ T cells that consist about 0.2% of blood T cells, are reported to recognize CD1d but not the other CD1 isoforms, and release Th1, Th2, Th17 cytokines. (Mangan et al., 2013)

T cell lines isolated from allergy patients were proved to recognize pollen lipids presented by CD1a in 2005. Most of the lines were TCRαβ+ CD4+ T cells, but one

TCRVδ1+ CD4+ CD161+ T cell line was also successfully isolated, which recognized 16:0/16:0 phosphatidyl-ethanolamin (PE) and PE from cypress pollen, brain and egg. This finding indicates the CD1a-reactivity of γδ T cells (Agea et al., 2005).

1.2.4 Role of CD1a-reactive T cells in diseases

The antibacterial response of CD1a-reactive T cells is suggested by their recognition of lipids derived from *M. tuberculosis*. Bacterial stimuli are also proved to up-regulate the CD1a expression on DCs (Yakimchuk et al., 2011). CD1a-autoreactive T cells are able to produce cytokines include IFNγ, IL-22, and IL-17A, which are involved in immune response against bacteria. (Cheung et al., 2016; Jarrett et al., 2016; Jong et al., 2014; Preihs, Christian Arambula et al., 2013)

IFNγ was the first detected and studied cytokine that CD1-reactive T cells are able to produce. The cytokine is known to participate in antimicrobial immunity by generating effective immune responses to eliminate pathogens (Kak, Raza, & Tiwari, 2018; Shtrichman & Samuel, 2001). IFNγ is known to control *M. tuberculosis* infection, in which IFNγ-producing CD4 T cells are essential for host survival and enhance CD8 T cell cytotoxicity (Green, DiFazio, & Flynn, 2013). IFNγ also stimulates intrinsic antibacterial activities to protect endothelial cells from virulent *S. aureus* strains, and contributes to the immune response against *S. aureus*. (Beekhuizen & Van De Gevel, 2007).

IL-22 and IL-17A are relevant to inflammation at various mucosal surface and epithelial barriers. These cytokines participant in host defense against microbes including *S. aureus* (Valeri & Raffatellu, 2016). IL-22 induces antimicrobial peptide expression to trigger the killing of pathogens, which further limits the growth of *S. aureus* (Fujita, 2013; Malhotra et al., 2016; Mulcahy, Leech, Renauld, Mills, & McLoughlin, 2016; Valeri & Raffatellu, 2016). IL-17A induces the expression of neutrophil-attracting chemokines and contributes to the clearance of *S. aureus*. The IL-17A producing epidermal γδ T cells are also essential for the anti-*S. aureus* immune response (Chan et al., 2015; J. S. Cho et al., 2010; Jin & Dong, 2013; Miller & Cho, 2011).

The role of CD1a-reactive T cells in inflammatory skin diseases has been investigated by the studies of bee and wasp venom allergy, atopic dermatitis, contact dermatitis and psoriasis. CD1a-reactive T cells at polyclonal level were proved to produce IFNγ, IL-17A, IL-22, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF). CD1a-autoreactive T cells in psoriasis patients and CD1a-reactive house dust mite-specific T cells in atopic dermatitis patients are much more activated than in healthy individuals. (Bourgeois et al., 2015; Cheung et al., 2016; Jarrett et al., 2016)

Besides directly binding and presenting lipid antigens, CD1a can also sense threats

through recognizing neoantigens generated *in vivo* by enzymes. A novel inflammatory pathway was found that phospholipase A2 (PLA2) generates lipid antigens *in vivo* and activates T cells through CD1a. Foreign PLA2 from bee venom and house dust mite, and host PLA2 from mast cells and ILC2 could all contribute to CD1a-reactive T cell response. In these systems, PLA2 is essential for generating CD1a ligands, and the CD1a-reactive T cell response is dependent on enzymatic bioactivity. Filaggrin could inhibit sPLA2 of house dust mite suggesting a role beyond barrier function of the skin. (Bourgeois et al., 2015; Cheung et al., 2016; Hardman et al., 2017; Jarrett et al., 2016)

1.3 Sphingomyelinase from *Staphylococcus aureus*

1.3.1 The sphingomyelinase family

Sphingomyelinase (SMase) was found in human spleen in 1967, and found to be absent in Niemann-Pick disease patients (Schneider & Kennedy, 1967). With the investigation of the roles of sphingomyelins and ceramides in physiological conditions, interest in SMase is increasing. (Goñi & Alonso, 2002)

The SMase family is divided into 5 sub-groups based on optimal pH and dependence of metal ions: lysosomal acid SMase, secretory acid SMase, neutral Mg^{2+}-dependent SMase, neutral Mg^{2+}-independent SMase, and alkaline SMase. (Samet & Barenholz, 1999) The lysosomal acid SMase and neutral Mg^{2+}-dependent SMase are most abundant, and considered as one of the major

sources of ceramide production in physiological conditions. (Goñi & Alonso, 2002)

The lysosomal acid SMase (aSMase) is Zn^{2+}-independent, and its optimal working pH is between pH 4.5-5. The enzyme mostly locates at the lysosome and/or late endosome. (Schneider & Kennedy, 1967; Schuchman, 2010) The association of aSMase, CD1d antigen SMs and CD1d-reactive iNKT cells in Niemann-Pick disease was proved in mouse models and humans, suggesting control and regulation roles of SMase to CD1-reactive T cells. (Melum et al., 2019) The secretory acid SMase (sSMase) is the secreted form of acid SMase with the same protein precursor but different trafficking pathway. Most of this enzyme is Zn^{2+}-dependent, but the sSMase from endothelial cells is partially Zn^{2+}-independent. The optimal working pH for sSMase is acidic, but sSMase can also function at extracellular neutral pH to hydrolyze certain substrates including atherogenic lipoproteins. This enzyme is considered to play a role in atherogenesis. (Goñi & Alonso, 2002; Schissel, Keesler, Schuchman, Williams, & Tabas, 1998; Tabas, 1999) Mg^{2+}-independent neutral SMase is a small group of enzymes isolated from HL-60 cell line, the function of which is still unclear. Alkaline SMase is Mg^{2+}-independent and isolated from intestinal tract, which is considered to digest the dietary SMs. (Clarke, Wu, & Hannun, 2011; Goñi & Alonso, 2002; Okazaki, Bielawska, Domae, Bell, & Hannun, 1994)

Neutral Mg^{2+}-dependent SMase (nSMase) is found in bacterial, yeast and

mammalian cells. The first mammalian nSMase was purified from rat brain, with the optimum pH around 7.5. (Liu, Hassler, Smith, Weaver, & Hannun, 1998; Llu & Hannun, 2000) Mammalian nSMase is commonly present but its activity is mainly found in brain. The enzyme is considered as central mediator of stress-induced ceramides. (Chatterjee, 1999; Goñi & Alonso, 2002)

The mammalian nSMase is divided into three groups: nSMase1, nSMase2 and nSMase3. The nSMase1 mainly locates at endoplasmic reticulum (ER), and there is debate about the role of nSMase1 as it does not seem to participate in SM metabolism. The nSMase2 is the most studied type of nSMase. It is brain-specific, and locates at Golgi, ER or plasma membrane, and increases the level of long chain ceramides. The nSMase2 is proved to be involved in apoptosis, inflammation, cell growth, and differentiation. While nSMase3 is a unique and distinct group that is linked to tumorigenesis. (Clarke et al., 2006a; Krut, Wiegmann, Kashkar, Yazdanpanah, & Krönke, 2006; Marchesini et al., 2004; Neuberger, Shogomori, Levy, Fainzilber, & Futerman, 2000)

The bacterial SMase was cloned from *S. aureus* in 1986. It is one of the first cloned nSMase and initially used as a substitute of poorly characterized mammalian nSMase. It is proved to be secreted by bacteria as virulence factor, which will be further discussed below. (Coleman, Arbuthnott, Pomeroy, & Birkbeck, 1986)

1.3.2 Bacterial SMase

Bacterial SMase is widely secreted by Gram-positive and Gram-negative bacteria. These SMase-producing bacteria do not synthesize sphingolipids by themselves, but use the SMase to take advantage of host sphingolipids and promote their pathogenicity. Based on the different cleavage sites of these enzymes, bacterial SMase can be generally divided into two groups: SMase C and SMase D. (Clarke et al., 2006b; Flores-Díaz, Monturiol-Gross, Naylor, Alape-Girón, & Flieger, 2016)

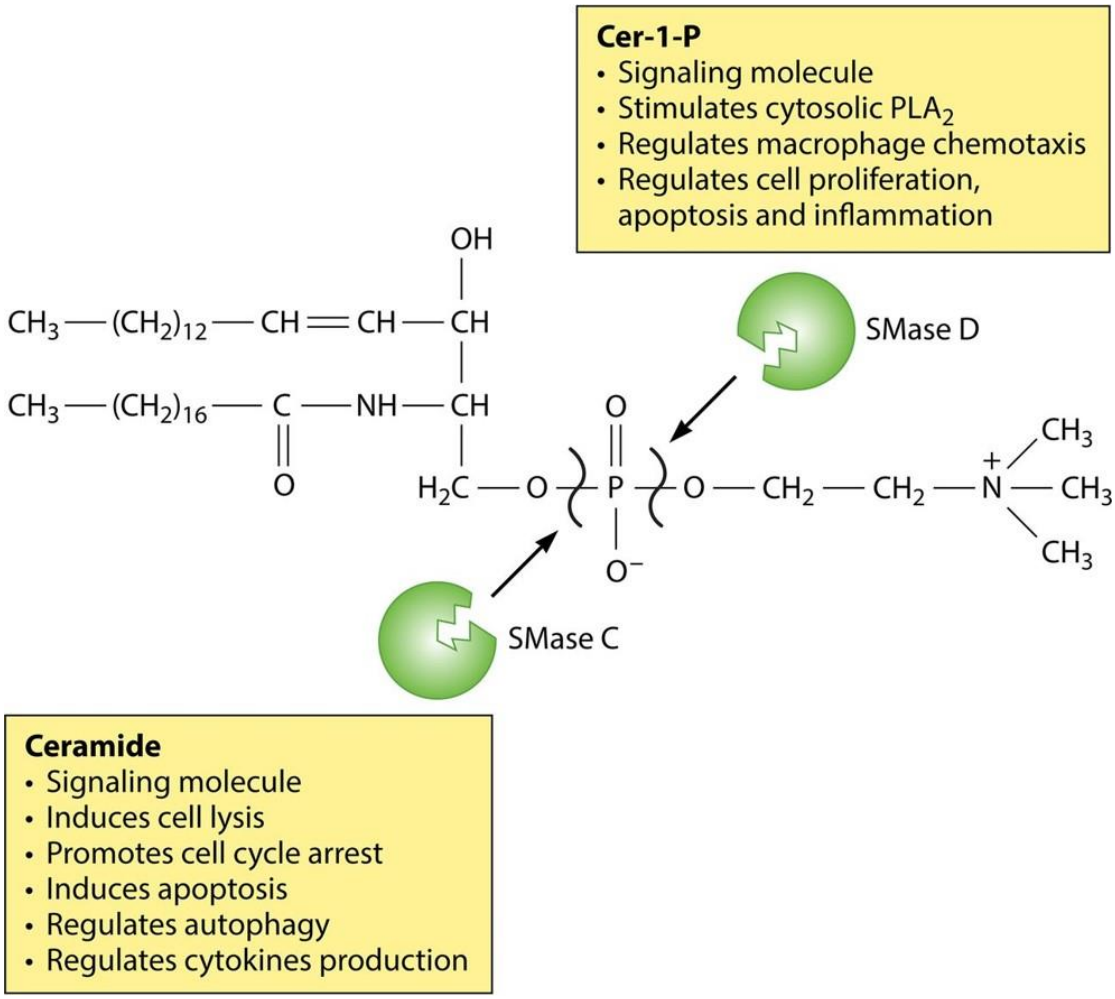


Figure 1.1 Enzymatic cleavage sites of SMase C and SMase D in sphingomyelin and cellular activities of the reaction products. From (Flores-

Díaz et al., 2016)

Sphingomyelin (SM) is the most abundant eukaryotic membrane sphingolipid in the outer leaflet of cell membranes and enriches in lipid rafts. The structure of SM is shown in Figure 1.1. SMs most commonly have a C18 sphingosine backbone, a N-linked fatty acid residue with variable length and saturation, and a phosphorylcholine. The SMase C cleaves SM to a ceramide and a phosphorylcholine, while SMase D cleavages to a Cer-1-phosphate and a choline. Ceramides are known as secondary signaling molecule and related to stress-induced apoptosis, inflammation, and differentiation. It is worth noting that SM, ceramide and CD1a self-antigen sulfatide share a common structure of C18 sphingosine, which perfectly suits the binding characteristics of the A' pocket of CD1a. (Flores-Díaz et al., 2016; Zajonc, Elsliger, Teyton, & Wilson, 2003b)

Bacterial SMase C includes the SMase from *B. cereus*, *L. ivanovii*, *S. aureus*, *Leptospirosis* and *M. tuberculosis*. Bacterial nSMase binds and lyses erythrocytes to aid iron acquisition from heme groups as well as to acquire nutrition for bacteria. Its cytotoxicity to various mammalian cells is also demonstrated. The treatment of SMase C increases the ceramides on cell membrane, which self-assemble to form ceramide-enrich domains that exclude cholesterol, and finally reduce membrane fluidity and cause fragility of plasma membrane. (Oda et al., 2010) Based on the study of *B. cereus* SMase C, the binding of SMase on the cell

surface is important for enzyme catalysis function. Replacement of key amino acids at binding area weakens the effect of the enzyme. (Ago et al., 2006; M. Huseby et al., 2007; Tomita, Taguchi, & Ikezawa, 1983)

Bacterial SMase D could be further divided into two groups based on TIM barrel structure. The nSMase D with TIM barrel structure is homologous to enzymes from fungi and spider venom. While the other nSMase D is produced by marine bacteria that could infect marine animals and humans. (Dias-Lopes et al., 2013; Rivas, Lemos, & Osorio, 2013)

1.3.3 The role of *S. aureus* SMase in diseases

S. aureus is a commonly found pathogen that causes skin and soft tissue infections, and can lead to endocarditis, pneumonia, meningitis, and sepsis. SMase from *S. aureus* is a typical example of bacterial SMase C, which is also called beta-toxin, and encoded by the *Hlb* gene. It is expressed by all virtually *S. aureus* strains, while the mutant bacteria strains that lack the *Hlb* gene are less virulent and have diminished pathogenicity. (Katayama, Baba, Sekine, Fukuda, & Hiramatsu, 2013; Salgado-Pabón et al., 2014) With the study of *Hlb* knockout strains using the mouse mammary gland model and rabbit corneal infection model, the SMase was proved to contribute to *S. aureus* infection and damage. (Bramley, Patel, O'Reilly, Foster, & Foster, 1989; O'Callaghan et al., 1997) The enzyme is cytotoxic to erythrocytes, keratinocytes and lymphocytes, helps with the bacterial colonization

and escape from phagosomes, and is involved in biofilm formation. Thus, nSMase has been linked with diseases caused by *S. aureus*. (Flores-Díaz et al., 2016)

The most-known function of SMase is its hemolytic activity to erythrocytes. The susceptibility of erythrocytes was related to their SM content; thus, the sheep erythrocytes were most sensitive to SMase, with 50% of SM in the total lipids. The SMase is not directly killing the cells, but weakens the cell membrane and makes it fragile to the low temperature, which is called hot-cold hemolysis. SMase is also cytotoxic to polymorphonuclear leukocytes, monocytes, and proliferating T lymphocytes without affecting viability. The cytotoxicity to these cells is not linked with SM content (Walev, Weller, Strauch, Foster, & Bhakdi, 1996)

SMase is proved to promote *S. aureus* colonization on skin by damaging keratinocytes, which is a common feature in many skin diseases especially atopic dermatitis. The colonization of bacteria is significantly higher than strains without *Hlb*. (Katayama et al., 2013) The enzyme also inhibits the IL-8 production of endothelial cells without harming the cells, which impairs the neutrophil migration and helps the escape of bacteria. (Tajima, Iwase, Shinji, Seki, & Mizunoe, 2009)

Furthermore, SMase is proved to induce neutrophil-mediated lung injury though its sphingomyelinase activity and the shedding of syndecan-1, the major heparan sulfate proteoglycan of alveolar epithelial cells. *S. aureus* causes the intense

inflammatory response in pneumonia, which leads to the development of lung injury. The central features of the disease are specifically increased neutrophilic inflammation, vascular leakage of serum proteins into the lung tissue, and leakage of proteins into the airway. All these features are significantly diminished in SMase-deficient *S. aureus* strains compared to wild-type strains. The ceramide production on epithelial cells induce the shedding of various cell surface proteins in ectodomain, including syndecan-1, which further contributes to the lung injury caused by *S. aureus*. (Hayashida, Bartlett, Foster, & Park, 2009; Park et al., 2004)

Although most of the pathogenic effect of SMase is based on its sphingomyelinase activity, the enzyme contributes to biofilm formation in a catalysis independent manner. It covalently cross-links to itself in the presence of DNA, and produces nucleoprotein matrix *in vivo*. (M. J. Huseby et al., 2010)

1.3.4 The difference between bacterial and mammalian nSMase

The sequence identity between bacterial, yeast and mammalian nSMase is low, being about 20% between nSMase from *B. cereus* and human nSMase2, and even lower between bacterial nSMase and human nSMase1, indicating a remote similarity. But the nSMase family shares a number of conserved residues at the catalytic region of the enzyme, suggesting a common catalytic mechanism. (Hofmann, Tomiuk, Wolff, & Stoffel, 2000) Here we focus on the comparison of bacterial SMase and mammalian nSMase2.

The nSMase2 in mammals are integral membrane proteins, at 71kDa with two putative transmembrane domains. It is intracellularly located at Golgi, ER and plasma membranes, and it is mainly activated in brain tissue. While the bacterial nSMase is soluble, 36-38kDa, and secreted by bacteria. (Chatterjee, 1999; Clarke et al., 2006a; Goñi & Alonso, 2002)

Most of the inhibitors of nSMase are tested by rat brain enzyme but not repeated with bacterial counterparts. However, it is worth noting that common inhibitors for human nSMase may not work for bacterial nSMase. The pharmacological inhibitors for human nSMase, scyphostatin, which was obtained from *Trichopeziza mollissima*, is proved to inhibit mammalian nSMase and aSMase, but not nSMase from *S. aureus* and *B. cereus*. (Nara et al., 1999a) Alutenusin, another nSMase inhibitor from *Penicillium sp*, has no antimicrobial activity to 12 bacterial strains. (Uchida, Tomoda, Dong, & Omura, 1999)

1.4 Atopic dermatitis and psoriasis

1.4.1 Introduction to atopic dermatitis

Atopic dermatitis (AD) also called atopic eczema is one of the most common chronic inflammatory skin diseases. Its prevalence is increasing in Europe, Asia and Africa (Deckers et al., 2012). AD is characterized by intense itch, eczematous lesions and a chronic disease course, the pathogenesis of which is complex and

not fully understood. The condition usually starts in infants and children, and can continue in adulthood. (Spergel & Paller, 2003) AD is extremely heterogeneous and has a wide range of clinical features, with may be related with age, ethnicity and disease severity, but clinical criteria are defined, with the support of skin biopsies analysis if needed. (Thomsen, 2014) AD is also strongly associated with food allergy and other atopic diseases including asthma and allergic rhinitis, but the underlying mechanism is still unclear. (Spergel & Paller, 2003; Weidinger, Beck, Bieber, Kabashima, & Irvine, 2018).

The essential features of AD include the epidermal barrier disruption, T cell subset activation, IgE-mediated reactivities, and imbalance of skin microbiota. Both genetic and environmental factors contribute to the disease. (Weidinger et al., 2018)

The strongest risk factor for AD is a family history of atopic diseases. The mutation of *FLG* gene is the best-known genetic risk factor of AD, which reduces the expression of *FLG* encoded epidermal protein filaggrin. Filaggrin mutation leads to abnormal keratinocyte differentiation and skin barrier dysfunction, and associate with dry and itchy skin. But *FLG* mutation is neither necessary nor sufficient to cause the disease. Many more genes associated with Th2 cytokines or antigen-processing could also contribute to genetic susceptibility of AD patients. (Irvine, McLean, & Leung, 2011; Weidinger et al., 2018)

Skin barrier dysfunction is one of the key features of AD. It is commonly observed in lesional skin, but several epidermal changes also occur in non-lesional skin of patients. The epidermal changes in AD include increased pH, altered surface microbiota, reduced water-holding function and increased trans-epidermal water loss, increased sensitivity to irritation and infection, and increased skin absorption of chemicals (Danby et al., 2018; Flohr et al., 2010; Halling-Overgaard et al., 2017; Jungersted et al., 2010; Kong et al., 2012). Reduced epidermal structural proteins and tight junction proteins, imbalance between proteases and their inhibitors, and an altered lipid profile are also observed. (Ishikawa et al., 2010; Janssens et al., 2012; Jungersted et al., 2010; Rawlings & Voegeli, 2013)

Studies of ceramide profiles in AD patients reported the close relationship of human stratum corneum (SC) ceramides and SC function. (Y. Cho, Lew, Seong, & Kim, 2004; Melnik, Hollmann, & Plewig, 1988) Total ceramide content including 11 species is downregulated in AD patients. We are specifically interested in the ceramide[NS] containing the non-hydroxy fatty acid chain and the sphingosine backbone, which is a known product of bacterial SMase. (Ishikawa et al., 2010)

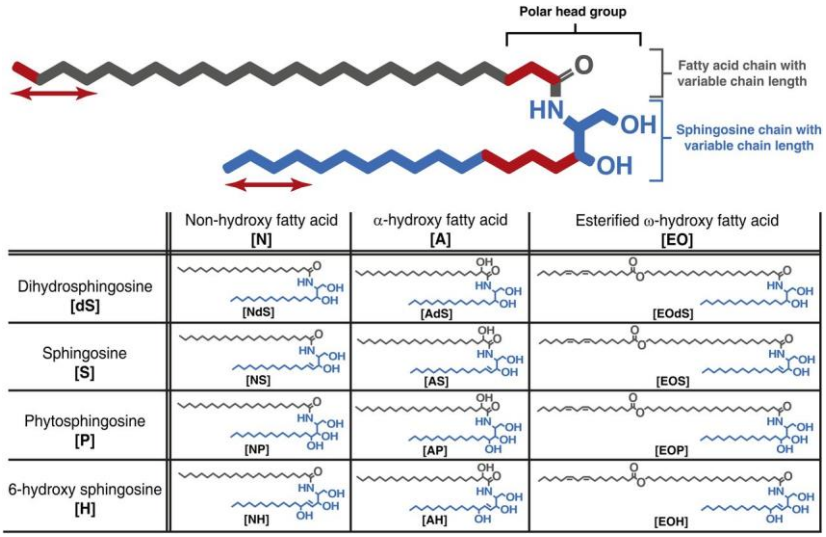


Figure 1.2 Structure and nomenclature of ceramide species. From (Janssens et al., 2012)

The total ceramide [NS] levels show no difference between AD patients and healthy controls, but short-chain ceramides (<40 total carbons) are abundant in AD patients compared to healthy individuals, while long-chain ceramides (>50 total carbons) significantly decrease, leading to an altered ceramide [NS] profile. Relative abundance of C34 ceramides were significantly increased in AD patients specifically. Strong positive correlation with SC trans-epidermal water loss, negative correlation with water-holding function, and negative correlation with barrier function were found with the expression levels of ceramide [NS]. (Ishikawa et al., 2010; Janssens et al., 2012)

AD shows distinct acute and chronic phases, and is associated with various T cell subsets. The cellular infiltration in AD lesions mainly consists of CD4+ T cells and increased number of DCs including LCs. T cell infiltration is also found in non-

lesional skin, but to a lesser extent. In the acute phase, the Th2 and Th22 cells are predominant, expressing Th2-associated cytokines (IL-13, IL-4, IL-31) and Th22-associated cytokine (IL-22). IL-4, IL-13 and other effects promote IgE class switching and induce IgE production. (Jujo, Renz, Abe, Gelfand, & Leung, 1992; Weidinger et al., 2018) While in chronic phase, the Th1 cells are progressively activated as well as Th2 and Th22, together with a modest activation of Th17 cells. (Gittler et al., 2012; Thepen et al., 1996) In addition to CD4+ T cells, CD8+ T cells can also be observed in patient skin, which represent an important source of IFNγ, IL-13 and IL-22. (Hijnen et al., 2013) However, the antigen specificity of skin-infiltrating T cells in AD is still not fully understood. T cell clones from AD lesional skin could recognize allergens, microbial antigens and autoantigens. The study of T cell clones in non-lesional and lesional skin proved the highly polyclonal TCR pattern in AD. (Bruijnzeel-Koomen & Mudde, 1997; Brunner et al., 2017; Roesner et al., 2016)

The imbalance of skin microbiota is also the key feature of AD patients, which will be discussed below.

1.4.2 Role of skin microbiota in AD

Predominant *Staphylococcus aureus* colonization is associated with AD, leads to an altered surface microbiota and reduced bacterial diversity. (Kong et al., 2012). In this study, *S. aureus* prevalence varied from 30% to nearly 100% in AD patients,

compared to 20% in healthy individuals; the prevalence of *S. aureus* colonization in patients was 70% for lesional skin, 39% for non-lesional skin and 62% for the nose. The density of the bacteria is associated with severity of AD. (Tauber et al., 2016; Totté, van der Feltz, Hennekam, et al., 2016)

The increased pH and expression of fibronectin and fibrinogen by keratinocytes promote the proliferation and binding of *S. aureus* on atopic skin. Besides, IL-4, IL-13 and IL-10 all decrease the host anti-microbial peptide production. Filaggrin breakdown products reduce the growth of *S. aureus*, and significantly reduce the bacterial secreted toxins by lowering the environment pH. (Miajlovic, Fallon, Irvine, & Foster, 2010) Thus, reduction of filaggrin expression weakens the host defense to *S. aureus*. All these factors enhance the colonization of the pathogen. However, it is still unclear whether these alterations lead to AD or are promoted by the disease. (S. H. Cho, Strickland, Boguniewicz, & Leung, 2001; Howell et al., 2005; Knor, Meholjic-Fetahovic, & Mehmedagic, 2011; Nomura et al., 2003)

S. aureus on AD skin secretes a variety of virulence factors that contribute to the disease, including superantigens (SAgs), α-toxin, δ-toxin, protein A, and SMase and more virulence factors are still being discovered. SAgs such as SEA/B induce non-specific APC-mediated T cell activation. The α-toxin is a hemolysin that triggers keratinocyte lysis. The δ-toxin contributes to the mast cell degranulation and inflammation. Protein A promotes the TNF-α production of keratinocytes.

(Williams & Gallo, 2015) SMase is found to be strongly secreted from the bacterial flora of AD patients (Ohnishi, Okino, Ito, & Imayama, 1999), which is one of the virulence factors of *S. aureus*. However, the role of SMase in AD pathogenesis is still unknown.

In AD patients, the predominant colonization of *S. aureus*, the increased short-chain ceramides in SC and the strongly secreted SMase suggest the participation of bacterial SMase in the disease. The studies of CD1a-reactive T cell activation to house dust mite and heat-killed *S. aureus* also demonstrate the potential role of CD1a in AD pathogenesis. (Hardman et al., 2017; Jarrett et al., 2016)

1.4.3 Pathogenesis of psoriasis

Psoriasis (PS) is a chronic, immune-mediated skin disorder with an estimated prevalence in adults at approximately 0.5%, and in children from 0% to 1.37%. One of the defining histological appearances of PS is the marked thickening of the epidermis, which is caused by increasing proliferation of keratinocytes and elongated epidermal rete. PS patients are more likely to develop cardiovascular disorders, diabetes, metabolic syndrome and depression, among which psoriatic arthritis (PsA) are the most frequent comorbidities. (Lowes, Bowcock, & Krueger, 2007; Michalek, Loring, & John, 2017)

PS is proved to have a strong genetic component, and family history of PS or PsA

is a high risk factor. (Rahman & Elder, 2005) The gene variations in PS susceptibility regions are largely involved in the disease, especially the variations of human leukocyte antigens (HLAs). (Sagoo, Cork, Patel, & Tazi-Ahnini, 2004)

Psoriasis is thought to be initially triggered by pathological inflammation and followed by amplification feedback loops between keratinocytes and immune cells. The central responding T cells in PS are thought to be the Th17 cells which are associated with IL-17A/F production and IL-23 response. The involvement of Th1 and Th22 cells are also key mediators in the disease. (Greb et al., 2016; Lowes et al., 2007) High percentages of CD8+ T cells also present in PS epidermal skin, which produce cytokines IL-17A/F and IL-22 (Hijnen et al., 2013).

1.4.4 Role of skin microbiota in PS

The prevalence of *S. aureus* colonization in patients with psoriasis is not as significant as in AD patients, but still higher than healthy individuals. The bacteria colonization is higher in lesional skin compared to non-lesional skin. (Totté, van der Feltz, Bode, et al., 2016)

S. aureus has been shown to perpetuate keratinocyte damage, activate Th1 and Th17 cells, inducing the production of TNFα and IFNγ, and associate with exacerbation of the disease. (Balci et al., 2009; Ng, Huang, Chu, Wu, & Liu, 2017; Totté, van der Feltz, Bode, et al., 2016) *S. aureus* SAgs induce T cell activation and

contributes to the pathogenesis of psoriasis. (ATEFI et al., 2012; D. Y. M. Leung, Walsh, Giorno, & Norris, 1993) *S. aureus* also secretes virulence factors protein A and induces IL-8 production. (Casas et al., 2011)

2. Aims and Objectives

The ability of CD1a-reactive T cells to recognize the CD1a-antigen complex is well established, but our understanding of the role of CD1a-reactive T cells in homeostasis and pathological conditions is still limited. Unlike CD1d proteins and NKT cells, research of CD1a and its responding T cells is relatively less, partially due to the lack of a widely available mouse model and the absence of efficient surface markers for CD1a-reactive T cells. Studies on polyclonal T cells, as well as the generation of new CD1a-reactive T cell lines and clones, will help us to investigate the functions, phenotypes and the underlying modulation mechanisms of CD1a-reactive T cells, which will extend our understanding of the human immune system.

CD1a-reactive T cells have been proved to be a normal component of human immune system, which participates in autoimmune disease and anti-microbial responses. CD1a-expressing APCs and CD1a-reactive T cells compose a rapid detection pathway to sense endogenous and exogenous lipids in the blood and at cutaneous sites. Besides directly presenting existing antigens, CD1a can also sense threats through recognizing neoantigens generated *in vivo* by PLA2. The co-localization of CD1a-expressing APCs and CD1a-reactive T cells in the skin suggests their potential contribution to interaction between immune system and skin microbiota.

We therefore postulated that CD1a-reactive T cells might contribute to anti-bacterial immunity, and sought to test the hypothesis that CD1a-reactive T cells recognize lipid antigens generated by *S. aureus* SMase as a method of bacterial sensing.

This hypothesis was tested by investigating the following aims:

Chapter 4: Identification of Bacterial Sphingomyelinase Responsive CD1a-reactive T Cells

Chapter 5: Investigation of the Role of Bacterial Sphingomyelinase in the Generation of CD1a Ligands

Chapter 6: Phenotypic and Functional Analysis of CD1a-reactive T Cell Lines and clones

Chapter 7: Identification of CD1a-reactive SMase-specific T cells in patients with skin diseases

3. Materials and Methods

3.1 Reagents

Sphingomyelinase: Sphingomyelinase from *Staphylococcus aureus* (Sigma-Aldrich, S8633)

Lipids from Sigma-Aldrich: Sphingomyelin from chicken egg yolk, ⩾95% (S0756)

Lipids from Avanti Polar Lipids: 36:2 SM (d18:1/18:1) (860587), 42:1 SM (d18:1/24:0) (860592), 42:2 SM (d18:1/24:1) (860593), Sphingomyelin (Milk, Bovine) (860063), 36:2 Ceramide (d18:1/18:1) (860519), 42:1 Ceramide (d18:1/24:0) (860524), 42:2 Ceramide (d18:1/24:1) (860525), 18:1 Lyso-PC (845875)

Anti-CD1a antibodies: Anti-CD1a antibody OKT6 (in house), Anti-CD1a antibody NA1/34 (in house), Purified anti-human CD1a Antibody, Clone HI149 (BioLegend 300101), Purified Mouse IgG1, κ Isotype Ctrl (BioLegend 400101)

Anti-MHC antibodies: Ultra-LEAF™ Purified anti-human HLA-A,B,C Antibody (311427), Ultra-LEAF™ Purified anti-human HLA-DR Antibody (307665), Ultra-LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody (401407), Ultra-LEAF™ Purified Mouse IgG2a, κ Isotype Ctrl (400263)

Flow Cytometry Antibodies (dilutions):

BioLegend: CD3 BV650 (1:100), CD3 FITC (1:100), CD3 PB (1:100), CD3 PerCP (1:100), CD3 BV785 (1:100), CD3 BV711 (1:100), CD4 AF488 (1:50), CD4 PE/Cy7 (1:100), CD4 AF700 (1:100), CD4 PE (1:100), CD4 FITC (1:00), CD4 PerCP (1:100), CD8 APC (1:100), CD8 PerCP (1:100), CD8 BV605 (1:100), CD8 PerCP/Cy5.5 (1:100)

TCRαβ PE/Cy7 (1:100), TCRαβ APC (1:100), TCRαβ BV605 (1:100), TCRαβ AF700 (1:100), TCRγδ APC (1:100), TCRγδ PE/dazzle 594 TxRd (1:100), TCRγδ PE (1:100), TCRγδ PE/Cy7 (1:100), TCR Vδ1 PE/Vio770 (1:10), TCR Vδ2 APC (1:100), CLA APC (1:100), CLA FITC (1:100), CLA PB (1:100), CD1a APC (1:100), CD1a BV421 (1:100), CD14 PerCP (1:100), CD11c PE (1:100), HLA-DR FITC (1:100), HLA-DR PE (1:100) Langerin PE (1:100), CD45 FITC (1:100), NKG2D FITC (1:100), NKG2D BV605 (1:100), CD69 PB (1:100), CD69 APC/Cy7 (1:100), CD69 AF700 (1:100), CD25 APC/Cy7 (1:100), CD28 APC (1:100), CD45RA FITC (1:100), CD45RO APC/Cy7 (1:100)

Invitrogen: Live/Dead Aqua (1:1000)

ThermoFisher: CellTrace Violet (1:1000)

Glass bottom cell culture plate: CELLview™ Culture dish, tissue culture treated, glass bottom (627860)

3.2 PBMC isolation from blood

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from the whole blood from donors with informed consent and ethics approval. Blood sample was diluted 1:1 with RPMI supplemented with 2mM L-glutamine, 100iu/ml penicillin, 100µg/ml streptomycin and Hepes (Life Tecnhologies) (RHepes) before layering over 15ml Lymphoprep (Nycomed, Roskilde, Denmark) in a 50ml Falcon tube. Sample was centrifuged for 20min at 2000rpm with the centrifuge brake switched off. The buffy coat layer of PBMCs was collected and washed twice in RHepes at 1800rpm for 10min and 1500rpm

for 5min, and then resuspended in complete media (RPMI supplemented with 2mM L-glutamine, 100iu/ml penicillin, 100µg/ml streptomycin, and 10% fetal calf serum (FCS) (Sigma-Aldrich), HEPES, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol (Life Technologies)).

3.3 Isolation of human T cells

PBMCs were isolated from fresh blood or cone samples. T-cells were purified from PBMCs using CD3 MACS separation beads (Miltenyi Biotec, Germany). PBMCs were resuspended in 80µl MACS buffer (0.5% PBS with 0.2mM EDTA) per 10 million cells and incubated with 20µl CD3 Microbeads per 10 million cells for 15min at 4°C. Cells were then washed in 1-2ml of MACS buffer per 10 million cells at 1500rpm, and resuspended in 500µl MACS buffer per 10 million cells. LS columns on a MACS magnet were pre-washed with 3ml of MACS buffer and then the cell suspension was passed through the LS column and washed 3 times with 3ml MACS buffer. The column was then removed from the magnet. 5ml MACS buffer was added and magnetically labelled cells were flushed out by pushing the plunger into LS column. Enriched cells were washed and resuspended in T cell medium (RPMI supplemented with 2mM L-glutamine, 100iu/ml penicillin, 100µg/ml streptomycin, and 5% heat-inactivated human serum, HEPES, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol (Life Technologies, CA, USA) in the presence of 1nM IL-2 (PeproTech, London, UK)) (TCM).

3.4 Monocyte-derived Dendritic cells (mDCs) culture

Autologous mDCs were differentiated from CD14+ monocytes. CD14+ human cells were isolated from PBMCS using MACS separation beads (Miltenyi Biotec Inc., Germany). CD14+ cells were then cultured in 6-well plates at 0.5 x 10^6 cells/ml in complete medium containing 50ng/ml GM-CSF (PeproTech, London, UK) and 1000iu/ml IL-4 (PeproTech, London, UK).

After 4 days, CD1a, CD11c and CD14 expressions were checked by Flow Cytometry. The differentiated mDCs were used as antigen presenting cells in ELISpot. The mDCs were incubated with 10μg/ml anti-HLA-A,B,C and anti-HLA-DR blocking antibodies overnight before co-culture with T cells to block the HLA-mediated T cell responses.

3.5 *Ex-vivo* ELISpot

CD1a-reactive T cell responses were tested by IFN-γ, IL-22 and IL-17A ELISpot (Mabtech AB, Sweden). T cells were isolated from PBMCs by CD3+ MACS separation. T cells were cultured in TCM to rest for 3-5 days.

On first day of the ELISpot, T cells were washed twice in RHepes and transferred to a new plate in complete media at about 1-2 x 10^6 cells/ml. K562 cells were washed twice with RHepes and resuspended in complete media at about 1 x 10^6

cells/ml, and added with 1μg/ml Sphingomyelinase (SMase) or heat-inactivated SMase overnight. K562 cells added with media only were used as control groups. T cells and K562 cells were cultured at 37°C and 5% CO_2. ELISpot plates (96-well multiscreen IP-plates Millipore Corp., MA, USA) were pre-treated with 15μl/well 35% ethanol for no more than one minute, and washing six times with distilled water. ELISpot plates were then coated with 70 μl/well capture antibody (MabTech) at 15μg/ml of anti-IFN-γ, or 10μg/ml anti-IL-22, or 10μg/ml anti-IL-17A overnight at 4°C.

On second day of the ELISpot, pre-treated K562 cells were washed in RHepes and resuspended in complete media at 0.25 x 10^6 cells/ml for plating out. The ELISpot plates were washed five times with RHepes and blocked with complete media for 30min to 1h at 37°C. The ELISpot plates were then added with 100μl K562 cells (25,000 cells/well). Polyclonal T cells were washed and resuspended in complete media at 0.5 x 10^6 cells/ml for plating out. T cell lines and clones were resuspended at 0.1 or 0.2 x 10^6 cells/ml for plating out. 100μl of T cells (50,000 cells/well for polyclonal T cells, and 10,000 or 20,000 cells/well for T cell lines and clones) were added into plates. Wells were set-up in duplicate or triplicate. PMA 20ng/ml (Sigma-Aldrich, St Louis, MO, USA) and Ionomycin 10ng/ml (Sigma-Aldrich, St Louis, MO, USA) were used as a positive control, and T cells only in the absence of K562 cells were used as a negative control. In some experiments 10μg/mL anti-CD1a blocking antibody or 10μg/mL isotype control were added to wells to test

the CD1a-dependency.

On third day of the ELISpot, the ELISpot plates were developed. After overnight incubation at 37°C and 5% CO_2, plates were washed 5 times with PBS and incubated with 50µl/well 1µg/ml of biotin-linked anti-IFN-γ, anti-IL-22 or anti-IL-17A monoclonal antibody (Mabtech AB, Sweden) for 2-4 hours at room temperature. Plates were then washed 5 times with PBS and incubated for 1-2 hours with 50µl/well 1µg/ml streptavidin-alkaline phosphatase (Mabtech AB, Sweden) at room temperature. Plates were washed 3 times with PBS and twice with distilled water. Spots were visualized using an alkaline phosphatase conjugate substrate kit at 100µl/well (Biorad, Hercules, CA, USA) and counted by automated ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic, Germany). Results were expressed as spots per number of T cells added per well.

3.6 Real-time PCR

T cells were isolated from PBMCs by CD3+ MACS separation. T cells were cultured in TCM to rest for 3-5 days. On first day of the real-time PCR, T cells were washed twice in RHepes and transferred to a new plate in complete media at about 1-2 x 10^6 cells/ml. K562 cells were washed twice with RHepes and resuspended in complete media at about 1 x 10^6 cells/ml, and incubated with 1µg/ml SMase. K562 cells added with media only were used as control groups. T cells and K562 cells were cultured at 37°C and 5% CO_2 overnight.

On second day of real-time PCR, pre-treated K562 cells were washed in RHepes and resuspended in complete media at 0.05 x 10^6 cells/ml for plating out. The 96-well-round-bottom plates were added with 100µl K562 cells (5,000 cells/well). Polyclonal T cells were washed and resuspended in complete media at 0.5 x 10^6 cells/ml for plating out. 100µl of T cells (50,000 cells/well) were added into plates. Wells were set-up in triplicate. Plates were cultured at 37°C and 5% CO_2 overnight.

On third day of real-time PCR, cells from triplicate wells were collected into one tube. Cells were washed and processed with mRNA extraction using Qiagen Turbocapture mRNA kit (Qiagen, Manchester, UK) and reverse transcription (Life Technologies). Real-time PCR was performed using the following Taqman gene expression assays (Applied Biosystems): *CD2* (Hs01040179_g1), *GAPDH* (Hs02786624_g1), *IFNγ* (Hs00989291_m1), *IL-22* (Hs01574154_m1), *IL-17A* (Hs00174383_m1), *IL-13* (Hs00174379_m1), *IL-10* (Hs00961622_m1), *IL-6* (Hs00174131_m1), *IL-17F* (Hs01028648_m1), *IL-4* (Hs00174122_m1), *IL-5* (Hs01548712_g1). Reactions were performed in a 7500 fast thermal cycler (Applied Biosystems).

3.7 Cytokine secretion assay in plate

CD1a-reactive T cell responses were tested by IFN-γ, IL-22 and IL-17A secretion assay (Miltenyi Biotec, Germany). T cells were isolated from PBMCs by CD3+

MACS separation. T cells were cultured in TCM to rest for 2-4 days.

On first day of the secretion assay, T cells were washed twice in RHepes and transferred to a new plate in complete media at about 1-2 x 10^6 cells/ml. K562 cells were washed twice with RHepes and resuspended in complete media at 0.5 x 10^6 cells/ml, and incubated with 1μg/ml SMase, heat-inactivated SMase, 100μM ceramides or 100μM sphingomyelins for 48 hours. K562 cells added with media only were used as control groups. T cells and K562 cells were cultured at 37°C and 5% CO_2.

On third day of the secretion assay, pre-treated K562 cells were collected and washed with RHepes. 2μl of CellTrace stock solution was added to 5ml of PBS for a final working solution (2mM). 1ml of CellTrace solution was used to resuspend the cell pellet in each condition, and incubated the cells for 10min at 37°C, protected from light. 5ml complete medium was added to the cells and incubated for a further 5min at 37°C. Cells were collected and washed, and resuspend in fresh pre-warmed complete media at about 2 x 10^6 cells/ml, then added 100μl (200,000 cells/well) in wells of 96-well-flat-bottom plates. T cells were collected and washed with RHepes, then resuspended with complete media at 1-2.5 x 10^6 cells/ml. 100μl (100,000-250,000 cells/well) of T cells was added in each well. No more than 250,000 T cells per well. T cells and K562 cells were cultured at 37°C and 5% CO_2 for 6h. In some experiments IL-12 was added at final concentration of

500pg/ml. PMA and Ionomycin were added in positive control group for 3h incubation.

After 6h incubation, cells were collected in 96-well-round-bottom plates. Cells were washed with 200µl cold media and centrifuged at 1500rpm for 5min at 4°C. Supernatant was completely removed, and cells were fully resuspended in cold catch-reagent mix (4µl of each cytokine catch reagent and top-up with cold complete media to 40µl for each condition), then incubated in 4°C fridge for 5 min. Cells were then transferred to 96-deep-well plate, and added with 1.5ml of warm detection-reagent mix (1.5ml of warm complete media with 4µl of each cytokine detection reagent for each condition). Cells were incubated for 45min at 37°C with slow rotation every 5min.

After 45min incubation, the cells were collected and washed by RHepes, then resuspended with 40µl of staining mix with live/dead stain (Invitrogen) and other staining antibodies. Cells were transferred to 96-well-round-bottom plate and incubated for 10min at 4°C in dark. Cells were then washed and ready for FACS analysis or sorting.

3.8 Rapid expansion method (feeders)

T cells were expanded by rapid expansion mix (REM). Two B cell lines CP and DG and PBMCs from one donor with IL-2 (BioLegend), PHA (eBioSciences) and anti-

CD3 (OKT-3) (BioLegend) in TCM were used to prepare REM.

CP and DG B cells and PBMCs from one donor were collected and irradiated. For each B cells, irradiated for 30min at 9000rad; for PBMCs, irradiated for 10min, 3000rad. Cells were collected and washed, then diluted with TCM. For each B cells, diluted to 0.6 x 10^6 cells/ml; for PBMCs, diluted to 6 x 10^6 cells/ml. Then equal volumes of CP B cells, DG B cells and PBMCs were fully mixed together, and the final solution had 0.2 x 10^6 cells/ml CP, 0.2 x 10^6 cells/ml DG and 2 x 10^6 cells/ml PBMCs. Cells were added with 200U/ml of IL-2, 2.5μg/ml of PHA and 50ng/ml of anti-CD3 (OKT-3).

For 0.1-1 x 10^6 cells/ml starting T cells to expanded in T25 flask, 5-10ml of REM was added. At Day5, 10ml of fresh TCM was added to the flask. T cells were refreshed with 10ml TCM every 2-3 days by gently removing the top 10ml. T cells were transferred to T75 flask when there was an obvious opaque lawn of cells on the bottom of the flask, and added with additional 10ml TCM. T cells were again refreshed with 10ml TCM every 2-3 days. The proliferation usually slowed down at around Day14, and cells were frozen or transferred to 48-well plates for experiments.

3.9 CD1a tetramerization

CD1a tetramers were prepared from CD1a monomers loaded with target lipids or

endogenous lipids, gifted from Harvard or from NIH. The stock concentration of CD1a monomers were 0.2μg/μl. 10μl of stock CD1a monomer (2μg) was used for CD1a tetramerization. The PE-Streptavidin (BioLegend, 405203) was diluted to 4μg/100μl in 50mM TBS pH8. 2μl of diluted PE-Streptavidin was added in CD1a monomer solution each time, fully mixed and rested at room temperature in dark for 10min. For 10μl of CD1a monomer, PE-Streptavidin was added for 5 times, so the final volume of CD1a-tetramer with PE fluorescent was 20μl. The concentration of assembled tetramer was about 0.1μg/μl.

3.10 CD1a tetramer staining

0.1-0.5 x 10^6 T cells were collected and washed in FACS buffer (PBS, 0.5% FCS, 2mM EDTA, pH7.2), then transferred to 96-well-round-bottom plate. Cells were incubated with 50μl live/dead stain (Invitrogen) at room temperature for 15min, then washed and resuspended in 40μl FACS buffer with 1μl CD1a-tetramer at room temperature for 25min. For 20μl of CD1a-tetramer prepared from monomer stock, 1μl of tetramer was used in each condition. Without washing, cells were added with 10μl anti-CD3 (OKT3) (BioLegend) and incubated at room temperature for 10min. 50μl of staining mix with other staining antibodies was added to cells to make a final 100μl solution, and incubated at 4°C for 15min. Cells were then washed twice in FACS buffer and incubated for 10min at 4°C with Fixation buffer (1x PBS solution contains 4% paraformaldehyde). After fixation, cells were washed and resuspend with FACS buffer. Data was acquired using Attune

NxT Flow Cytometer (Life Technologies, USA)

3.11 Flow cytometry

0.1-0.5 x 10^6 cells were washed twice in FACS buffer and then incubated for 30 minutes at 4°C with live/dead stain (Invitrogen) and staining antibodies. The list of antibodies and titrations used can be found in Chapter 3.1. Cells were then washed with FACS buffer and incubated for 10min at 4°C with Fixation buffer. After fixation, cells were washed and resuspend with FACS buffer. Data was acquired using Attune NxT Flow Cytometer (Life Technologies, USA)

3.12 PBMC isolation from skin

Skin samples from clinical were obtained from Oxford hospitals, from patients with informed consent and ethics approval. Hair and fatty tissue were removed. Samples were finely diced and incubated overnight at 37℃ 5% CO_2 in 1mg/ml collagenase in complete media. After incubation, 2-3 ml cold 10mM PBS/EDTA was added and clumps were dispersed by repeated pipetting. The sample was then passed through a 70 µm nylon cell strainer and washed through with 50 ml PBS/EDTA. Sample was then passing through a 40 µm nylon cell strainer and washed for 20min at 1500rpm and 4℃. Cells were resuspended in 25 ml RHepes and layered over 15ml Lymphoprep (Nycomed, Roskilde, Denmark) and centrifuged for 20min at 2000rpm with the centrifuge brake switched off. The buffy coat layer of cells was collected and washed twice in RHepes at 1800rpm for

10min and 1500rpm for 5min, and then resuspended in complete media. CD3+ cells were then isolated from cells using CD3 MACS separation beads (Miltenyi Biotec, Germany).

3.13 Statistics

Statistical analyses were performed using GraphPad Prism 6. Cohorts of healthy donors, AD and PS patients investigated for CD1a-reactive T cell responses were analyzed using two-tailed unpaired or paired t test. For tetramer binding and CLA expression, the log-transformed value of fold changes was used for statistics. Significance of log-fold change was calculated by one-sample t test.

4. Result I: Identification of Bacterial Sphingomyelinase Responsive CD1a-reactive T Cells

Introduction and aims

Major Histocompatibility Complex (MHC) molecules can present peptide antigens to stimulate T cell receptor (TCR) and trigger downstream effector function. T cell activation through TCR-peptide-MHC complex is known as one of the most important discoveries in Immunology and has many translational applications including vaccines, therapeutic manipulation or diagnostic tests (Lau & Dunn, 2018; Skwarczynski & Toth, 2016). However, as previous discussed, recent studies show that MHC is not the only antigen-presenting system.

CD1 proteins are a family of MHC-I-like glycoproteins, first identified in a paper published in 1979 (McMichael et al., 1979). The genes of this family were cloned later (F Calabi & Milstein, 1986). With the finding of CD1-reactive T cells and CD1 antigens, it became appreciated that CD1 proteins have the ability to present lipid antigens to activate T cells (Beckman et al., 1994; Porcelli et al., 1989). Their antigens include mammalian self-lipids, such as skin oils (Jong et al., 2014), and foreign lipids that are unique to specific microorganisms, such as mycolic acid (Beckman et al., 1994).

Based on sequence analysis, the CD1 family could be classified into three groups:

group 1 includes CD1a, CD1b and CD1c, which are expressed in humans, and present lipids to specific T cells; group 2 is the CD1d protein, which is expressed in both humans and mice, and presents antigens to natural killer T cells; group 3 is CD1e, a soluble intracellular protein thought to be important for lipid loading (Barral & Brenner, 2007).

CD1a is the CD1 isoform that highly expressed by epidermal Langerhans cells, and by subsets of dendritic cells in epithelia of various tissues. It can also be induced by other cells including human group 2 innate lymphoid cells (ILC2). Its tissue-distribution suggests a role of CD1a in antigen-presentation at the skin (Hardman et al., 2017; Huang et al., 2001; Peña-Cruz, Ito, Dascher, Brenner, & Sugita, 2003). Like other CD1 isoforms, CD1a is able to present self-lipids as well as exogenous lipids. CD1a could bind to endogenous ligands like sphingolipids, phospholipids, sulfatide, and headless skin oils (Hardman et al., 2017; Shamshiev et al., 2002; Zajonc et al., 2003a). It is also demonstrated to bind DDM from *Mycobacterium tuberculosis*, and urushiol from plants (J. H. Kim et al., 2016; Moody et al., 2004).

CD1a-reactive T cells were one of the first known forms of CD1-reactive T cells. Recent studies also proved that CD1a-reactive T cells are a normal component of the human T cell repertoire, and commonly present in human blood and skin. CD1a-autoreactive T cells are able to produce cytokines include IFNγ and IL-22, and contribute to skin disease such as atopic dermatitis and psoriasis (Cheung et

al., 2016; Jarrett et al., 2016; Jong et al., 2014; Preihs, Christian Arambula et al., 2013). The existence of CD1a protein, CD1a ligands and CD1a-reactive T cells at skin suggests a key role of CD1a in the skin immune system.

Besides directly binding and presenting exogenous lipids, CD1a can also sense threats through recognizing neoantigens generated *in vivo* by foreign enzymes. A series of papers from our lab demonstrate a previously unknown immune response that was induced by phospholipase A2 (PLA2). PLA2 from bee venom and house dust mite generate lipid antigens *in vivo* and activate T cells through CD1a. Host PLA2 from mast cells and ILC2 could also contribute to CD1a-reactive T cell responses. In these systems, PLA2 is an essential component for CD1a ligand generation, and the CD1a-reactive T cell response is dependent on enzymatic bioactivity (Bourgeois et al., 2015; Cheung et al., 2016; Hardman et al., 2017; Jarrett et al., 2016)

We therefore postulated that CD1a-reactive T cells might contribute to anti-bacterial immunity, and sought to test the hypothesis that CD1a-reactive T cells recognize lipid antigens generated by *S. aureus* SMase *in vivo* as a method of bacterial sensing.

The key aims of this chapter were to identify and quantify CD1a-reactive SMase-specific T cells in the peripheral blood of healthy individuals, to characterize their

functional phenotype as determined by cytokine secretion, and to investigate the relationship between T cell response and enzymatic bioactivity. K562 cells that lacked surface expression of MHC protein were used as antigen-presenting cells, which had been engineered to highly express human CD1a proteins (K562-CD1a), and were kindly donated by Branch Moody (Harvard). This system allows the parallel testing of unrelated donors without the influence of MHC alloreactivity. T cell responses were examined at both transcriptional and translational level. The CD1a-reactive SMase-specific T cell phenotype was further tested by flow cytometry. Heat-inactivation of SMase was used to investigate the influence of enzymatic bioactivity to T cell response. And anti-CD1a antibody was used to confirm CD1a specificity. The experiments were conducted to examine responses to potential neoantigens generated by bacterial SMase.

4.1 Recognition of bacterial SMase by ex-vivo CD1a-reactive T cells

To investigate whether ex-vivo CD1a-reactive T cells from healthy donors were able to response to bacterial sphingomyelinase, T cells from PBMCs were isolated by MACS CD3+ beads separation. SMase from *Staphylococcus aureus* was obtained from Sigma-Aldrich.

K562 cells transfected with human CD1a molecules (K562-CD1a) were used as antigen-presenting cells. This cell line has low HLA expression on the surface, and thus may avoid the influence of MHC-TCR interaction. It also largely avoided

alloreactivity, so we may test T cells from unrelated donors in parallel. K562 cells transfected with empty vector (K562-Ev) were used as negative control conditions.

In agreement with previous published studies, we first used interferon-γ (IFNγ) as a 'readout' of CD1a-reactive T cell activity. (Cheung et al., 2016; Jong et al., 2014; Preihs, Christian Arambula et al., 2013)

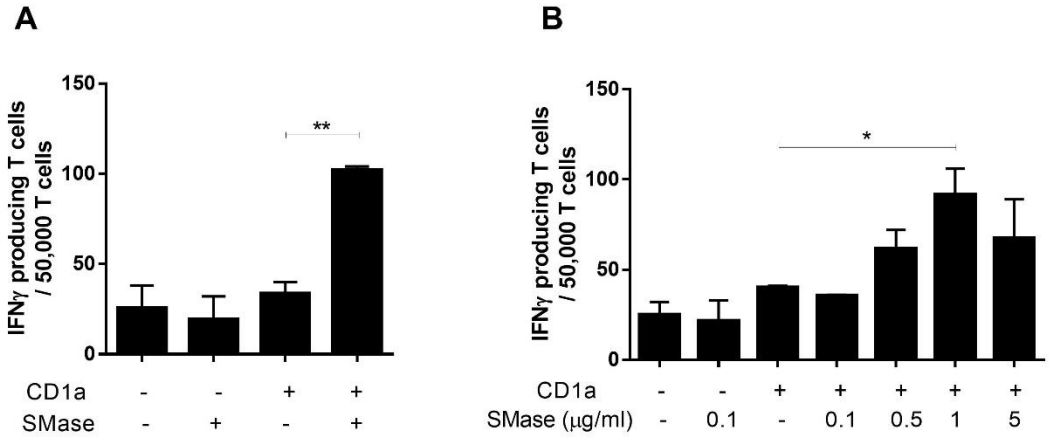


Figure 4.1 Circulating CD1a-reactive SMase-specific T cells produce IFNγ. T cells were isolated from healthy donor PBMCs (A – donor C1742; B – donor C1757) and incubated overnight with K562-CD1a or K562-Ev cells pre-treated with or without *S. aureus* SMase. IFNγ production was measured by ELISpot. Data representative of at least three donors from different experiments (A). The titration of the IFNγ response to increasing concentration of SMase is shown (B). Bars represent standard error. * $P<0.05$, ** $P<0.01$.

As seen previously(Bourgeois et al., 2015), a general background of non-specific

response to K562 cells was observed, at a rate of about 4 in 10,000. There was a significant T cell activation against K562-CD1a cells with SMase pre-treated overnight, compared with SMase only group and CD1a only group, indicating that the T cell response was dependent on the presence of both CD1a protein and SMase enzyme. (Figure 4.1A)

The titration of SMase concentration was also tested. With the increasing of enzyme concentration from 0.1μg/ml to 1μg/ml, the T cell response also increased (Figure 4.1B) The concentration of 1μg/ml SMase was used in future experiments.

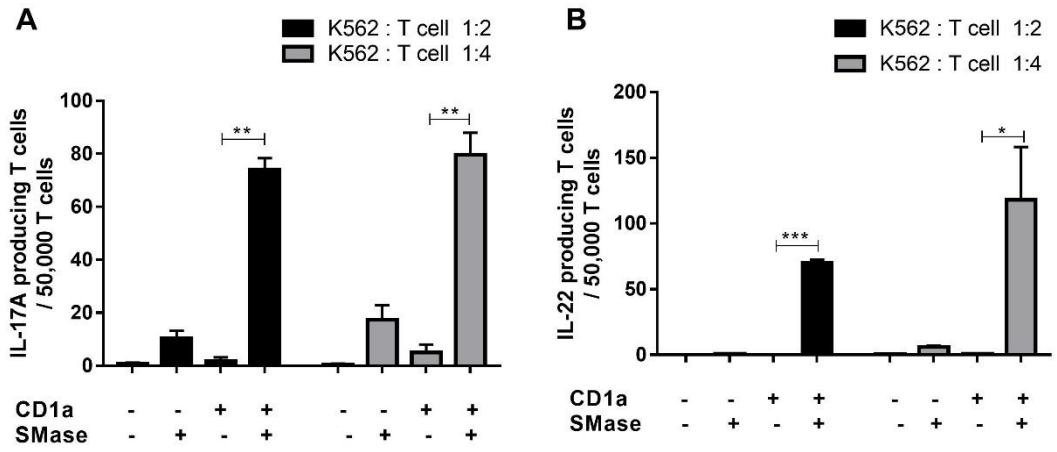


Figure 4.2 Circulating CD1a-reactive SMase-specific T cells produce IL-17A and IL-22 under different K562/T cell ratios. T cells were isolated from the same healthy donor PBMCs (donor 210116-C1) and incubated overnight with K562-CD1a or K562-Ev cells pre-treated with or without *S. aureus* SMase. IL-17A (A) and IL-22 (B) production were measured by ELISpot. Two different ratios of K562 and T cells were tested. Data representative of at least three donors from different

experiments. Bars represent standard error. * P<0.05, ** P<0.01, *** P<0.001.

Based on previous studies, CD1a-reactive T cells in human blood were able to produce IL-22 and IL-17A (Cheung et al., 2016; Preihs, Christian Arambula et al., 2013), and the role of these two cytokines in human skin diseases has been proved (Fujita, 2013; Jin & Dong, 2013). So, besides IFNγ, the IL-17A and IL-22 responses of T cells against K562-CD1a pre-treated with SMase overnight were also tested by ELISpot. Consistent with IFNγ, significant production of IL-17A and IL-22 were observed separately from the same donor, compared to SMase only and CD1a only group, indicating a broad contribution of CD1a-dependent SMase-specific responses. (Figure 4.2) Two ratios of K562 and T cells were tested, and in both conditions the CD1a-reactive SMase-specific response was observed. Thus, the ratio K562 cell: T cell 1:2 was used in future experiments, consistent with ratio in IFNγ experiments. Sometimes, above the non-specific background against K562 cells, a small (and not significant) T cell response was also observed against K562-Ev cells pre-treated with SMase. This suggested the CD1a may not be the only pathway involved in this response.

4.2 CD1a-reactive SMase-specific T cell responses are regulated at the transcription level

In order to further confirm the cytokine up-regulation of CD1a-reactive SMase-specific T cells, and also in an attempt to reduce T cell requirements necessary for

experiments, we tested the T cell response at the transcriptional level by real-time PCR. Polyclonal T cells were isolated by CD3+ MACS beads. K562-Ev and K562-CD1a cells were pre-treated with or without SMase overnight. T cells were cultured with K562 cells, and mRNA extracted and were further used in real-time PCR.

In the previous research (Preihs, Christian Arambula et al., 2013), polyclonal T cells were cultured with K562 cells for 6 hours at a K562 to T cell ratio of 1:10. Samples were normalized to β-actin. At this ratio the cytokines produced by K562 cells were not detectable compared with T cells. Based on this paper, we kept the same K562 to T cell ratio of 1:10 in future experiments.

However, according to RNA expression data from the Human Protein Atlas (https://www.proteinatlas.org), K562 cells also express low level of general reference genes including *GAPDH* and *β-actin.* (Figure 4.3) Here, we used *CD2*, which is a widely used pan-T-cell marker, as reference gene in real-time PCR. (Naeim, Nagesh Rao, Song, & Phan, 2018)

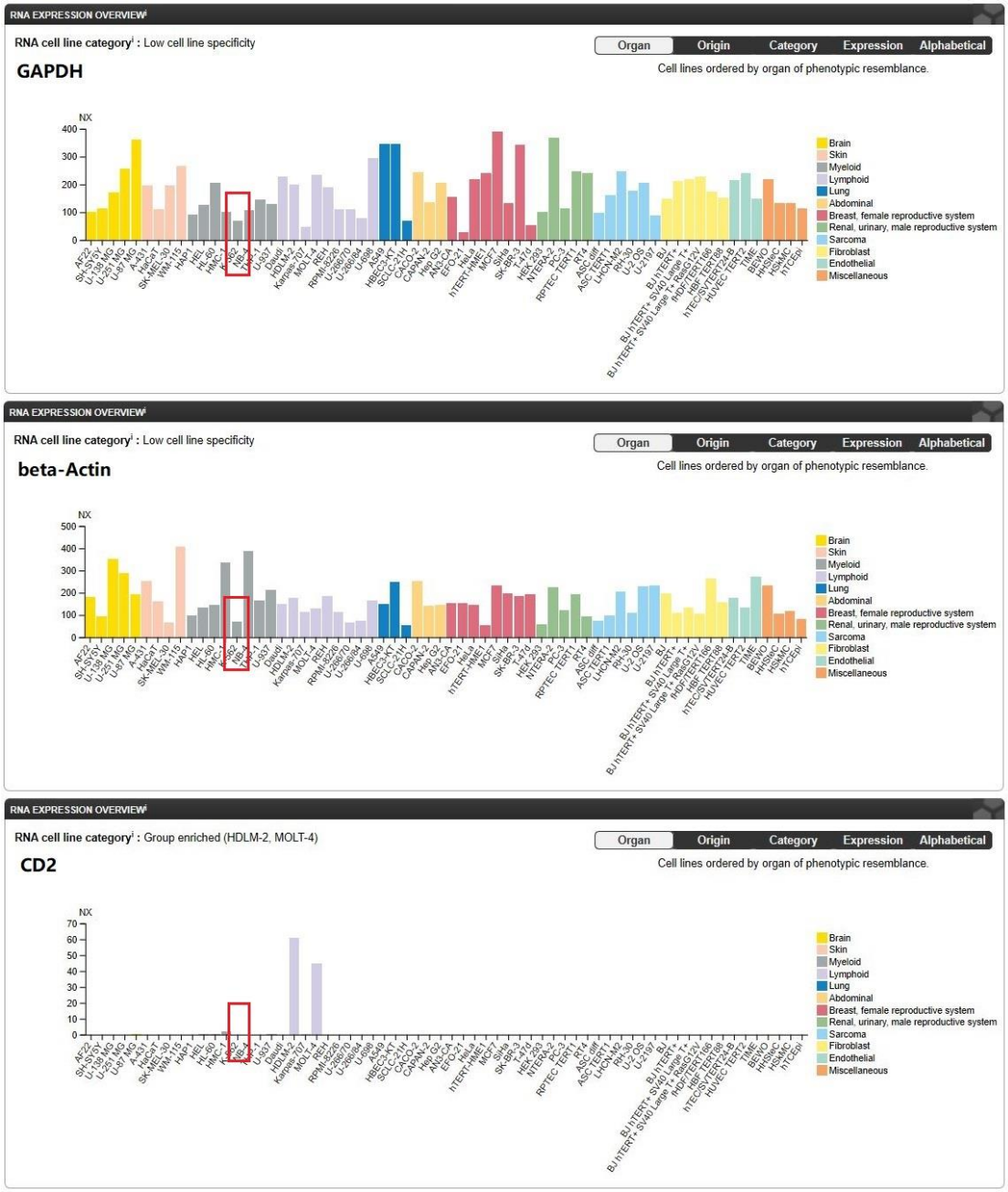


Figure 4.3 RNA expression overview of *GAPDH, β-actin* and *CD2* from the Human Protein Atlas. The RNA expression level in K562 cells is labelled in red.

According to the RNA expression result from the Human Protein Atlas, K562 cells do not express *CD2.* (Figure 4.3) We also tested the RNA expression level of *CD2* and other cytokines, including *IFNγ, IL-17A, IL-22* and *IL-13*, in K562-Ev and

K562-CD1a cells by real-time PCR. None of these genes was detectable. Thus, the K562 cells in mixed samples did not influence the RNA expression results in experimental conditions.

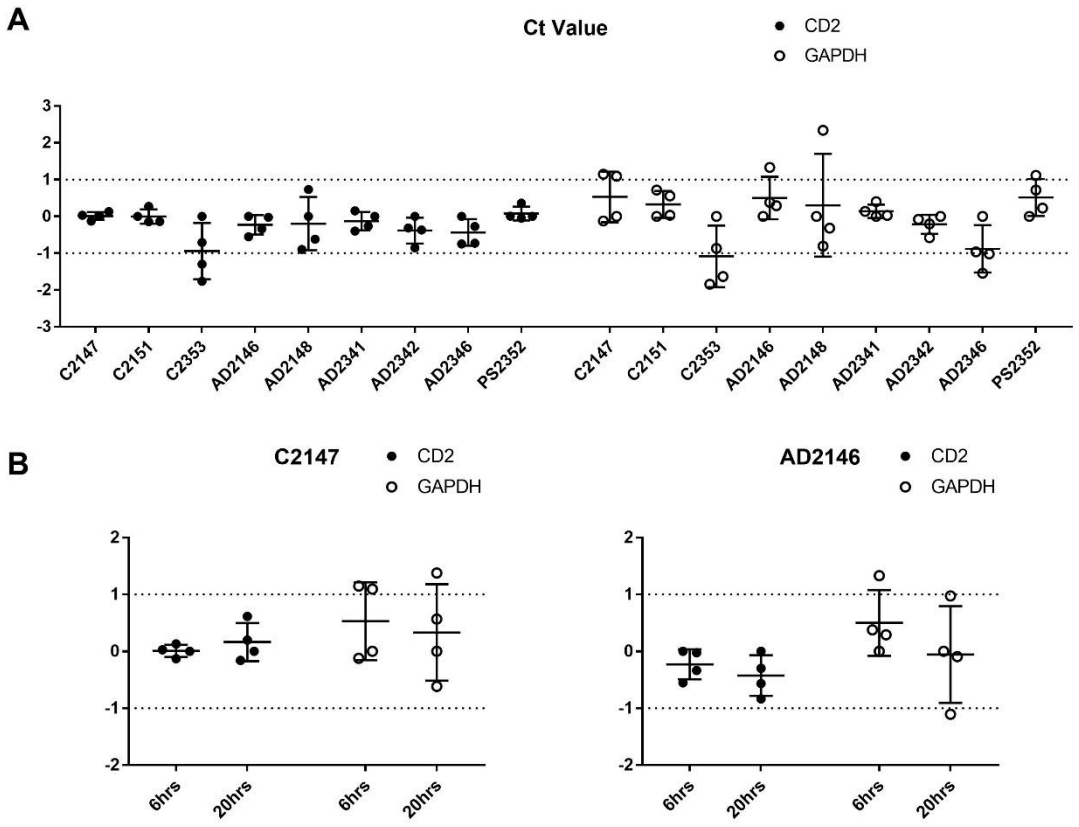


Figure 4.4 *CD2* expression was stable in experiment conditions. The RNA expression level of *CD2* was tested by real-time PCR in healthy donors, AD and PS patients. Four experiment conditions were tested, which were K562-Ev and K562-CD1a cells with or without SMase separately, and each spot in every donor represent one condition. Difference value of Ct value was used as an indicator of Ct value variation, which was measured by raw Ct value in each condition minus Ct value of K562-Ev without SMase. The difference value of Ct value of *CD2* (black) and *GAPDH* (red) were shown. (A) The difference value of Ct value in 9 donors. (B)

The difference value of Ct value under two co-culture time in two selected donors (C2147, AD2146).

To confirm that *CD2* could be used as reference gene, we first tested the Ct value of *CD2* and *GAPDH* in different experimental conditions. Polyclonal T cells isolated from healthy donors and patients with AD and PS were cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with or without SMase separately. RNA expression level of *CD2* and *GAPDH* in every condition were tested by real-time PCR. We used the difference value of Ct value as an indicator to measure the variation of gene expression. In 9 different donors, irrespective of skin diseases, the difference value of Ct value of *CD2* was in the range between -1 to 1, indicating the *CD2* expression was stable in experimental conditions. Compared to the results of *GAPDH*, *CD2* expression was stable. (Figure 4.4A)

The difference value of Ct value was also tested in two co-culture periods. T cells were cultured with K562 cells for 6hrs and 20hrs, and *CD2* gene expression level was tested. (Figure 4.4B) *CD2* expression under two co-culture time was stable among all four conditions, compared to the results of *GAPDH*.

All of these results indicated that *CD2* may use as a stable reference gene; and so, in the future experiments, samples were normalized to *CD2*.

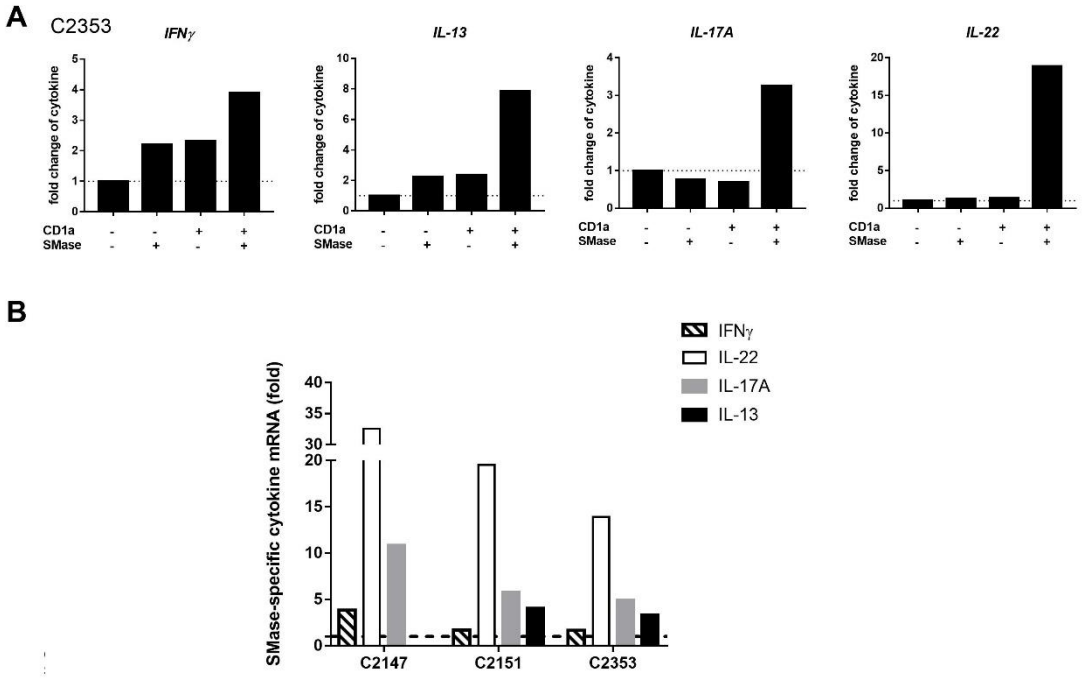


Figure 4.5 Circulating CD1a-reactive SMase-specific T cells up-regulate cytokines at the transcriptional level. (A) Polyclonal T cells isolated from healthy PBMCs cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with or without SMase. Cytokine gene upregulation was measured by real-time PCR. Cytokine mRNA fold change results of one selected donor (C2353) are shown. (B) Polyclonal T cells from healthy PBMCs cultured with K562-CD1a with or without SMase pre-treatment. Summary results of SMase-specific cytokine gene upregulation in three healthy donors. Samples were normalized to *CD2*.

The *IFNγ*, *IL-13*, *IL-17A* and *IL-22* gene upregulations of one selected healthy donor C2353 were measured by real-time PCR (Figure 4.5A). Consistent with the ELISpot result, *IL-22* expression was up-regulated 20 fold against K562-CD1a with SMase, and much higher than CD1a only and SMase only group. We also detected

an 8 fold upregulation of *IL-13*, and 3~4 fold upregulation of *IFNγ* and *IL-17A*.

The real-time PCR was repeated in three healthy donors to expand the pilot study (Figure 4.5B). In all these three donors, the SMase-specific *IL-22* and *IL-17A* gene upregulation was observed when CD1a present. There was also an upregulation of *IL-13* gene expression, which suggested the involvement of type 2 cytokine in CD1a-reactive SMase-specific T cell response. The *IFNγ* upregulation was only 1.5~3 fold. However, based on previous studies (S. Kim et al., 2013), the *IFNγ* mRNA expression upregulated from 30mins and peaked at 4 hours after stimuli, suggesting that the 6 hours culture might be too long.

Real-time PCR offered a quick test for cytokines involved in CD1a-reactive SMase-specific T cell responses in single experiment. The approach reduced total T cell requirements and experimental time. We therefore used real-time PCR as a pilot testing method in future experiments.

4.3 The CD1a-reactive SMase-specific T cell responses were repeatable in large healthy cohort

To confirm the CD1a-reactive SMase-specific T cells were a normal component of human blood repertoire, the T cell responses were subsequently tested in a larger cohort of healthy donors by ELISpot.

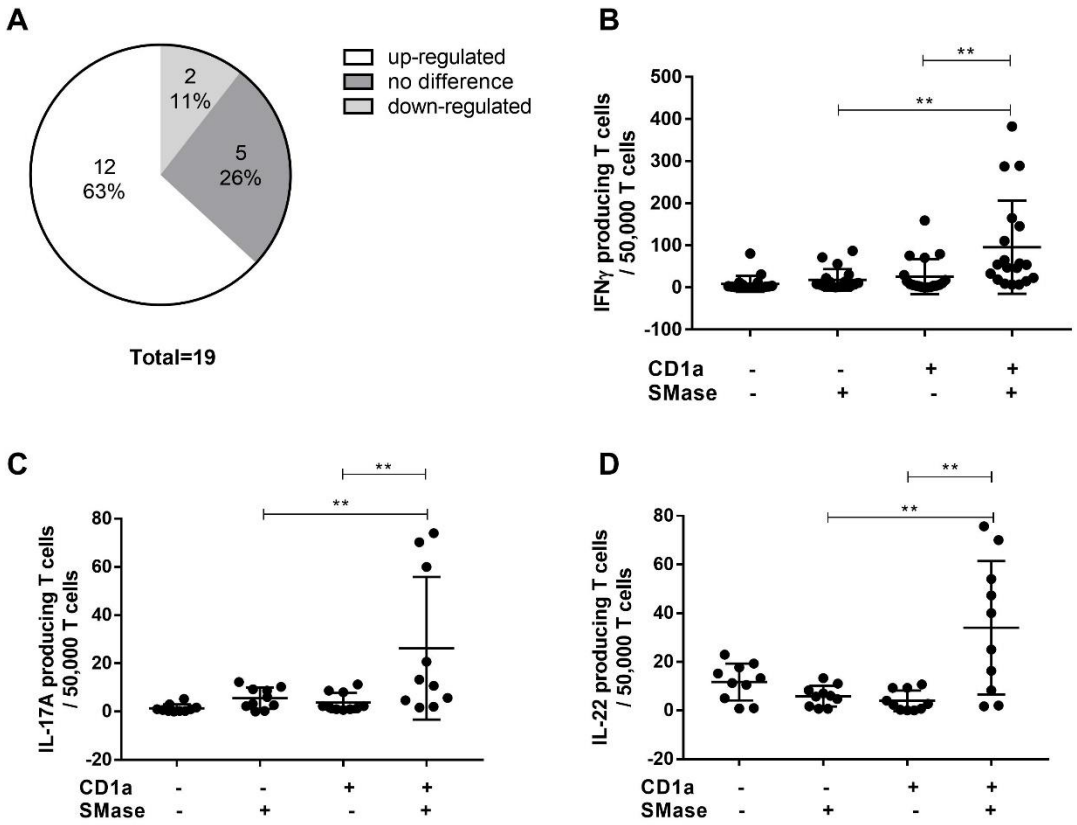


Figure 4.6 CD1a-reactive SMase-specific T cell responses were repeatable in larger healthy cohort. (A) T cells were isolated by CD3 MACS beads from healthy donor PBMCs (n=19), and incubated overnight with K562-CD1a or K562-Ev cells pre-treated with or without *S. aureus* SMase. (B) IFNγ production was tested by ELISpot and presented. IL-17A (C) and IL-22 (D) production were also measured by ELISpot in a healthy cohort (n=11). Bars represent standard error. ** P<0.01.

The IFNγ production was first tested in a healthy cohort containing 19 donors, which included cone samples and fresh blood samples. (Figure 4.6A) Around two third of total donors (12 out of 19) showed significant higher IFNγ response against K562-CD1a pre-treated with SMase, indicating the responding T cells were commonly present. The summary results of total donors were consistent with

previous findings (Figure 4.6B).

Also, IL-17A and IL-22 production were further tested in a healthy cohort including 11 donors (Figure 4.6C, 4.6D). Similarly, the IL-17A and IL-22 T cell responses were significantly higher against K562-CD1a pre-treated with SMase, compared to CD1a only group or SMase only group, consistent with previous findings.

All these results demonstrated that CD1a-reactive SMase-specific T cells were commonly present in peripheral blood of healthy individuals.

4.4 The CD1a-reactive SMase-specific T cells can associate with different T cell subsets

Although ELISpot and real-time PCR offered consistent and reliable results, we wanted to further investigate the characteristics of CD1a-reactive SMase-specific T cells, including their T cell subsets and surface markers. Thus, we used cytokine-secretion assay from Miltenyi Biotec; with the combination of flow cytometry, it allows us to sensitively detect cytokine-secreting cells as well as study their co-expressed markers. Also, the secretion assay allows us to sort living cells and further generate specific T cell lines and clones, which is an advantage compared to other techniques such as intracellular staining.

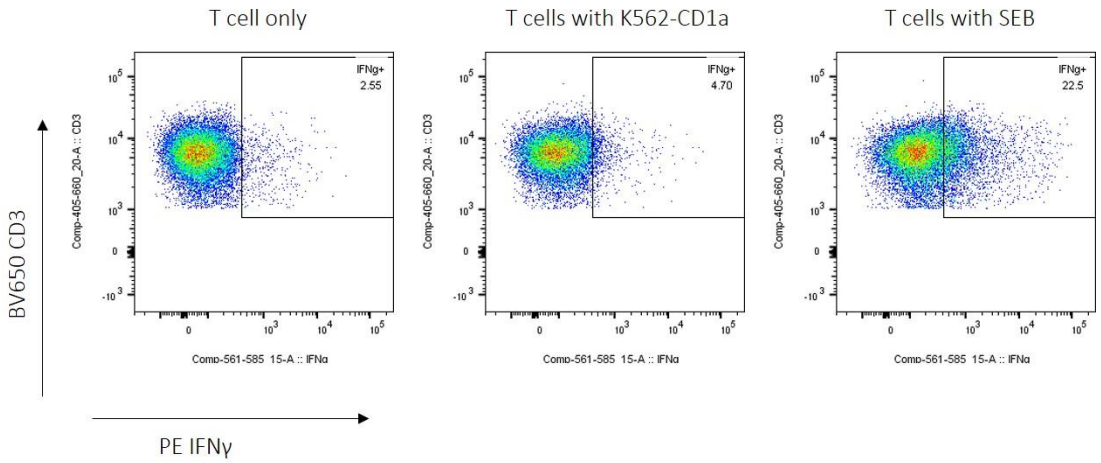


Figure 4.7 Detection of IFNγ secreting cells by cytokine secretion assay. Polyclonal T cells isolated from healthy PBMC cultured with K562-CD1a cells for 10hrs. T cells stimulated by Staphylococcal enterotoxin B (SEB) was used as positive control. T cell only was used as negative control. FACS plots were gated on live cells and singlets.

The CD1a-autoreactive T cell response was first tested by cytokine secretion assay (Figure 4.7). Polyclonal T cells isolated from healthy PBMC were cultured with K562-CD1a cells. Consistent with ELISpot results, a background response (2.5%) was found in T cell only negative control. The percentage of IFNγ secreting T cells of all CD3+ cells increased against K562-CD1a cells (4.7%), which suggested the CD1a-autoreactive T cell response. A much higher increase of IFNγ secreting T cells (22.5%) above background was observed against SEB, which is known as a good stimulus for inducing the production of human IFNγ (Lee, Lee, Jay, & Rozee, 1990). These results confirmed the cytokine secretion assay was successful and was sensitive enough to detect IFNγ secreting T cells against K562 cells.

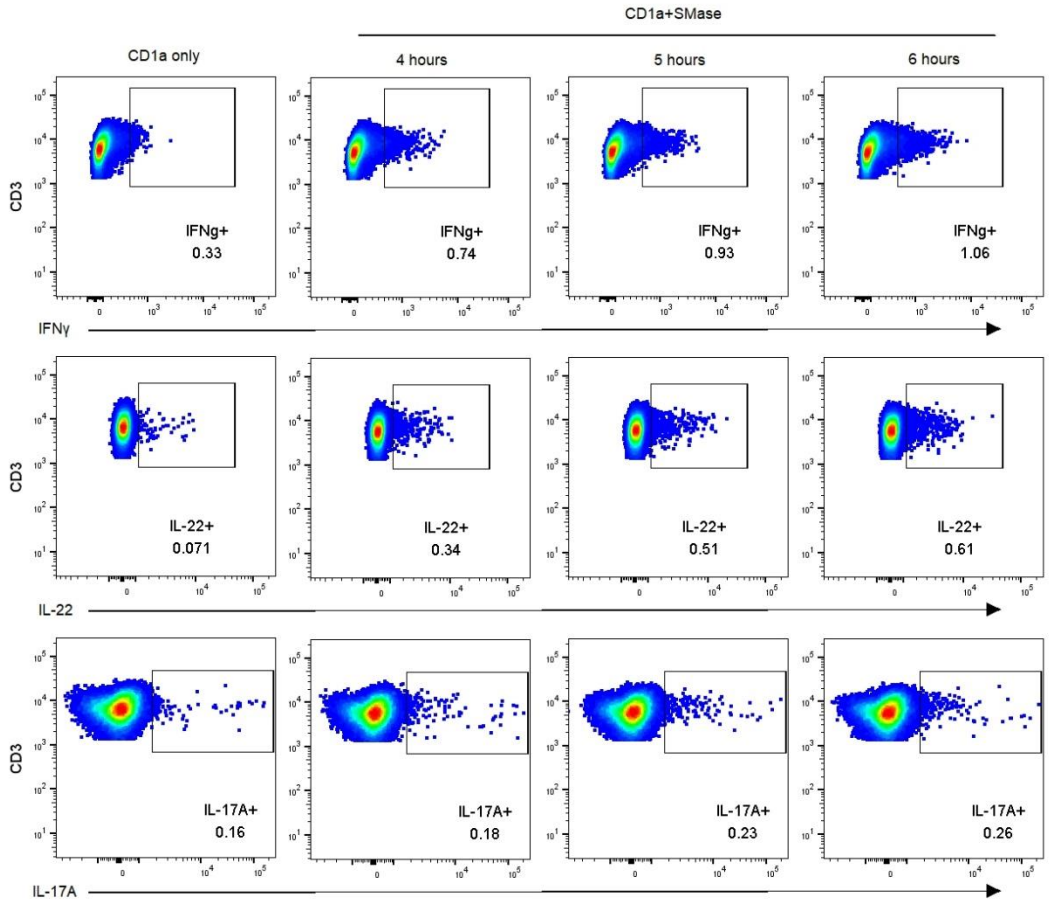


Figure 4.8 The percentage of cytokine-secreting T cells increases with increasing stimulation time. Polyclonal T cells isolated from healthy PBMC (donor W032) cultured for 4, 5, and 6hrs separately with K562-CD1a cells pre-treated with or without SMase. IFNγ, IL-22 and IL-17A producing T cells were tested in the same cytokine secretion assay. FACS plots gated on CD3+, live cells and singlets.

In a pilot experiment, 10 hours stimulation was found to be too long and caused a high background of CD1a autoreactive responses. We reduced the co-culture period to 4, 5, and 6 hours separately. We also pre-treated K562 cells with SMase

for two days rather than one day before co-cultured with T cells, which provided better results, and found the frequencies of cytokine-producing cells were higher compared to ELISpot results. Two-day pre-treatment was used in future cytokine secretion assay. IFNγ, IL-22 and IL-17A production were measured in the same experiment (Figure 4.8).

Consistent with previous results, a CD1a-autoreactive background was detected in this donor: 0.33% for IFNγ, 0.071% for IL-22, and 0.14% for IL-17A. After 4hrs culture, we observed a 2 fold IFNγ response and a 5 fold IL-22 response against K562-CD1a with SMase compared to background. The percentage of IFNγ and IL-22 secreting cells raised with the increasing stimulation time. IL-17A response was noted from 5hrs, and also showed an increasing trend. Although there were a few highly cytokine producing cells, the general IL-17A response was relatively weak, compared to IFNγ and IL-22 (Figure 4.8). Based on these results, polyclonal T cells were cultured with K562 cells for 6 hours of stimulation in future experiments.

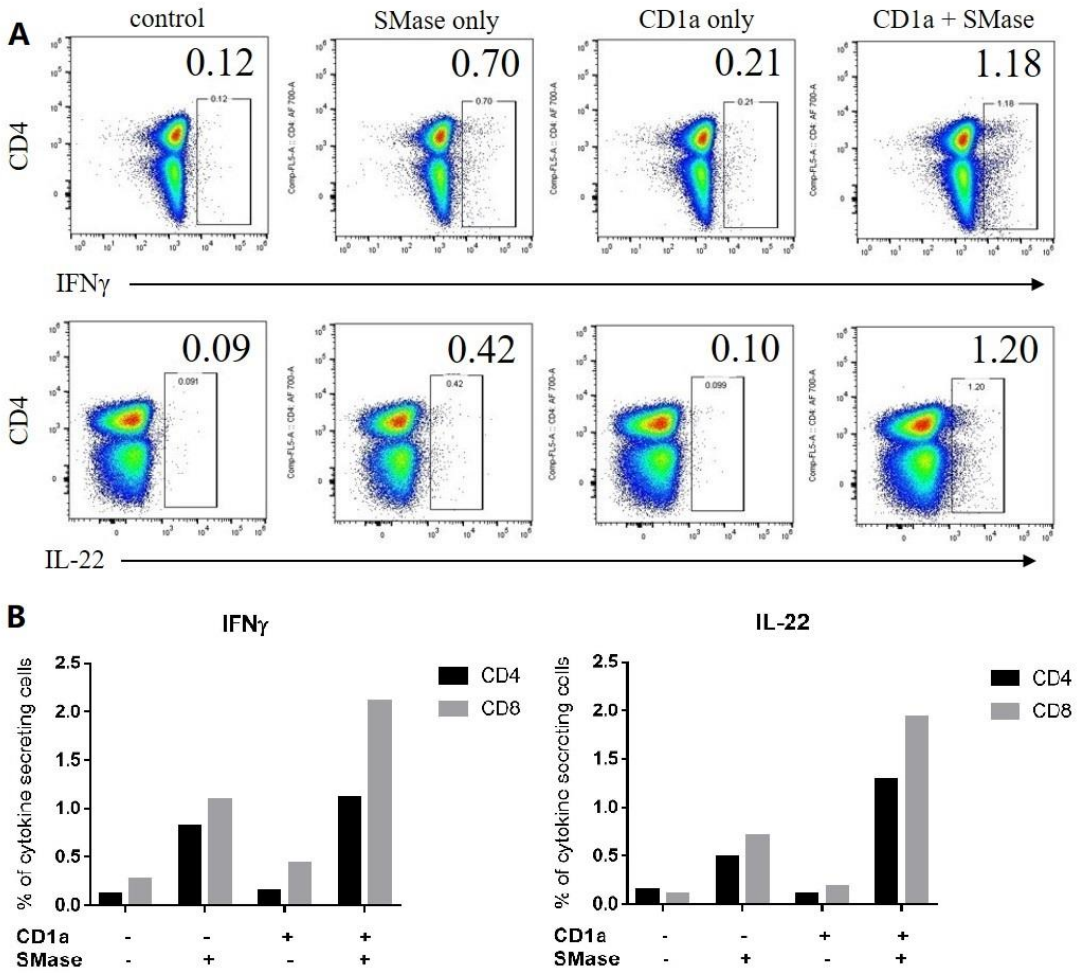


Figure 4.9 CD1a-reactive SMase-specific response in both CD4 and CD8 T cell subsets. Polyclonal T cells isolated from healthy PBMC cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with or without SMase. Cytokine secreting T cells were detected in same experiment by cytokine secretion assay. Results from one selected donor (W032) are shown, which was representative of at least three donors from different experiments. (A) The percentage of IFNγ and IL-22 producing T cells in each condition. FACS plots gated on CD3+, live cells and singlets. (B) The summary of the percentage of IFNγ (left) and IL-22 (right) producing T cells in CD4+ and CD8+ T cell subsets separately from the same experiment.

Consistent with what we found in ELISpot and real-time PCR experiments, the percentage of IFNγ and IL-22 secreting T cells increased against K562-CD1a cells pre-treated with SMase (about 1.2%), much higher than that in CD1a only or SMase only group, further confirming the presence of CD1a-reactive SMase-specific T cells in peripheral blood. In both CD4 positive and CD4 negative populations, a group of highly cytokine-secreting T cells was observed against K562-CD1a cells pre-treated with SMase (Figure 4.9A).

We further analyzed the percentage of cytokine producing T cells in CD4+ and CD8+ T cell subsets separately. The summary results from the same donor (W032) are shown (Figure 4.9B). Similar to total CD3+ T cells, both CD4+ and CD8+ T cells showed CD1a-reactive SMase-specific response, suggesting that both CD4+ and CD8+ T cells can respond.

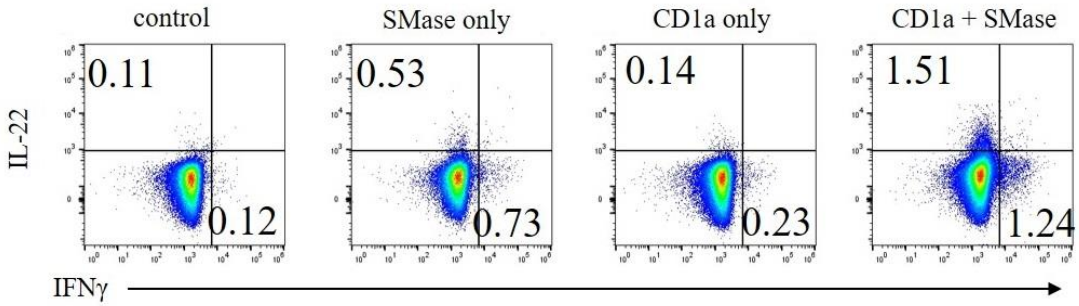


Figure 4.10 IFNγ and IL-22 were produced by different CD1a-reactive SMase-specific T cell subsets. Polyclonal T cells isolated from healthy PBMC (donor W032) co-cultured with K562-Ev and K562-CD1a cells pre-treated with or

without SMase. The IFNγ and IL-22 secreting T cells were tested by cytokine secretion assay. FACS plots gated on CD3+, live cells and singlets.

Based on previous studies (Alam et al., 2010; Behrends, Renauld, Ehlers, & Holscher, 2013), IL-22 can be co-expressed by IFNγ-secreting Th1 cells. We investigated whether these IFNγ and IL-22 secreting CD1a-reactive SMase-specific cells were derived from the same population of T cells. The cytokine secretion assay allowed us to stain two cytokines with different fluorescent antibodies in the same experiment. The data from the healthy donor W032 is shown (Figure 4.10). Two separate T cell subsets producing IFNγ and IL-22 against K562-CD1a with SMase were identified.

4.5 The cell surface marker expression of CD1a-reactive SMase-specific T cells

Based on previous research, CD1a-autoreactive T cells proved to express skin-specific homing receptors including cutaneous lymphocyte antigen (CLA), CCR6, CCR4 and CCR10 (Preihs, Christian Arambula et al., 2013). CLA is a skin lymphocyte homing receptor that directs T cells to skin, and CCR6 is proved to direct activated T cells to skin in inflammatory conditions (Paradis, Cole, Nelson, & Gladue, 2008; Zhang, Borges, Fan, Harris, & Turka, 2015). We tested the co-expression of CCR6 and CLA with cytokines to detect whether CD1a-reactive SMase-specific T cells potentially home to skin.

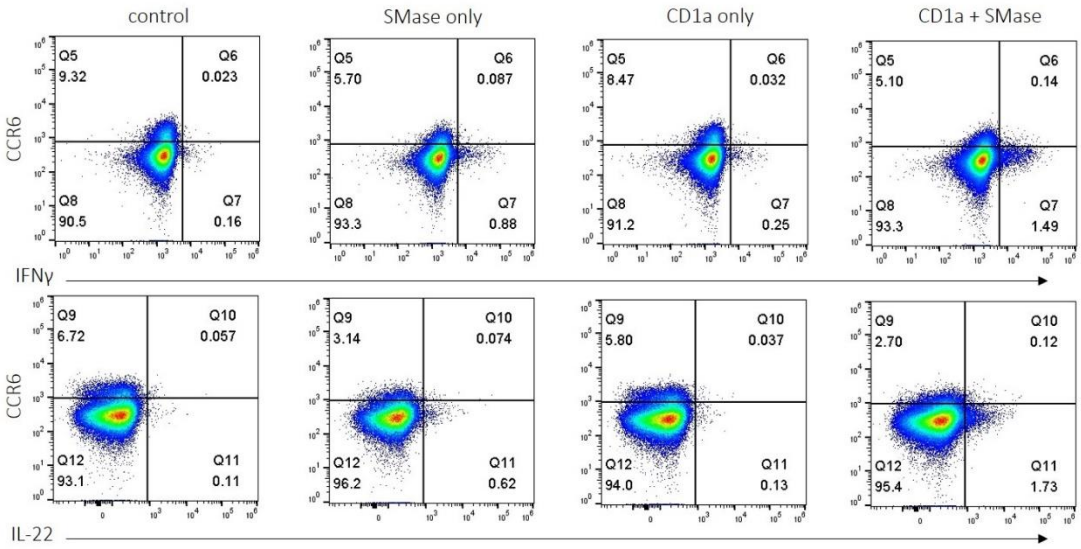


Figure 4.11 CD1a-reactive SMase-specific T cells did not express CCR6 on surface. Polyclonal T cells isolated from healthy PBMC (donor W032) co-cultured with K562-Ev and K562-CD1a cells pre-treated with or without SMase. T cells were stained for IFNγ, IL-22 and CCR6. FACS plots gated on CD3+, live cells and singlets.

Majority of IFNγ or IL-22 secreting cells did not co-express significant CCR6, based on the FACS results (Figure 4.11). However, some CCR6+ cytokine-secreting cells were found (0.12-0.14%).

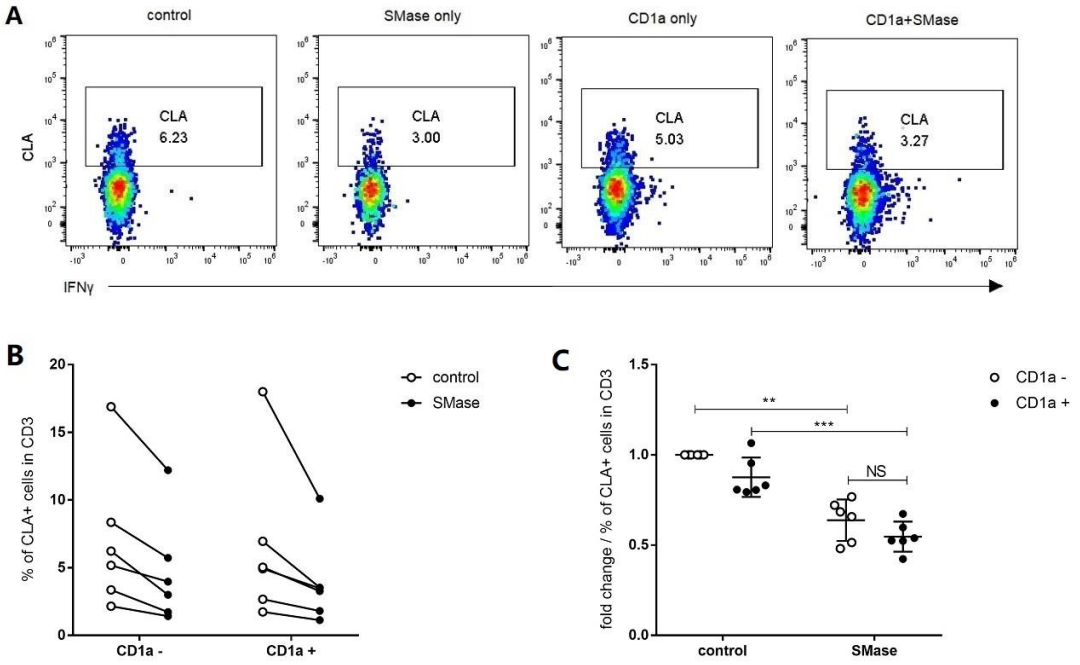


Figure 4.12 cutaneous lymphocyte antigen (CLA) expression on T cells decreased against K562 cells pre-treated with SMase. Polyclonal T cells isolated from a healthy cohort (n=6) co-cultured with K562-Ev and K562-CD1a cells pre-treated with or without SMase. (A) Representative FACS plots are shown (donor W006). T cells were stained with IFNγ and CLA. FACS plots gated on CD3+, live cells and singlets. (B) The percentage of CLA+ T cells in each condition (n=6). (C) The percentage of CLA+ T cells was normalized to that in group of K562-Ev cells without SMase pre-treatment and fold changes are shown. The log-transformed value of fold change was used for statistics. Significance of log-fold change was calculated by One-sample t test. Bars represent standard error. ** P<0.01 *** P<0.001.

The percentage of CLA+ T cells decreased against K562 cells pre-treated with

SMase (Figure 4.12A), and this finding was repeatable in 6 healthy donors (Figure 4.12B). We calculate the fold change of CLA+ T cells percentage by normalizing to that in K562-Ev group. The percentage of CLA+ T cells in SMase-treated group was only half of the amount of those in groups without SMase treatment. The percentage of CLA+ T cells against K562-Ev or K562-CD1a showed no difference, indicating the decrease was independent of the presence of CD1a (Figure 4.12C).

The reason for this unexpected decrease needs further investigation, and will be tested in future experiments. As the living cells were > 95% in each condition, it may not be the cell viability that influenced CLA or CCR6 expression. Also, K562 cells were washed multiple times to remove SMase before co-culture. SMase did not directly contact T cells, and was not able to digest skin-homing receptors on T cell surface. Thus, CLA may be regulated by interaction between T cells and K562 cells pre-treated with SMase. As CLA could be up-regulated by IL-12 and down-regulated by IL-4 (D. Y. Leung et al., 1995; Seneviratne et al., 2005), it is worth testing these two cytokines in future experiments. Furthermore, cell-culture is known to modulate CLA expression through unknown mechanisms that could confound the interpretation of this data, which is also worth to take into consideration.

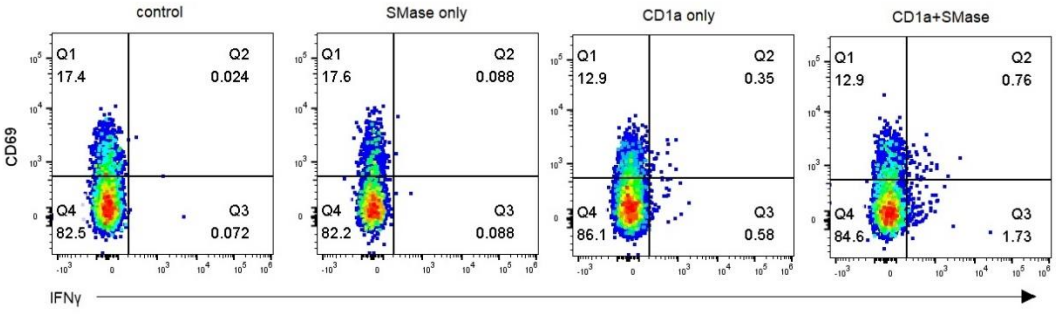


Figure 4.13 CD1a-reactive SMase-specific T cells partially co-expressed T cell activation marker CD69. Polyclonal T cells isolated from healthy donor (donor W006) co-cultured with K562-Ev and K562-CD1a cells pre-treated with or without SMase. T cells were stained for IFNγ and CD69. FACS plots gated on CD3+, live cells and singlets. Data representative of at least three donors from different experiments.

CD69 is known as an early T cell activation marker (Ziegler, Ramsdell, & Alderson, 1994). After co-culture with SMase-treated K562-CD1a cells, a proportion of the responding T cells co-expressed CD69 and IFNγ. The IFNγ-CD69 co-expressing T cells increased in CD1a+SMase group, compared to CD1a autoreactive group, from 0.35% to 0.76% in the example, representative of three donors.

4.6 The CD1a-reactive SMase-specific T cell response depends on SMase enzymatic activity

As described in Introduction, the bacterial SMase from *S. aureus* is a Mg^{2+}-dependent neutral sphingomyelinase (nSMase), which shares a low level of

identity to its mammalian counterparts (Clarke et al., 2006a; Hayashida et al., 2009). And the available mammalian nSMase inhibitors did not show inhibition to bacterial SMase (Nara et al., 1999b). Based on previous papers, heat-inactivation or presence of ion competitor EDTA reduced SMase enzymatic activity and influenced its functions (Coll, Morales, Fernández-Checa, & Garcia-Ruiz, 2007). Thus, we heat-inactivated *S. aureus* SMase 10mins at 80℃, to test the influence of enzymatic activity on CD1a-reactive SMase-specific response.

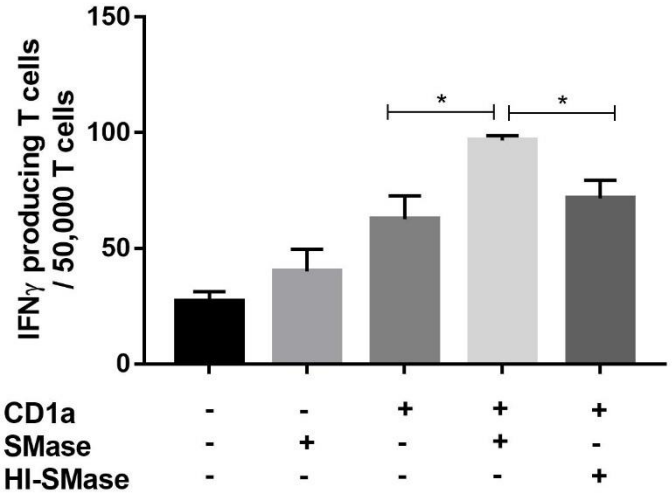


Figure 4.14 Heat-inactivation of SMase influenced the CD1a-reactive SMase-specific response. Polyclonal T cells isolated from healthy donor (donor C210917-2) co-cultured with K562-Ev and K562-CD1a cells pre-treated with SMase, heat-inactivated SMase (HI-SMase) or media. IFNγ producing T cells were measured by ELISpot. Data representative of at least three donors. Bars represent standard error. * $P<0.05$.

The influence of enzymatic activity was first tested by ELISpot. The T cell response against K562-CD1a treated with heat-inactivated SMase was significantly decreased compared to normal SMase, and almost to the level of CD1a-autoreactive response (Figure 4.14).

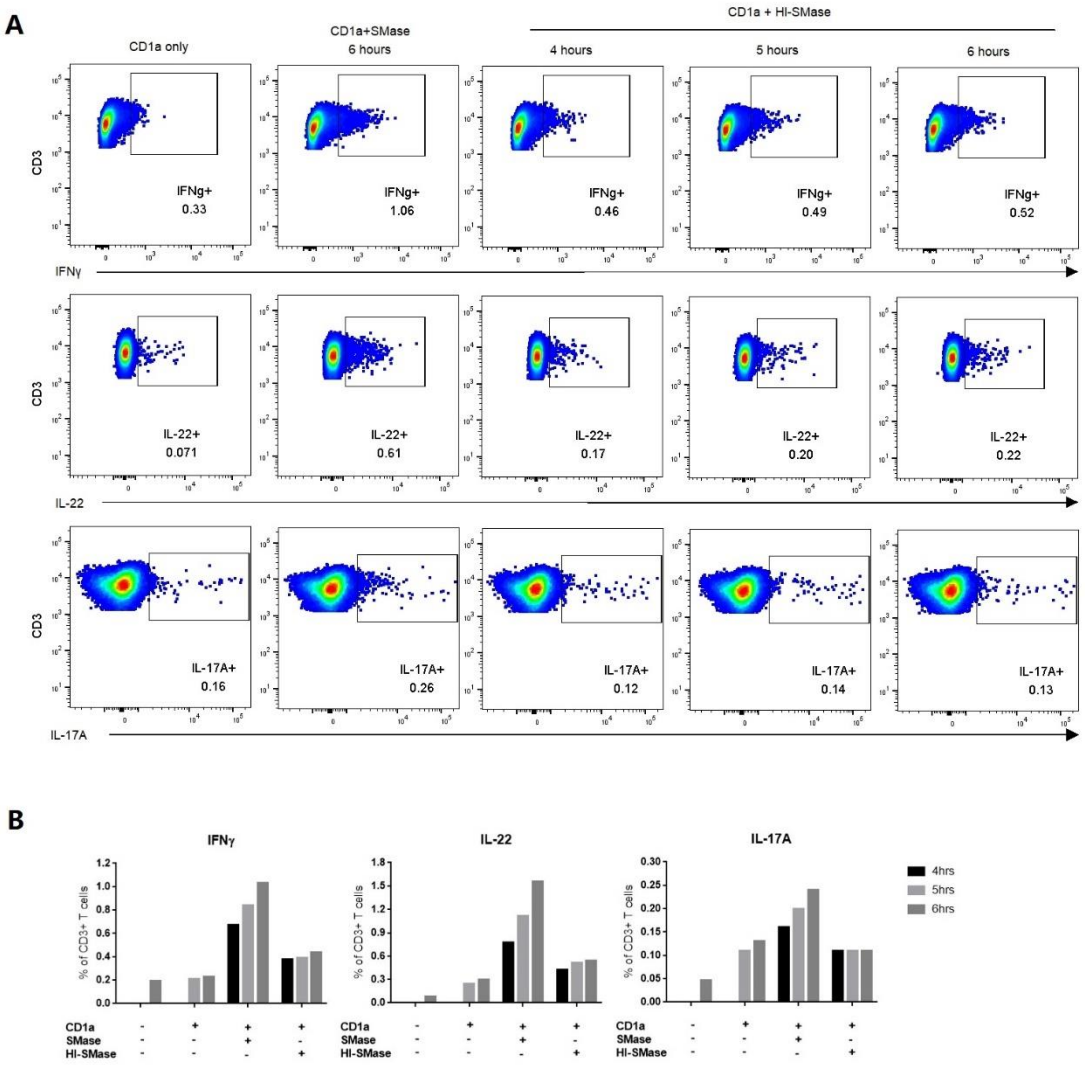


Figure 4.15 Heat-inactivation of SMase reduced the CD1a-reactive SMase-specific cytokine productions of IFNγ, IL-22 and IL-17A. Polyclonal T cells isolated from healthy donor (W032) co-cultured with K562-CD1a cells pre-treated with SMase, HI-SMase, or media for 4, 5, and 6 hours. Cytokine producing T cells were measured by secretion assay. (A) FACS plots gated on CD3+, live cells

and singlets. (B) The percentage of IFNγ, IL-22 and IL-17A secreting T cells in each condition.

The heat-inactivated SMase response was further tested by cytokine secretion assay with different co-culture time of T cells and K562 cells. T cell response against HI-SMase showed reduced production of IFNγ, IL-22 and IL-17A. Compared to the previous results in Figure 4.8, in which the percentage of cytokine secreting T cells raised with increasing stimulation time, the T cell response against HI-SMase did not change over a similar time course (Figure 4.15).

These results demonstrated that the CD1a-reactive SMase-specific T cell response depended on SMase enzymatic activity. The IFNγ, IL-22 and IL-17A production were all reduced if the SMase was heat-inactivated.

4.7 The CD1a-reactive SMase-specific T cell response demonstrates CD1a specificity

In order to test CD1a specificity of SMase-specific T cell response, SMase pre-treated K562-CD1a cells were incubated with anti-CD1a antibodies or control IgG before co-cultured with polyclonal T cells. IFNγ production was tested by ELISpot. The anti-CD1a antibodies from three different clones were tested: HI149, OKT-6, and NA1/34.

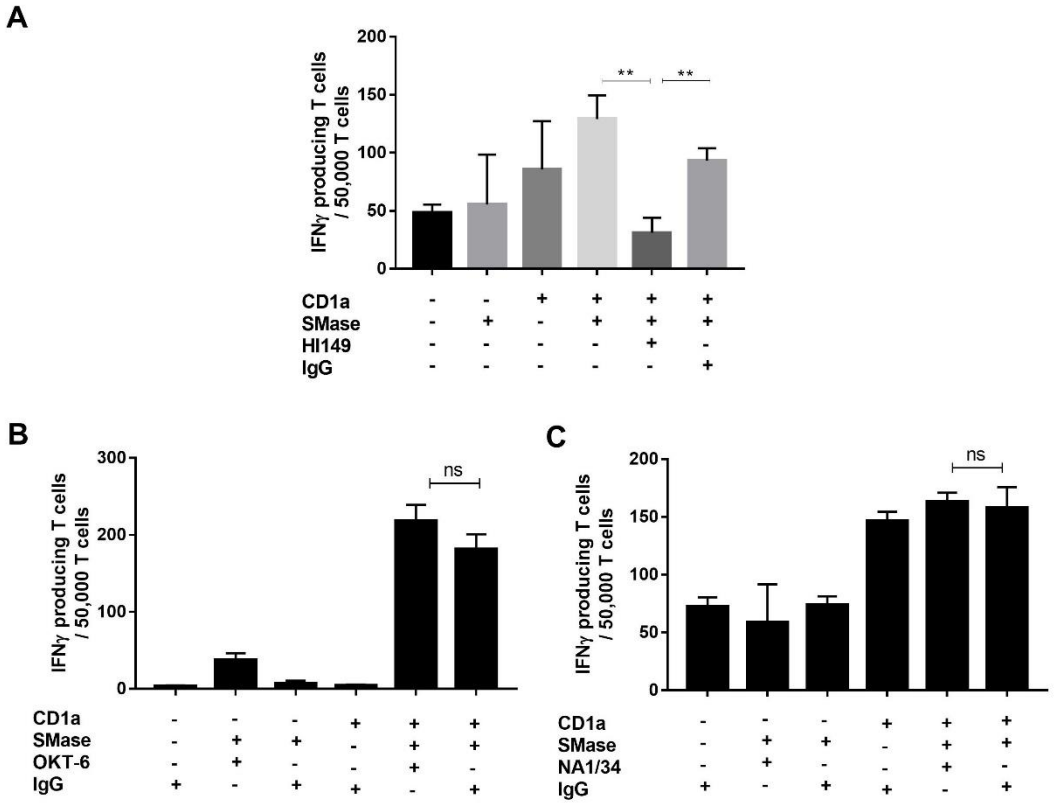


Figure 4.16 The CD1a-reactive SMase specific T cell response was reduced by anti-CD1a antibody HI149. Polyclonal T cells isolated from healthy donors co-cultured with K562-Ev and K562-CD1a cells pre-treated with or without SMase. K562 cells were incubated with anti-CD1a antibodies or IgG control before co-culture with T cells. IFNγ producing T cells were measured by ELISpot. Three anti-CD1a antibodies were tested: HI149 (A), OKT-6 (B) and NA1/34 (C). Data representative of at least three donors. Bars represent standard error. ** $P<0.01$.

The response was inhibited by anti-CD1a antibody HI149 but not by IgG control, indicating the CD1a dependence (Figure 4.16A). However, the antibodies OKT-6 and NA1/34 did not show the blocking effect (Figure 4.16B, 4.16C). It may be explained by varying inhibitory effects of anti-CD1a antibodies due to their

different binding sites with CD1a and has been observed by others in the group, with HI149 being the only antibody showing consistent inhibition.

4.8 Polyclonal T cells respond to primary autologous *in vitro* derived CD1a positive antigen presenting cells

K562-CD1a cells have the advantage of being able to be used with T cells from any donor. However, these cells might not mimic CD1a-mediated presentation by native CD1a+ antigen-presenting cells such as myeloid dendritic cells. Nevertheless, in work from the lab, others have shown that K562-CD1a cells can accurately predict mDC ability to present CD1a antigens to T cells (Bourgeois et al., 2015; Cheung et al., 2016; Jarrett et al., 2016). Thus, to investigate CD1a-reactive SMase-specific T cells response closer to more physiological presentation conditions, we used monocyte-derived myeloid dendritic cells (mDCs) as antigen presenting cells.

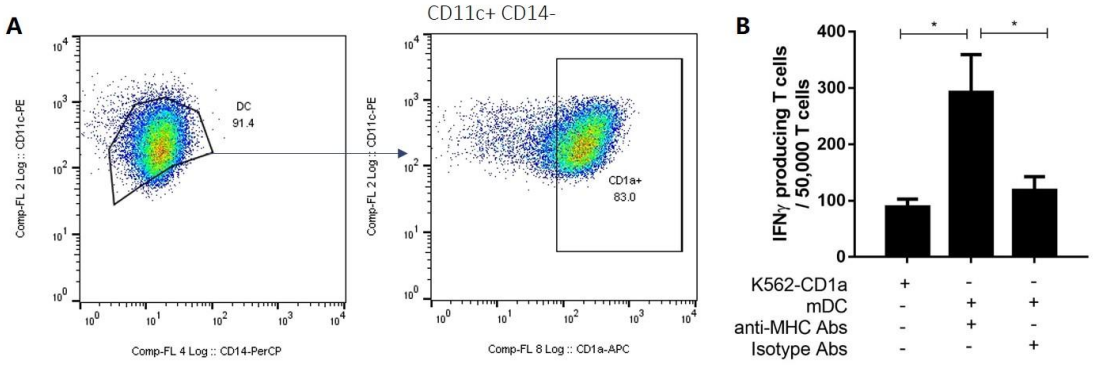


Figure 4.17 CD1a expression by mDCs and the auto-reactive T cell response to mDCs. (A) Monocytes were differentiated into mDCs. Representative FACS

plots of CD1a expression on cell surface. FACS plots gated on live cells and singlets (left), and further gated on CD11c+ CD14- population (right). (B) Polyclonal T cells isolated from same donor, cultured with mDCs and K562-CD1a cells as control. mDCs were incubated with anti-MHC antibodies (anti-MHC Abs) or isotype controls (Isotype Abs) before co-cultured with T cells. T cell response was measured by IFNγ ELISpot. Data representative of at least three donors. Bars represent standard error. * $P<0.05$.

Monocytes were isolated from healthy PBMC by CD14+ MACS separation, and further differentiated with GM-CSF and IL-4 to produce mDCs. The differentiation of mDCs were confirm by FACS before experiments, with high CD1a expression (Figure 4.17A).

Polyclonal T cells from the same donor were isolated by CD3+ MACS separation. mDCs were incubated with anti-MHC antibodies or isotype controls before co-cultured with T cells, and K562-CD1a cells were used as control. The IFNγ response was measured in all three conditions. However, after anti-MHC antibodies treatment, the mDCs induced a significant higher T response rather than blocking effect.

This failure of MHC blocking was consistent in at least three donors, and caused T cell over-response against mDCs treated with SMase. The concentration and

incubation time of anti-MHC antibodies need titration as the experiment may be associated with Fc-dependent binding which may be less relevant at lower concentrations of anti-MHC antibody. Antibodies from other sources will also be tested in future experiments. The mDCs will be incubated with additional anti-CD1a antibody to test the CD1a dependence of the SMase-specific T cell response.

Discussion

Overall these data show that CD1a-reactive SMase-specific T cells exist in the peripheral blood at high frequencies and produce IFNγ, IL-22, and IL-17A in response to SMase challenge in healthy individuals. This CD1a-reactive SMase-specific response was regulated at both the transcriptional and translational levels. The CD1a-reactive SMase-specific T cell response was shown to be dependent on SMase enzymatic bioactivity, and the polyclonal T cell response decreased to heat-inactivated SMase challenge. It suggested the role of enzymatic contribution to the SMase-specific response, and further supported our hypothesis. The T cell response was also shown to be CD1a-dependent. Cytokine secretion assay demonstrated that the majority of IFNγ and IL-22 cytokine producing cells were comprised of two unique subsets. However, the majority CD1a-reactive SMase-specific T cells did not co-express skin homing markers including CLA and CCR6 after short incubation, and the total percentage of CLA+ T cells decreased in SMase challenge.

This is the first time that CD1a-reactive bacterial SMase-specific T cells have been described in the literature. These T cell responses were dependent on enzymatic activity, which suggests that they recognize lipid antigens generated by *S. aureus* SMase *in vivo*. This could be through a number of mechanisms, for example the bacterial SMase modifies the lipid profile in the skin, increasing the CD1a activating ligands and/or decreasing CD1a inhibitory ligands.

As detailed in introduction, IFNγ is known to play an important role in antimicrobial immunity, and generates effective immune responses for eliminating pathogens (Kak et al., 2018; Shtrichman & Samuel, 2001). One of the undoubted anti-bacterial effects of IFNγ is its ability to control *Mycobacterium tuberculosis* infection, in which IFNγ-producing CD4+ T cells are essential for host survival and enhance CD8+ T cell cytotoxicity (Green et al., 2013). IFNγ also contributes to anti-*Staphylococcus aureus* immune responses; it protects venule endothelial cells from severe injury by virulent clinical *S. aureus* strains through stimulation of intrinsic antibacterial activities (Beekhuizen & Van De Gevel, 2007). IL-22 and IL-17A are induced at various mucosal surface and epithelial barriers, and are essential for host defense against microbes including *S. aureus* (Valeri & Raffatellu, 2016). IL-22 is important for limiting the growth of *S. aureus* and inducing antimicrobial peptide expression to trigger the killing of pathogens (Fujita, 2013; Malhotra et al., 2016; Mulcahy et al., 2016; Valeri & Raffatellu, 2016). IL-17A drives the expression of neutrophil-attracting chemokines and is

important for the clearance of *S. aureus*. In addition, IL-17A producing epidermal γδ T cells are proved to be essential for host defense against *S. aureus* (Chan et al., 2015; J. S. Cho et al., 2010; Jin & Dong, 2013; Miller & Cho, 2011). All these cytokines may therefore link the CD1a-reactive SMase-specific T cells to anti-*S. aureus* immunity.

In summary, CD1a-reactive SMase-specific T cell responses were detected in multiple unrelated healthy individuals, suggesting these cells are important in CD1a biology and the immune response to bacteria. Consistent with previous research about CD1a-reactive T cell response mediated by enzymatic activity (Bourgeois et al., 2015; Jarrett et al., 2016), heat inactivation of SMase significantly decreased the T cell response. This association will be further explored in following chapters.

Investigation of the potential antigens generated by SMase will be important to further study in order to define the underlying mechanism of this CD1a-reactive SMase-specific T cell response, and this will be addressed in Chapter 5. It will be key to functionally phenotype these cells and investigate their TCR sequence, which will be the focus of Chapter 6. Finally, investigation of these CD1a-reactive SMase-specific T cells in skin diseases such as AD and PS will contribute an understanding of disease pathogenesis, and this will be concentrated on in Chapter 7.

5. Result II: Investigation of the Role of Bacterial Sphingomyelinase in the Generation of CD1a Ligands

Introduction and aims

As described, CD1a is able to present lipid antigens to T cells. So far, several lipids have been described to be presented by CD1a, including mammalian sulfatide, natural headless skin oils, phospholipids, glycerophospholipids and sphingomyelin (Birkinshaw et al., 2015; Jong et al., 2014; Zajonc et al., 2003a). The CD1a proteins are stabilized by lipids, which are thought to be exchanged on the surface by ligands present in the microenvironment. They predominantly carry lipids with large hydrophilic head groups including SM (Manolova et al., 2006).

CD1a ligands have been divided into two groups: the permissive ligands and non-permissive ligands. The working mechanism of these two groups of ligands was further suggested by crystal structures of CD1a loaded with LPC, oleic acid, SM, sulfatide and mycobactin-like lipopeptide. SM is a non-permissive ligand which interfered with BK6 TCR contact by disrupting the CD1a roof and TCR and CD1a binding sites. BK6 permissive ligands included LPC and oleic acid, which permitted TCR binding with CD1a in the absence of interference. (Birkinshaw et al., 2015; Van Rhijn et al., 2015)

SMase from *S. aureus* is also called beta-toxin or beta-lysin, and was one of the

first cloned bacterial SMases (Coleman et al., 1986). It is a Mg2+ dependent neutral SMase, which can cleave sphingomyelins, the most abundant cell membrane sphingolipids. The bacterial SMases are divided into two groups based on their hydrolyzing position, and *S. aureus* SMase belongs to the SMase C group, which produces ceramides and phosphorylcholine as products. (Flores-Díaz et al., 2016; Hayashida et al., 2009)

In chapter 4, we identified the presence of CD1a-reactive SMase-specific T cells in the blood of healthy individuals, the functions of which were dependent on SMase enzymatic bioactivity. We therefore sought to identify the modulation of CD1a ligands by SMase.

The aims of this chapter were to investigate the potential lipid neoantigens generated by SMase during the CD1a-reactive SMase-specific T cell response, and to further study the underlying mechanisms. Two groups of lipids were studied, sphingomyelins (SM) and ceramides, which are the substrates and primary products of SMase. This was investigated by CD1a tetramers loaded with various synthetic lipids, kindly donated by Branch Moody from Harvard, and cytokine secretion assays as functional readouts. Consistent with previous chapter, the K562 cells with low MHC molecules and transfected to express CD1a protein were used as APCs, to study the polyclonal CD1a-reactive SMase-specific T cells isolated from unrelated healthy individuals. The correlation of SMase-specific and

ceramide-specific T cell response was further investigated.

5.1 CD1a tetramers

The possible TCR interactions with CD1a-lipid complexes have not been well-studied due to the limited experimental tools and reagents. However, CD1a-tetramers can be used to stain CD1a-reactive T cells, which could be further sorted for functional analyses (Benlagha, Weiss, Beavis, Teyton, & Bendelac, 2000; Kasmar et al., 2013; M. H. Young & Gapin, 2011).

We used soluble biotinylated CD1a monomers, which were produced by NIH to prepare tetramers. CD1a proteins were incubated with synthetic lipids and complexed with streptavidin coupled with fluorescent dye. CD1a-tetramer bound with endogenous lipids was named “CD1a-endo tetramer” and used as control.

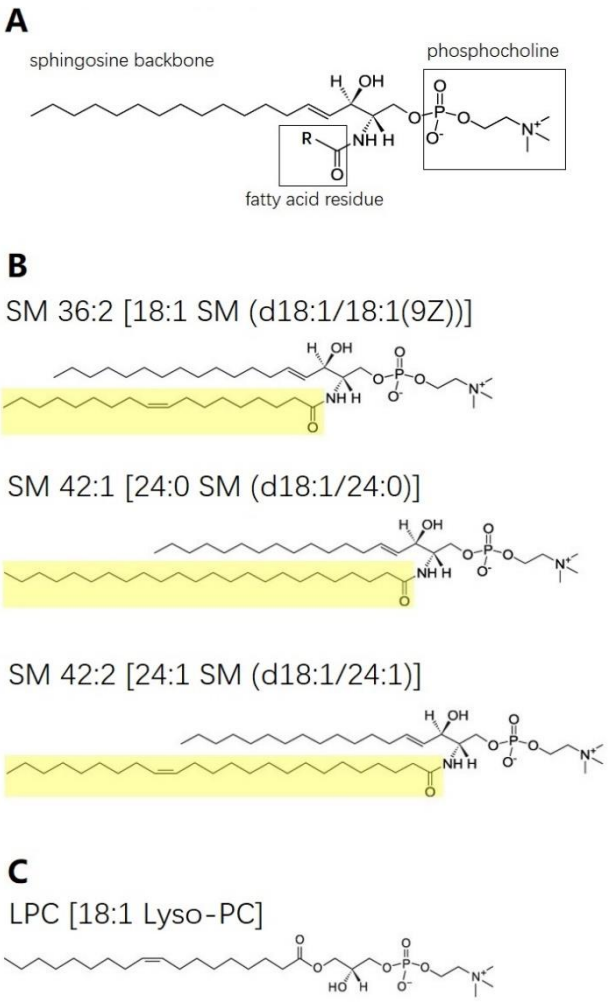


Figure 5.1 Synthetic lipids used for tetramer preparation. (A) The general structure of sphingomyelin (SM). (B) Three synthetic sphingomyelins with different acyl chains, SM 36:2, SM 42:1, SM 42:2. The acyl chain of each SM is labelled in yellow, and has variable length and unsaturation. (C) The structure of LPC (18:1 Lyso-PC). All lipids were from Avanti Polar Lipids.

Sphingomyelin (SM) consists of a phosphocholine, a sphingosine d18:1, and a fatty acid/acyl chain (Figure 5.1A). In previous studies based on BK6 TCR, SM has been shown to be non-permissive ligand of CD1a, while LPC (Figure 5.1C) is a known CD1a permissive ligand for the BK6 TCR. (Birkinshaw et al., 2015) This

interference effect of SM is thought to be influenced by the length and unsaturation of the fatty acid residue. Based on recent unpublished work of Rachel Cotton and TanYun Cheng from Branch Moody's lab (presented at CD1/MR1 Conference Oxford 2019), the SM 42:1 and SM 42:2 (Figure 5.1B) were found enriched in CD1a eluents.

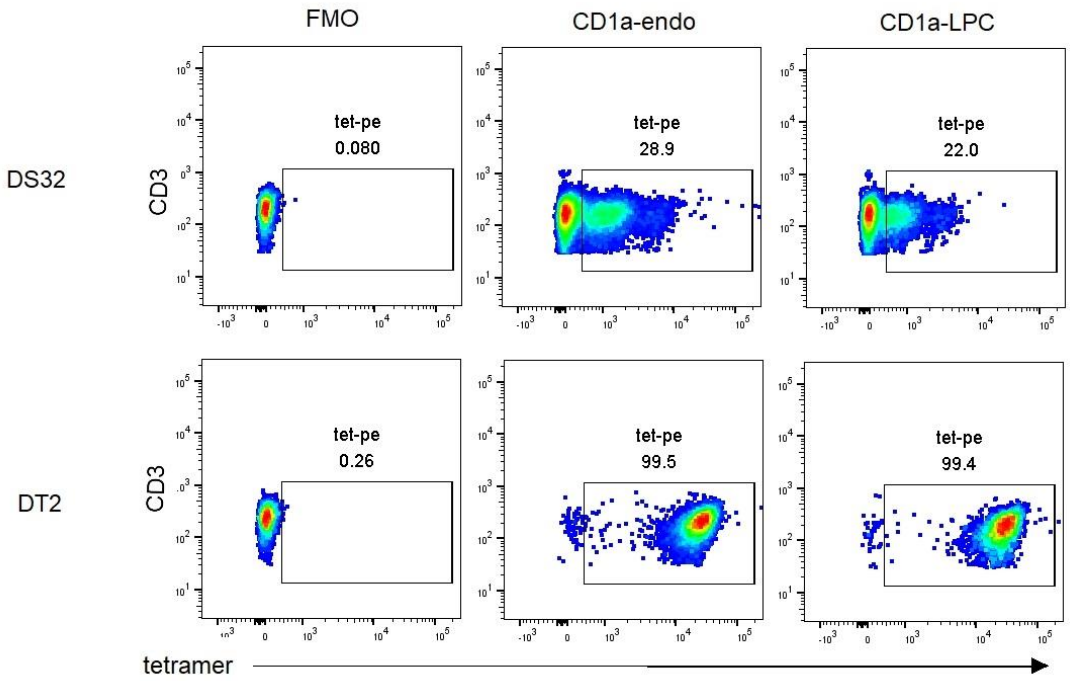


Figure 5.2 Tetramer staining of CD1a-autoreactive T cell lines DS32 and DT2.

The CD1a-autoreactive T cell lines DS32 and DT2 (provided by Rachel Cotton) stained with CD1a-endo and CD1a-LPC tetramers. Fluorescence Minus One (FMO) of tetramer was used as negative control. FACS plots were gated on live cells and singlets.

The tetramer staining process was first tested by two CD1a-autoreactive T cell

lines, DS32 and DT2, which were kindly donated by Rachel Cotton and Branch Moody (Harvard). DS32 and DT2 are lines sorted based on CD1a-LPC tetramer and could be stained with CD1a-endo tetramer. DS32 is a mixture of tetramer positive and negative cells, while DT2 is a tetramer positive line.

We stained these two cell lines with CD1a-endo and CD1a-LPC tetramers. Both of the cell lines were positively stained, proving the tetramer staining experiment was successful (Figure 5.2).

5.2 CD1a-tetramer staining in healthy individuals

We used CD1a-endo tetramer as well as CD1a tetramer loaded with SM 36:2, SM 42:1, SM 42:2 and LPC, to investigate the percentage of tetramer-positive T cells in PBMCs.

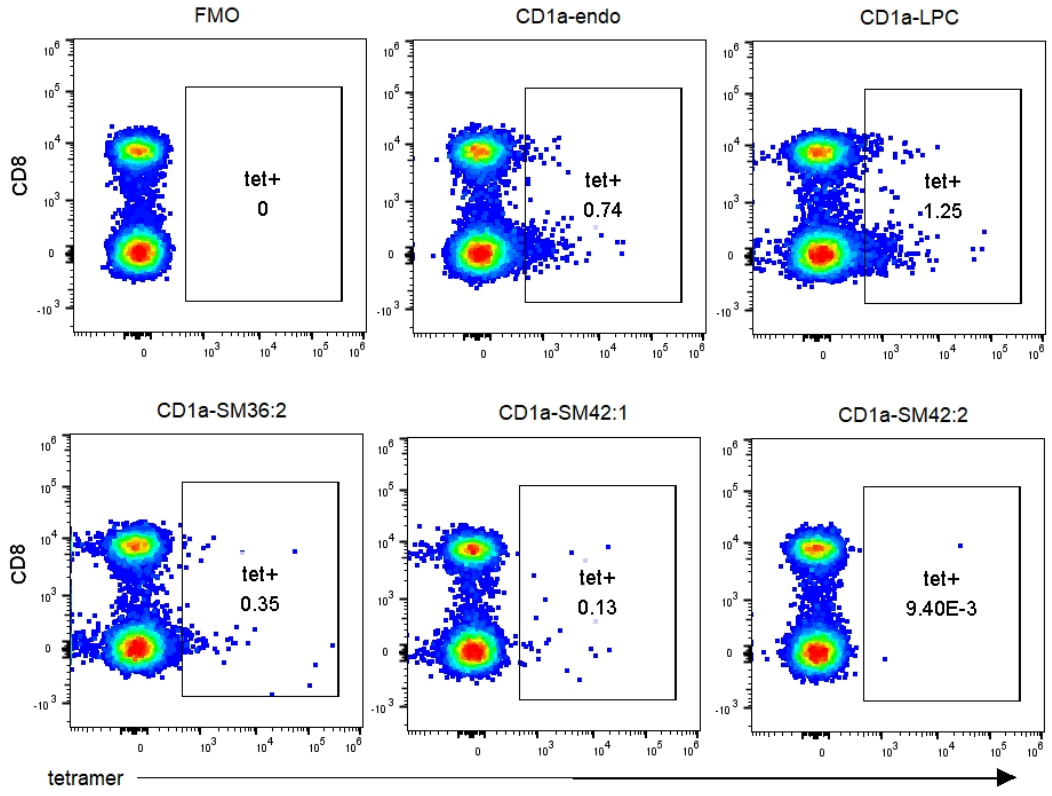


Figure 5.3 Tetramer staining of polyclonal T cells from healthy individuals. Polyclonal T cells were isolated from healthy PBMCs (donor W016) by CD3+ MACS separation. T cells were stained with CD1a-tetramers and other surface markers. FMO of tetramer was used as negative control. Data representative of at least three donors from different experiments. FACS plots were gated on CD3+, live cells and singlets.

Polyclonal T cells isolated from healthy PBMCs were stained with CD1a tetramers as well as other surface markers. CD1a tetramer-positive cells were found in both CD4+ and CD8+ positive T cell subsets. The CD1a-endo and CD1a-LPC tetramer positive cells were approximately 1% of total T cells, while the percentage of CD1a-SM tetramers positive cells were decreased, only 0.35% for SM 36:2, 0.13% for SM

42:1, and not detectable for SM 42:2 (Figure 5.3).

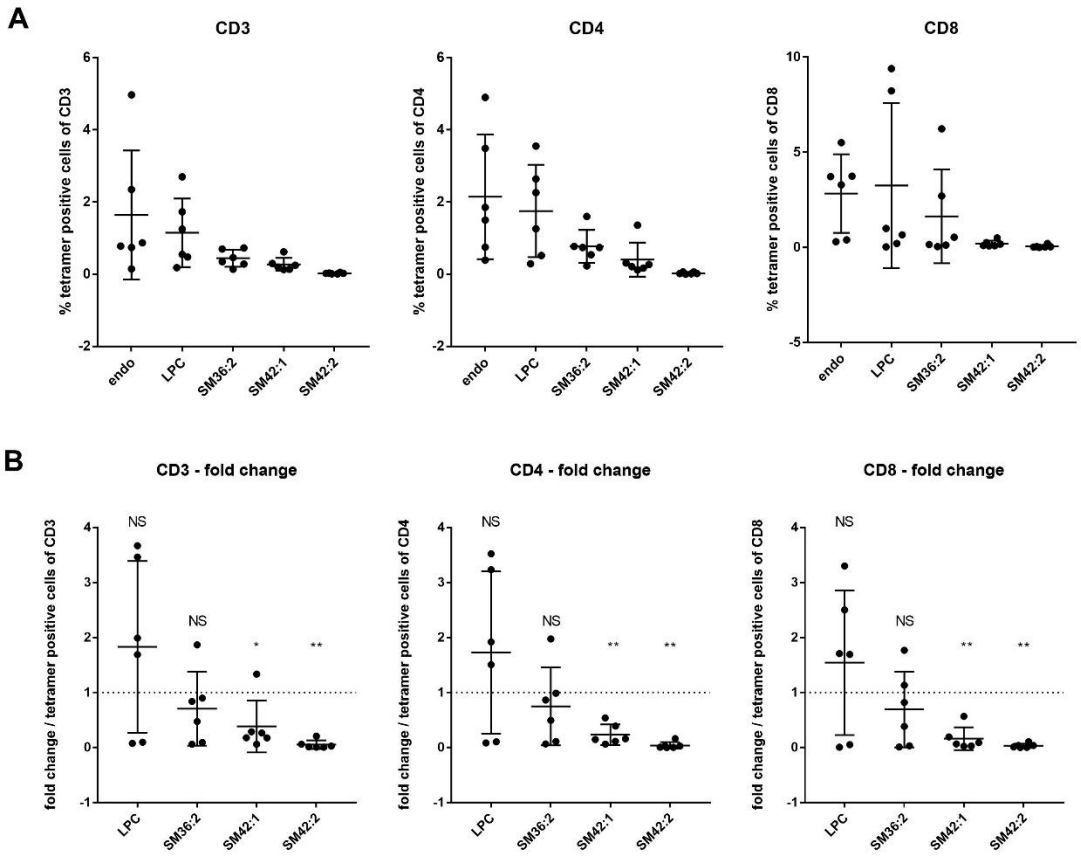


Figure 5.4 Percentage of tetramer-positive T cells in CD3+, CD4+ and CD8+ populations. The polyclonal T cells were isolated from healthy donors (n=6). T cells were stained with CD1a-tetramers and other surface markers. Percentage of tetramer-positive T cells in CD3+, CD4+ and CD8+ populations are shown (A). Fold changes of CD1a-LPC and CD1a-SMs normalized to CD1a-endo are presented (B). The log-transformed value of fold change was used for statistics. Significance of log-fold change was calculated by One-sample t test. Bars represent standard error. * $P<0.05$ ** $P<0.01$.

The percentage of tetramer-positive T cells was tested in CD3+, CD4+ and CD8+ populations in 6 healthy individuals. The range of CD1a-endo tetramer positive cells varied from 0.1 to 5% in total CD3+ T cells, and had similar range in CD4+ and CD8+ T cell subsets, indicating the donor variation. The percentage of CD1a-LPC tetramer positive cells was 0.1-3% in CD3+ T cells and CD4+ T cell subsets, but 0-10% in CD8+ T cells (Figure 5.4A).

However, the range of three CD1a-SM tetramer positive cells was lower, especially in CD3+ and CD4+ populations. For SM 36:2, the tetramer-positive cells were approximately 0.5% in CD3+ cells, 0.7% in CD4+ cells, and 0-6% in CD8+ cells. For SM 42:1, the percentage of tetramer positive cells was much reduced in CD3+ and CD4+ cells, and was almost undetectable in CD8+ populations. For SM 42:2, the tetramer-positive cells were lower than 0.1% in all three populations (Figure 5.4A). In general, the CD4+ and CD8+ T cell populations shared a similar pattern of tetramer recognizing.

To reduce the influence of donor variation, we normalized the percentage of CD1a-LPC and CD1a-SM tetramer positive cells to that of CD1a-endo tetramer. The percentage of CD1a-LPC and CD1a-SM36:2 tetramer positive cells showed no difference between that of CD1a-endo, while the percentage of CD1a-SM42:1 and CD1a-SM42:2 tetramer positive cells was significantly lower (Figure 5.4B). These results suggested the blocking effect of SM 42:1 and SM 42:2 but not SM

36:2, which indicated that the length and unsaturation of CD1a ligands may influence the TCR binding.

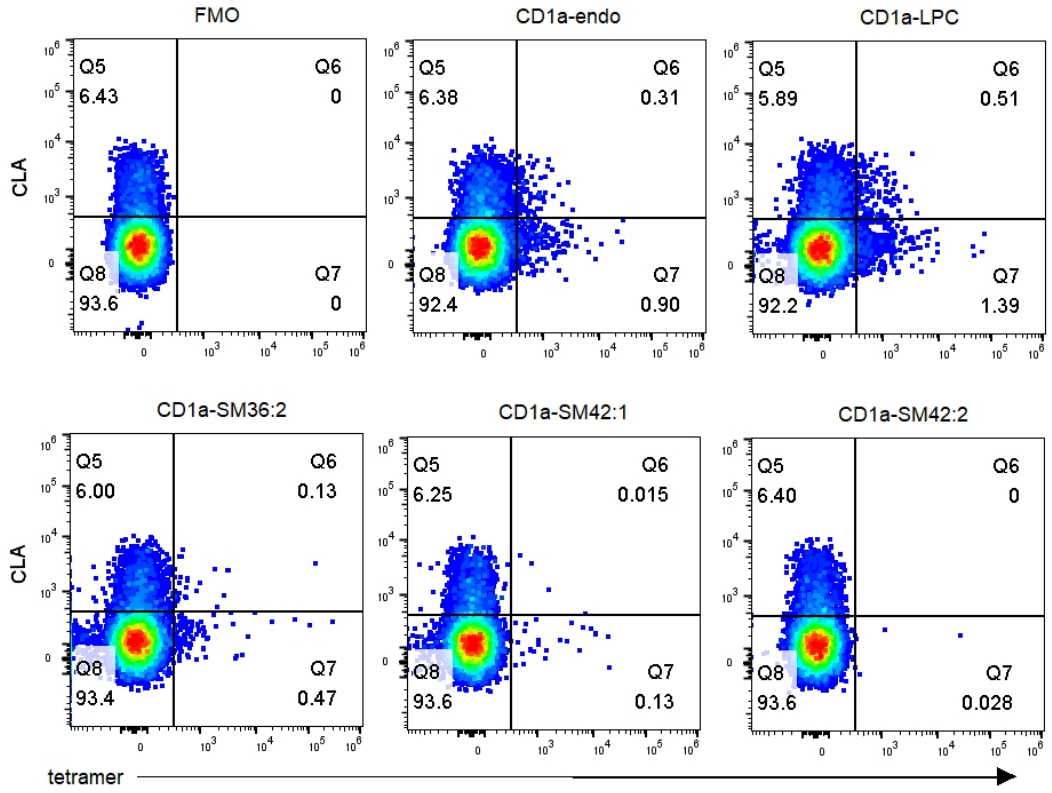


Figure 5.5 CLA expression on tetramer positive cells. Polyclonal T cells were isolated from healthy PBMCs (donor W016) by CD3+ MACS separation. T cells were stained with CD1a-tetramers and CLA. FMO of tetramer was used as negative control. Data representative of at least three donors from different experiments. FACS plots were gated on CD3+, live cells and singlets.

Polyclonal T cells were stained with CD1a-tetramers and CLA. The total percentage of CLA+ T cells was approximately 6.4%. In all tetramer positive cells, a proportion expressed CLA on the surface, indicating they may have the capacity

to migrate to skin (Figure 5.5).

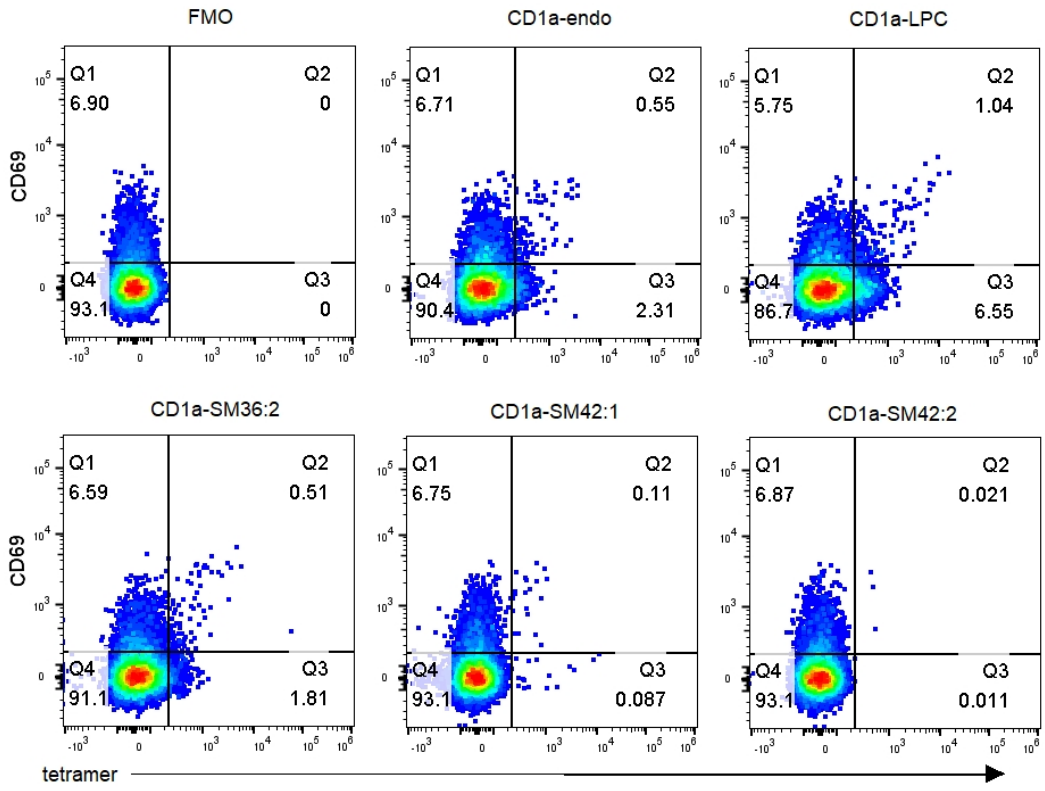


Figure 5.6 CD69 expression on tetramer-positive cells. Polyclonal T cells were isolated from healthy PBMCs (donor W006) by CD3+ MACS separation. T cells were stained with CD1a-tetramers and CD69. FMO of tetramer was used as negative control. Data representative of at least three donors from different experiments. FACS plots were gated on CD3+, live cells and singlets.

Polyclonal T cells were also stained with CD1a-tetramers and CD69. Some tetramer-positive cells were also expressed CD69, indicating their activation status (Figure 5.6).

5.3 Synthetic ceramides as products of SMase

The SMase from *Staphylococcus aureus* cleaves SM and generates ceramide and phosphorylcholine (Flores-Díaz et al., 2016). Thus, we could predict the products of SM 36:2, SM 42:1 and SM 42:2 after SMase treatment (Figure 5.7).

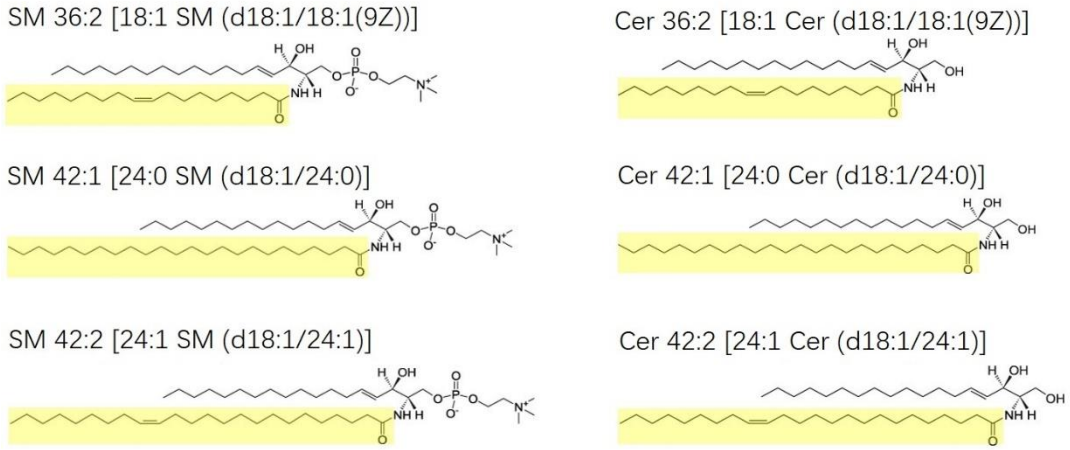


Figure 5.7 Structure of synthetic ceramides. Based on the enzymatic mechanism of SMase, we predicted the structure of ceramide 36:2, 42:1 and 42:2 (right) as the products of synthetic SMs (left) after SMase treatment. All lipids are from Avanti Polar Lipids.

As ceramide is a group of hydrophobic lipids with long fatty-acid carbon chains, we used chloroform/methanol 2:1 as solvent (and suggested by Avanti Polar Lipids). The toxic effect of this solvent to K562 cells was tested.

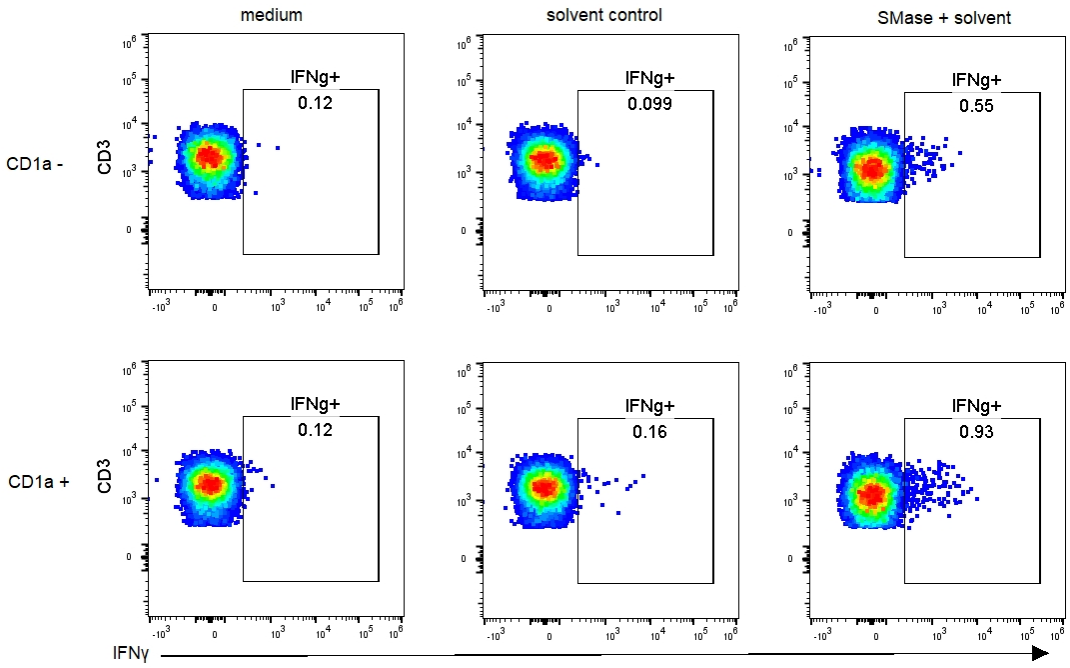


Figure 5.8 Chloroform/methanol solvent did not influence CD1a-reactive SMase-specific T cell response. Polyclonal T cells isolated from healthy PBMC (donor W016) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with or without SMase. Chloroform/methanol solvent was added at 10µl/ml final concentration. Medium only groups were used as negative control. IFNγ producing T cells were tested in cytokine secretion assay. FACS plots gated on CD3+, live cells and singlets.

We used IFNγ secretion assay to test the CD1a-reactive SMase-specific T cell response. K562 cells were incubated with 10µl/ml chloroform/methanol solvent (the same volume as ceramide solution), then treated with or without SMase over 48 hours. K562 cells cultured in medium only were used as negative control. After pre-treatment, K562 cells were co-cultured with polyclonal T cells, and IFNγ

producing cells were tested. The background T cell responses to K562-Ev and K562-CD1a were the same in solvent control groups and medium only groups. T cell responses to K562-CD1a treated with SMase was higher than groups without SMase treatment. These results were consistent with previous experiments, demonstrating the addition chloroform/methanol solvent did not influence the CD1a-reactive SMase-specific response (Figure 5.8).

5.4 Synthetic ceramides as potential CD1a lipid antigens

The three synthetic ceramides were dissolved in chloroform/methanol solvent at high concentration, and added to a final concentration of 100μM into K562-Ev or K562-CD1a cells. In negative control groups, K562 cells were added with equivalent 10μl/ml solvent. In SMase-treated groups, K562 cells were added with 10μl/ml solvent and 1μg/ml SMase. K562 cells in all conditions were treated for 48 hours before co-culture with T cells.

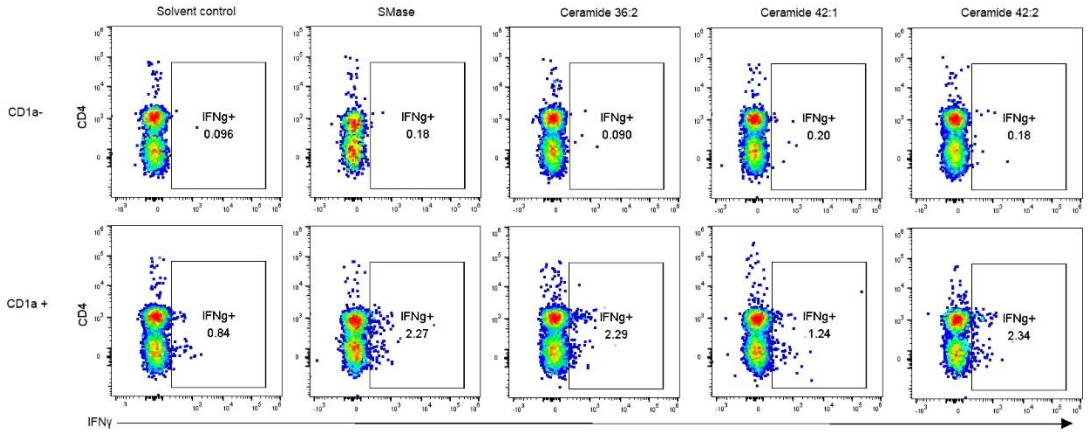


Figure 5.9 Synthetic ceramides as potential CD1a ligands that induced CD1a-

reactive T cell response. Polyclonal T cells isolated from healthy PBMC (donor W006) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. IFNγ producing T cells were tested in cytokine secretion assay. Data representative of at least three donors from different experiments. FACS plots gated on CD3+, live cells and singlets.

Based on cell counting (not shown), the K562 cells after ceramide treatment had good viability, demonstrating the lipids at tested concentration did not influence the cell viability. A CD1a autoreactive response was detected, at approximately 0.8%. The T cell responses to K562-Ev treated with SMase or three synthetic ceramides were consistent, and all lower than 0.2%. The CD1a-reactive SMase-specific T cell response was significant higher, consistent with previous findings. The T cell responses against K562-CD1a pre-treated with ceramide 36:2, 42:1 and 42:2 were all higher compared to related K562-Ev groups. These results suggested that ceramides, as lipid products generated by SMase, may serve as potential CD1a antigens that induced CD1a-reactive T cell responses. Comparative responses across the cohort are shown below.

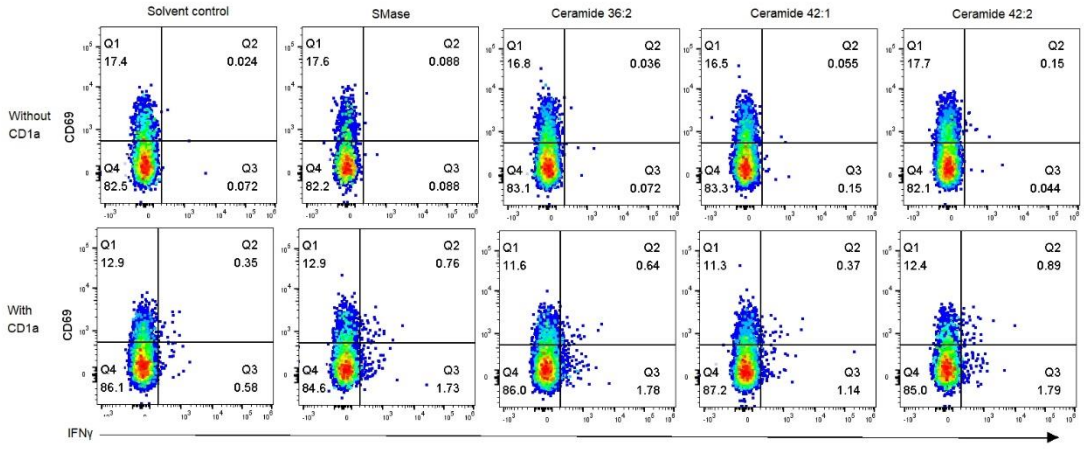


Figure 5.10 Proportion of CD1a-reactive ceramide-specific T cells express CD69. Polyclonal T cells isolated from healthy PBMC (donor W006) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. T cells stained with IFNγ and CD69. Data representative of at least three donors from different experiments. FACS plots gated on CD3+, live cells and singlets.

Consistent with what was observed for CD1a-reactive SMase-specific T cells, the percentage of total CD69+ T cells was not altered in all conditions, but a group of CD69 and IFNγ co-expressing cells was observed. In all IFNγ producing cells, a proportion expressed CD69, indicating their activation (Figure 5.10).

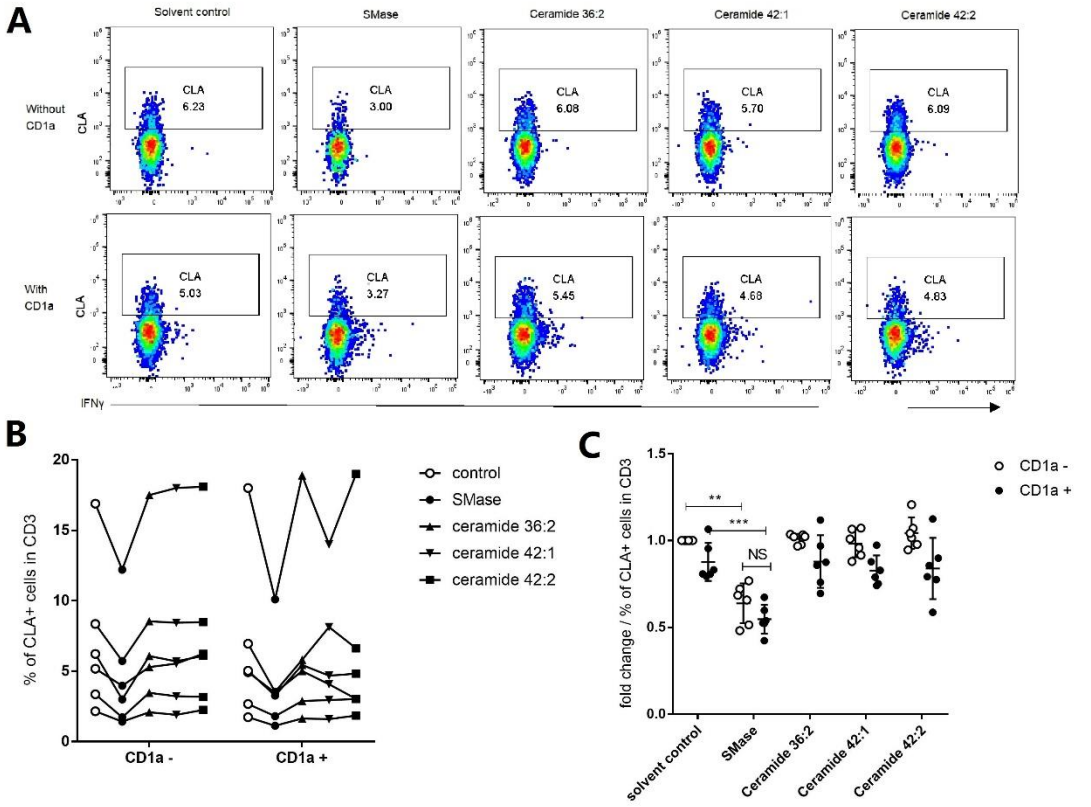


Figure 5.11 CLA expression on CD1a-reactive ceramide-specific T cells. Polyclonal T cells isolated from healthy PBMC (donor W006) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. (A) Representative FACS plots are shown (donor W006). T cells were stained with IFNγ and CLA. FACS plots gated on CD3+, live cells and singlets. (B) The percentage of CLA+ T cells in each condition (n=6). (C) The percentage of CLA+ T cells was normalized to that in group of K562-Ev cells pre-treated with medium only and fold changes are shown. The log-transformed value of fold change was used for statistics. Significance of log-fold change was calculated by One-sample t test. Bars represent standard error. ** P<0.01 *** P<0.001.

Consistent with CD1a-reactive SMase-specific T cells, the majority of ceramide-

specific T cells did not express CLA on the cell surface. The percentage of total CLA+ T cells was not changed among ceramide-treated groups (Figure 5.11A), and the fold changes had no significant difference compared to SMase untreated groups (Figure 5.11C). However, we found in some donors, the percentage of CLA+ T cells in certain K562-CD1a ceramide-treated groups was lower than K562-CD1a untreated group (Figure 5.11B), indicating that regulation of CLA might be complex. As discussed in previous chapter, cytokine production such as IL-12 and IL-4, and the modulation effect of cell-culture on CLA expression, are worthy to take into consideration in future work.

5.5 The correlation of SMase-specific response and ceramide-specific response

To further investigate the CD1a-reactive T cell response to ceramides, we repeated experiments in 5 healthy donors, and found the correlation between SMase-specific and ceramide-specific responses.

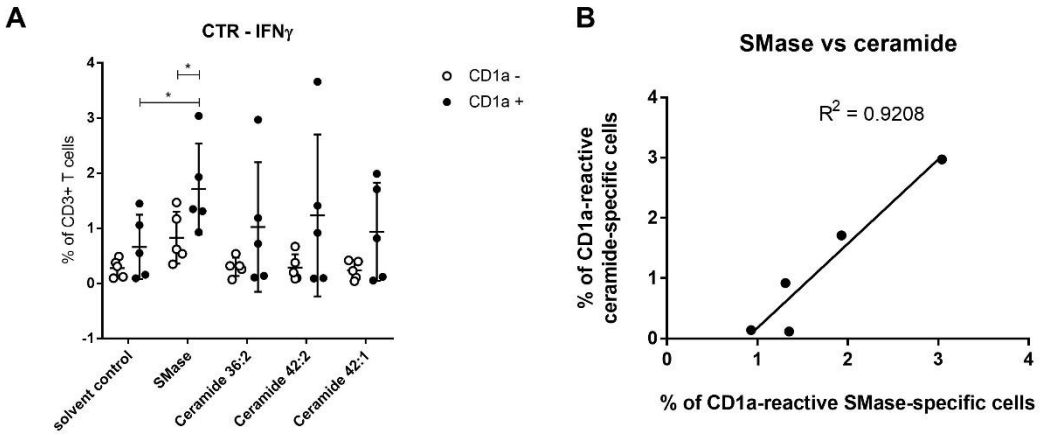


Figure 5.12 The correlation of SMase-specific response and ceramide-specific response. Polyclonal T cells isolated from healthy PBMCs (n=5) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. IFNγ producing T cells were tested in cytokine secretion assay. (A) The percentage of IFNγ producing T cells in each condition. Bars represent standard error. * $P<0.05$ (B) The linear correlation between CD1a-reactive SMase-specific response and CD1a-reactive ceramide-specific response. $R^2=0.9208$, $P=0.0097$.

CD1a-reactive SMase-specific and ceramide-specific responses were tested in 5 healthy individuals. In summary, the CD1a-reactive SMase-specific response was significantly higher than the CD1a-autoreactive response, consistent with previous findings in Chapter 4. Different individuals showed preference to different ceramides, which indicated that ceramides with various length and unsaturation fatty-acid chains may all serve as CD1a ligands. Although the three synthetic ceramides did not have a significantly higher response compared to untreated group so far, they showed trends of up-regulation. It is worth increasing the donor numbers to test the CD1a-reactive ceramide-specific T cell response in larger cohort (Figure 5.12A).

The correlation of CD1a-reactive SMase-specific and ceramide-specific response was analyzed. As some of the donors responded to several kinds of ceramides, for each donor, we selected the highest one as the comparative ceramide-specific

response. We found a linear correlation between SMase-specific and ceramide-specific responses. The R^2 was 0.9208, indicating the donors with stronger SMase-specific responses also had higher responses to at least one of the synthetic ceramides (Figure 5.12B).

5.6 Sphingomyelin as CD1a non-permissive ligands

Proved by previous research (Birkinshaw et al., 2015; Jong et al., 2014) and tetramer staining results presented here, SM could interrupt the TCR-CD1a interaction, and reduce the percentage of CD1a-tetramer positive staining T cells. SM also inhibited the CD1a-autoreactive T cell response of certain T cell lines (Jong et al., 2014). Thus, we proceeded to test the blocking effect of SM for polyclonal T cells. SMs with various fatty-acid residue were tested.

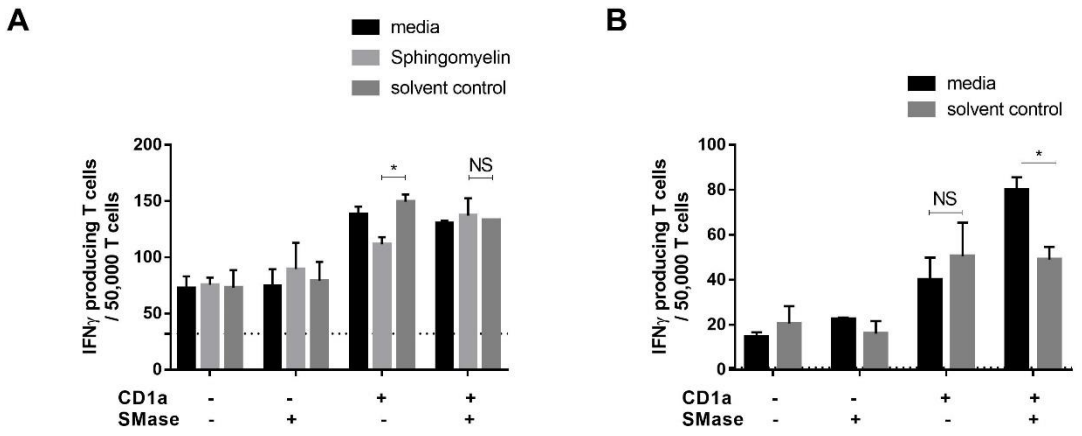


Figure 5.13 SM from egg yolk showed blocking effect to CD1a-autoreactive. Polyclonal T cells isolated from healthy donors PBMCs (A – donor W006; B – donor C050517-1) and incubated overnight with K562-CD1a or K562-Ev cells pre-

treated with or without SMase. K562 cells were incubate with SM, solvent control or medium before co-culture. IFNγ production was measured by ELISpot. (A) SM from egg yolk blocked CD1a-autoreactive response. (B) Variability of solvent influence on CD1a-reactive SMase-specific T cell response was observed in some donors. Bars represent standard error. * $P<0.05$.

The SM from egg yolk was purchased from Sigma-Aldrich, which is predominantly the SM with C16:0 fatty-acid form. The SM was first dissolved in ethanol, and further diluted in PBST. The final lipid concentration was 100μM. The SM from egg yolk showed a significant blocking effect on the CD1a-autoreactive T cell response, which could be abrogated by SMase treatment (Figure 5.13A). The experiment was repeated in further 3 healthy individuals. However, we found in some of the donors, the ethanol-PBST solvent may influence SMase and caused a significant reduction of CD1a-reactive SMase-specific response (Figure 5.13B). We were also suggested by collaborators that ethanol-PBST may influence lipid-loading of CD1a. Thus, we further tested chloroform/methanol solvent to dissolve SM.

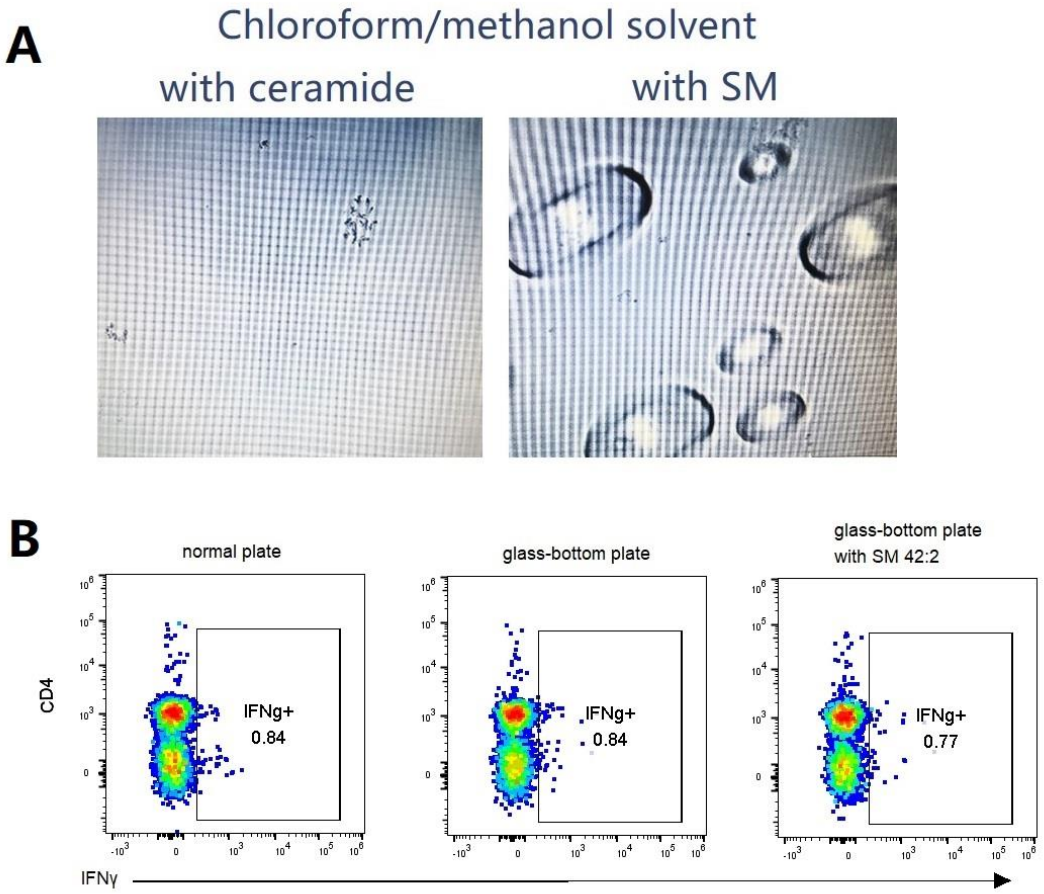


Figure 5.14 Toxicity test of SM dissolved in chloroform/methanol on plastic- and glass-bottom plate. SMs and ceramides were dissolved in chloroform/methanol solvent, and added at 10μl/ml into K562-CD1a cells, with a final lipid concentration of 100μM. T cell response against K562-CD1a cells was tested. (A) Normal plastic cell-culture plate showed structural disruption after adding SM but not ceramide chloroform/methanol solution. (B) K562-CD1a cells cultured in normal plastic cell-culture plate and glass-bottom cell-culture plate, and further treated with or without SM 42:2. T cell responses to these K562-CD1a was tested by secretion assay (donor W006).

However, on testing the toxicity of SM solution to K562 cells, we noted that at

100μM, SM dissolved in chloroform/methanol was toxic to K562 cells, and led to >90% cell death. This was partly explained by the damage of plastic cell-culture plates caused by SM solution (Figure 5.14A). However, the same volume and concentration of chloroform/methanol solution or ceramide solution did not cause similar problems.

We further cultured K562 cells in glass-bottom cell-culturing plate. K562-CD1a cells cultured in normal plastic plate and glass-bottom plate, and cultured with 10μl/ml of chloroform/methanol. Cells in both plates had similar viability and induced same level of CD1a-autoreactive T cell response. SM chloroform/methanol solution added in glass-bottom plate did not influence K562 cells viability. T cell response against K562-CD1a treated with SM was similar to CD1a-autoreative response (Figure 5.14B).

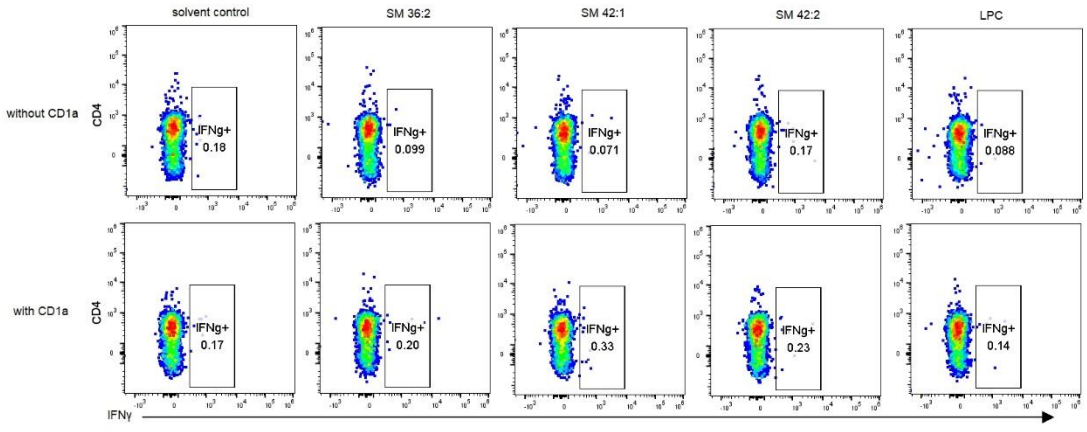


Figure 5.15 Testing the blocking effect of synthetic SMs. Polyclonal T cells isolated from healthy PBMC (donor W024) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMs, LPC, or medium only in glass-bottom

plate. IFNγ producing T cells were tested by cytokine secretion assay. Data representative of at least three donors from different experiments. FACS plots gated on CD3+, live cells and singlets.

In the pilot experiments we tested blocking effect of SM36:2, SM42:1 and SM42:2 in three different healthy individuals. The three synthetic SMs were added 10µl/ml into K562-Ev or K562-CD1a cells in glass-bottom plate at a final lipid concentration of 100µM. In LPC-treated groups, K562 cells were cultured with 10µg/ml LPC dissolved in chloroform/methanol. In negative control groups, K562 cells were cultured with 10µl/ml solvent. K562 cells in all conditions were treated for 48 hours before co-culture with T cells.

None of the synthetic SMs showed the blocking effect of CD1a-autoreactive, and LPC did not induce a T cell response (Figure 5.15). This may be explained by non-effective dissolving of long-chain hydrophobic lipids. In previous ELISpot experiments, SM was ultrasonic treated and heated before added to K562 cells, which would help with lipid loading. In future experiments, we would treat the synthetic SMs with ultrasound/heating in glass tubes; and it would also be important to titrate the concentration of SM before conclusions can be made.

Discussion

Overall, by using CD1a-tetramers loaded with endogenous lipids, LPC and

synthetic sphingomyelins, these data demonstrate that SMs with long-chains could interrupt TCR-CD1a interaction, and reduce the percentage of CD1a-tetramer positive staining T cells in polyclonal T cells from healthy individuals. The length and unsaturation of fatty-acid residues of SM are likely to significantly influence the effect. The SM 42:2, which has long carbon-chain and two double bonds, showed the best blocking effect. The blocking effect of SM is also suggested by cell functional assays, in which the CD1a-autoreactive response was reduced by SM from egg yolk and recovered by SMase. It would be important to further modify the experimental conditions and investigate the effect of SM on the CD1a-autoreactive response. Furthermore, synthetic ceramides, which have same fatty-acid residues with related SMs, could serve as CD1a ligands and induced CD1a-reactive ceramide-specific T cell response. The SMase-specific and ceramide-specific T cell response showed a linear correlation, implicating the role of ceramides in explaining the SMase response.

As discussed in Chapter 4, SMase from *S. aureus* induced the CD1a-reactive SMase-specific T cell response and induced production of IFNγ, IL-22 and IL-17A. The response could be reduced by enzyme heat-inactivation, suggesting it is dependent on enzyme bioactivity. The function of SMase is known to cleave SM and generates ceramide and phosphorylcholine (Flores-Díaz et al., 2016). SMase contributes to the reduction of SMs, the ligands with blocking effect, and to the generation of ceramides, the lipid antigens that could induce T cell activation *ex*

vivo.

Although the length and unsaturation of fatty-acid residues may influence the blocking effect of SMs, all three ceramides with different fatty-acid chains seem to be able to induce a T cell response, which is important to further investigate in a larger healthy cohort. In addition, it would be important to investigate whether responses are altered in those with inflammatory skin disease, and to establish T cell clones for more detailed functional analyses. Lastly, the role of ceramides and SMs can be further investigated through biophysical and structural approaches.

Also, the generation of ceramides as CD1a ligands may not be the only way that SMase involved in CD1a-reactive T cell responses. As SMs are enriched in CD1a protein eluate, and have been shown to serve as non-permissive ligands to block the T cell recognition, it could be that the SMase modifies the relative proportion of SMs *in vivo* and allowing access for permissive ligands to load on CD1a proteins. This mechanism may be tested in future experiments.

The above studies were conducted using polyclonal T cells derived from the blood, testing T cell responses against bacterial SMase (results chapter 4), or potential neoantigens (results chapter 5). Besides the study of CD1a-reactive response to SMase and its potential lipid products in polyclonal T cells, it is also important to generate CD1a-reactive T cell lines and clones, which may further help to

investigate the phenotypic and functional characterization of these responding T cells. The TCR sequencing of reactive T cell clones would contribute to the understanding of CD1a-TCR interaction as well. We also aim to investigate the CD1a-reactive SMase-specific T cell responses in skin T cells by using CD3+ skin T cell lines. Therefore, we generated CD1a-reactive T cell lines and clones from blood and skin, which will be discussed in the next chapter.

6. Result III: Phenotypic and Functional Analysis of CD1a-reactive T Cell Lines and clones

Introduction and aims

A series of studies have revealed the existence of CD1a-reactive T cells that recognize self-lipids or exogenous ligands, such as lipids from pollen or mycobacteria (Agea et al., 2005; Birkinshaw et al., 2015; Van Rhijn et al., 2015). By taking advantage of K562 cells that highly express CD1 but lack MHC on the surface, previous studies have found a high frequency of CD1a-autoreative T cells in all tested healthy donors. The CD1a-autoreactive T cells comprised 0.3-10% circulating memory T cells, and did not show highly conserved TCR sequences. The data demonstrate that CD1a-reactive T cells are a normal component of the human T cell repertoire. These T cells are able to produce various cytokines, including IFNγ, IL-13, and IL-22, and involved in multiple immune pathways. (De Lalla et al., 2011; Preihs, Christian Arambula et al., 2013; Vincent, Xiong, Grant, Peng, & Brenner, 2005)

CD1a is constitutively expressed by Langerhans cells that are located in the epidermis of the skin, and CD1a-autoreactive T cells have been shown in some studies to express skin-homing markers including CLA, CCR4, CCR6 and CCR10, indicating their ability to migrate to skin. CD1a-autoreative T cells can also be directly isolated from normal human dermis samples. The co-localization of

CD1a-expressing cells and CD1a-reactive T cells suggests their roles in skin immunity. (Jong et al., 2014; Preihs, Christian Arambula et al., 2013)

The first CD1a-reactive T cell line was the CD4- CD8- αβ T cell line BK6, which directly lysed CD1a-expression MOLT-4 cells in a CD1a-dependent manner (Porcelli et al., 1989). BK6 is a CD1a-autoreactive T cell line, and its TCR was subsequently used for studying TCR-CD1a-ligand interactions (Birkinshaw et al., 2015). Another modeled crystal structure of TCR-CD1a-ligand was the TCR CD1a-lipopeptide trimolecular complex of CD1a-DDM specific αβ T cell CD8-2 (Zajonc et al., 2005). More CD1a-reactive T cell lines were observed, most of which were αβ T cells, but one human Vδ1 γδ T cell line was also proved to recognize phospholipid presented by CD1a (Agea et al., 2005). Although the first CD1a-reactive T cell clone was CD4/CD8-double negative, later samples of CD1a-autoreactive clones were found in CD4+ and CD8+ subsets too (Agea et al., 2005; Preihs, Christian Arambula et al., 2013; Vincent et al., 2005).

Interestingly, one αβ CD1a-reactive T cell clone was proved to recognize both self-lipids and foreign antigens from microbial extracts, but the self-reactivity was weaker than bacterial response, demonstrating the dual reactive T cell clones existed (Vincent et al., 2005).

However, compared to group 2 CD1-reactive T cells (NKT cells that recognize

CD1d), our understanding of CD1a-reactive T cells is still limited, as group 1 CD1 proteins are not expressed in mice. Also, there is no specific surface marker for CD1a-reactive T cells so far. The number of known CD1a-reactive T cell clones is still limited; thus, the generation of CD1a-reactive T cell clones is important for extending our knowledge of the phenotype, function, and antigen specificity of CD1a-reactive T cells. T cell clones are also efficient tools to investigate the underlying mechanisms of the CD1a-dependent activation, CD1a-TCR interaction, downstream signaling pathways, and relative gene regulation.

The aims of this chapter are to generate CD1a-reactive SMase-specific T cell lines and clones, as well as CD1a-autoreactive T cell lines and clones, from healthy individuals. Besides blood T cells, we also sought to generate CD1a-reactive T cell lines from healthy skin. IFNγ secretion assay was used for generating T cell lines. The phenotypes of CD1a-reactive T cell lines were further tested by flow cytometry. Some T cell lines/clones were sent for TCR sequencing to our collaborator David Price from Cardiff University.

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

In order to further study the underlying mechanism of CD1a-reactive T cells, the CD1a-reactive SMase-specific T cell lines and CD1a-autoreactive T cell lines were generated by using IFNγ secretion assay.

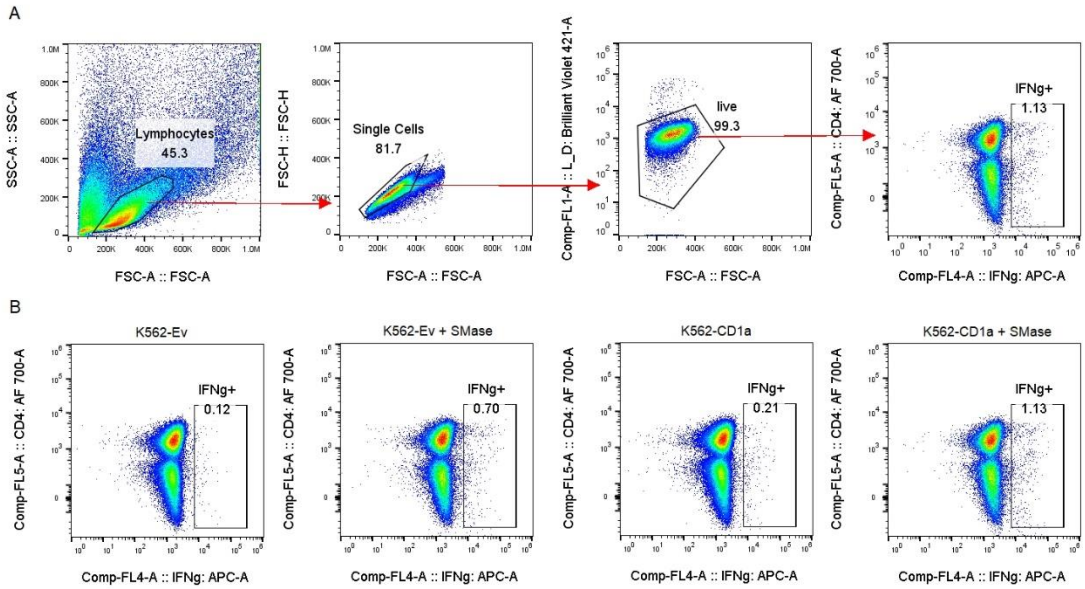


Figure 6.1 Flow cytometry gating strategy for the generation of CD1a-reactive T cell lines. Polyclonal T cells isolated from healthy PBMCs cultured overnight with K562-Ev and K562-CD1a cells pre-treated with or without SMase. IFNγ highly producing T cells were detected and sorted by cytokine secretion assay. Results from one selected donor (W032) are shown, which were representative of at least three donors from different experiments. (A) Flow cytometry gating strategy for IFNγ-producing CD1a-reactive SMase-specific T cells. These T cells are gated on lymphocytes/singlets/live cells/IFNγ+. (B) Population of IFNγ-producing T cells under each condition are shown. The K562-Ev, K562-Ev + SMase, K562-CD1a groups were used as control for gate setting, and IFNγ-producing T cells were sorted from K562-CD1a + SMase group.

Polyclonal T cells from peripheral blood were isolated and cultured with K562-CD1a cells pre-treated with or without SMase, and with K562-Ev cells as control

(Figure 6.1). The CD1a-reactive T cell response was detected by IFNγ secretion assay, and cytokine-producing cells were sorted in 96-well-round-bottom plates at 1 cell, 10 cells or 50 cells per well. Cell sorting was done by FACS facility team of the department or by lab member Yi-ling Chen. The T cells were further expanded by rapid expansion mix.

Cell lines	Cell per well	Well number	Growing lines	ratio
W032 IFNγ CD1a-auto	50	1	1	100%
	10	9	9	100%
	1	20	9	45%
W032 IL-22 CD1a-SMase	50	1	1	100%
	10	9	9	100%
	1	31	13	42%
W032 IL-22 CD1a-auto	10	10	8	80%

Figure 6.2 T cell number in each well influenced the growing ratio of T cell lines. Results from selected donor (W032) are shown, which were representative of at least three donors from different experiments. CD1a-reactive cytokine producing T cells were initially sorted in 96-well-round-bottom plate at 1 cell, 10 cells or 50 cells per well. The ratio of wells with growing T cell lines in total wells was calculated.

Cytokine-producing cells were very activated. When sorted by single cell per well followed with expansion by rapid expansion mix, T cells in 40-45% of total wells were expanded. However, when sorted by 10 cells or 50 cells per well, T cells in >80% of total wells were expanded (Figure 6.2). Thus, first sorting by 10 cells per

well, then re-sorting after expansion, might be an ideal strategy for generating T cell lines and clones, considering the balance between specificity and yielding efficiency.

6.2 Blood CD1a-reactive SMase-specific T cell lines

All IFNγ producing T cell lines generated were further tested for CD1a reactivity and SMase specificity by ELISpot (Figure 6.3A). Of all the lines generated, five CD1a-reactive SMase-specific T cell lines showed strong responses, which were isolated from two different healthy individuals: T cell lines 5-G9, 6-C11, 1-B5, 1-C11 were isolated from donor W016 and expanded from single cell seeded per well; while W006B2 line was isolated from donor W006 and expanded from 10 cells per well. The promising lines were subsequently expanded by rapid expansion mix for future experiments. CD1a reactivity and SMase specificity of T cell lines were confirmed after every expansion. The CD1a-reactive SMase-specific response remained consistent (Figure 6.3B). Sometimes we noticed variability in response to SMase in the absent of CD1a, suggesting CD1a-independent effect in addition to CD1a-dependent effect.

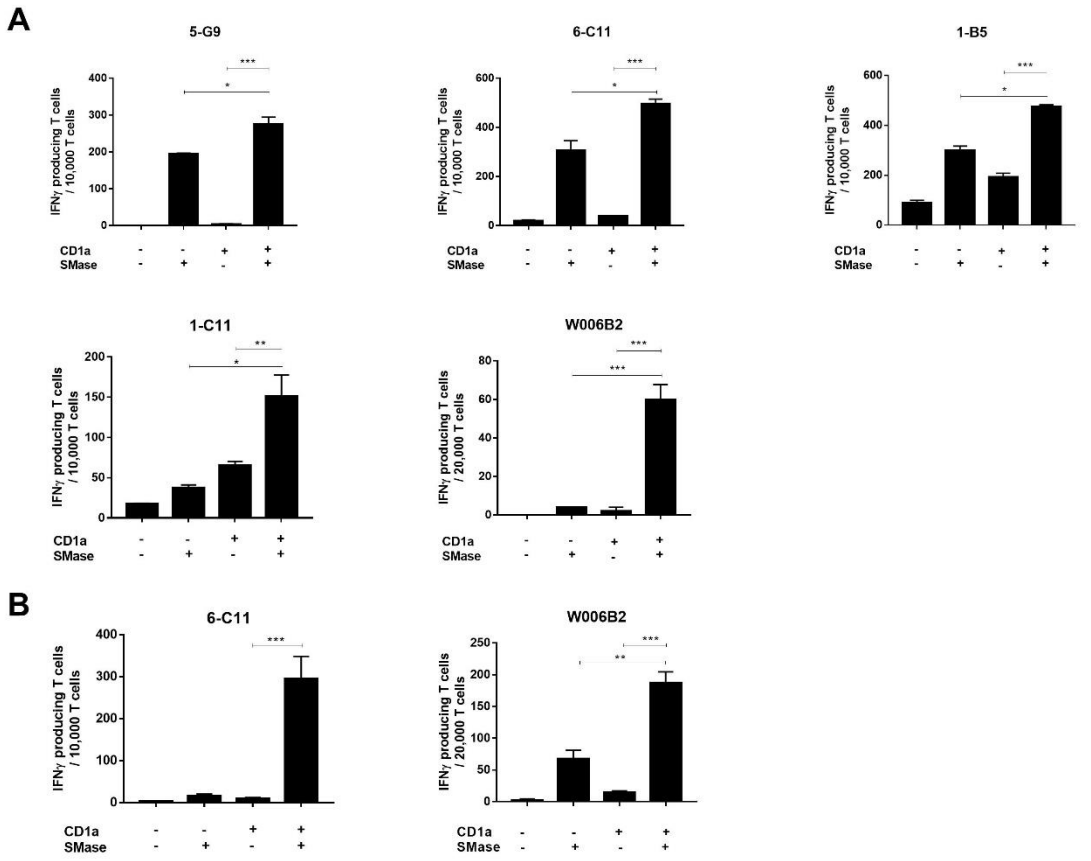


Figure 6.3 CD1a-reactive SMase-specific T cell lines generated by IFNγ secretion assay from healthy PBMCs. T cell lines generated by cytokine secretion assay and expanded by rapid expansion mix. T cells co-cultured with K562-Ev or K562-CD1a cells pre-treated with or without SMase overnight. IFNγ production was measured by ELISpot. The CD1a-reactive SMase-specific response of T cell lines was examined after one expansion (A) and two expansions (B). Bars represent standard error, * P<0.05, ** P<0.01; ***P<0.001.

After first expansion, the surface marker expressions of each T cell lines were tested, for further study of the phenotype of these CD1a-reactive SMase-specific T cells.

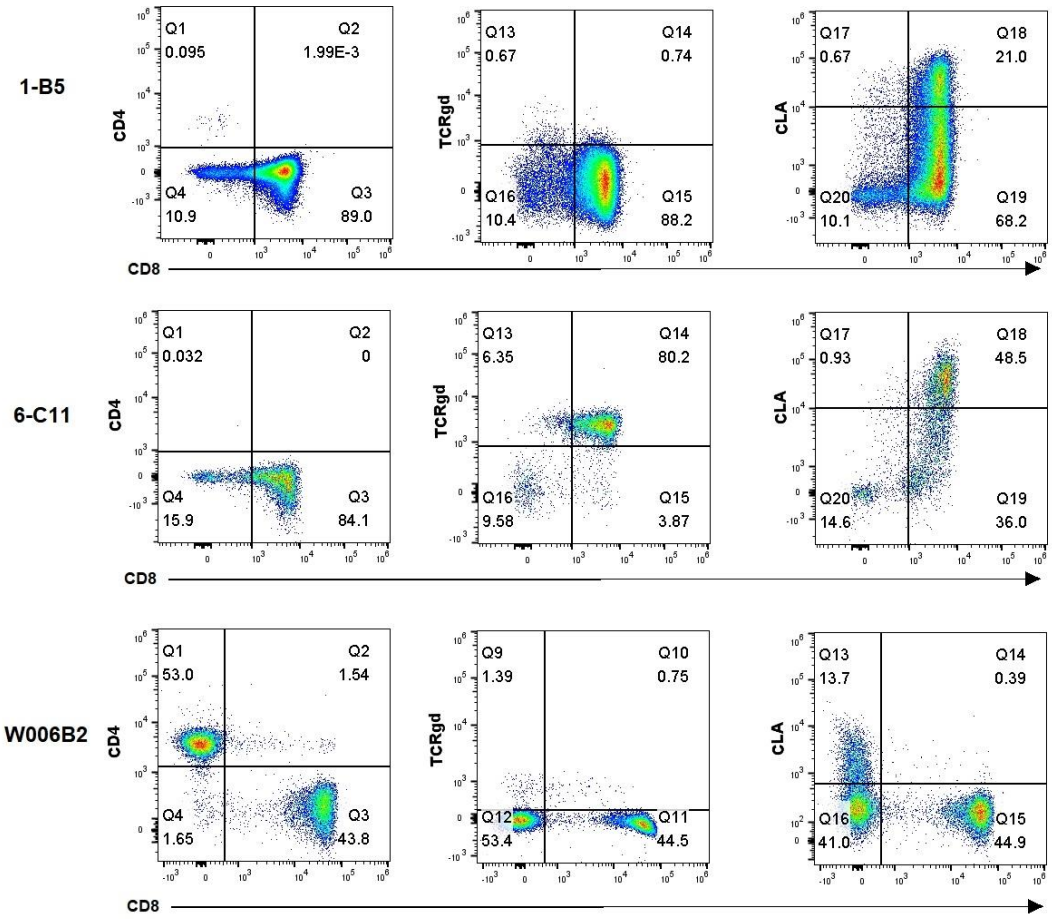


Figure 6.4 Surface expression of cell surface markers on 1-B5, 6-C11 and W006B2 CD1a-reactive SMase-specific T cell lines. Flow cytometric analysis of CD4, CD8, γδ TCR, and CLA on CD1a-reactive SMase-specific T cell lines. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using Fluorescence Minus One (FMO).

Three CD1a-reactive SMase-specific T cell lines were stained with key surface markers. T cell line 1-B5 was composed of dominantly CD8+ αβ T cells; T cell line 6-C11 was composed of dominantly CD8+ γδ T cells; T cell line W006B2 was a

mixture of CD4+ and CD8+ αβ T cells. Interestingly, a proportion of these CD1a-reactive SMase-specific T cell lines express CLA, indicating the capability of these T cells to migrate to skin (Figure 6.4). Although the majority of CD1a-reactive SMase-specific T cells in PBMC discussed in previous chapters did not express CLA, these T cell lines started to express CLA on surface after expansion over two weeks, suggesting the expression of skin homing marker may be influenced by cell culture.

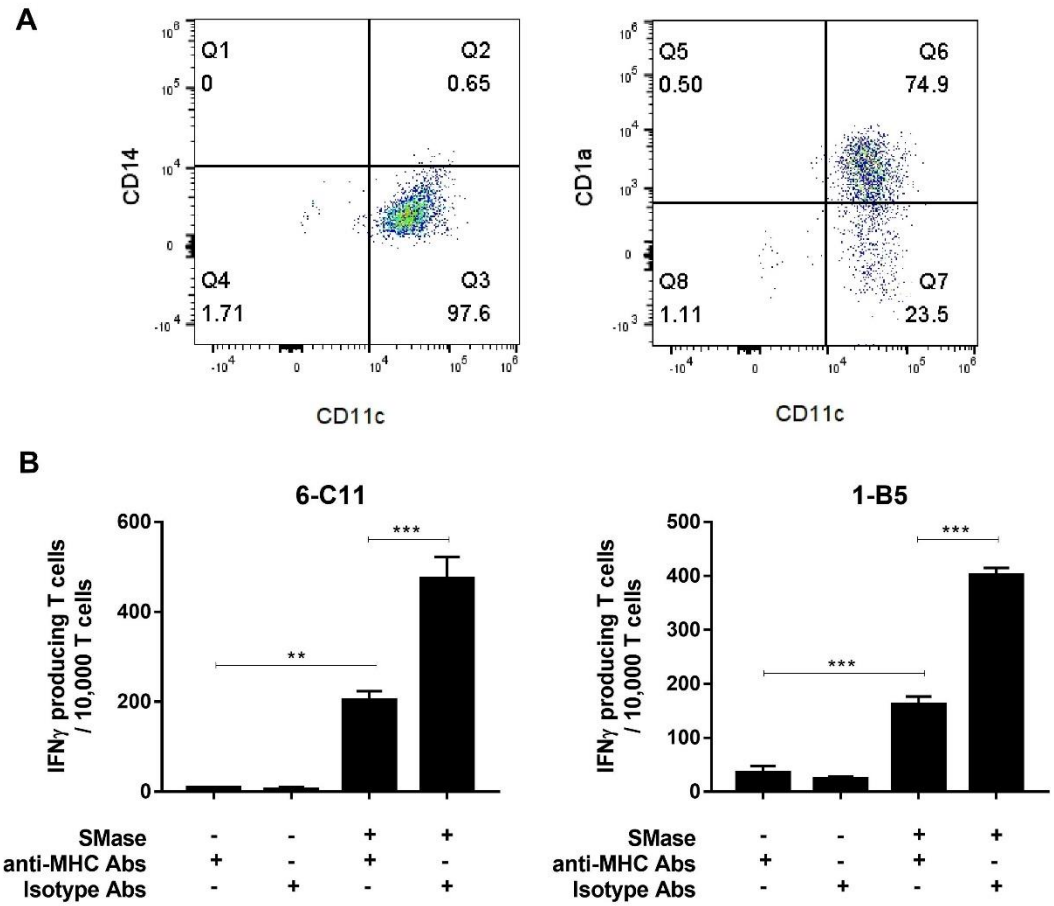


Figure 6.5 CD1a-reactive SMase-specific T cells responded to mDCs and showed SMase specificity. (A) The differentiation and CD1a expression of the

monocyte-derived myeloid dendritic cells were confirmed. Plots gated on singlets and live cells. (B) T cell line 6-C11 and 1-B5 were incubate with CD1a-expressing *in vitro* derived mDCs from the same donor. The mDCs were pre-treated with or without SMase overnight, and incubated with anti-MHC antibodies or isotypes before co-culture with T cells. IFNγ production was detected by ELISpot. Bars represent standard error, ***P<0.001.

CD14+ cells were isolated by MACS separation and differentiated in media containing IL-4 and GMCSF to produce monocyte-derived myeloid dendritic cells (mDCs). The differentiation and CD1a expression of the mDCs were confirmed before experiments (Figure 6.5A). The mDCs were pre-incubated with anti-MHC I and anti-MHC II antibodies or isotype controls, and treated with or without SMase overnight. T cell lines generated from the same donor were co-cultured with mDCs, and IFNγ production was tested by ELISpot. With the presence of anti-MHC antibodies, the T cell response to mDC pre-treated with SMase was significantly higher than the untreated group (Figure 6.5B). The CD1a dependence of this response need further tested by using anti-CD1a antibody.

The CD1a-reactive SMase-specific T cell line W006B2 was further sorted by single cell sorting and expanded by rapid expansion mix, in order to generate T cell clones. The specificity of T cell lines generated were further tested by IFNγ secretion assay. Of all the lines generated, three W006B2-resorted T cell lines,

W006B2R2, W006B2R7 and W006B2R9 showed strong responses, and the percentage of responding T cells increased from ~0.75% (150 cells in 20,000 cells) to 2-3%. Although the lines respond to CD1a-expressing K562 cells, CD1a dependence will be further confirmed in future experiments using CD1a-blocking antibodies. These three T cell lines were sent for TCR sequencing (Figure 6.6).

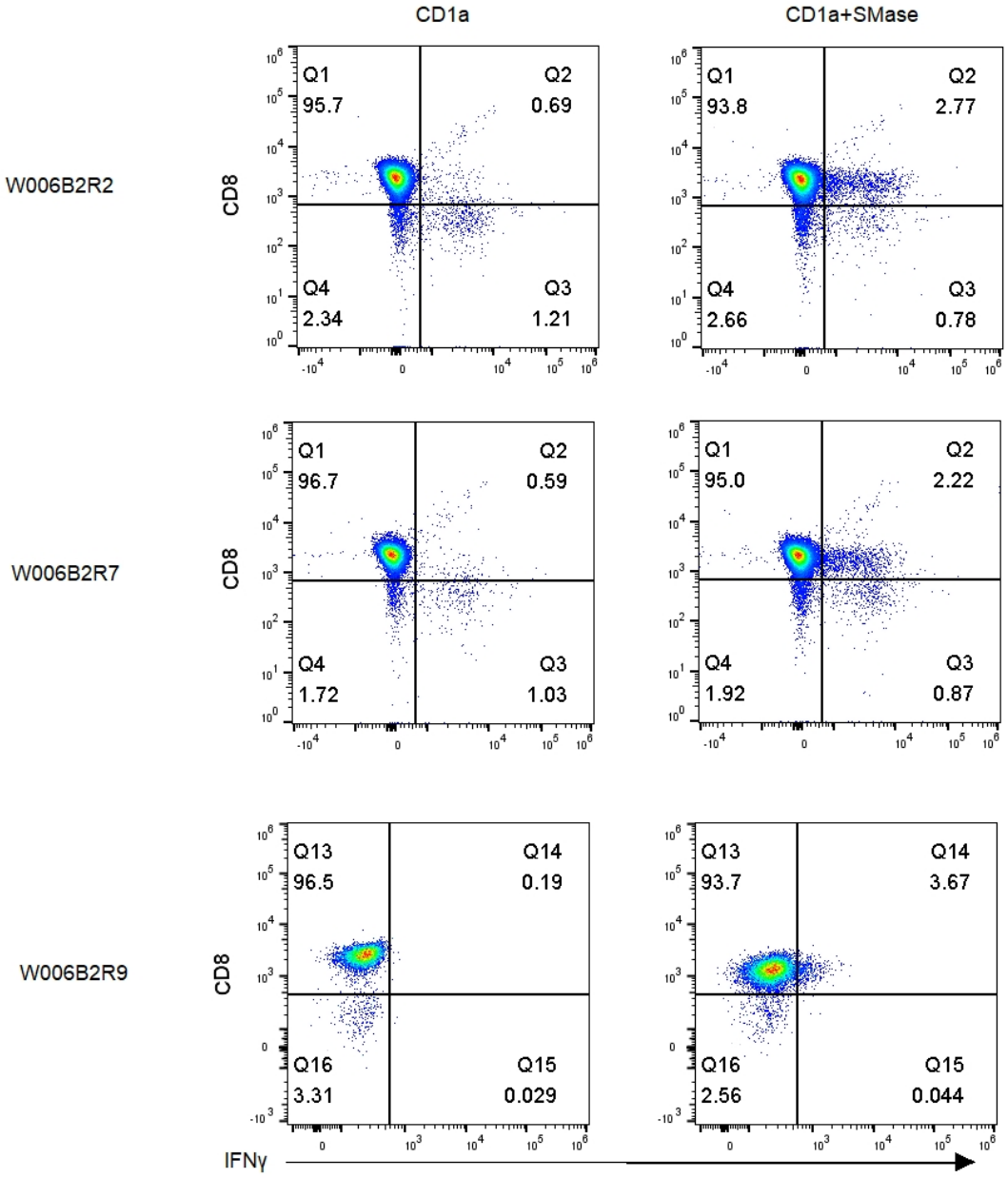


Figure 6.6 Three selected W006B2 re-sorted CD1a-reactive SMase-specific T cell lines. T cell line W006B2 was re-sorted by single cell per well and expanded by rapid expansion mix. T cells co-cultured with K562-CD1a cells pre-treated with or without SMase overnight. IFNγ production was measured by secretion assay. Plots gated on singlets, live cells, and CD3+ cells.

The key surface markers of W006 re-sorted T cell lines were stained. All these three T cell lines were CD8+ αβ T cells (Figure 6.7).

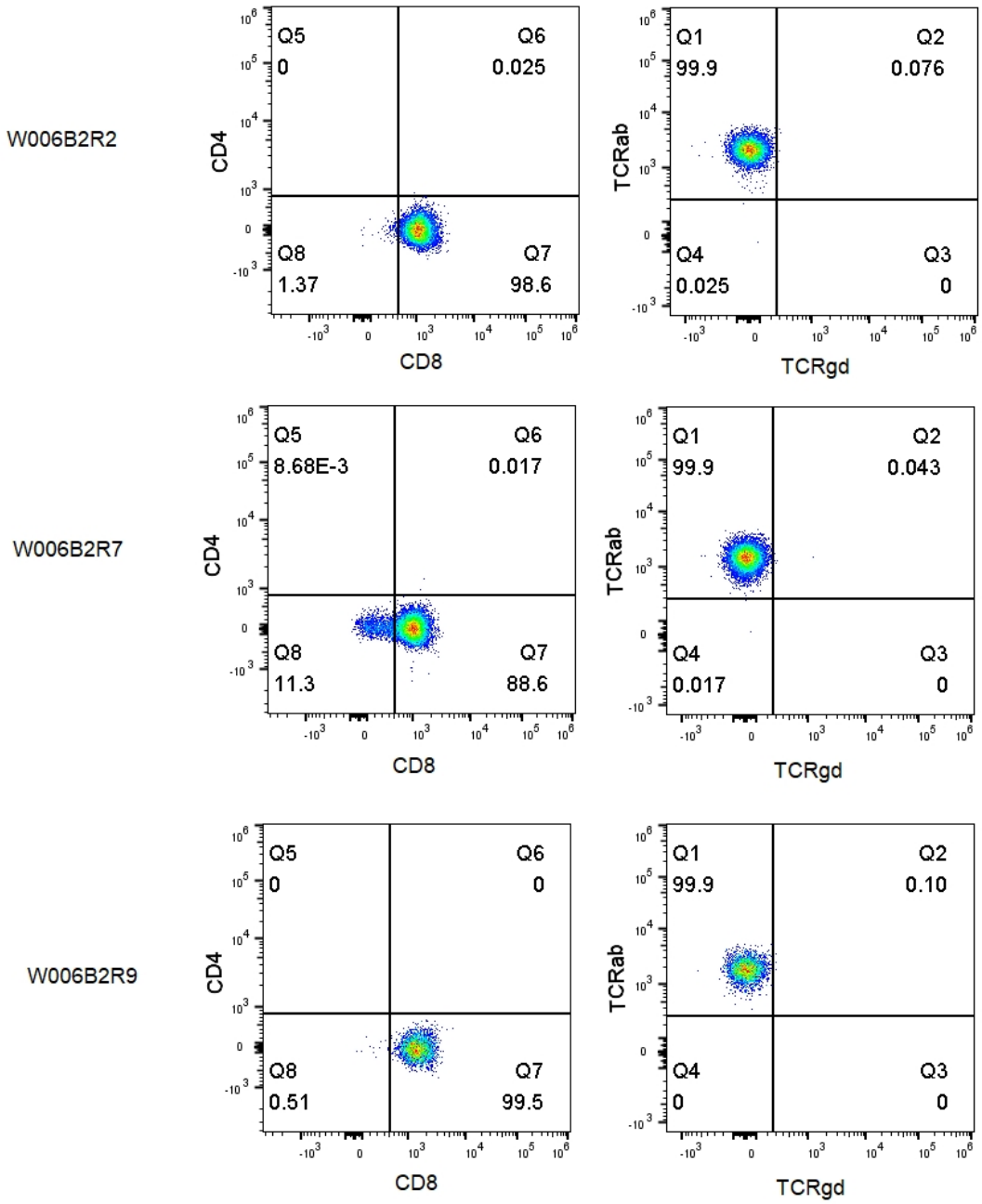


Figure 6.7 Surface expression of cell surface markers on W006B2 re-sorted CD1a-reactive SMase-specific T cell lines. Flow cytometric analysis of CD4, CD8, γδ TCR and αβ TCR on W006B2 re-sorted T cell lines. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using Fluorescence Minus One (FMO).

6.3 Blood CD1a-autoreactive T cell lines

In addition to CD1a-reactive SMase-specific T cell lines, we were able to generate CD1a-autoreactive lines as well, in order to enhance the understanding of CD1a-mediated immunity.

Of all the lines generated, six CD1a-autoreactive T cell lines showed strong response, which were isolated from three different healthy individuals: T cell line 7-C8 was isolated from donor W016 and expanded from single cell per well; T cell lines W006B1, W006B3, W006B4, W006B5 were isolated from donor W006 and expanded from 10 cells per well; T cell line W015B6 was isolated from donor W015. The promising lines were subsequently expanded by rapid expansion mix for future experiments (Figure 6.8).

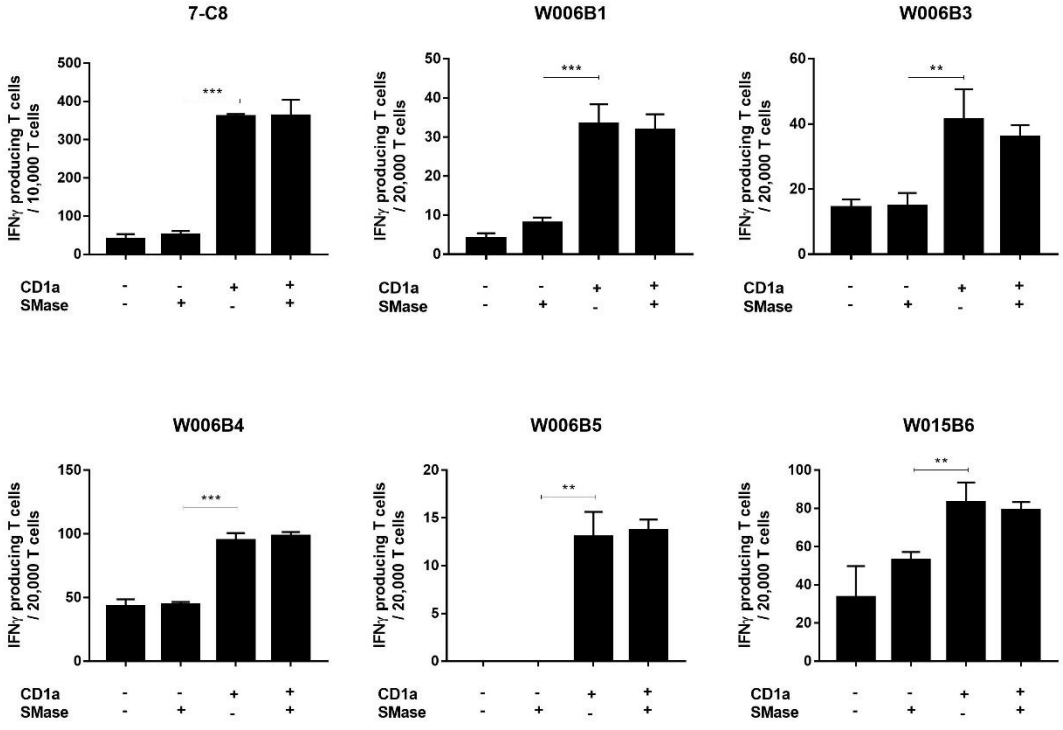


Figure 6.8 CD1a-autoreactive T cell lines generated by IFNγ secretion assay

from healthy PBMCs. T cell lines generated by cytokine secretion assay and expanded by rapid expansion mix. T cells co-cultured with K562-Ev or K562-CD1a cells pre-treated with or without SMase overnight. IFNγ production was measured by ELISpot. Bars represent standard error, * P<0.05, ** P<0.01; ***P<0.001.

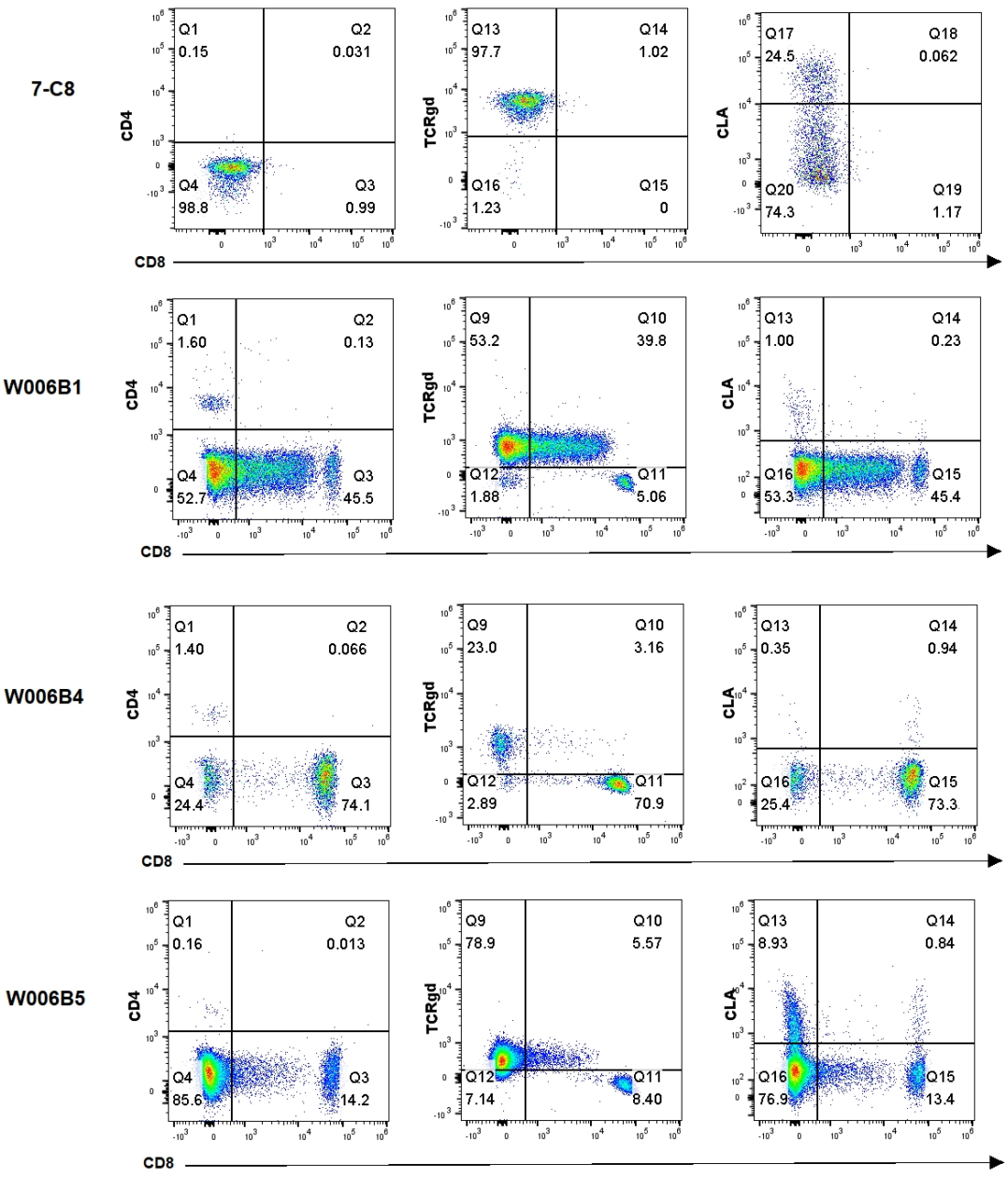


Figure 6.9 Surface expression of cell surface markers on 7-C8, W006B1,

W006B3, W006B4, W006B5 CD1a-autoreactive T cell lines. Flow cytometric analysis of CD4, CD8, γδ TCR, and CLA on CD1a-autoreactive T cell lines. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using Fluorescence Minus One (FMO).

Four CD1a-autoreactive T cell lines were stained with key surface markers. T cell line 7-C8 was composed of dominantly CD4 CD8 double negative γδ T cells; T cell line W006B1, W006B4, W006B5 were a mixture of CD4+ and CD8+ T cells, and a mixture of αβ and γδ T cells. Based on previous studies, CD1a-reactive T cells have been found in CD4+, CD8+ and DN components, and most of the known CD1a-autoreactive T cell lines are αβ T cells, although γδ CD1a-reactive T cells have been identified too (Agea et al., 2005; Preihs, Christian Arambula et al., 2013). Interestingly, proportion of these CD1a-autoreative lines we generated were γδ T cells, indicating the γδ T cells might play an important role in CD1a autoimmunity. In addition, a proportion of these T cell lines expressed CLA, suggesting their ability to home to skin (Figure 6.9).

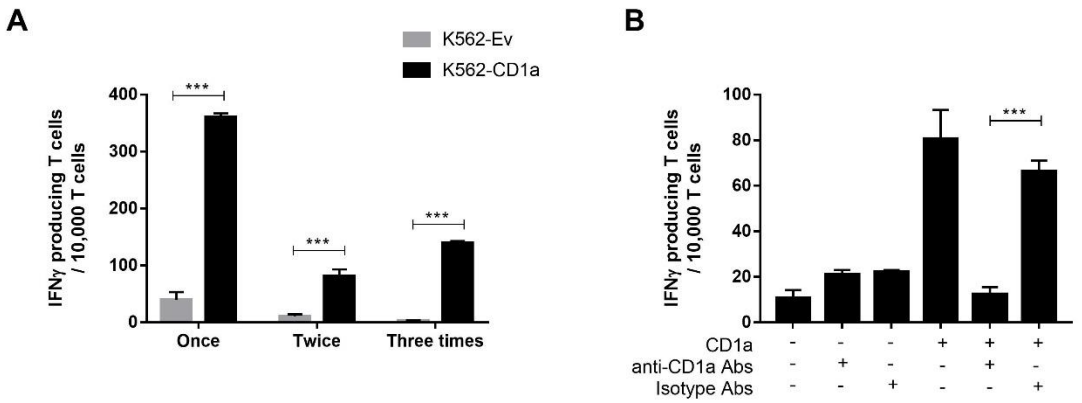


Figure 6.10 Blood CD1a-autoreactive T cell line 7-C8 responded consistently after multiple expansions, and in a CD1a-dependent manner. T cells co-cultured with K562-Ev or K562-CD1a cells overnight. IFNγ production was measured by ELISpot. (A) T cell line 7-C8 was expanded by rapid expansion mix for three times. CD1a reactivity of T cells was tested after each expansion. (B) K562-Ev and K562-CD1a cells were incubated with anti-CD1a antibodies or isotype control before co-cultured with T cells. Bars represent standard error, ***P<0.001.

T cell line 7-C8 was selected for further expansion by rapid expansion mix for future experiments. The cell line was expanded for three times, and after each expansion, the CD1a activity was tested. The CD1a-autoreactive response of 7-C8 was stable through multiple expansions, indicating it could be a long-term T cell line (Figure 6.10A). T cell response of 7-C8 to K562-CD1a cells was blocked by anti-CD1a antibody, confirming its CD1a specificity.

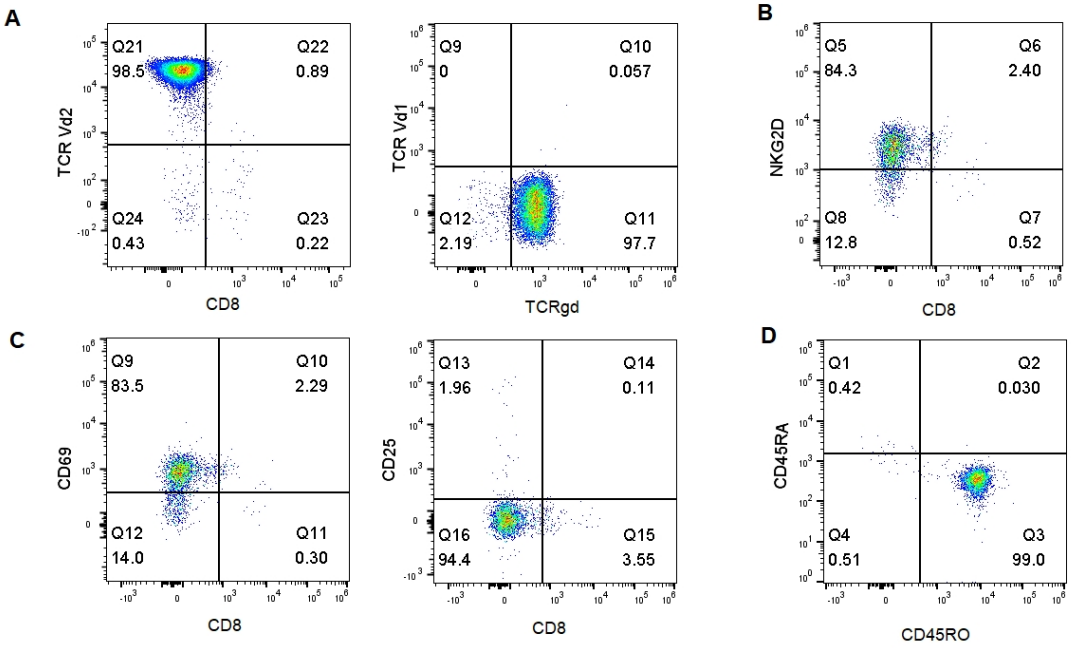


Figure 6.11 Surface expression of cell surface markers on 7-C8 CD1a-autoreactive T cell lines. Flow cytometric analysis of (A) TCR Vδ1, TCR Vδ2, (B) NKG2D, (C) CD69, CD25, (D) CD45RO, CD45RA on CD1a-autoreactive T cell line 7-C8. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using Fluorescence Minus One (FMO).

To further study the phenotype of the 7-C8 cell line, the key surface marker expressions were tested. 7-C8 line positively stained with TCR Vδ2 but not TCR Vδ1, indicating it belongs to Vδ2 γδ T cells, the predominant circulating γδ T cell subset in human blood (Fisher et al., 2014; Wu et al., 2015). It also expressed NKG2D, the costimulatory receptor constitutively expressed by γδ T cells (Rincon-Orozco et al., 2005; Wensveen, Jelenčić, & Polić, 2018). The T cell line expressed CD69 but not CD25 on the cell surface, and it was CD45RA-/CD45RO+, suggesting it belongs to memory cells subset; these characteristics fit the standard

phenotype of cultured T cell lines (Figure 6.11).

6.4 Generation of CD1a-reactive T cell lines from skin

Based on previous studies, CD1a-reactive T cells are able to infiltrate to skin (Jarrett et al., 2016; Preihs, Christian Arambula et al., 2013). The T cell lines we generated were also able to express skin-homing marker CLA. We further sought to generate T cell lines from skin to investigate the skin CD1a-reactive T cell response.

Skin immune cells were isolated from healthy adult skin based on previous study (Salimi et al., 2016), and CD3+ T cells were sorted (undertaken by lab member Yi-ling Chen). Skin CD3+ T cells were expanded by rapid expansion mix. All skin T cell lines generated were further tested for CD1a reactivity and SMase specificity by ELISpot (Figure 6.12). Of all the lines generated, one CD1a-reactive SMase-specific T cell line (SN200818#5) and one CD1a-autoreative T cell line (SN200818#4) strongly responded, which were isolated from donor SN200818.

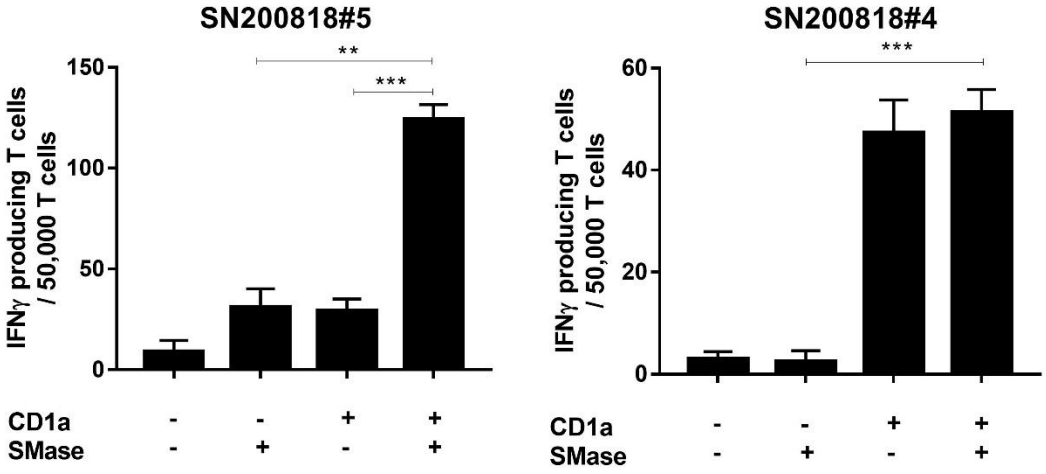


Figure 6.12 Skin CD1a-reactive SMase-specific T cell line SN200818#5 and skin CD1a-autoreactive T cell line SN200818#4 were generated from healthy individual. Skin CD3+ T cell lines sorted and expanded by rapid expansion mix. T cells co-cultured with K562-Ev or K562-CD1a cells pre-treated with or without SMase overnight. IFNγ production was measured by ELISpot. Bars represent standard error, ** P<0.01; ***P<0.001.

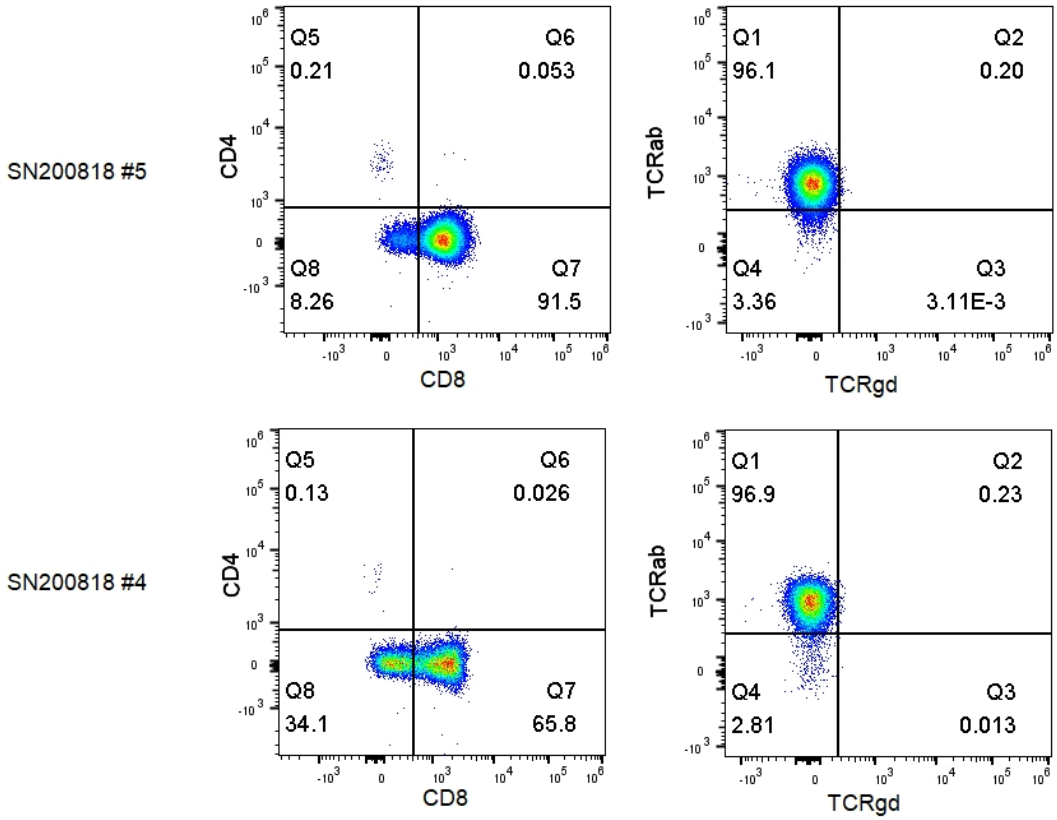


Figure 6.13 Surface expression of cell surface markers on SN200818#5 and SN200818#4 skin CD1a-reactive T cell lines. Flow cytometric analysis of CD4, CD8, αβ TCR and γδ TCR on skin CD1a-reactive T cell lines. Plots gated on singlets, live cells, and CD3+ cells. Gates determined by using Fluorescence Minus One (FMO).

The two selected skin CD1a-reactive T cell lines were stained with key surface markers. The CD1a-reactive SMase-specific T cell line SN200818#5 was a dominant CD8+ αβ T cell line, whereas the CD1a-autoreactive T cell line SN200818#4 was a mixture of CD4+ and CD8+ αβ T cells.

6.5 TCR sequencing of T cell clones

The promising CD1a-reactive SMase-specific and CD1a-autoreactive T cell lines that were sorted as single cells were sent for TCR sequencing. The TCRs were sequenced by David Price from Cardiff University.

7-C8 CD1a-autoreactive clone	TRGV	CDR3
	9	CALWEVSELGKKIKVF
	TRDV	CDR3
	2	CACDPLLGDDKLIF
1-B5 CD1a-reactive SMase-specific clone	TRAV	CDR3
	19	CALSNAGGTSYGKLT
	TRBV	CDR3
	27	CASKISGTSGSNEQF

Figure 6.14 TCR sequence of 7-C8 and 1-B5 T cell lines.

Consistent with previous staining results, 7-C8 CD1a-autoreactive T cell clone belonged to Vγ9/Vδ2 T cell subset, which is the majority of circulating human γδ T cells in most healthy individuals. This T cell subset is able to recognize nonpeptide antigens, and known for its antimicrobial and anti-tumor function, although the mechanism is still unclear (Fisher et al., 2014; Luoma, Castro, & Adams, 2014). The generation of 7-C8 T cell clone and successful TCR sequencing will allow the further study of CD1a and γδ T cells, and the 7-C8 TCR is currently being established in a Jurkat T cell line by lab member Rachel Etherington.

1-B5 CD1a-reactive SMase-specific T cell clone was αβ T cells with TCAV19 and TCBV27. This represents the first TCR sequencing of CD1a-reactive T cells that specifically respond to bacterial SMase. From these and other TCR sequenced, the αβ TCR did not show common sequence features consistent instead with a diverse repertoire, but it will be important to increase the numbers sequenced.

6.6 CD1a as activation marker

The generated T cell lines were co-cultured with K562-Ev and K562-CD1a to test CD1a reactivity and SMase specificity. Unexpectedly, we found the T cell line positively stained with anti-CD1a antibodies after incubation with K562-CD1a.

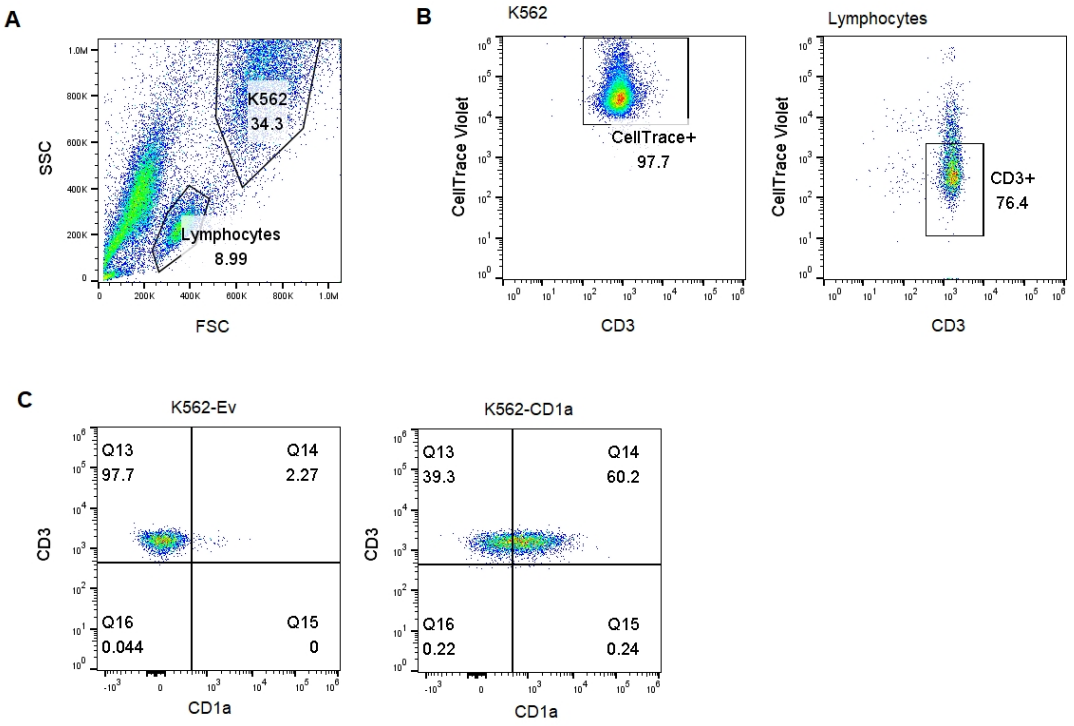


Figure 6.15 7-C8 CD1a-autoreactive T cell line expressed CD1a on surface after co-cultured with K562-CD1a. CD1a-autoreactive T cell line 7-C8 was co-

cultured with K562-Ev or K562-CD1a cells overnight. Flow cytometric analysis of CD1a on 7-C8. (A) The populations of K562 cells and lymphocytes on FACS plot. (B) Flow cytometric analysis of CellTrace Violet on K562 cells, and CD3 on 7-C8. Plots gated on singlets and live cells. (C) Flow cytometric analysis of CD3 and CD1a on 7-C8. Plots gated on singlets, live cells and CD3+ cells. Dara representative for at least three different experiments.

In order to avoid the influence of K562 cells, we labelled the K562 cells with CellTrace Violet dye, and separated T cells (CellTrace-, CD3+) from K562 cells (CellTrace+) (Figure 6.15 A, 6.15B). 7-C8 CD1a-autoreactive T cell line was CD3+ and CD1a- when co-cultured with K562-Ev cells. But a proportion of the autoreative line co-expressed CD1a and CD3 when cultured with K562-CD1a (Figure 6.15C).

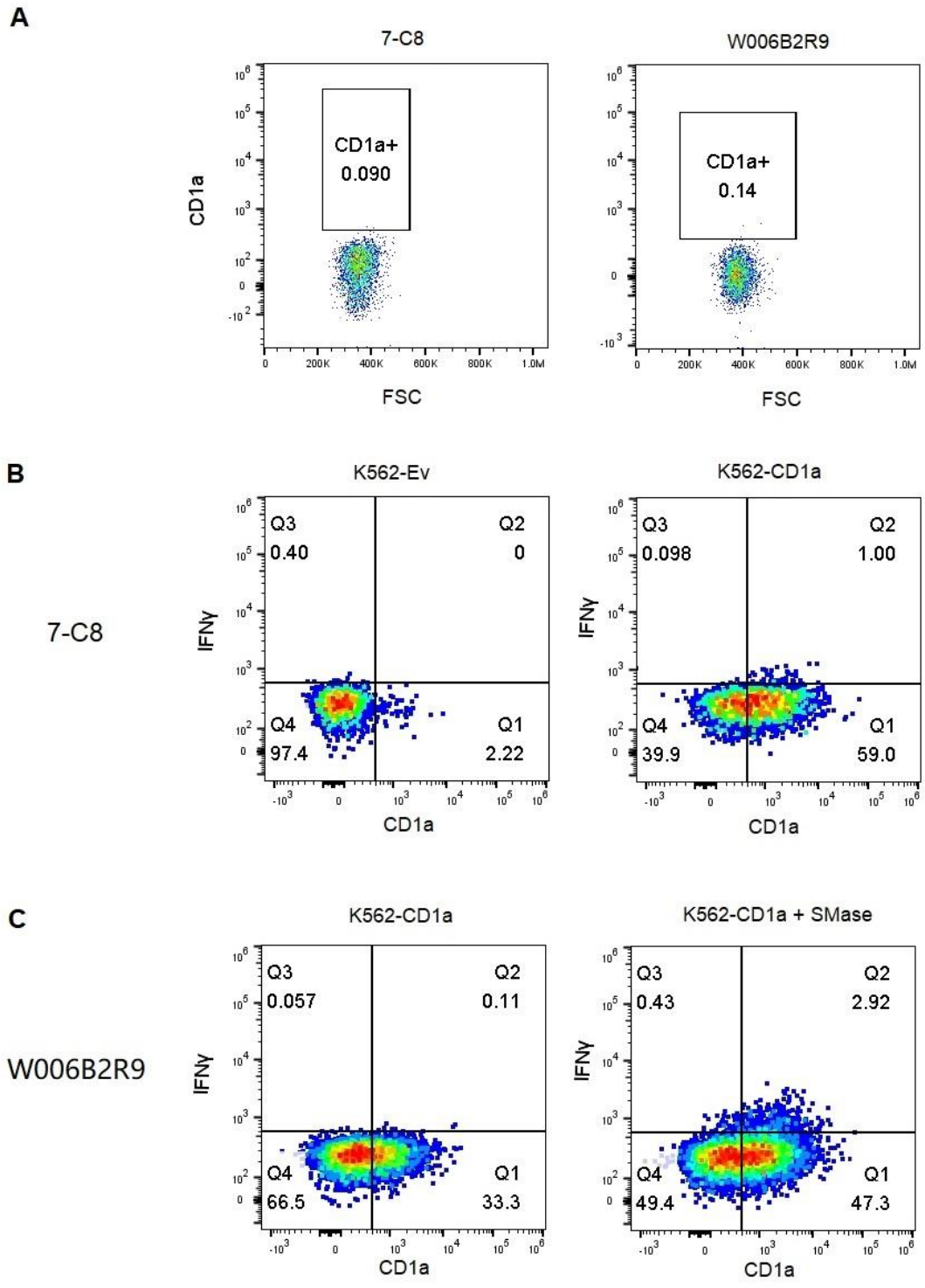


Figure 6.16 CD1a may function as an activation marker. CD1a-autoreactive T cell line 7-C8 was co-cultured with K562-Ev or K562-CD1a cells overnight. CD1a-reactive SMase-specific T cell line W006B2R9 was co-cultured with K562-CD1a cells pre-treated with or without SMase. Flow cytometric analysis of CD1a on T

cells only (A), Flow cytometric of IFNγ production and CD1a expression on T cell line 7-C8 (B) and W006B2R9 (C) after incubation with K562 cells. Plots gated on singlets, live cells and CD3+ cells. Gates determined by using Fluorescence Minus One (FMO).

The CD1a-autoreactive T cell line 7-C8 and CD1a-reactive SMase-specific T cell line W006B2R9 did not express CD1a at static state. (Figure 6.16A)

7-C8 was co-cultured with K562-Ev or K562-CD1a cells overnight. 7-C8 T cells did not express CD1a, and did not produce IFNγ when incubated with K562-Ev. However, the T cell line started to express CD1a after incubation with K672-CD1a cells despite limited IFNγ production in this experiment. (Figure 6.16B)

Similarly, T cell line W006B2R9 was co-cultured with K562-CD1a cells pre-treated with or without SMase. In both conditions, W006B2R9 started to express CD1a when incubated with K562-CD1a cells, and the responding T cells co-expressed IFNγ and CD1a after incubation with K562-CD1a cells pre-treated with SMase. (Figure 6.16C)

Further experiments were necessary to determine whether T cell lines were induced to express CD1a, similar to ILC2 (Hardman et al., 2017), or they received the CD1a through trogocytosis, which involved the exchange of plasma

membrane fragments between antigen presenting cells and lymphocytes (Joly & Hudrisier, 2003). The co-expression of CD1a and IFNγ suggested CD1a may work as a potential activation marker for T cells.

6.7 Tetramer staining with clones

As described in previous chapter, the CD1a-tetramers loaded with various lipids were used to study CD1a-TCR interaction. We used CD1a-tetramers bound with endogenous lipids or LPC, which were produced by NIH and kindly donated by Branch Moody from Harvard, to stain with T cell lines generated by IFNγ secretion assay.

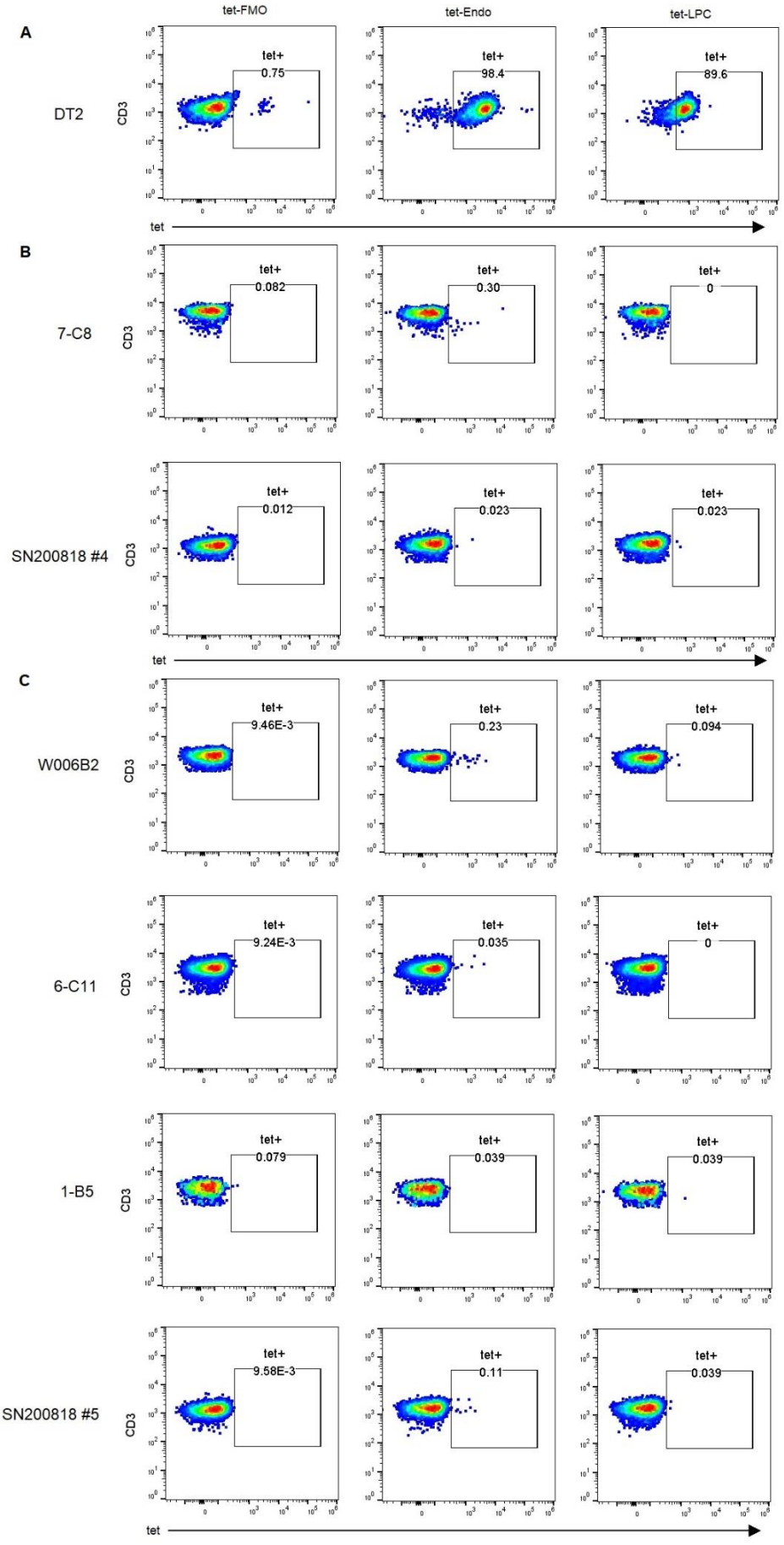

tet-FMO
tet-Endo
tet-LPC
A
DT2
tet+
0.75
tet+
98.4
tet+
89.6
CD3
tet
B
7-C8
tet+
0.082
tet+
0.30
tet+
0
SN200818 #4
tet+
0.012
tet+
0.023
tet+
0.023
C
W006B2
tet+
9.46E-3
tet+
0.23
tet+
0.094
6-C11
tet+
9.24E-3
tet+
0.035
tet+
0
1-B5
tet+
0.079
tet+
0.039
tet+
0.039
SN200818 #5
tet+
9.58E-3
tet+
0.11
tet+
0.039

Figure 6.17 Tetramer staining of CD1a-autoreactive T cell lines and CD1a-reactive SMase-specific T cell lines from blood and skin. The CD1a-reactive T cell lines generated by IFNγ secretion assay were stained with CD1a-endo and CD1a-LPC tetramers. Fluorescence Minus One (FMO) of tetramer was used as negative control. FACS plots were gated on CD3+ cells, live cells and singlets. (A) The CD1a-autoreactive T cell line DT2 as positive control. (B) The CD1a-autoreactive T cell lines. 7-C8 generated from blood, and SN200818#4 generated from skin. (C) The CD1a-reactive SMase-specific T cell lines. W006B2, 6-C11 and 1-B5 generated from blood, and SN200818#5 generated from skin.

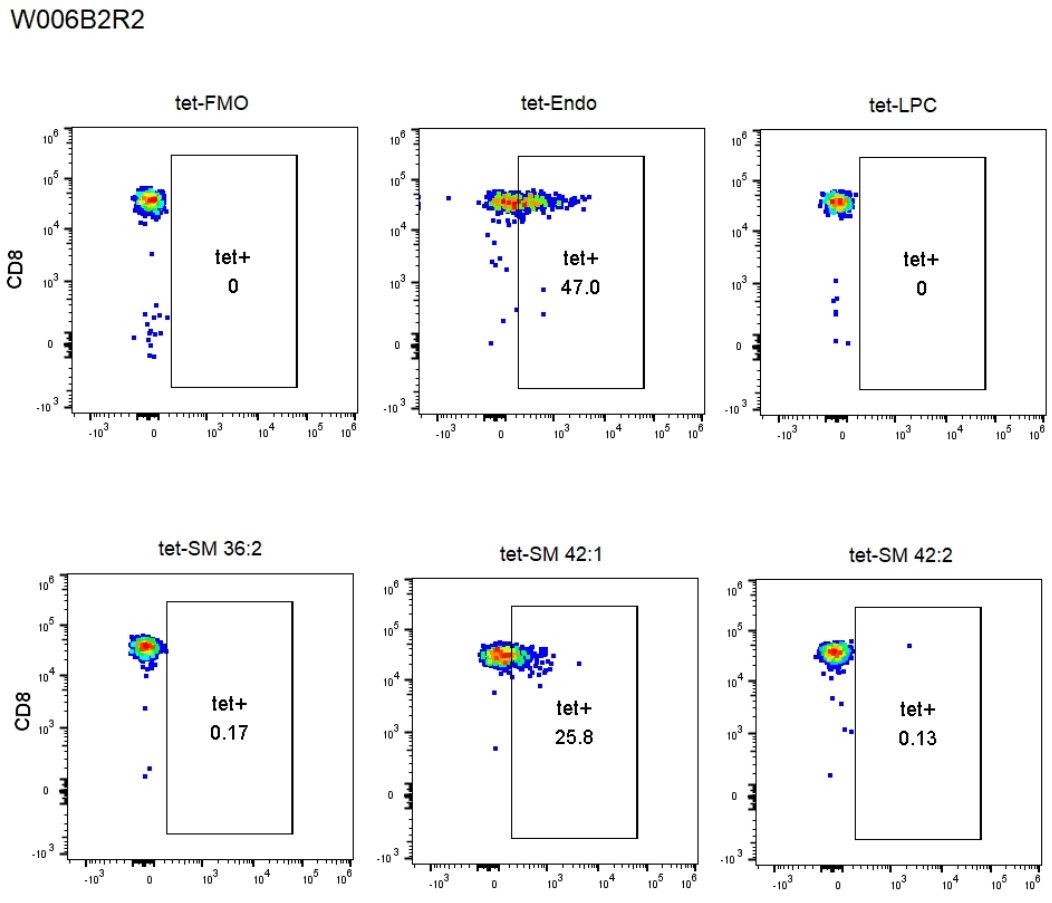


Figure 6.18 Tetramer staining of CD1a-reactive SMase-specific T cell line

W006B2R2. The CD1a-reactive SMase-specific T cell line W006B2R2 generated by IFNγ secretion assay were stained with CD1a-endo tetramer, CD1a-LPC tetramer, and CD1a tetramers with synthetic SMs. Fluorescence Minus One (FMO) of tetramer was used as negative control. FACS plots were gated on CD3+ cells, live cells and singlets.

The tetramer staining process was first tested using a CD1a-autoreactive T cell line DT2, which was kindly donated by Rachel Cotton and Branch Moody (Harvard). DT2 was sorted based on CD1a-LPC tetramer and could be stained with CD1a-endo tetramer. The T cell line was used as positive control for tetramer staining (Figure 6.17A).

Two CD1a-autoreactive T cell lines, 7-C8 generated from blood and SN200818#4 generated from skin, were tested by CD1a-endo and CD1a-LPC tetramers, and none of the lines positively stained with tetramers (Figure 6.17B). Similarly, four CD1a-reactive SMase-specific T cell lines, W006B2, 6-C11, 1-B5 generated from blood and SN200818#5 generated from skin, were tested with tetramers. None of them stained with CD1a-endo or CD1a-LPC tetramers (Figure 6.17C).

However, the CD1a-reactive SMase-specific T cell line W006B2R2, which was re-sorted from line W006B2, was 47% stained with CD1a-endo tetramer, and 26% stained with CD1a-SM42:1 tetramer (Figure 6.18). As in cytokine secretion assay,

W006B2R2 showed a low level CD1a-autoreactive response, which was much lower compared to T cell response against K562-CD1a pretreated with SMase (Figure 6.6). The weak CD1a-autoreactive response of W006B2R2 may explain its partial positive staining of CD1a-endo tetramer.

CD1a tetramer staining was dependent on the structure of the added ligands and was specific for TCRs (Kasmar et al., 2014). The antigens recognized by each T cell line generated by IFNγ secretion assay were still unknown, and lipid profiles of CD1a endogenous ligands were varied. The affinity of TCRs on each T cell line for CD1a proteins with endogenous lipids or LPC could be too weak to detect, which may explain the negative tetramer staining of T cell lines. Also, for some of the CD1a-reactive SMase-specific T cell lines, such as 6-C11 and W006B2, their CD1a-autoreactive responses to K562-CD1a cells were close to baseline. Thus, it is reasonable that these T cell lines did not positively stain with CD1a-endo tetramer.

Discussion

The lack of readily detectable surface markers for CD1a-reactive T cells limits the further study of their phenotypes. Besides the generation of CD1a-tetramer positive T cell lines, like DT2 or DS32 from Harvard, the IFNγ secretion assay provides an efficient tool to generate CD1a-reactive T cell lines based on their immune functions. It helps us further investigate their phenotypes and characters,

and increase our understanding of the physiological functions of CD1a-reactive T cells. Similarly, the cytokine secretion assay could be used to generate CD1a-reactive T cell lines that producing IL-22, IL-17A or IL-13, too.

S. aureus SMase was proved to induce CD1a-reactive SMase-specific T cell response, and the responding T cell lines and clones were isolated from blood of different healthy individuals. The T cell lines identified were αβ and γδ T cells, indicating both of the T cell subsets may contribute to the CD1a-reactive SMase-specific response. TCR of the 1-B5 T cell clone was sequenced, which belonged to αβ T cells. Interestingly, both of CD4+ and CD8+ T cells in polyclonal T cells showed CD1a-reactive SMase-specific response, as described in previous chapters, and CD4+, CD8+ and DN cells were all sorted for T cell line generation. However, the 1-B5 clone, 6-C11 line, and W006B2 re-sorted lines (W006B2R2, W006B2R7, W006B2R9) were all CD8+ cells. This potential bias may result from selected expansion by rapid expansion method, and it will be important to generate T cell lines using different methods. The CD1a-reactive SMase-specific T cell lines were able to express CLA on the surface after expansion, indicating their ability to migrate to skin. CD1a-reactive SMase-specific T cell lines were also found to respond to primary CD1a+ APCs with the present of anti-MHC antibodies. These T cell lines could be further expanded or sorted for future experiments, and their CD1a-reactive SMase-specific response remained consistently.

CD1a-autoreactive lines were also generated by IFNγ secretion assay from PBMC of different healthy individuals. Most of CD1a-autoreactive lines were predominately γδ T cells, suggesting the role of γδ T cells in CD1a autoimmunity. These T cell lines were able to express skin-homing marker CLA on the surface too. In human there are mainly two types of γδ T cells, named based on their TCR variable segments. The Vδ1 γδ T cells are abundant at skin and large intestine, while Vδ2 γδ T cells are mainly resident at blood. (Chien et al., 2014; Deusch et al., 1991; Ebert et al., 2006; Toulon et al., 2009) Human Vδ1 T cells are able to recognize lipid antigens presented by CD1a, CD1c and CD1d, while the CD1-reactivity of Vδ2 T cells remains unclear. The most well-studied antigens of human Vδ2 T cells are the phosphoantigens, including isopentenyl pyrophosphate (IPP) from mammalian cells and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) from bacteria (Hintz et al., 2001), but the recognition mechanisms are still unknown.

We successfully isolated a CD1a-autoreactive Vδ2 γδ T cell clone 7-C8, TCR of which was sequenced. 7-C8 belonged to the Vγ9/Vδ2 T cell subset, and was a CD45RA-/CD45RO+ memory T cell clone. It expressed skin-homing marker CLA, and also expressed NKG2D, the costimulatory receptor constitutively expressed by γδ T cells (Rincon-Orozco et al., 2005; Wensveen et al., 2018). 7-C8 remained a stable CD1a-autoreactive T cell response after multiple rounds of expansion,

which could be abrogated by anti-CD1a antibody, demonstrating its CD1a dependency. CD1a-autoreactive T cell lines identified previously were predominantly αβ T cells, while one Vδ1 γδ T cell line was found to be able to recognize CD1a as well (Agea et al., 2005; Porcelli et al., 1989; Preihs, Christian Arambula et al., 2013). It is of interest to generate a novel Vδ2 γδ T cell clone from blood that shows CD1a reactivity, which will extend our understanding of human CD1a-reactive T cells.

Besides CD1a-reactive SMase-specific T cells identified from blood, the skin T cell lines generated from healthy donor were proved to respond to SMase through CD1a. One skin CD1a-reactive SMase-specific T cell line and one CD1a-autoreactive T cell line were generated from healthy skin. Both of the lines were predominately αβ T cells. The skin CD1a-reactive SMase-specific line was mainly CD8+ cells, while the CD1a-autoreactive line was a mixture of CD4+ and CD8+ cells.

During the study of 7-C8 and W006B2R9 lines, T cells did not express CD1a at the steady state, but were found to express CD1a on the surface after incubation with K562-CD1a cells. The IFNγ producing T cells were found co-expressing CD1a, suggesting CD1a might be a potential activation marker upon stimulation. During the life-history of T cells in the thymus, the up-regulation of CD1a on cell surface at early stage marks the commitment of thymocytes to T cell lineage. At later stage,

only CD1a+ CD3+ thymocytes proliferate upon stimulation. At the final stage, the functionally mature immunocompetent cells start to down-regulate CD1a. CD1a is proved to be expressed on mature human thymocytes but not peripheral T cells. And CD1a expression on DCs and blood-derived ILC2 is known to be downregulated by cardiolipin and lysophosphatidic acid within human serum. We hypothesis that T cells are potentially able to express CD1a upon stimulation, which may be inhibited by serum lipids. While in the secretion assay, the removal of human serum and replacement of fetal calf serum (FCS) release the inhibition of CD1a expression. Further investigation is needed to test this hypothesis. (Hardman et al., 2017; Leslie et al., 2008; Res & Spits, 1999; Sotzik et al., 1993) Also, it is still unclear whether T cells were induced to express CD1a or received the molecule through trogocytosis, which is worth taking into consideration.

Two CD1a-autoreactive T cell lines and five CD1a-reactive SMase-specific T cell lines were tested with CD1a-endo and CD1a-LPC tetramers, and one of them stained with CD1a tetramers. Based on previous study of CD1a-reactive DDM-specific CD8-2 T cell line, CD1a tetramer staining was dependent on the added ligands and was specific for TCR. CD1a-reactive T cell lines with specific ligands may not be stained with general CD1a-endo tetramer (Kasmar et al., 2014). As lipids profile of CD1a endogenous ligands is varied, and the specific antigens of the functional CD1a-reactive T cell lines we generated were still unclear, it is reasonable that the other T cell lines did not stain with CD1a-endo or CD1a-LPC

tetramers. CD1a-tetramers loaded with different lipids, for example ceramides, may be tested in future experiments.

In summary, the generation of CD1a-reactive SMase-specific and CD1a-autoreactive T cell lines and clones provided efficient tools for further studying their phenotypes and physiological functions. The finding of Vδ2 γδT cell clone also extends our understanding of CD1a-autoreactive T cells. Besides circulating T cells, the CD1a-reactive SMase-specific skin T cell line demonstrated the presence of these T cells in skin, indicating they may contribute to immune response at cutaneous sites. *Staphylococcus aureus* is one of the commensal bacteria of humans, and has been proved to associate with skin inflammatory disease atopic dermatitis. Psoriasis patients were also proved to have higher risk of *S. aureus* colonization than healthy individuals. (K. Iwamoto, Moriwaki, Miyake, & Hide, 2019; Ng et al., 2017) SMase is one of the virulence factors produced by the bacteria. (Hayashida et al., 2009) We are interested to investigate whether CD1a-reactive SMase-specific T cells play a role in the pathogenesis of skin inflammatory diseases including atopic dermatitis and psoriasis, which will be discussed in next chapter.

7. Result IV: Identification of CD1a-reactive SMase-specific T cells in patients with skin diseases

Introduction and aims

In previous chapters we identified CD1a-reactive SMase-specific T cells in healthy individuals, which were present in most of donors, and were able to produce IFNγ, IL-22 and IL-17A in response to bacterial SMase challenges. These cytokines contribute to bacterial clearance and keratinocyte proliferation (J. S. Cho et al., 2010; Fujita, 2013; Shtrichman & Samuel, 2001). The CD1a-reactive SMase-specific T cell lines and clones were isolated from healthy PBMCs, and a proportion expressed the skin-homing marker CLA on the surface. CD1a-reactive SMase-specific T cell lines were also generated from skin CD3+ T cells. These data indicate the CD1a-reactive SMase-specific T cells are commonly present in humans and may contribute to skin immunity. We sought to investigate the role of these T cells in the skin diseases atopic dermatitis (AD) and psoriasis (PS).

AD, also called atopic eczema, is one of the most common chronic inflammatory skin diseases, and its prevalence is increasing in Europe, Asia and Africa (Deckers et al., 2012). It is characterized by intense itch and eczematous lesions, the pathogenesis of which is complex and not fully understood. Both genetic and environmental factors contribute to the disease, including the epidermal barrier disruption, T cell subset activation, IgE-mediated reactivities, and imbalance of

skin microbiota. AD is also strongly associated with food allergy and other atopic diseases including asthma and allergic rhinitis, but the underlying mechanism is still unclear. (Spergel & Paller, 2003; Weidinger et al., 2018).

AD shows distinct acute and chronic phases, and is associated with various T cell subsets. The Th2 and Th22 cells are predominant in acute phase, expressing type-2 cytokines (IL-13, IL-4 and IL-31) and IL-22. While in chronic phase, the Th1 cells are progressively activated as well as Th2 and Th22, together with a modest activation of Th17 cells. (Gittler et al., 2012; Thepen et al., 1996) CD4+ T cells are present in AD lesional skin and are considered to contribute to inflammation; in addition, CD8+ T cells can also be observed in patient skin, which represent an important source of IFNγ, IL-13 and IL-22. (Hijnen et al., 2013)

An altered surface microbiota colonization is associated with AD, usually with dominant *Staphylococcus aureus* colonization and reduced bacterial diversity (Kong et al., 2012). In this study, *S. aureus* prevalence varied from 30% to nearly 100% in AD patients, compared to 20% in healthy individuals; the prevalence of *S. aureus* colonization in patients was 70% for lesional skin, 39% for non-lesional skin and 62% for the nose. The density of the bacteria is associated with severity of AD. (Tauber et al., 2016; Totté, van der Feltz, Hennekam, et al., 2016) *S. aureus* secretes a variety of virulence factors that contribute to the disease, including superantigens, hemolysin-α, δ-toxin and protein A, and more virulence factors

are still being discovered. (Williams & Gallo, 2015) SMase is found to be strongly secreted from the bacterial flora of AD patients (Ohnishi et al., 1999), which cleaves SM into ceramide and phosphocholine. The role of SMase in AD pathogenesis is still unknown.

Studies of ceramide profiles in AD patients reported the close relationship of human stratum corneum (SC) ceramides and SC function. Although total ceramide content (including 11 species) is downregulated in AD, short-chain ceramides are abundant in AD patients compared to healthy individuals, especially C34 ceramide species. Strong positive correlation with SC trans-epidermal water loss and negative correlation with water-holding function were found with the expression levels of ceramides with non-hydroxy fatty acid and sphingosine backbone. (Ishikawa et al., 2010; Janssens et al., 2012)

Psoriasis is a chronic, immune-mediated skin disorder with an estimated prevalence in adults at approximately 0.5%, and in children from 0% to 1.37%. Psoriasis patients are more likely to develop cardiovascular disorders, diabetes, metabolic syndrome and depression, and have a reduced quality of life. (Michalek et al., 2017) The marked thickening of epidermis is one of the defining histological appearances of psoriasis, which is caused by increasing proliferation of keratinocytes and elongated epidermal rete. (Lowes et al., 2007)

Psoriasis is thought to be initially triggered by pathological inflammation and followed by amplification feedback loops between keratinocytes and immune cells. Th17 cells are considered as central responding T cells in PS, as well as the involvement of Th1 and Th22 cells. (Greb et al., 2016; Lowes et al., 2007) High percentages of CD8+ T cells are present in PS epidermal skin, which are also able to produce cytokines IL-17A/F and IL-22 (Hijnen et al., 2013).

The prevalence of *S. aureus* colonization in patients with psoriasis is higher at lesional skin compared to non-lesional skin; *S. aureus* has been shown to perpetuate keratinocyte damage, activate Th1 and Th17 cells, inducing the production of TNFα and IFNγ, and associate with exacerbation of disease. (Balci et al., 2009; Ng et al., 2017; Totté, van der Feltz, Bode, et al., 2016)

The key aims of this chapter were to investigate CD1a-reactive SMase-specific T cells in the peripheral blood of AD and PS patients. ELISpot, cytokine secretion assays and real-time PCR were used to study their functional phenotype. SMs were studied by CD1a tetramers loaded with various synthetic lipids, kindly donated by Branch Moody from Harvard. Ceramides were studied by cytokine secretion assays as functional readouts.

7.1 Information of patients and donors

Blood of AD and PS patients and healthy individuals were collected from clinic

and processed in the lab. Polyclonal T cells were isolated for experiments. The information of patients and donors were shown.

Donor	Gender	Age	Clinical Status
AD1866	F	28	Severe AD
AD1995	M	51	Mild AD
AD2146	F	57	Mild AD
AD2148	F	36	Mild AD
AD2209	F	62	Severe AD
AD2341	F	25	Mild AD
AD2342	M	46	Moderate AD
AD2344	F	23	Moderate AD
AD2346	M	36	Moderate AD
AD2436	F	22	Mild AD
AD2499	N/A	51	Mild AD

Donor	Gender	Age	Clinical Status
PS2004	F	32	Mild PS
PS2005	M	64	Moderate PS
PS2083	F	56	Severe PS
PS2092	F	60	Moderate PS
PS2579	M	61	Mild PS

PS2352	M	65	Severe PS

Donor	Gender	Age	Clinical Status
C2147	F	39	HC
C2151	F	35	HC
C2565	M	42	HC
W006	F	32	HC
W015	M	35	HC
W016	F	33	HC
W024	M	36	HC
W032	F	40	HC

Figure 7.1 Patients and donors list. The severity of AD and PS patients is based on Investigator's Global Assessment (IGA) scale (Eichenfield et al., 2002; Langley, Feldman, Nyirady, van de Kerkhof, & Papavassilis, 2015). Abbreviations: AD, atopic dermatitis; PS, psoriasis; HC, healthy control; F, female; M, male; N/A, not available.

7.2 CD1a-tetramer staining in AD patients

In previous chapters we used CD1a tetramers loaded with various lipids to analyze the percentage of T cells that were able to bind with CD1a protein. This method demonstrated the existence of CD1a-reactive T cells in humans *ex vivo* (Kasmar et al., 2014). We further analyzed the CD1a-reactive T cells in AD patients by CD1a-tetramers, and compared with healthy individuals. In previous studies,

CD1a autoreactive T cells in blood of AD patients were detected by IFNγ and IL-13 ELISpot, the percentage of which was similar with healthy individuals (Jarrett et al., 2016). While CD1a-autoreactive T cells were proved to produce other cytokines such as IL-22 and IL-17A (J. H. Kim et al., 2016; Preihs, Christian Arambula et al., 2013), ELISpot that detects single/limited cytokines may not reflect the whole population of CD1a-autoreactive T cells. CD1a-tetramers would potentially capture all antigen-binding cells regardless of cytokine production capacity.

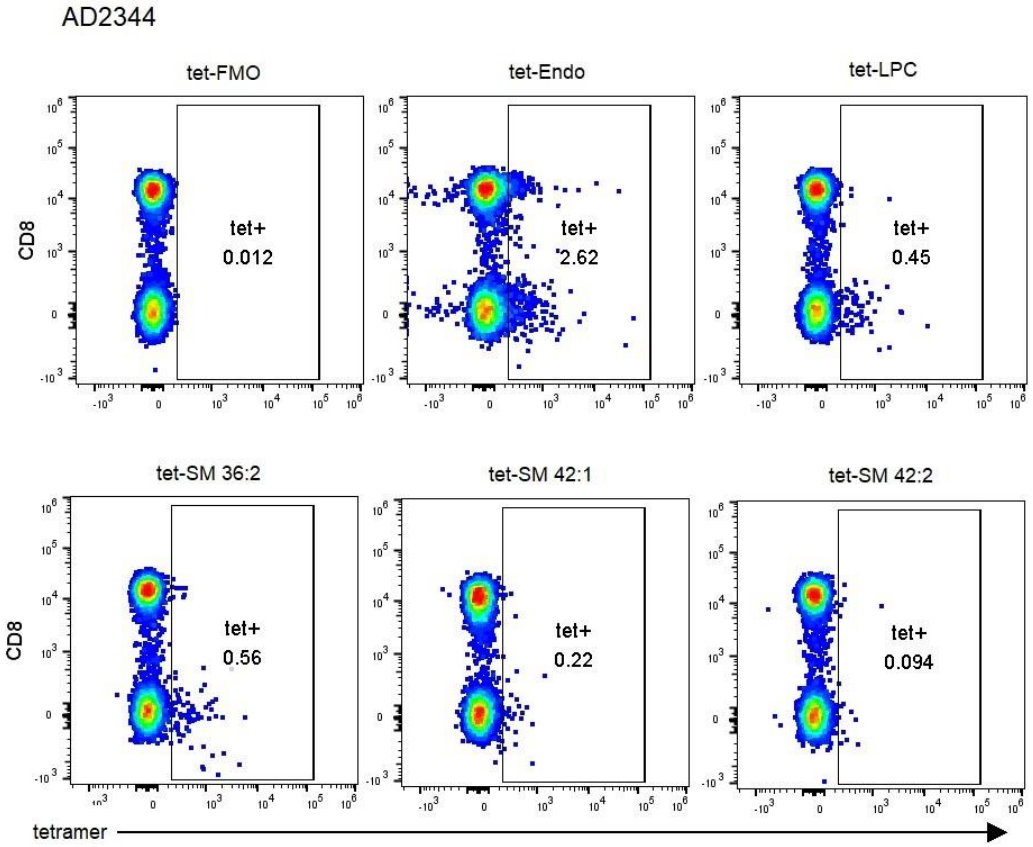
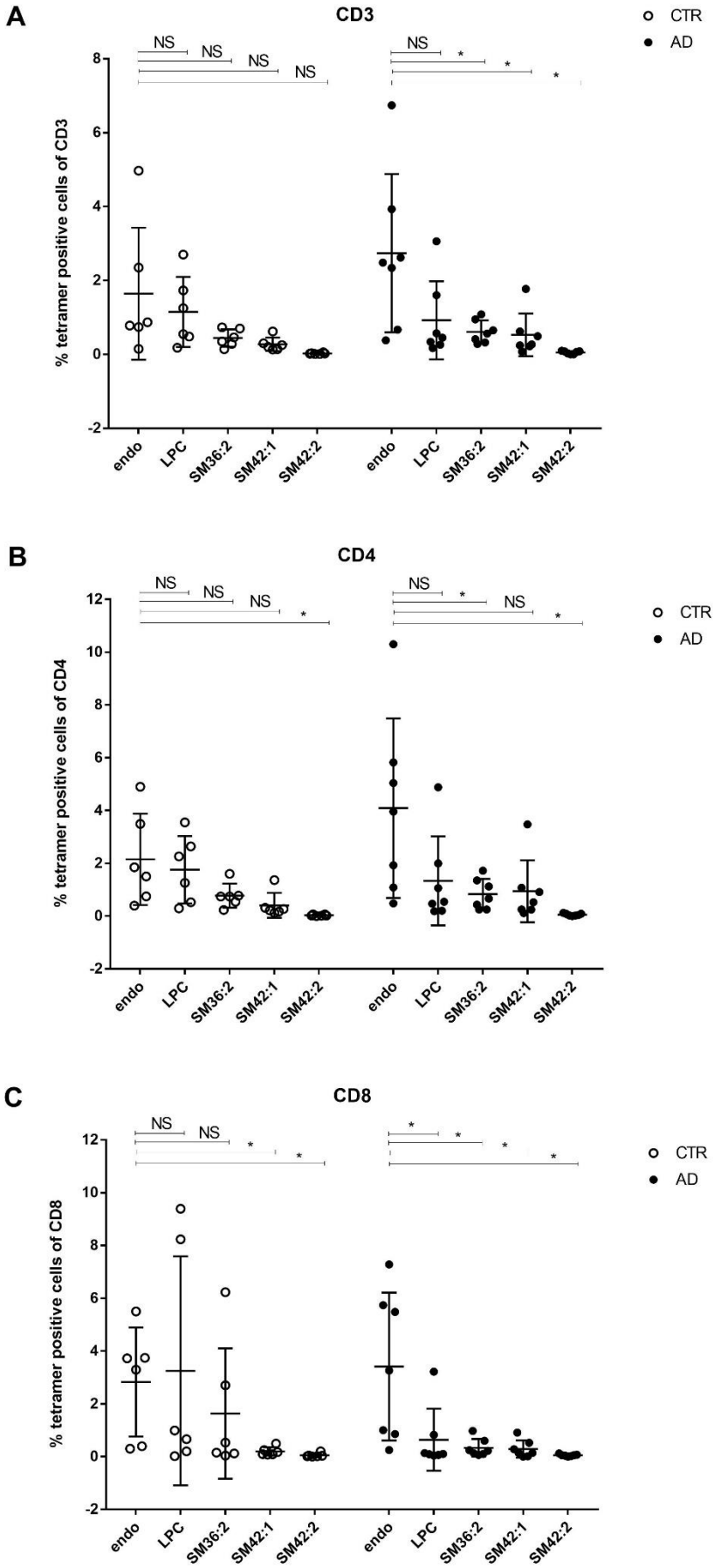


Figure 7.2 Tetramer staining of polyclonal T cells from AD patients. Polyclonal T cells were isolated from AD patient PBMCs (donor AD2344) by CD3+ MACS

separation. T cells were stained with CD1a-tetramers and other surface markers. FMO of tetramer was used as negative control. Data representative of at least three donors from different experiments. FACS plots were gated on CD3+, live cells and singlets.

We used CD1a-endo tetramer as well as CD1a tetramer loaded with SM 36:2, SM 42:1, SM 42:2 and LPC, which were kindly donated by Branch Moody (Harvard), to investigate the percentage of tetramer-positive T cells in PBMCs. Polyclonal T cells isolated from AD patient PBMCs were stained with CD1a tetramers as well as other surface markers. CD1a tetramer-positive cells were found in both CD4+ and CD8+ positive T cell subsets. In this example, the percentage of CD1a-endo and CD1a-LPC tetramer positive cells were approximately 2.5% of total T cells. The percentage of CD1a-SM36:2 tetramer positive cells were similar to CD1a-LPC, while the percentage of the other two CD1a-SM tetramers positive cells were decreased, only 0.2% for SM 42:1, and less than 0.1% for SM 42:2 (Figure 7.2).


A
CD3
CTR
AD
NS
NS
NS
NS
NS
*
*
*
% tetramer positive cells of CD3
8
6
4
2
0
-2
endo
LPC
SM36:2
SM42:1
SM42:2
endo
LPC
SM36:2
SM42:1
SM42:2
B
CD4
CTR
AD
NS
NS
NS
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NS
*
NS
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% tetramer positive cells of CD4
12
10
8
6
4
2
0
-2
endo
LPC
SM36:2
SM42:1
SM42:2
endo
LPC
SM36:2
SM42:1
SM42:2
C
CD8
CTR
AD
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% tetramer positive cells of CD8
12
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6
4
2
0
-2
endo
LPC
SM36:2
SM42:1
SM42:2
endo
LPC
SM36:2
SM42:1
SM42:2

Figure 7.3 Percentage of tetramer-positive T cells in CD3+, CD4+ and CD8+ populations in healthy individuals and AD patients. The polyclonal T cells were isolated from healthy donors (n=6) and AD patients (n=7). T cells were stained with CD1a-tetramers and other surface markers. Percentage of tetramer-positive T cells in CD3+ (A), CD4+ (B) and CD8+ (C) populations are shown. Bars represent standard error. * $P<0.05$.

The percentage of tetramer-positive T cells was tested in CD3+, CD4+ and CD8+ populations in 7 AD patients and 6 healthy individuals, and the patterns of tetramer staining were similar. Polyclonal T cells had higher staining with CD1a-endo tetramer, and reduced staining with CD1a-SM tetramers (Figure 7.3).

The range of CD1a-endo tetramer positive cells in AD patients varied from 0.3 to 7% in total CD3+ T cells and CD8+ cells, and 0.5 to 10% in CD4+ cells, which were slightly higher than healthy donors, but were not significantly different.

In AD patients, the percentages of CD1a-LPC tetramer positive cells were similar compared to CD1a-endo tetramer-positive cells in CD3+ and CD4+ populations; but in CD8+ cells, the percentage of CD1a-LPC tetramer was significantly lower than CD1a-endo, which was not observed in healthy individuals.

The range of three CD1a-SM tetramer positive cells was lower than CD1a-endo

tetramer-positive cells in AD patients. For SM 36:2, the tetramer-positive cells were approximately 0.6% in CD3+ cells, 0.8% in CD4+ cells, and 0.3% in CD8+ cells. For SM 42:1, the percentage of tetramer positive cells was much reduced in CD3+ and CD8+ cells, but was not significantly different to CD1a-endo tetramer-positive cells in CD4+ populations. For SM 42:2, the tetramer-positive cells were lower than 0.1% in all three populations. In summary, the SM 42:2 significantly reduced the CD1a-tetramer binding in both healthy individuals and AD patients, while healthy donors and AD patients showed different patterns for SM 42:1 and SM 36:2.

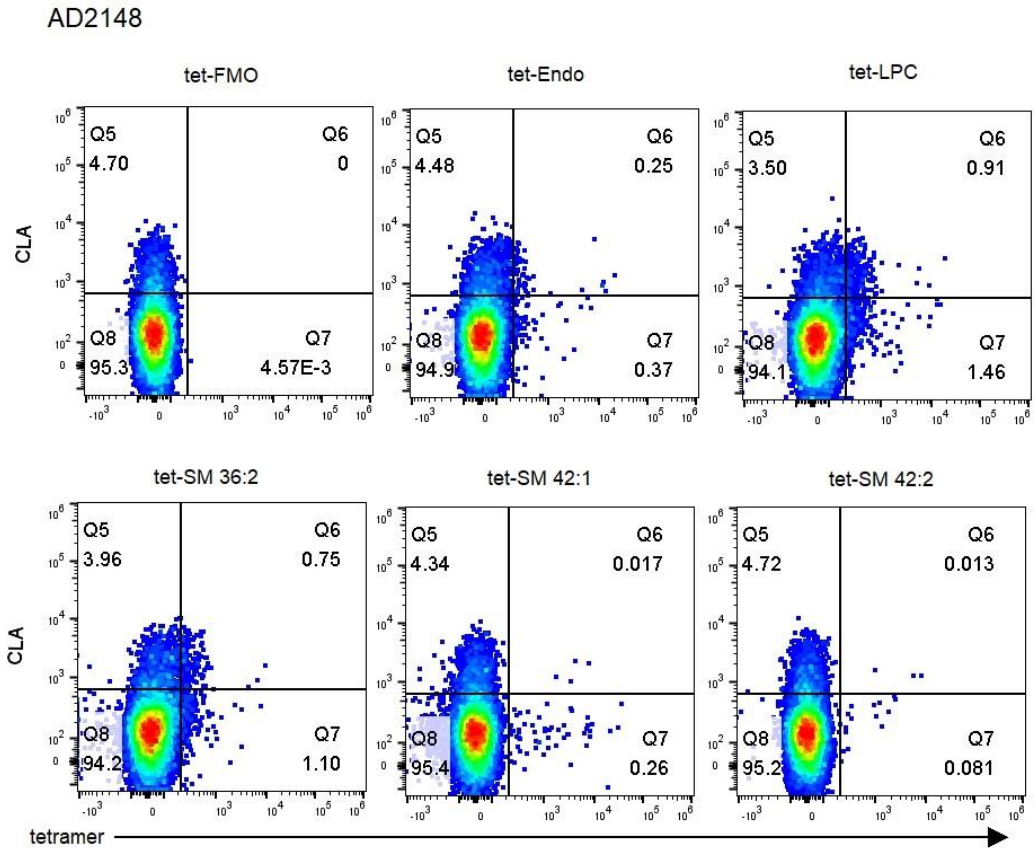

Figure 7.4 CLA expression on tetramer positive cells. Polyclonal T cells were

isolated from AD patients PBMCs (donor AD2148) by CD3+ MACS separation. T cells were stained with CD1a-tetramers and CLA. FMO of tetramer was used as negative control. Data representative of at least three donors from different experiments. FACS plots were gated on CD3+, live cells and singlets.

Polyclonal T cells from AD patients were stained with CD1a-tetramers and CLA. A proportion of all tetramer positive T cells expressed CLA on the surface, indicating they may have the capacity to migrate to skin, similar to healthy individuals. (Figure 7.4)

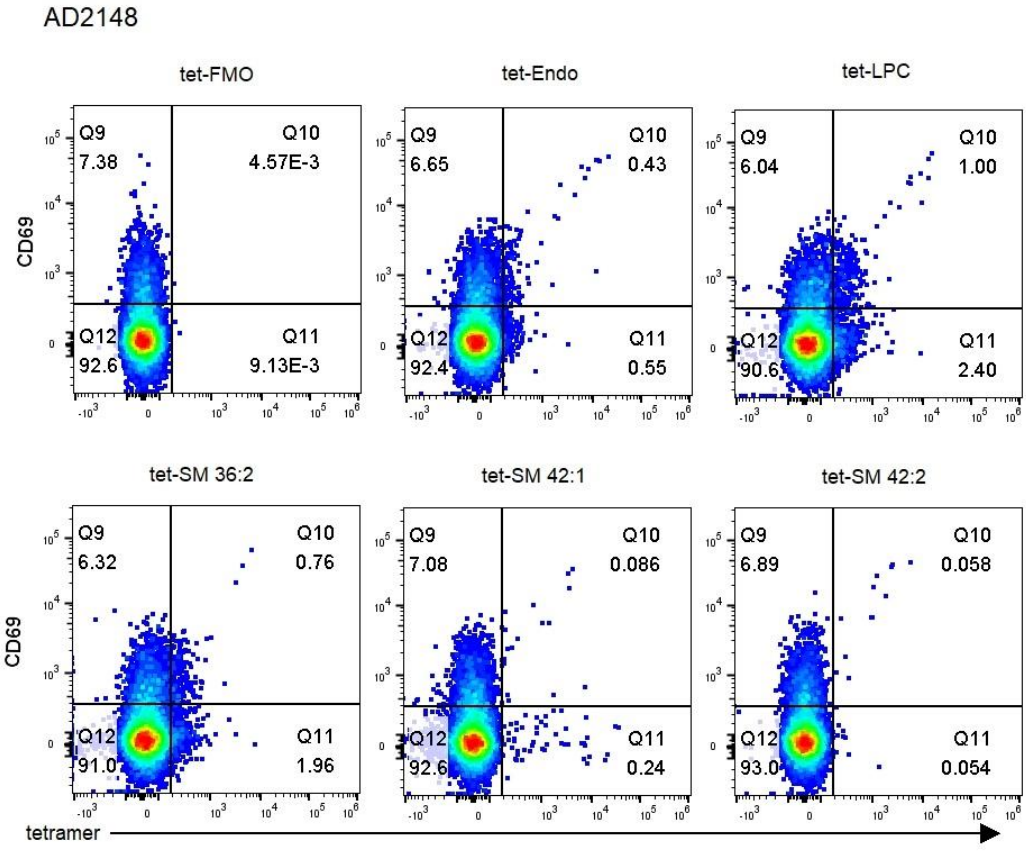

Figure 7.5 CD69 expression on tetramer-positive cells. Polyclonal T cells were

isolated from AD patients PBMCs (donor AD2148) by CD3+ MACS separation. T cells were stained with CD1a-tetramers and anti-CD69. FMO of tetramer was used as negative control. Data representative of at least three donors from different experiments. FACS plots were gated on CD3+, live cells and singlets.

Polyclonal T cells from AD patients were stained with CD1a-tetramers and anti-CD69. Similar to the findings in healthy individuals, a proportion of tetramer positive cells expressed CD69 on the surface, indicating their activation status. (Figure 7.5)

7.3 Investigation of CD1a-reactive SMase-specific T cells in AD patients

In previous chapters, we have demonstrated the existence of CD1a-reactive SMase-specific T cells in healthy PBMCs. As *Staphylococcus aureus* is one of the commensal bacteria of humans, and has been proved to associate with AD (K. Iwamoto et al., 2019), we sought to investigate the CD1a-reactive SMase-specific T cells in AD patients.

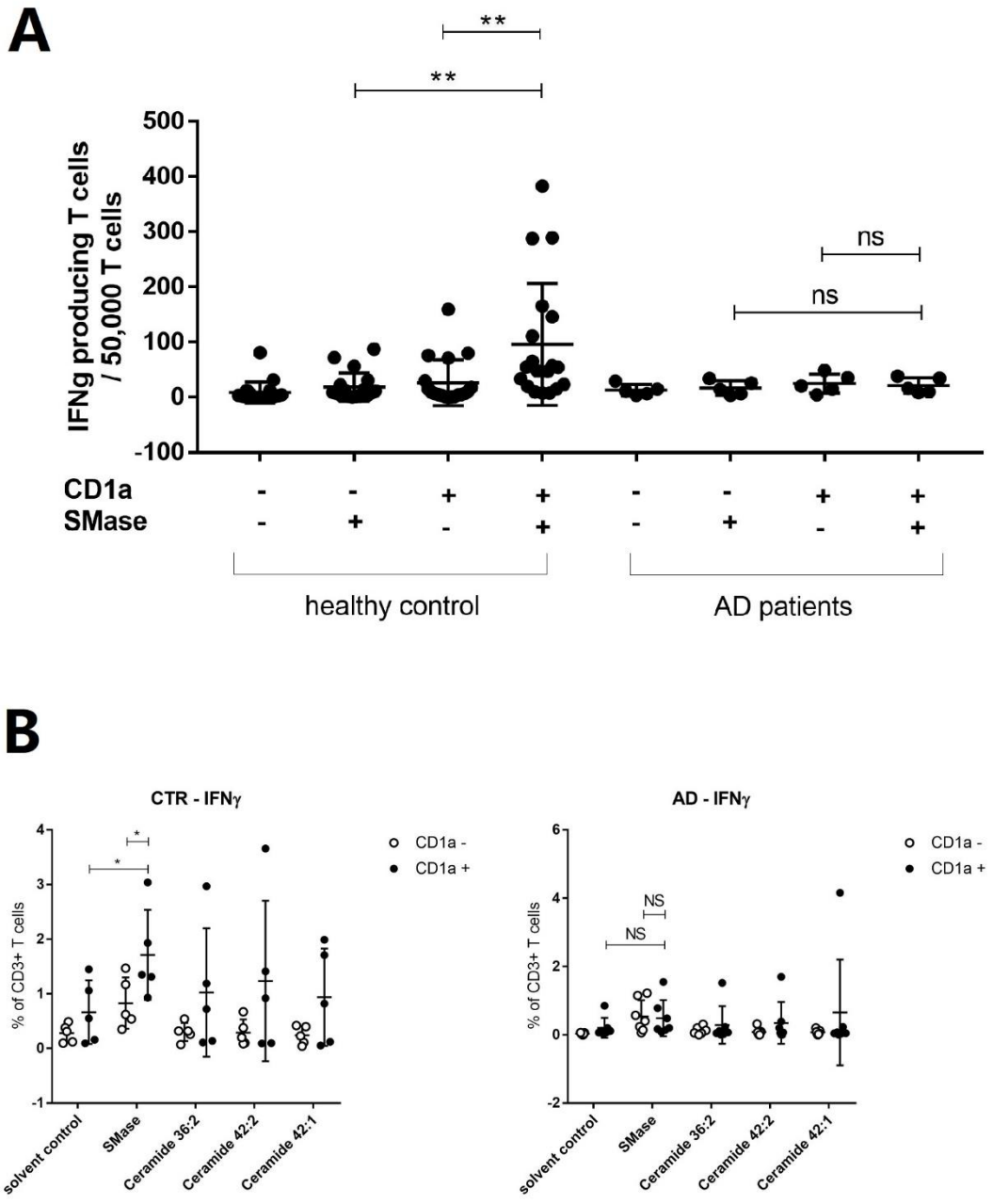

Figure 7.6 IFNγ-producing CD1a-reactive SMase-specific T cells were not detected in AD patients. (A) T cells were isolated by CD3 MACS beads from healthy donor (n=19) and AD patients (n=5) PBMCs. T cells incubated overnight with K562-CD1a or K562-Ev cells pre-treated with or without *S. aureus* SMase. IFNγ production was tested by ELISpot and presented. (B) Polyclonal T cells

isolated from healthy (n=5) and AD patients (n=7) PBMCs cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. IFNγ producing T cells were tested by IFNγ secretion assay. Bars represent standard error. * P<0.05, ** P<0.01.

The IFNγ production by CD1a-reactive SMase-specific T cells was first tested in 5 AD patients by ELISpot (Figure 7.6A). The percentage of IFNγ producing CD1a-autoreactive T cells is known to be lower than 0.05% (Jarrett et al., 2016), and a low level of CD1a-autoreactive response was also detected here. No IFNγ producing T cell response against K562-CD1a pre-treated with SMase was observed, which was different from the results in healthy individuals.

The IFNγ-producing CD1a-reactive T cells were further tested by cytokine secretion assay in 7 AD patients (Figure 7.6B), and the results were consistent with ELISpot. In AD patients, the CD1a-reactive SMase-specific T cell response was not significantly different to the CD1a-autoreactive response, while healthy individuals showed significant IFNγ upregulation to SMase through CD1a.

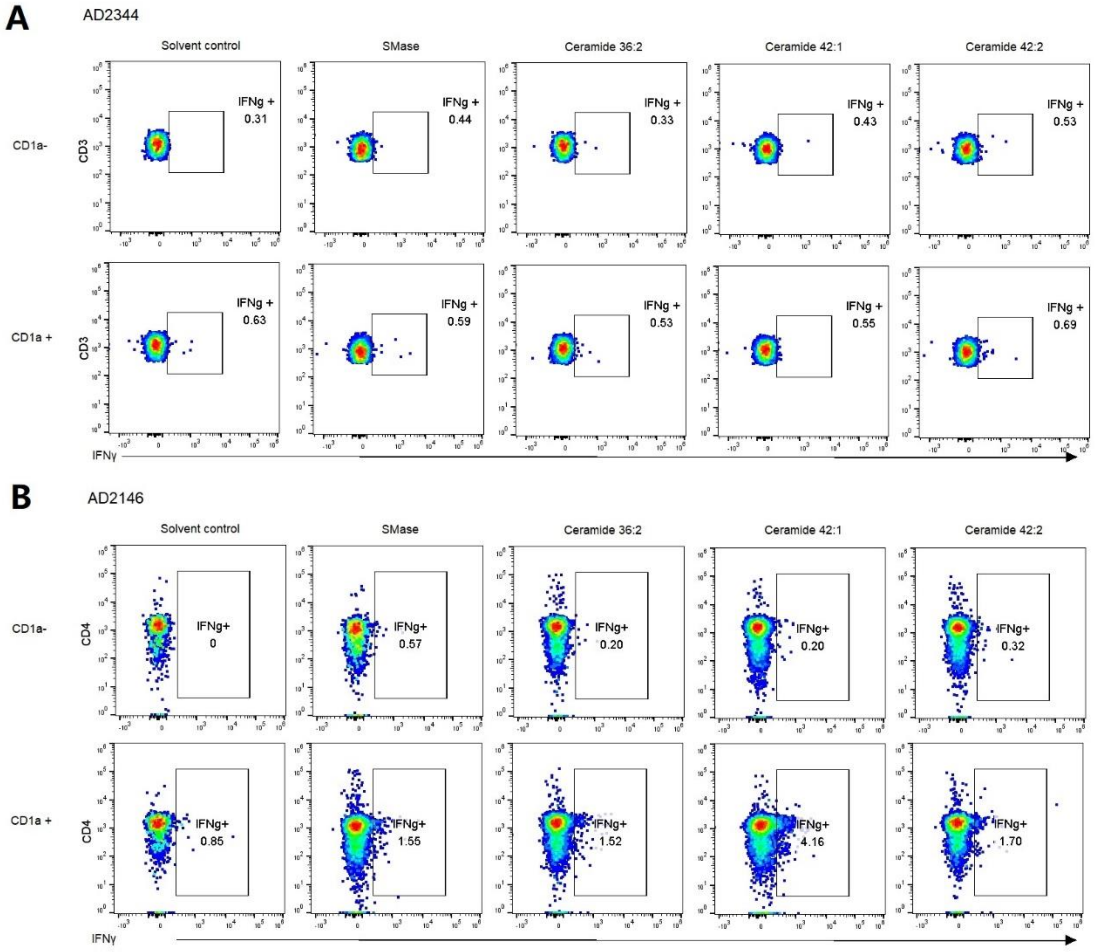

Figure 7.7 AD patients showed two different pattens of CD1a-reactive T cell responses to SMase and ceramide treatment. Polyclonal T cells isolated from AD patients PBMCs (donor AD2344 and AD2146) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. IFNγ producing T cells were tested in cytokine secretion assay. Data representative of at least three donors from different experiments. FACS plots gated on CD3+, live cells and singlets.

In the 7 AD patients tested, two patterns of CD1a-reactive T cell response to SMase and ceramide treatment were noted. In most of the AD patients, a CD1a-

autoreactive response was detected, at approximately 0.6%. The T cell responses to K562-CD1a cells treated with SMase or three synthetic ceramides were similar to CD1a-autoreactivity (Figure 7.7A).

However, we noticed that in patient AD2146, a significantly higher CD1a-reactive SMase-specific response (1.55%), which was two-fold that of the CD1a autoreactive response (0.85%). T cells from this patient also highly responded to three synthetic ceramides, especially ceramide 42:1. This patient has moderate AD with asthma and hay fever to tree pollen. Another patient with moderate AD and asthma (AD2148) did not present a similar pattern. As AD is strongly associated with allergy and other atopic diseases, it is worth taking these factors into consideration, and carefully documenting patients with different sub-types of AD in future experiments. (Figure 7.7B).

Based on previous studies of AD, Th2 and Th22 activation appears dominant in acute AD with progressive activation of Th1 and Th17 cells in chronic stages. Typical cytokines, including Th2-associated IL-4 and IL-13, Th22-associated IL-22, and Th1-associated IFNγ and IL-12, are associated with these different stages of AD. Thus, it is important to test other cytokines besides IFNγ in future experiments, such as IL-22 and IL-17A that were also shown to be expressed by CD1a-reactive SMase-specific T cells. Also, a further classification of patients dependent on severity and disease progression might be necessary (Gittler et al.,

2012; Weidinger et al., 2018).

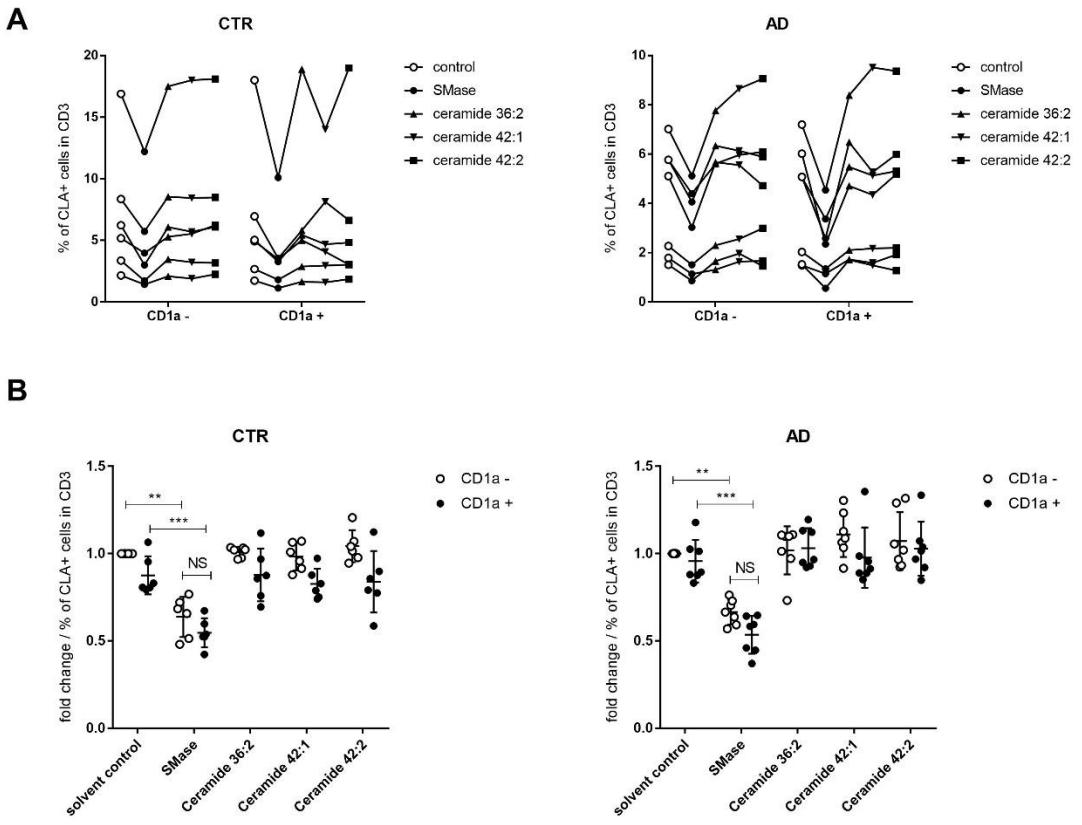

Figure 7.8 CLA expression on CD1a-reactive T cells in AD patients and healthy individuals. Polyclonal T cells isolated from AD patients PBMCs (n=7) and healthy PBMCs (n=6) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. (A) The percentage of CLA+ T cells of healthy individuals (left) and AD patients (right) in each condition. (B) The percentage of CLA+ T cells of healthy individuals (left) and AD patients (right) was normalized to that in group of K562-Ev cells pre-treated with medium only and fold changes are shown. The log-transformed value of fold change was used for statistics. Significance of log-fold change was calculated by One-sample t test. Bars represent standard error. ** P<0.01 *** P<0.001.

The percentage of total CLA+ T cells in AD patients ranged from 1-8%, and was similar to that observed in healthy individuals. Consistent with CD1a-reactive T cells in healthy donors, the CLA expression on T cells from AD patients in the SMase-treated group was only half of the amount of those in groups without SMase treatment, while the percentage of total CLA+ T cells was not changed among ceramide-treated groups (Figure 7.8A). The fold changes were calculated, and the pattern remained the same (Figure 7.8B). In summary, this down-regulation of CLA after SMase treatment was consistent in healthy individuals and AD patients, and was independent with CD1a. As discussed in previous chapter, cytokine production and the modulation effect of cell-culture on CLA expression are worth taking into consideration in future work.

7.4 Investigation of CD1a-reactive SMase-specific T cells in PS patients

Psoriasis patients have also been shown to have higher risk of *S. aureus* colonization than healthy individuals. CD1a-reactive T cells were enriched in PS patients and were able to produce IFNγ, IL-22 and IL-17A (Cheung et al., 2016; Ng et al., 2017) . Thus, we sought to investigate the CD1a-reactive SMase-specific T cells in PS patients.

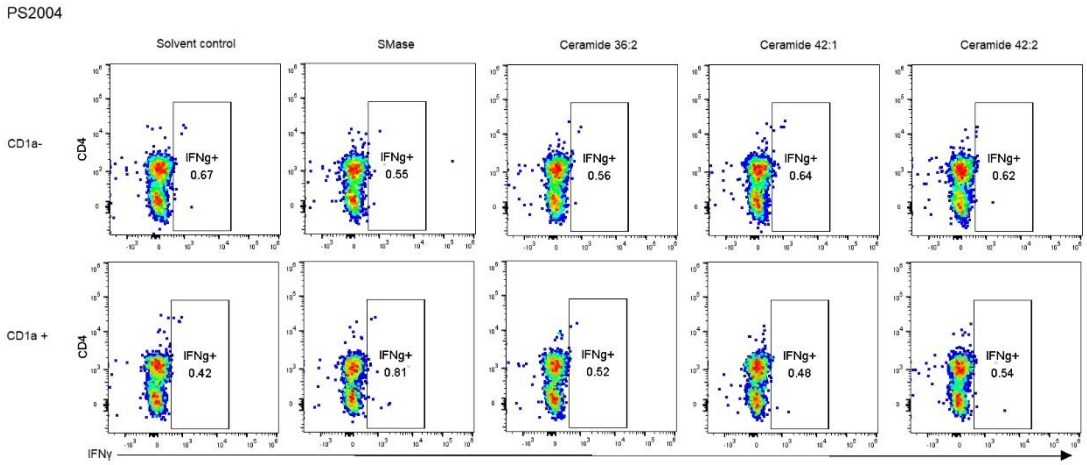

Figure 7.9 CD1a-reactive T cell responses to SMase and synthetic ceramides in PS patients. Polyclonal T cells isolated from PS patients PBMC (donor PS2004) cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. IFNγ producing T cells were tested in cytokine secretion assay. Data representative of at least three donors from different experiments. FACS plots gated on CD3+, live cells and singlets.

The CD1a-reactive T cells response to SMase and three synthetic ceramides in PS patients were tested by IFNγ cytokine secretion assay. The background of T cell response was about 0.5%, and a slightly up-regulation of IFNγ to K562-CD1a pre-treated with SMase was observed (0.8%). The T cell responses to ceramides were similar to background. (Figure 7.9)

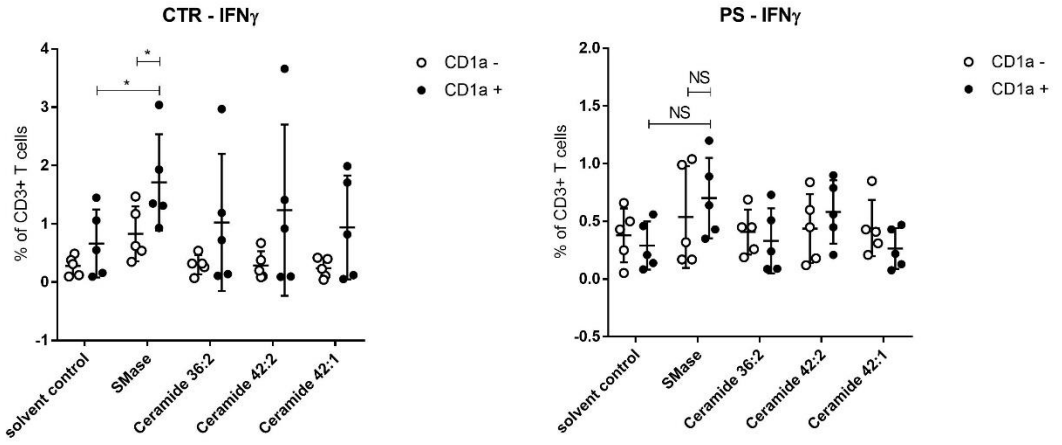

Figure 7.10 IFNγ-producing CD1a-reactive SMase-specific T cells in PS patients. T cells were isolated by CD3 MACS beads from healthy donor (n=5) and PS patients (n=5) PBMCs. T cells cultured for 6hrs with K562-Ev or K562-CD1a cells pre-treated with SMase, ceramides, or medium only. IFNγ producing T cells were tested by IFNγ secretion assay. Bars represent standard error. * $P<0.05$.

The IFNγ production of CD1a-reactive SMase-specific T cells was further tested in 5 PS patients by cytokine secretion assay (Figure 7.10). As the percentage of IFNγ producing CD1a-autoreactive T cells was around 0.04% in PS patients (Cheung et al., 2016), a low level of CD1a-autoreactive response was expected. In PS patients, the CD1a-reactive SMase-specific T cell response was higher than K562-Ev pre-treated with SMase or CD1a-autoreactivity, and indicated a trend of IFNγ upregulation, although no statistically significant difference was found so far. It will be important to repeat the experiments in a larger patient cohort, as well as testing other cytokines such as IL-17A and IL-22.

7.5 Real-time PCR testing

In previous results, IFNγ-producing CD1a-reactive T cells response to SMase was not significantly different in AD and PS patients compared to healthy controls.

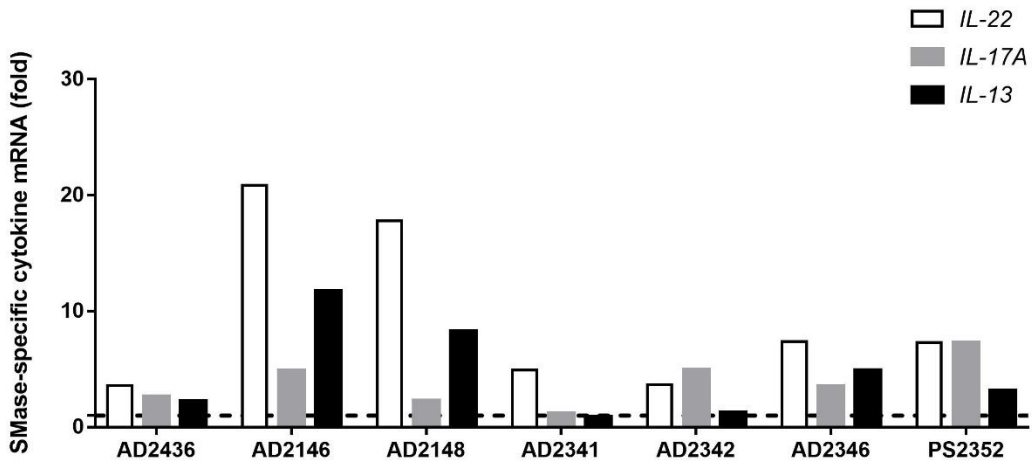

Figure 7.11 Circulating CD1a-reactive SMase-specific T cells in AD and PS patients up-regulate cytokines at the transcriptional level. Polyclonal T cells isolated from PBMCs of 6 AD patients and 1 PS patient cultured for 6hrs with K562-CD1a cells pre-treated with or without SMase. Cytokine gene upregulation was measured by real-time PCR. Summary results of SMase-specific cytokine gene upregulation of CD1a+SMase compared to CD1a-autoreactivity in 7 donors. Samples were normalized to *CD2*.

The SMase-specific *IL-22*, *IL-17A* and *IL-13* gene upregulation by T cells derived from 6 AD patients and 1 PS patient were measured by real-time PCR. Compared to the CD1a-autoreactive response, AD patients had a 4 to 20 fold up-regulation of *IL-22* gene expression in response to K562-CD1a cells treated with SMase. We

also detected *IL-17A* and *IL-13* gene up-regulation in different AD patients. In the PS patient (donor PS2352), a CD1a-reactive SMase-specific *IL-22* (7 fold), *IL-17A* (7 fold) and *IL-13* (3 fold) gene up-regulations were detected. These data suggested the involvement of Th2-associated and Th17-, Th22-mediated cytokines in CD1a-reactive SMase-specific T cell response in AD and PS patients. Real-time PCR offered a quick test for cytokines involved at transcriptional level. We would therefore further investigate the real time PCR and cytokine production by functional assay in future experiments.

Discussion

In summary, by using CD1a-tetramers loaded with endogenous lipids, LPC and synthetic sphingomyelins, we demonstrate the interruption of long-chains SMs to TCR-CD1a interaction in both healthy controls and AD patients. The percentages of T cells recognizing CD1a-endo and CD1a-LPC tetramers showed no difference within each cohort, and showed no difference between healthy individuals and AD patients. However, the percentage of CD1a-tetramer positive T cells in AD patients showed different preference to the length and unsaturation of SM compared to healthy individuals. SM 42:2 showed the best blocking effect in both AD and healthy donors. SM 36:2 significantly influenced tetramer binding in AD patients but not in healthy donors; while the blocking effect of SM 42:1 was not as significant in AD patients as in healthy individuals.

Furthermore, CD1a-reactive T cell response to SMase and ceramides were tested in AD and PS patients. In contrast to healthy individuals, the IFNγ-producing CD1a-reactive SMase-specific T cells were not detected in AD PBMCs, shown by ELISpot and secretion assay. In PS patients, the CD1a-reactive SMase-specific T cell response was higher than control groups, although no statistically significant difference was noted. By using real-time PCR, the up-regulation of *IL-22*, *IL-17A* and *IL-13* genes were found in AD and PS patients.

We noted that the patient AD2146, who has moderate AD with asthma and hay fever to tree pollen, had a significantly higher CD1a-reactive SMase-specific response compared to CD1a-autoreactive response. T cells from this patient also highly responded to three synthetic ceramides, especially ceramide 42:1. This pattern to SMase or ceramides was not found in another patient with moderate AD and asthma (AD2148). In real-time PCR, significantly high *IL-22* and *IL-13* gene expressions to CD1a+SMase were also observed in both of these two patients. AD is strongly associated with allergy and other atopic diseases, which should be taken into consideration. The careful documentation of patients with different sub-types of AD is necessary in future experiments. (Weidinger et al., 2018)

As described in the introduction, AD is considered as multiphasic disease, with stage-dependent progressive upregulation of Th2, Th22, Th1 and Th17 cytokines.

The immune response in AD is skewed towards Th2-associated pathways, which promote B cell IgE class switching and production, and reduce filaggrin expression of keratinocytes. (Gittler et al., 2012; Howell et al., 2009; Weidinger et al., 2018) Also, in previous studies, the PBMCs from AD children showed significantly higher proliferative response to *S. aureus*. Despite the enhanced proliferation, the PBMCs tended to produce more IL-4 and less IFNγ in response to *S. aureus*. (Campbell & Kemp, 1997) This may be explained by the activation-induced apoptosis of Th1 circulating activated memory/effector T cells in AD patients, while Th2 cells survived (AKDIS et al., 2003). These data may explain the down-regulation of IFNγ-producing CD1a-reactive SMase-specific T cells in AD. We are interested to further test cytokine expressions of IL-22, IL-17A and IL-13 in CD1a-reactive SMase-specific T cells in AD, which are suggested by the real-time PCR results. Further testing of these cytokines by functional assay will be an additional next step of the project.

A general up-regulation of 0.1-0.3% IFNγ-producing T cell response to K562-CD1a treated with SMase were found in PS patients by cytokine secretion assay, which was slightly diminished compared to healthy individuals. The less production of IFNγ may result in insufficient eradication of *S. aureus*. We cannot claim the presence of elevated IFNγ-producing CD1a-reactive SMase-specific T cells in the blood of PS patients so far. Repeated experiments in larger cohort are necessary, as well as the measurement of other cytokines including IL-17A and

IL-22. In addition, it will be of interest to examine response in lesional skin.

It is interesting to note the increasing prevalence of *S. aureus* on skin and the diminished production of IFNγ by CD1a-reactive SMase-specific T cells in healthy individual, PS patients and AD patients. IFNγ is known to play an important role in antimicrobial immunity, and generates effective immune responses for eliminating pathogens (Kak et al., 2018; Shtrichman & Samuel, 2001). It contributes to anti-*Staphylococcus aureus* immune responses by stimulating intrinsic antibacterial activities (Beekhuizen & Van De Gevel, 2007). We hypothesise the CD1a-reactive SMase-specific T cells in healthy individuals as a novel pathway for sensing bacteria such as *S. aureus*. The underlying mechanisms could be investigated by CD1a-reactive SMase-specific T cell lines and clones, as described in previous chapters.

8. Discussion

As discussed, CD1a is an MHC-like antigen-presenting molecule that is mainly located at the skin. (McMichael et al., 1979) It belongs to CD1 protein family which is able to present lipid antigens to T cells, including endogenous lipids such as sulfatides and skin oils, and exogenous lipids such as lipopeptide from *M. tuberculosis*. (Jong et al., 2014; Moody et al., 2004; Zajonc et al., 2003b) CD1a is markedly expressed on human epidermal Langerhans cells (LCs) as well as antigen-presenting cells (APCs) in epithelia of various tissues. The function of CD1a to bind lipids and interact with TCR is now well-established. (Brigl & Brenner, 2004; Dougan et al., 2007; Sotzik et al., 1993)

CD1a-reactive T cells have been proved to be a normal component of human αβ T cell repertoire, the percentage of which is 0.3-10% (De Lalla et al., 2011). These T cells express skin-homing markers, and could be also isolated from skin. CD1a-reactive T cells express multiple cytokines, including IFNγ, IL-22, IL-17A, IL-13, GM-CSF, and contribute to immune response to *M. tuberculosis* as well as skin inflammatory diseases. (Beckman et al., 1994; Cheung et al., 2016; De Jong et al., 2010; Jarrett et al., 2016) The co-localization of CD1a-expressing APCs and CD1a-reactive T cells at cutaneous sites suggests their participation in immune response to skin microbiota, the study of which is still very limited.

The binding groove of CD1a is consisted of A' and F' pockets, which are capable of holding hydrophobic chains of lipid antigens. Based on the crystal structure of CD1a with sulfatide, the sphingosine chain with 18 carbons could ideally fit in A' pocket; while the F' pocket, which is closer to protein surface, accommodates lipids with various length and headgroup. (Zajonc et al., 2003a) The sphingomyelin (SM) that also has 18-carbon sphingosine backbone is proved to bind with CD1a by crystal structure, but the large headgroup disrupts the interaction between BK6 TCR and CD1a-ligand complex. Thus, SM is termed the CD1a non-permissive ligand for BK6. The blocking effect of SM is further proved by functional assays based on BC2 cell line. (Birkinshaw et al., 2015; Jong et al., 2014) Sphingomyelin (SM) is the most abundant eukaryotic membrane sphingolipid in the outer leaflet of cell membrane and is enriched in lipid rafts. It is the substrate of sphingomyelinase (SMase). The enzyme cleaves the phosphocholine headgroup from SM and produces ceramides, which also share the C18 sphingosine backbone, and may potentially serve as CD1a antigens. (Flores-Díaz et al., 2016)

SMase is one of the virulence factors secreted by multiple bacteria including *B. cereus*, *L. ivanovii*, and *S. aureus.* Its counterpart in mammalian cells is the neutral SMase 2, which is an integral membrane protein and mainly activated at brain tissue. (Clarke et al., 2011) SMase lyses erythrocytes for iron and nutrition acquisition for bacteria. The treatment of *S. aureus* SMase on mammalian cell surface increases ceramides and causes fragility of plasma membrane. (M. Huseby

et al., 2007) *S. aureus* is a commonly found pathogen that cause skin and soft tissue infections, leading to endocarditis, pneumonia, meningitis, and sepsis. It is commonly localized on lesional skin of patients with atopic dermatitis (AD) and psoriasis (PS). (K. Iwamoto et al., 2019; Totté, van der Feltz, Bode, et al., 2016) SMase is expressed by all virtually *S. aureus* strains, and is proved to contribute to *S. aureus* infection, damage and colonization. The effect of SMase in lung injury caused by *S. aureus* is reported, but its roles in skin immune diseases are still unclear. (Hayashida et al., 2009; Katayama et al., 2013; Salgado-Pabón et al., 2014)

This thesis aimed to address several questions regarding the functions and phenotype of CD1a-reactive T cells in bacterial sensing through CD1a-ligand modulation by *S. aureus* SMase, and further investigate their role in homeostasis and pathological conditions. In these studies, we have identified the presence of CD1a-reactive SMase-specific T cells in blood of healthy individuals, which were detected in most of the donors. These T cells were found in CD4+, CD8+ and double negative populations, perhaps indicating the response might be less dependent on MHCs. These T cells were able to produce cytokines IFNγ, IL-22 and IL-17A, which are thought to contribute to anti-*S. aureus* immune responses. (Beekhuizen & Van De Gevel, 2007; Valeri & Raffatellu, 2016) The cytokines were regulated at both the transcriptional and translational levels. In these studies, we have shown that the functions of CD1a-reactive SMase-specific T cells were dependent on SMase enzymatic activity. The products of SMase, including

ceramides, may activate these T cells at the polyclonal level and potentially serve as new lipid antigens generated *in vivo* in this immune response. The SMase-specific and ceramide-specific T cell response showed a linear correlation, implicating the role of ceramides in explaining the SMase response. However, the T cell responses to ceramides with different length of carbons and unsaturation were donor dependent; it would be interesting to investigate the ceramide-specific T cell response in a larger cohort, and further study the influence of length and unsaturation of carbon chains on lipid recognition.

This is the first time that CD1a-reactive bacterial SMase-specific T cells have been described in the literature. As a lipid-antigen presenting molecule, CD1a is not able to directly recognize proteins or peptides. However, recent studies have found a novel pathway that CD1a recognizes lipid neoantigens generated by bee venom-derived or house dust mite-derived PLA2 *in vivo*. The exogenous PLA2 digests phospholipids into lyso-phospholipids and antigenic free fatty acids, which may activate T cells via CD1a. Thus, CD1a was able to recognize presence of enzymes secreted from pathogens. (Bourgeois et al., 2015; Jarrett et al., 2016) Consistent with these findings, we found that CD1a also sensed the presence of bacterial SMase likely through generation of neolipids, in an analogous pathway to PLA2.

CD1 proteins have different trafficking pathways, which allow them to sample lipid

contents from different cellular compartments. (Ly & Moody, 2014; Odyniec et al., 2010) As CD1a is only internalized through early endosome pathway, this protein quickly comes back to plasma membrane, and is enriched at the cell surface. CD1a is able to exchange lipids directly at cell surface without cellular internalization, and the lipid loading of CD1a may not require low pH. (Barral et al., 2008; Ly & Moody, 2014; Manolova et al., 2006) All these unique characters make CD1a sensitive to the lipids in immediate extracellular environment.

SMase-secreting bacteria cannot generate sphingolipids by themselves, so they take advantage of host sphingolipids through SMase and promote their pathogenicity. It is also a method for them to acquire iron through lysing erythrocytes. Thus, SMase is one of the key enzymes for bacterial survival. (Flores-Díaz et al., 2016) Based on unpublished data from Rachel Cotton and Tanyun Cheng at Harvard (presented at CD1/MR1 Conference Oxford 2019), long-chain SMs are enriched in CD1a eluate, which are hypothesized to block the CD1a-autoreactive response. The presence of SMase would be predicted to alter the lipid component at the cell surface, changing the ratio of permissive and non-permissive ligands by decreasing the amount of SM and increasing ceramides. CD1a is then functionally activated and may trigger a T cell response. Also, under normal conditions, CD1a-expressing APCs are located at the epidermis, while CD1a-reactive T cells locates predominantly at the dermis. The distribution of these cells at different layers further prevents CD1a autoreactive response.

(Bourgeois et al., 2015) The presence of bacterial SMase indicates a break at the skin surface, which disrupts the layer structure and allows the contact of APCs and T cells. The CD1-reactive T cells, which belong to the unconventional T cell family, are able to respond rapidly within hours after stimulation. (Godfrey et al., 2015) Thus, the CD1a-expressing APCs and CD1a-reactive T cells may serve as a skin barrier sensing system that responds at an early stage. This system is a complement for the MHC-peptide pathway, as the CD1a pathway not only sensing the exogenous lipids but also sensing foreign enzyme secreted by pathogens.

The bacterial SMase is very different from its mammalian counterparts. Human SMases are divided into five groups based on their optimum pH and ion-dependency. The acid SMase mostly locates at lysosome and/or late endosome. (Schneider & Kennedy, 1967; Schuchman, 2010) Based a recent paper about acid SMase and CD1d antigen SMs, the association between cellular sphingolipid metabolism and CD1d-reactive iNKT cells in Niemann-Pick disease was proved in a mouse model and humans, suggesting the control and regulation roles of SMase to CD1-reactive T cells. (Melum et al., 2019) Also, the co-localization of acid SMase and CD1d in late endosomes and lysosomes allows the CD1d to contact with lipids that acid SMase modulated. Similarly, bacterial SMase is secreted by *S. aureus* and released to the environment. The enzyme binds and cleaves the SM on outer layer of cell membrane. The co-localization of bacterial

SMase and CD1a at cell surface increases the chance of CD1a to bind the enzyme modulated neoantigens.

SMs share a very similar structure with ceramides. Both of the lipids have a sphingosine backbone with 18 carbons and a N-linked fatty acid residue with various lengths and unsaturation, but SMs have an extra phosphorylcholine compared to ceramides. The two carbon chains of SM accommodate in A' and F' pockets of CD1a separately, and allow lipid presentation by CD1a protein. (Birkinshaw et al., 2015) This suggests the possibility of ceramides to fit in CD1a binding curve using the similar structure of SMs. Based on crystal structure of CD1a-SM complex, the charged phosphorylcholine headgroup of the lipids protrudes out from CD1a surface, which disrupts the interaction between CD1a and TCR. (Birkinshaw et al., 2015; Van Rhijn et al., 2015) This charged headgroup could be removed by SMase. We accordingly hypothesise that SMase may potentially alter the non-permissive ligands into permissive ligands. This hypothesis is worth testing by CD1a plate-bound assay using CD1a-reactive T cells as well as crystal structures.

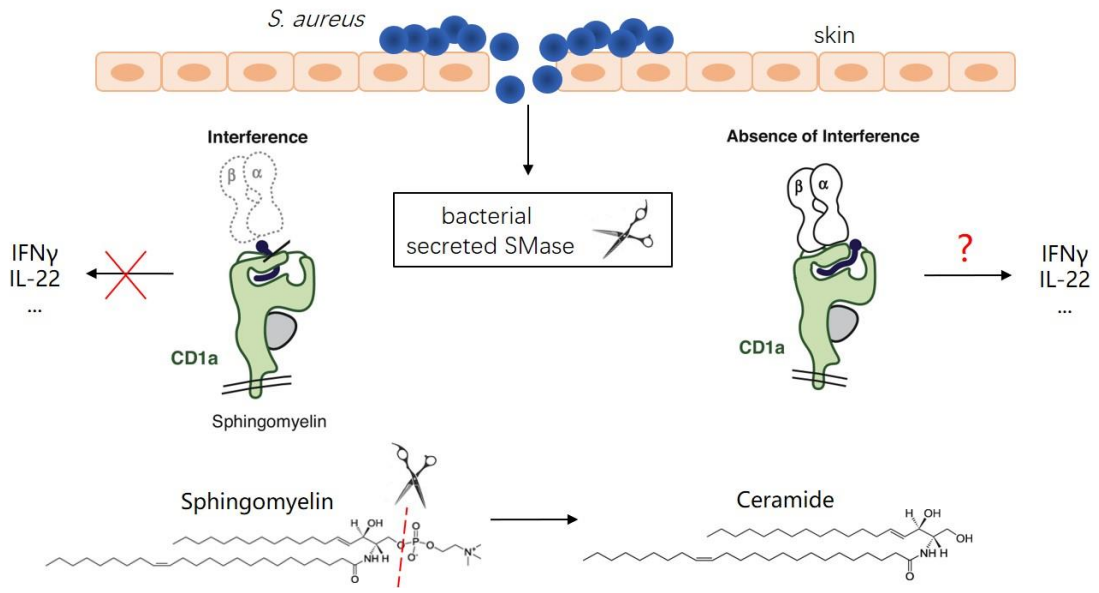

Figure 8.1 Proposed mechanism of CD1a-reactive SMase-specific T cell stimulation. Adapted from (Moody & Cotton, 2017). The localization of *S. aureus* on skin surface produces secreted SMase. The impaired skin barrier leads to increasing exposure to bacterial SMase and causes the hydrolysis of SMs. SMs as non-permissive ligands block the activation of T cells and cytokine production, while the presence of foreign SMase alters the SMs to ceramides, which may serve as permissive CD1a ligands and trigger the CD1a-reactive T cell responses.

The lack of readily detectable surface markers for CD1a-reactive T cells is one of the impediments to further study the function and phenotype of these T cells. Most of the research in the field relies on the study of several CD1a-reactive T cell lines, which allows us to investigate in detail the underlying mechanism of the regulation, function, and phenotype of these cells. But the limited number of T cell lines may not fully describe the general characteristics of the whole population of CD1a-reactive T cells. Thus, it will be important to increase the numbers of lines

generated. CD1a-tetramers are an efficient method to detect and isolate specific reactive T cells, but the tetramer-binding is influenced by lipid loading and its affinity with TCR. Also, the association of tetramer-binding with functional activation is still unclear. (Kasmar et al., 2014) This indicates the importance of generating CD1a-reactive T cell lines based on their immune functions.

In these studies, we have generated CD1a-reactive SMase-specific T cell lines and clones from several blood of healthy individuals. The T cell lines identified were αβ and γδ T cells, and most of the lines were CD8+ cells. CD1a-autoreactive T cell lines were also generated, most of which were predominately γδ T cells, suggesting the role of γδ T cells in CD1a autoimmunity. The SMase-specific and autoreactive T cells were able to express the skin-homing marker CLA, indicating their ability to migrate to skin. Besides, lines of CD1a-reactive SMase-specific and CD1a-autoreactive T cells were found from healthy skin samples. The TCRs of CD1a-reactive SMase-specific αβ T cell clone 1-B5 and CD1a-autoreactive clone 7-C8 were sequenced. One of the CD1a-reactive SMase-specific T cell line was stained with CD1a tetramer. All these T cell lines allow us to further investigate the phenotype characters and physiological functions of CD1a-reactive T cell population.

Importantly, we successfully isolated a CD1a-autoreactive Vδ2 γδ T cell clone 7-C8. This is the first time that human CD1a-reactive T cells have been found in Vδ2

γδ T cell population in the literature. The 7-C8 belonged to the Vγ9/Vδ2 T cell subset, and was a CD4 CD8 double negative memory T cell clone. 7-C8 remained a stable CD1a-autoreactive T cell response after multiple rounds of expansion, which could be blocked by anti-CD1a antibody, demonstrating its CD1a dependency. It expressed skin-homing marker CLA, and also expressed NKG2D, the costimulatory receptor constitutively expressed by γδ T cells (Rincon-Orozco et al., 2005; Wensveen et al., 2018).

Most of the CD1a-autoreactive T cell lines generated so far belong to the αβ T cell repertoire. The γδ T cells were found to recognize pollen lipids via CD1a, CD1c and CD1d, and one Vδ1 γδ T cell clone was generated at 2005. (Agea et al., 2005; De Jong et al., 2010) The recognition mechanisms of Vγ9/Vδ2 T cells, the most abundant γδ T cell subset in human blood, is still unclear. The most well-studied antigens of human Vδ2 T cells are the small molecular phosphoantigens. (Fisher et al., 2014; Wu et al., 2015) The Vγ9/Vδ2 T cells is well known for rapid and potent response to acute infection and tumorigenesis. (Bouet-Toussaint et al., 2008; Palakodeti et al., 2012) A recent study of Vγ9Vδ2 T cells reports a small population of CCR6+ skin-homing Vγ9Vδ2 T cell subset, which produce IL-17A. The population of these T cells decreased in PS patients and recovered after treatment. (Laggner et al., 2011) Our finding of CLA+ CD1a-autoreactive Vγ9Vδ2 T cell clone further support this hypothesis that these T cell subsets participate in skin immune response. The γδ T cells are known to be activated via NKG2D, which was

expressed on 7-C8 cell line too. But NKG2D triggers the production of TNF-α but not of IFN-γ, and this activation is antigen-independent. (Rincon-Orozco et al., 2005) As the CD1a-autoreactive response of 7-C8 was not detected in K562-Ev control groups, this response is likely independent with NKG2D. The generation of 7-C8 T cell clone and successful TCR sequencing will allow the further study of CD1a and γδ T cells, and the 7-C8 TCR is currently being established in a Jurkat T cell line by lab member Rachel Etherington.

Besides, to further support our hypothesis, the role of SMs as non-permissive ligands at polyclonal level was tested by CD1a-tetramers loaded with various lipid antigens, gifted from Harvard. The percentage of CD1a-endo tetramer positive cells in CD3+ cells was around 0.5-6%. Although the percentage was slightly higher in AD patients, generally it was at a similar level and there was no statistical difference between AD patients and healthy individuals. The CD1a permissive ligand LPC loaded on tetramers stained similar frequency of tetramer positive staining T cells amongst total CD3+ cells compared to CD1a-endo tetramer, and showed no difference between AD patients and healthy individuals. By replacement of endogenous lipids with synthetic SMs, the population of CD1a-tetramer positive T cells significantly reduced. The percentage of CD1a-tetramer positive T cells in AD patients showed different preferences to the length and unsaturation of SM compared to healthy individuals, but in summary, the SM 42:2 with long carbon chains and two double-bonds showed the strongest blocking

effect. The increasing length of carbon chains and numbers of double-bonds reduces the flexibility of lipids and pushes them to certain orientations. This finding may be further explained by the unpublished crystal structures from our collaborator in Australia (presented at CD1/MR1 Conference Oxford 2019). The blocking effect of SM 42:2 is further supported by its enrichment in CD1a eluate, proved by our collaborator in Harvard. The long chain SMs bind with CD1a proteins and generally block TCR recognition at polyclonal level, which reduces the CD1a-autoreactive immune response in normal conditions. We noted that CD4+ and CD8+ cells generally showed a similar pattern with CD3+ cells as a whole, which is consistent with the finding of CD1a-reactive T cells in both CD4+ and CD8+ populations at the polyclonal level. The difference between CD1a-reactive T cell response in CD4+ and CD8+ subsets may be further investigated.

CD1a-reactive T cell responses to SMase and ceramides were further tested in patients with two *S. aureus*-associated skin inflammatory diseases, AD and PS. The prevalence of *S. aureus* on skin is low in healthy individuals, moderate in PS patients, and remarkably high in AD patients. The percentage of IFNγ-producing CD1a-reactive T cells is enriched in healthy individuals but not detected in PBMCs of AD patients. In PS patients, the CD1a-reactive SMase-specific T cell response was higher than CD1a only or SMase only groups, although no statistically significant difference was noted so far. However, by using real-time PCR, we detected the CD1a-reactive SMase-specific up-regulation of *IL-22*, *IL-17A* and

IL-13 genes in AD and PS patients, which is worth repeating in a larger cohort.

These data suggest the different cytokine profile in homeostasis and pathological condition of the diseases, which is consistent with previous studies. Although Th1 cells are increasingly activated at chronic stage of AD, the role these cells are still unclear, and the immune response in AD is skewed towards Th2-associated pathways. (Gittler et al., 2012; Greb et al., 2016; Weidinger et al., 2018) The IL-4 and IL-13 are believed to contribute to the disease. It is worth testing these Th2-associated cytokines in future experiments. Additionally, it is necessary to carefully document patients with different sub-types of AD, as the comorbidities and different genetic background may have overlapping immune responses.

The lipid profile of atopic dermatitis has been well studied, and changes in ceramides are linked with functions of human stratum corneum (SC). (Y. Cho et al., 2004; Melnik et al., 1988) The total amount of 11 ceramides species is reduced, and linked with increasing SC trans-epidermal water loss and decreasing SC water-holding function. However, the ceramides with sphingosine backbone and non-hydroxy fatty acid chain shows no difference of amount between AD patients and healthy individuals. In contrast, AD skin has an altered component of ceramides with varied length of carbon chains. The short-chain ceramides (<40 total carbons) of this species are abundant, while the long-chains ceramides (>50 total carbons) significantly decrease. (Ishikawa et al., 2010; Janssens et al., 2012;

Jungersted et al., 2010) As human SMase is proved to be reduced in AD lesional skin and may be less involved in the generation of ceramides, we hypothesis that the increased short-chain ceramides may be the products of bacterial SMase. (Jensen et al., 2004)

Predominant *Staphylococcus aureus* colonization is associated with severity of AD, leading to an altered surface microbiota and reduced bacterial diversity. (Kong et al., 2012). An increased pH promotes the proliferation and binding of *S. aureus* on atopic skin, and the production of IL-4, IL-13 and IL-10 all decrease the host anti-microbial peptides. The dysfunction of filaggrin also weakens the host defense to *S. aureus*. (Miajlovic et al., 2010) SMase enzymatic activity is significantly high in microbiota of AD patients (Ohnishi et al., 1999), which may result from the increased secretion of bacterial SMase from *S. aureus*. In summary, the predominant colonization of *S. aureus*, the increased short-chain ceramides in SC and the strongly secreted SMase suggest the participation of bacterial SMase in the disease. The studies of CD1a-reactive T cell activation to house dust mite and heat-killed *S. aureus* also demonstrate the role of CD1a in AD pathogenesis. (Hardman et al., 2017; Jarrett et al., 2016) In previous studies, PBMCs from AD patients produced more IL-4 and less IFNγ after *S. aureus* stimulation. (Campbell & Kemp, 1997) The predominant IL-4 is necessary for IgE class-switching, which contributes to the disease progression, and inhibits the generation of Th1 cells. The activation-induced apoptosis of circulating Th1 but

not Th2 cells in AD patients might also explain the Th2 predominance. (AKDIS et al., 2003) These data may explain the lack of IFNγ-producing CD1a-reactive SMase-specific T cells in AD patients, which could impair the efficient clearance of invaded bacteria and could further contribute to the pathogenesis of the disease. While the pathogenesis of AD involves the activation of Th2, Th22, Th1 and Th17 T cell subsets, the cytokine-network is complex and need further investigation.

In conclusion, we have identified polyclonal CD1a-reactive SMase-specific T cells, which are enriched in the blood of healthy individuals and found in skin. They produce a number of cytokines to participate in anti-bacterial immune responses, identifying a novel pathway of bacterial sensing, which plays a potential role in AD. Ceramides produced by bacterial SMase may serve as neoantigens generated *in vivo*. Ceramide-specific T cell response shows a linear correlation with SMase-specific response via CD1a. We demonstrate the blockage effect of long-chain SMs to CD1a-tetramer positive T cells at polyclonal level. These data indicate the lipid alteration from non-permissive ligands to permissive ligands potentially caused by invading bacterial SMase. We successfully generated CD1a-reactive SMase-specific T cells from blood and skin samples from different individuals. We are also able to identify CD1a-autoreactive T cells in human Vγ9/Vδ2 T cell subsets, and successfully generated and sequenced a stable CD1a-autoreactive Vγ9/Vδ2 T cell clone. All these data extend our understanding of immune function

and phenotype characteristics of CD1a-reactive T cells.

8.1 Future work

Further work is needed to further investigate the questions that the results in this thesis have raised.

First, the current studies of CD1a-reactive SMase-specific T cell response were focused on IFNγ as well as IL-22 and IL-17A. These are the key cytokines associated with Th1, Th22 and Th17 cells, but the Th2-assocated cytokines such as IL-4 and IL-13 production need to be tested and analyzed as well, which will be particularly important in AD patients. IL-4 and IL-13, as well as their receptors, have been reported to associate with atopic disorders including AD. IL-13 is considered as key mediator of Th2 response in the skin, and affects keratinocytes, immune cells, neurons and fibroblasts. It leads to the decreased production of barrier proteins and antimicrobial peptides from keratinocytes, supports *S. aureus* colonization, and induces inflammation. (Bieber, 2020) Previous studies have proved the ability of CD1a-reactive T cells to produce IL-13 in AD, but the study of IL-4 is still less understood. (Jarrett et al., 2016) The production of IL-13 by CD1a-reactive SMase-specific T cells was also suggested by real-time PCR results. It is worth testing this cytokine production in functional assays, and expanding the experiments to AD patients. The activation of Th17 cells and the role of IL-17A are proved to be key mediators in PS pathogenesis. (Greb et al., 2016) We have

demonstrated the IL-17A production of CD1a-reactive SMase-specific T cells in healthy individuals, and this initial analysis should be expanded to PS patients too. Additionally, IL-22 is produced by CD1a-autoreactive T cells, and the cytokine contributes to keratinocyte proliferation and participation in AD and PS. It is worth testing IL-22 production of CD1a-reactive SMase-specific T cells in AD and PS patients.

Second, CD1a-reactive ceramide-specific and SM-specific T cell responses were tested in several healthy individuals, and focused on IFNγ by far. The ceramide-specific T cell response was higher than control group, indicating the potential up-regulation effect, which should be further tested in a larger cohort. The length and unsaturation of fatty acid chains in ceramides influence the level of T cell response in different healthy donors. Currently we used ceramide 36:2, ceramide 42:1 and ceramide 42:2. The two ceramides with 42 carbons are products of SM 42:1 and SM 42:2 after SMase treatment, which are enriched in CD1a eluate (presented in CD1/MR1 conference Oxford 2019) and preferred substrates of human neutral SMase 2 in the brain (Clarke et al., 2006b). The ceramide 36:2 is the product of SM 36:2 after SMase treatment, which is used as sample of short-chain SM and tested in tetramer staining experiments. In addition, the ceramide with 34 carbons is worth testing in future experiments, as it is known to be abundant in skin of AD patients and is linked with the loss of SC function. (Ishikawa et al., 2010; Janssens et al., 2012) The blocking effect of SM was suggested by

tetramer staining results, and should be further investigated in cellular functional assay. More specifically, the blocking effect of SM 42:2, SM 42:1, SM 36:2, and SM with 34 carbons should be first studied. Besides IFNγ, other cytokines including IL-22 and IL-17A should be tested in future experiments too. Further, to take advantage of CD1a-reactive T cell lines and clones, the ceramide-specific response is worth testing in CD1a-reactive SMase-specific T cells, in order to provide more definitive evidence of ceramide as neoantigens generated by SMase; and the blocking effect of SM could be tested in different CD1a-autoreactive T cell lines/clones for investigating the interaction between CD1a-SM complex and specific TCRs.

Finally, we currently have proved the 7-C8 as a stable CD1a-autoreactive Vδ2 γδ T cell clone. Similar with previous applications of CD1a-reactive T cell clones BK6 and BC2, the sequenced TCR allows us to study the interaction of 7-C8 TCR with different CD1a-ligand complexes by crystal structure, which may extend our understanding of CD1a antigen recognition. Also, this functional stable T cell clone provides an effective tool to screen and identify the permissive and non-permissive ligands of CD1a. Besides, based on this Vγ9/Vδ2 cell line we can investigate the activation mechanism of this T cell subset, which still remains unclear. (Godfrey et al., 2015) The generation of a 7-C8 TCR transfected Jurkat cell line is underway.

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