

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Exploiting non-polymorphic MHC molecules in cancer therapy

by Rita Szoke-Kovacs

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND IMMUNE BIOLOGY

DEBRECEN, 2026

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Exploiting non-polymorphic MHC molecules in cancer therapy

by Rita Szoke-Kovacs

Supervisors: Dr. Péter Gogolák, Dr. Mariolina Salio



UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND
IMMUNE BIOLOGY

DEBRECEN, 2026

Contents

1. LIST OF ABBREVIATIONS	2
2. INTRODUCTION	5
2.1 T cell driven immune responses	5
2.2 Unconventional MHC molecules recognized by unconventional T cell populations	6
2.3 Cluster of differentiation 1 (CD1) molecules.....	7
2.3.1 CD1 recycling and antigen presentation	8
2.3.2 CD1 structure drives antigen selectivity	9
2.3.3 The CD1c-associated lipid-antigen repertoire.....	11
2.3.4 TCR recognition of CD1-lipid complexes	12
2.4 CD1 restricted T cells in disease.....	12
2.5 Harnessing Soluble T Cell Receptors for Therapeutic Use.....	14
2.6 Targeting HLA molecules limits patient reach	15
3. AIMS.....	16
3.1 Dissect the lipid antigen repertoire associated with CD1 complexes in mammalian cells and cancer cell lines	16
3.2 Molecular recognition of CD1c.....	17
4. MATERIALS AND METHODS	19
4.1 Lipids used.....	19
4.3 In-vitro ligand exchange.....	21
4.4 Isoelectric focusing.....	22
4.5 Native mass spectrometry	22
4.6 CD1c-endo tetramers and dextramers.....	23
4.7 Immunoprecipitation (IP) and Western Blot Analysis of CD1c from cell lines.	23
4.8 TCR discovery by Phage display	24
4.9 MAGPIX peptide screening.....	26
4.10 TCR and ImmTAC Construct design	27
4.11 TCR and ImmTAC expression	27
4.12 TCR and ImmTAC refold procedure	28
4.13 TCR and ImmTAC purification.....	29
4.14 SPR analysis.....	30
4.15 Affinity Maturation of TCR chains.....	31
4.16 Co-purification of ImmTAC-CD1c complexes for lipidomic studies.....	35
4.17 Co-purification of TCR-CD1c complexes for crystallography	36
4.18 X-ray crystallography.....	36

4.19	Generating antibody-based CD1c-specific T cell engagers.....	37
4.20	Generation of CD1c-expressing cell lines.....	38
4.21	Generation of CD1c restricted effector cell lines	39
4.22	T cell re-direction assay using anti-CD1c/anti-CD3 bispecific reagents.....	39
4.23	T cell activation assay	39
4.24	T cell cloning	40
	Cell isolation	40
	Expansion of CD1c- and CD1d- ganglioside specific T cell clones.....	41
4.25	Flow Cytometry and Cell Sorting.....	41
4.26	Lipid extraction for mass spectrometry	41
4.27	Mass Spectrometry (MS) data collection.....	42
4.28	Analyzing and post-processing the MS data	42
4.29	Study approval.....	44
5.	RESULTS	45
5.1	Mammalian expression of CD1 molecules.....	45
5.2	Lipid-ligand exchange method development.....	46
5.3	Validation of ligand exchange.....	47
5.3.1	Isoelectric Shift Assay.....	47
5.3.2	Native mass spectrometry.....	48
5.3.3	Efforts to establish TCR binding assay to validate ligand exchange	52
5.3.4	Tetramer staining.....	55
5.4	The CD1-associated lipid repertoire	57
5.4.1	Analyzing the lipid repertoire of CD1 isoforms.....	57
5.4.2	Widespread lipid capture by CD1 isoforms in mammalian cells.....	57
5.4.3	The shared lipid profile of CD1 isoforms	59
5.4.4	CD1 isoform specific features.....	61
5.4.5	Characterization of lipid chain lengths bound by CD1 isoforms.....	63
5.5	Binding mode of CD1c-specific TCRs.....	68
5.5.1	Generating CD1c-specific TCRs.....	68
5.5.2	Affinity enhanced CD1c restricted TCRs	69
5.5.3	Structural basis of CD1c lipid independent recognition.....	73
5.5.4	Increased number of hydrophobic interactions drive high-affinity of S2c ^{a5b6}	79
5.5.6	TCR-tap experiments to study CD1c restricted TCR specificity.....	81
5.6	Harnessing TCR-CD3 bispecifics to redirect T-cell activity toward CD1c-expressing cells	85
5.7	T cell redirection via CD1c-specific antibody-based T cell engagers.....	89
5.8	Antigen presentation by CD1 molecules.....	91
5.8.1	mLPA, a novel CD1c-associated antigen.....	91
5.8.2	Modelling the CD1c lipid loading pathways.....	91
5.9	Generating lipid-specific T cell clones	95

6. DISCUSSION.....	100
6.1 Lipid-loaded CD1 complexes allow the development of bispecific molecules..	100
6.2 CD1-associated Lipid Repertoire	101
6.3 Antigen presentation by CD1c.....	104
6.4 Targeting CD1c via bispecific engagers.....	105
7. SUMMARY.....	109
8. HUNGARIAN SUMMARY.....	112
9. LIST OF KEYWORDS.....	115
10. ACKNOWLEDGEMENTS	116
11. BIBLIOGRAPHY.....	117
12. PUBLICATION LIST	133
13. SUPPLEMENT	135
13.1 Vector maps.....	135
13.2 Supplementary Figures and tables	138

1. LIST OF ABBREVIATIONS

α GalCer	a-Galactosylceramide
AIEX	Anion-exchange chromatography
AP	Adaptor Protein
APC	Antigen-presenting cell
ARF6	ADP-ribosylation factor 6
B-ALL	B-cell acute lymphoblastic leukaemia
BiTE	Bi-specific T-cell engager
CD	Cluster of Differentiation; a system used to classify cell marker molecules, commonly used to define immune cell subsets
CDR	Complementary Determining Regions
CE	Cholesteryl ester
Cer	Ceramide
CHAPS	3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CHO	Chinese Hamster Ovary
Chol	Cholesterol
CIEX	Cation-exchange chromatography
CL	Cardiolipin
DAG	diacylglycerol
DC	Dendritic cell
DiHexCer	Dihexosylceramide
DTT	dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
GB4	Globosides
GD	Disialoganglioside
GM	Monosialoganglioside
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnTI	N acetylglucosaminyltransferase I
GQ1b	Ganglioside GQ1b
GT1b	Trisialoganglioside-GT1b
HexCer	Hexosylceramide
HFE	Hemochromatosis Protein
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HTA	Human Tissue Act
IEC	Intestinal epithelial cells
IEF	Isoelectric Focusing
IEX	Ion-exchange chromatography
IL 1 β	Interleukin 1 β

ILC	Innate lymphoid cells
ILT2	Ig-like Transcript 2 molecule, an NK cell receptor with type Ia and type Ib MHC binding properties
ImmTAC	Immune Mobilising Monoclonal TCR Against Cancer
iNKT cells	invariant Natural Killer T cells
IP	Immunoprecipitation
K _D	Equilibrium binding constant
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPCO-	Lysoalkyl-phosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPEO-	Lysoalkyl-phosphatidylethanolamine
LPG	Lysophosphatidylglycerol
LPI	Lysophosphatidylinositol
LPS	Lysophosphatidylserine
MHC	Major Histocompatibility Complex
mLPA	Methyl-lysophosphatidic acid
moDCs	Monocyte-derived dendritic cells
MR1	MHC class I-related protein
MS	Mass Spectrometry
NKT cells	Natural Killer T cells
PA	Phosphatidic acid
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PC-O	Ether-linked phosphatidyl-choline
PDB	Protein Data Bank
PE	Phosphatidylethanolamine
PEO-	Ether-linked phosphatidylethanolamine
PG	Phosphatidylglycerol
pHLA	peptide-Human Leukocyte Antigen Complex
PI	Phosphatidylinositol
pMHC	peptide-Major Histocompatibility Complex
PNGase F	Peptide:N-glycosidase F
PS	Phosphatidylserine
scFv	Single-chain fragment variable
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion
SM	sphingomyelin
SPR	Surface Plasmon Resonance
Sulf	Sulfatide
TAG	Triacylglycerol
T-ALL	T-cell acute lymphoblastic leukaemia

TCR
vdWs
 β 2m

T cell receptor
van der Waals
 β 2-microglobulin

2. INTRODUCTION

2.1 T cell driven immune responses

The innate and the adaptive immune systems are two complementary parts of the immune system. The innate immune system initiates quick, non-specific immune responses, recognizing signs/patterns of danger or damage, initiating further response and neutralizing pathogens. It consists of physical barriers, immune cells and inflammatory mediators. The adaptive immune system in contrast provides highly specific immune responses to a large variety of materials, as antigens through T and B lymphocytes, which results in immunological memory (1,2). T lymphocytes have a crucial role in maintaining health and combating disease (2–4). The recognition of peptide-major histocompatibility complex (pMHC) molecules by T cell receptor (TCR) on infected and malignant cells triggers strong immune responses (3,5). The major histocompatibility complex (MHC) molecules are highly polymorphic and can accommodate and present various peptide antigens (6,7). Similarly, T Cell Receptors (TCRs) show exceptional diversity, which is the result of the T Cell Receptor Gene rearrangement and random chain pairing, which occur during thymic development of T cells. This process generates 1×10^{18} possible $\alpha\beta$ TCR variants, establishing immense diversity of the immune system (8,9). Thymocytes, after undergoing TCR rearrangement, proceed through positive and negative selection in the thymic microenvironment, based on TCR recognition of self-peptides presented by MHC molecules (10–12). The selected double positive cells differentiate into MHC class II restricted CD4⁺ or MHC class I restricted CD8⁺ single-positive cells. The mature CD4⁺ T cells have a role in immune regulation and help other innate and adaptive cells of the immune system including the CD8⁺ T cells. The cytotoxic CD8⁺ T cells are responsible for eliminating infected or cancerous cells (13,14). The thymic selection during T cell maturation reduces the initial TCR diversity to 2.5×10^7 possible $\alpha\beta$ TCR variants within an individual (15,16). Naive T cells that express these antigen-specific TCRs continuously circulate between the bloodstream and the lymphoid organs. They can get activated by the cognate pMHC complex, however, due to their low numbers, they cannot provide immediate protection (3). Instead, the activation triggers the T cell clonal expansion and differentiation, generating a large effector T cell population within 7-10 days of antigen exposure (3,16). Some T cells differentiate towards memory T cells, which will respond quickly and strongly during subsequent antigen exposure (3).

2.2 Unconventional MHC molecules recognized by unconventional T cell populations

Since the MHC system was discovered in the 1940s, the MHC molecules are considered as the main driver of T cell activity. The MHC molecules are responsible for presenting antigens to T cells and initiating adaptive T cell responses. In mammals, the MHC molecules are categorized into Class I and Class II, based on their structure, expression patterns, and functional roles (17,18). Class II molecules are expressed on professional antigen presenting cells (APCs) and present exogenous antigens to CD4⁺ T cells (18). Class I is further divided into Class Ia and Ib (19). The human MHC class Ia molecules include HLA-A, HLA-B, and HLA-C. These molecules are highly polymorphic and ubiquitously expressed on almost all nucleated cells. Class Ia molecules present endogenous peptides to CD8⁺ T cells. Class Ib molecules contain HLA-E, HLA-F, HLA-G, and molecules such as CD1 family and MR1. These molecules have limited polymorphism and more restricted tissue expression. Class Ib molecules present a wide range of antigens, like lipids, metabolites and viral leader sequences (18). The field has gradually broadened with the discovery of certain $\alpha\beta$ and $\gamma\delta$ T lymphocytes, which are capable of recognizing non-polymorphic antigen presenting molecules (20–22). The genes encoding the Cluster of Differentiation 1 (CD1) family and MHC class I-related protein (MR1) antigen-presenting molecules are found outside the MHC locus (23,24), while Human Leukocyte Antigen E (HLA-E), Hemochromatosis Protein (HFE), the mouse H2-M3, and H2-Q9 are found within the MHC locus (25). These non-conventional antigen-presenting molecules do not presenting ‘conventional’ peptide antigens (with the exception of HLA-E), but they capture metabolites or lipids and are recognized by ‘unconventional’ T cells (20,22). Unconventional T cells can respond quickly and are widely distributed in the peripheral circulation as memory cells (26). Since they recognize non-polymorphic MHCs, they are not donor restricted. Unlike conventional T cells, antigen-specific unconventional T cells have a memory phenotype, mainly localise in non-lymphoid organs and have a smaller diversity of TCRs (26,27).

T cell biology research and immunotherapy strategies mainly target peptide antigens on the surface of the major histocompatibility complexes, such as MHC class I and II molecules (13). The development of tools such as tetramers (28–30) has expanded our knowledge of unconventional T cells and harnessing of monomorphic antigen presenting molecules represents an appealing universal immunotherapy strategy.

2.3 Cluster of differentiation 1 (CD1) molecules

A family of MHC class I-like antigen-presenting molecules called the Cluster of differentiation 1 (CD1) molecules presents exogenous and endogenous lipid antigens to T cells (31,32). Phylogenetically, CD1 molecules first appeared along with MHC molecules, B cell and T cell receptors in cartilaginous fish (33). Five isoforms (CD1a, CD1b, CD1c, CD1d and CD1e) evolved as a result of repeated duplication processes and the number of isoforms varies across vertebrate species (33). They are encoded on chromosome 1 of primates (34). They share a similar overall structure with MHC molecules, with heavy chains that have three extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) that bind to $\beta 2$ -microglobulin ($\beta 2m$) non-covalently (35). The lipid antigens are accommodated (35,36) in the hydrophobic amino acid lined antigen-binding pocket created by the $\alpha 1$ and $\alpha 2$ domains. The heavy chains of CD1 possess multiple N-linked glycosylation sites, which become glycosylated as they are transported through the Golgi apparatus (35,37). The CD1 family is divided into two major subgroups. The subgroups are based on structural and functional characteristics. CD1a, CD1b, and CD1c in group 1 activate adaptive T cells, while group 2 includes only CD1d, which activates subsets of T cells that exhibit innate-like functions (38). The fifth isoform, CD1e doesn't appear on the cell surface but assists in lipid loading on CD1b(39). While conventional MHC class Ia molecules are expressed on all nucleated cells, CD1 expression is predominantly restricted to "professional" antigen-presenting cells (APCs) and double-positive (CD4+CD8+) thymocytes (40). CD1a, CD1b, and CD1c show inducible expression in response to stimuli like GM-CSF or IL -1 β (41). CD1a molecules are present in high levels on dermal dendritic cells and Langerhans cells in the skin (42). CD1b is predominantly found on dendritic cells migrating to the lymph nodes and on myeloid-derived dendritic cells (43). CD1c shows the broadest expression pattern of the family. CD1c can be found on monocyte-derived dendritic cells (moDCs), Langerhans cells, and B cells under normal conditions (44,45). CD1d is expressed in several tissues (41), and a large variety of cells, including monocytes, dendritic cells (DCs), B cells, thymocytes, and innate lymphoid cells (ILCs). It can also be found on non-hematopoietic cells like intestinal epithelial cells (IECs), hepatocytes and keratinocytes (46). Although a few studies revealed minor polymorphisms of CD1a located in the promoter region, affecting the expression levels of CD1a, further polymorphism of CD1 isoforms have not been described (47). The NCBI SNP database lists several single nucleotide polymorphisms (SNPs) for CD1 genes both in regulatory regions (promoters) and coding regions (exons). Variants in exons that change amino acids

(missense) could theoretically alter CD1 protein structure or function, but whether they do, depend on their location and effect on folding, ligand binding, or TCR interaction. These variants all have a MAF < 0.05%, which are termed rare allele frequency, so they might represent population-specific or individual differences rather than common polymorphisms. Han et al. analyzed genetic polymorphism in all five human CD1 genes using PCR-SSCP and sequencing in 110 unrelated healthy donors. All five genes were polymorphic in exon 2, that encodes part of the antigen binding domain, however, the substitutions in CD1c gene were silent (did not change the amino acid sequence), meaning the protein structure is likely conserved (48). The non-polymorphic nature means that there are no genetic variations in humans - apart from CD1a that has two different alleles that differ by two amino acids - therefore the T cell mediated immune responses directed against the CD1 family members are not constrained by the genetic background of the patient. Furthermore, due to the inherently low mutation rate of lipid antigens, targeting them poses a reduced risk of tumour immune evasion compared to peptide-HLA-based approaches.

2.3.1 CD1 recycling and antigen presentation

The CD1 isoforms are synthesized in the endoplasmic reticulum (ER) and traffic through the Golgi before reaching the cell surface (35). The molecules are internalized and recycled through various endosomal compartments (35, 41). However, there are differences in their capacity to bind to lipids and traffic in the endosomal network. CD1a molecules can bind exogenous lipids at the cell surface, while CD1b, CD1c and CD1d are less efficient at lipid loading on the cell surface and recycle into the endosomes for lipid exchange (41). The cell surface-expressed CD1 molecules bind to Adaptor Protein (AP) complexes on the plasma membrane facing towards the cytoplasm. The cytoplasmic tails of CD1c and CD1d contain tyrosine-based motifs, specifically YXXZ (Y represents tyrosine, any amino acids can be at the X positions, and at the last position, Z stands for a bulky hydrophobic amino acid). These are required for binding to adaptor protein-2 (AP2) (35). CD1b is the only isoform, whose YXXZ motif can bind AP3 (35). The APs recognize the isoform-specific motif on the cytoplasmic tail, enabling the CD1 isoforms to be sorted and directed to the different sub compartments within the endosomal network (35). The CD1a cytosolic tail lacks the recycling motif utilized by AP-2 and AP-3; therefore, the molecules remain mainly at the surface and undergo ARF-6 dependent superficial recycling only in the early endosomes (49, 41). CD1a doesn't require acidic pH for lipid loading. CD1b traffics deeply into late

endosomes and lysosomes using both AP-2 and AP-3 (35, 41). CD1c recycles amongst the early and late endosomal compartments using AP-2 (41). CD1d traffics to late endosomes and lysosomes using AP-2 and possibly AP-3 (41, 35). CD1b uniquely requires acidic pH for loading long-chain lipids (41,50).

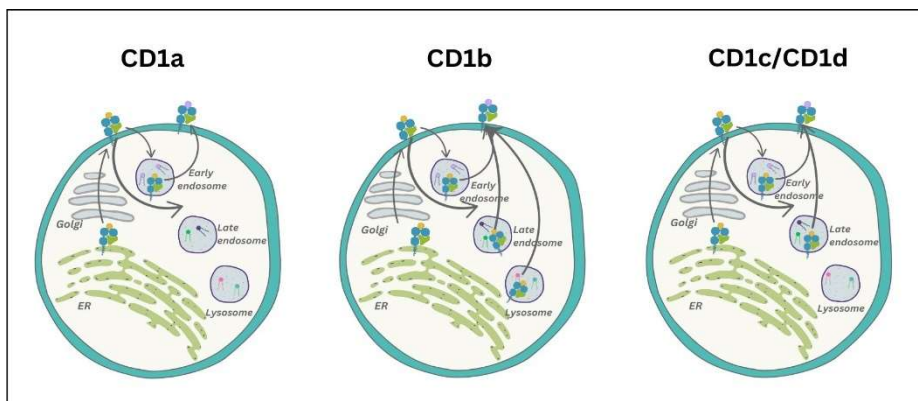


Figure 2.1: Intracellular trafficking of human CD1 isoforms.

The CD1 isoforms are synthesized, refolded and first loaded with endogenous lipids in the endoplasmic reticulum. They are then transferred to the cell surface through the Golgi. CD1a can primarily be found at the cell surface. CD1a recycles into the sorting and early endosomes. CD1c and CD1d are recycled into the early and late endosomes along with CD1b, which also enters the lysosomes. The overlap between the recycling route of CD1 isoforms might impact on the lipidome presented by the individual isoforms.

2.3.2 CD1 structure drives antigen selectivity

The antigen binding pocket of the different CD1 isoforms varies in depth and numbers of channels, properties that influence presentation of different lipids. All CD1 molecules have A', F' pockets and isoform specific additional "accessory" pockets and tunnels, each contributing to how lipids are bound and presented. The specific cellular distribution patterns of these proteins suggest that they utilize both separate and parallel pathways to survey lipid antigens in endosomal structures. The differences in groove size and architecture correlate with the types and sizes of lipids each isoform can present (37, 51) (Figure 2.2). CD1a has the smallest groove volume (1,350 cubic Ångström, Å³), followed by CD1d (1,400 Å³), CD1c (1,780 Å³), and CD1b with the largest volume (2,200 Å³) (52,53). The CD1a binding groove consists of two chambers, which are the A' and F' pockets. The A' pocket is formed by electrostatic interactions between the side chains of the A' roof. The Valine28 amino acid closes the bottom of the pocket, limiting the size of the antigen's alkyl chain, therefore, CD1a typically presents lipids with short or single hydrocarbon chains (51,54,55). Apart from CD1c, no other isoforms possess this roof structure. The CD1b antigen-binding groove is structurally unique, having four interconnected pockets (A', F', C', and T') compared to the

two pockets (A' and F') of other isoforms (52,56) The first CD1 crystal structure solved with bound lipid ligand was of human CD1b, revealing that the hydrophobic alkyl chains of the lipids are positioned well inside the binding pockets, while the hydrophilic head groups are exposed to the surface (57). The CD1b ATF super channel enables the binding of extremely long lipids which can reach 72 carbons in length (52). The CD1b groove is predicted to accommodate C72-76 lipids, but surprisingly, it can also bind C80-86 glucose mycolates, suggesting that lipids may protrude through the C' portal (58,59). CD1b can also bind multiple lipids simultaneously, with smaller lipids having a spacer/scaffold role (60) The two hydrophobic antigen-binding pockets, A' and F', are also present in CD1c. The solved structure in complex with mannosyl-phosphomycoketide demonstrated that the branched lipid is located within the CD1c A' pocket with its five methyl branches in the alkyl chains pointing outwards (61,62). This unique binding mechanism allows CD1c to specifically recognize and present two known mycoketide lipids, mannosyl-phosphomycoketide and phosphomycoketide, as mycobacterial antigens. CD1c can also bind a lipopeptide with a single alkyl chain (63), which is a common feature of the three known exogenous antigens that are captured by CD1c. The A' pocket and the F' pocket are essentially continuous, and open externally across an accessory portal called the D portal, which is a distinctive feature of CD1c (52). Crystal structure revealed that the binding groove is suitable for co-presenting two lipids, with the mycoketide backbone accommodated in the A' pocket and a C12 hydrocarbon chain found in the F' pocket (62). The CD1d binding groove consists of an A' and an F' pocket without accessory portals. It was crystallized first in complex with the high-affinity agonist, α -galactosylceramide (64). The positioning of the alkyl chain is driven by conserved polar contacts between specific amino acids of the CD1c main chain (Asp80, Asp153, Thr156) and the polar residues of the lipid backbone. Hence, the acyl chain is situated within the F' pocket, irrespective of its length. Alkyl chains of 16-18 carbon length can ideally be accommodated in the F' pocket, like sphingosine, however, it can also harbor slightly longer chains as well. α GalCer analogues with shorter alkyl chains require co-presentation with spacer lipids in both A' and F' pockets, thereby ensuring optimal lipid binding (65).

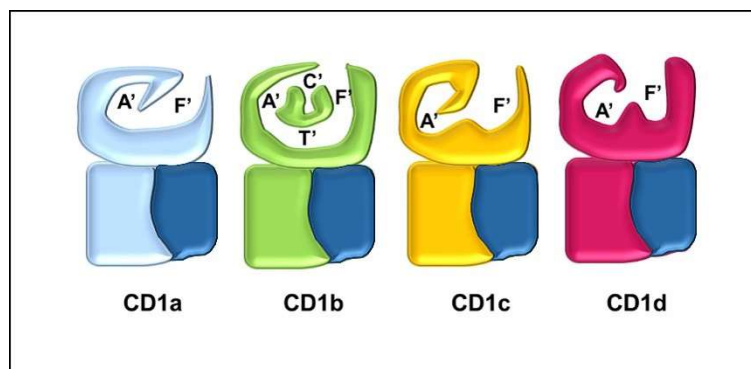


Figure 2.2 Structural differences in the CD1 lipid-binding grooves

The figure demonstrates the lipid-binding grooves of CD1 isoforms (CD1a, CD1b, CD1c, CD1d). B2m is depicted with dark blue. All isoforms feature A' and F' pockets. CD1a has a relatively shallow groove with an A' roof. CD1b features deep, interconnected pockets, accessory portals and a T' tunnel. CD1c has a flexible binding groove also featuring a roof structure and an accessory portal open externally called the D portal (not shown). CD1d has an A' and F' pocket without accessory portals.

2.3.3 The CD1c-associated lipid-antigen repertoire

A major unmet challenge in the CD1c field is to understand the range of lipids that can be presented by this molecule. In particular, whether there exist any cancer specific/selective lipid that might allow the development of more targeted cancer drugs. To date, the only CD1c cancer-associated lipid antigen identified is methyl-lysophosphatidic acid (mLPA) (66). It is a self-lipid that was found in leukemic cells such as primary human leukaemia, and importantly, it was not present in healthy monocytes and B-cells (66). Although CD1c-mLPA is a possible target in leukaemia, it is not known how heterogeneous this molecule is in terms of expression across different patients, and expression within individual tumors. Thus, a deeper insight into the CD1-associated lipidome will reveal the pattern of lipid presentation on tumors versus healthy cells and may uncover other possible lipid targets. Currently there are conflicting reports about the CD1c-associated lipid repertoire. Several research groups have applied different experimental methodologies to isolate CD1 complexes from cells, and to dissect the CD1-associated lipidome. Self-lipid antigens presented by CD1a, b and c molecules to T-cells were reported, however, the studies only characterised glycosphingolipids, sulfatides, and gangliosides (GM1, GD1a, GD1b, GT1b and GQ1b) (58,60,62). In contrast, Huang et al. identified a much larger variety of self-lipids, including multiple phospho- and glycolipids, captured by soluble CD1 molecules produced in human cells (50). The lack of consensus on CD1-associated antigen repertoire means there is still a need to develop a better system for characterization of CD1-associated lipid antigens.

2.3.4 TCR recognition of CD1-lipid complexes

T cell receptors on T cells recognize CD1-lipid complexes through two possible mechanisms: either dual recognition of CD1 and lipid called the Head Group Discrimination Model - where TCRs recognize the protruding polar head groups of the lipids, similarly to peptide-MHC recognition by $\alpha\beta$ TCRs - or direct TCR contact with CD1, called the Absence of Interference Model, where TCR binding occurs without the classical lipid head group interactions (32,67,68). CD1a-restricted TCRs often recognize the CD1a molecule itself without directly contacting the lipid antigen (49). This has been shown with TCRs that bind over the A' roof of CD1a without contacting the lipid head, in the "absence of interference" model (49). Certain lipids, like sulfatide and sphingomyelin, can block TCR interactions by altering the structure of the A', thus acting as non-permissive (35). A notable population of autoreactive $\alpha\beta$ T cells recognizing CD1a is present, estimated at 0.02% to 0.4% of memory T cells in the blood. These are involved in skin homeostasis, secreting IL-22, which promotes the release of antimicrobial peptides from epidermal keratinocytes (33). CD1b plays an important role in presenting mycobacterial antigens (50). Tetramer technology has revealed a specific T cell population that recognizes mycobacterial lipids, in a CD1b restricted manner (50). Autoreactive CD1b specific T cells also exist but are less frequent compared to those responding to CD1a (33). Like CD1a and CD1b, CD1c also elicits autoreactive T cell responses, with higher frequencies among T cells derived from both adult and cord blood. Some CD1c reactive T cell clones recognizing various mycobacterial glycolipids were identified by Roy et al (51). CD1d lipid-specific iNKT cells are well studied, and evidence is emerging of other T cell populations reactive to CD1d, like $\gamma\delta$ T cells (52). For example, there is a significant number of $\gamma\delta$ T cells in blood that are reactive to CD1d-sulfatide complexes (55). In contrast to TCRs showing high specificity towards a single antigen, some CD1c reactive TCRs are polyspecific and recognize several CD1c-bound self-lipid antigens (56). These TCRs contact the CD1c protein's surface rather than the presented lipids. Wun and colleagues in 2018 demonstrated that the 3C8 $\alpha\beta$ TCR binds to CD1c itself rather than exposed lipid headgroups, showing a deviation from traditional models of TCR-antigen recognition.

2.4 CD1 restricted T cells in disease

Several studies have been focusing on the role of autoreactive T cells in diseases. CD1-restricted autoreactive T cells have been reported to be activated by self-lipids presented by

the CD1 family (40,69–71). De Lalla et al. found an unexpectedly high frequency (between 1 in 10 and 1 in 300) of circulating T cell being CD1-autoreactive (72). Subsets of CD1c-restricted T cells recognise self-lipids as well as bacterial lipids which has been speculated to have implications in autoimmune diseases (73). CD1-autoreactive T cells were reported to infiltrate the thyroid organs in Graves' disease and Hashimoto's disease (74). CD1c positive B-cells and CD1a, CD1b and CD1c positive dendritic cells can infiltrate diseased thyroid glands, leading to tissue damage, due to inflammation. CD1 autoreactive T-cells can lyse CD1a and CD1c positive thyroid target cells (37,74). CD1a restricted T cells have also been involved in allergic skin diseases and psoriasis (75–77). Several studies have demonstrated a role for CD1c-mediated responses in host immunity to TB infection (47,61,78,79). It has also been shown that CD1-restricted T-cells are also involved in tumour surveillance (66,80). These T-cell clones that have been found in multiple donors, were able to recognise CD1c-expressing leukaemia cell lines presenting methyl-lysophosphatidic acid (mLPA), a self-lipid enriched in leukaemic cells but not present in healthy tissue such as monocytes or B-cells. The CD1c mLPA- specific TCRs identified by Lepore et al. is being developed to treat acute myeloid leukaemia by adoptive T-cell therapy (66).

A previous study suggests that autoimmune diseases, for example systemic lupus erythematosus, psoriasis, diabetes, and rheumatoid arthritis, can be associated with altered blood lipid content or dyslipidaemia (81). Self-peptide specific autoreactive T cells can aggravate or attenuate the inflammation commonly cause by altered lipid levels and autoimmune diseases, however, the function of self-lipid-specific T cells and their role in dyslipidaemia related autoimmune diseases is not well understood (81,82). Some data suggest that in systemic lupus erythematosus patients, pathogenic autoantibody responses caused by Immunoglobulin G production are triggered by the help given by CD1c-restricted T cells to CD1c+ B cells (83,84). Psoriasis patients also often exhibit altered lipid levels (85,86), where CD1-restricted T cells could be driving inflammation in response to lipid antigens (77,81,87). Similarly, in Rheumatoid Arthritis, CD1-restricted T cells may be responsible for disease progression by contributing to the inflammation (81,88). Targeting lipid metabolism or modulating the CD1-restricted T cell activity could be a strategy for treating diseases with altered lipid metabolism (81).

2.5 Harnessing Soluble T Cell Receptors for Therapeutic Use

The natural receptor of peptide-Human leukocyte Antigen complexes (pHLA) and CD1 molecules is the T cell receptor (TCR). The TCRs are transmembrane proteins consisting of an α and a β chain. These chains comprising two domains, the constant domain (C) and the variable domain (V) (89,90). Structural studies revealed that the TCR's α and β variable domains directly interact with the α -helices that surround the binding groove of the pHLA, and the presented protruding peptide-antigen (91,92). The specificity of the TCR interaction is determined by three variable regions, the complementary determining regions (CDR) of CDR1, CDR2, and CDR3. The CDR1 and CDR2 regions are coded by the V gene segment in the germline, whereas the CDR3 hyper-variability is generated by adding or removing nucleotide bases at V(D)J junctions (93). In general, the CDR3 loop contacts the peptide surface and the CDR1 and CDR2 loops bind to the pHLA helices (94). TCRs can be harnessed as therapeutics either as cellular therapies or as off-the shelf soluble bispecific engagers (95–99). A major challenge in engineering soluble TCRs for therapy is that natural TCRs have poor stability in soluble format and show low affinity for pHLA complexes (94). Furthermore, in order to develop them as therapeutic molecules, their biochemical properties such as low expression yields, misfolding and aggregation have required development. Immunocore Ltd. solved the stability and expression quality issues by introducing an intermolecular disulfide bond between the TCR constant domains (94), and employed a phage-display based affinity maturation technique to enhance the TCR binding affinity (94,100,101).

The engineering of soluble TCRs led to the development of soluble therapeutic molecules targeting pHLA complexes by Immunocore Ltd. The ImmTAC (Immune-mobilizing monoclonal TCRs Against Cancer) molecules were developed to contain a monovalent affinity-enhanced TCR (KD ~ pM range) joined to a monovalent anti-CD3 single-chain Fc antibody fragment of the UCHT1 antibody (102,103). The ImmTAC targeting arm (high-affinity TCR) binds to the pHLA on the target cell surface, thus decorating the tumor cells with anti-CD3 effector molecules. The anti-CD3 molecules engage with the T cells, regardless of their endogenous TCR specificity, resulting in the redirected killing of tumor cells even at low-level presentation of the target pHLA (103). The first ImmTAC, Tebentafusp, has been approved by the FDA in 2022 for the treatment of uveal melanoma and targets gp100-HLA-A2 complexes (104–106).

2.6 Targeting HLA molecules limits patient reach

Human leukocyte antigens (HLA) are attractive therapeutic targets because they can display short peptides from the majority of all cellular proteins on the cell surface, providing a window into cellular states and functions, such as infection or dysregulation such as what is observed in cancer (107,108). T cell receptors (TCRs) can initiate potent immune responses against infected or dysregulated cells through their recognition of peptide–HLA complexes (93). As a result, multiple therapeutic strategies—both cellular and protein-based—have been developed to exploit HLA–peptide complexes as targets for redirecting T cell activity toward diseased cells (107,108). However, the extreme polymorphism of HLA molecules poses significant challenges. The majority of HLA-targeted therapies have been developed with a focus on HLA-A*02:01, the most prevalent allele among individuals of Caucasian descent, expressed by approximately 47% of this population, restricting their applicability to a limited patient cohort (109). In contrast, CD1c presents a potentially advantageous target for therapeutic development. Its restricted expression pattern and monomorphic nature make it a suitable therapeutic target candidate (110). CD1c is mainly present on thymocytes, B-lymphocytes and monocyte derived antigen-presenting cells. While this restricted expression may limit the types of cancers that can be targeted through CD1c, it represents a promising target for blood cancers where its limited expression should reduce the likelihood of toxicity towards healthy tissues. Recent studies indicated that CD1c-restricted T cells can specifically recognize leukemic cell lines through a CD1c-dependent mechanism, and CD1c has been shown to be present in a significant number of acute myeloid leukaemia patient samples, as well as in T-cell acute lymphoblastic leukaemia (T-ALL) and B-cell acute lymphoblastic leukaemia (B-ALL) patients, with expression rates ranging from 50 to 75 percent (57). Because CD1c is not present on hematopoietic progenitor cells, targeting the CD1c-expressing populations therapeutically would enable bone marrow-derived precursors to regenerate the hematological compartment. In conclusion, the development of TCR based bispecific T cell engagers that target CD1c molecules could lead to a new generation of novel therapeutics that bypasses HLA restriction and has minimal off-target toxicity that would otherwise compromise the overall health of the individual.

3. AIMS

The overall aim of this PhD was to develop CD1c targeting bispecific engagers to target hematological malignancies. To achieve this, an initial characterization of the CD1 lipidome was performed.

3.1 Dissect the lipid antigen repertoire associated with CD1 complexes in mammalian cells and cancer cell lines

This part of the study is focused on the lipid antigen repertoire associated with CD1 complexes in mammalian cells and cancer cell lines. CD1 molecules were produced in mammalian expression system, where they are associated with a naturally occurring mixture of lipids. A comparative lipidomics analysis was conducted, by using lipid mass spectrometry to identify and quantify the lipids associated with CD1 isoforms. The aim of the study is to gain insights into the range of lipids that can be presented by the CD1 isotypes and to determine if any cancer specific lipids can be identified in the CD1c lipidome.

The project followed a systematic approach composed of three main steps:

1. Produce soluble CD1 molecules in Expi293F cells. Optimize the mammalian expression system to produce soluble CD1 molecules in mammalian cells (secreted into the media), ensuring the elution of diverse lipids that can be associated with these CD1 complexes.
2. Generate CD1c-expressing cell lines in relevant cancer cells and purify CD1c for mass spectrometry.
3. Mass spectrometry analysis of CD1-associated lipidome. Perform mass spectrometry analysis to identify the CD1-associated lipidome isoforms and CD1c molecules expressed in cancer cells.

This data will contribute to our current knowledge of antigen presentation by the CD1 family and its role in cancer immunology.

3.2 Molecular recognition of CD1c

The molecular basis of TCRs recognition of CD1c-lipid complexes is poorly understood. In fact, there is currently only one TCR-CD1c co-complex crystal structure published (111). There is evidence that T lymphocytes recognize CD1-lipid complexes following the MHC-peptide co-recognition paradigm (i.e. CD1c restricted TCRs require a specific lipid for binding) (112). However, other studies suggest that any APC, expressing the correct CD1 molecules, can directly activate T cells in a ligand dependent or independent manner (111,113). Other data suggest something in between. For example, Chancellor and colleagues (111), have shown that lipid binding induces major changes in the CD1c protein structure suggesting that CD1c bound lipids serve primarily to remodel TCR binding surfaces on CD1c, although direct structural evidence for this model is lacking. These assumed different modes of TCR-CD1c-lipid recognition have important implications in the development of molecules that target CD1c for therapeutic purposes.

The project's methodical approach:

4. Generate soluble CD1c and CD1d molecules bound to specific lipids. Develop methods to load specific lipids into CD1 molecules generated by mammalian expression (which will present endogenous lipids). Validate the ligand loading by using biochemical methods and cellular assays.
5. Isolate CD1c lipid-specific TCRs. Select TCRs in TCR phage libraries by using soluble CD1-lipid complexes and employ lipid-loaded CD1c tetramers to identify antigen-specific T cell clones. The identified TCRs were further characterized as possible candidates for TCR affinity enhancement.
6. Uncover the molecular basis of CD1-lipid TCR recognition. Investigate the molecular mechanism of TCR engagement with CD1-lipid complexes and study the biophysical properties of the TCR-CD1c interaction. Crystal structures of TCR- CD1 complexes provided a powerful tool to study the principles of CD1-TCR binding. Specifically, to better understand the interaction between the TCR and the CD1c lipid-cargo and uncover whether such interactions enable TCRs to be specific to a single lipid, or a family of lipids. This will be important for selecting TCRs for therapeutic purposes, as well as for better understanding the nature for CD1c antigen recognition in general.

Generate CD1c lipid-specific high affinity TCRs. This aim provided a deeper understanding of the mechanisms underpinning CD1c-lipid antigen presentation and recognition by lipid-antigen-specific TCRs. The information generated by the previous aims was used to design structurally guided phage panning libraries to mutate CD1c lipid-specific TCRs to generate enhanced affinity TCRs. The tools and understanding generated during this project allowed assessment of the lipid-specificity of these engineered TCRs during affinity maturation.

Ultimately, the vision of this work is to build up a better understanding of the nature of CD1c-lipid presentation, and TCR recognition, and translate this knowledge into the generation of bispecific TCR based reagents that can discriminate between healthy cells and cancer cells by targeting cancer specific CD1c-lipid antigens during T-cell re-direction.

4. MATERIALS AND METHODS

4.1 Lipids used

Abbreviation	Explanation	Distributor	Cat. No.
PC	Phosphatidylcholine	Avanti Polar Lipids	840055C
PI	Phosphatidylinositol	Avanti Polar Lipids	840042C
PG	Phosphatidylglycerol	Avanti Polar Lipids	841138C
PS	Phosphatidylserine	Merck Life Science UK Limited	840032C
16:0-17:0 CYCLO PC	16:0-17:0 CYCLO Phosphatidylcholine	Merck Life Science UK Limited	857501P
SM	Sphingomyelin	Avanti Polar Lipids	860584P
GD1a	Disialoganglioside GD1a	Sigma	G2392
GD2	Disialoganglioside GD2(NH ₄ +salt)	Matreya LLC	1527
GD3	Disialoganglioside GD3(NH ₄ +salt)	Matreya LLC	1504
GM1	Monosialoganglioside GM1 (NH ₄ +salt)	Matreya LLC	1061
GM2	Monosialoganglioside GM2 (NH ₄ +salt)	Matreya LLC	1502
GM3	Monosialoganglioside GM3 (NH ₄ +salt)	Matreya LLC	1503
GT1b	Trisialoganglioside-GT1b	Sigma	G3767
GB4	Globosides	Matreya LLC	1068
α GalCer	α -Galactosyl ceramide	Abcam	ab144262
KRN7000	Synthetic α -Galactosyl ceramide	Merck Life Science UK Limited	867000P
C16 LPA	Palmitoyl lysophosphatidic acid	Avanti Polar Lipids	857223

4.2 Molecular cloning and expression of soluble CD1c molecules

The $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains of human CD1a, b, c and d proteins were N-terminally joined to the human $\beta 2m$ using glycine-serine linker (GGGGSGGSGSGGGSS). The CD1 heavy chain and $\beta 2m$ was linked to a C-terminal Avi-TagTM (GLNDIFEAQKIEWHE) followed by a 6xHis tag. The construct was produced in a single chain construct using GeneArt Gene Synthesis (Invitrogen) services and cloned into the mammalian expression vector (pCDNA3.1) (Figure 4.1). The vector contains the BirA enzyme gene that enables the biotinylation of the expressed CD1c protein (114,115) (Supplementary Figure 14.1). 1 μ g plasmid per ml of culture was used to transfect the Expi293F cells (Thermo Fisher Scientific A14527) according to the ExpiFectamineTM 293 Transfection Kit manufacturer's instructions (Life Technologies A14636). The optimal expression time for CD1c monomers bound to endogenous lipids (CD1c-endo) were five days post-transfection. The protein was purified from the medium by affinity chromatography using 5 ml HisTrap excel column (Cytiva 17371205) and a subsequent Size Exclusion chromatography purification using a Superdex200 10/300 increase column (Cytiva 28990944) as a final polishing step for separating aggregates.

CD1c and CD1d molecules for in vitro lipid-ligand loading were expressed in Expi293FGnTI- cells (Thermo Fisher Scientific A39240). The Expi293F GnTI- cell line is derived from Expi293F cell line that is N-acetylglucosaminyltransferase I (GnTI) deficient, therefore the CD1 molecules expressed in these cells lacking complex N-glycans on the four glycosylation sites. CD1 molecules with less complex glycosylation are more suitable for partial denaturation at low pH that makes the ligand binding groove more accessible for ligand exchange.

For crystallography purposes soluble CD1c was produced in specific cellular protein expression system and post treatment, since while glycosylation is often essential for proper protein folding and stability, its structural complexity poses challenges for glycoprotein crystallization (116,117). To overcome the challenge presented by glycosylation during crystallization, the CD1c protein was transiently expressed in mammalian cells alongside Mannosidase I enzyme inhibitor Kifunensine (Merck, K1140-1MG), yielding homogeneous high mannose type N-linked glycans. This approach promotes proper protein folding while rendering the glycan chains susceptible to enzymatic trimming using PNGaseF (NEB P0704S). PNGase F cleaves nearly all N-linked glycans by hydrolyzing the bond between the innermost N-acetylglucosamine (GlcNAc) and the asparagine residue in both high-

mannose and complex glycan structures. CD1c construct for crystallization was generated with a C-terminal 6xHis tag that was cleaved off by TEV protease (NEB P8112S) after protein purification.



Figure 4.1 Schematic overview of the dual expression constructs of CD1 isoforms with BirA enzyme to produce soluble CD1 molecules.

CD1 construct N term/C term: CMV promoter; IL2 leader sequence; B2m microglobulin, glycine-serine linker (GGGSGSGSGSGGSS); CD1a/b/c/d protein $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains, rigid peptide linker (PPTPSTPPT); Avi-TagTM; 6xHis-tag. BirA construct N term/C term: Ig K leader sequence; FLAG-tag; glycine-serine linker (GGGSGSGSGSGGSS); BirA biotin ligase, KDEL ER retention signal sequence. The BirA ligase is retained into the ER providing increased biotinylation efficiency of the produced CD1 proteins.

4.3 In-vitro ligand exchange

50 molar excess of lipids were added into glass vials, and the organic solvent was evaporated using a nitrogen stream. The lipids were re-solubilized in ligand exchange buffer (0.5% CHAPS containing 50nM Sodium Citrate buffer, pH 6.0) and were sonicated at 37°C for 45 minutes 45KHz, normal mode. CD1c-endo or CD1d-endo monomers were added to each vial and incubated at 37°C O/N. The next day the volume of each vial was concentrated in Vivaspin 500 concentrators (Sartorius, VS0102) and the CD1c and CD1d molecules were purified on Zebra Desalting spin columns (Thermo Fisher, 89882). The success of ligand exchange was assessed by Isoelectric Mobility Shift Assay (IEF gel pH 3-7; Invitrogen 10405935). The pH of the ligand exchange buffer had a great effect on the efficiency of the ligand exchange, and best ligand exchange results were observed at pH 6.0.

To validate the ligand loading success by TCR binding, CD1c molecules were pre-treated with ligand exchange buffer (vehicle) and subsequently loaded with CD1a lipid. Three consecutive days of pre-treatment and CD1a loading were performed and the molecules were analysed by SPR. The CD1 molecules were purified on desalting columns before the SPR experiments. Binding analysis was conducted on a CM5 sensor chip using a BIAcore® 3000 (GE Healthcare, Buckinghamshire, U.K.). All flow cells were coated with 5000 response units (RU) of streptavidin. Approximately 2000 RU of CD1c-lipid complex

was bound to the sensor chip at a 10 $\mu\text{L}/\text{min}$ flow rate for even coverage of the chip surface. One flow cell was left blank for a negative control. 8 serial dilutions (1/2) of the TCR stock of 0.7mg/ml were injected into the sensor chips at a flow rate of 30 $\mu\text{L}/\text{min}$ at 25°C via kinetic injections. The results were analysed using BIAevaluation 3.1 (GE Healthcare).

4.4 Isoelectric focusing

Isoelectric focusing was performed as described before by Kempna et al. (118,119). CD1 protein was mixed 1 : 1 with isoelectric focusing (IEF) loading buffer and equal amounts were loaded on isoelectric focusing gels (Fisher Scientific, Ready gel IEF-polyacrylamide gels, pH 3 ± 7 , 10405935). Gels were run for 1 h at 100 V, 1 h at 250 V and 30 min at 500 V, fixed with 12 % of Trichloroacetic acid (Sigma-Aldrich; T6399), and stained using Coomassie Blue Stain (Abcam; ab119211).

4.5 Native mass spectrometry

Native mass spectrometry describes the analysis of protein and protein complexes under conditions where the analytes in solution are in their native state throughout the analysis (120–122). This allows mass spectrometry (MS) to be coupled to existing non-denaturing analytical chromatographic separations such as size-exclusion (SEC) and ion-exchange (IEX) techniques – both anion-exchange chromatography (AIEX) and cation-exchange chromatography (CIEX) - which historically have not been routinely coupled together. 10 μg protein/sample in standard phosphate buffered saline (PBS) was pipetted into glass vials. 5 μg protein/sample was loaded onto the SEC column. The SEC-MS Method LC Parameters are listed in table 4.1.

Multi-wavelength detection	214 nm, 280 nm
Column temperature	25°C
Flow rate	0.3 mL/min
Mobile phase	25 mM ammonium acetate
Injection volumes	5 μg column load (20-30 μL at 0.2 mg/mL); 5 μL for mobile phase blanks
Run time	20 min
Column	Waters Protein BEH SEC (4.6 x 300 mm, 1.7 μm , 200 Å)
Sample temperature	5°C

Table 4.1: SEC-MS Method LC Parameters

The SEC peaks from 4ml to 14ml are sent to MS for analysis. The MS analysis parameters are listed in table 4.2.

Polarity	Positive
In-source CID	80 eV
Microscans	10
Resolution	17,500
AGC target	3e6
Maximum IT	200 ms
Scan range	m/z 2500-6000
Sheath gas flow	20
Aux gas flow	5
Spray voltage	4.00 kV
Capillary temp	225°C
Aux gas heater temperature	150°C
S-lens RF	100

Table 4.2: SEC-MS Method MS Parameters

Raw data was deconvoluted using PMI-Byos (Protein Metrics) software. The data was processed using Chromeleon software (Thermo Fisher Scientific).

4.6 CD1c-endo tetramers and dextramers

Fluorescently labelled CD1c and CD1d tetramers (123) were generated by incubating biotinylated CD1c and CD1d monomers with PE-conjugated streptavidin (Invitrogen 12-4317-87) or Allophycocyanin-conjugated streptavidin (Biolegend 405207) tetrameric protein. The streptavidin subunits were oversaturated with 25% excess of biotinylated CD1 monomers. CD1 dextramers were generated using APC-labelled SA-Dextramer (Immudex DX01K AP 1000). The dextramer backbones were oversaturated with CD1 monomers.

4.7 Immunoprecipitation (IP) and Western Blot Analysis of CD1c from cell lines

Antibody coupling to Dynabeads: 20µg of CD1c specific capture antibody (L161, Abcam, ab190305) were coupled to 50µl of Protein G magnetic beads (Thermo Fisher, 88848) and the volume was adjusted to a final of 200µl with PBS pH7.4. The binding was left in rotator at 4°C, overnight. The IP was performed with PBS buffer. (It is a concern that Tween 20 can remove lipid ligands from the CD1c groove, hence it is preferable to avoid using detergents during IP.)

Soluble CD1c IP: 10µg of CD1c-endo produced in Expi293F cells was diluted into 200µl of PBS and transferred to the antibody coupled beads. 10µl of input material were kept for

analysis. The beads were incubated for 1 hour. After incubation, 10 μ l of reciprocal flow-through material was kept and the beads were washed three times with 1ml of PBS. The beads were re-suspended in 20 μ l Laemmli buffer (Bio-Rad, #1610737EDU) containing dithiothreitol (DTT), boiled at 95°C/5min to elute the immunoprecipitated protein.

Cell lysis: 500 μ l of 2x Lysis buffer (1% NP-40, 300mM NaCl, 100mM Tris-HCl, pH 8.0) with Roche protease inhibitors; 2tablets/50ml was used directly to the cell pellet and cells were left on ice for 60 minutes. The extracts were spun at 17,000 x g for 10 min and the supernatant transferred to fresh tubes. 10 μ l of input material were kept for analysis and 100 μ l of cell lysate was transferred to pre-coupled and washed magnetic beads.

IP from cell lysate: The magnetic beads were incubated for 1hr at 4°C in rotator. 10 μ l of reciprocal flow-through material was kept for analysis and washes of 1ml followed with buffers A to D using a magnetic rack. Buffer A (150mM NaCl, 50mM Tris, 5mM EDTA, 0.05% NP-40, pH 8.0), buffer B (150mM NaCl, 50mM Tris, pH 8.0), buffer C (450mM NaCl, 50mM Tris, pH 8.0), buffer D (50mM Tris pH8.0). Beads were resuspended with 20 μ l of Buffer D and equal volume Laemmli buffer containing DTT. To elute, the beads were boiled at 95°C/5min.

Western blots: The samples were run on 4-12% SDS-PAGE gel and the gel was blotted for 1hr/25V; the PVDF membrane then was blocked with 6 ml of PBS, 0.1% Tween-20 (PBST) and 5% skimmed milk powder for 1h. The membrane was washed 3x with PBST. CD1c was detected by staining with anti- β 2m-HRP antibody in 1:1000 dilution overnight at 4°C and developed with Pierce ECL Western Blotting Substrate (Fisher Scientific, 10590624).

4.8 TCR discovery by Phage display

To obtain TCRs specific to CD1c, phage display technology was employed as a powerful method for screening and isolating antigen-specific T cell receptors (100,124,125). Phage display involves the generation of random TCR α - and β chain libraries of TCRs displayed on the surface of bacteriophages, which are then screened against CD1c-endo molecules to find TCRs binding specifically to the antigen. 100 nM of biotinylated CD1c-endo molecules were captured on streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin-coated magnetic beads, Invitrogen, 11206D). A library of purified phage particles was

incubated with the beads to screen for specific phage particles that bind to the CD1c molecules. The phage particles were pre-blocked in a 3% MPBS buffer (PBS containing 3% Marvel milk powder), which helps to minimize non-specific binding. The beads were then washed to remove non-bound phage. Electrocompetent TGI *E. coli* cells (Biosearch Technologies, 60502) in early log phase were infected with the trypsin eluted CD1c-endo-bound phage particles. The TGI *E. coli* cells are commonly used in phage display due to their rapid growth and the presence of an F' episome, which enables infection by the M13 bacteriophage. Furthermore, these cells carry the *supE* amber suppressor mutation, enabling them to read UAG “amber” stop codons as glutamine. This allows fusion to the phage viral coat protein (PIII) and the expression of full-length proteins from phagemid vectors. The infected cells were then plated onto YTEag plates and incubated at 30°C for 16 hours. YTEag plates contain YTE (10 g tryptone, 5 g yeast extract, 8 g NaCl, 15 g Bacto-Agar in 1 L MQ-water) and 100 µg/ml ampicillin, and 2% glucose. Following infection, the cells were plated out at different dilutions on titration plates to calculate the size of the libraries. The rest of the library was plated on large, square YTEag plates. The large surface area of these plates maximizes the number of clones and therefore the diversity of the library. This process was repeated for a total of three rounds of selection, allowing for the enrichment of phage particles that have a higher affinity for the CD1c-endo molecules. After each round of panning a small number of clones were sequenced and the library titres were calculated indicating the nature of the phage population being enriched. Performing multiple rounds of selection increased the chances of identifying phage particles that specifically interact with CD1c receptors (Figure 4.2).

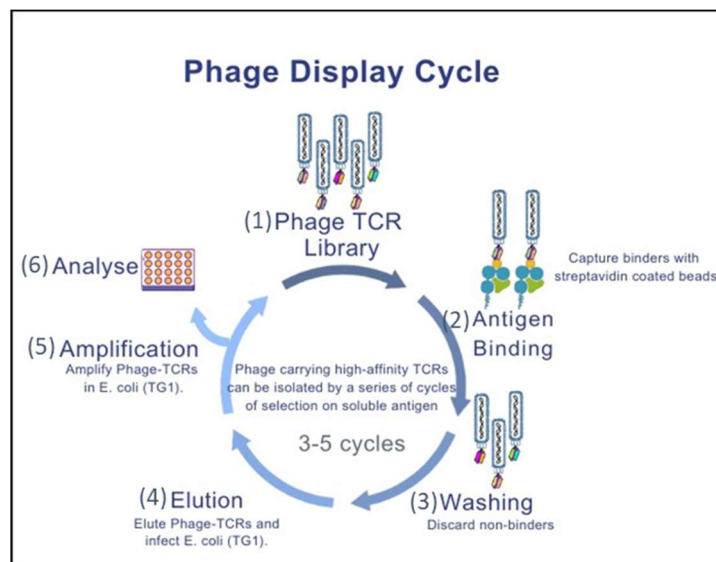


Figure 4.2: Phage panning of TCR libraries.

(1) Biotinylated-pHLA is incubated with purified phage, which displays the TCRs on their surface. (2) Phage (TCR):biotinylated-pHLA (antigen) complexes are formed. Magnetic beads coated with streptavidin are added and bind to ('rescue') the phage (TCR):biotinylated-pHLA complexes. (3) Washing the bead-bound phage removes non-specific binders. (4) Following trypsin cleavage, the eluted phage is used to (5) re-infect TG1 cells and plated on suitable media. (6) This enables panning output determination, colony PCR (cPCR) amplification and sequencing of clones.

4.9 MAGPIX peptide screening

To assess TCR cross-reactivity, we employed a MagPLEX bead-based assay (Invitrogen, UK), following previously established protocols (126). To thoroughly examine TCR specificity and selectivity, the receptors were challenged with a broad spectrum of HLA-peptide and CD1-lipid complexes. Biotinylated HLA-A*02:01 and HLA-A*01:01 molecules were refolded with representative peptides, while CD1 molecules containing endogenous lipids were produced in Expi293F cells. The pre-selected bacterial virus M13 displayed phagemid-encoded CD1c specific TCR molecules fused to capsid protein pIII on its surface, which then bound to the biotinylated peptide-HLA complexes and CD1 isomers immobilized on neutravidin-conjugated MagPLEX™ magnetic beads and were used in the assay. Positive beads were detected with M13 phage specific R-Phycoerythrin (PE) -conjugated monoclonal antibody (RL-ph2, 2B Scientific Limited, cat# MUB0604). Positive binders were identified by generating signals that exceeded background levels. The background threshold was defined as three times the median signal intensity of bead regions bound by the native helper phage. Positive binding was determined by calculating the percentage of signal attributable to binding with the index peptide. The experiment was performed using triplicates and mean values were calculated.

4.10 TCR and ImmTAC Construct design

The TCR chains were cloned into the pLAS0729 and pLAS0730 vectors. Vector maps can be found in supplementary data (Supplementary Figures 14.2 and 14.3). TCR constructs contain the variable and constant domains of the α and β chains and the chains are stabilized with a disulphide bridge (127). TCR constructs for functional experiments were reformatted into ImmTAC format (Figure 4.3). In the ImmTAC constructs, the TCR β - chain is fused with an anti-CD3 scFv, derived from UCHT1 mouse monoclonal antibody, using pLAS536 vector (Supplementary Figures 13.4) as previously described Liddy and colleagues (ImmTAC molecules, total size 75kDa) (103).

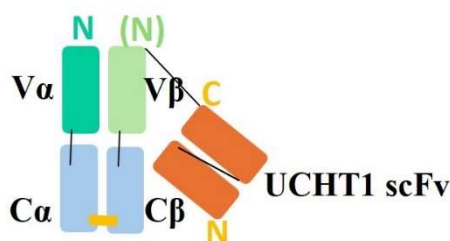


Figure 4.3 ImmTAC molecule structure.

The TCR α and β chains are stabilized by a disulphide bond (orange stick) in the constant region (light blue). The CD3scFV (orange) domain is fused to the N-terminal of the TCR β chain. TCR α (V α) and β (V β) variable domains are depicted in light and dark green.

4.11 TCR and ImmTAC expression

The TCR chains were expressed in the BL21(DE3)RosettaLysS *E. coli* strain as previously described by Cole and colleagues (128). These competent cells allow high-efficiency protein expression driven by the T7 promoter. The *E. coli* cells were transformed with the expression vectors by heat-shock and plated onto LB-Ampicillin plates. TYP starter cultures (1g Potassium Phosphate Dibasic, 5g Sodium Chloride, 16g Yeast extract, 16g Tryptone per 1l) with one streak of cells from the plate were grown for three hours at 37°C at 220 rpm in 250ml Erlenmeyer flasks until the density of the bacterial culture reach approximately 0.5 optical density at 600nm. 250 ml Auto induction Media (55.85g AI terrific broth powder per 1l and 10 ml glycerol (1%). Ampicillin) was inoculated with 3.75 ml pre-culture. Baffled flasks were then incubated at 37°C at 220 rpm for 2 hours and then 30°C overnight. TCR and ImmTAC α and β chains contain disulfide bonds. The cytosol of bacterial cells is a reducing

environment that prevents disulfide bonds from forming, therefore the expressed protein aggregates as inclusion bodies (IBs) during expression. The IBs were separated from the *E. coli* cells by centrifugation at 4000 rpm for 30 minutes and supernatant was discarded. 200µg/g pellet DNaseI (New England Biolabs, M0303L) were added to 40ml dH₂O containing MgCl₂ to resuspend the pellet. The sample was incubated at 37°C/30min in a shaker incubator. The IBs were then disrupted at 28 kpsi twice and 2 rounds of washes were performed with equal amounts of Triton Wash Buffer (0.5% Triton-X 100, 50mM Tris, 100mM NaCl, 10mM EDTA) and re-suspension Buffer (50mM Tris, 100mM NaCl, 10mM EDTA). The IBs were re-suspended in 2 ml of re-suspension Buffer. The quality and purity of the IBs were assessed on SDS-PAGE gel and Bio-Rad Gel Doc EZ Imager Gel Documentation System estimated the purity. The IB concentration was calculated based on Molecular weight (MW), Extinction coefficient (ϵ) and Absorbance of IBs at 280 nm (A₂₈₀).

$$\text{Concentration(mg/mL)} = A_{280} * \frac{\text{mol. wt(Da)}}{\epsilon_{280}(\text{mM}^{-1}\text{cm}^{-1})} * \text{dilution f actor}$$

The α and β chains expressed this way are denatured and refolded after IB extraction.

4.12 TCR and ImmTAC refold procedure

45 mg of α and 30 mg of β inclusion bodies were denatured in Denaturation Buffer (6M Guanidine, 50mM Tris, 100mM NaCl, 10mM EDTA, 20mM DTT) to unfold and dissociate the aggregated polypeptides from one another. This amount corresponds to 1.5:1 (α : β) molar ratio to limit β homodimer formation. ImmTAC α and β chains were mixed in an equimolar chain ratio (α : β 1.0:1.0), which corresponds to approximately 25 mg of α chain and 60 mg of β chain. β - β formation is much less likely due to the fused scFv (the effector domain sterically prevents β - β homodimer formation). DTT was added to the denaturation buffer to reduce the cysteine residues and to inhibit the formation of any non-native disulphide bonds. The unfolded α and β chains were mixed in Refold buffer (4M Urea, 0.4M L-Arginine, 100mM Tris, 2mM EDTA, 1.9mM Cystamine, (6.5mM Cysteamine). L-Arginine has been shown to reduce aggregation events, and it is often found, along with low concentrations of urea and/or guanidine, in refolding buffers (120). The proteins fold into their native state during dialysis.

4.13 TCR and ImmTAC purification

TCRs were purified in two steps – anion exchange chromatography and size exclusion chromatography. ImmTAC molecules require an additional cation exchange chromatography step after the initial anion exchange chromatography step. The two purification steps, ion exchange and size exclusion chromatography were undertaken using an ÄKTA Pure system (cytivalifesciences.com; Cytiva, MA, US).

Anion and Cation exchange chromatography separates molecules according to their net charge (129,130). The anion exchange chromatography employs positively charged resin that binds molecules with a negative net charge. In contrast, cation exchange utilizes a negatively charged ion exchange resin that binds molecules with a positive net charge. A protein's net surface charge will change, depending on the pH of the buffer. The protein's isoelectric point (pI) is where the protein has a net zero charge. Therefore, every charged protein is going to bind to the resin at a particular pH, with an affinity driven by their pI. This affinity difference enables the separation of the proteins. Soluble TCRs were purified by anion exchange chromatography with Poros 50 HQ column (Thermo Fisher, 1255907). ImmTAC molecules than were purified by cation exchange chromatography with a Poros 50 HS column (Thermo Fisher, 1335909). The column was equilibrated with binding buffer before the bound proteins were fractionated by the application of an increasing concentration gradient of NaCl. During the salt gradient application, the salt ions compete with the protein for charged binding sites displacing the bound protein. The stronger the charge of a protein the higher the concentration of NaCl that is required for its elution.

Size exclusion chromatography (SEC) separates proteins by size, filtrating through a gel matrix (131). The gel beads contain specific size distribution of pores. Small molecules can pass through the pores, therefore running through the column more slowly, while large proteins cannot infiltrate the pores and are eluted at an earlier volume. Accordingly, proteins are separated based on their size and are eluted isocratically based on their molecular weight (MW). Even though SEC cannot discriminate similar MW species particularly well, it is efficient in removing aggregates and unfolded protein. For the final purification step of TCR and ImmTAC molecules, Superdex® 200 10/300 GL (Cytiva 17-5175-01) column was used with DPBS buffer (Gibco, 14040133).

4.14 SPR analysis

The binding analysis of the purified TCRs was performed using a BIAcore® T- 200 and BIAcore® 8K (GE Healthcare, Buckinghamshire, U.K.) with CM5 sensor chip (Cytiva, 29149604) and Series S CM5 sensor chip (Cytiva, 29149603) (132,133). Before streptavidin binding, the chips were activated with CM5 chip amine coupling solutions (100 mM NHS and 400 mM EDC). EDC stands for 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. It's a carbodiimide crosslinker, often used to activate carboxyl groups (-COOH) so they can react with primary amines (-NH₂) to form amide bonds. NHS stands for N-hydroxysuccinimide. It's used to stabilize the reactive intermediate formed by EDC. Streptavidin molecules (5000 response units (RU)) were covalently bound to the chip surface and the remaining reactive groups were deactivated by 1 M ethanol-amine hydrochloride. Between 100 and 1000 RU of CD1c-endo molecules were immobilized on the CM5 sensor chip at a flow rate of 10 µL/min. A blank flow cell was included as a negative control

Steady state affinity measurement of low affinity TCRs

Wild type, low affinity TCRs have fast on and off rates that cannot be accurately measured. The affinities (K_D) of these TCRs are therefore determined by measuring steady state binding across a range of concentrations (steady state affinity). The concentrations cover at least 20%-80% saturation of the CD1c-endo molecules bound on the chip. Following concentration to 100 µM, TCRs and ImmTAC molecules were injected to the sensor chip at 25°C using a series of eight two-fold dilutions, via kinetic injections at 30 µL/min flow rate. Results were analyzed using Biacore T-200 Evaluation software, Biacore Insight Evaluation Software (GE Healthcare, Buckinghamshire, U.K.), and GraphPad Prism software program. The equilibrium binding constant (K_D) values were calculated presuming a 1:1 interaction ($A + B \leftrightarrow AB$) of the ligand and the analyte. Binding responses at equilibrium were plotted against the protein concentrations and analyzed using non-linear regression based on the Langmuir binding equation $AB = (B \times AB_{max}) / (K_D + B)$ (134).

Single cycle kinetics (SCK) analysis of medium to high-affinity TCRs and ImmTACs

Single cycle kinetics was used to measure kinetic parameters of relatively high affinity mutants. It consists of multiple measurements of the on-rate (k_{on} , k_a) through five injections of increasing concentrations and the single measurement of the off-rate (k_{off} , k_d) over a long dissociation time. This method allows accurate kinetic measurements of medium to high affinity TCRs and

ImmTAC molecules. Conditions: CD1c-endo captured: 100 RU; TCR dilution: 10 nM, 5 nM, 2.5 nM, 1.25 nM and 0.625 nM; contact time: 200s dissociation time: 7200s at 10µl/min, temperature: 25 °C, flow rate: 60µl/min. The results were analyzed using Biacore T-200 Evaluation software.

4.15 Affinity Maturation of TCR chains

The wild-type S2 TCR was subjected to affinity maturation process to enhance the TCR's affinity towards CD1c, as described by Yi et.al (100). Phage libraries containing degenerate complementarity-determining regions (CDRs) were generated using the IMGT tool (http://www.imgt.org/IMGT_vquest/vquest). The workflow of the library preparation is described in Figure 4.4.

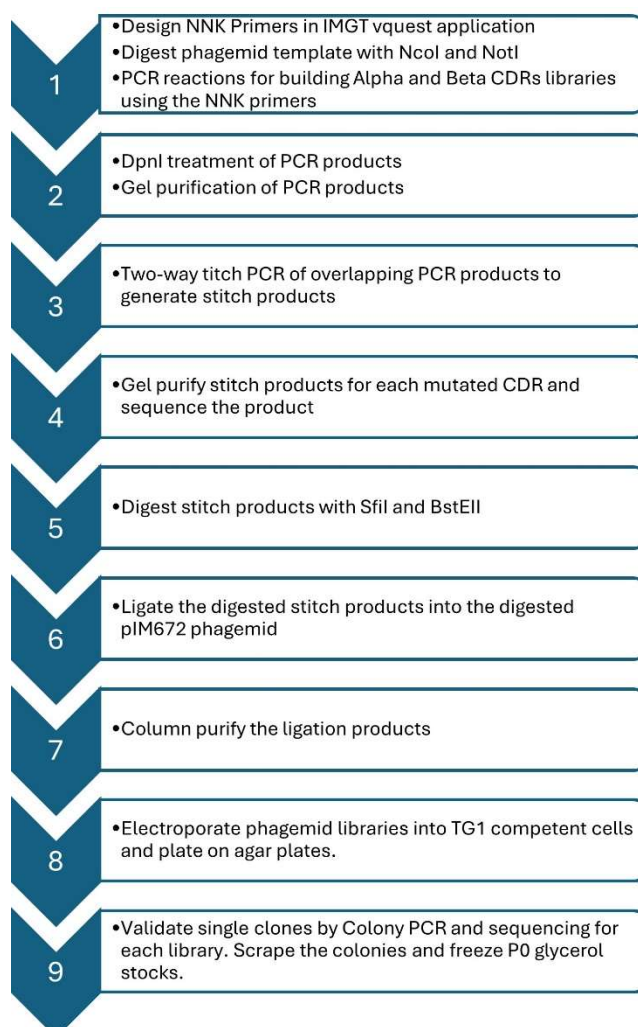


Figure 4.4 Workflow diagram for affinity maturation library preparation.

TCR-phages binding to immobilized CD1c-endo were selected through three rounds. High-affinity TCRs containing mutations in the α and/or the β chains were selected. The wild-type TCR α and β templates were cloned into the pIM672 phagemid. The phagemid templates were digested with either *NcoI* or *NotI* to be subsequently used as templates in the PCRs for the building of the affinity maturation libraries. Primary PCRs using YOL22 as an external primer were set up using the *NcoI*-digested template and YOL13 primers were used with the *NotI*-digested template. Random mutagenesis was used to build the first-generation libraries. 3% randomization rate was chosen to target the three CDRs of one chain in each library. The 3% rate determines a 3% chance of a wild-type nucleotide being replaced by each of the three other nucleotides. Mutants resulting from random mutagenesis diversification retain a very high % of the wild-type protein sequence and are biased towards a minimal number of changes. Therefore, random libraries are likely to deliver functional mutants with lower mutational loads. Two libraries were constructed, one library of the alpha chain and a second one for the beta chain, using NNK codon templates. NNK codon templates are degenerate DNA codons, introducing all 20 amino acids plus a stop codon. N can be any of the four bases (A,T,C or G) and K represents Guanine (G) or Thymine (T). These were used to generate libraries with diverse amino acid sequences. In each library, CDR1, CDR2 and CDR3 of one TCR chain are randomized leaving the partner chain unchanged. Primers used for generating libraries are shown in Table 4.3, below. Primer sequences can be found in the supplementary materials (Supplementary table 13.1)

TCR chain	template	Fragment 1		Fragment 2		Fragment 3	
		Fw_primer	Rw_primer	Fw_primer	Rw_primer	Fw_primer	Rw_primer
alpha	NotI	YOL13	428	422	429	423	430
beta	NcoI	425	432	426	433	-	-

Table 4.3: Primers used for generating soft mutagenesis library.

50 nM CD1c-endo was used for panning below the K_D which favors the binding of higher affinity clones. To preserve the integrity of the lipid ligand, panning was conducted using buffers free of detergent. The final steps—re-suspension and blocking—were performed in PBS supplemented with 2% BSA. Following three rounds of phage panning an inhibition phage ELISA was carried out to screen the affinity maturation outputs. In this procedure, binding of phage to immobilized cognate CD1c-endo is inhibited by 100nM of cognate

CD1c-endo in solution. Phage is produced and blocked with skimmed milk powder solution with and without non-biotinylated cognate CD1c-endo (the inhibitor). Biotinylated cognate CD1c-endo is immobilized onto a streptavidin coated 96-well Maxisorp NUNC-IMMUNO plate (Thermo Scientific, 442404) and incubated with the blocked phage, washed and treated with an HRP conjugated anti-M13 monoclonal antibody (GE Healthcare, 27-9421-01) and visualized using TMB Peroxidase Substrate (TMB Microwell peroxidase substrate system, KPL, 50-76-00). The absorbance values for phage incubated with and without non-biotinylated cognate CD1c-endo were used to calculate the percentage reduction in signal or level of inhibition. The phage inhibition ELISA was used to rank TCRs based on affinity, since TCRs with higher affinity show a greater reduction in ELISA signal (called inhibition). This occurs because TCRs with higher affinity and slower off-rates are captured by the non-biotinylated cognate CD1c-endo in solution and prevented from binding to the immobilized cognate CD1c-endo on the assay plate. Following ELISA data collection, clones with best signals or inhibition rates were sequenced. The mutated TCRs were refolded as ImmTACs for screening in cellular assays and cross-reactivity. A TCR with appropriate affinity (typically nanomolar with a half-life of ~1 min to allow for selection of higher affinity mutants), yield and specificity was chosen as a template for second generation libraries. At this stage, hard mutational load is typically used. Here, six libraries were built, one for each CDR. In each library, only one CDR is randomized, over a stretch of five consecutive amino acids, with the remaining five CDRs unchanged from the template sequence. In addition to hard mutational load libraries, structural libraries were designed based on the crystal structure to target interactions with the CD1c heavy chain. The libraries built based on the wild type crystal structure is shown in Table 4.4.

Three rounds of panning were performed, with 100pM, 10pM and 1pM CD1c-endo respectively. Inhibition ELISA was performed to rank the best mutants. The best candidates were then cloned into mammalian expression vector in ImmTAC format and expressed as soluble molecule. Primer design for NNK mutagenesis is shown in Supplementary Table 13.3.

Library	CDR1a	CDR2a	CDR3a	CDR1b	CDR2b	CDR3b
Template S2-a2b1	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
1) aCDR1 NNK	XXXXXS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	AXXXXX	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
2) aCDR2 NNK	ATGYPS	XXXXXADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKXXXXXDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATXXXXXGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKAXXXXX	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
3) aCDR3 NNK	ATGYPS	LKATKADDDKGS	CXXXXXYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALXXXXXWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGXXXXXKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYXXXXXQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYXXXXXF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
4) bCDR1 NNK	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	XXXXX	TYFQNEAQL	CASSPRMVRGAEAFF
5) bCDR2 NNK	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	XXXXXEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYXXXXXQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQXXXXXE	CASSPRMVRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNXXXXX	CASSPRMVRGAEAFF
6) bCDR3 NNK	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CXXXXXMV RGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASXXXXXRGAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPXXXXXAEAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMXXXXXAFF
	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRXXXXXF
7) Exp1	XXXXPS	LKATKADDDKGS	CALSGXXGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
8) Exp2	ATGYPS	LKATKADDDKGS	CALSGQYGWGKLQF	SEHNR	TXFXXEXQXX	CASSPRMVRGAEAFF
9) Exp3	ATGYPS	LXAXXADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMXXXXAEAFF
10) Exp4 (loop ext 1aa)	ATGYPS	LKXXXXXDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKAXXXXXDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKXXXXXGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
11) Exp5 (loop ext 2aa)	ATGYPS	LKXXXXXADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATXXXXXDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKAXXXXXGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
12) Exp6 (loop ext 3aa)	ATGYPS	LKXXXXXKADDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATXXXXXDDKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKAXXXXXKGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF
	ATGYPS	LKATKADXXXXXGS	CALSGQYGWGKLQF	SEHNR	TYFQNEAQL	CASSPRMVRGAEAFF

Table 4.4: Second generation mutagenesis libraries

4.16 Co-purification of ImmTAC-CD1c complexes for lipidomic studies

The CD1 monomers produced in mammalian cells are bound to a wide range of endogenous lipids from the mammalian cells. The aim of the project is to identify the lipid ligands bound by the wild-type and high-affinity ImmTAC molecules as described by Cheng et al. who analyzed lipids positioned between CD1d and NKT T cell receptors (135). Soluble CD1c monomers were produced in Expi293F cells and ImmTACs were expressed in CHO cells. After purification of the molecules, 1mg of CD1c-endo was mixed with 1mg of ImmTAC. The ImmTAC+CD1c-endo mix was purified on Superdex200 10/300 increase size exclusion chromatography column (as was mentioned in chapter 4.13). Sample application: 0.5ml/min. Elution: 0.4ml/min with 1.2 CV and 500µl fractions were collected. The fractions bound and not bound to the ImmTACs were sent for lipidomic analysis (Figure 4.5).

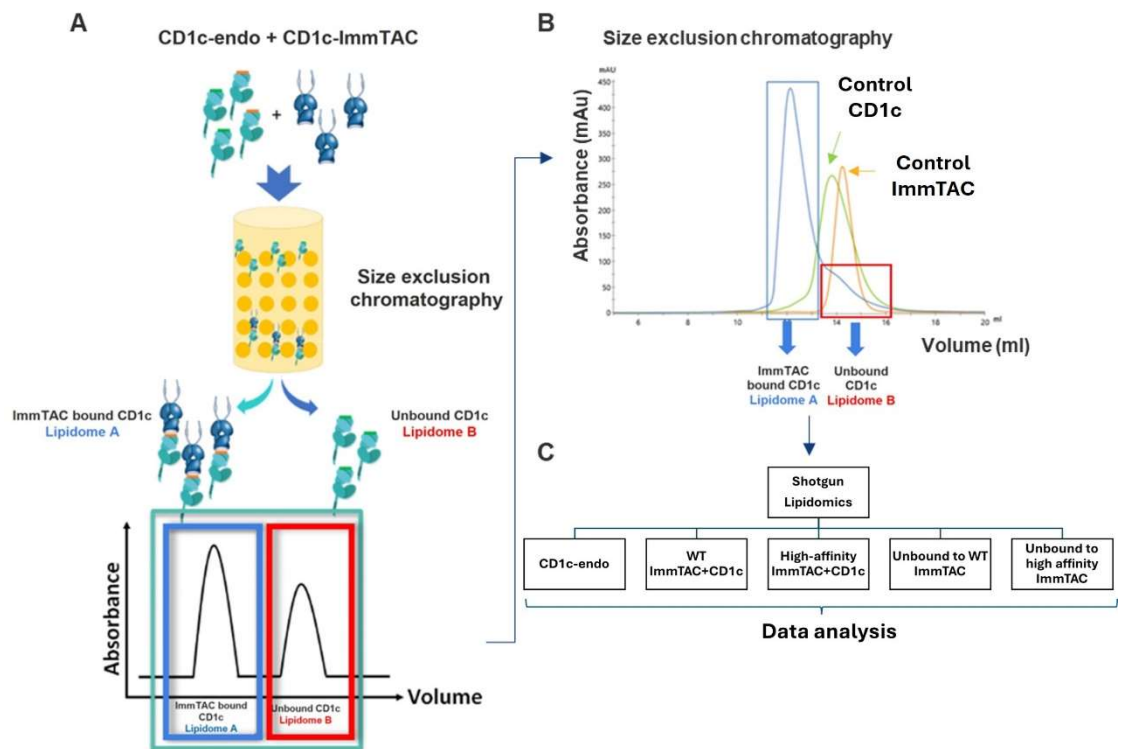


Figure 4.5. Lipidomic analysis of CD1c-lipid complexes trapped by CD1c-specific ImmTACs. (Adopted from Szoke-Kovacs, 2025, p. 9, Figure 4.)

A) CD1c-endo proteins were expressed in Expi293F cells and incubated with CD1c-targeting ImmTACs. The resulting mixtures were subjected to size exclusion chromatography to separate CD1c-ImmTAC complexes from free CD1c molecules. **B)** The separation of CD1c-ImmTAC complexes was performed using a size exclusion chromatography column equipped with a UV detector for monitoring elution profiles. **C)** Complexes formed between CD1c-endo, wild-type, and high-affinity ImmTACs, along with unbound fractions, were analysed via shotgun mass spectrometry. HLA-A2 and ImmTACs alone served as background controls for comparative analysis.

4.17 Co-purification of TCR-CD1c complexes for crystallography

The aim of this section of the project was to prepare CD1c-TCR complexes for crystallography. Soluble CD1c monomers were produced in Expi293FGnTI- cells with homogeneous glycosylation pattern. CD1c-endo was treated with PNGaseF for 2h at 37°C and then overnight at 4°C to remove the remaining glycosylation followed by 1h incubation at 37° C on the next morning (see further details in chapter 4.2). The CD1c monomers were then concentrated on VIVASPIN TURBO 4 10000 MWCO columns to 500µl before size exclusion purification on Superdex 75 10/300 column. The TCRs were expressed in *E. coli*., refolded and purified by Anion exchange chromatography and a subsequent Size exclusion chromatography. 1mg of CD1c-endo was mixed with 1mg of TCR. The TCR+CD1c-endo mix was purified on Superdex200 increase column and eluted into crystallization buffer (10mM Tris pH 8.0, 20 mM NaCl). Sample application: 0.5ml/min. Elution: 0.4ml/min with 1.2 column volume (CV). Fractions of 500µl were collected and subsequently concentrated to a final protein concentration of 10 mg/ml for crystallography.

4.18 X-ray crystallography

To obtain crystals of the wild type and affinity matured TCRs in complex with CD1c, S2c^{WT} and S2c^{a5b6} TCRs were combined with CD1c-endo at a molar ratio of 1:1.1 (TCR to CD1c), concentrated to approximately 10 mg/ml, and subsequently buffer-exchanged into 10 mM Tris (pH 8.0) with 20 mM NaCl. Crystallization trials were initiated using sitting drop vapor diffusion method at 20°C, with drops composed of 150 nl protein complex and 150 nl reservoir solution (Figure 4.6). Briefly, the sitting drop vapor diffusion method is a protein crystallization technique, where the protein and reservoir solution drop (crystallization droplet) is placed on a pedestal of a well containing reservoir solution in the bottom of the sealed well. The reservoir solution contains higher concentration of the precipitant, therefore over the time, water evaporates from the crystallization droplet into the reservoir. The protein concentration gradually increases in the drop leading to supersaturation where the protein molecules organize into a crystal lattice. Multiple Crystallization reagents and concentrations were tested in the crystal screen. Crystals for the two complexes formed under the following conditions:

- S2cWT-CD1c: 0.1 M sodium cacodylate trihydrate (pH 6.4), 0.2 M calcium chloride dihydrate, 16% (w/v) PEG 3350, and 3% (w/v) 1,6-hexanediol.

- S2ca5b6-CD1c: 0.2 M sodium thiocyanate and 20% (w/v) PEG 3350.

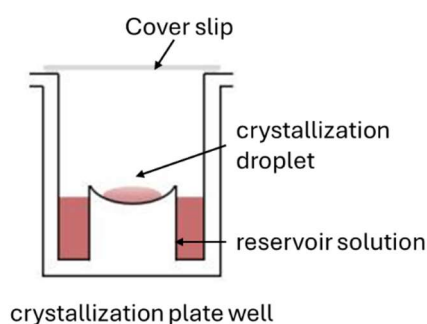


Figure 4.6 Sitting Drop Vapor Diffusion Crystallization

The best crystals that were chosen for analysis were soaked with 30% ethylene glycol to protect from freezing and flash-cooled in liquid nitrogen in Litho loops (Molecular Dimensions, UK). The X-ray diffraction data were collected at the Diamond Light Source (UK) beamlines I03 and I04. The data were processed using the standard software of autoPROC staraniso(136) for data integration and xia2 3dii (137) for scaling for S2c^{WT} - CD1c and S2c^{a5b6}-CD1c. The structure of S2c^{WT} -CD1c complex was solved by molecular replacement (starting from known structures of similar protein) using the PDB codes 3OV6 (for CD1c-B2m), 4P4K (for TCR α) and 5FK9 (for TCR β) as search models in Phaser (138) within the CCP4 suite (139). The S2c^{a5b6}-CD1c complex was solved by molecular replacement using a single copy of the S2c^{WT} -CD1c complex as the search model. The models were built with repeated cycles of manual model building in COOT (140) and computer refinement using Refmac (141). Molprobity (142) was used to assess the stereochemical properties and validation of the models. Pymol (Schrödinger) was used to generate the structures. The structure factors and refined atomic coordinates were deposited in the PDB. (Accession code 9QWJ and 9QWK.)

4.19 Generating antibody-based CD1c-specific T cell engagers

Bispecific T cell engagers (BiTEs) are antibody based immunotherapeutic molecules, which redirect T cells to surface tumour antigens (97,143,144). 13 in-house identified clones of anti-CD1c antibodies of various origin (llama, chicken and human) were reformatted as Bispecific T cell Engagers (BiTE molecules are engineered fusion proteins composed of two single-chain variable fragments (scFvs) linked in tandem via a flexible glycine-serine (GS) linker (145). The variable light (VL) and variable heavy (VH) chains were fused in two possible orientations (Figure 4.7A).

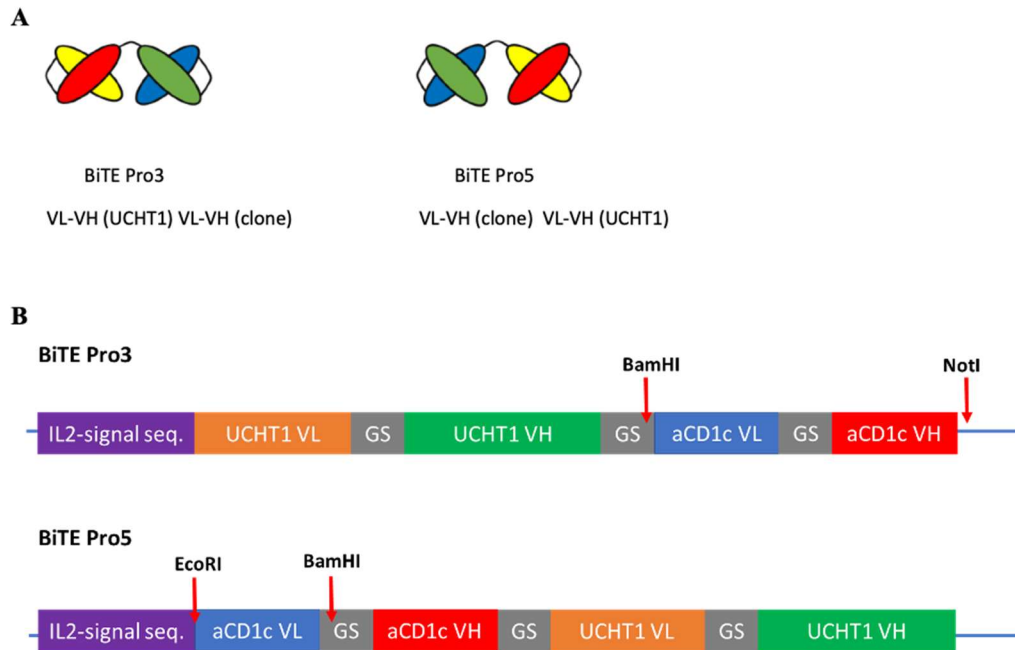


Figure 4.7 Structure of a BiTE.

(A) The VH and VL domains fused in two orientations. Pro 3: UTHC1 VL/VH-aCD1c VL/VH. Pro5: aCD1c VL/VH- UTHC1 VL/VH. (B) Structure of the BiTE constructs.

One of the scFvs binds to T cells via the CD3 receptor (UCHT1), and the other to a CD1c molecule on the cell surface (145) (Figure 4.7 B). The BiTE molecules were expressed in CHO cells and purified by protein L affinity chromatography.

4.20 Generation of CD1c-expressing cell lines

The CD1c constructs were cloned into pSF vector (Supplementary Figure 13.5). HEK 293T cells were transiently co-transfected to generate lentiviral particles with a third-generation system. The system contains a lentiviral transfer vector plasmid, packaging plasmids (PMDLg/pRRE) and (pRSV-Rev) envelope plasmid (pMDG.2modified). DNA was mixed in 150 mM NaCl. The transfection was mediated by polyethylenimine (PEI) (146). THP1(CTIIAB2mKO), K562 and MOLT4(B2mKO) cells were transduced with single-chain CD1c by lentiviral transduction. CIITA KO cells were generated by knocking out the Class II transactivator (CIITA) gene, crucial for the expression of MHC class II (MHC-II) molecules. Therefore, these cells do not express MHC II molecules. B2M KO cells were generated by knocking out the Beta-2-microglobulin (B2M) gene, crucial component of Major Histocompatibility Complex class I (MHC-I) molecules. Therefore, these cells do not express MHC type I molecules, including those type Ib molecules that forms complexes with

β 2 microglobulins. In order to achieve efficient transduction, concentrated lentivirus was used with protamine sulphate in 48-well plates while centrifuging ('spinfection') (147). The success of the transduction was accessed by flow cytometry.

4.21 Generation of CD1c restricted effector cell lines

Lentiviruses were generated and NFAT.Luc.Jurkat.TCRKO and JRT3 Jurkat cell lines were transduced with TCR alpha and beta chains by the same method as described above for the generation of CD1c-expressing cell lines.

4.22 T cell re-direction assay using anti-CD1c/anti-CD3 bispecific reagents

A dilution series ($1-1 \times 10^{-6}$ nM) of the bispecific engagers (BiTE or ImmTAC molecules) was prepared in Jurkat assay Media which is Jurkat Culture Media without antibiotics and Hygromycin B (RPMI 1640, 10% FBS, 2mM L-Glut) and added to the relevant wells in a 384 flat bottom assay plate. To avoid discrepancies due to manual handling, all pipetting tasks were performed via Viaflo 384 liquid handling robot. 25,000 effector cells (Jurkat.NFAT.Luc) were plated in each relevant well in 8 μ l Jurkat Assay Media. 12,500 target cells were added in 8 μ l Jurkat Assay Media in each relevant well. The assay plates were incubated at 37°C, 5% CO₂ for overnight (~22h). The next day Bio-Glo reagent (Promega, WI, USA, G7940) was prepared. The assay plate and the BioGlo reagent were equilibrated to room temp for 5 - 10 min and an equal amount of BioGlo reagent was added to the culture wells. The NFAT(Luc) Jurkat reporter cells are engineered with the NFAT response element, which drives the luciferase-expressing system. Upon T cell activation the Jurkat cells express firefly luciferase under the control of the NFAT response elements which is one of the main parts of the TCR signalization pathways (148). The assay plates were incubated for 5 min and the luminescence signal was measured by using a Clariostar plate reader at 574nm. The data were analyzed using Microsoft Excel and GraphPad Prism software.

4.23 T cell activation assay

Target cells were washed and resuspended in Jurkat Assay Media which is Jurkat Culture Media without antibiotics and Hygromycin B (RPMI 1640, 10% FBS, 2mM L-Glut). 50,000

target cells were plated in each relevant well in a flat bottom 96 well plate. 10µg/ml antiCD1c blocking monoclonal antibody (clone L161) was added to the control wells and incubated for 30min. 50,000 effector cells (Jurkat.NFAT.Luc) were washed in Jurkat Assay Media and added to each relevant well, bringing the final volume to 200µl in each well. Assays with primary T cells were performed in R10 media (RPMI 10% FCS, supplemented with glutamine, sodium pyruvate, Hepes, non-essential amino acids and pen streps). The assay plates were incubated at 37°C, 5% CO₂ for overnight (~16h). On the following day, cells were washed and initially stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain for 20 minutes at room temperature in the dark. After another wash, Fc receptors were blocked using Human TruStain FcX™ (BioLegend, 422302) for 15 minutes. The cells were stained for activation markers CD69 PerCP/Cy5.5 (Biolegend, 310926) and CD25 PECF594 (BD Bioscience, 562403) for 30min on ice. After washing the cells, flow cytometry was performed on BD LSRFortessa™ Cell Analyzer. Data were analysed using Flowjo V10.

4.24 T cell cloning

Cell isolation

Peripheral blood mononuclear cells (PBMCs) and CD2⁺ cells were isolated from blood (100-200mL) from healthy internal donors from Immunocore Ltd., following an Health Technology Assessment regulation approved protocol. 15mL Lymphoprep was added to Leucosep tubes and were centrifuged at 350×g for 1 minute. The blood was diluted 1:2 in HBSS and 35ml was added to the Leucosep tubes. The tubes were centrifuged at 800×g for 15 min with the brake off. The cells were carefully removed from the interface and transferred to a fresh tube. The cells were washed with 50ml of Hanks' Balanced Salt Solution (HBSS) and centrifuged at 350×g for 8 min with the brake on. Subsequently, the pellet was washed twice with 50ml of HBSS and centrifuged at 240×g for 8 min with the brake on. The cells were counted and CD2⁺ cells were purified using Miltenyi CD2 MicroBeads (Miltenyi Biotec, 130-091-111). The PBMCs were washed in MACS buffer (PBS, 10% FCS, 10mM EDTA) and labelled with 20µl of CD2 MicroBeads per 10⁷ cells in 1 ml volume. The cells were incubated for 15 minutes in the refrigerator and were briefly mixed after the first 7 minutes. The cells were purified on LS columns and washed three times with 3ml of MACS Buffer to remove unlabeled cells. The labelled cells were eluted with 5 ml of MACS buffer.

Expansion of CD1c- and CD1d- ganglioside specific T cell clones

After purification, 12×10^6 CD2⁺ cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) cell tracer dye. 2×10^6 THP1.DKO and THP1-CD1c and CD1d high cells were collected in R10 media, in common 25cm² growth surface area (T25) flasks and were irradiated at 66Gy. After irradiation, 0.5×10^6 cells were transferred into common 24 well tissue culture plate and pulsed with lipid-ligands (GD3, GM3 or Neu5GM3) at 25µg/ml. The cells were incubated at 37°C, 5% CO₂ for 3 hours to aid the loading of lipids onto CD1c molecules. THP1-CD1d cells were incubated with 10ug/ml anti-HLA-A, B, C and L243.2 antibodies to block class one and class two HLAs for 30min. 2×10^6 labelled T cells were added to each well and incubated 37°C, 5% CO₂ for 3 days. 5 drops of HSIL2 media (IMDM 5% pooled human AB serum and 500IU IL2) were added to each well every day and the cells were split when they reached confluence. The cells were analyzed for the loss of CFSE, looking for dividing cells. The proliferating cells with low CFSE fluorescence were sorted on the Sony Sorter.

4.25 Flow Cytometry and Cell Sorting

Cells were washed in FACS buffer (PBS supplemented with 10% FCS) and centrifuged at $400 \times g$ for 5 minutes using a V-bottom 96-well plate. The pellets were resuspended in LIVE/DEAD™ Fixable Aqua Dead Cell Stain and incubated for 20 minutes at room temperature in the dark. Following another wash, Fc receptors were blocked for 15 minutes using Human TruStain FcX™ (BioLegend, 422302). The pellets were then stained with antibody master mix for 30 minutes on ice. After washing, the cells were resuspended in 400µl of FACS Buffer in tubes, and flow-cytometry was performed on BD LSRFortessa™ Cell Analyzer (149). For cell sorting, the cells were resuspended in a 3ml FACS Buffer and the sorting was performed on a Sony SH800S Sorter. Results were analyzed by FlowJo software.

4.26 Lipid extraction for mass spectrometry

Lipidomic analysis using mass spectrometry was carried out by Lipotype GmbH (Dresden, Germany) following the method outlined by Surma et al. (150). The method was described previously in our publication (151). A two-step chloroform/methanol procedure was used to extract the lipids (152). An internal lipid standard mixture was spiked into the

samples prior to lipid extraction. The list of lipids contained by the standard mix can be found in supplementary material. (Supplementary Table 13.4) Gangliosides were isolated from the aqueous phase following chloroform/methanol extraction using a solid-phase extraction technique. The organic layer was evaporated on an infusion plate using a speed vacuum concentrator. The dried material was then reconstituted in a chloroform/methanol/propanol mixture (1:2:4, V/V/V) containing 7.5 mM ammonium acetate, followed by drying in a 33% ethanol solution of methylamine in chloroform/methanol (0.003:5:1, V/V/V). All steps involving organic solvents were automated using the Hamilton Robotics STARlet platform equipped with Anti Droplet Control.

4.27 Mass Spectrometry (MS) data collection

The data collection method was described previously in our publication (151). The samples were subjected to direct infusion analysis on a QExactive mass spectrometer (Thermo Scientific) with a TriVersa NanoMate ion source (Advion Biosciences). All samples were subjected to positive and negative ion mode analysis in a single acquisition. The resolution of the experiments was $R_{m/z=200}=280000$ for MS and $R_{m/z=200}=17500$ for MSMS (tandem MS). MSMS was activated by an inclusion list including corresponding MS mass ranges that were scanned in 1 Da increments (153). The MS and MSMS data were combined to observe the ammonium adducts of CE, Chol, DAG and TAG ions; the acetate adducts of PC and PC O⁻; and the deprotonated anions of CL, PA, PE, PE O⁻, PG, PI and PS. The deprotonated anions of Gb3, Gb4, GM4, GM3, GM2, GM1, GD3, GD2, GD1, GT3, GT2, GT1, GQ1, Sulf, LPA, LPE, LPE O⁻, LPI and LPS, and the acetate adducts of Cer, HexCer, DiHexCer, SM, LPC and LPC O⁻ were monitored by MS only. The full list of lipids analyzed can be found in the Supplementary material (Supplementary Table 13.2).

4.28 Analyzing and post-processing the MS data

The data analysis method was described previously in our study (151). The mass spectrometry data collected was analysed with Lipotype's LipidXplorer software for lipid identification (154, 155). (The LipidXplorer software was developed by Lipotype GMBH. The software is Lipotype's property and not open sourced.) In the first step, the lipid

identification from the unprocessed (*.raw format) mass spectra was performed by LipotypeXplorer. The molecular weights of the whole molecules were used to identify lipids by MS-only mode. Lipids were identified in MSMS mode by using the intact masses and the collision-induced fragmentation generated masses of the fragments. Lipid identifications were performed using filters of mass accuracy, occupancy threshold, signal-to-noise ratio, and background levels, followed by normalization and statistical analysis. The lipids and lipid intensities were saved in a database and lipid quantification was performed using the intensities obtained from lipid class-specific internal standards. Lipotype's data management system was employed to perform the data post-processing and normalization. Data were filtered to retain lipids exhibiting a signal-to-noise ratio above 5 and signal intensities at least fivefold higher than those observed in corresponding blank controls. Prior to analysis, the range of variability in the cell pellet samples was established (150) and the quantification limits and coefficient variations for the lipid classes were set. Quantification limits ranged from low micromolar to sub-micromolar concentrations, with average coefficients of variation across quantified lipid classes falling between 10–15%, depending on the lipid type. Blank samples were included in each run to monitor background noise, and reference quality control samples were used to detect sample-specific issues and ensure consistency across runs. Lipid quantification was performed by normalizing to internal standards specific to each lipid class. The total amount of lipid classes was calculated by summing the pmoles values of the individual lipid molecules (species or subspecies). To determine mol% relative to total lipids, the quantities of individual lipid classes were normalized against the overall lipid content. For lipids analyzed semi-quantitatively, peak intensities were first normalized to an internal standard that did not belong to the same lipid class. These values were then further adjusted based on the total lipid content of each sample to yield relative abundances. The dataset provided by Lipotype was processed using a custom R-based workflow (R version 4.3.2), developed in-house. The full source code is openly available on GitHub at: <https://github.com/ritaszokekovacs/CD1-lipidome17052024.git> and <https://github.com/ritaszokekovacs/CD1c-restricted-TCR-lipidome.git>. To assess the structure of the complex data, an unsupervised learning method was performed with consideration for the connections between the variables. For exploratory data analysis, principal component analysis (PCA) was employed. Visualizations were generated using ggplot2, with the viridis colour palette applied where feasible to maintain accessibility and consistency across colour-blind and greyscale formats (145–147).

4.29 Study approval

Blood was sourced from local donors. All human blood was obtained and processed following the guidelines of Immunocore's Human Tissue Act compliance team, to comply with the United Kingdom Human Tissue Act 2004.

5. RESULTS

5.1 Mammalian expression of CD1 molecules

Soluble single-chain CD1a, CD1b, CD1c and CD1d molecules were produced in ExpiCHO and Expi293F mammalian cell lines. The pCDNA3.1 dual expression plasmid was introduced into mammalian cells via transfection, including the single chain CD1 construct and BirA biotin ligase (Figure 5.1A see also in Chapter 4.2, Figure 4.1). The co-expression of the BirA enzyme allows for the “in-cell” biotinylation of the CD1 monomers. After being expressed and biotinylated, the CD1 monomers are secreted to the media while the BirA enzyme is retained within the endoplasmic reticulum by a KDEL motif. The best yield of CD1 monomers was observed at five days post-transfection in Expi293F cells, therefore, this expression system was chosen for protein production (Figure 5.1B). After affinity- and SEC purification, ILT2 binding to CD1c was tested by the surface plasmon resonance (SPR) method to assess the quality of CD1c monomers (Figure 5.1C). ILT2 protein was cloned and refolded in-house by Immunocore and used to quality control the folding of HLA and HLA-like molecules. ILT2 binds to the CD1c heavy chain and $\beta 2m$, confirming the molecule’s correct folding. Furthermore, the quality of the monomers was assessed in SPR assays by testing their binding properties to TCRs previously reported in the literature (Figure 5.1D).

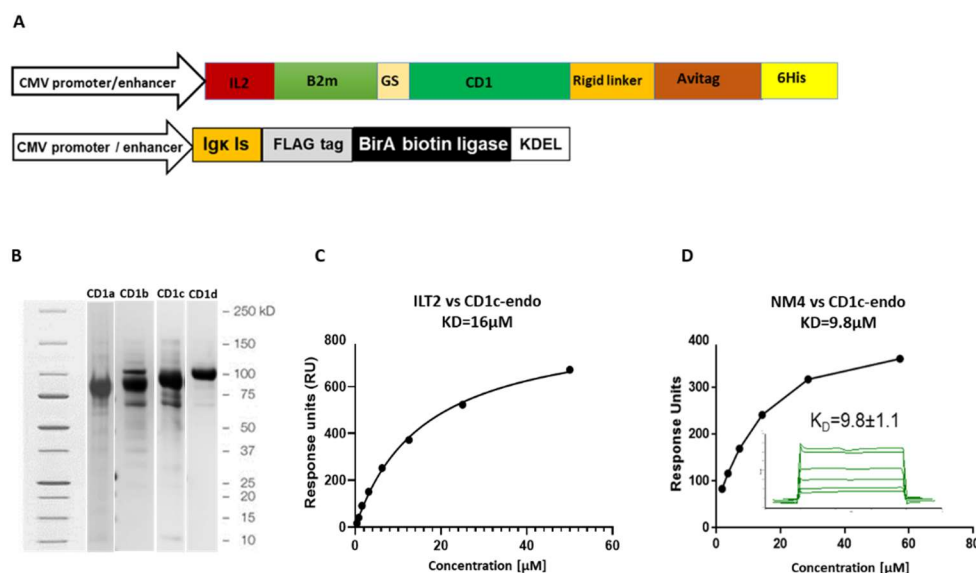


Figure 5.1: Mammalian expression of CD1 molecules

(A) Construct for mammalian expression (B) Purified CD1 proteins on SDS-PAGE (C) Binding affinity of ILT2 to CD1c-endo (D) NM4 TCR binding to CD1c-endo measured by SPR.

The CD1c molecules have four glycosylation sites. The appearance of the molecule on SDS-PAGE and IEF gels is not homogenous (Figure 5.1B), it consists of several bands. The bands can represent different glycosylation states of CD1c. Silencing the glycosylation sites on both CD1b and CD1c led to reduced stability and unsuccessful expression in mammalian cells (not shown) and was therefore not used.

5.2 Lipid-ligand exchange method development

The aim was to generate lipid loaded CD1c molecules (with self-lipid targets from a disease of interest). This would allow for the isolation and characterization of TCR candidates, and the generation of enhanced affinity TCRs. CD1c monomers produced in mammalian cells are bound to lipids from the cells (CD1c-endo). Researchers employ different strategies to facilitate lipid antigen loading to CD1 molecules, however clear validation of lipid exchange by biochemical assays has not been shown so far (62,156–160). Loading self-lipids into soluble recombinant CD1 molecules is challenging because lipids generally have poor solubility, and a lipid occupying the CD1-lipid binding pocket stabilizes the CD1 protein structure. Therefore, exchanging lipids in CD1 molecules requires partial unfolding of the CD1 protein. The goal was to partially unfold the CD1c molecules and remove the endogenous ligands from the binding pockets using a detergent-containing ligand exchange buffer at pH 6.0. The CHAPS (Sigma, C3023-25G) detergent used in this protocol is a mild surfactant. CHAPS is an amphiphilic molecule, having both hydrophilic and hydrophobic regions. This amphiphilic quality enables the detergent to break protein-protein, protein-lipid and lipid-lipid links, and denatures proteins and macro-molecules. Lipids were added at 50× molar excess to the ligand exchange buffer and solubilized by sonication. The CD1c-endo and CD1d endo molecules were incubated with the solubilized lipids overnight at 37°C. To validate the ligand exchange, Isoelectric focusing (IEF), Native Mass Spectrometry, tetramer staining and lipid-specific TCR binding measured by SPR were performed. The correct conformation of CD1 monomers was assessed by ILT2 binding.

5.3 Validation of ligand exchange

5.3.1 Isoelectric Shift Assay

Isoelectric Shift Assay can be employed to analyze interactions between proteins and ligands such as lipids (118). This method is suitable for testing proteins with multiple ligands semi-quantitatively. Isoelectric focusing gels separate proteins by their isoelectric point (pI). The isoelectric focusing method was adopted to validate the ligand exchange on CD1 molecules. The CD1 protein migrated on the gel during isoelectric focusing based on the charge of the protein, including the lipid ligand. The captured negatively charged lipid ligand can shift the pI, causing the CD1-lipid complex to migrate to a lower pH region. Similarly, replacing the negatively charged lipid ligands with neutral lipids shifts the protein to its neutral pI. CD1c- and CD1d-endo proteins appear as multiple bands on isoelectric focusing (IEF) gel (Figure 5.2). CD1c and CD1d molecules were loaded with neutral lipids (phosphatidylcholine (PC) and KRN7000), and negatively charged lipids (Disialoganglioside (GD2), Disialoganglioside (GD3), Monosialoganglioside (GM3) and Globoside (Gb4)). A shift in the pI of the CD1 molecules loaded with negatively charged lipids was observed, while no shift was seen when loaded with neutral lipids (Figure 4.2). The observed pI shift suggests successful lipid loading. This method is not suitable for quantifying the ratio of successfully loaded molecules. To gain a better understanding of the proportion of loaded and endogenous lipid-containing molecules, Native Mass Spectrometry was performed.

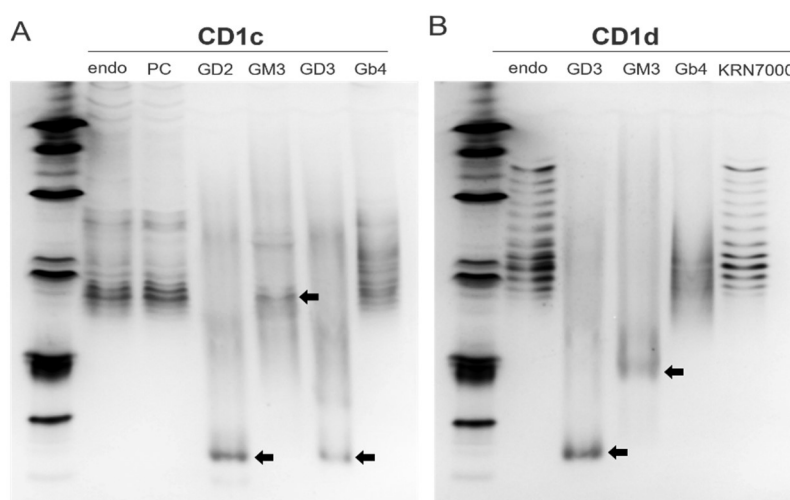


Figure 5.2: Isoelectric mobility shift assay

(A) CD1c-endo, CD1c-PC, CD1c-GD2, CD1c-GM3, CD1c-GB4 **(B)**CD1d-endo, CD1d-GD3, CD1d-GM3, CD1d-Gb4, CD1d-KRN7000. The pI shift is shown by arrows.

5.3.2 Native mass spectrometry

The CD1c/CD1d-lipid complexes were analysed by native electrospray ionization (ESI)-MS described before by Garcia et al. (60,161), to characterize the lipid content bound to the CD1c- and CD1d-endo molecules and quantitate the ligand loading process. Native MS doesn't require the extraction of the lipid ligands from the protein before detection. The CD1-lipid complexes can be detected without the need for any chemical or physical manipulation, allowing for the detection of all types of lipid molecules that are bound to the CD1 proteins (162,163). However, complex glycosylation can result in a noisy MS signal that makes it difficult to interpret the mass spectra. CD1c and CD1d molecules were expressed in Expi293FGnTI⁺ cells to obtain homogenous glycosylation. CD1c and CD1d molecules were then loaded with lipid ligands, and the quality control of the CD1-lipid complexes was performed by SDS-PAGE and IEF electrophoresis (Figure 5.3). Size exclusion chromatography was performed on CD1-lipid complexes ahead of mass spectrometric evaluation and the peaks of 7.5ml to 9ml and 9ml to 9.5ml of CD1d-endo (Figure 5.3A) and 7.5 to 9ml of CD1c-endo (Figure 5.3B) were sent for mass spectrometry analysis. The native mass spectrum of CD1c and CD1d with their endogenously loaded lipid ligands is shown in Figure 5.4.

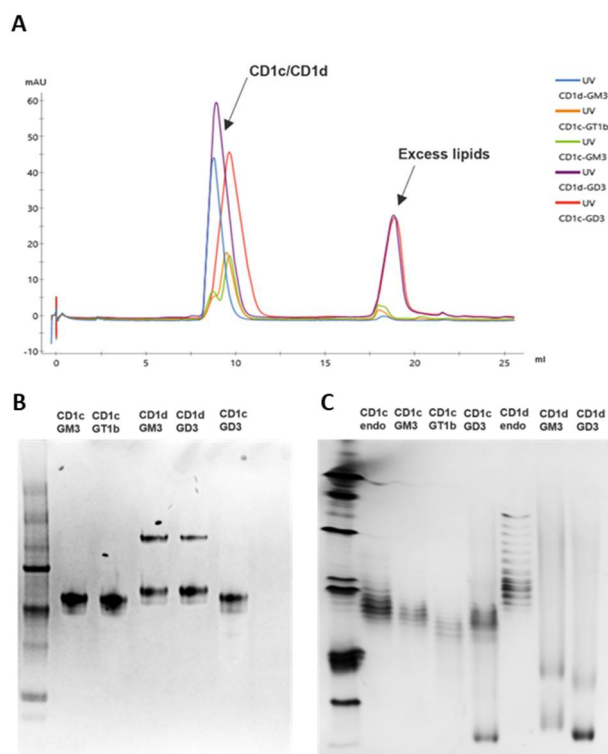


Figure 5.3: Native Mass Spectrometry sample preparation

(A) SEC purification of CD1-lipid complexes (B) SDS-PAGE analysis of CD1-lipid complexes (C) IEF gel analysis of CD1-lipid complexes

The shift observed on the IEF gel Figure 5.3 C and the native mass spectrometry data suggests some level of ligand exchange, however, the mass spectrum is difficult to interpret. The CD1 molecules contain a wide variety of endogenous lipids from the Expi293FGnTI- cells. This is reflected in the observed charge distributions, suggesting that several CD1d-endo (Figure 5.4A) and CD1c-endo (Figure 5.4B) species could be extracted.

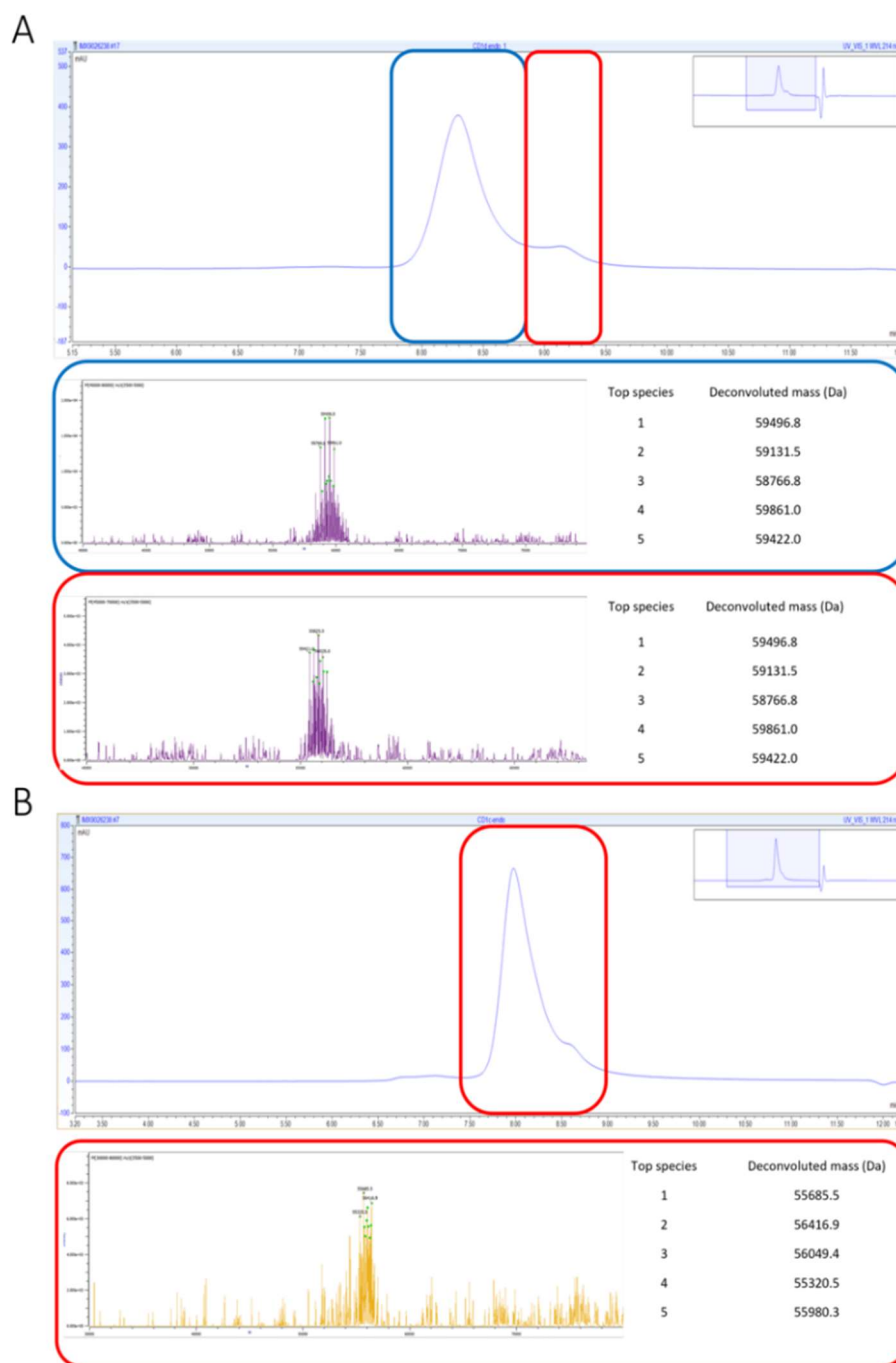


Figure 5.4: Native mass spectrometry of CD1c-endo and CD1d

(A) SEC peaks collected for native MS and native mass spectrum of CD1d-endo **(B)** SEC peaks collected for native MS and native mass spectrum of CD1c-endo.

After lipid loading, the average molecular masses of the top five peaks ranged between 55320.5 Da and 56416.9 Da for CD1c-endo Figure 5.4A and between 58766.8 Da and 59861.0 Da for CD1d-endo Figure 5.4B. The theoretical molecular weight of CD1c and CD1d is 48000, which assumes no lipid in the binding groove. The lipids' average size range is below 1000 Da which makes the CD1-lipid complexes expected to be around 49000 Da. Additionally, the CD1 molecules are less stable without full glycosylation which could also contribute to the noise detected in the data. Some changes were observed in the SEC and MS profile of the lipid-loaded CD1c- and CD1d complexes but could not determine the species due to the high complexity of the data (Figure 5.5 and 5.6). Thus, this method would require a significant amount of optimization to set up a quantitative method for ligand exchange.

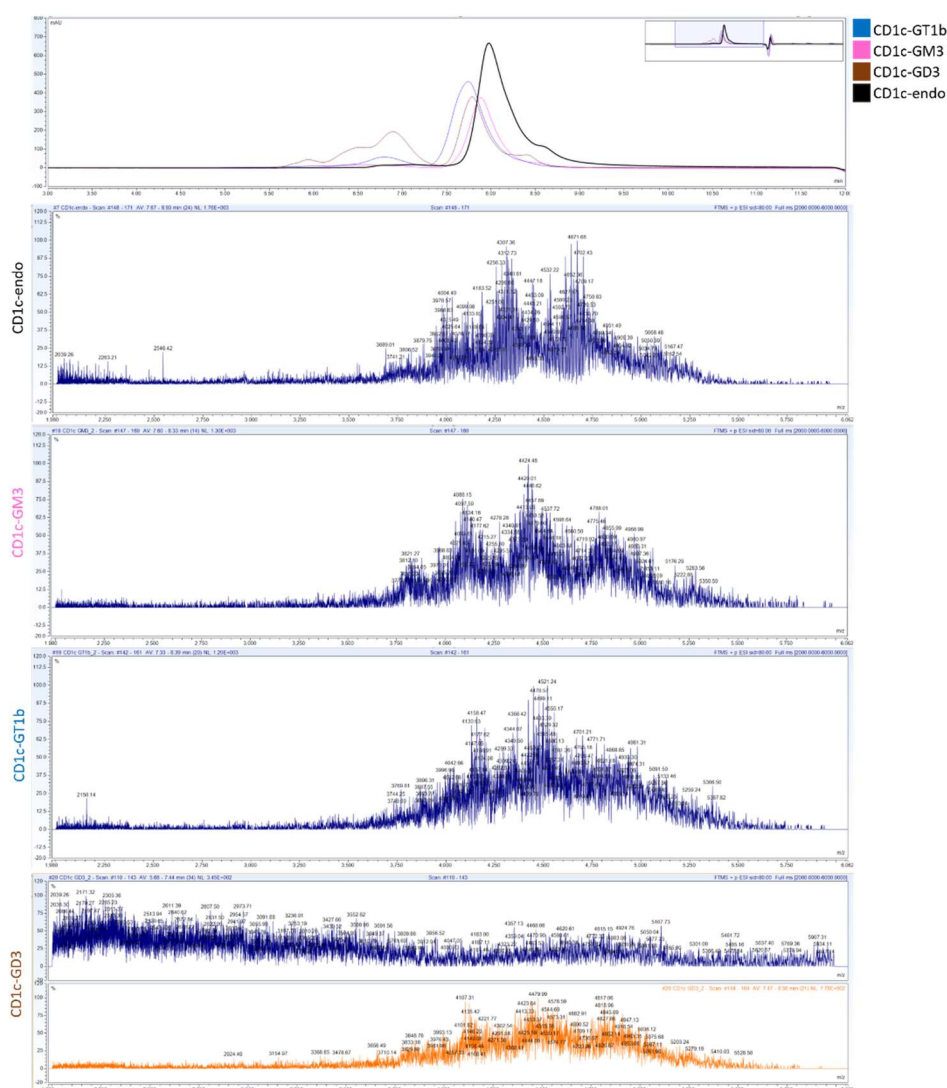


Figure 5.5: Native MS profile of CD1 molecules after ligand exchange

The fractions of the SEC peaks (top figure) were analysed by MS. The top figure shows an overlay of the SEC profiles. One peak of CD1c-endo, CD1c-GM3, CD1c-GT1b were analyzed by MS. Two peaks of CD1c-GD3 were analyzed (blue and orange MS profiles derived from two SEC peaks).

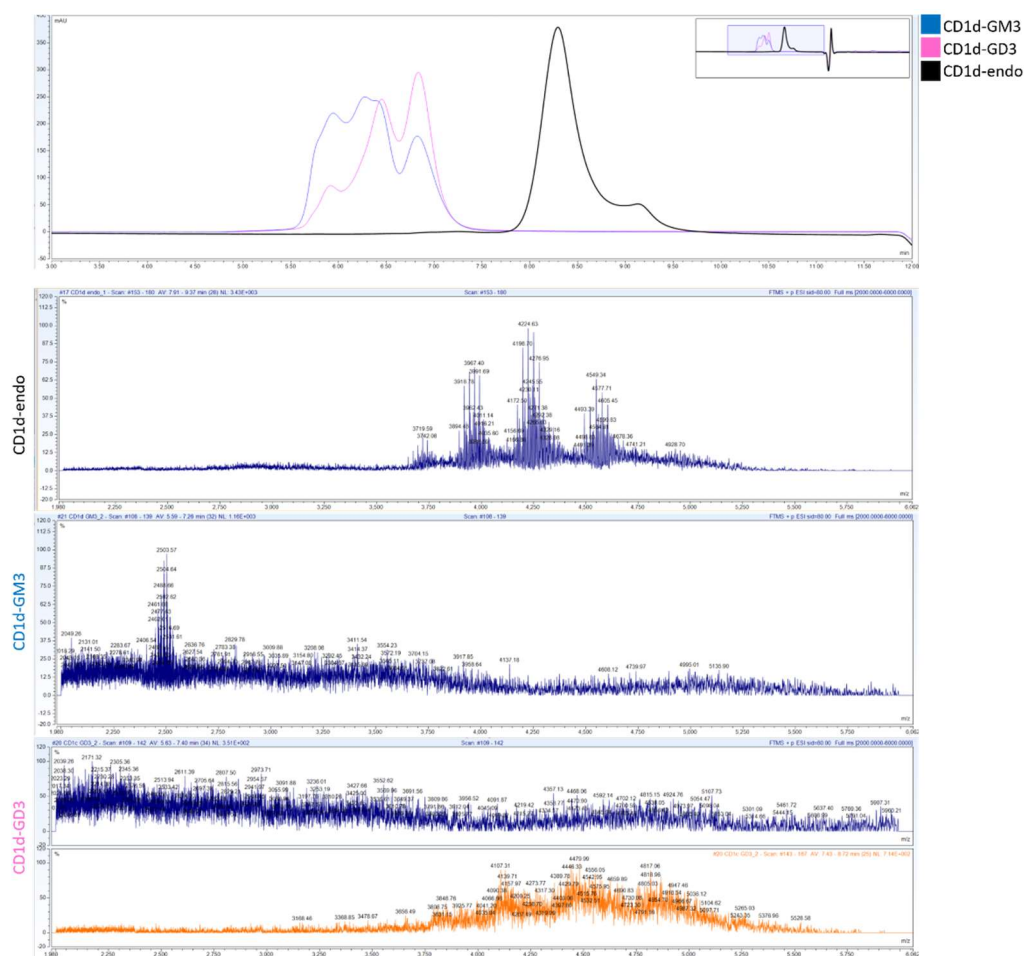


Figure 5.6: Native MS profile of CD1 molecules after ligand exchange

The fractions of the SEC peaks (top figure) were analysed by MS. The top figure shows an overlay of the SEC profiles. One peak of CD1d-endo and CD1d-GM3 were analyzed. Two peaks of CD1d-GD3 were analysed (the blue and orange MS profiles derived from two SEC peaks).

5.3.3 Efforts to establish TCR binding assay to validate ligand exchange

TCR binding-based assay was also developed to optimize and validate ligand exchange. This assay was built on our current knowledge of the TCR-CD1c co-recognition model that implies that lipid ligands with large polar headgroups disrupt TCR binding (Figure 5.7A) (55).

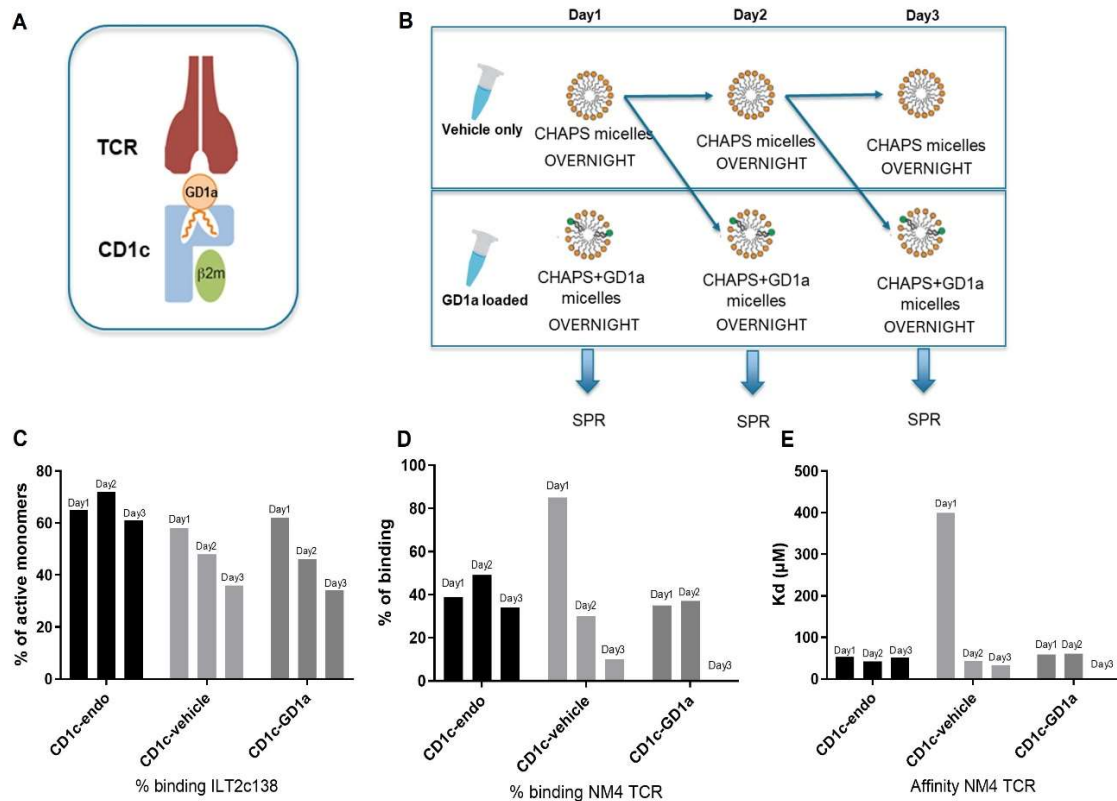


Figure 5.7: Validation of ligand exchange by TCR binding

(A) TCR binding model to CD1c-GD1a (B) Schematic of the ligand loading experiment (C) Percentage of functional CD1c molecules after ligand exchange. ILT2 binding was tested by SPR to confirm the functionality of CD1c molecules after ligand exchange (D)(E) NM4 binding to CD1c-GD1a was lost after two vehicle treatments followed by GD1a loading.

A ganglioside, GD1a with a large headgroup was chosen to test our hypothesis. The soluble TCR format of the NM4, a T cell clone exhibiting reactivity to CD1c, described by Mansour et al. (164) was refolded. The refolded NM4 TCR was tested in SPR experiments to measure TCR binding and affinity against lipid loaded CD1c molecules. CD1c-endo molecules were treated with ligand exchange buffer containing CHAPS, to remove endogenous lipids overnight and then loaded with 50x molar excess of GD1a solubilized in ligand exchange buffer overnight. Three sets of CD1c molecules were loaded with GD1a (Figure 5.7 B). On day 1, the first batch was either treated with “vehicle” (micelles containing CHAPS in ligand

exchange buffer, created by sonication) or loaded overnight with CD1a in ligand exchange buffer containing CHAPS micelles. On day 2, the CD1c protein was purified on Zebra desalting columns and the GD1a loaded molecules were analysed by SPR. The “vehicle” treated molecules were subsequently treated again with vehicle or loaded with GD1a. The molecules were treated similarly on day 3 and analysed. The ligand exchange buffer treatment facilitates the removal of the endogenous ligands before GD1a loading. The CD1c molecules were purified on desalting columns before the SPR experiments.

ILT2 binding to CD1c molecules was measured to assess the functionality of the CD1c molecules after ligand exchange. The R_{max} was used to calculate the percentage of properly folded, active monomers (Figure 5.7 C). To analyze TCR binding, blank flow cell 1 was subtracted from the flow cells containing CD1c monomers. The R_{max} was corrected according to the stoichiometric ratios and normalized to ILT2 binding. NM4 bound to a bigger proportion of CD1c treated with ligand exchange buffer only (vehicle), an artefact which may arise from the non-specific binding of the CHAPS detergent to the CD1c surface, making it "sticky" on the chip surface. The low affinity binding to CD1c-vehicle also indicates that the observed binding is likely an artefact. NM4 binding to CD1c, treated twice with the vehicle before loading with GD1a was lost (Figure 5.7 D and E).

The effect of CD1c glycosylation on TCR binding was tested, which might be the cause of the poor reproducibility and batch-to-batch variation. The CD1c molecules produced with short, more homogenous glycosylation were less stable and lost ILT2 binding after two nights of vehicle treatment (Figure 5.8).

Testing these molecules against NM4 TCR after the same ligand exchange regimen, we found that NM4 bound with a higher affinity to the CD1cGnTI- molecules produced with homogeneous glycosylation. However, the day 3 GD1a loading data wasn't reproduced due to the instability of CD1c molecules with low glycosylation patterns (Figure 5.9). Hence, this method is not suitable for routine validation of ligand exchange.

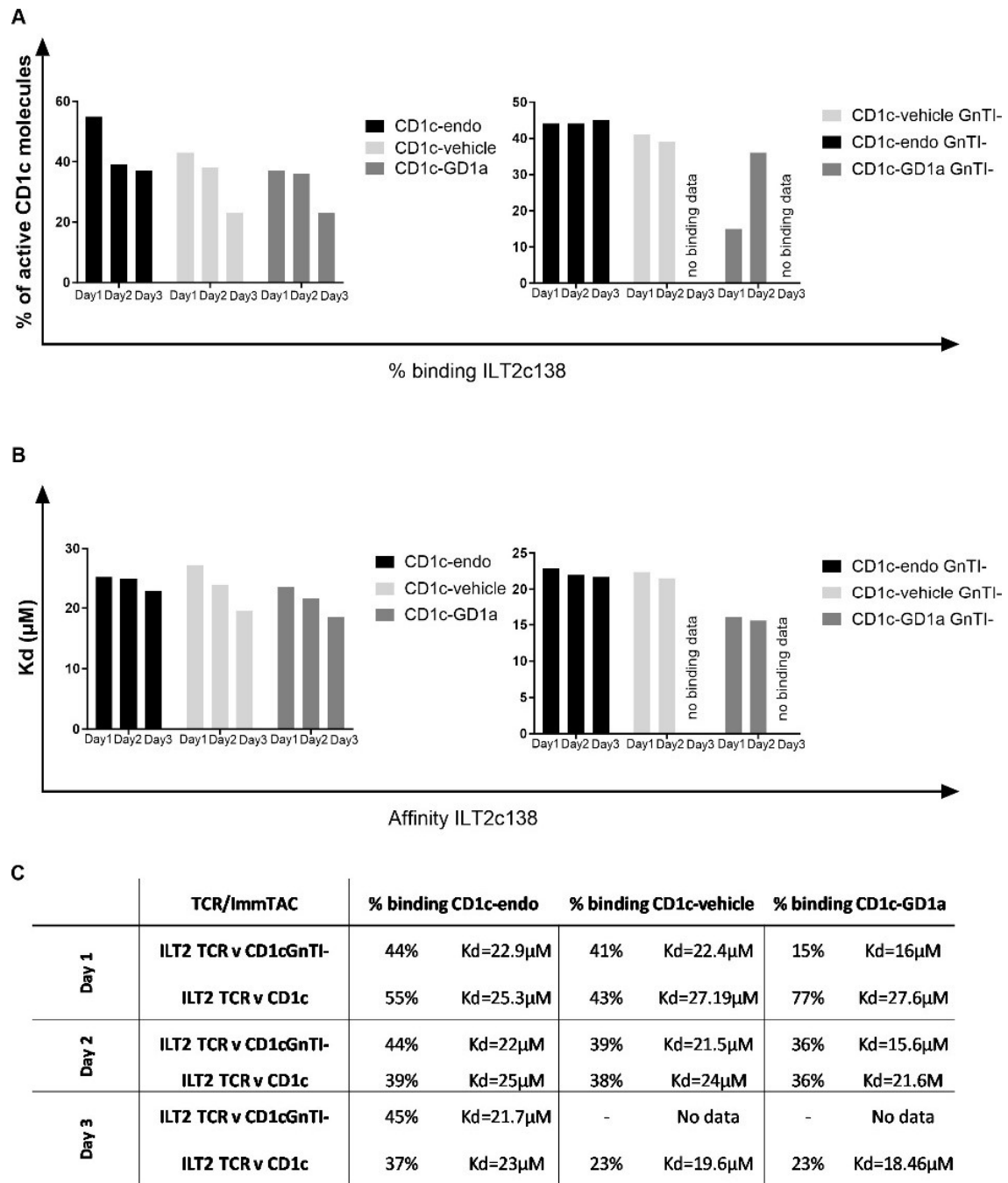


Figure 5.8: The effect of CD1c glycosylation on CD1c stability

(A) % of ILT2 binding to CD1c molecules after ligand exchange. (B) ILT2 binding affinity to CD1c molecules after ligand exchange. (C) Summary of ILT2 binding percentages and affinities.

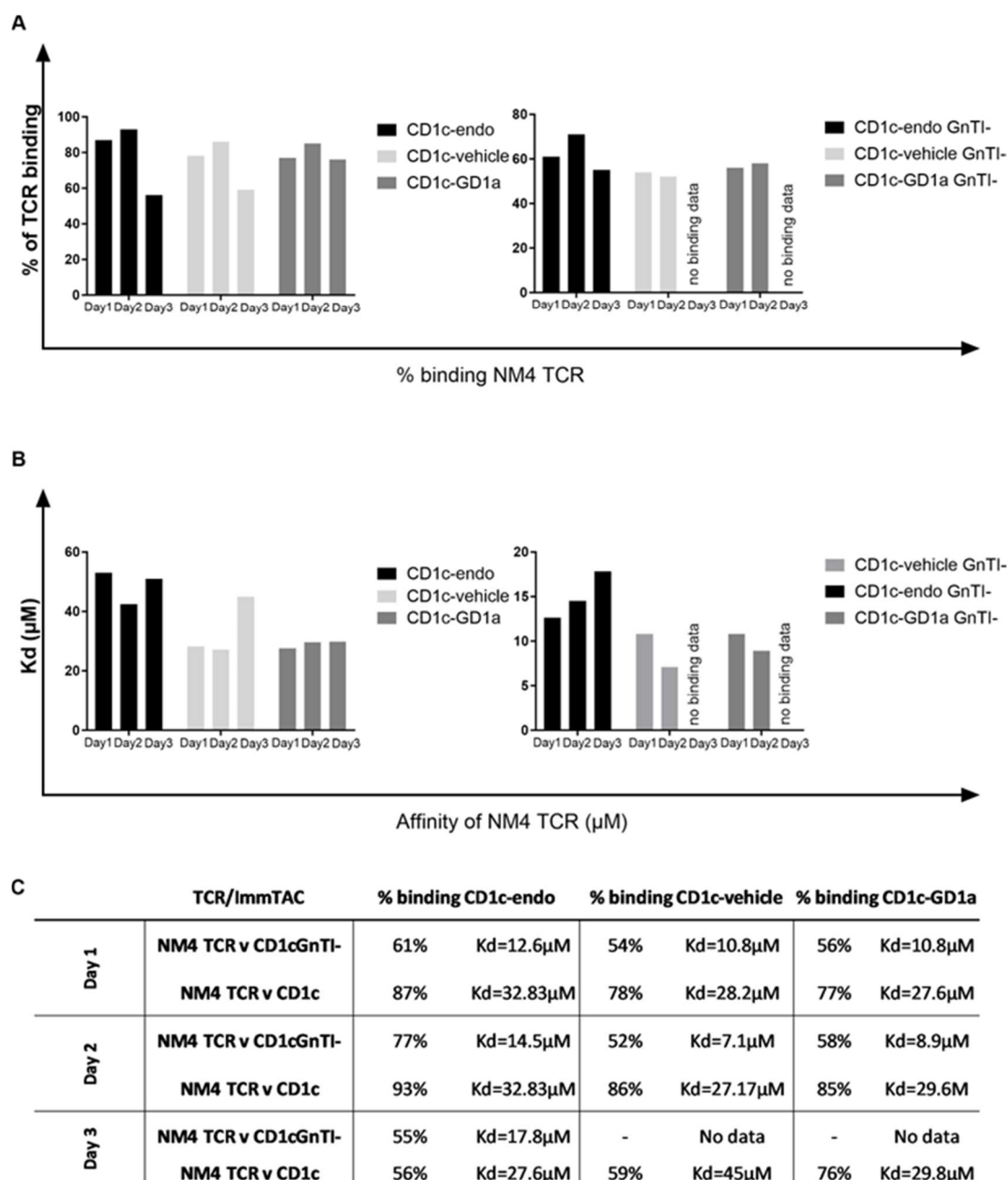


Figure 5.9: The effect of CD1c glycosylation on TCR binding

(A) % of NM4 binding to CD1c molecules after ligand exchange. (B) NM4 TCR binding affinity to CD1c molecules after ligand exchange. (C) Summary of NM4 binding percentages and affinities.

5.3.4 Tetramer staining

It was described previously that CD1d- α GalCer tetramers bind human NKT cells (165). The hypothesis was that NKT cells would recognize CD1d- α GalCer tetramers but not CD1d-endo (unsuccessful loading) tetramers, therefore positive staining indicates successful lipid loading. In this experiment, CD1d-endo molecules were loaded with KRN7000 (a synthetic

form of α GalCer) and generated tetramers for cell staining. Tetramer molecules are composed of a fluorescently labelled tetrameric streptavidin and four biotinylated CD1d-lipid molecules. CD1d molecules refolded with α GalCer were used as a positive control, kindly provided by Mariolina Salio. The in-vitro refolded molecules are mixed with the α GalCer ligand during refolding, therefore these CD1d molecules only contain α GalCer in their binding groove. Like MHC class I, CD1d cannot be refolded empty (166). The reagents were titrated to achieve optimal staining and to reduce background. Figure 5.10 illustrates the staining of previously sorted iNKT cells using CD1d- α GalCer tetramers. The iNKT cells were stained by both reagents, suggesting that the ligand exchange was successful and the CD1d- α GalCer molecules were functional.

Some level of lipid exchange was observed with both validation methods, which however is difficult to quantify. The most reliable method seems to be the detection of antigen-specific T cells via tetramer staining, which however requires the availability of specific T cell clones.

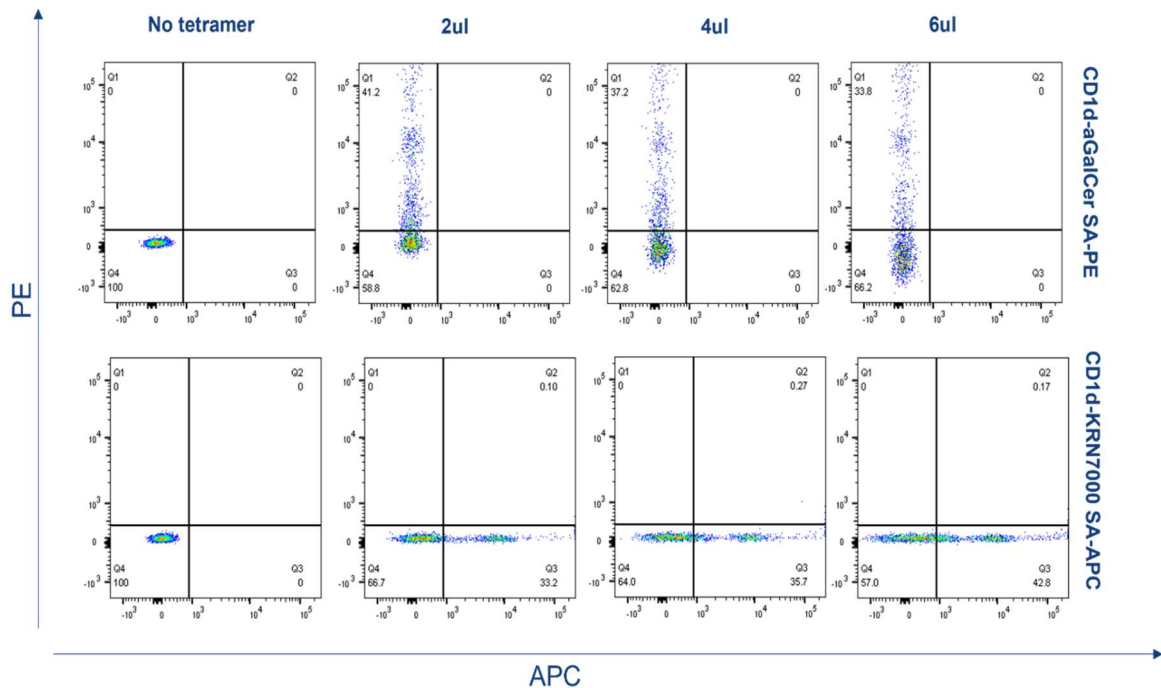


Figure 5.10: Validation of ligand loading with tetramer staining

iNKT cells were stained with SA-PE labelled CD1d- α GalCer tetramers (Y-axis) and SA-APC labelled CD1d-KRN7000 tetramers (X-axis).

5.4 The CD1-associated lipid repertoire

5.4.1 Analyzing the lipid repertoire of CD1 isoforms

The expression of the CD1 molecules in soluble format allows purification of the proteins without detergent, minimizing the loss of lipid ligands. Recent results published by Huang et al. (2023) validated the use of soluble proteins as a substitute model for transmembrane proteins (50). Therefore, soluble CD1 isoforms (CD1a, CD1b, CD1c and CD1d) were produced in Expi293F cells (50). The eluted lipid ligands were detected by mass spectrometry. HLA-A2 was also expressed in Expi293F cells and used the eluted lipids as a control to minimize false positive lipid detection. HLA-A2 is an ideal control due to its similar size, structure and function, however, since HLA-A2 binds to peptide antigens any lipids eluted from it should be accounted as background. The concern of abundance is a frequent problem of MS methods. Since several lipid species can have similar m/z ratios, only the most abundant can be detected with high confidence. Therefore, the most abundant lipid species of the sample dominate the mass spectrum, and less abundant species could be lost at the noise level (167–169). To overcome this limitation, CD1 protein replicates of different amounts (100 μ g and 500 μ g) derived from one protein production were analysed. To identify the optimal amount of CD1 required for mass-spectrometry an initial experiment was conducted by analyzing a titration series of CD1 molecules by mass spectrometry. The feasibility test determined that 500 μ g of CD1 protein is optimal for the mass spectrometry analysis. To discover less frequent lipid species, an additional 100 μ g replicates of CD1 were included. The purified protein samples were analyzed by mass spectrometry. An R-based bioinformatics analysis pipeline was created in-house to analyze the self-lipid binding capacities of the CD1 isoforms. 81 features were found, accounting for less than 6% of the lipid features detected in HLA-A2 controls, therefore the lipids identified in the CD1 samples are assumed to be derived from the lipid-binding pockets.

5.4.2 Widespread lipid capture by CD1 isoforms in mammalian cells

To investigate whether the CD1 isoforms are selective for certain lipids or rather sample the whole cellular lipidome, the total lipids eluted from Expi293F cells were compared with the lipidome eluted from the CD1 isoforms. The data was filtered to the top 293 lipids captured by each isotype. CD1 isoforms surprisingly bound the vast majority of the cellular lipids. While some lipids significantly diverged from the cellular background, the lipids that

were most dominant in the cellular lipidome were found to be enriched in all CD1 lipidomes. (Figure 5.11). The lipid profile of the CD1a and CD1c closely followed the cellular lipid composition, most abundantly capturing phospholipids (Figure 5.11A). CD1c followed the trend of the cellular lipidome in lipid chain length as well, while CD1a skewed towards C34-C38 lipids (Figure 5.11B). The lipidome of CD1b and CD1d showed bigger variance to the cellular lipid profile and, apart from the dominant PC, showed main enrichment in sphingolipids (Figure 5.11A). CD1b over-captured short-chain length lipids of C16-C20 and CD1d captured C42 lipids above the cellular profile (Figure 5.11B).

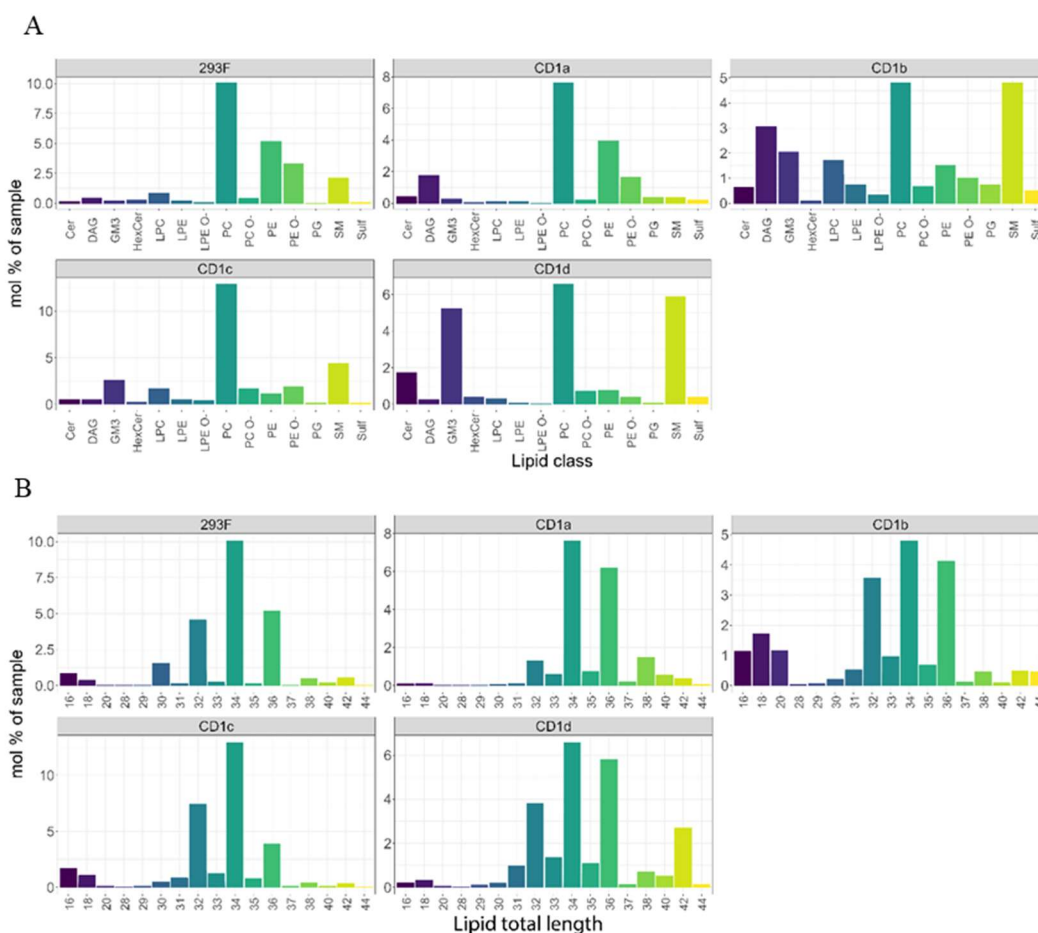


Figure 5.11: Common cellular lipids found captured by the CD1 isoforms (Szoke-Kovacs, 2024, p.5, Figure 2 and p75, Figure 5)

Comparison of the lipid classes (293) shared by the CD1 isoforms in and the 293F cells' total lipidome. Only the lipid fractions that were found in all CD1 isoforms are shown. The lipid intensities were normalized to a class-specific internal standard. The values are expressed in mol percentage (mol%), calculated by summing the amounts of individual lipids (species of subspecies) of a lipid class in pmoles to produce the total amount of the lipid class and then normalized to the overall lipid amount producing the mol% per total lipids **(A)** Lipid classes eluted from the CD1 isoforms. **(B)** Lipid chain length distribution of the CD1 isoforms.

5.4.3 The shared lipid profile of CD1 isoforms

A recent study by Huang et al. 2023 (29) has indicated that the CD1 isotypes capture an overlapping lipidome and act redundantly, presenting the cellular lipidome. Our data, produced by using a different mass spectrometry technique further confirmed these findings. To explore relationships among the variables, principal component analysis (PCA), an unsupervised learning model was applied to data standardized by molar percentage (Figure 5.12A). To evaluate the similarity of the CD1 isoform lipidomes, the lipid data was divided into bins according to the occurrence of the features in the CD1 isoforms and plotted on a Venn diagram (Figure 5.12B). As a result, a significant overlap in lipid composition was found amongst the CD1 isoforms, indicating that CD1 isoforms broadly sample the cellular lipidome, similarly reported by Huang et al. (29). The greatest deviation was found in the CD1a lipidome (Figure 5.12A).

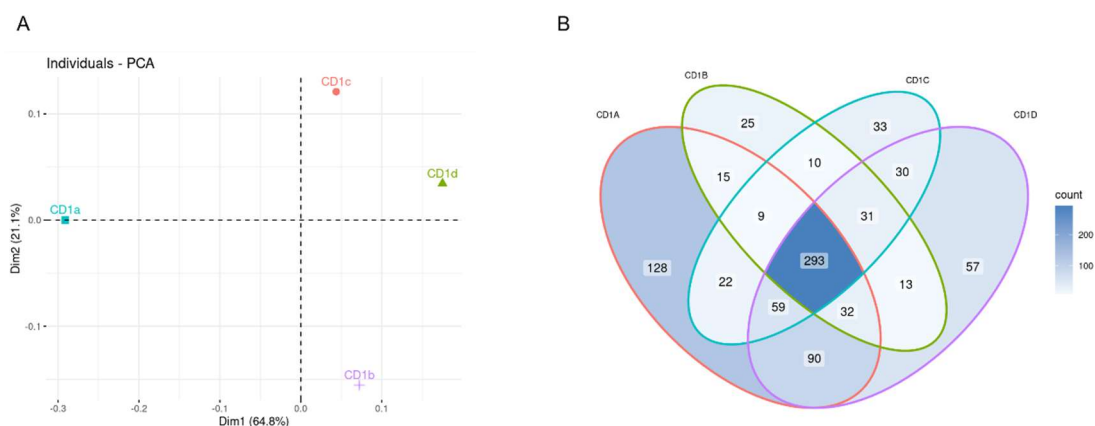


Figure 5.12: Lipidome of CD1 isoforms (Szoke-Kovacs, 2024, p.5, Figure 1)

(A) PCA of lipid classes captured by the CD1 isoforms. (B) Overlap of lipid classes captured by the CD1 isoforms.

An Individuals PCA analysis was performed to determine which individual lipids are the main contributors to the principal components. The individuals and variables PCA analysis merged into a biplot highlights the lipids that most significantly impact the principal components and illustrates their correlation with the variables (isoforms) (Figure 5.13) (170). 1040 lipids in total were identified from the lipidome eluted from the isoforms, wherein 293 lipids were shared across the isoforms. The lipid classes present in the eluted CD1 lipidomes show different distributions of the classes in the four isoforms (Figure 5.14). 12 Ceramides (Cer), 15 diacylglycerols (DAG), 3 hexosylceramides (HexCer), 4 lyso-phosphatidylcholines (LPC), 4 lyso-phosphatidyl-ethanolamines (LPE), 3 ether-linked lyso-phosphatidyl-ethanolamines (LPE O-), 74 phosphatidylcholines (PC), 68 ether-linked

The CD1a specific features were enriched in PI and PS lipids and headless lipid species of DAG (Figure 5.15). PG(C34-36), ether-PC, LPC, ether-LPC, LPE, ether-LPE, DAG and sulfatide within the CD1b-specific lipids were identified. Looking at the CD1c-specific lipidome, the most prominent species were found were GM2 (36:1;2), GD1 and GD2, along with phospholipids (PI, PS PC, PE O-, PC O-), lysolipids (LPC, LPA. LPI), ceramide and cardiolipin (C68-72). Cholesterol was most prominently enriched in CD1d (Figure 5.15). The full range of unique lipids identified on species and subspecies level can be found in supplementary material (Supplementary Figure 13.6).

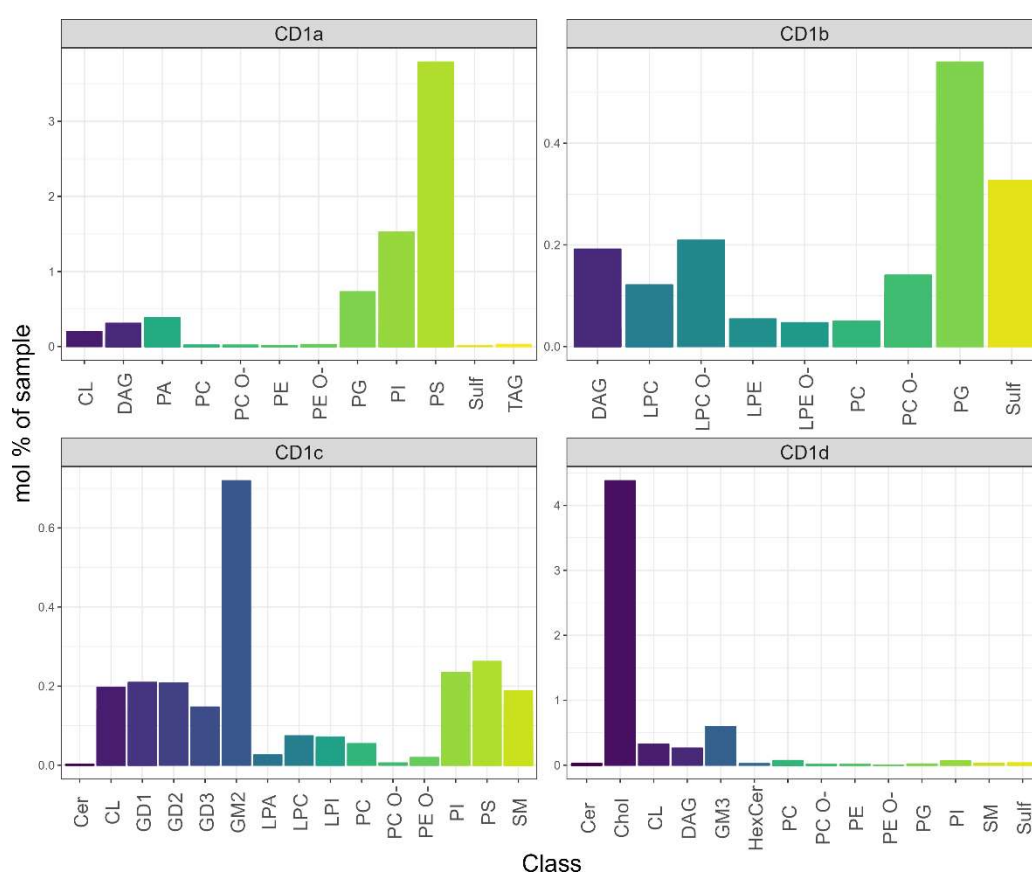


Figure 5.15: Mol% of CD1 isoform specific lipids.

(Szoke-Kovacs, 2024, p.7, Figure 4)

PI and PS were the most abundant CD1a specific lipids; PG and sulfatide were found abundantly in the CD1b specific lipidome; GM2 (36:1;2), GD1 and GD2 species were prominent in the CD1c specific lipidome; Cholesterol was uniquely found in the CD1d specific lipidome.

5.4.5 Characterization of lipid chain lengths bound by CD1 isoforms

Lipids are typically built up of glycerol, acyl-chains and phosphate groups. The acyl-chain length is interpreted as the total number of carbon atoms in the single chain lipids or the sum of two fatty acyl chains (e.g. attached to a glycerol backbone). The lipid antigens are captured in the CD1 molecule's hydrophobic binding groove (36). The CD1 isoforms' binding grooves have distinctive structures, which can affect the binding affinity to lipids with different acyl chain length and saturation level (37,50) (Figure 2.2). To get an insight into the lipid chain lengths present in the CD1 isoform lipidomes, the data was analyzed for acyl-chain length. Phospholipids and glycolipids were analysed in species and subspecies levels and sphingolipids were analysed at species level. CD1 isoforms show a preference towards lipids with different chain lengths (Figure 5.16).

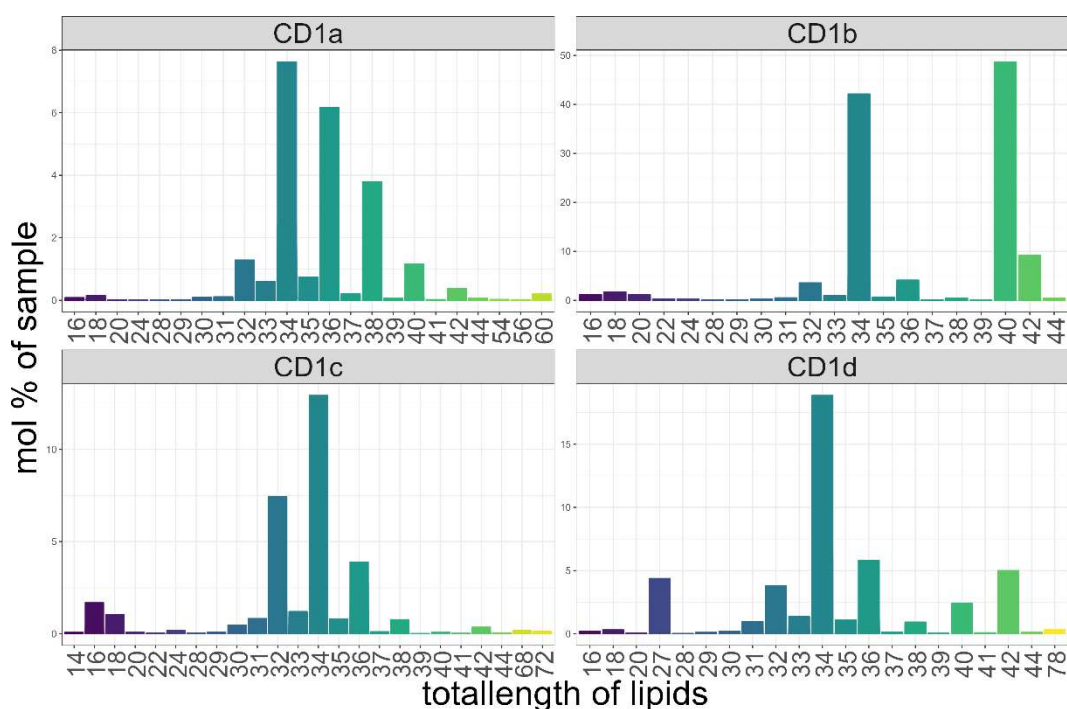


Figure 5.16: Mol% of lipid chain length within CD1 isotypes of shared lipids.

(Szoke-Kovacs, 2024, p.8, Figure 6)

The graphs depict the mol percentage of lipid classes within the 293 lipids shared by all isoforms. CD1a shows a preference towards mid-sized lipids (C34-C38). CD1b captures larger lipids (C40-C42) and shorter chain-length lipids (C34-C36). CD1d shows bias towards shorter lipid length (C34-C36) and accommodates some longer chains (C40-C42). The CD1c lipidome, on the other hand, has an even chain length distribution.

We found midsized lipid species (C34-C38) in the CD1a specific lipid pool; therefore, we suggest that CD1a shows preference towards mid-sized lipids. The CD1a isoform also accommodates larger cardiolipin (C60) and triacylglycerol (C56) species. CD1b captured

larger lipids (C40-C42) and shorter chain-length lipids (C34-C36). More sphingolipids captured than phospholipids in the lipidome eluted from CD1b, and the CD1b isotype-specific lipidome was enriched in PG and Sulfatide (C34-C38). CD1b has been previously reported to capture two lipids in its binding groove, a shorter lipid, possibly serving as a spacer and a larger lipid that can protrude from the binding groove (60). Short chain-length lipids (C16-C24) were also identified in the CD1b lipidome that could act as spacer lipids aiding the presentation of another lipid. CD1d shows bias towards shorter lipid length (C34-C36) and accommodates some longer chains (C40-C42) but captures mainly sphingolipids and PC. The CD1c lipidome, on the other hand, has an even chain length distribution, reflecting its structural flexibility (111). Most CD1c isoform-specific lipids range from C32 to C34. This isoform has also been thought to harbor smaller spacer lipids together with a larger antigenic lipid (62). Short (C14-C18) lysolipids were identified in the CD1c lipidome which could be accommodated in the F' pocket, supporting the presentation of an antigenic lipid. While the CD1c lipidome was heavily enriched in phospholipids, the isoform-specific lipids were C36 Gangliosides and shorter, C32 and C34 SM and phospholipids.

The most abundant phospholipid species (PC and PC O-) and the most commonly presented SM and GM3 were chosen to investigate whether the acyl-chain length is the driver of the selective capturing of the CD1 isoforms (Figure 5.17. and Figure 5.18).

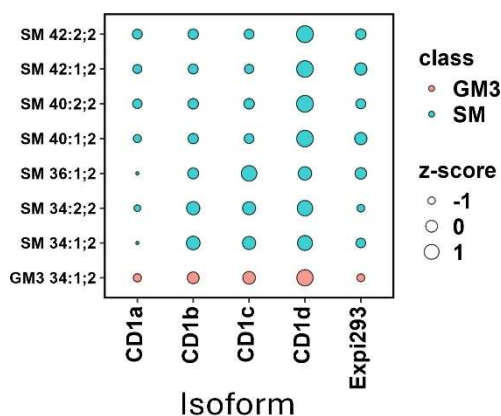


Figure 5.17: Sphingolipids captured by the CD1 isoforms. (Szoke-Kovacs, 2024, p.9, Figure 8)

The abundance of lipids indicated by the bubble size in the isoform's lipidome. CD1a captured larger SM species (C40-C42); CD1b bound short chain SMs (C34); CD1c preferred C34-C36 SMs. CD1d did not exhibit a preference for any specific sphingomyelin (SM) chain length. GM3 was enriched by CD1d.

Z-scores were assigned to the lipid abundances (representing the relative distance from the average) expressed in mol% values, which then were plotted and showed on a heat map. The lipids abundance is indicated by the bubble sizes on the heatmap. CD1a captured middle length (C38–C40) PC chains and SM species of C40–C42 carbon length. CD1b bound to PC species of short chain length (C30–C38) and SMs (C34). CD1d skewed towards large PCs (C42–C46) while it did not show tendency for capturing SM of any chain length preferentially. CD1c captured SMs of C34–C36 length and bound the whole variety of PC species (C30–C46). This analysis confirmed the preference of the CD1 isoforms towards optimal total chain length, but bias towards certain acyl chains or chain pairing were not found, or any indication of favored saturation levels in our data.

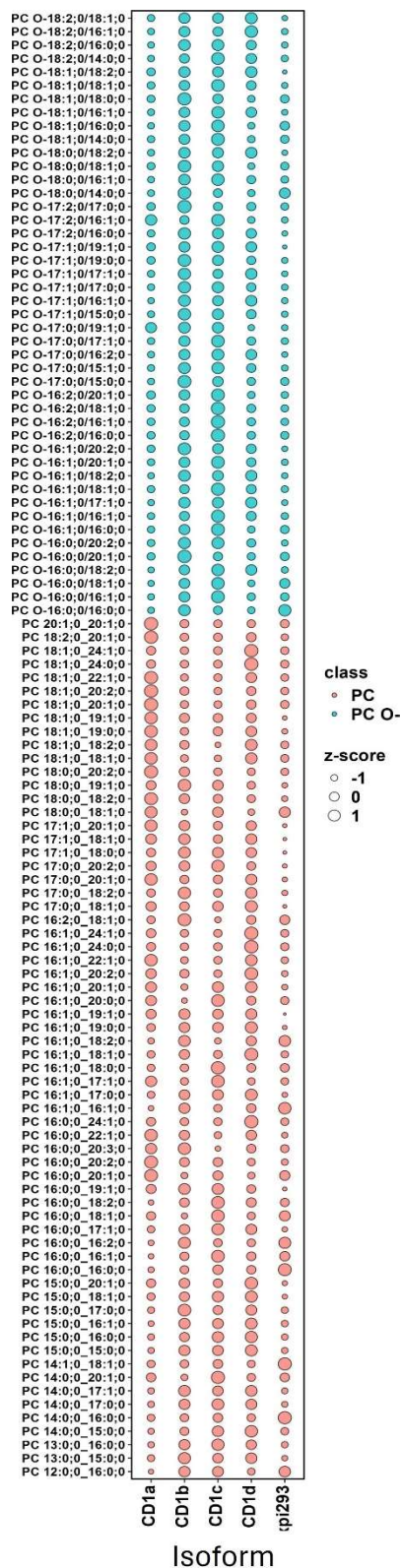


Figure 5.18: PC and PC O- species captured by CD1 isoforms (Szoke-Kovacs, 2024, p.9, Figure 7)

The abundance of lipids is indicated by the bubble size in the isoform's lipidome. CD1a captured middle length (C38–C40) PC species; CD1b bound PC (C30–C38); CD1d preferred PCs (C42–C46); CD1c captured the whole variety of PC species (C30–C46).

To measure cellular influence on lipid-ligand presentation by the CD1 isoforms, the mol% of lipids captured by each isoform to the cellular background were compared (Figure 5.19).

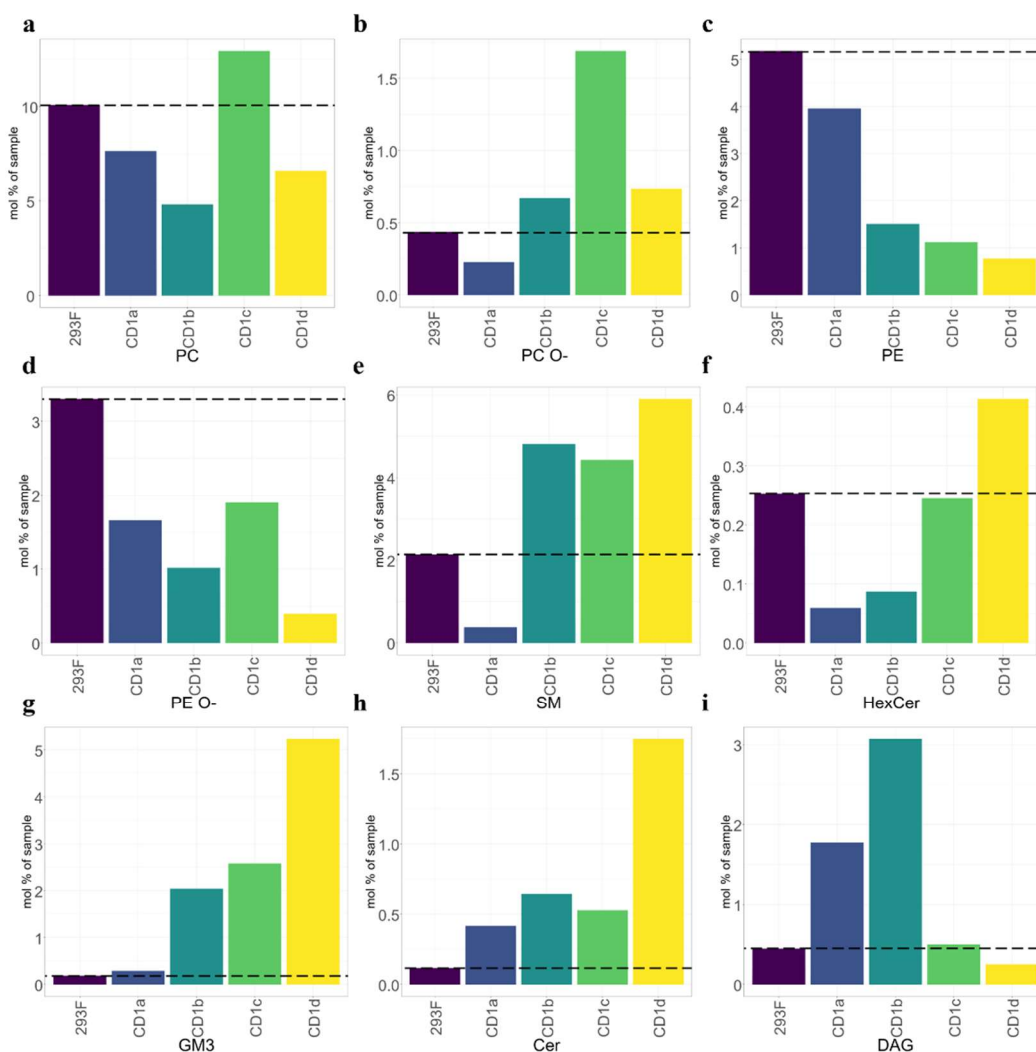


Figure 5.19: Shared lipids over captured by the CD1 isoforms

(Szoke-Kovacs, 2024, p.6, Figure 3)

Comparison of the over-capture of nine lipid classes by the CD1 molecules to the cellular lipidome. **(3a) (3b)(3c)(3d)** Phospholipids. **(3e) (3f)(3g)** Headed sphingolipids. **(3h) (3i)** Headless lipids. Ceramides were highly over-captured by CD1d. SMs shown over-capture by CD1b, CD1c and CD1d. Common under-capture of phospholipids (PE, EPE, and EPC). GM3 over-captured by CD1b, CD1c and CD1d.

All CD1 isoforms demonstrated strong enrichment for the most abundant PC and ether-PC (PC O⁻) were highly enriched in all CD1 molecules, but interestingly, PE and ether-PE (PE O⁻) species were consistently underrepresented. Experimental evidence suggests that presenting headless lipids facilitates autoreactive T cell recognition, while headed sphingolipids captured by CD1 molecules block autoreactive T cell activation (55). Modest

levels of SM were over captured in most CD1 lipidomes. Surprisingly, CD1a didn't over-capture SMs but selectively bound long-chain SMs (C40-C42) (See also Figure 5.17). This has also been reported by Cotton et al. 2021 (32). HexCer was somewhat over-captured by CD1d, with GM3, another headgroup bearing sphingolipid, was found to be 26-fold over-captured by CD1d. GM3 was also 10-fold over-captured by CD1b and CD1c. Lipids lacking polar head groups, such as ceramides, showed 2–3-fold enrichment in CD1a, CD1b, and CD1c, and a 9-fold enrichment in CD1d. CD1a over captured DAG by 3-fold and similarly DAG was 6-fold over captured by CD1b.

5.5 Binding mode of CD1c-specific TCRs

5.5.1 Generating CD1c-specific TCRs

A CD1c-specific TCR ($S2c^{WT}$ TCR) was isolated from in-house naïve TCR phage display libraries. The $S2c^{WT}$ TCR has an average affinity of $K_D = 16.7\mu M$ (measured by surface plasmon resonance) for CD1c bound to endogenous lipids (CD1c-endo) produced in Expi293F cells (Figure 5.20). This is within the expected range observed for conventional TCR-pHLA interactions (101,171). In SPR experiments, the $S2c^{WT}$ TCR achieved around 75% binding of the theoretical R_{max} (determined by dividing the experimentally observed R_{max} by the theoretical R_{max} , calculated using the number of CD1c on the chip), indicating a largely lipid-independent binding mode and consistent with the data of previously reported CD1c-reactive TCRs (111).

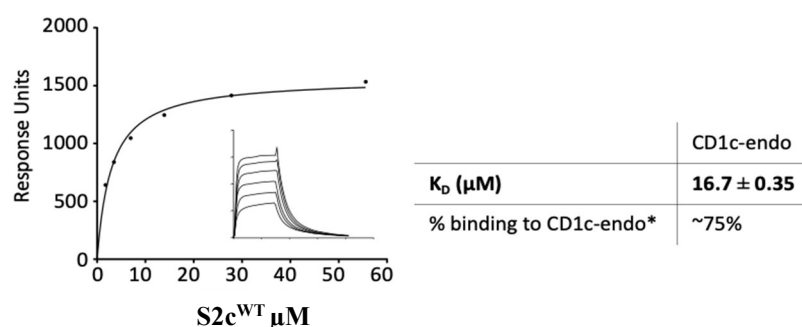


Figure 5.20: $S2c^{WT}$ TCR binding affinity (Adopted from Szoke-Kovacs, 2025, Suppl. Figure 1.)

Surface plasmon resonance was performed to measure the binding dissociation constant (affinity) of the $S2c^{WT}$ TCR binding to CD1c-endo (CD1c bound to lipids from Expi293F cells).

5.5.2 Affinity enhanced CD1c restricted TCRs

Like pHLA-peptide complexes, target of TCR bispecific engagers, CD1-lipid complexes are naturally presented at a low level on the cell surface. Bispecific engagers with 1 to 100 μM affinity generally have short half-lives (172), therefore are not suitable for targeting molecules with low levels of surface expression. To overcome this limitation, affinity-enhanced TCRs were generated towards CD1c by subjecting the S2c^{WT} TCR to phage display as detailed earlier (173,174). For the 1st generation affinity maturation, soft mutagenesis libraries (3% randomization rate of each nucleotide position) were built, targeting the three CDRs of one chain in each library. Three rounds of phage panning were carried out using an antigen concentration below the K_D which favors the binding of higher affinity clones. The antigen concentration was reduced across the subsequent pans to select for higher affinity binders, 50nM, 5nM and 0.25nM CD1c-endo, respectively. A fourth panning round of off-rate selection was performed. Here, after incubation of the phage library with the immobilized, biotinylated CD1c-endo, the phage library was incubated with an excess of (100 nM) non biotinylated CD1c-endo for two hours. The clones with a faster off rate fall off the biotinylated CD1c, bind to the non-biotinylated CD1c in solution and are removed by washing, biasing the selections towards clones with a longer half-life.

Inhibition ELISAs were carried with or without pre-incubation of the phage with 100 nM non biotinylated CD1c. The absorbance values for phage incubated with and without non-biotinylated CD1c were used to calculate the percentage reduction in signal or level of inhibition. The phage inhibition ELISA data were used to rank TCRs based on affinity, as TCRs with higher affinity show a greater reduction in ELISA signal (inhibition). The a2bWT showed an inhibition of 83 and 85% and aWTb1 showed an inhibition of 82%. The best candidate, CD1c-S2-a2b1-U with affinity of 1.05nM and half-life of 37min towards CD1c-endo was chosen as template for the second round of affinity maturation (Figure 5.21).

In the second round of affinity maturation, the 1st generation output CD1c-S2-a2b1 TCR was used as template. Three phage panning rounds were performed, using reduced antigen concentration to select for higher affinity binders, 100pM, 10pM and 1pM CD1c-endo respectively. Six hard mutagenesis libraries were used, containing mutations across the CDR chains and six structurally guided libraries. The first structurally guided library included mutations of the CDR1 α and CDR3 α targeting the CD1c helix 1 and 2.

A) CD1c-S2 alpha and beta mutants- affinity against CD1c-endo

Affinity mutants TCR name	CDR1 α	CDR2 α	CDR3 α	CDR1 β	CDR2 β	CDR3 β	k_a	k_d	K_D to CD1c-endo (HEK)	T1/2
Rjp2c-S2 wt	ATGYPS	ATKADDK	CALSDQYGWGKLQF	SEHNR	FQNEAQ	CASSPRTGRGAEAFF	-	-	16.7 μ M	x
CD1c-S2-a1bWT-U	ATGYPS	ATKADDK	CALSDAYGWGELQF	SEHNR	FQNEAQ	CASSPRTGRGAEAFF	-	-	22.9 μ M	x
CD1c-S2-a2bWT-U	ATGYPS	ATKADDK	CALSGQYGWGKLQF	SEHNR	FQNEAQ	CASSPRTGRGAEAFF	-	-	200 μ M	x
CD1c-S2-aWTb1-U	ATGYPS	ATKADDK	CALSDQYGWGKLQF	SEHNR	FQNEAQ	CASSPRMVRGAEAFF	-	-	600 μ M	x

B) CD1c-S2 alpha and beta combination mutants- affinity against CD1c-endo

Affinity mutants TCR name	CDR1 α	CDR2 α	CDR3 α	CDR1 β	CDR2 β	CDR3 β	k_a	k_d	K_D to CD1c-endo (HEK) M	T1/2
CD1c-S2-a1b1-U	ATGYPS	ATKADDK	CALSDAYGWGELQF	SEHNR	FQNEAQ	CASSPRMVRGAEAFF	4.94E+05	7.67E-04	1.55 nM	15 min
CD1c-S2-a2b1-U	ATGYPS	ATKADDK	CALSGQYGWGKLQF	SEHNR	FQNEAQ	CASSPRMVRGAEAFF	4.10E+05	4.31E-04	1.05 nM	37 min

Figure 5.21 First generation affinity maturation outputs

(A) The top molecules from the inhibition ELISA were expressed as ImmTAC molecules and their affinity was tested against CD1c-endo by SPR. (B) The affinity of α and β -chain combinations were tested for binding to CD1c-endo. The best candidate with the highest affinity and half-life (in red bracket) was chosen as template for the second generation of affinity maturation.

The second library included mutations of the CDR2 β targeting direct contacts to the CD1c helix 1. Library 3 was designed to stabilize the TCR binding to CD1c helix 2 by allowing co-variance of the CDR2 α and CDR3 β . Three loop extension libraries were designed with 1, 2 or 3 additional amino acid residues of the CDR2 α , targeting CD1c helix2. Phage inhibition ELISAs were carried out with 0.5nM non-biotinylated CD1c. Clone S2-a5b1 showed an inhibition of 60%. The panning outputs were screened by using phage-ELISA for cross-reactivity and 28 chain combinations were expressed as ImmTAC molecules in a mammalian expression system To assess cross-allele reactivity, the affinity-matured ImmTACs were profiled using surface plasmon resonance against CD1a, CD1b, CD1d, and a diverse panel of peptide-HLA complexes, including HLA-A2, HLA-A11, HLA-B7, HLA-B35, and HLA-Cw304 (Figure 5.22). The ImmTACs with cross-reactivity and half-life shorter than the template were removed. The top 11 clones are shown in Figure 5.22. The S2-a5 alpha chain derived from the 2nd generation affinity maturation aCDR1 NNK library and S2-b6 beta chain derived from first generation affinity maturation output were the best chain combination. This mutant reached 100% binding of the CD1c-endo molecules measured by SPR and gained high specificity to CD1c compared to the template molecule S2-a2b1 which shown cross-reactivity to CD1b. The affinity of the S2c^{WT} TCR increased from the initial K_D value of 16.7 μ M to 15 pM. The half-life of the high affinity mutant, S2c^{a5b6} reached 9.6 hours. The mutations obtained through the affinity maturation process were modelled onto the S2c^{WT} structure (Figure 5.23).

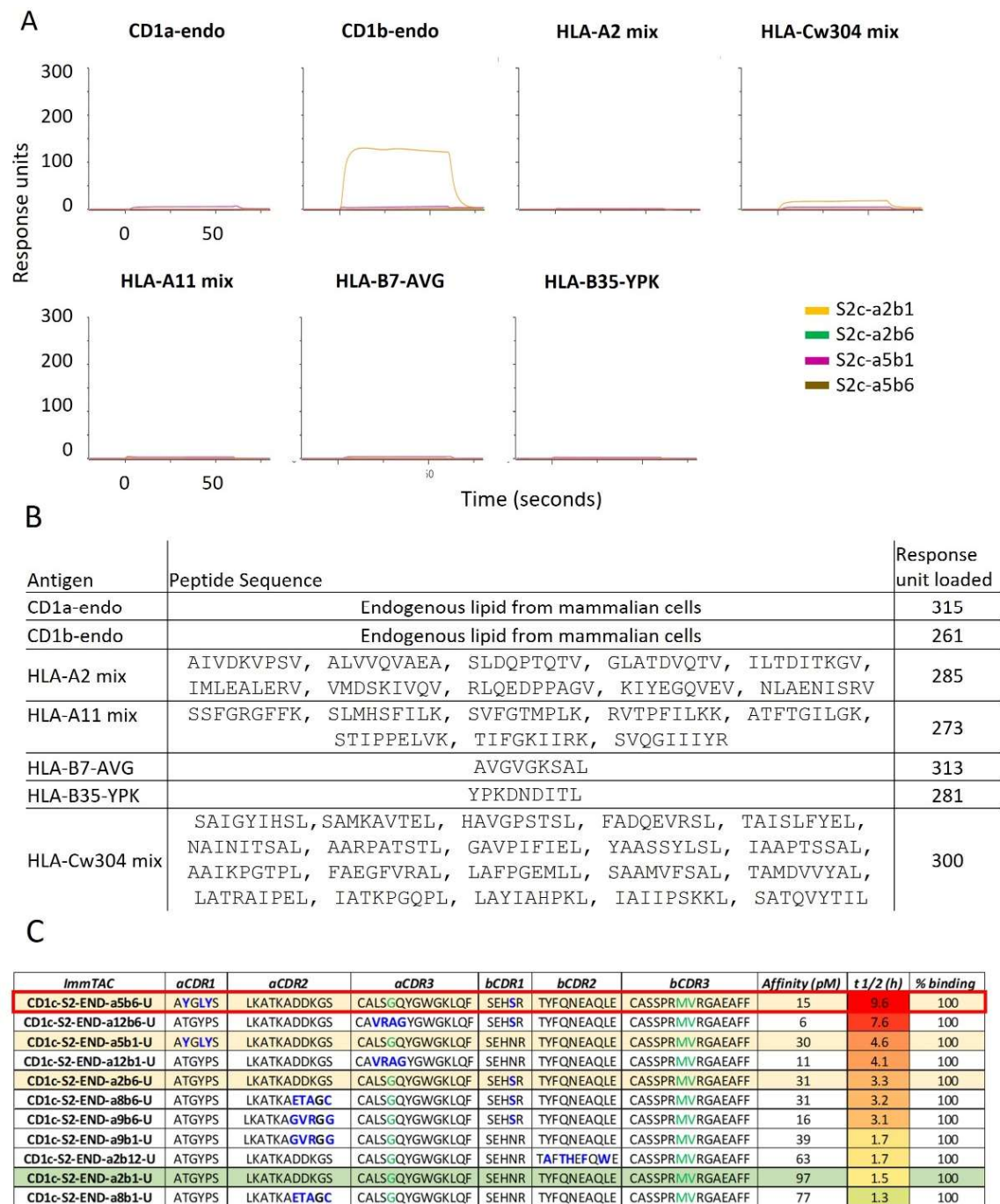


Figure 5.22 Biophysical characterization of the affinity maturation outputs (Adopted from Szoke-Kovacs, 2025, Suppl. Figure 3.)

Surface plasmon resonance was applied to measure the binding of the TCRs against CD1a, CD1b, CD1d, and HLA-A2, HLA-A11, HLA-B7, HLA-B35 and HLA-Cw304 in complex with common peptide antigens.

(A) Association curves of the TCRs to CD1-endo isoforms and common HLA-peptide panel (B) Table of molecules used in cross-reactivity testing. (C) The binding dissociation constant (affinity) of the top candidates was tested by SPR against CD1c-endo molecules. CD1c-endo binding by S2cWT TCR was estimated by dividing observed R_{max} by theoretical R_{max} based on CD1c loading. Data shown represents three experiments with different CD1c-endo batches. The lead molecule chosen is shown in red bracket.

Based on the modelling, the mutations are either positioned within the CDR loops facing internally, or towards the surface of the CD1c heavy chain (Figure 5.23). The 100% binding of CD1c-endo observed by SPR, and the modelled mutations suggest that the affinity maturation changes might have resulted in higher affinity binding to the CD1c heavy chain, allowing high affinity, lipid-agnostic recognition of CD1c-endo.

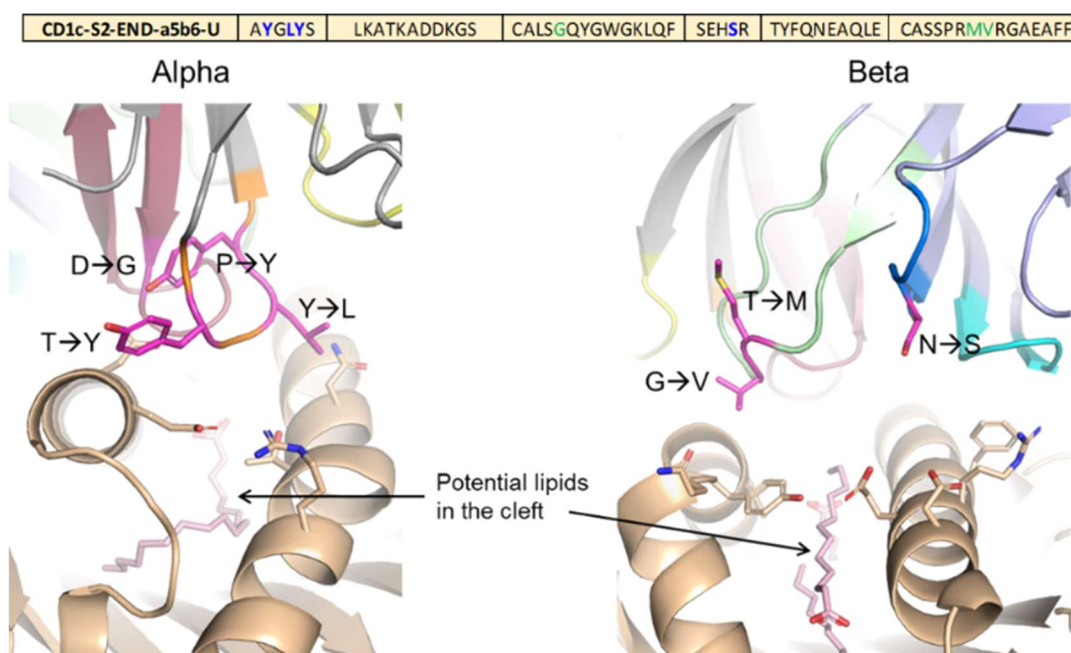


Figure 5.23 Affinity maturation changes predominantly target CD1c

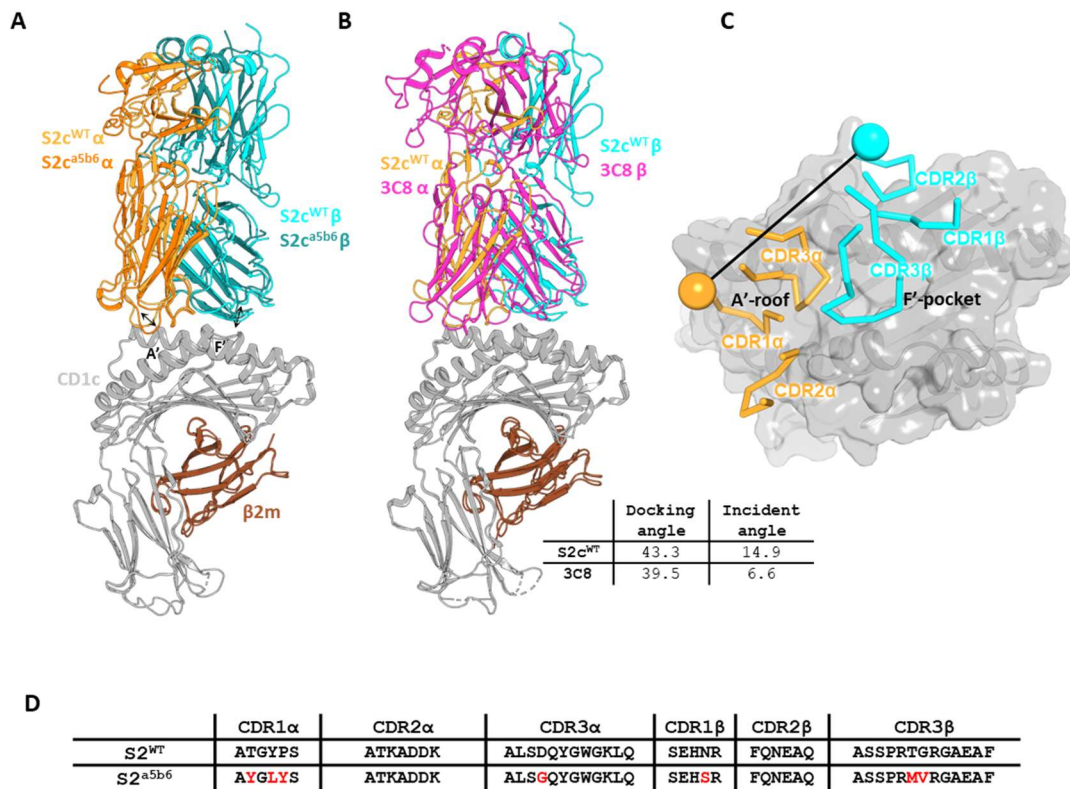
Mutations of S2c^{a5b6} modelled onto S2c^{WT} -wt structure. Residue changes are depicted with black letters.

5.5.3 Structural basis of CD1c lipid independent recognition

To gain a deeper understanding of the CD1-lipid recognition of TCRs, the crystal structure of the S2c^{WT}-CD1c-endo trimolecular complex at a resolution of 2.04Å and the S2c^{a5b6}-CD1c complex at a resolution of 2.27 Å were solved. (Figure 5.24).

Figure 5.24. Crystal structures of CD1c-S2c TCR complexes. (Szoke-Kovacs, 2025, p. 5, Figure 1.)

(A) Structural overlay of the S2c^{WT} TCR (PDB: 9QWJ) and the affinity-enhanced S2c^{a5b6} variant (PDB: 9QWK) bound to CD1c-endo. TCRα, TCRβ, CD1c, and β2m chains are rendered in orange, cyan, grey, and brown, respectively, with the S2ca5b6 chains shown in deeper hues. Arrows highlight conformational shifts in the CDR1α and CDR3β loops between the two TCRs. (B) Comparison of CD1c-S2c^{WT} and CD1c-3C8 (PDB: 6C09) complexes, with the 3C8 TCR chains depicted in magenta. (C) Top-down view illustrating CD1c as a grey surface and S2c^{WT} CDRs as ribbon structures. Disulphide bonds within the TCR variable domains are marked by spheres, with a connecting line representing the vector used to determine the docking angle. (D) Alignment of CDR sequences from S2c^{WT} and S2c^{a5b6} TCRs, with mutations introduced in the S2c^{a5b6} variant highlighted in red.



This structural analysis provides important insights into the interaction mode, contact footprint, and lipid accommodation, enhancing our knowledge of the mechanisms behind CD1c-restricted TCR recognition. The analysis of the overlaid crystal structures of S2c^{a5b6}, S2c^{WT} and 3C8 TCRs in complex with CD1c revealed similar central binding to CD1c and diagonal docking angle 43.3° of the S2c^{a5b6}, the S2c^{WT} and 39.5° the previously reported CD1c-restricted 3C8

TCR (111) (Figure 5.24 A, B and C). The S2c^{WT} and 3C8 exhibit interactions with overlapping groups of CD1c residues and similar docking orientations, while S2c^{a5b6} exhibits increased interaction with residues along CD1c helix 1, whereas 3C8 shows a stronger engagement with residues on helix 2. The Asp65, Glu80 and Glu157 residues of CD1c were contacted by the TCRs, establishing several van der Waals (vdWs), and electrostatic contacts (hydrogen bonds and/or salt bridges). 3C8 exclusively contacted the Gln151, Glu153 and Ser169 residues with multiple vdWs and electrostatic interactions and S2c^{WT} uniquely established contact with Arg71 and Leu162. The TCR contact analysis revealed that the majority of contacts were made across spanning the CD1c helix 1, concentrating above the CD1c A'-roof, resulting of a 1015 Å² buried surface area. This value is consistent with the established range typically reported for TCR-pHLA and TCR-CD1 interactions (67). Analysis of the interface indicated that the TCR α -chain predominantly mediates the interaction, contributing approximately 63% of the contact points. The CDR1 and CDR2 loops are aligned over the CD1c α -helices, while the CDR3 β loop is positioned directly above the CD1c F' portal (Figure 5.24 C and Figure 5.25 A-D). Contacts made by TCR β -chains (35%) were largely directed by CDR2 β towards the CD1c helix 1 and further contacts made by CDR3 β towards both helices. The CDR1 β made no contribution to the interface (Figure 5.25 E-H). There was no clear density observed for the lipid head group(s), which might be attributed to the mixture of lipids occupying the CD1c binding groove. Contacts between the S2c^{WT} TCR and the lipid component was not resolved in our structure. The main contacts were focused on the CD1c α 1 residues of Asp65, Leu68, Arg79, and Glu80 by the S2c^{WT} TCR CDR1 α (Tyr30), CDR3 α (Gln95 and Tyr96), CDR2 β (Glu52) and CDR3 β (Arg97 and Arg100) residues (Figure 5.25 A-H). Structural studies of TCR-pHLA complexes have identified specific HLA surface residues that are frequently engaged by TCRs, particularly the restriction triad residues at positions 65, 69, and 155 (175). Numerous CD1d-TCR structures exist for murine and human systems. Invariant iNKT TCRs consistently use a nearly identical binding footprint on CD1d (63). The comparison of the S2c^{WT} TCR contact footprint with that of the 3C8 TCR, the only other reported TCR-CD1c co-complex structure (110), revealed similar binding features. Both TCRs engage with the CD1c surface through a high number of salt bridges (5 for S2c^{WT} and 6 for 3C8). These may help stabilize the interactions with the lipid cargo (Tables 5.2 and 5.3). The CD1c residues of Asp65 and Glu80 were critical for the interactions with both TCRs.

CD1c	S2c ^{WT}	S2c ^{a5b6}
Ser59	Thr28α 1	Tyr28α 1HB
Glu61		Tyr28α 4
Glu62	Thr28α 5, 1HB	Tyr28α 10
Asp65	Asp94α 5	Gly94α 2
	Gln95α 9, 2HB	Gln95α 8, 4HB
	Tyr96α 2	Tyr96α 2
Leu66	Tyr96α 1	Tyr96α 3
Leu68	Gln95α 4	Gln95α 5
	Glu56β 1	Glu56β 3
Leu69	Gln95α 3	Gln95α 2
	Tyr96α 2	Tyr96α 1
	Trp98α 1	Trp98α 1
Arg71	Leu55α 3, 2HB	Leu55α 2, 2HB
Phe72	Gln95α 1	Gln95α 2
	Tyr48β 5	Tyr48β 6
	Gln50β 7, 1HB	Gln50β 5, 1HB
	Leu55β 1	Leu55β 3
	Glu56β 1	Glu56β 1
Phe75	Gln50β 3	Gln50β 2
	Asn51β 1	Asn51β 1
	Glu52β 1	Glu52β 1
	Ala53β 2	Ala53β 2
	Leu55β 2	Leu55β 1
Gly76	Gln50β 1, 1HB	Gln50β 2
Arg79	Asn51β 3	Asn51β 2
	Glu52β 4, 3HB, 1SB	Glu52β 4, 3HB, 1SB
Glu80	Arg97β 3, 3HB, 1SB	Arg97β 5, 3HB, 1SB
Tyr152	Arg97β 1HB	Arg97β 1HB
		Met98β 1
Gly154	Trp98α 2	Trp98α 2
		Arg100β 2
Val155		Trp98α 1
Glu157		Leu30α 3
	Lys52α 3, 1HB, 1SB	Lys52α 1, 1HB, 1SB
	Arg100β 1, 1HB	Arg100β 3
Thr158	Tyr96α 2	Tyr96α 1
	Trp98α 3, 1HB	Trp98α 4, 1HB
	Arg100β 2	Arg100β 1
Asn161	Tyr30α 3	Leu30α 4
		Tyr96α 2
Leu162	Tyr96α 4	Tyr96α 4
Ser165	Tyr30α 1, 1HB	
Thr166	Tyr30α 1	
	Tyr96α 3, 1HB	Tyr96α 4, 1HB

Table 5.2: List of contacts between S2c^{WT}/S2c^{a5b6} TCR and CD1c (Szoke-Kovacs, 2025, Suppl. Table 3.). Van der Waals, hydrogen bond (HB) and salt bridge (SB) contacts up to 4Å distance are listed. Interface mutated residues in S2c^{a5b6} are highlighted in red.

CD1c	3C8 TCR (PDB 6C09)	
Glu62	Ser28α	3, 1HB
	Met29α	3
Asp65	Asn27α	3, 1HB
	Val108α	1
	Lys111α	2, 1HB, 1SB
Leu66	Met29α	2
Leu68	Val108α	1
	Asp110α	2
	Lys111α	3
Leu69	Gly109α	2
Phe72	Gly109α	1
	Asp110α	5
	Gln57β	2
	Tyr109β	1
	Met114β	1
Phe75	Gln57β	16
	Gly58β	1
Gly76	Tyr109β	2, 1HB
Arg79	Thr30β	4
	Tyr109β	2
Glu80	Tyr109β	5, 1HB
	Arg110β	2, 2HB, 1SB
Gln151	Arg110β	3, 1HB
	Gly111β	4, 1HB
Tyr152	Tyr109β	2
	Arg110β	4, 1HB
Glu153	Gly111β	1HB
	Pro112β	3
Gly154	Gly111β	1, 1HB
	Pro112β	2, 1HB
	Met114β	3
Val155	Tyr109α	1
Glu157	Tyr32α	4, 1HB
	Ser57α	2, 1HB
	Ile59α	5
	Pro112β	1
Thr158	Asp31α	1
	Met114β	2
Tyr160	Ile59α	3
Asn161	Asp31α	1
	Ser58α	1, 2HB
Thr166	Met29α	1
Ser169	Ser28α	1, 3HB
	Met29α	1

Table 5.3: List of contacts between 3C8 TCR and CD1c. (Szoke-Kovacs, 2025, Suppl. Table 4.) Van der Waals, hydrogen bond (HB) and salt bridge (SB) contacts up to 4Å distance are listed

These residues form multiple van der Waals interactions and electrostatic contacts, including hydrogen bonds and/or salt bridges. The residues, Leu68 and Arg79 were also contacted by both TCRs, but they contributed significantly only in the case of S2c^{WT}. The residue Gln151 was a key contact residue for 3C8 but was not for S2c^{WT}. This indicates that while the S2c^{WT} and 3C8 TCRs present similar contact interfaces, their binding modes differ and are supported by distinct networks of unique interactions. (Tables 5.2 and 5.3).

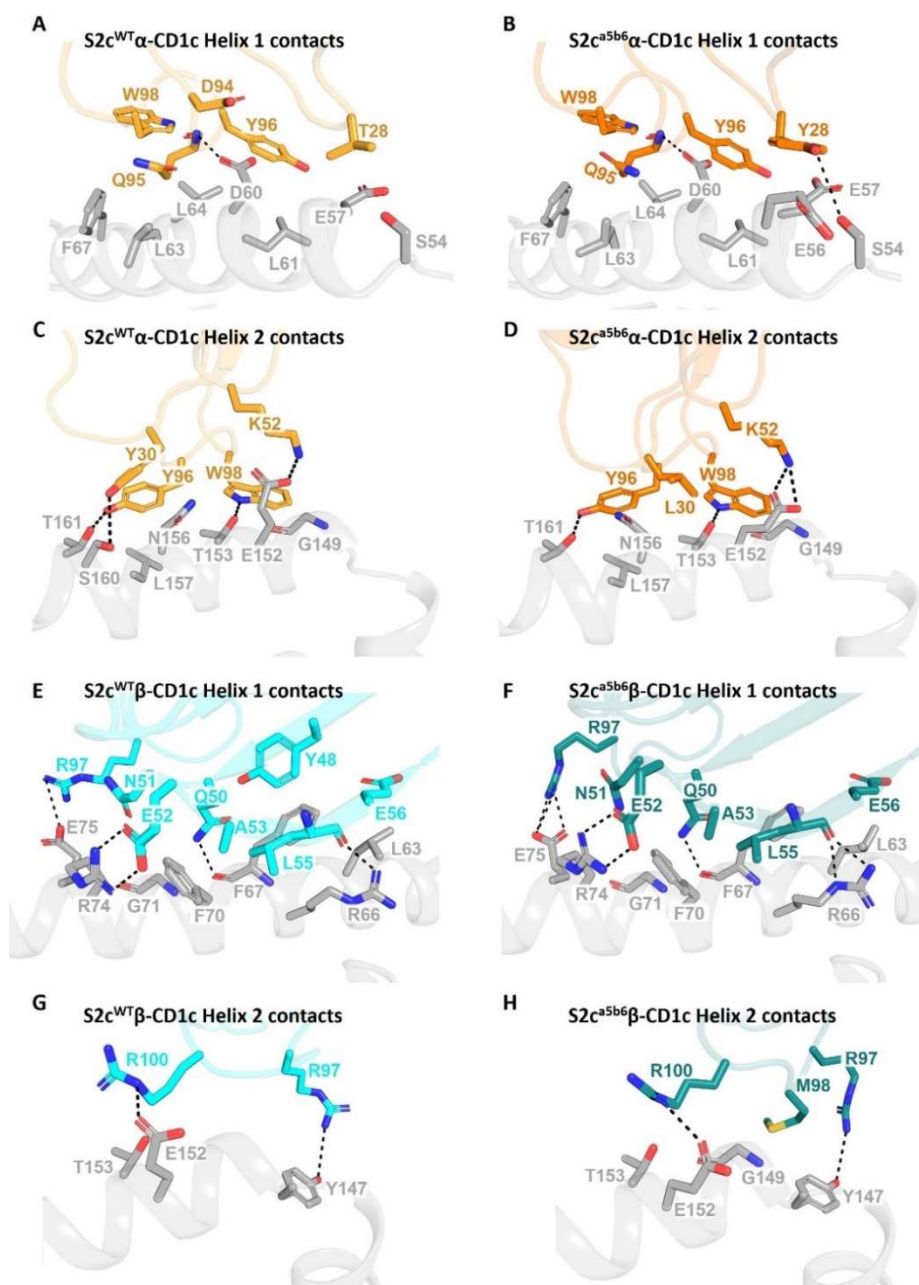


Figure 5.25 Contact analysis of S2c^{WT} and S2ca5b.
(Szoke-Kovacs, 2025, p. 6, Figure 2.)

The high affinity was driven by Y28^α, L30^α and M98^β (B, D, H). Y28^α makes additional van der Waals contacts to CD1c Glu61 and Glu62, L30^α to Glu157 and M98^β to Tyr152 (B, D, H).

Given current insights into CD1-restricted TCRs, the observed binding mode most closely resembles that of CD1b-restricted TCR structures. In contrast to CD1c-restricted TCRs, known CD1a-restricted TCR have a distinct tilt angle, and CD1d-restricted TCRs bind with a distinct parallel crossing angle (Figure 5.26).

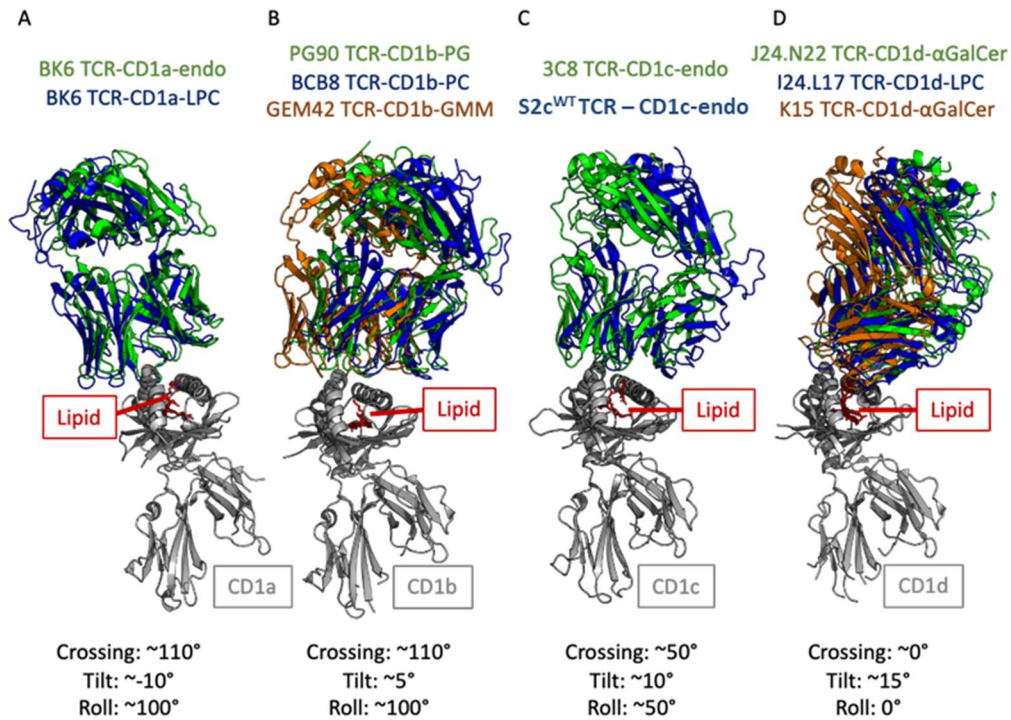


Figure 5.26 Structural comparison of S2c^{WT}-CD1c-endo with CD1a, b, c and d restricted TCRs

Side view superpositions aligned on the CD1 molecules. CD1 molecules are shown as grey cartoon and lipid cargos are shown as red sticks. A) BK6 TCR (4X6D) (green and blue cartoon) binding to CD1a-endo or CD1a-LPC; B) PG90 (5WKI) (green cartoon), BCB8 (8DV4) (blue cartoon), and GEM42 (5L2K) (orange cartoon) TCRs binding to various CD1b-lipid complexes; C) 3C8 (6C09) (green cartoon), S2c^{WT} (9QWJ) (blue cartoon) TCRs binding to CD1c-endo; D) J24.N22 (3SDX) (green cartoon), J24.L17 (3TZV) (blue cartoon) and NK15 (3HUJ) (orange cartoon) TCRs binding to various CD1d-lipid complexes. Average crossing, tilt and roll angles for TCRs binding to each class of CD1 are shown. **Crossing angle:** angle between the TCR's longitudinal axis and the axis of the antigen bound in the CD1 binding groove. This has an effect on which CDR loop contacts the antigen; **Tilt:** Indicates how much the TCR leans toward one end of the CD1 binding groove, which affects which TCR chain dominates the binding interaction; **Roll:** Indicates the rotation of the TCR around its own axis while binding to the CD1 molecule, which can impact on signalling and the accessibility of CD3 molecules.

5.5.4 Increased number of hydrophobic interactions drive high-affinity of S2c^{a5b6}

Among the seven mutations introduced (Figure 5.24 D), the increased affinity of S2c^{a5b6} for CD1c was mainly influenced by three hydrophobic residues: Y28 α , L30 α , and M98 β . Y28 α establishes further van der Waals interactions with CD1c Glu61 and Glu62, L30 α interacts with Glu157, and M98 β engages with Tyr152 (Figure 5.25 B, D, H). In contrast, D94 α diminishes interactions with CD1c Asp65 compared to S2c^{WT} (Figure 5.27 A). The increased number of hydrophobic interactions improves the affinity for CD1c and improves lipid selectivity, particularly through S30 β (Figure 5.27 C) and M98 β (Figure 5.25 H and Figure 5.27 C). Overall, our results indicate that TCR affinity can be enhanced by targeted hydrophobic mutations, facilitating improved interaction with both CD1c and the lipids it presents.

5.5.5 Lipid Accommodation and CD1c F67 Conformational Flexibility

The diverse nature of lipids captured by CD1c-endo made it difficult to detect clear density for the lipid head groups. Despite the structural data suggesting that the S2c^{WT} TCR made possible contacts with the lipid, proof of the contact was not evident in our structure. However, the structural analysis revealed two copies of the S2c^{WT}-CD1c complex in the asymmetric unit, which is the smallest unique portion of the crystal lattice, that generates the crystal. Having more than one complexes in the asymmetric unit indicates some degree of structural variability. In this case, the two copies shown significant differences in density within the lipid head group region (Figure 5.27 A, B), indicating variability in lipid binding. The CD1c F67 exists in two distinct side-chain conformations, which affects how lipids are accommodated. Larger lipid head groups likely need the outward-facing conformation of F67. The S2c^{WT} TCR adapts to these variations by modifying side chain conformations near the CD1c F67 and F'-portal region. The Q95 α and Y48 β residues flip to fit CD1c F67 in its external-facing position, which in turn shifts the positions of R31 β , Q50 β , and N30 β residues in the loop, facilitating interactions with larger lipid head groups (Figure 5.27 C). Moreover, two density fragments were detected within the CD1c groove, resembling previously identified 'spacer lipids' (111,164). Computational modeling and refinement of decanes provided a good match with the observed density. We observed four similar molecules of the complex in the asymmetric unit of the S2c^{a5b6}-CD1c crystal structure. These contained similar lipid density protruding from the F'-portal and surrounded by the TCR β residues

(Figure 5.27 C). The CD1c F67 was found in one outward facing configuration, similar to the alternative conformation seen in the S2c^{WT}-CD1c complex (Figure 5.27 C). The Y48 β , R31 β and Q50 β side chain conformations remained consistent with the S2c^{WT} alternate conformation. The S30 β and M98 β residues acquired in the affinity maturation process create a larger space for the lipid head groups and encompass the lipid density, implying a preference for larger lipid head groups. These findings were confirmed by lipid mass spectrometry analysis of the lipids eluted from the S2c^{a5b6}-CD1c complex (Figure 5.29).

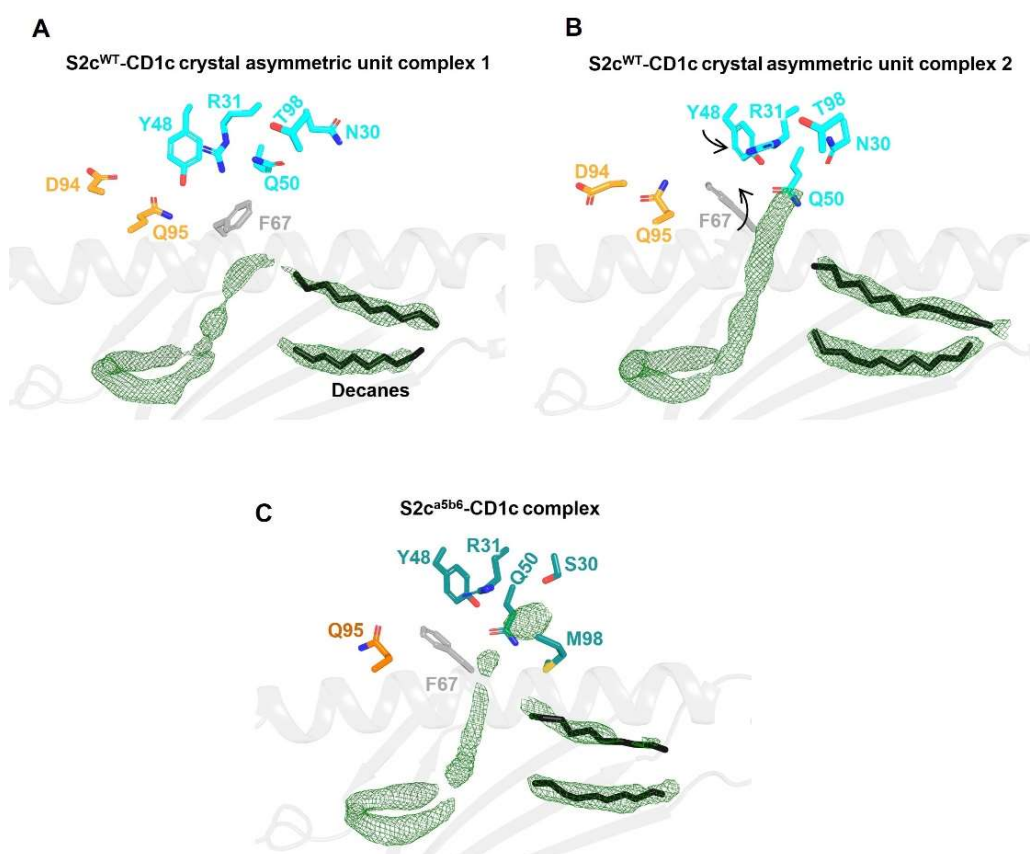


Figure 5.27 Structural insights into lipid binding pockets via Fo-Fc density comparison in CD1c-S2 TCR Complexes (Szoke-Kovacs, 2025, p. 7, Figure 3.)

(A–B) Structural analysis of the S2^{WT}-CD1c complex reveals differences in lipid occupancy between the two molecules present in the asymmetric unit. A conformational flip of CD1c residue F67 and the resulting shift in TCR Y48 β are highlighted with arrows (B). The TCR residues close to the F-pocket adopt distinct conformations across the two units are shown with sticks. The Fo–Fc omit map, visualized as a green netting contoured at 3 σ , highlights regions of electron density, while spacer lipids (decanes) are rendered in black to mark their inferred positions. (C) In contrast, the S2a5b6-CD1c complex displays consistent lipid density and residue conformations across all four asymmetric unit molecules.

Our structural analyses of the S2c^{WT}-CD1c-endo and S2c^{a5b6}-CD1c complexes offer important insights into how TCRs recognize CD1c. While there are some shared binding

features between S2c^{WT} and 3C8, each TCR also has its own distinct contact networks. Moreover, the S2c^{a5b6} TCR illustrates how mutations that enhance hydrophobic interactions can refine lipid selectivity and binding strength. These discoveries deepen our understanding of CD1c-restricted TCR recognition and provide a basis for future research into CD1c-mediated immune responses.

5.5.6 TCR-trap experiments to study CD1c restricted TCR specificity

To understand whether the affinity maturation of the of the S2c^{WT} TCR changed the repertoire of CD1c-associated lipids recognized by the TCR, lipid mass spectrometry was conducted of the CD1c bound ligands co-purified in complex with the wild-type and affinity enhanced TCRs. Soluble CD1c molecules were expressed in mammalian Expi293F cells (50) containing endogenous lipids from the Expi293F cell line (CD1c-endo). Huang et al. 2023 (50) previously validated the use of secreted CD1 proteins as a surrogate model for lipidomic analysis of transmembrane proteins. The expression of the CD1 molecules in soluble format allows detergent-free protein purification methods, therefore minimizing the reduction in lipid-ligand availability. Wild-type (S2c^{WT}) and high-affinity (S2c^{a5b6}) TCRs in ImmTAC format were produced in ExpiCHO cells and purified as explained in methods. CD1c-endo was mixed with S2c^{WT} or CD1c-S2-a5b6 TCR at 1:1 ratio. Complexes were subjected to purification via gel filtration chromatography, using a Superdex 200 Increase 10/300 GL column to achieve separation based on molecular size. The ImmTAC-CD1c complexes and CD1c fractions unbound by the ImmTAC were separated, and the fractions were sent for lipid mass spectrometry analysis. The lipid content derived from HLA-A2 samples was analyzed, expressed and purified similarly to CD1c to avoid false positive lipid detection. S2c^{WT} and S2c^{a5b6} ImmTACs on their own were expressed and purified with the same method and the lipid content eluted from the samples were analyzed and subtracted as background. A total of 813 lipid features were identified, of which 101 appeared in the HLA-A2 samples. The 101 features equaled for 184 pmoles of lipids, of which 104 pmoles were made up by four lipid features that were not present in the TCR-trap samples (GM1 40:2;2, GM1 42:1;2, GM1 42:2;2, GM3 42:2;2). The lipids eluted from the ImmTAC only samples accounted for 2-4% of the total lipidome extracted from the ImmTAC-CD1c complexes. The most predominant lipid (GD2 42:1;2), eluted from the ImmTAC-only control was absent in the complexes but was detected from the unbound CD1c fraction. In contrast, GM3 42:2;2 was present at comparable levels across all samples and controls, suggesting it represents a

false-positive signal. Subtracting these features leads to negligible background ($\sim 0.4\%$). The TCR-trap samples shared 402 lipid features. 14 Ceramides, 7 HexCer, 2 DiHexCer, 16 DAG, 6 TAG, 1 GD1, 1 GD2, 1 GD3, 2 GM1, 1 GM2, 3 GM3, 3 LPA, 7 LPC, 4 LPC O-, 9 LPE, 3 LPE O-, 4 LPI, 1 LPG, 2 LPS, 2 PA, 94 PC 95 PC O-, 35 PE, 62 PE O-, 3 PG, 39 PI, 20 PS, 11 SM and 16 Sulf species.

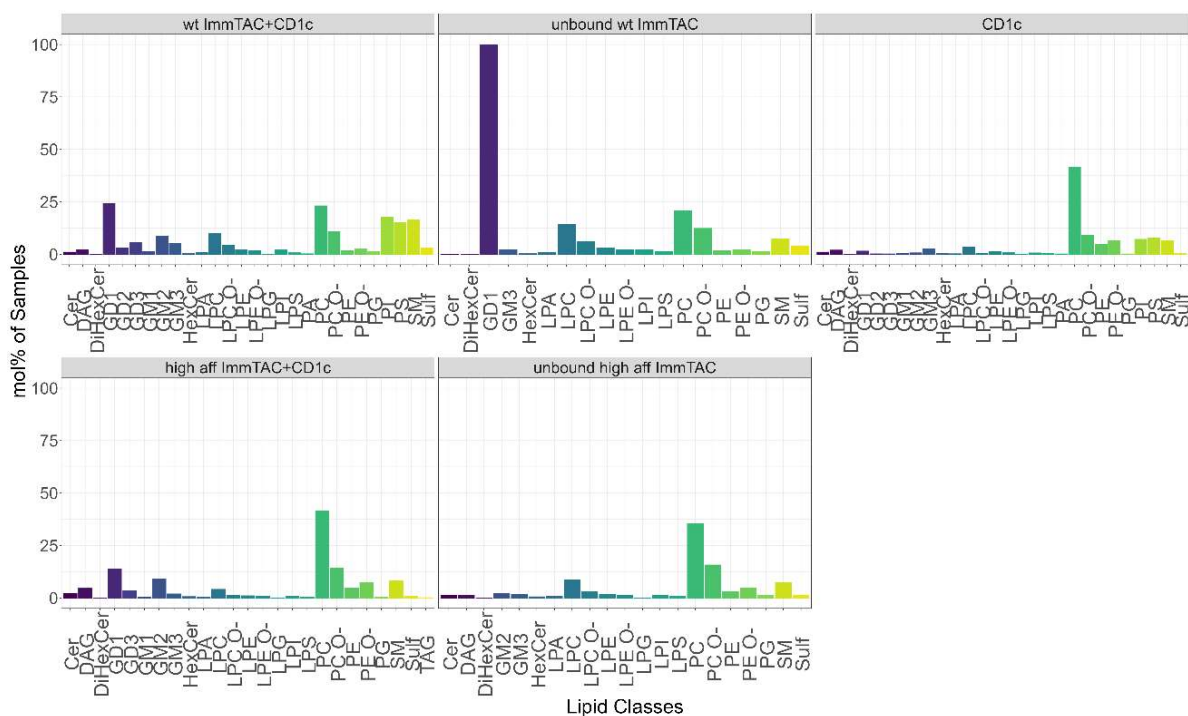


Figure 5.28. Mol% of the Lipid classes eluted from the wt- and high-affinity ImmTACs

Mol% of the lipid classes eluted from the TCR-taps, the unbound fractions in comparison with the total lipidome of the CD1c-endo. Lipid class-specific internal standards were used to normalization. The values are expressed in mol percentage (mol%), calculated by summing the amounts of individual lipids (species of subspecies) of a lipid class in pmoles to produce the total amount of the lipid class and then normalized to the overall lipid amount producing the mol% per total lipids.

Similar lipid content was eluted from the S2c^{WT} (wt ImmTAC+CD1c) and high affinity S2c^{a5b6} (high aff ImmTAC+CD1c) ImmTACs complexes (Fig 5.28), which suggests that the affinity enhancement resulted by the affinity maturation process was mainly directed towards the CD1c heavy chains, without great impact on lipid specificity. The most abundant lipids after affinity maturation remained the phospholipids, however, the chain length profile of the lipids eluted from the high affinity ImmTAC-CD1c complex shifted slightly towards larger lipids (Figure 5.29 A green and red rectangles). The complexes contained similar acyl-chain pairings of PC and PE. PI and PS lipid species were missing from the high-affinity ImmTAC-CD1c samples. This observation implies that the ImmTAC binding is most likely influenced by the lipid headgroup rather than the acyl chain lengths, although

it has been shown they can influence CD1 recognition by T cells (176). Interestingly, the lipids missing from the high-affinity complex exhibit negatively charged head groups (PI, PS) (Figure 5.28).

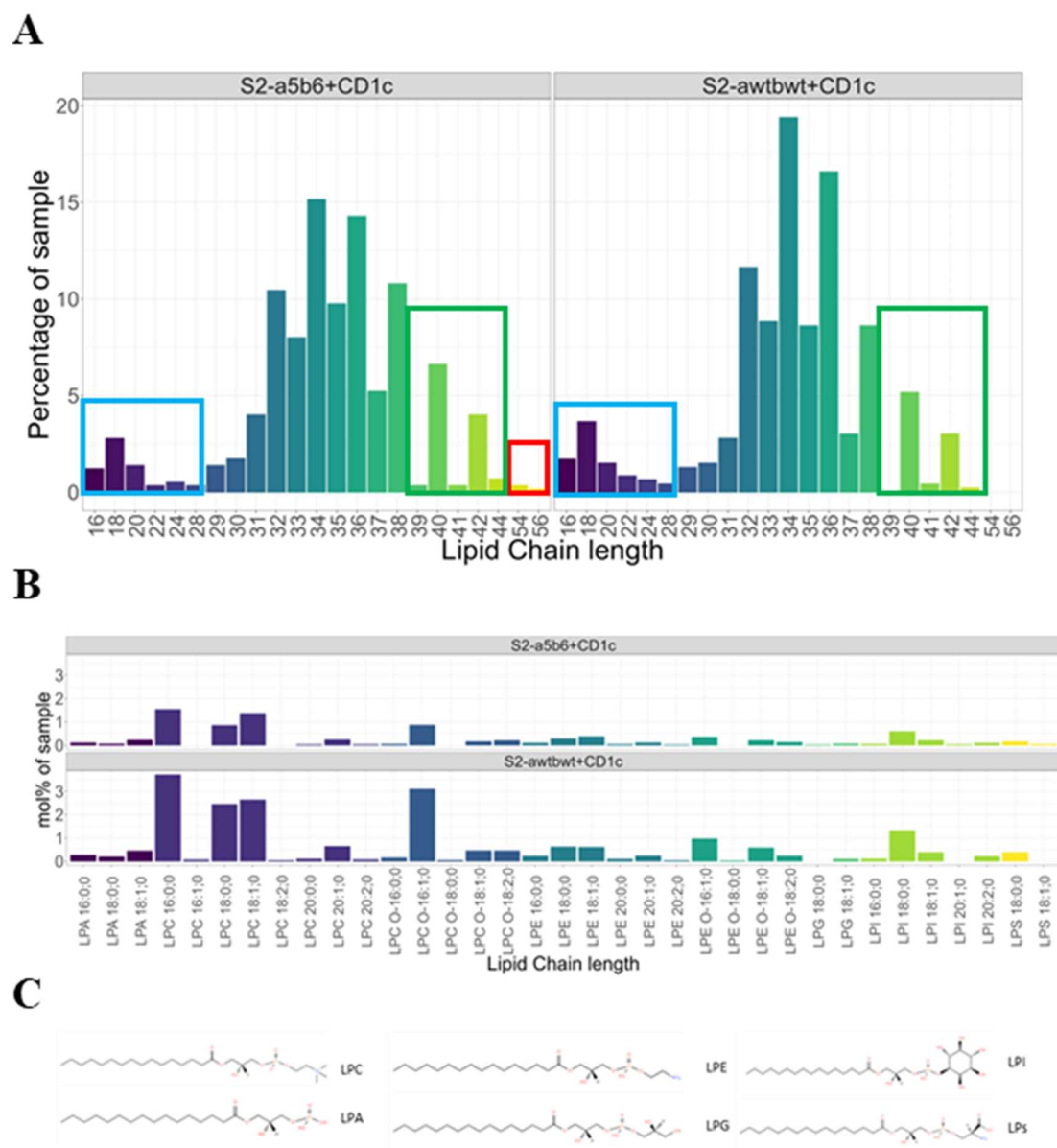


Figure 5.29 Changes of the lipid repertoire in the CD1c-ImmTAC interface. (Adopted from Szoke-Kovacs, 2025, p. 9, Figure 4.)

(A) Lipid chain length of the lipid repertoire in the CD1c-ImmTAC binding interface. Spacer lipids (C16-C28) (blue rectangle), Large lipids C39-C44 (green rectangle), Large lipids C54-C56 (red rectangle)(B) Short-chain lipids might act as spacer lipids. (C) Lyso-lipid species identified from the CD1c-ImmTAC lipidome.

Some larger lipids, TAG 54:1;0, TAG 54:2;0, TAG 56:2;0, and GM2 42:2;2 (Figure 5.29 A, red rectangle) were identified only from the high affinity complex and were not present in the wt ImmTAC-CD1c lipidome. Short-chain lyso-lipids (C16–C28) were also detected within the complexes' lipidome and are potential candidates for occupying the A' pocket within the CD1c binding groove. (Figure 5.29 A, blue rectangle). These lipids, with C16–C18 carbon chain length might be optimal as a spacer lipid, maintaining the structure of the CD1c binding groove and assisting in the co-presentation of another lipid in the F' pocket (Figure 5.29 B and C).

5.6 Harnessing TCR-CD3 bispecifics to redirect T cell activity toward CD1c-expressing cells

The affinity matured CD1c-targeting TCR, S2c^{a5b6} was engineered to ImmTAC format. The β chain of the S2c^{a5b6} TCRs was fused to an anti-CD3 scFv ($K_D=10\text{nM}$, half-life =2 minutes). A dilution series (10^{-1} – 10^{-6} nM) of the bispecific engagers was added to the co-cultures. The ImmTAC potency was measured by redirecting T cells against CD1c-expressing and CD1c negative cell lines. In order to abrogate cell surface expression of CD1, MHC class I and class II molecules and generate the THP-DKO cell line, the B2M and CIITA genes were knocked out from THP1 wild-type cell lines using CRISPR/Cas9.

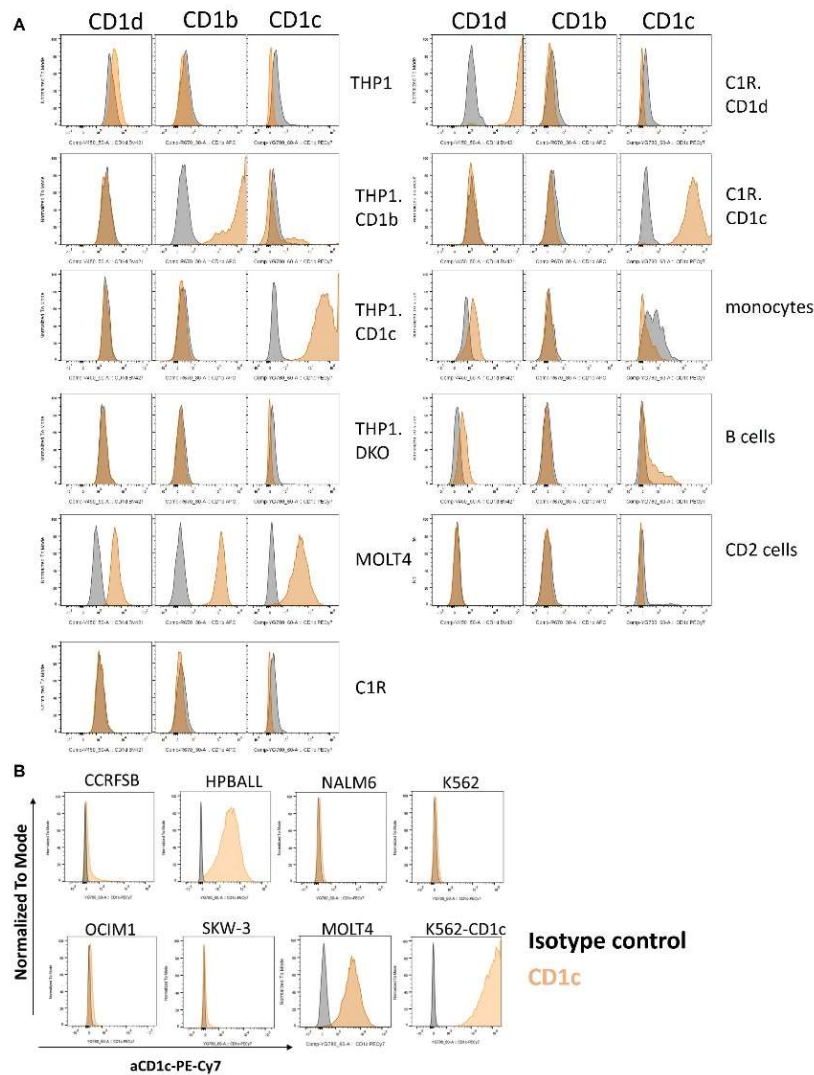


Figure 5.30. CD1c expression levels on CD1c-expressing cells and primary cells
(Adopted from Szoke-Kovacs, 2025, Suppl. Figure 4.)

(A) Histograms of CD1b, CD1c or CD1d expression on cell lines and primary cells, and (B) CD1c expression on cancer cell lines. Isotype control depicted in grey and CD1 staining with orange colours.

CD1c or CD1b was stably expressed on the cell surface using lentiviral transduction of the gene construct of single chain CD1c- β 2m (THP1-CD1c) or single chain CD1b- β 2m (THP1-CD1b) into THP1-DKO. In addition, lentiviral transduction was used to express CD1c- β 2m or CD1d- β 2m in C1R cells (human B-cell lymphoblastoid), and CD1c- β 2m in the K562 cell line (chronic myelogenous leukaemia). Cells were labeled using the L161 anti-CD1c antibody, and CD1c surface expression was subsequently measured via flow cytometry. High CD1c levels were measured on THP1-CD1c, K562-CD1c, and C1R-CD1c, the parental cells (THP1, C1R and K562) shown undetectable levels of CD1c and the THP1-DKO was negative to CD1c staining (Figure 5.29 A and B). CD1c levels were also confirmed by FACS on cancer cell lines, CCRFSB, NALM6, HPB-ALL, SKW3, and OCIM1 (Figure 5.30 B).

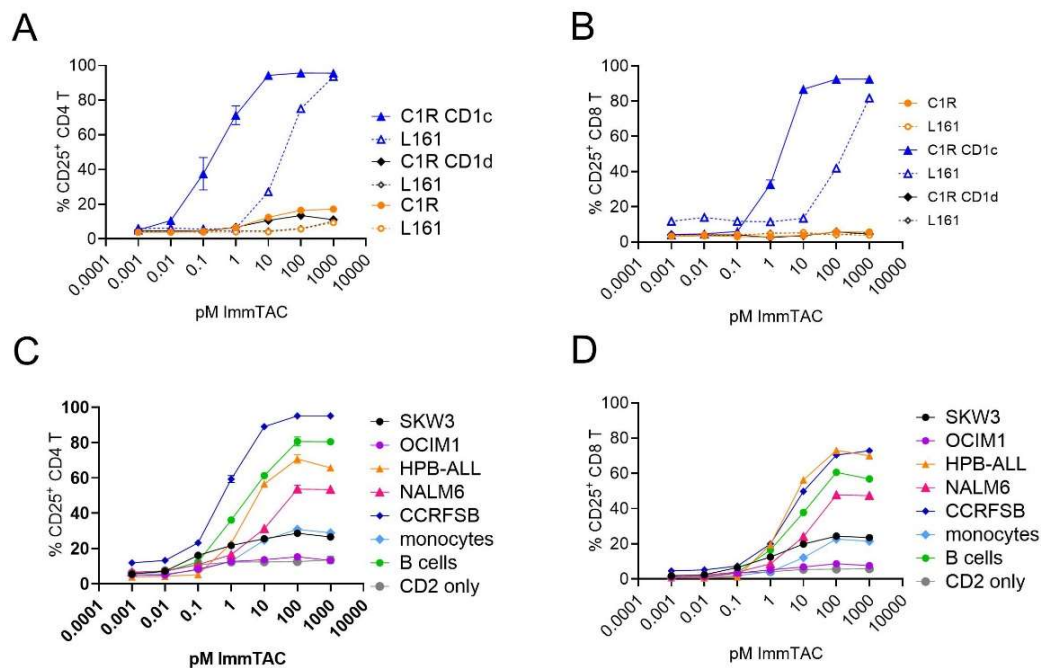


Figure 5.31. ImmTACs targeting CD1c elicit pan-T cell activation against both primary cells and cell lines with varying CD1c expression levels.

(Szoke-Kovacs, 2025, p. 10, Figure 5.)

To measure the potency of the S2c^{a5b6} ImmTAC molecule, CD2⁺ T-cells and cancer cell lines were co-cultured. **(A and B)** Dose-dependent activation curve of CD4 T cells **(A)** or CD8 T cells **(B)** Dose-dependent activation curve against C1R, C1R CD1c or C1R CD1d target cells. **(C and D)** Dose-dependent activation curve of CD4 T cells **(C)** or CD8 T cells **(D)** T cells against SKW3, OCIM1, HPB-ALL, NALM6, CCRFSB, monocytes and B cells and control of CD2⁺ T cells without target cells. Panels A and B illustrate the proportion of CD25-expressing T cells (an indicator of activation) in co-cultures treated with (dotted lines) or without (solid lines) the anti-CD1c blocking antibody L161. Data shown represent one of two independent experiments, each performed in triplicate.

T cell activation was measured by CD25 upregulation. The S2c^{a5b6} ImmTAC triggered CD4⁺ and CD8⁺ T cell responses against CD1c-expressing cell lines. (Figure 5.31A and B, Figure 5.32A, B, C and D). The addition of L161 anti-CD1c antibody partly blocked this T cell activation (Figure 5.31 A and B, Figure 5.32A, B, C and D). The S2c^{a5b6} ImmTAC was then challenged against a haematological cell line panel (Table 5.4) expressing various levels of CD1c (Figure 5.32B) and assessed the level T cell redirection.

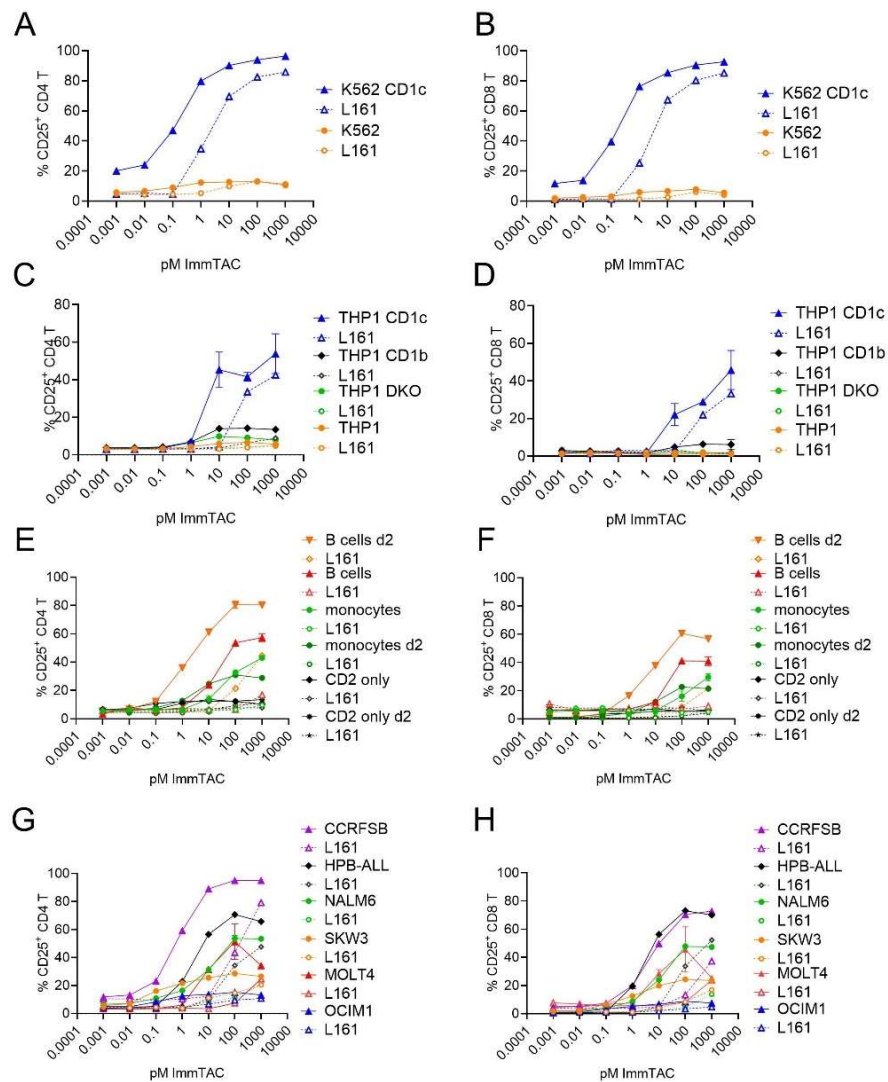


Figure 5.32 S2c^{a5b6} ImmTAC demonstrate both selective target recognition and broader reactivity in driving pan-T cell activation (Szoke-Kovacs, 2025, Suppl. Figure 5.)

To measure the potency of the S2c-a5b6 ImmTAC molecule, CD2⁺ T-cells and cancer cell lines were co-cultured. (A–B) Dose-dependent activation of CD4 (A) and CD8 (B) T cells in response to K562 and K562-CD1c target cells.

(C–D) Activation profiles of CD4 (C) and CD8 (D) T cells exposed to THP1, THP1 DKO, THP1-CD1b, and THP1-CD1c targets. (E–F) CD4 (E) and CD8 (F) T cell responses to primary monocytes and B cells across a dose range. (G–H) Activation curves for CD4 (G) and CD8 (H) T cells challenged with a panel of cell lines including SKW3, OCIM1, HPB-ALL, NALM6, CCRFSB, and MOLT4. Data shown represent one of two independent experiments, each performed in triplicate.

T cells were activated against the CCRFSB, NALM6, HPB-ALL, SKW3 target cell lines but were no activation against OCIM1 (Figure 5.31C and D). CD1c dependent activation was validated by almost full blockade by the L161 anti-CD1c antibody (Figure 5.32G and H). On-target versus off-tumor effect of high-affinity CD1c specific ImmTACs was assessed by measuring T cell activation against monocytes and B cells from two donors. Reactivity was observed to primary B cells and mild reactivity was observed to monocytes, which was blocked by the anti-CD1c antibody, L161 (Figure 5.31 C and D, Figure 5.32E and F). These findings suggest that CD1c-targeting ImmTACs hold potential for immunotherapeutic intervention in hematologic malignancies, although further molecular refinement is required to mitigate on-target, off-tumour effects in primary monocytes and B cells.

Cell line	Indication	Vendor	Cat. number
THP1	Acute monocytic leukaemia (AML)	ATCC	TIB-202 TM
C1R	Lymphoblastoid cell line	(Suckling et al., 2025) Frontiers in Immunology, 16, 1547664.	
K562	Chronic myeloid leukaemia (CML)	DSMZ/ATCC	DSMZ (ACC 10)/ ATCC (CCL 243)
MOLT4	Acute lymphocytic leukaemia (ALL)	ECACC	85011413
CCRFSB	Acute lymphocytic leukaemia (ALL)	ATCC	CCL 120
HPB-ALL	Acute lymphocytic leukaemia (ALL)	DSMZ	ACC 438
NALM6	Acute lymphocytic leukaemia (ALL)	DSMZ	ACC 128
OCIM1	Acute monocytic leukaemia (AML)	DSMZ	ACC529
SKW-3	Chronic lymphocytic leukaemia (CLL)	DSMZ	ACC 53

Table 5.4: Cancer Cell lines (Szoke-Kovacs, 2025, Suppl. Table 1.)

Cancer cell lines and indications used in T cell redirection experiments.

5.7 T cell redirection via CD1c-specific antibody-based T cell engagers

The affinity of Bispecific T cell engagers (BiTEs) to CD1c-endo molecules was tested by SPR (Table 5.5). The affinities ranged from low nM to high pM. The highest percentage of binding was achieved by the human antibody-based BiTEs with high pM affinities. Slight cross-reactivity towards CD1b-endo was observed with all BiTE molecules.

Molecule name	Variable domain species	ligand	1:1 binding			ligand	1:1 binding		
			% of binding	KD (M)	t 1/2 (min)		% of binding	KD (M)	t 1/2 (min)
AB001-pro50	Llama	CD1c-endo	67.18	1.88E-10	36.44	CD1b-endo	6.85	1.18E-10	65.62
AB001-pro51	Llama	CD1c-endo	59.59	8.45E-10	15.36	CD1b-endo	4.95	2.64E-10	33.06
AB002-pro50	Llama	CD1c-endo	62.26	1.34E-09	3.44	CD1b-endo	5.88	5.85E-10	3.98
AB002-pro51	Llama	CD1c-endo	60.57	4.69E-09	2.30	CD1b-endo	5.36	1.45E-09	0.62
AB003-pro50	Llama	CD1c-endo	64.71	3.55E-10	157.98	CD1b-endo	5.87	2.55E-10	119.71
AB003-pro51	Llama	CD1c-endo	64.49	6.31E-10	70.69	CD1b-endo	5.85	7.89E-10	55.49
AB004-pro50	Llama	CD1c-endo	0.39	7.15E-14	9619.60	CD1b-endo	0.37	8.82E-14	408377.65
AB004-pro51	Llama	CD1c-endo	69.97	4.21E-11	157.69	CD1b-endo	5.83	1.90E-11	298.77
AB006-pro50	Llama	CD1c-endo	67.90	1.78E-10	80.93	CD1b-endo	5.31	6.32E-11	87.63
AB006-pro51	Llama	CD1c-endo	67.60	4.29E-10	55.69	CD1b-endo	5.92	1.25E-10	70.38
AB007-pro50	Llama	CD1c-endo	66.02	4.31E-10	105.67	CD1b-endo	5.41	1.35E-10	122.53
AB007-pro51	Llama	CD1c-endo	67.65	9.28E-10	78.54	CD1b-endo	5.30	4.09E-10	81.08
AB009-pro50	Llama	CD1c-endo	65.72	1.16E-10	32.25	CD1b-endo	4.94	5.75E-11	43.78
AB009-pro51	Llama	CD1c-endo	65.22	2.99E-10	55.37	CD1b-endo	5.31	4.39E-11	44.99
AB012-pro50	Llama	CD1c-endo	66.47	2.06E-09	23.42	CD1b-endo	0.43	1.86E-12	21939.62
AB012-pro51	Llama	CD1c-endo	57.53	1.16E-08	14.54	CD1b-endo	0.44	1.57E-13	219392.16
AB015-pro50	Llama	CD1c-endo	56.74	3.57E-09	3.63	CD1b-endo	0.41	4.33E-13	33615.33
AB015-pro51	Llama	CD1c-endo	60.03	6.72E-09	4.61	CD1b-endo	0.31	1.80E-13	124696.52
AB019-pro50	Llama	CD1c-endo	62.86	7.48E-10	9.29	CD1b-endo	0.34	2.20E-13	113810.58
AB019-pro51	Llama	CD1c-endo	57.84	1.43E-09	8.24	CD1b-endo	0.18	7.81E-13	30416.09
AB022-pro03	Chicken	CD1c-endo	46.63	9.10E-10	0.00	CD1b-endo	0.23	3.14E-13	29335.09
AB022-pro05	Chicken	CD1c-endo	67.47	2.04E-09	0.00	CD1b-endo	0.13	6.48E-14	1.28
AB023-pro03	Chicken	CD1c-endo	3.04	1.13E-09	0.00	CD1b-endo	0.03	2.92E-15	3.32
AB023-pro05	Chicken	CD1c-endo	46.16	9.08E-09	81.87	CD1b-endo	0.11	5.99E-11	1612.07
AB032-pro03	Human	CD1c-endo	83.14	1.79E-10	97.89	CD1b-endo	0.51	1.46E-14	0.45
AB032-pro05	Human	CD1c-endo	84.03	1.24E-10	97.20	CD1b-endo	0.18	1.58E-09	2.30

Table 5.5. Binding properties of the BiTEs.

The binding dissociation constant (affinity) of the BiTE molecules were tested by SPR against CD1c-endo and CD1b-endo molecules. The proportion of CD1 bound by BiTEs was estimated by calculating the ratio of the experimentally observed R_{max} to the theoretical R_{max}, derived from the amount of CD1 immobilized.

Next, the BiTEs' ability to redirect T cells was tested in T-cell redirection assay and compared their potency to CD1c specific ImmTACs. Briefly, a dilution series ($1-1 \times 10^{-6}$ nM) of the bispecific engagers (BiTE or ImmTAC molecules) was added to Jurkat effector cells (Jurkat.NFAT.Luc), expressing CD3 in co-culture with CD1c or CD1b expressing THP1 target cells overnight as described in Chapter 4.22. If T-cell engager mediated T-cell activation occurs, the Jurkat cells express firefly luciferase and luminescence signal can be measured on a plate reader at 574nm (Figure 5.33).

Both bispecific formats showed specific T-cell redirection, with no Jurkat activation observed against a cell line not expressing the CD1c target (THP1.DKO) (Figure 5.33 A). Enhanced levels of Jurkat activation were observed with the BiTEs compared to affinity matured CD1c specific ImmTAC (S2c^{a5b6}), while the wild type ImmTAC underperformed all bispecific engagers (Figure 5.33 B). Most engagers exhibited a low level of cross-reactivity towards CD1b (Figure 5.33 C). A background noise of 500 RLU was measured in the presence of all cell types as well as without target cells, which can be accounted for by CD1 expression by the Jurkat cell line (Figure 5.33 C).

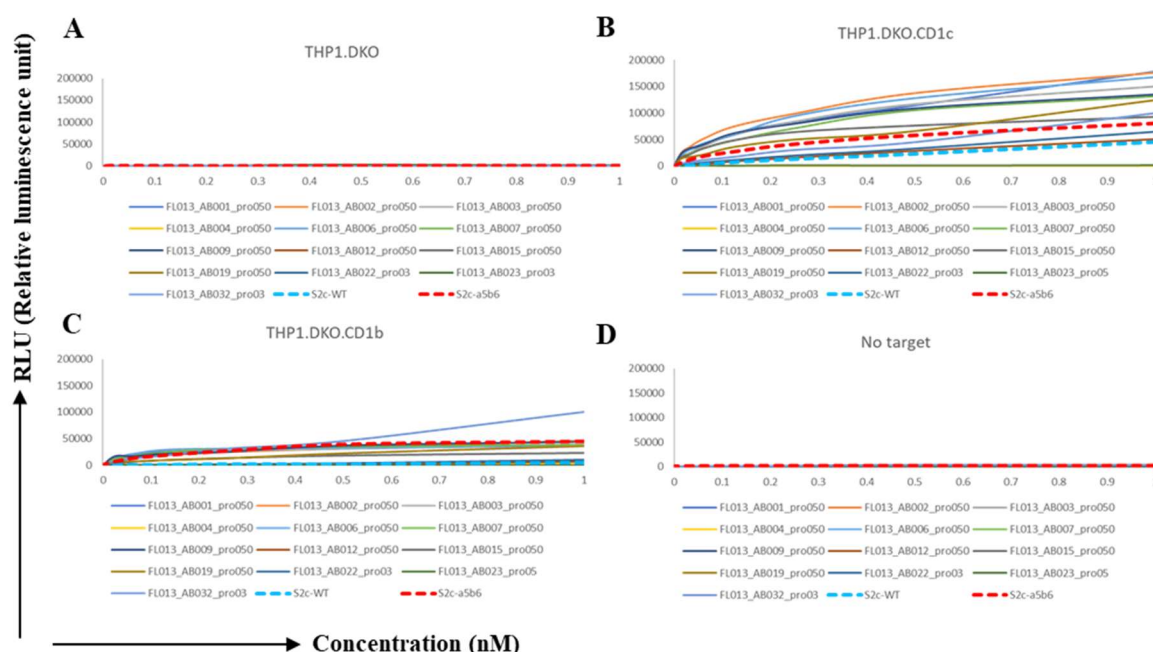


Figure 5.33 T-cell redirection assay by BiTEs and ImmTACs

T-cell redirection assay using CD1c-specific Bispecific T-cell Engagers (BiTEs) and TCR based bispecific molecules (ImmTACs). **(A)** THP1.DKO is the background cell line, with no CD1 expression. **(B)** THP1.DKO.CD1c is the background cell line expressing CD1c. **(C)** THP1.DKO.CD1b is the background cell line expressing CD1b. **(D)** No target cell line added to the culture.

The development of CD1c-specific Bispecific T-cell Engagers is a promising strategy for targeting CD1c-expressing cells. The human antibody-based BiTE molecules showed the best binding affinities, while maintaining minimal cross-reactivity to CD1b. The T-cell redirection assay demonstrated their capability to activate T cells in a CD1c-dependent manner, with greater potency to the affinity-matured TCR based bispecific molecule (ImmTAC) and better than the wild-type ImmTAC. There was no significant off-target activation, which reinforces the specificity of these molecules. These results emphasize the potential of BiTEs as effective immunotherapeutic agents for CD1c-targeted applications, indicating a need for further in vivo validation and optimization.

5.8 Antigen presentation by CD1 molecules

5.8.1 mLPA, a novel CD1c-associated antigen

The mLPA (methyl-lysophosphatidic acid) lipid antigen was identified by Lepore et.al. (66) from acute myeloid leukaemia patient samples. They also identified T-cell clones specific to mLPA in a CD1c-restricted manner. While they discussed the mLPA-specific TCRs and showed their potency in mouse AML xenograft models, they did not investigate the origin and loading of the mLPA antigen onto CD1c molecules. mLPA is a modified phospholipid. It has an ether-bonded alkyl chain and possesses intriguing structural characteristics, as the methyl group on the phosphate moiety. This ether bond between the alkyl chain and the glycerol backbone, is a known peroxisomal hallmark of the ether-bonded lipid synthesis (177). To better understand the nature of this lipid antigen, I aimed to identify the pathway where the mLPA lipid is loaded onto CD1c.

5.8.2 Modelling the CD1c lipid loading pathways

Leukaemia cell lines that express CD1c with different recycling properties were created to explore where the mLPA lipid is loaded onto the CD1c molecule (Figure 5.34).

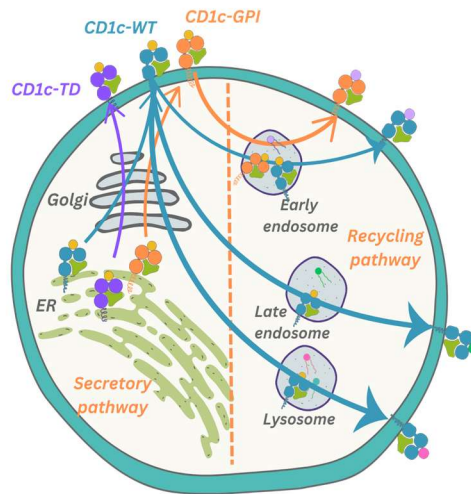


Figure 5.34: Modelling the intracellular recycling of CD1c molecules.

CD1c mutants follow different recycling pathways. Wild-type CD1c (scCD1c-WT) recycles to the early- and late-endosomes and lysosomes. GPI anchored (scCD1c-GPI) molecules are localized mainly in the early endosomes while the tail deleted scCD1c-TD molecules are not able to recycle into the intracellular compartments.

Wild-type CD1c (scCD1c-WT) molecules assemble in the ER with self-lipids and traffic to the cell surface. They then recycle to the early- and late- endosomes and lysosomes (35,37,46,178,179), where the bound self-lipid ligand can be exchanged for other lipids including external lipid ligands. CD1c molecules are recycled within the endo-lysosomal compartment through association with the Adaptor Protein 2 (AP2) (54). In a study, Jackman et al. reported that the YXXZ motif in the CD1d cytosolic tail is responsible for recycling into the intracellular compartments (180). They showed similar recycling properties by mutating the tyrosine residue or deleting the cytosolic tail.

CD1c format	cytosolic tail
scCD1c-WT	KKHCSYQDIL
scCD1c-TD	KK*
scCD1c-GPI	STSGVHWIAAWLVVTL SIIPSILLA

Table 5.6: CD1c cytosolic tail variants

Asterix (*) depicts deletion after KK residues.

Similarly, as described by Jackman et al. (180), a tail deleted (scCD1c-TD) was created by deleting the cytosolic tail (HCSYQDIL) to impede the recycling of the molecule into the intracellular compartments (Table 5.6). Several cell surface proteins are anchored to the cell surface by glycosylphosphoinositol (GPI) (181) and GPI-anchored proteins are internalized and retained in sorting and early-endosomes (182). Geho et al. utilized GPI re-anchoring to study whether the CD1 transmembrane and cytoplasmic domains direct the lipid antigen capture and presentation of the CD1 isoforms (183). GPI-anchored variant of CD1c was engineered by swapping the cytosolic tail to a GPI sequence adopted from the decay-accelerating factor (DAF) protein. Leukaemia cell lines (THP1 and K562) carrying the CD1c variants were generated by lentiviral transduction. To confirm similar cell surface levels of CD1c, the transduced cell lines were sorted. The GPI anchoring of the CD1c molecules of the scCD1c-GPI expressing cell line was confirmed by PI-PLC cleavage (Figure 5.35).

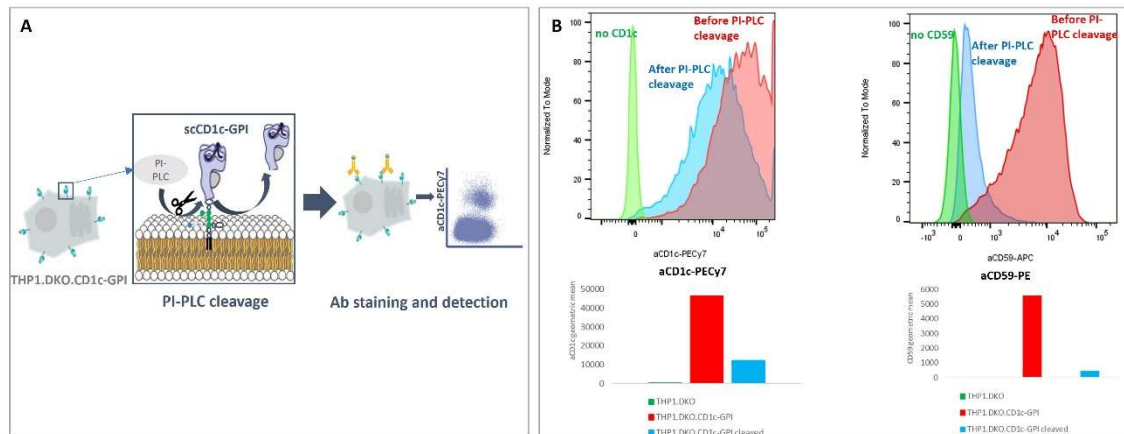


Figure 5.35: Validation of GPI anchored CD1c expression on target cells.

(A) The target cell line was treated with PI-PLC to cleave the GPI molecules and release the CD1c molecules from the cell surface. (B) Following cleavage, cells were labeled with the anti-CD1c antibody L161 and subjected to flow cytometric analysis to assess surface expression. aCD59, a known GPI anchored molecule was also stained as a control. After cleavage the amount of CD1c and CD59 detected on the cell surface were reduced.

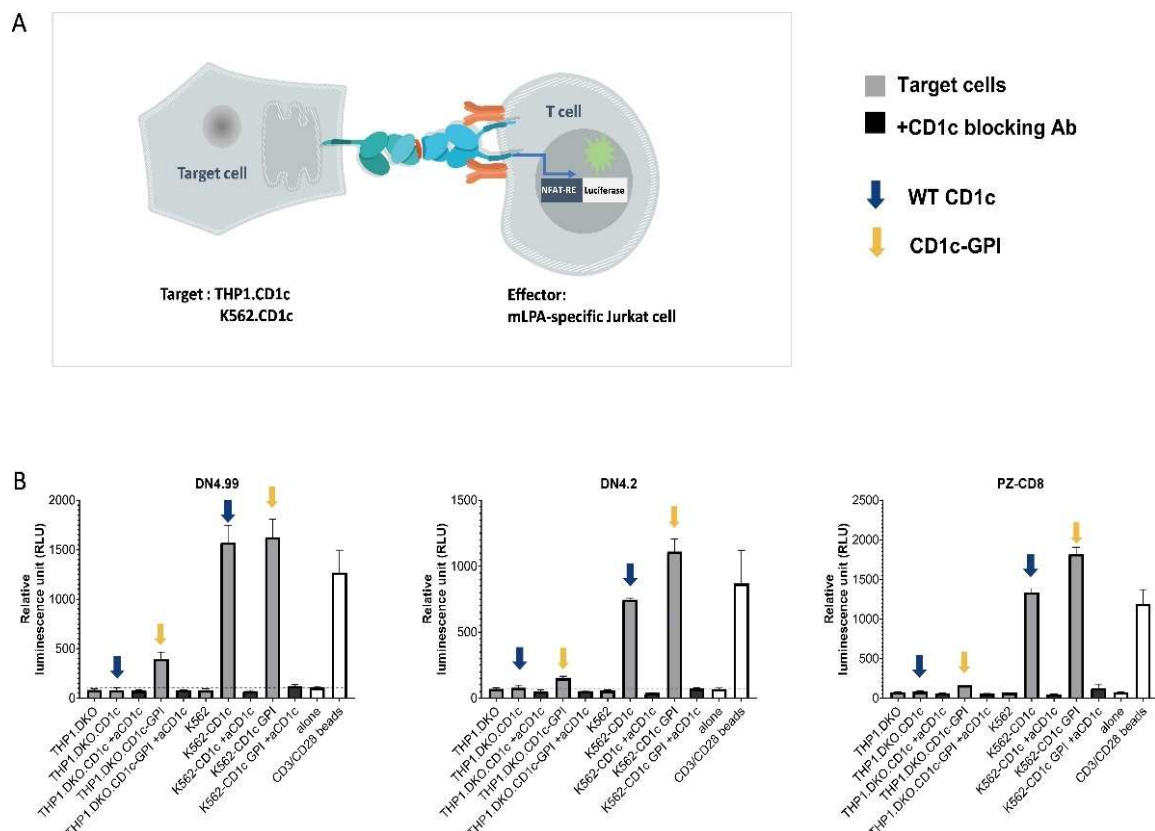


Figure 5.36: mLPA specific Jurkats recognize WT and CD1c-GPI molecules expressed on THP1 and K562 cell lines.

(A) The target cell lines were co-cultured with mLPA-specific Jurkat cells. Upon T-cell activation the NFAT mediated Luciferase enzyme is generated, and the signal can be measured by plate reader at 260nm. (B) Jurkat activation levels were doubled in the presence of CD1c-GPI molecules compared to WT CD1c. The response was blocked by adding anti-CD1c antibody to the culture.

mLPA-specific T cells described by Lepore et al. recognized CD1c expressed on cancer cell lines generated in our lab (66). Jurkat cell lines were generated, expressing the mLPA-specific TCRs (DN4.99, DN4.2, PZ-CD8) and tested CD1c-restricted recognition of leukaemia cell lines. mLPA-specific Jurkats recognized CD1c expressed on cancer cell lines such as THP1 and K562. Interestingly, their activation level increased two-fold against both THP1 and K562 cell lines expressing CD1c-GPI (Figure 5.36). This response was shown to be CD1c-dependent, as it was effectively inhibited by the addition of the blocking antibody L161 to the co-culture. It was previously described that GPI-anchored proteins widely spread in the endocytic organelles, with a preference for localization in early-, sorting-, and recycling endosomes (183–185). Our Jurkat activation data suggests that the mLPA lipid might be loaded in the secretory pathway or early endosomes. To test this hypothesis, the lysosome function was blocked by Bafilomycin A1 (BafA1) treatment in all target cell lines (186,187).

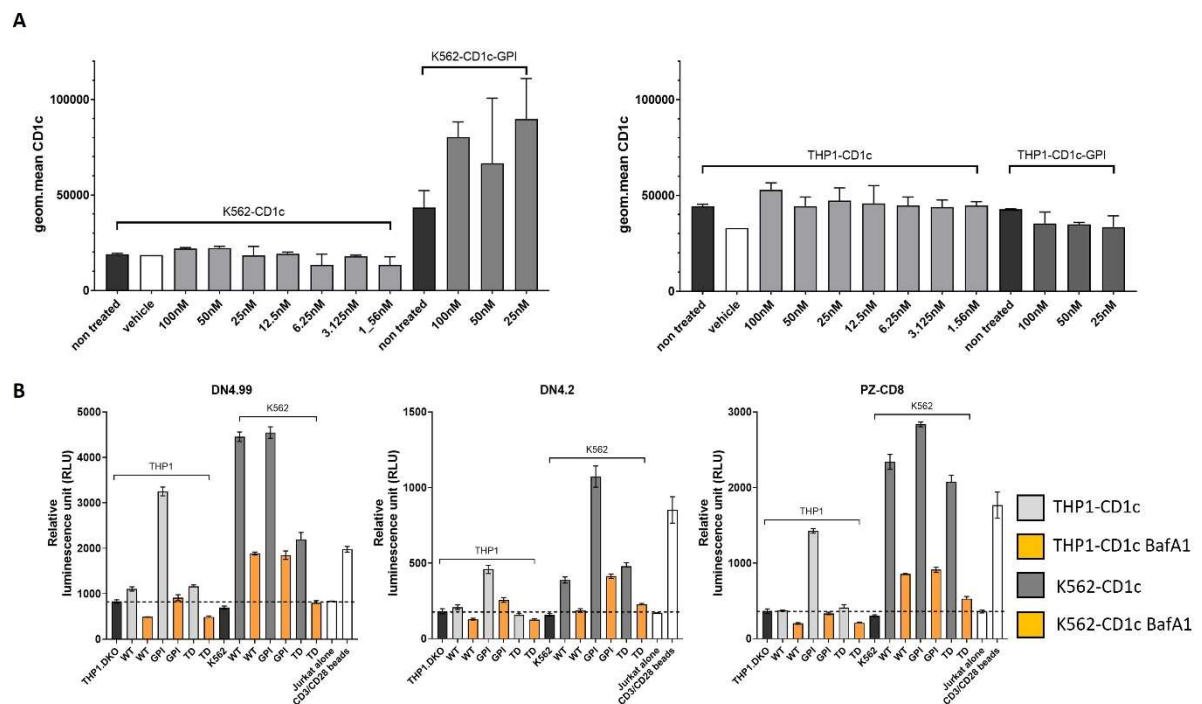


Figure 5.37: THP1 expressing CD1c-GPI triggers enhanced level of Jurkat activation
(A) Bafilomycin A1 treatment has no effect on CD1c cell surface expression **(B)** The target cells were treated with BafA1 and subsequently were co-cultured with mLPA-specific Jurkat cells. Upon T-cell activation the NFAT mediated Luciferase enzyme is generated, and the signal can be measured by plate reader at 260nm.

Bafilomycin A1 treatment did not affect the cell surface expression of CD1c in THP1 cell lines, however, the cell surface expression of non-recycling CD1c almost doubled in K562

cell line (Figure 5.37 A) The elevated expression levels may stem from differences in endosomal trafficking and recycling pathways in K562 cells. Notably, these cells lack Na^+/K^+ -ATPase-mediated regulation of endosomal acidification, which could further influence lipid presentation dynamics. (188). Blocking the acidification and lysosomal trafficking by BafA1 treatment reduced Jurkat activation levels in all CD1c formats, with some Jurkat activation still observed with the CD1c- GPI expressing cell lines (Figure 5.37 B). Since BafA1 treatment affects lysosomal acidification and peroxisomal gene expression including peroxisomal lipid metabolizing enzymes as well, the disruption of peroxisomal function might lead to altered lipid metabolism. As a consequence, the lipid content of intracellular compartments might change.

5.9 Generating lipid-specific T-cell clones

As an alternative method to find lipid-specific TCRs, a T-cell cloning approach was explored. This approach selectively expands a specific population of T-cells that recognize a particular antigen, creating a pool of clones with the same antigen-binding specificity. Previous studies used various methods to expand and isolate unconventional T-cell clones: T-cell expansion with APCs, tetramer/dextramer based cell sorting by flow cytometry, and sorting activated T-cells using activation markers or cytokine release (78,79,189). To expand CD1d restricted, GD3 and αGalCer specific T-cell clones artificial APCs (aAPC) were loaded with CD1d-GD3/CD1d- αGalCer and autologous DCs with GD3 and αGalCer . Fresh peripheral blood mononuclear cells (PBMCs) were isolated from 200mL of blood from internal donors. The monocytes were separated using CD14 Micro Beads and Pan T-cells were purified by negative selection.

5.9.1 Stimulation and expansion of lipid-specific T-cell clones using artificial APCs

Streptavidin magnetic beads (SPHERO™ Streptavidin magnetic particles, 4.0-4.5 μm , Spherotech, SVM-40-10) were coated with biotinylated CD1d-lipid complexes and for co-stimulation, biotinylated anti-CD28 antibodies (Biolegend, 302904) to create aAPCs. Pan T-cells were co-cultured with aAPCs in a ratio of T cells to aAPC (2:1) with IL-2 (PeproTech EC, 200-02), IL-7 (Miltenyi, 130-095-364) and IL-15 (PeproTech, 200-15) for ten days. After ten days, the cells were stained with CD1d-lipid-coated CD1d-lipid-coated and APC-conjugated dextramers (Immudex, Klickmer®, empty loadable dextran backbone, DX01K) and anti-CD3 (PE anti-human CD3 Antibody, clone UTHC1, Biolegend, 303408), anti-CD4

(CD4-PerCPCy5.5, clone SK3, Biolegend, 344607), anti-CD8 (PE/Cyanine7 anti-human CD8, clone SK1, Biolegend, 980910) and anti-CD16 (Alexa Fluor® 700 anti-human CD16 Antibody, clone 3G8, Biolegend, 302026), anti-CD14(Brilliant Violet 510™ anti-human CD14 Antibody, clone 63D3, Biolegend, 367124) and anti-CD19 (Brilliant Violet 510™ anti-human CD19 Antibody, clone HIB19, Biolegend, 302242) antibodies.

5.9.2 Stimulation and expansion of lipid-specific T-cell clones using moDCs

Dendritic Cells (DC) were differentiated from monocytes by culturing them in differentiation media (RPMI 1640, 10% FBS, L-Glut (2mM), Pen/strep, 100ng/ml GM-CSF (PeproTech EC, 300-03) 3% IL4 (PeproTech EC, 200-04)) in six well tissue culture plates (COSTAR, 3516). MoDCs were detached by day 5 and were pulsed with lipid antigens (GD3 and α GalCer) overnight. 100 μ g/ml lipid stocks were prepared in DMSO and working stocks were diluted with culture media. The dendritic cells were cultured overnight with 100nM of lipids added to the culture media. Next morning the Pan T cells were co-cultured with moDCs in a ratio of T cells to moDC (3:1 and 5:1) in the presence of IL2, IL7 (Miltenyi, 130-095-364) and IL15 (Peprotech, 200-15) for ten days. After ten days, the cells were stained with CD1d-lipid-coated and APC-conjugated dextramers (Immudex, Klickmer®, empty loadable dextran backbone, DX01K) and with anti-CD3 (PE anti-human CD3 Antibody, clone UTHC1, Biolegend, 300408), anti-CD4 (CD4-PerCPCy5.5, clone SK3, Biolegend, 344607), anti-CD8 (PE/Cyanine7 anti-human CD8, clone SK1, Biolegend, 980910) and anti-CD16 (Alexa Fluor® 700 anti-human CD16 Antibody, clone 3G8, Biolegend, 302026), anti-CD14(Brilliant Violet 510™ anti-human CD14 Antibody, clone 63D3, Biolegend, 367124) and anti-CD19 (Brilliant Violet 510™ anti-human CD19 Antibody, clone HIB19, Biolegend, 302242) antibodies. The base level of CD1d-restricted T-cell frequency in the separated T-cell fraction was determined before stimulation (Figure 5.38 and Figure 5.39). Viability staining was used to gate on live cells after defrosting the cells and CD19 and CD14 staining with the same color (“dump channel”) was used to exclude contaminating monocytes and B cells. The live population contained 4.9% of CD3+, CD16+ Natural killer cells (NK cells). The CD3+ population was separated into 15.8% of CD8+ T cells and 74.5% CD4+ T cells. The gates to detect dextramer positive cells were set on these negative populations (Figure 5.38 A). With CD1d-endo dextramer staining negligible amount of CD1d autoreactive T cells were detected. (0.004% CD4+, 0.014% CD8+ and 0.013% NK cells) (Figure 5.38 B). The CD1d-GD3 dextramer staining revealed

similar levels of CD1d-GD3 reactive T cells (0.006% CD4+, 0.02% CD8+ and 0.003% NK cells) (Figure 5.39 A) while the level of CD1d-aGalCer reactive population (1.7% CD4+, 0.12% CD8+ and 0.79% NK cells) were slightly higher, and consistent with the literature (Figure 5.39 B). These levels measured again after ten days of antigen stimulation (Figure 5.40).

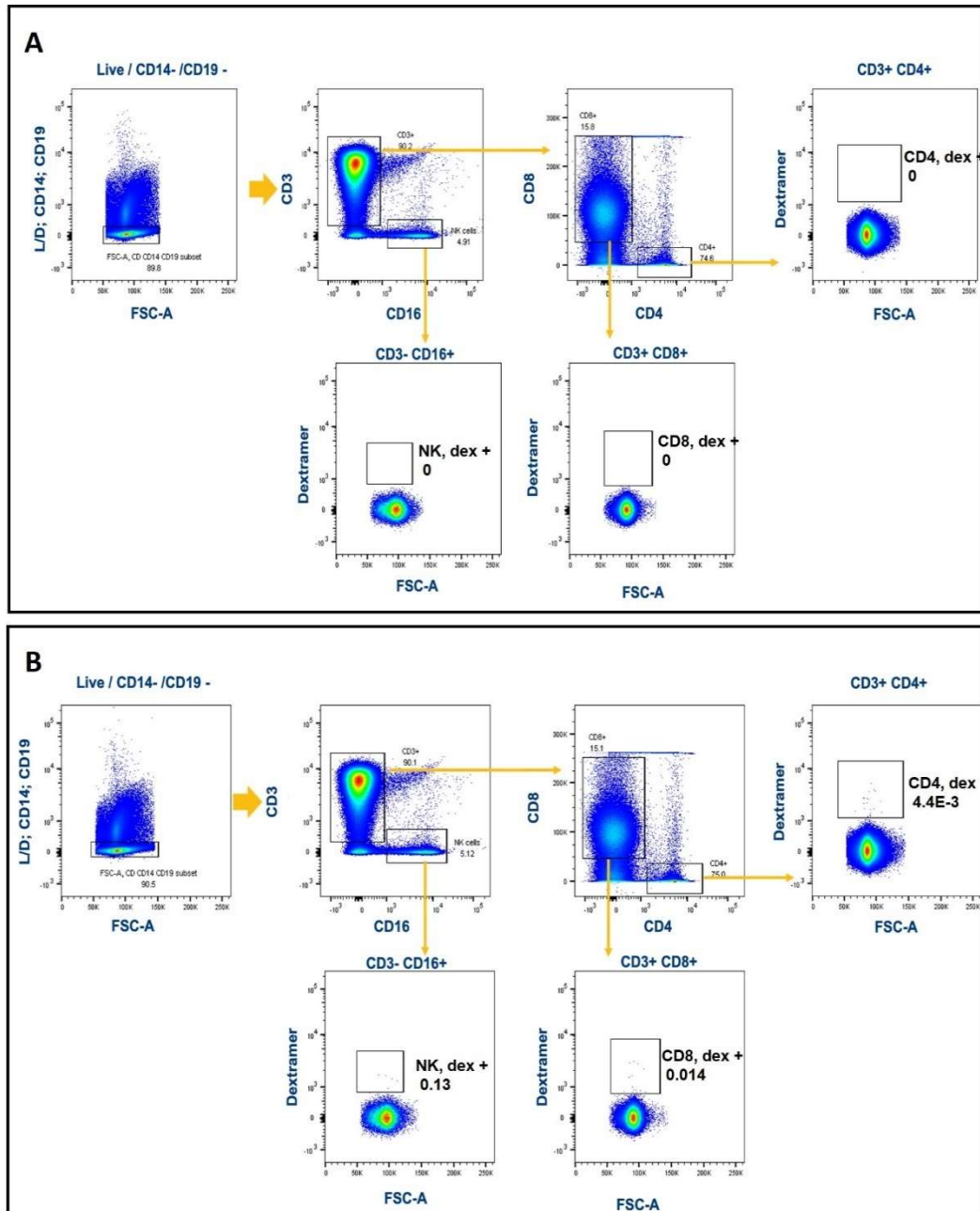


Figure 5.38 Phenotyping CD1d-restricted T-cell populations pre-stimulation

T negative fraction containing T cells were stained with Live/Dead Aqua cell viability stain and anti-CD14 and CD19 with the same color to exclude contaminating monocytes and B. The CD3 positive cells were stained with anti-CD4, anti-CD8 and anti-CD16 antibodies for phenotyping. CD1d-endo dextrans were used to identify lipid-specific T-cell clones. (A) No dextramer (B) CD1d-endo dextramer

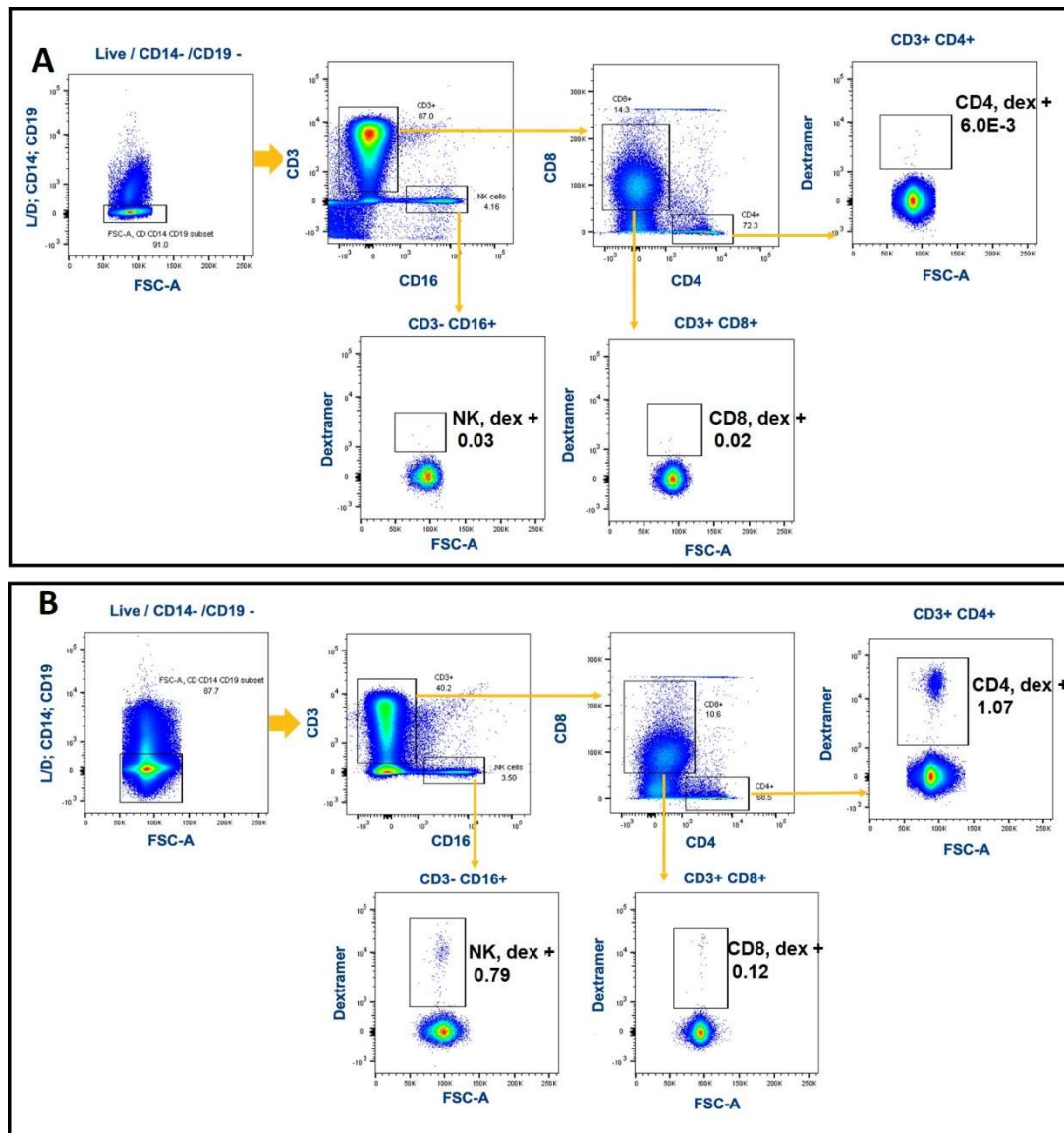


Figure 5.39 Phenotyping CD1d-restricted T-cell populations pre-stimulation

T negative fraction containing T cells were stained with Live/Dead Aqua cell viability stain and anti-CD14 and CD19 with the same color to exclude contaminating monocytes and B. The CD3 positive cells were stained with anti-CD4, anti-CD8 and anti-CD16 antibodies for phenotyping. CD1d-GD3 and CD1d-aGalCer dextrans were used to identify lipid-specific T-cell clones. **(A)** CD1d-GD3 dextramer **(B)** CD1d-aGalCer dextramer

No detectable change in the frequency of GD3-reactive T cells was observed across any of the methods employed. Both GD3 loaded DC (Figure 5.40 A) and GD3-aAPC (Figure 5.40 B) stimulation resulted in poor levels of CD1d-GD3 and CD1d-endo dextramer staining. A 6-fold increase was observed in the frequency of α GalCer reactive T cells (6.83% of CD4+) in the α GalCer pulsed DC stimulated culture (Figure 5.40 D), while no change was observed in the aAPC stimulated cultures (Figure 5.40 C).

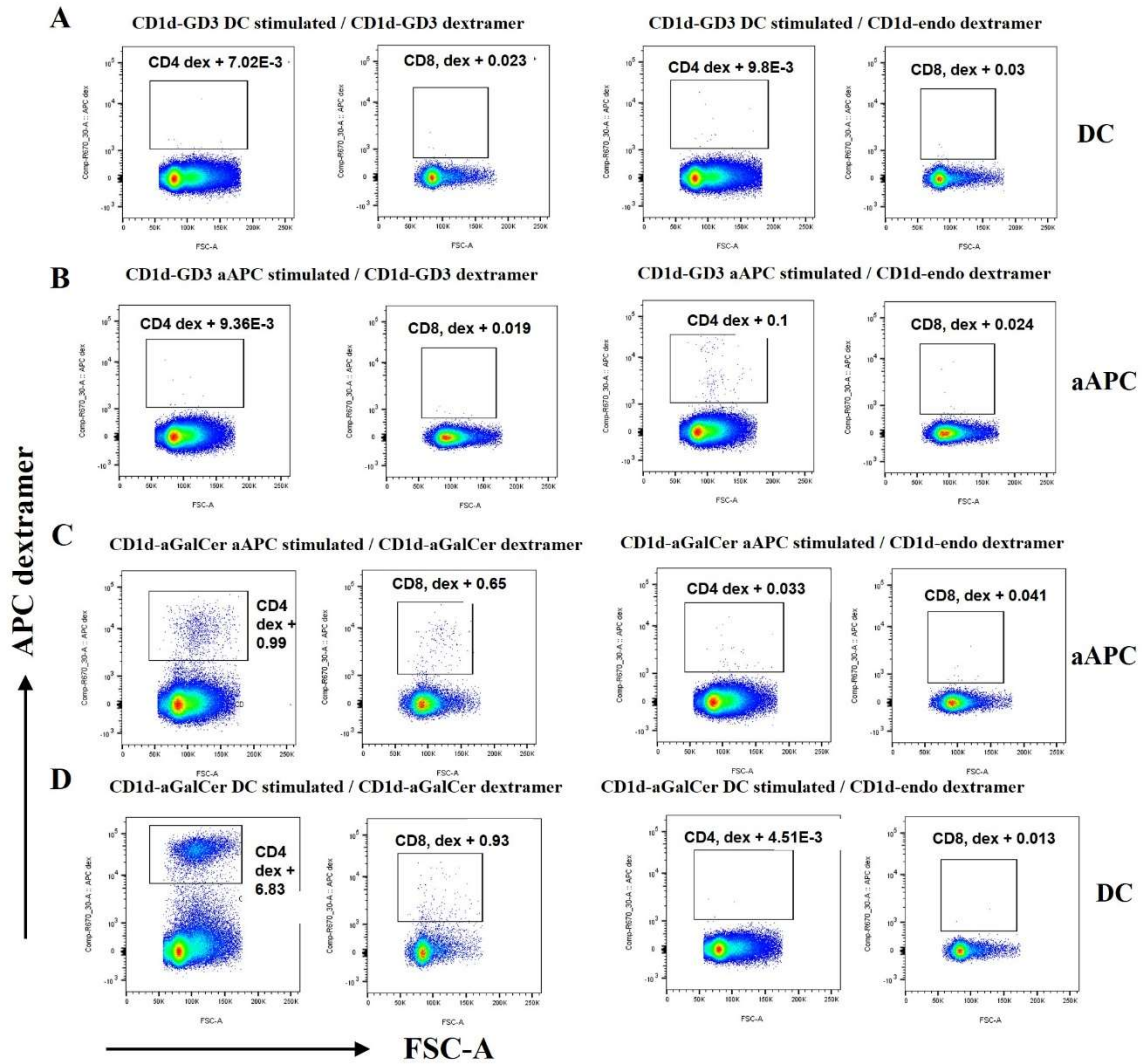


Figure 5.40 CD1d-restricted T-cell populations post-stimulation

T-cell cultures were stained with CD1d-GD3 and CD1d-endo dextramers. **(A)** GD3 loaded DC stimulated T-cells **(B)** GD3-aAPC stimulated T-cells **(C)** αGalCer loaded DC stimulated T-cells **(D)** αGalCer-aAPC stimulated T-cells.

These results demonstrated that while the CD1d-GD3 and CD1d-endo reactive T-cell populations remained unchanged after stimulation, the αGalCer-pulsed DCs were successful in expanding αGalCer-reactive CD4⁺ T-cells by sixfold. In contrast, the artificial APCs did not induce a significant response. The higher success in T-cell expansion of DCs could be attributed to their ability to present lipid antigens in a more physiologically relevant manner, their natural expression of co-stimulatory molecules, and their capacity to form immunological synapses that effectively engage T-cells. These findings highlight the differential efficacy of stimulation methods in generating unconventional T-cell clones and provide valuable insights for future studies aiming to harness lipid-specific T-cells.

6. DISCUSSION

6.1 Lipid-loaded CD1 complexes allow the development of bispecific molecules

I demonstrated the feasibility of the expression of functional, soluble single-chain CD1a, CD1b, CD1c, and CD1d molecules in mammalian cell lines. The correct folding and functionality of CD1c monomers were validated by ILT2 and TCR binding assays. However, the heterogeneity observed on SDS-PAGE and IEF gels is likely due to glycosylation variants. Silencing these glycosylation sites led to unsuccessful expression, suggesting reduced stability of the CD1 molecules without adequate glycosylation. The CD1 molecules expressed in mammalian cells capture heterogeneous lipid ligands in their binding pockets from the cells. To generate CD1c molecules loaded with a single lipid, I developed a lipid-ligand exchange protocol. Since glycosylation has a major effect on CD1 stability, CD1 molecules were expressed with reduced, more homogeneous glycosylation to aid partial unfolding of the molecules for ligand exchange. To overcome the challenges of lipid solubility and the stabilising effect of the endogenous lipids in the binding pockets, a detergent-based exchange method was implemented, using CHAPS and 50 molar excess of lipids in a ligand exchange buffer at pH 6.0. The partial unfolding of CD1 protein achieved by this method, allowed for the replacement of endogenous lipids. The efficiency of the ligand exchange was validated by multiple approaches, such as IEF, Native Mass Spectrometry, tetramer staining, and lipid-specific TCR binding assays. While most of these methods indicated some level of lipid exchange, quantifying the fraction of successfully loaded molecules remained challenging. The most effective method confirming the success of the lipid exchange was the tetramer staining of iNKT cells with CD1d- α GalCer tetramers, where the positive staining of iNKT cells indicated that the exchanged lipid ligands were correctly presented by CD1d. However, this method is only applicable when antigen-specific T-cell clones are accessible.

Although the lipid exchange protocol facilitated the exchange of endogenous lipids in the CD1 binding pocket to varying extents, further optimisations are needed to improve reproducibility and quantification. Future work could focus on refining these methods to develop a standard method for lipid loading and lipid-antigen discovery.

6.2 CD1-associated Lipid Repertoire

In this study, I analysed the lipid ligands eluted from CD1 isoforms expressed in mammalian cells. Our findings showed that CD1 molecules broadly sample the cellular lipidome, demonstrating considerable overlap between isoforms, in agreement with previously published data (50), while also exhibiting distinct preferences for certain lipid classes and chain lengths. I demonstrated that CD1 isoforms are capable of capturing almost all cellular lipids, although there are small differences in their binding preferences. CD1a and CD1c displayed a lipid profile that closely mirrors the cellular background, which is primarily rich in phospholipids. On the other hand, CD1b and CD1d preferred sphingolipids, setting them apart from the cellular lipidome, supporting the results published by Rudolph et al (190). Remarkably, cholesterol was only found to be over-captured by CD1d. This finding aligns with a reported function of CD1d, which acts as a surface receptor for oxidized cholesterol, thereby promoting the initiation and activation of the peroxisome proliferator-activated receptor- γ (PPAR γ) (191). This indicates that while CD1 molecules do not strictly target specific lipid types, their structural differences play a significant role in shaping their lipid-binding capabilities. As crystallography results suggested before, the binding pockets of CD1 isoforms are likely to determine the preferences for lipid chain lengths (52). Our analysis of lipid chain length distributions showed that CD1a tends to bind mid-sized lipids (C34-C38), CD1b can accommodate both short (C16-C20) and long (C40-C42) chain lipids, CD1c binds a wide range of lipid lengths (C30-C46), and CD1d prefers shorter lipids (C34-C36) but is also capable of binding long-chain lipids (C40-C42). These results reinforce the concept that CD1 molecules exhibit lipid-binding versatility, driven by the structural characteristics of their antigen-binding grooves. Cotton et al. reported endogenous sphingomyelins (C42:2) captured by CD1a, blocking tetramer binding to T-cell receptors of autoreactive T cells (55). My dataset did not confirm this SM over capture by CD1a, but larger SM species (C40-C42) were selectively captured by CD1a. Similarly, SM was over captured by CD1b, c and d. Rudolph et al. (190) also detected long chain sphingolipids (SM42:2) in a similar study, analysing the CD1d bound lipidome, which are in line with the SMs (C40:1,2; C40:2,2; C42:1,2; C42:2,2) found to be selectively bound to CD1d in our study. The concept of long chain SMs selectively binding as natural T-cell inhibitors is supported by our data and is consistent with previously reported findings (190,192).

The presence of short-chain lipids in the CD1b lipidome indicates that these molecules

could serve as stabilising spacers for longer antigenic lipids. Likewise, CD1c capacity to accommodate short lysolipids reinforces the theory of a two-lipid presentation system, highlighting the functional versatility of CD1 molecules. Similar findings based on crystal structures were reported before (60,164). Lipids like phosphatidylcholine (PC) were found in high abundance across all isoforms, highlighting their abundance in cellular membranes, while in contrast, all CD1 molecules under-capture phosphatidylethanolamine (PE) and ether-PE, suggesting a selective capture mechanism. Mansour et al. reported that captured cholesteryl esters maintain the CD1c conformation and promote the recognition by CD1c restricted autoreactive T cells(164). Surprisingly, gangliosides (GD1, GD2, GD3 and GM2) were the most abundant lipids captured by CD1c. These results share similarities with intriguing unpublished results showed at the CD1 MR1 conference in 2024, suggesting, that gangliosides could bind around the CD1c alpha-helices and possibly support the presentation of other lipid ligands. In 2008, GM3 was proposed as an inhibitory ligand of natural killer T-cells (193), modulating the Th2 NKT-cells reactivity to self-glycolipids. Our dataset confirmed GM3 over-captured by CD1b, CD1c and CD1d. These results not only support previous reports (50) but also extend our understanding of the structural and functional implications of lipid presentation by CD1 molecules. Future research should aim to explore how environmental factors and disease-related lipid changes influence CD1 function to further understand their role in immunity and for therapeutic purposes.

Analysis the lipid profile eluted from CD1c molecules from relevant cancer cells would enable the screening for cancer-associated lipid antigens. Only a few studies have investigated the lipid content eluted from recycling CD1 molecules (46,163). Huang et al. (46) showed a significant overlap of the lipids obtained from recycling and non-recycling versions of CD1a and CD1b, suggesting that recycling CD1 molecules capture exogenous antigens. Rudolph et al. (163) analysed the lipid content of recycling and non-recycling versions of murine CD1d molecules and reported similar findings. There are two major challenges in studying the CD1c-associated lipidome of relevant cancer cell lines. The naturally occurring low expression level of CD1c limits the yield of purified protein from the cell lysates, and the protein purification method can alter the lipid content associated with the CD1c molecules. To overcome the low CD1c expression levels, single-chain CD1c was overexpressed in cell lines of different lineages. Different strategies were employed to capture CD1c molecules from the cell lysates. A CD1c immunoprecipitation method was developed, using commercial and in-house developed antibodies and tested affinity

purification techniques from cell lysate by adding 6xHistidine or streptavidin tags to the C terminal end of the single-chain CD1c molecules. However, the enrichment of CD1c from cancer cell lines proved to be challenging. To overcome the limitations of the low natural expression of CD1c, I developed CD1c overexpressing cancer cell lines and tested protein enrichment strategies to isolate CD1c in sufficient quantities for downstream analyses. First, affinity purification method was tested, using 6xHis-tagged CD1c expressed in THP1 cells. His-Trap purification is a widely used method for recombinant protein isolation due to its high specificity for histidine residues and compatibility with physiological conditions, however, our attempts to purify CD1c-6xHis using His-Trap columns were unsuccessful. This may be attributed to low levels of expression, misfolding, or poor binding to the column. More success was achieved by using StrepTactinXT-based purification, leveraging the high-affinity interaction between the Strep-tag®II peptide and Strep-TactinXT resin. Two formats of streptavidin-tagged CD1c were developed: a full-length membrane-bound version and a truncated soluble format. While membrane-bound CD1c purification was unsuccessful, soluble CD1c was successfully isolated from THP1 and MOLT4 cell lines. Despite the successful purification, the yield was too low for subsequent mass spectrometry analysis. Due to the limitations of affinity purification, immunoprecipitation (IP) method was developed, as an alternative for enriching CD1c. 32 antibodies were tested developed in-house and found several with promising binding characteristics. A Protein G magnetic bead-based IP assay was developed with these antibodies and were able to capture soluble CD1c-endo. The IP method was also applied to lysates from CD1c-expressing THP1 cells, successfully enriching membrane-bound CD1c. Antibody AB012 was identified as the best performer, which captured CD1c from cell lysates. Other antibodies, such as AB005, AB006, and AB032, also showed potential but will need further validation in more CD1c-expressing cell lines to establish their broader applicability. This study highlights the challenges and progress made in enriching CD1c from cancer cell lines. Although affinity purification using His-Trap did not yield successful results, the StrepTactinXT-based method allowed for the isolation of soluble CD1c, though the yields were low. Immunoprecipitation appeared as a more effective strategy for capturing both soluble and membrane-bound CD1c.

Future research could aim to optimize purification conditions to enhance protein yields and validate the most effective antibodies across various cell lines. These initiatives will improve the ability to study CD1c-associated lipidomes and their roles in disease. Huang et al. (50)

recently reported a substantial lipidomic overlap between the recycling and non-recycling versions of CD1a and CD1b molecules, while Rudolph et al. (190) compared the lipid capture of recycling and non-recycling murine CD1d molecules with similar results. These findings suggest that CD1 recycling might be a specialised way of capturing and presenting exogenous lipid antigens.

6.3 Antigen presentation by CD1c

The identification of mLPA as a novel, cancer associated CD1c-bound antigen represented an important milestone in the field of unconventional T-cell targets (66) . While previous studies demonstrated mLPA-specific T-cell recognition in a CD1c-restricted manner (22,66,102), our study aimed to elucidate the pathway of mLPA loading onto CD1c molecules and its implications for immune activation. To investigate the intracellular loading pathway of mLPA onto CD1c, leukaemia cell lines were developed, expressing CD1c molecules with distinct recycling properties. THP1 cell lines were generated expressing wild-type CD1c (scCD1c-WT) that recycles through early and late endosomes as well as lysosomes, tail-deleted variant (scCD1c-TD) that remains at the cell surface without intracellular trafficking and GPI-anchored CD1c (scCD1c-GPI) that is retained in early endosomes. Jurkat cell lines expressing mLPA-specific TCRs were established and evaluated their ability to recognize CD1c-expressing leukaemia cells. Notably, Jurkat activation levels increased two-fold when co-cultured with cells expressing CD1c-GPI, suggesting that mLPA might be loaded onto CD1c within early endosomes or the secretory pathway. The activation of Jurkat cells was significantly reduced by blocking CD1c with specific antibodies, confirming CD1c restriction. To further investigate the lipid-loading pathway, the cells were treated with a lysosomal acidification inhibitor, Bafilomycin A1 (BafA1). While BafA1 treatment did not affect CD1c surface expression in THP1 cells, it led to increased CD1c expression in K562 cells, likely due to differences in endosomal sorting mechanisms. Jurkat activation levels decreased in all CD1c-expressing cell lines following BafA1 treatment, reinforcing the hypothesis that lysosomal and peroxisomal pathways contribute to mLPA processing. The disruption of lysosomal and peroxisomal function by BfA could alter lipid metabolism, affecting intracellular lipid antigen presentation.

Our study provides evidence that mLPA loading onto CD1c likely occurs in the secretory pathway or early endosomes, as supported by elevated Jurkat activation with CD1c-GPI. The effect of BafA1 treatment further suggests that lysosomal and peroxisomal processes influence mLPA presentation. Future studies could focus on dissecting the molecular mechanisms behind mLPA synthesis, trafficking, and presentation by CD1c. This study could broaden our understanding of the lipid synthesis and lipid metabolism pathways in cancer cells, generating lipid antigens and open new opportunities for therapeutic strategies targeting lipid antigens in leukaemia and other malignancies.

6.4 Targeting CD1c via bispecific engagers

This study demonstrates the potential of targeting CD1c with bispecific engagers, utilizing the S2c^{a5b6} TCR capability to recognize CD1c-lipid complexes in a largely lipid agnostic manner. Through comprehensive biophysical and structural analyses, the S2c^{WT} TCR bound to CD1c-endo with an affinity ($K_D = 16.7\mu\text{M}$) comparable to that of classical TCR-pHLA interactions (101,128). Our structural analysis indicated that the S2c^{WT} TCR binds primarily in a lipid-independent manner, with similar docking angle to the 3C8 CD1c-reactive TCR that has been characterized previously (111). The crystallographic analysis revealed a binding footprint that covers both the α -helices and the A' roof of CD1c, which is comparable to CD1b-restricted TCRs (68). The significant role of the TCR α -chain, accounting for 63% of the interaction, points to a strong engagement strategy that depends on electrostatic and van der Waals forces with specific CD1c surface residues, particularly Asp65, Leu68, Arg79, and Glu80. The absence of a distinct density for lipid head groups in the structural data further reinforces the idea that S2c^{WT} primarily interacts with CD1c through its heavy chain rather than through lipid-specific interactions. Although the contact analysis suggested possible contact with the lipid headgroup, lipid density was not observed, supporting this finding. The contact analysis revealed that asymmetric unit of the crystal structure contained two variants of the S2c^{WT}-CD1c-endo complex with substantial difference in the lipid head group density. The structural data confirmed that the two variants contain distinctive side chain conformations of CD1c F67 allowing for the accommodation of different lipid head groups. The S2c^{WT} was able to bind to both variants, by the flexibility of the Q95 α and Y48 β side chains surrounding the CD1c F67 residue and subsequent movement of the R31 β , N30, Q50, R31 β and 30 β residues, accommodating larger headgroups. This finding is underpinning this TCR lipid agnostic binding mode. The

presence of ‘spacer lipids’ was also confirmed by modelling decanes into the observed density, similar to the results reported by Roy and Mansour et al. (111,164). Due to the naturally low surface expression of CD1c-lipid complexes, the initial affinity of the S2c^{WT} TCR (KD = 16.7 μ M) was not ideal for developing bispecific engagers. Therefore, phage display based affinity maturation was performed, increasing the affinity to 15pM and the half-life to 9.6 hours, which is essential for therapeutic applications (102). To further explore the determinants of the high-affinity interactions between the S2c^{a5b6} TCR and CD1c-endo, the crystal structure of the S2c^{a5b6}-CD1c complex was obtained. The asymmetric unit of the complex contained four variants, all of them holding very similar lipid densities, protruding out of the F’ pocket. Only one conformation of the CD1c F67 side chain was found, similar to the one observed in the S2c^{WT}-CD1c-endo complex containing densities of larger lipid head groups. Similarly, the Y48 β , R31 β and Q50 β side chains maintained the conformation of the S2c^{WT} TCR, allowing binding to a larger lipid headgroup containing CD1c variant. This increased flexibility of lipid recognition is attributed to the S30 β and M98 β residues, derived from the affinity maturation process. These mutated residues wrap around the larger lipid density, generating space to accommodate larger lipid headgroups. This finding suggests that the affinity maturation process resulted in more flexibility to bind to CD1c presenting lipids with larger head groups.

To further understand the specificity of the TCRs, a lipidomic analysis of the ImmTAC-CD1c-endo complexes was performed. CD1c-endo complexes, co-purified with both wild-type and high-affinity S2c ImmTAC variants, showed a significant overlap in lipid composition. This finding supports that while affinity maturation of the TCR did not significantly change lipid specificity, it enhanced interactions with the CD1c heavy chain. Notably, the high-affinity TCR showed a tendency to bind larger lipids, whereas negatively charged lipids (PI, PS) were less represented. This implies that the characteristics of lipid headgroups might affect ImmTAC binding, which could have implications for therapeutic effectiveness. Furthermore, specific larger lipids (TAG 54:1;0, TAG 54:2;0, TAG 56:2;0, and GM2 42:2;2) were found only in the high-affinity complexes, indicating that a wider range of lipids may be recognized by the high-affinity ImmTAC which in agreement with the structural results. The MS analysis found evidence of C16-C18 lyso-lipids in the CD1c-endo binding groove of both complexes. This result agrees with the lipid density observed in the CD1c binding groove of the structures. These lipids could act as spacer lipids, maintaining the CD1c structure and assisting in the presentation of other lipids. These findings contribute to our understanding of lipid recognition by CD1c restricted TCRs

and lay the groundwork for designing TCR based therapeutic agents targeting CD1c.

The affinity enhanced TCR was reformatted to a bispecific engager (ImmTAC) format, containing an anti-CD3 effector arm, allowing the redirection of CD3 expressing T cells to CD1c positive target cells. Potent, on-target specificity was observed against CD1c-expressing cell lines, however, on-target, off-tumor reactivity to CD1c-expressing primary cells, such as B cells and monocytes was also observed, due to the TCRs lipid-agnostic feature. While the lipid agnostic approach to target CD1c on leukaemia is not feasible, the discovery of tumour specific lipid antigens in leukemic cells in the future will allow the development of lipid-specific TCR based T-cell engagers.

To explore alternative methods to target CD1c, CD1c-specific antibody based Bispecific T-cell Engagers (BiTEs) were developed. In this research, 13 anti-CD1c antibody clones were reformatted into BiTE molecules. BiTEs were evaluated for their capacity to redirect T-cells towards CD1c-expressing target cells and were compared with CD1c-specific ImmTACs. Both BiTEs and ImmTACs successfully triggered T-cell activation in a CD1c-dependent manner, with no activation noted in the absence of CD1c expression. The potency of the BiTEs was enhanced to that of the affinity-matured ImmTAC (S2c^{a5b6}), while the wild-type ImmTAC showed inferior performance relative to both bispecific formats (as expected). Additionally, most BiTEs displayed minimal cross-reactivity to CD1b, highlighting their specificity for CD1c. Neither the BiTEs nor the ImmTACs resulted in significant off-target activation. Our functional assays did not confirm the superiority of any bispecific format. However, some therapeutic applications might benefit from the use of a CD1c-specific engager, in either BiTE or ImmTAC format, therefore exploring the use of both formats in new therapeutic applications is beneficial.

The development of high-affinity bispecific engagers targeting CD1c could become a promising tool for immunotherapy. TCR- and antibody-based bispecific engagers restricted to CD1c can identify cells that express CD1c, without relying on specific lipid antigens, which increases their therapeutic versatility. Furthermore, the longer half-life of these affinity-enhanced ImmTACs supports sustained immune activation, potentially boosting cytotoxic responses of the redirected effector cells. However, maintaining specificity while limiting off target toxicity is crucial. Although efforts were made to reduce cross-reactivity with other CD1 isoforms during the affinity maturation process, additional functional assays are needed to verify selectivity and to explore the risk of off-target effects that may arise

from interactions with non-malignant CD1c-expressing cells in preclinical studies.

This study shows that CD1c can be effectively targeted with bispecific engagers. Future research should aim to optimize these molecules for clinical use, evaluate their safety, and confirm their effectiveness in disease models.

T-cell cloning methods, such as T-cell expansion with APCs, tetramer/dextramer based cell sorting by flow cytometry, and sorting activated T-cells using activation markers or cytokine release to discover antigen-specific TCRs were employed in previous studies (95–97). In order to find additional lipid-specific TCRs, we used a T-cell cloning approach to expand lipid-specific T-cell populations. The CD1d-restricted T-cell cloning experiments highlighted differences in effectiveness between artificial antigen-presenting cells (aAPCs) and monocyte-derived dendritic cells (moDCs) in expanding lipid-specific T-cells. aAPCs were created by coating streptavidin magnetic beads with biotinylated CD1d-lipid complexes and biotinylated CD28 specific antibodies, to stimulate T-cells. Despite co-culturing pan T-cells with aAPCs in the presence of IL2, IL7, and IL15, the results indicated that aAPCs did not significantly enhance the expansion of antigen-specific T-cell populations. Conversely, the moDC-based stimulation protocol demonstrated superior efficacy in expanding α GalCer-reactive T-cells. Differentiated DCs were pulsed with lipid antigens and co-cultured with pan T-cells, leading to a sixfold increase in the frequency of α GalCer-reactive CD4⁺ T-cells. This outcome suggests that moDCs, which naturally express co-stimulatory molecules and effectively present lipid antigens, are better suited for stimulating lipid-specific T-cell responses than aAPCs. However, similar to the aAPC approach, moDC stimulation did not lead to an expansion of CD1d-GD3 or CD1d-endo reactive T-cells. These findings suggest that the presentation of lipid antigens by moDCs is more physiologically relevant and effective for T-cell activation compared to synthetic aAPCs. The lack of response in CD1d-GD3 and CD1d-endo reactive T-cells may indicate inherent limitations in their antigen recognition or a need for further optimization of the stimulation conditions. The significant expansion of α GalCer-reactive CD4⁺ T cells support the potential of moDCs in generating lipid-specific T-cell clones. Future research could explore optimizing the antigen-loading process and refining the culture conditions to improve the expansion of other lipid-reactive T-cell populations. Additionally, understanding the molecular mechanisms behind the differential responses to aAPC and moDC stimulation could further contribute to the development of effective tools for unconventional T-cell cloning.

7. SUMMARY

This PhD thesis aimed to create bispecific engagers that target CD1c for treating hematological malignancies. To lay the groundwork for this research, an initial characterization of the CD1 lipidome was conducted, which helped to understand how CD1c-lipid complexes interact with T-cell receptors. The study was structured around two primary research objectives: (1) analyzing the lipid antigen repertoire linked to CD1 complexes in both mammalian and cancer cells, and (2) exploring how TCRs recognize CD1c at a molecular level.

To investigate the lipid antigen repertoire presented by CD1 molecules, CD1 isoforms were generated in a mammalian expression system, capturing a mixture of endogenous lipids. Lipid mass spectrometry was utilized to identify and quantify the lipids linked to CD1 isoforms. CD1 molecules were found to broadly interact with the cellular lipidome, exhibiting both overlapping and unique binding preferences. CD1a and CD1c primarily captured a profile rich in phospholipids, whereas CD1b and CD1d preferred sphingolipids. Cholesterol was mainly associated with CD1d, consistent with its established role in recognizing oxidized cholesterol. Analyzing lipid chain lengths indicated that CD1a binds to mid-sized lipids, CD1b accommodates a broad range, CD1c has a wide binding capacity, and CD1d favors shorter lipids but can also capture long-chain variants. The presence of long-chain sphingolipids suggests a natural inhibitory function for autoreactive T cells. Interestingly, CD1c showed a strong affinity for gangliosides, hinting at potential roles in antigen presentation and immune modulation. Future studies should investigate how environmental factors and diseases influence CD1 function and its therapeutic applications. Aiming to reveal cancer-specific lipid signatures within the CD1c lipidome, CD1c-expressing cancer cell lines were developed, and CD1c enrichment methods were tested to isolate CD1c from cancer cells for lipidomic analysis. Although it was possible to purify soluble CD1c from THP1 cells, the yield was too low for subsequent mass spectrometry analysis. To better understand cancer-associated lipid antigens, the loading of the only known cancer-associated lipid, mLPA presented by CD1c was investigated. mLPA loading to CD1c is crucial for the recognition by specific, unconventional T cells. CD1c-expressing leukaemia cell lines were created with different recycling characteristics to evaluate where mLPA was loaded onto CD1c. The increased activation of specific Jurkat T cells to CD1c-GPI expressing cells indicated that the early endosomes in secretory pathway are likely to be the location of mLPA loading. Moreover, experiments that inhibited lysosomal function

suggested that both lysosomal and peroxisomal processes play a role in mLPA presentation. This project provided insights into the lipid repertoire that can be presented by the CD1 molecules and aimed to determine whether cancer-specific lipids exist within the CD1c lipidome.

The second objective of the study aimed to explore how TCRs identify CD1c-lipid complexes, a topic that is still not well understood due to the scarcity of structural data. There is evidence to suggest that TCRs restricted by CD1c might need a specific lipid for binding, similar to MHC-peptide interactions, or they could recognize CD1c molecules in a ligand-independent manner. To explore the molecular basis of CD1c-lipid TCR recognition, soluble CD1c-lipid complexes were used to select TCRs from phage display libraries. As a result, CD1c restricted TCRs were isolated that recognize CD1c on cancer cell lines and reformatted them as bispecifics to enable redirection of T cells to kill CD1c-expressing cancer cells. Structural analysis revealed that the wild type TCR was mainly binding to the CD1c protein rather than having lipid specificity. Leveraging the insights from structural studies phage display libraries were designed for the engineering of affinity-enhanced TCRs. By affinity maturation technique, picomolar level affinity and a half-life of 9.6 hours were achieved, which makes the TCR a suitable candidate for therapeutic use. Structural and lipidomic analysis of the wild-type and high-affinity TCR-CD1c complexes indicated that the high-affinity TCR could accommodate larger lipids, therefore suitable to recognize a wide range of CD1c-associated self-antigens. Anti-CD1c antibody based Bispecific T-cell engagers were also developed. The BiTEs effectively activated T cells and showed comparable efficacy to affinity-matured TCR based ImmTACs. We demonstrated that the CD1c specific ImmTAC was potent to redirect T cells to kill CD1c positive cancer cell lines, however on-target, off-tumor reactivity was also observed to healthy B cells and monocytes, limiting its therapeutic application in a lipid-agnostic approach.

In the search for lipid-specific TCRs, CD1c and CD1d molecules were generated loaded with lipid antigens. To ensure the loading of a single lipid, a lipid-ligand exchange protocol was developed using detergent-based methods. The efficiency of this exchange was validated through IEF, mass spectrometry, and tetramer staining assays. While lipid exchange was successful, further optimization is necessary to improve reproducibility and quantification for lipid-antigen discovery. The antigen-loaded CD1 molecules were used for tetramers and dextramers in T-cell cloning experiments. CD1d lipid-specific T-cell populations were expanded by stimulating them with lipid loaded monocyte-derived dendritic cells (moDCs) and artificial APCs (aAPCs). The moDCs proved to be more

effective than artificial aAPCs in expanding antigen-specific T cells. While stimulation with moDCs led to a significant increase in α GalCer-reactive T cells, there was no expansion of CD1d-GD3 and CD1d-endo reactive T cells under the conditions tested. These results underscore the benefits of using moDCs for unconventional T-cell stimulation and suggest the need for further refinement of antigen-loading protocols.

The successful development of high-affinity bispecific engagers targeting CD1c has potential as a tool for immunotherapy. While the lipid agnostic approach to target CD1c on leukaemia is not feasible, the identification of tumour specific lipid antigens in leukemic cells in the future will allow the development of lipid-specific TCR based T-cell engagers. Future research should aim to identify cancer specific CD1-associated lipid-targets and optimize lipid-specific molecules for clinical use, evaluate their safety, and confirm their effectiveness in disease models.

8. HUNGARIAN SUMMARY

Jelen PhD értekezés célja olyan bispecifikus molekulák létrehozása volt, amelyek a CD1c-t célozzák meg a hematológiai rosszindulatú daganatok kezelésében. A kutatás alapjaként elvégeztük a CD1 lipidóm jellemzését, amely segített megérteni, hogy a CD1c-lipid komplexek hogyan lépnek kölcsönhatásba a T-sejt receptorokkal. A tanulmány két elsődleges kutatási cél köré épült: (1) a CD1 komplexekhez kapcsolódó lipid antigén repertoár elemzése normál- és rákos sejtekben, és (2) annak feltárása, hogy a TCR-ek hogyan ismerik fel a CD1c-t molekuláris szinten.

A CD1 molekulák által prezentált lipid antigén repertoár vizsgálatához emlős expressziós rendszerben CD1 izoformákat állítottunk elő, melyek endogén lipidek keverékét prezentálják. A CD1 izoformákhoz kapcsolódó lipidek azonosítására és mennyiségi meghatározására lipid tömegspektrometriát használtunk. Azt találtuk, hogy a CD1 molekulák széles körben kölcsönhatásba lépnek a celluláris lipidómmal, átfedő és egyedi kötési preferenciákat mutatva. A CD1a és CD1c elsősorban a foszfolipidekben gazdag profilt mutatott, míg a CD1b és a CD1d a szfingolipideket részesítette előnyben. A koleszterin főként a CD1d-hez kötődött, ami összhangban van az oxidált koleszterin felismerésében betöltött szerepével. A lipidláncok hosszának elemzése azt mutatta, hogy a CD1a közepes méretű lipidekhez kötődik, a CD1b a lipidek széles tartományát prezentálja, a CD1c széles kötőkapacitással rendelkezik, a CD1d pedig a rövidebb lipideket részesíti előnyben, de képes megragadni a hosszú láncú változatokat is. A hosszú láncú szfingolipidek jelenléte az autoreaktív T-sejtek természetes gátló funkciójára utal. Érdekes módon a CD1c erős affinitást mutatott a gangliozidokhoz, ami az antigénprezentációban és az immunmodulációban betöltött lehetséges szerepekre utal. A jövőbeni tanulmányoknak meg kell vizsgálniuk, hogy a környezeti tényezők és betegségek hogyan befolyásolják a CD1 működését és terápiás alkalmazásait. A CD1c lipidómon belüli rákspecifikus lipidek feltárása érdekében CD1c-t expresszáló rákos sejtvonalakat fejlesztettünk ki. A CD1c rákos sejtekből történő izolálása céljából CD1c dúsítási módszereket teszteltünk. Habár oldható CD1c-t sikeresen tisztítottunk THP1 sejtekből, a protein mennyisége túl alacsony volt a későbbi tömegspektrometriás analízishez. A rákos megbetegedésekkel összefüggő lipid antigének megértése érdekében megvizsgáltuk az egyetlen ismert rákkal kapcsolatos lipid, az mLPA CD1c-hez való kötődésének módját. Az mLPA CD1c-hez kötése kulcsfontosságú a specific's, nem hagiomania T-setter alkali felismeréshez. Az mLPA CD1c-be való

betöltődésének vizsgálatára különböző sejten belüli lokalizációra képes CD1c-t expresszáló leukémiás sejtvonalakat hoztunk létre. A specifikus Jurkat T-sejtek fokozott aktiválódása CD1c-GPI-t expresszáló sejtekhez azt mutatta, hogy a szekréciós útvonal korai endoszómái valószínűleg az mLPA terhelés helyei. Ezenkívül a lizoszómális funkciót gátló kísérletek arra utaltak, hogy mind a lizoszómális, mind a peroxiszómális folyamatok szerepet játszanak az mLPA prezentációjában.

Ez a tanulmány betekintést nyújtott a CD1 molekulák által bemutatható lipidrepertoárba, és hogy léteznek-e rákspecifikus lipidek a CD1c lipidómon belül.

A tanulmány második célja a TCR-ek CD1c-lipid komplexekhez való kötődésének tanulmányozása volt. Ez a téma a szerkezeti adatok szűkössége miatt még mindig nem teljesen ismert. Bizonyítékok vannak arra vonatkozóan, hogy a CD1c által korlátozott TCR-eknek specifikus lipidre lehet szükségük a kötődéshez, hasonlóan az MHC-peptid kölcsönhatásokhoz. Más források a CD1c molekulák ligandum-független módon való felismerésére utalnak. A CD1c-lipid TCR felismerés molekuláris alapjainak feltárásához specifikus TCR-eket állítottunk elő oldható CD1c-lipid komplexeket segítségével, fág display könyvtárakból. Ennek eredményeként izoláltunk CD1c-korlátozott TCR-eket, amelyek képesek felismerni a CD1c molekulákat rákos sejtvonalakon. Ezeket TCR-okat bispecifikus molekulákká alakítottuk, melyek lehetővé tették a T-sejtek átirányítását a CD1c-t expresszáló rákos sejtek elpusztítására. A krisztallográfiai szerkezeti elemzés kimutatta, hogy a vad típusú TCR elsősorban a CD1c fehérjéhez kötődött, és nem rendelkezett lipid specificitással. A szerkezeti vizsgálatokból származó ismereteket felhasználva fág könyvtárakat terveztünk, affinitás-növelt TCR-ek előállítására. Az affinitás-érési technikával pikomoláris szintű affinitást és 9,6 órás felezési időt értünk el, ami a TCR-t alkalmas jelöltté teszi terápiás használatra. A vad típusú és nagy affinitású TCR-CD1c komplexek szerkezeti és lipidomikus analízise azt bizonyította, hogy a nagy affinitású TCR nagyobb lipideket képes befogadni, ezért alkalmas a CD1c-hez kapcsolódó antigének széles körének felismerésére. A tanulmány részeként, anti-CD1c antitest alapú bispecifikus T-sejt bispecifikumokat is kifejlesztünk. A BiTE-k hatékonyan aktiválták a T-sejteket, és az affinitás érlelt TCR-alapú ImmTAC-okhoz hasonló hatékonyságot mutattak. Kimutattuk, hogy a CD1c-specifikus ImmTAC hatékonyan képes a T-sejteket átirányítani a CD1c-pozitív rákos sejtvonalak elpusztítására, azonban az egészséges B-sejtek és monociták esetében is megfigyeltünk on-taget off-tumor reaktivitást, ami korlátozza az ImmTAC terápiás alkalmazását, lipid-agnosztikus megközelítésben.

A lipidspecifikus TCR-ek kifejlesztésére lipid-antigénekkel feltöltött CD1c és CD1d

molekulákat hoztunk létre. Az egyetlen lipiddel feltöltött CD1 molekulák előállításához egy detergens alapú lipid-ligandum csere módszert dolgoztunk ki. A lipid csere a hatékonyságát IEF, tömegspektrometria és tetramer festési vizsgálatokkal validáltuk. Míg a lipidcsere sikeres volt, további optimalizálás szükséges a reprodukálhatóság és a mennyiségi meghatározás javításához. Az antigénnel töltött CD1 molekulákat tetramerek és dextramerek előállítására használtuk T-sejt klónozási kísérletekben. CD1d-lipidspecifikus T-sejt-populációkat expandálunk lipiddel töltött monocita eredetű dendritikus sejtekkel (moDC) és mesterséges APC-kkel (aAPC-kkel) stimulálva. A moDC-k hatékonyabbnak bizonyultak, mint a mesterséges aAPC-k az antigén-specifikus T-sejtek szaporításában. Míg a moDC-kkel végzett stimuláció az α GalCer-reaktív T-sejtek számának jelentős növekedéséhez vezetett, a CD1d-GD3 és CD1d-endo-reaktív T-sejteket nem találtunk a vizsgált körülmények között. Ezek az eredmények alátámasztják a moDC-k nem hagyományos T-sejt-stimulációra való alkalmazásának előnyeit és az antigén-betöltési protokollok további finomításának fontosságát.

A CD1c-t megcélzó, nagy affinitású bispecifikus molekulák sikeres előállítása arra utal hogy ezek a molekulák alkalmasak immunterápiás eszközök fejlesztésére. Bár a CD1c célzása leukémiában lipid agnosztikus megközelítéssel nem megvalósítható, a tumorspecifikus lipid antigének azonosítása a leukémiás sejtekben a jövőben lehetővé teszi lipid-specifikus, TCR-alapú T-sejt-engagerek kifejlesztését. A jövőbeli kutatásoknak a rákspecifikus CD1-hez kapcsolódó lipidek azonosítására és a lipid-specifikus molekulák klinikai alkalmazásra való optimalizálására, biztonságosságuk értékelésére és hatékonyságuk megerősítésére kell irányulniuk a betegségmodellekben.

9. LIST OF KEYWORDS

T-cell engager, immunotherapy, bispecifics, CD1, self-lipid antigen, antigen presentation, lipidome, lipid mass spectrometry, crystal structure

10. ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance, support, and encouragement of many people, to whom I am deeply grateful.

I would like to express my sincere gratitude to my PhD supervisor, **Dr Peter Gogolak at the University of Debrecen**, for his invaluable guidance, patience, and insightful advice throughout this journey. His academic expertise and continuous support have been instrumental in shaping this research.

I am also immensely grateful to my PhD supervisor **at Immunocore Ltd, Dr Mariolina Salio**. Mariolina took over my supervision at the company halfway through my PhD, providing mentorship, academic and technical expertise, which have been crucial in developing my research. Her unwavering support, encouragement, and belief in my abilities gave me the confidence and motivation to persevere through the challenges I encountered. I truly appreciate her guidance and kindness.

I would also like to extend my gratitude to **Immunocore Ltd.** for allowing me to conduct my research within the organization and for providing the necessary resources and environment to carry out my work.

To all my colleagues at the **Experimental Immunology Department**, thank you for the stimulating discussions, collaboration, and friendship. Your support and encouragement have made this journey more enriching and enjoyable. Special thanks to **Richard Suckling** for his invaluable support and critically reading my thesis. His insightful feedback and thoughtful suggestions greatly improved my work. I deeply appreciate his time and encouragement throughout this process.

Finally, my deepest gratitude goes to **my husband and my family**, who encouraged me to start my PhD. Your love, patience, and support have been my greatest source of strength. Thank you for always believing in me and for standing by my side through every challenge.

This PhD journey has been both challenging and rewarding, and I am grateful to everyone who has been a part of it. I would like to express my heartfelt gratitude to my Ph.D. supervisors, Dr. Mariolina Salio and Dr. Peter Gogolak for their continuous support.

11. BIBLIOGRAPHY

1. Vivier E, Malissen B. Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nature immunology* (2005) 6:17–21.
2. Netea MG, Schlitzer A, Placek K, Joosten LAB, Schultze JL. Innate and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens. *Cell Host Microbe* (2019) 25:13–26. doi: 10.1016/j.chom.2018.12.006
3. Sun L, Su Y, Jiao A, Wang X, Zhang B. T cells in health and disease. *Signal transduction and targeted therapy* (2023) 8:235.
4. Cenerenti M, Saillard M, Romero P, Jandus C. The Era of Cytotoxic CD4 T Cells. *Front Immunol* (2022) 13:867189. doi: 10.3389/fimmu.2022.867189
5. Liu B, Kolawole EM, Evavold BD. Mechanobiology of T Cell Activation: To Catch a Bond. *Annu Rev Cell Dev Bi* (2021) 37:1–23. doi: 10.1146/annurev-cellbio-120219-055100
6. Trowsdale J, Knight JC. Major histocompatibility complex genomics and human disease. *Annual review of genomics and human genetics* (2013) 14:301–323.
7. Renier G. [Role of the HLA system in the functioning of the immune system]. *Allerg Immunol* (1992) 24:123–6.
8. Attaf M, Legut M, Cole DK, Sewell AK. The T cell antigen receptor: the Swiss army knife of the immune system. *Clin Exp Immunol* (2015) 181:1–18. doi: 10.1111/cei.12622
9. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature* (1988) 334:395–402. doi: 10.1038/334395a0
10. Griesemer AD, Sorenson EC, Hardy MA. The Role of the Thymus in Tolerance. *Transplantation* (2010) 90:465–474. doi: 10.1097/tp.0b013e3181e7e54f
11. Palmer E. Negative selection — clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* (2003) 3:383–391. doi: 10.1038/nri1085
12. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* (2014) 14:377–391. doi: 10.1038/nri3667
13. He Q, Jiang X, Zhou X, Weng J. Targeting cancers through TCR-peptide/MHC interactions. *Journal of hematology & oncology* (2019) 12:1–17.
14. Jones H, Molvi Z, Klatt M, Dao T, Scheinberg D. Empirical and Rational Design of T Cell Receptor-Based Immunotherapies., 585385. (2020)

15. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A Direct Estimate of the Human $\alpha\beta$ T Cell Receptor Diversity. *Science* (1999) 286:958–961. doi: 10.1126/science.286.5441.958
16. Ashby KM, Hogquist KA. A guide to thymic selection of T cells. *Nat Rev Immunol* (2024) 24:103–117. doi: 10.1038/s41577-023-00911-8
17. Martin R, Kaufman J. Some thoughts about what non-mammalian jawed vertebrates are telling us about antigen processing and peptide loading of MHC molecules. *Curr Opin Immunol* (2022) 77:102218. doi: 10.1016/j.coi.2022.102218
18. Wieczorek M, Abualrous ET, Sticht J, Álvaro-Benito M, Stolzenberg S, Noé F, Freund C. Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. *Front Immunol* (2017) 8:292. doi: 10.3389/fimmu.2017.00292
19. Hofstetter AR, Sullivan LC, Lukacher AE, Brooks AG. Diverse roles of non-diverse molecules: MHC class Ib molecules in host defense and control of autoimmunity. *Curr Opin Immunol* (2011) 23:104–110. doi: 10.1016/j.coi.2010.09.009
20. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nature immunology* (2015) 16:1114–1123.
21. Godfrey DI, Nours JL, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T cell targets for cancer immunotherapy. *Immunity* (2018) 48:453–473.
22. Lepore M, Mori L, Libero GD. The conventional nature of non-MHC-restricted T cells. *Frontiers in immunology* (2018) 9:1365.
23. Rogers SL, Kaufman J. Location, location, location: the evolutionary history of CD1 genes and the NKR-P1/ligand systems. *Immunogenetics* (2016) 68:499–513.
24. Reinink P, Rhijn IV. Mammalian CD1 and MR1 genes. *Immunogenetics* (2016) 68:515–523. doi: 10.1007/s00251-016-0926-x
25. Ryan SO, Cobb BA. Roles for major histocompatibility complex glycosylation in immune function. *Seminars in immunopathology*. p. 425–441 doi: 10.1007/s00281-012-0309-9
26. Lv M, Zhang Z, Cui Y. Unconventional T cells in brain homeostasis, injury and neurodegeneration. *Frontiers in Immunology* (2023) 14:1273459.
27. Mayassi T, Barreiro LB, Rossjohn J, Jabri B. A multilayered immune system through the lens of unconventional T cells. *Nature* (2021) 595:501–510.
28. Altman JD, Moss PAH, Goulder PJR, Barouch DH, McHeyzer-Williams MG, Bell JL, McMichael AJ, Davis MM. Phenotypic Analysis of Antigen-Specific T Lymphocytes. *Science* (1996) 274:94–96. doi: 10.1126/science.274.5284.94

29. Tiwari R, Singh VK, Rajneesh, Kumar A, Gautam V, Kumar R. MHC tetramer technology: Exploring T cell biology in health and disease. *Adv Protein Chem Struct Biol* (2024) 140:327–345. doi: 10.1016/bs.apcsb.2024.02.002
30. Skinner PJ, Haase AT. In situ tetramer staining. *J Immunol Methods* (2002) 268:29–34. doi: 10.1016/s0022-1759(02)00197-7
31. Salio M, Silk JD, Jones EY, Cerundolo V. Biology of CD1-and MR1-restricted T cells. *Annual review of immunology* (2014) 32:323–366.
32. Dutronc Y, Porcelli SA. The CD1 family and T cell recognition of lipid antigens. *Tissue Antigens* (2002) 60:337–353. doi: 10.1034/j.1399-0039.2002.600501.x
33. Dascher C. Evolutionary biology of CD1. *T Cell Activation by CD1 and Lipid Antigens* (2007)3–26.
34. Rhijn IV, Godfrey DI, Rossjohn J, Moody DB. Lipid and small-molecule display by CD1 and MR1. *Nature reviews Immunology* (2015) 15:643–654.
35. Moody DB, Porcelli SA. Intracellular pathways of CD1 antigen presentation. *Nature Reviews Immunology* (2003) 3:11–22.
36. Schiefner A, Wilson I. Presentation of Lipid Antigens by CD1 Glycoproteins. *Curr Pharm Des* (2009) 15:3311–3317. doi: 10.2174/138161209789105108
37. Chancellor A, Gadola SD, Mansour S. The versatility of the CD 1 lipid antigen presentation pathway. *Immunology* (2018) 154:196–203.
38. Iwabuchi K, Kaer LV. Role of CD1-and MR1-restricted T cells in immunity and disease. (2019)
39. Salle HD la, Mariotti S, Angenieux C, Gilleron M, Garcia-Alles L-F, Malm D, Berg T, Paoletti S, Maître B, Mourey L, et al. Assistance of microbial glycolipid antigen processing by CD1e. *Science* (2005) 310:1321–1324.
40. Siddiqui S, Visvabharathy L, Wang C-R. Role of group 1 CD1-restricted T cells in infectious disease. *Frontiers in immunology* (2015) 6:337.
41. Moody DB, Cotton RN. Four pathways of CD1 antigen presentation to T cells. *Current opinion in immunology* (2017) 46:127–133.
42. Wollenberg A, Kraft S, Hanau D, Bieber T. Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema. *Journal of Investigative Dermatology* (1996) 106:446–453.
43. Olivier M, Foret B, Vern YL, Kerboeuf D, Guilloteau LA. Plasticity of migrating CD1b+ and CD1b-lymph dendritic cells in the promotion of Th1, Th2 and Th17 in response to Salmonella and helminth secretions. *PLoS One* (2013) 8:e79537.

44. Sugita M, Wel N van der, Rogers RA, Peters PJ, Brenner MB. CD1c molecules broadly survey the endocytic system. *Proceedings of the National Academy of Sciences* (2000) 97:8445–8450.
45. Milne P, Bigley V, Gunawan M, Haniffa M, Collin M. CD1c+ blood dendritic cells have Langerhans cell potential. *Blood, The Journal of the American Society of Hematology* (2015) 125:470–473.
46. Evans L, Barral P. CD1 molecules: Beyond antigen presentation. *Molecular Immunology* (2024) 170:1–8.
47. Taheri M, Danesh H, Bizhani F, Bahari G, Naderi M, Hashemi M. Association between genetic variants in CD1A and CD1D genes and pulmonary tuberculosis in an Iranian population. *Biomedical Reports* (2019) 10:259–265.
48. Han M, Hannick LI, DiBrino M, Robinson MA. Polymorphism of human CD1 genes. *Tissue Antigens* (1999) 54:122–127. doi: 10.1034/j.1399-0039.1999.540202.x
49. Barral DC, Cavallari M, McCormick PJ, Garg S, Magee AI, Bonifacino JS, Libero GD, Brenner MB. CD1a and MHC class I follow a similar endocytic recycling pathway. *Traffic* (2008) 9:1446–1457.
50. Huang S, Shahine A, Cheng T-Y, Chen Y-L, Ng SW, Balaji GR, Farquhar R, Gras S, Hardman CS, Altman JD, et al. CD1 lipidomes reveal lipid-binding motifs and size-based antigen-display mechanisms. *Elsevier* (2023) 186:4583–4596. doi: 10.1016/j.cell.2023.08.022
51. Adams EJ. Lipid presentation by human CD1 molecules and the diverse T cell populations that respond to them. *Current opinion in immunology* (2014) 26:1–6.
52. Ly D, Moody DB. The CD1 size problem: lipid antigens, ligands, and scaffolds. *Cellular and molecular life sciences* (2014) 71:3069–3079.
53. Shahine A. The intricacies of self-lipid antigen presentation by CD1b. *Mol Immunol* (2018) 104:27–36. doi: 10.1016/j.molimm.2018.09.022
54. Barral DC, Brenner MB. CD1 antigen presentation: how it works. *Nature Reviews Immunology* (2007) 7:929–941.
55. Cotton RN, Wegrecki M, Cheng T-Y, Chen Y-L, Veerapen N, Nours JL, Orgill DP, Pomahac B, Talbot SG, Willis R, et al. CD1a selectively captures endogenous cellular lipids that broadly block T cell response. *Journal of Experimental Medicine* (2021) 218:e20202699.
56. Shahine A. The intricacies of self-lipid antigen presentation by CD1b. *Molecular immunology* (2018) 104:27–36.
57. Gadola SD, Zaccai NR, Harlos K, Shepherd D, Castro-Palomino JC, Ritter G, Schmidt RR, Jones EY, Cerundolo V. Structure of human CD1b with bound ligands at 2.3 Å, a maze for alkyl chains. *Nature immunology* (2002) 3:721–726.

58. Cheng T-Y, Relloso M, Rhijn IV, Young DC, Besra GS, Briken V, Zajonc DM, Wilson IA, Porcelli S, Moody DB. Role of lipid trimming and CD1 groove size in cellular antigen presentation. *The EMBO journal* (2006) 25:2989–2999.
59. Shahine A. The intricacies of self-lipid antigen presentation by CD1b. *Mol Immunol* (2018) 104:27–36. doi: 10.1016/j.molimm.2018.09.022
60. Garcia-Alles LF, Versluis K, Maveyraud L, Vallina AT, Sansano S, Bello NF, Gober H-J, Guillet V, Salle H de L, Puzo G, et al. Endogenous phosphatidylcholine and a long spacer ligand stabilize the lipid-binding groove of CD1b. *The EMBO journal* (2006) 25:3684–3692.
61. Moody DB, Ulrichs T, Mühlecker W, Young DC, Gurcha SS, Grant E, Rosat J-P, Brenner MB, Costello CE, Besra GS, et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature* (2000) 404:884–888.
62. Scharf L, Li N-S, Hawk AJ, Garzón D, Zhang T, Fox LM, Kazen AR, Shah S, Haddadian EJ, Gumperz JE, et al. The 2.5 Å structure of CD1c in complex with a mycobacterial lipid reveals an open groove ideally suited for diverse antigen presentation. *Immunity* (2010) 33:853–862.
63. Rhijn IV, Young DC, Jong AD, Vazquez J, Cheng T-Y, Talekar R, Barral DC, León L, Brenner MB, Katz JT, et al. CD1c bypasses lysosomes to present a lipopeptide antigen with 12 amino acids. *Journal of Experimental Medicine* (2009) 206:1409–1422.
64. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, Fersht AR, Besra GS, Schmidt RR, Jones EY, et al. The crystal structure of human CD1d with and without α -galactosylceramide. *Nature immunology* (2005) 6:819–826.
65. Girardi E, Zajonc DM. Molecular basis of lipid antigen presentation by CD 1d and recognition by natural killer T cells. *Immunological reviews* (2012) 250:167–179.
66. Lepore M, Lalla C de, Gundimeda SR, Gsellinger H, Consonni M, Garavaglia C, Sansano S, Piccolo F, Scelfo A, Häussinger D, et al. A novel self-lipid antigen targets human T cells against CD1c⁺ leukemias. *Journal of Experimental Medicine* (2014) 211:1363–1377.
67. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. *Annual review of immunology* (2015) 33:169–200.
68. Gras S, Rhijn IV, Shahine A, Cheng T-Y, Bhati M, Tan LL, Halim H, Tuttle KD, Gapin L, Nours JL, et al. T cell receptor recognition of CD1b presenting a mycobacterial glycolipid. *Nat Commun* (2016) 7:13257. doi: 10.1038/ncomms13257
69. Dellabona P, Consonni M, Lalla C de, Casorati G. Group 1 CD1-restricted T cells and the pathophysiological implications of self-lipid antigen recognition. *Tissue Antigens* (2015) 86:393–405. doi: 10.1111/tan.12689

70. Roura-Mir C, Moody DB. Sorting out self and microbial lipid antigens for CD1. *Microbes Infect* (2003) 5:1137–1148. doi: 10.1016/j.micinf.2003.08.003
71. Jong de, Annemieke. Activation of human T cells by CD 1 and self-lipids. *Immunological reviews* (2015) 267:16–29.
72. Lalla C de, Lepore M, Piccolo FM, Rinaldi A, Scelfo A, Garavaglia C, Mori L, Libero GD, Dellabona P, Casorati G. High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells. *Eur J Immunol* (2011) 41:602–610. doi: 10.1002/eji.201041211
73. Guo T, Koo MY, Kagoya Y, Anczurowski M, Wang C-H, Saso K, Butler MO, Hirano N. A Subset of Human Autoreactive CD1c-Restricted T Cells Preferentially Expresses TRBV4-1+ TCRs. *J Immunol* (2018) 200:500–511. doi: 10.4049/jimmunol.1700677
74. Roura-Mir C, Catálfamo M, Cheng T-Y, Marqusee E, Besra GS, Jaraquemada D, Moody DB. CD1a and CD1c Activate Intrathyroidal T Cells during Graves' Disease and Hashimoto's Thyroiditis. *J Immunol* (2005) 174:3773–3780. doi: 10.4049/jimmunol.174.6.3773
75. Alshenawy HA, Hasby EA. Immunophenotyping of dendritic cells in lesional, perilesional and distant skin of chronic plaque psoriasis. *Cell Immunol* (2011) 269:115–119. doi: 10.1016/j.cellimm.2011.03.015
76. Ogg GS, Rossjohn J, Clark RA, Moody DB. CD1a and bound lipids drive T-cell responses in human skin disease. *Eur J Immunol* (2023) 53:e2250333. doi: 10.1002/eji.202250333
77. Ye JH, Chen Y-L, Ogg G. CD1a and skin T cells: a pathway for therapeutic intervention. *Clin Exp Dermatol* (2024) 49:450–458. doi: 10.1093/ced/llad460
78. DeWitt WS, Yu KKQ, Wilburn DB, Sherwood A, Vignali M, Day CL, Scriba TJ, Robins HS, Swanson WJ, Emerson RO, et al. A Diverse Lipid Antigen-Specific TCR Repertoire Is Clonally Expanded during Active Tuberculosis. *J Immunol* (2018) 201:888–896. doi: 10.4049/jimmunol.1800186
79. Ly D, Kasmar AG, Cheng T-Y, Jong A de, Huang S, Roy S, Bhatt A, Summeren RP van, Altman JD, Jacobs WR, et al. CD1c tetramers detect ex vivo T cell responses to processed phosphomycoketide antigens. *J Exp Med* (2013) 210:729–741. doi: 10.1084/jem.20120624
80. Swann JB, Coquet JMC, Smyth MJ, Godfrey DI. T Cell Activation by CD1 and Lipid Antigens. *Curr Top Microbiol Immunol* (2007) 314:293–323. doi: 10.1007/978-3-540-69511-0_12
81. Bagchi S, Genardi S, Wang C-R. Linking CD1-Restricted T Cells With Autoimmunity and Dyslipidemia: Lipid Levels Matter. *Front Immunol* (2018) 9:1616. doi: 10.3389/fimmu.2018.01616
82. Ehteshamfar S, Akhbari M, Afshari JT, Seyedi M, Nikfar B, Shapouri-Moghaddam A, Ghanbarzadeh E, Momtazi-Borojeni AA. Anti-inflammatory and immune-modulatory

- impacts of berberine on activation of autoreactive T cells in autoimmune inflammation. *J Cell Mol Med* (2020) 24:13573–13588. doi: 10.1111/jcmm.16049
83. Sieling PA, Porcelli SA, Duong BT, Spada F, Bloom BR, Diamond B, Hahn BH. Human Double-Negative T Cells in Systemic Lupus Erythematosus Provide Help for IgG and Are Restricted by CD1c. *J Immunol* (2000) 165:5338–5344. doi: 10.4049/jimmunol.165.9.5338
84. Yuan X, Qin X, Wang D, Zhang Z, Tang X, Gao X, Chen W, Sun L. Mesenchymal stem cell therapy induces FLT3L and CD1c⁺ dendritic cells in systemic lupus erythematosus patients. *Nat Commun* (2019) 10:2498. doi: 10.1038/s41467-019-10491-8
85. Wójcik P, Biernacki M, Wroński A, Łuczaj W, Waeg G, Žarković N, Skrzydlewska E. Altered Lipid Metabolism in Blood Mononuclear Cells of Psoriatic Patients Indicates Differential Changes in Psoriasis Vulgaris and Psoriatic Arthritis. *Int J Mol Sci* (2019) 20:4249. doi: 10.3390/ijms20174249
86. Greve AM, Wulff AB, Bojesen SE, Nordestgaard BG. Elevated plasma triglycerides increase the risk of psoriasis: a cohort and Mendelian randomization study. *Br J Dermatol* (2024) 191:209–215. doi: 10.1093/bjd/ljae089
87. Genardi S, Morgun E, Wang C-R. CD1-Restricted T Cells in Inflammatory Skin Diseases. *J Invest Dermatol* (2021) 142:768–773. doi: 10.1016/j.jid.2021.03.033
88. Mortier C, Govindarajan S, Venken K, Elewaut D. It Takes “Guts” to Cause Joint Inflammation: Role of Innate-Like T Cells. *Front Immunol* (2018) 9:1489. doi: 10.3389/fimmu.2018.01489
89. Mariuzza RA, Agnihotri P, Orban J. The structural basis of T-cell receptor (TCR) activation: An enduring enigma. *J Biol Chem* (2019) 295:914–925. doi: 10.1074/jbc.rev119.009411
90. Rudolph MG, Stanfield RL, Wilson IA. HOW TCRS BIND MHCS, PEPTIDES, AND CORECEPTORS. *Annu Rev Immunol* (2006) 24:419–466. doi: 10.1146/annurev.immunol.23.021704.115658
91. Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* (1996) 384:134–141. doi: 10.1038/384134a0
92. Garcia KC, Degano M, Stanfield RL, Brunmark A, Jackson MR, Peterson PA, Teyton L, Wilson IA. An $\alpha\beta$ T Cell Receptor Structure at 2.5 Å and Its Orientation in the TCR-MHC Complex. *Science* (1996) 274:209–219. doi: 10.1126/science.274.5285.209
93. Szeto C, Lobos CA, Nguyen AT, Gras S. TCR Recognition of Peptide–MHC-I: Rule Makers and Breakers. *Int J Mol Sci* (2020) 22:68. doi: 10.3390/ijms22010068
94. Robinson RA, McMurran C, McCully ML, Cole DK. Engineering soluble T-cell receptors for therapy. *Febs J* (2021) 288:6159–6173. doi: 10.1111/febs.15780

95. Klebanoff CA, Chandran SS, Baker BM, Quezada SA, Ribas A. T cell receptor therapeutics: immunological targeting of the intracellular cancer proteome. *Nat Rev Drug Discov* (2023)1–22. doi: 10.1038/s41573-023-00809-z
96. Litzow MR, Sun Z, Mattison RJ, Paietta EM, Roberts KG, Zhang Y, Racevskis J, Lazarus HM, Rowe JM, Arber DA, et al. Blinatumomab for MRD-Negative Acute Lymphoblastic Leukemia in Adults. *N Engl J Med* (2024) 391:320–333. doi: 10.1056/nejmoa2312948
97. Tian Z, Liu M, Zhang Y, Wang X. Bispecific T cell engagers: an emerging therapy for management of hematologic malignancies. *J Hematol Oncol* (2021) 14:75. doi: 10.1186/s13045-021-01084-4
98. Thieblemont C, Karimi YH, Ghesquieres H, Cheah CY, Clausen MR, Cunningham D, Jurczak W, Do YR, Gasirowski R, Lewis DJ, et al. Epcoritamab in relapsed/refractory large B-cell lymphoma: 2-year follow-up from the pivotal EPCORE NHL-1 trial. *Leukemia* (2024) 38:2653–2662. doi: 10.1038/s41375-024-02410-8
99. Moreau P, Garfall AL, Donk NWCJ van de, Nahi H, San-Miguel JF, Oriol A, Nooka AK, Martin T, Rosinol L, Chari A, et al. Teclistamab in Relapsed or Refractory Multiple Myeloma. *N Engl J Med* (2022) 387:495–505. doi: 10.1056/nejmoa2203478
100. Li Y, Moysey R, Molloy PE, Vuidepot A-L, Mahon T, Baston E, Dunn S, Liddy N, Jacob J, Jakobsen BK, et al. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nature biotechnology* (2005) 23:349–354.
101. Aleksic M, Liddy N, Molloy PE, Pumphrey N, Vuidepot A, Chang K, Jakobsen BK. Different affinity windows for virus and cancer-specific T-cell receptors: Implications for therapeutic strategies. *Eur J Immunol* (2012) 42:3174–3179. doi: 10.1002/eji.201242606
102. Lowe KL, Cole D, Kenefeck R, OKelly I, Lepore M, Jakobsen BK. Novel TCR-based biologics: mobilising T cells to warm ‘cold’ tumours. *Cancer Treat Rev* (2019) 77:35–43. doi: 10.1016/j.ctrv.2019.06.001
103. Liddy N, Bossi G, Adams KJ, Lissina A, Mahon TM, Hassan NJ, Gavarret J, Bianchi FC, Pumphrey NJ, Ladell K, et al. Monoclonal TCR-redirected tumor cell killing. *Nature medicine* (2012) 18:980–987.
104. Dhillon S. Tebentafusp: First Approval. *Drugs* (2022) 82:703–710. doi: 10.1007/s40265-022-01704-4
105. Howlett S, Carter TJ, Shaw HM, Nathan PD. Tebentafusp: a first-in-class treatment for metastatic uveal melanoma. *Ther Adv Med Oncol* (2023) 15:17588359231160140. doi: 10.1177/17588359231160140
106. Chen LN, Carvajal RD. Tebentafusp for the treatment of HLA-A*02:01–positive adult patients with unresectable or metastatic uveal melanoma. *Expert Rev Anticanc* (2022) doi: 10.1080/14737140.2022.2124971

107. Pearlman AH, Hwang MS, Konig MF, Hsiue EH-C, Douglass J, DiNapoli SR, Mog BJ, Bettegowda C, Pardoll DM, Gabelli SB, et al. Targeting public neoantigens for cancer immunotherapy. *Nat Cancer* (2021) 2:487–497. doi: 10.1038/s43018-021-00210-y
108. Shafer P, Kelly LM, Hoyos V. Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects. *Front Immunol* (2022) 13:835762. doi: 10.3389/fimmu.2022.835762
109. Hurley CK. Naming HLA diversity: A review of HLA nomenclature. *Hum Immunol* (2021) 82:457–465. doi: 10.1016/j.humimm.2020.03.005
110. Aquino A, Graziani G, Franzese O, Prete SP, Bonmassar E, Bonmassar L, D'Atri S. Exogenous Control of the Expression of Group I CD1 Molecules Competent for Presentation of Microbial Nonpeptide Antigens to Human T Lymphocytes. *J Immunol Res* (2011) 2011:790460. doi: 10.1155/2011/790460
111. Wun KS, Reijneveld JF, Cheng T-Y, Ladell K, Uldrich AP, Nours JL, Miners KL, McLaren JE, Grant EJ, Haigh OL, et al. T cell autoreactivity directed toward CD1c itself rather than toward carried self lipids. *Nature immunology* (2018) 19:397–406.
112. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T Cell Antigen Receptor Recognition of Antigen-Presenting Molecules. *Annu Rev Immunol* (2014) 33:1–32. doi: 10.1146/annurev-immunol-032414-112334
113. Cotton RN, Shahine A, Rossjohn J, Moody DB. Lipids hide or step aside for CD1-autoreactive T cell receptors. *Curr Opin Immunol* (2018) 52:93–99. doi: 10.1016/j.coi.2018.04.013
114. Mechold U, Gilbert C, Ogryzko V. Codon optimization of the BirA enzyme gene leads to higher expression and an improved efficiency of biotinylation of target proteins in mammalian cells. *J Biotechnol* (2005) 116:245–249. doi: 10.1016/j.jbiotec.2004.12.003
115. Parrott MB, Barry MA. Metabolic Biotinylation of Secreted and Cell Surface Proteins from Mammalian Cells. *Biochem Biophys Res Commun* (2001) 281:993–1000. doi: 10.1006/bbrc.2001.4437
116. Yu C, Crispin M, Sonnen AF -P., Harvey DJ, Chang VT, Evans EJ, Scanlan CN, Stuart DI, Gilbert RJC, Davis SJ. Use of the α -mannosidase I inhibitor kifunensine allows the crystallization of apo CTLA-4 homodimer produced in long-term cultures of Chinese hamster ovary cells. *Acta Crystallogr Sect F* (2011) 67:785–789. doi: 10.1107/s1744309111017672
117. Chang VT, Crispin M, Aricescu AR, Harvey DJ, Nettleship JE, Fennelly JA, Yu C, Boles KS, Evans EJ, Stuart DI, et al. Glycoprotein Structural Genomics: Solving the Glycosylation Problem. *Structure* (2007) 15:267–273. doi: 10.1016/j.str.2007.01.011
118. Kempná P, Cipollone R, Villacorta L, Ricciarelli R, Zingg J. Isoelectric Point Mobility Shift Assay for Rapid Screening of Charged and Uncharged Ligands Bound to Proteins. *IUBMB Life* (2003) 55:103–107. doi: 10.1080/1521654031000095756

119. Farmerie L, Rustandi RR, Loughney JW, Dawod M. Recent advances in isoelectric focusing of proteins and peptides. *J Chromatogr A* (2021) 1651:462274. doi: 10.1016/j.chroma.2021.462274
120. Keener JE, Zhang G, Marty MT. Native Mass Spectrometry of Membrane Proteins. *Anal Chem* (2021) 93:583–597. doi: 10.1021/acs.analchem.0c04342
121. Tamara S, Boer MA den, Heck AJR. High-Resolution Native Mass Spectrometry. *Chem Rev* (2022) 122:7269–7326. doi: 10.1021/acs.chemrev.1c00212
122. Karch KR, Snyder DT, Harvey SR, Wysocki VH. Native Mass Spectrometry: Recent Progress and Remaining Challenges. *Annu Rev Biophys* (2022) 51:1–23. doi: 10.1146/annurev-biophys-092721-085421
123. Dolton G, Tungatt K, Lloyd A, Bianchi V, Theaker SM, Trimby A, Holland CJ, Donia M, Godkin AJ, Cole DK, et al. More tricks with tetramers: a practical guide to staining T cells with peptide–MHC multimers. *Immunology* (2015) 146:11–22. doi: 10.1111/imm.12499
124. Ledsgaard L, Kilstrup M, Karatt-Vellatt A, McCafferty J, Laustsen AH. Basics of Antibody Phage Display Technology. *Toxins* (2018) 10:236. doi: 10.3390/toxins10060236
125. Saw PE, Song E-W. Phage display screening of therapeutic peptide for cancer targeting and therapy. *Protein Cell* (2019) 10:787–807. doi: 10.1007/s13238-019-0639-7
126. Holland CJ, Crean RM, Pentier JM, Wet B de, Lloyd A, Srikannathasan V, Lissin N, Lloyd KA, Blicher TH, Conroy PJ, et al. Specificity of bispecific T cell receptors and antibodies targeting peptide-HLA. *The Journal of clinical investigation* (2020) 130:2673–2688.
127. Boulter JM, Glick M, Todorov PT, Baston E, Sami M, Rizkallah P, Jakobsen BK. Stable, soluble T-cell receptor molecules for crystallization and therapeutics. *Protein engineering* (2003) 16:707–711.
128. Cole DK, Pumphrey NJ, Boulter JM, Sami M, Bell JI, Gostick E, Price DA, Gao GF, Sewell AK, Jakobsen BK. Human TCR-binding affinity is governed by MHC class restriction. *The Journal of Immunology* (2007) 178:5727–5734.
129. Bollag DM. Peptide Analysis Protocols. *Methods Mol Biol* (1994) 36:11–22. doi: 10.1385/0-89603-274-4:11
130. Wallace RG, Rochfort KD. Protein Chromatography, Methods and Protocols. *Methods Mol Biol* (2023) 2699:161–177. doi: 10.1007/978-1-0716-3362-5_9
131. Uliyanchenko E. Size-exclusion chromatography—from high-performance to ultra-performance. *Anal Bioanal Chem* (2014) 406:6087–6094. doi: 10.1007/s00216-014-8041-z
132. Malmqvist M. BIACORE: an affinity biosensor system for characterization of biomolecular interactions. *Biochem Soc Trans* (1999) 27:335–340. doi: 10.1042/bst0270335

133. Leonard P, Hearty S, Ma H, O’Kennedy R. Protein Chromatography, Methods and Protocols. *Methods Mol Biol* (2016) 1485:339–354. doi: 10.1007/978-1-4939-6412-3_17
134. Lipschultz CA, Li Y, Smith-Gill S. Experimental Design for Analysis of Complex Kinetics Using Surface Plasmon Resonance. *Methods* (2000) 20:310–318. doi: 10.1006/meth.1999.0924
135. Cheng T-Y, Praveena T, Govindarajan S, Almeida CF, Pellicci DG, Arkins WC, Rhijn IV, Venken K, Elewaut D, Godfrey DI, et al. Lipidomic scanning of self-lipids identifies headless antigens for natural killer T cells. *Proc Natl Acad Sci* (2024) 121:e2321686121. doi: 10.1073/pnas.2321686121
136. Vonrhein C, Flensburg C, Keller P, Sharff A, Smart O, Paciorek W, Womack T, Bricogne G. Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr Sect D: Biol Crystallogr* (2011) 67:293–302. doi: 10.1107/s0907444911007773
137. Gildea RJ, Beilsten-Edmands J, Axford D, Horrell S, Aller P, Sandy J, Sanchez-Weatherby J, Owen CD, Lukacik P, Strain-Damerell C, et al. xia2.multiplex: a multi-crystal data-analysis pipeline. *Acta Crystallogr Sect D* (2022) 78:752–769. doi: 10.1107/s2059798322004399
138. Bunkóczi G, Echols N, McCoy AJ, Oeffner RD, Adams PD, Read RJ. Phaser.MRage: automated molecular replacement. *Acta Crystallogr Sect D* (2013) 69:2276–2286. doi: 10.1107/s0907444913022750
139. Agirre J, Atanasova M, Bagdonas H, Ballard CB, Baslé A, Beilsten-Edmands J, Borges RJ, Brown DG, Burgos-Mármol JJ, Berrisford JM, et al. The CCP4 suite: integrative software for macromolecular crystallography. *Acta Crystallogr Sect D* (2023) 79:449–461. doi: 10.1107/s2059798323003595
140. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr Sect D: Biol Crystallogr* (2004) 60:2126–2132. doi: 10.1107/s0907444904019158
141. Winn MD, Murshudov GN, Papiz MZ. Macromolecular TLS Refinement in REFMAC at Moderate Resolutions. *Methods Enzym* (2003) 374:300–321. doi: 10.1016/s0076-6879(03)74014-2
142. Chen VB, Wedell JR, Wenger RK, Ulrich EL, Markley JL. MolProbity for the masses—of data. *J Biomol NMR* (2015) 63:77–83. doi: 10.1007/s10858-015-9969-9
143. Ravi G, Costa LJ. Bispecific T-cell engagers for treatment of multiple myeloma. *Am J Hematol* (2023) 98:S13–S21. doi: 10.1002/ajh.26628
144. Simão DC, Zarrabi KK, Mendes JL, Luz R, Garcia JA, Kelly WK, Barata PC. Bispecific T-Cell Engagers Therapies in Solid Tumors: Focusing on Prostate Cancer. *Cancers* (2023) 15:1412. doi: 10.3390/cancers15051412
145. Huehls AM, Coupet TA, Sentman CL. Bispecific T-cell engagers for cancer immunotherapy. *Immunol Cell Biol* (2015) 93:290–296. doi: 10.1038/icb.2014.93

146. Gutiérrez-Granados S, Cervera L, Kamen AA, Gòdia F. Advancements in mammalian cell transient gene expression (TGE) technology for accelerated production of biologics. *Crit Rev Biotechnol* (2018) 38:918–940. doi: 10.1080/07388551.2017.1419459
147. Berggren T, Lutz M, Modesto V. General Spinfection Protocol. *StemBook* (2012) doi: 10.3824/stembook.1.85.1
148. Grailer J, Cheng ZJ, Hartnett J, Slater M, Fan F, Cong M. A Novel Cell-based Luciferase Reporter Platform for the Development and Characterization of T-Cell Redirecting Therapies and Vaccine Development. *J Immunother* (2023) 46:96–106. doi: 10.1097/cji.0000000000000453
149. Cossarizza A, Chang H, Radbruch A, Akdis M, Andrä I, Annunziato F, Bacher P, Barnaba V, Battistini L, Bauer WM, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies*. *Eur J Immunol* (2017) 47:1584–1797. doi: 10.1002/eji.201646632
150. Surma MA, Gerl MJ, Herzog R, Helppi J, Simons K, Klose C. Mouse lipidomics reveals inherent flexibility of a mammalian lipidome. *Scientific reports* (2021) 11:19364.
151. Szoke-Kovacs R, Khakoo S, Gogolak P, Salio M. Insights into the CD1 lipidome. *Frontiers in Immunology* (2024) 15:1462209.
152. Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, Simons K, Shevchenko A. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proceedings of the National Academy of Sciences* (2009) 106:2136–2141.
153. Surma MA, Herzog R, Vasilj A, Klose C, Christinat N, Morin-Rivron D, Simons K, Masoodi M, Sampaio JL. An automated shotgun lipidomics platform for high throughput, comprehensive, and quantitative analysis of blood plasma intact lipids. *European Journal of Lipid Science and Technology* (2015) 117:1540–1549.
154. Herzog R, Schwudke D, Schuhmann K, Sampaio JL, Bornstein SR, Schroeder M, Shevchenko A. A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. *Genome biology* (2011) 12:1–25.
155. Herzog R, Schuhmann K, Schwudke D, Sampaio JL, Bornstein SR, Schroeder M, Shevchenko A. LipidXplorer: a software for consensual cross-platform lipidomics. *PloS one* (2012) 7:e29851.
156. Garcia-Alles LF, Giacometti G, Versluis C, Maveyraud L, Paepe D de, Guiard J, Tranier S, Gilleron M, Prandi J, Hanau D, et al. Crystal structure of human CD1e reveals a groove suited for lipid-exchange processes. *Proc Natl Acad Sci* (2011) 108:13230–13235. doi: 10.1073/pnas.1105627108
157. Facciotti F, Cavallari M, Angénieux C, Garcia-Alles LF, Signorino-Gelo F, Angman L, Gilleron M, Prandi J, Puzo G, Panza L, et al. Fine tuning by human CD1e of lipid-specific immune responses. *Proc Natl Acad Sci* (2011) 108:14228–14233. doi: 10.1073/pnas.1108809108

158. Paepe DC-D, Layre E, Giacometti G, Garcia-Alles LF, Mori L, Hanau D, Libero G de, Salle H de la, Puzo G, Gilleron M. Deciphering the Role of CD1e Protein in Mycobacterial Phosphatidyl-myo-inositol Mannosides (PIM) Processing for Presentation by CD1b to T Lymphocytes*. *J Biol Chem* (2012) 287:31494–31502. doi: 10.1074/jbc.m112.386300
159. Shahine A, Rhijn IV, Cheng T-Y, Iwany S, Gras S, Moody DB, Rossjohn J. A molecular basis of human T cell receptor autoreactivity toward self-phospholipids. *Sci Immunol* (2017) 2:eaao1384. doi: 10.1126/sciimmunol.aao1384
160. Reinink P, Souter MNT, Cheng T, Gorkom T van, Lenz S, Kubler-Kielb J, Strle K, Kremer K, Thijsen SFT, Steere AC, et al. CD1b presents self and *Borrelia burgdorferi* diacylglycerols to human T cells. *Eur J Immunol* (2019) 49:737–746. doi: 10.1002/eji.201847949
161. Garcia-Alles LF, Giacometti G, Versluis C, Maveyraud L, Paepe D de, Guiard J, Tranier S, Gilleron M, Prandi J, Hanau D, et al. Crystal structure of human CD1e reveals a groove suited for lipid-exchange processes. *Proceedings of the National Academy of Sciences* (2011) 108:13230–13235.
162. Benesch JL, Aquilina JA, Ruotolo BT, Sobott F, Robinson CV. Tandem mass spectrometry reveals the quaternary organization of macromolecular assemblies. *Chemistry & biology* (2006) 13:597–605.
163. Heuvel RH van den, Heck AJ. Native protein mass spectrometry: from intact oligomers to functional machineries. *Current opinion in chemical biology* (2004) 8:519–526.
164. Mansour S, Tocheva AS, Cave-Ayland C, Machelett MM, Sander B, Lissin NM, Molloy PE, Baird MS, Stübs G, Schröder NW, et al. Cholesteryl esters stabilize human CD1c conformations for recognition by self-reactive T cells. *Proceedings of the National Academy of Sciences* (2016) 113:E1266–E1275.
165. Gadola SD, Dulphy N, Salio M, Cerundolo V. V α 24-J α Q-independent, CD1d-restricted recognition of α -galactosylceramide by human CD4⁺ and CD8 α β ⁺ T lymphocytes. *The Journal of Immunology* (2002) 168:5514–5520.
166. Karadimitris A, Gadola S, Altamirano M, Brown D, Woolfson A, Klenerman P, Chen J-L, Koezuka Y, Roberts IAG, Price DA, et al. Human CD1d–glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci* (2001) 98:3294–3298. doi: 10.1073/pnas.051604498
167. Cimino J, Calligaris D, Far J, Debois D, Blacher S, Sounni N, Noel A, Pauw ED. Towards Lipidomics of Low-Abundant Species for Exploring Tumor Heterogeneity Guided by High-Resolution Mass Spectrometry Imaging. *Int J Mol Sci* (2013) 14:24560–24580. doi: 10.3390/ijms141224560
168. Schwudke D, Schuhmann K, Herzog R, Bornstein SR, Shevchenko A. Shotgun Lipidomics on High Resolution Mass Spectrometers. *Cold Spring Harb Perspect Biol* (2011) 3:a004614. doi: 10.1101/cshperspect.a004614

169. Köfeler HC, Ahrends R, Baker ES, Ekroos K, Han X, Hoffmann N, Holčapek M, Wenk MR, Liebisch G. Recommendations for good practice in MS-based lipidomics. *J Lipid Res* (2021) 62:100138. doi: 10.1016/j.jlr.2021.100138
170. Smirnov D, Mazin P, Osetrova M, Stekolshchikova E, Khrameeva E. The Hitchhiker's Guide to Untargeted Lipidomics Analysis: Practical Guidelines. *Metabolites* (2021) 11:713. doi: 10.3390/metabo11110713
171. Coles CH, McMurrin C, Lloyd A, Hock M, Hibbert L, Raman MCC, Hayes C, Lupardus P, Cole DK, Harper S. T cell receptor interactions with human leukocyte antigen govern indirect peptide selectivity for the cancer testis antigen MAGE-A4. *J Biol Chem* (2020) 295:11486–11494. doi: 10.1074/jbc.ra120.014016
172. Ashfield R, Jakobsen BK. Making high-affinity T-cell receptors: a new class of targeted therapeutics. *IDrugs: The Investigational Drugs Journal* (2006) 9:554–559.
173. Li Y, Moysey R, Molloy PE, Vuidepot A-L, Mahon T, Baston E, Dunn S, Liddy N, Jacob J, Jakobsen BK, et al. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nature biotechnology* (2005) 23:349–354.
174. Frick R, Høydahl LS, Hodnebrug I, Vik ES, Dalhus B, Sollid LM, Gray JJ, Sandlie I, Løset GÅ. Affinity maturation of TCR-like antibodies using phage display guided by structural modeling. *Protein Eng Des Sel* (2022) doi: 10.1093/protein/gzac005
175. Zareie P, Farenc C, Gruta NLL. MHC Restriction: Where Are We Now? *Viral Immunol* (2020) 33:179–187. doi: 10.1089/vim.2019.0195
176. McCarthy C, Shepherd D, Fleire S, Stronge VS, Koch M, Illarionov PA, Bossi G, Salio M, Denkberg G, Reddington F, et al. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *J Exp Med* (2007) 204:1131–1144. doi: 10.1084/jem.20062342
177. Wanders R, Ferdinandusse S, Brites P, Kemp S. Peroxisomes, lipid metabolism and lipotoxicity. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* (2010) 1801:272–280.
178. Briken V, Jackman RM, Watts GF, Rogers RA, Porcelli SA. Human CD1b and CD1c isoforms survey different intracellular compartments for the presentation of microbial lipid antigens. *The Journal of experimental medicine* (2000) 192:281–288.
179. Sugita M, Peters PJ, Brenner MB. Pathways for Lipid Antigen Presentation by CD1 Molecules: Nowhere for Intracellular Pathogens to Hide. *Traffic* (2000) 1:295–300. doi: 10.1034/j.1600-0854.2000.010401.x
180. Jackman RM, Stenger S, Lee A, Moody DB, Rogers RA, Niazi KR, Sugita M, Modlin RL, Peters PJ, Porcelli SA. The tyrosine-containing cytoplasmic tail of CD1b is essential for its efficient presentation of bacterial lipid antigens. *Immunity* (1998) 8:341–351.

181. Kinoshita T. Glycosylphosphatidylinositol (GPI) Anchors: Biochemistry and Cell Biology: Introduction to a Thematic Review Series. *J Lipid Res* (2016) 57:4–5. doi: 10.1194/jlr.e065417
182. Chatterjee S, Smith ER, Hanada K, Stevens VL, Mayor S. GPI anchoring leads to sphingolipid-dependent retention of endocytosed proteins in the recycling endosomal compartment. *The EMBO journal* (2001)
183. Geho DH, Fayen JD, Jackman RM, Moody D, Porcelli SA, Tykocinski ML. Glycosyl-phosphatidylinositol reanchoring unmasks distinct antigen-presenting pathways for CD1b and CD1c. *The Journal of Immunology* (2000) 165:1272–1277.
184. Maxfield FR, Mayor S. ADP-Ribosylation in Animal Tissues, Structure, Function, and Biology of Mono (ADP-ribosyl) Transferases and Related Enzymes. *Adv Exp Med Biol* (1997) 419:355–364. doi: 10.1007/978-1-4419-8632-0_47
185. Mayor S, Sabharanjak S, Maxfield FR. Cholesterol-dependent retention of GPI-anchored proteins in endosomes. *EMBO J* (1998) 17:4626–4638. doi: 10.1093/emboj/17.16.4626
186. Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* (1991) 266:17707–12.
187. Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. *Autophagy* (2015) 11:1437–1438. doi: 10.1080/15548627.2015.1066957
188. Sipe DM, Jesurum A, Murphy RF. Absence of Na⁺, K⁺ (+)-ATPase regulation of endosomal acidification in K562 erythroleukemia cells. Analysis via inhibition of transferrin recycling by low temperatures. *Journal of Biological Chemistry* (1991) 266:3469–3474.
189. Rhijn IV, Berlo T van, Hilmenyuk T, Cheng T-Y, Wolf BJ, Tatituri RVV, Uldrich AP, Napolitani G, Cerundolo V, Altman JD, et al. Human autoreactive T cells recognize CD1b and phospholipids. *Proc Natl Acad Sci* (2016) 113:380–385. doi: 10.1073/pnas.1520947112
190. Rudolph M, Wang Y, Dai L, Zeissig S. Sortase A-cleavable CD1d identifies sphingomyelins as major class of CD1d-associated lipids. *Frontiers in immunology* (2022) 13:897873.
191. Rosales C, Tang D, Geng Y-J. CD1d serves as a surface receptor for oxidized cholesterol induction of peroxisome proliferator-activated receptor- γ . *Atherosclerosis* (2015) 239:224–231. doi: 10.1016/j.atherosclerosis.2015.01.004
192. Melum E, Jiang X, Baker KD, Macedo MF, Fritsch J, Dowds CM, Wang J, Pharo A, Kaser A, Tan C, et al. Control of CD1d-restricted antigen presentation and inflammation by sphingomyelin. *Nat Immunol* (2019) 20:1644–1655. doi: 10.1038/s41590-019-0504-0

193. Park J-E, Wu DY, Prendes M, Lu SX, Ragupathi G, Schrantz N, Chapman PB. Fine specificity of natural killer T cells against GD3 ganglioside and identification of GM3 as an inhibitory natural killer T-cell ligand. *Immunology* (2008) 123:145–155.

12. PUBLICATION LIST



UNIVERSITY of
DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN

H-4002 Egyetem tér 1, Debrecen

Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number:

DEENK/518/2025.PL

Subject:

PhD Publication List

Candidate: Rita Szőke-Kovács

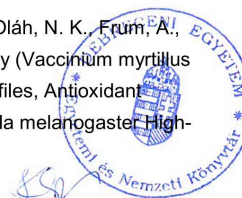
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Szőke-Kovács, R.**, Khakoo, S., Rangel, V. L., Della Cristina, P., Pentier, J., Khanolkar, R., El-Ajouz, S., Simmons, R. A., Cole, D. K., Gogolák, P., Salio, M., Karupiah, V.: A CD1c lipid agnostic T cell receptor bispecific engager redirects T cells against CD1c+ cells.
Front. Immunol. 16, 1-14, 2025.
DOI: <http://dx.doi.org/10.3389/fimmu.2025.1614610>
IF: 5.9 (2024)
2. **Szőke-Kovács, R.**, Khakoo, S., Gogolák, P., Salio, M.: Insights into the CD1 lipidome.
Front. Immunol. 15, 1-11, 2024.
DOI: <http://dx.doi.org/10.3389/fimmu.2024.1462209>
IF: 5.9

List of other publications

3. Wet, B. d., Simmons, R. A., Suckling, R. J., **Szőke-Kovács, R.**, Mansour, S., Lepore, M., Cole, D. K., Jaworski, J., Chapman, A. L., Aleksic, M.: Characterization of Human CD8αβ Interaction With Classical and Unconventional MHC Molecules.
Eur. J. Immunol. 55 (1), 7, 2024.
DOI: <http://dx.doi.org/10.1002/eji.202451230>
IF: 3.7
4. Neamtu, A. A., **Szőke-Kovács, R.**, Mihok, E., Georgescu, C., Turcuş, V., Oláh, N. K., Fium, A., Tita, O., Neamtu, C., Szőke-Kovács, Z., Cziáky, Z., Máthé, E.: Bilberry (*Vaccinium myrtillus* L.) Extracts Comparative Analysis Regarding Their Phytonutrient Profiles, Antioxidant Capacity along with the In Vivo Rescue Effects Tested on a *Drosophila melanogaster* High-Sugar Diet Model.
Antioxidants. 9, 1-33, 2020.
DOI: <http://dx.doi.org/10.3390/antiox9111067>
IF: 6.312





5. Szőke-Kovács, Z., Móré, E. C., **Szőke-Kovács, R.**, Máthé, E., Frecska, E.: Selective Inhibition of the Serotonin Transporter in the Treatment of Depression: Sertraline, Fluoxetine and Citalopram.
Neuropsychopharmacol Hung. 22 (1), 1-14, 2020.
6. Szőke-Kovács, Z., Móré, E. C., **Szőke-Kovács, R.**, Máthé, E., Frecska, E.: Selective inhibition of the serotonin transporter in the treatment of depression: sertraline, fluoxetine and citalopram.
Neuropsychopharmacol. Hung. 22 (1), 4-15, 2020.
7. Máthé, E., Moş, L., Szőke-Kovács, Z., **Szőke-Kovács, R.**, Vigh, S., Pribac, C., Czapár, M., Oláh, C.: Plant extracts affect dose-dependently the hatching rate of *Drosophila melanogaster* but do not induce chromosome loss and/or aneuploidy.
Anal. Univ. Oradea Fac. Ecotoxicologie, Zootehnie şi Tehnologii de Industrie Alimentară. 11B, 399-404, 2012.

Total IF of journals (all publications): 21,812

Total IF of journals (publications related to the dissertation): 11,8

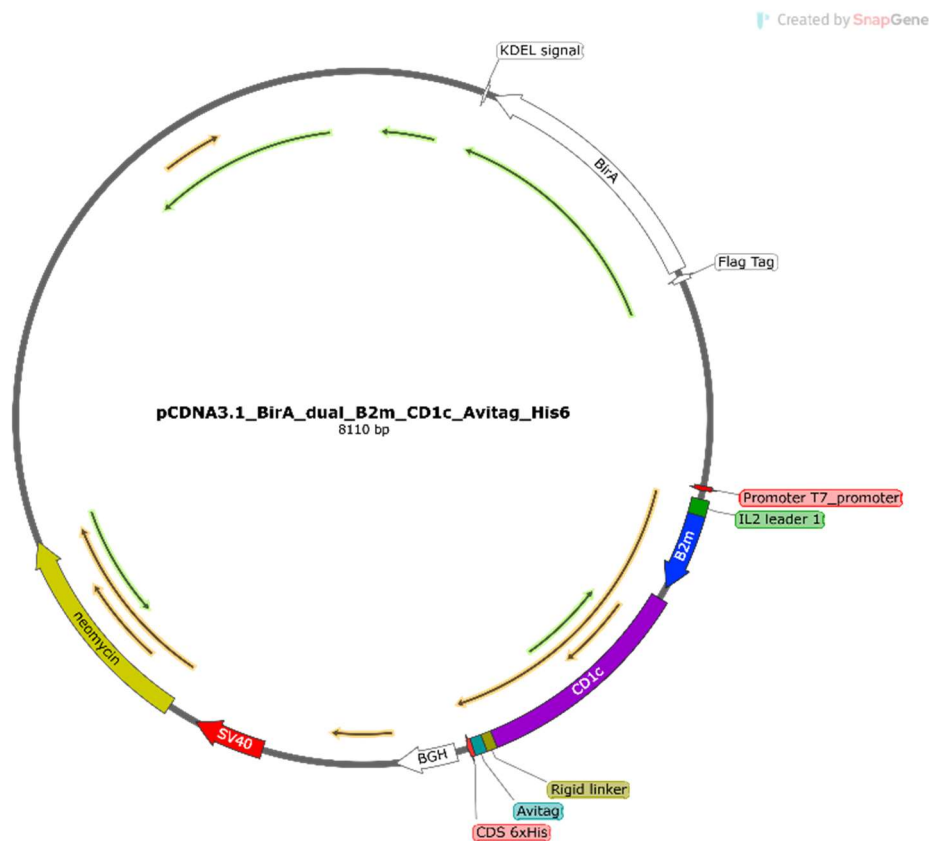
The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

10 September, 2025



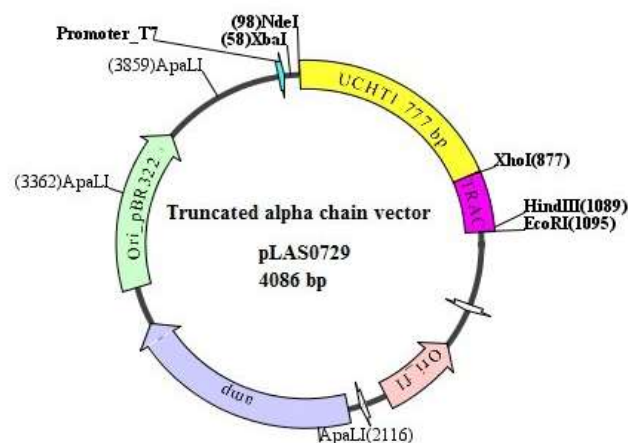
13. SUPPLEMENT

13.1 Vector maps



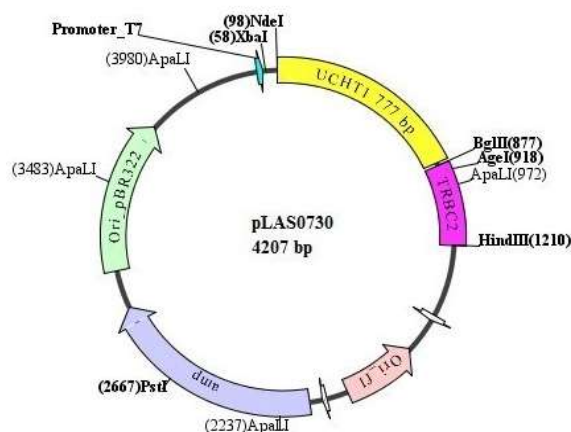
Supplementary Figure 13.1: pCDNA3.1 vector map containing single chain CD1c

The pCDNA3.1 is a dual expression vector designed to express biotinylated protein in mammalian expression system. The expression of the gene of interest is driven by T7 promoter. The vector contains a BirA construct under the CMV promoter to attach a biotin molecule to the expressed protein. An AviTag sequence was inserted into the C-terminal of the full length CD1c construct. As a result, the AviTag located in the C-terminal of the CD1c protein is ligated to a single biotin by BirA.



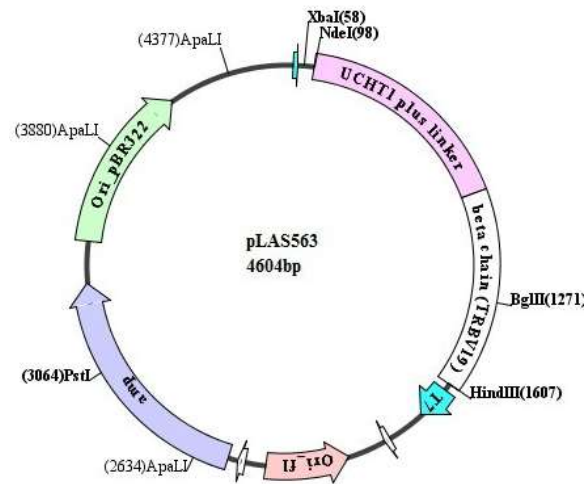
Supplementary Figure 13.2: pLAS0729 vector map

Alpha chain expression vector. Each alpha chain consists of a variable domain containing the CDR regions (T-cell receptor alpha variable gene– TRAV) followed by a junction region (T-cell receptor alpha junction gene - TRAJ) and finally the constant domain (T-cell receptor alpha constant gene - TRAC). Variable domains may be one of many (as defined by IMGT) and the same variable chain can be used by different TCRs. The variable chains and thus individual TCRs are unique due to their CDR regions. pLAS0729 holds only the alpha constant domain. pLAS0729 has a truncated constant domain missing the amino acid sequence FFPSPESS at the C-terminus. pLAS0729 was constructed after it had been shown that the C-terminus of the alpha constant domain is subject to proteolysis.



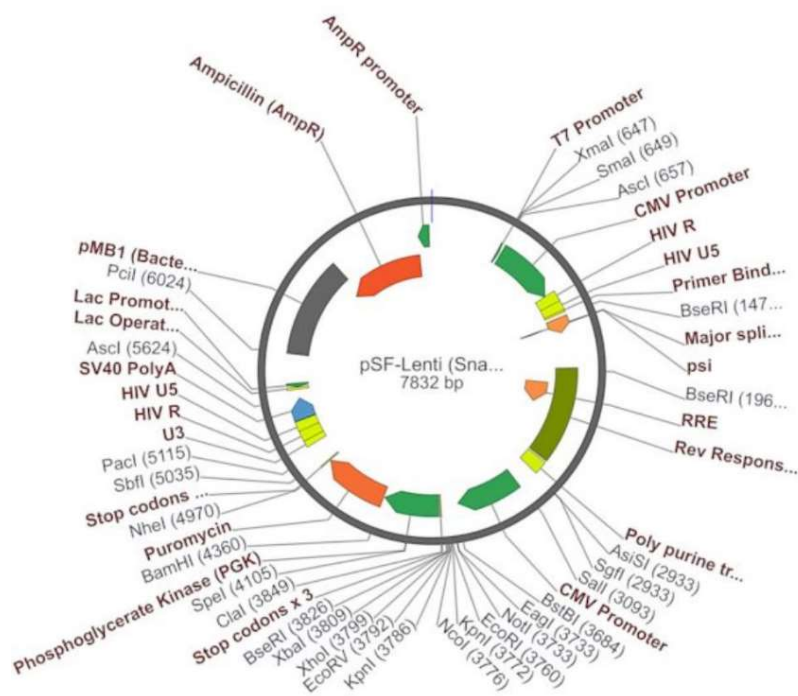
Supplementary Figure 13.3: pLAS0730 vector map

Beta chain expression vector. Each beta chain consists of a variable domain containing the CDR regions (T-cell receptor beta variable gene - TRBV) followed by a junction region (T-cell receptor beta junction gene – TRBJ) and finally the constant domain (T-cell receptor beta constant gene – TRBC). Variable domains may be one of many (as defined by IMGT) and the same variable chain can be used by different TCRs. The variable chains and thus individual TCRs are unique due to their CDR regions. There are two genes for the beta chain constant domain (TRBC1 and TRBC2) which differ in only three amino acids in the region of the beta chain that is used to form our TCRs.



Supplementary Figure 13.4: pLAS563 vector map

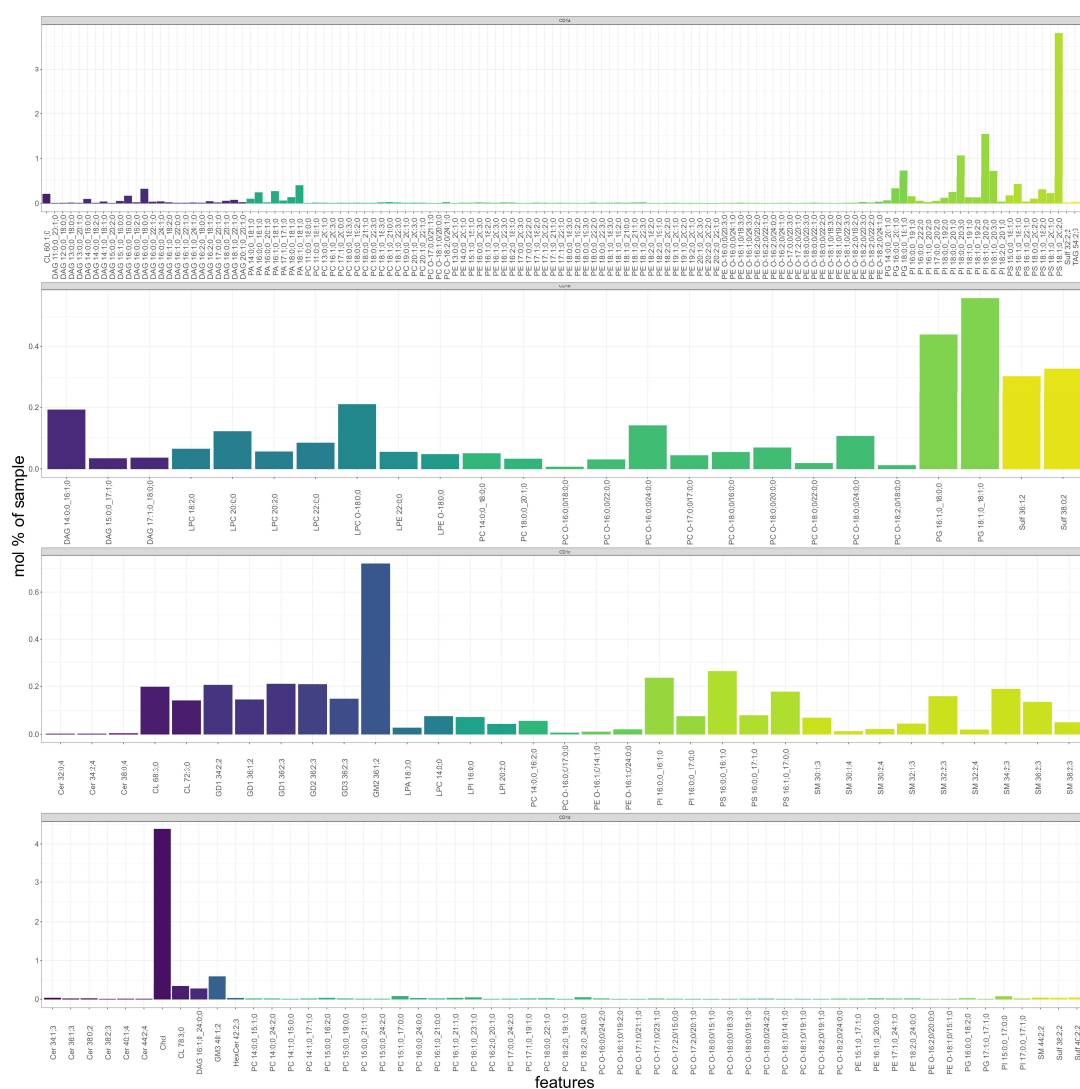
Beta chain expression vector. pLAS0563 contains the beta chain (TRBV19) fused to a humanized anti-CD3 single chain antibody fragment (UCHT1). The anti-CD3 antibody fragment binds to CD3 molecules on T-cells leading to a potent redirected T-cell response. pLAS0563 should be used to express beta chains to refold as ImmTACs.



Supplementary Figure 13.5: pSF-Lenti - Puromycin CMV Plasmid

The HIV1 based lentivirus expression vector, pSF-Lenti can be used for the stable integration of genes into both dividing and non-dividing cells. The CMV promoter drives the expression of a gene of interest followed by a multiple cloning site and the PGK promoter to drive the puromycin resistance.

13.2 Supplementary Figures and tables



Supplementary Figure 13.6: Mol% of unique lipids of the CD1 isotypes.

The CD1a unique features were enriched in PI and PS; the CD1b lipidome were enriched in PG and sulfatide; the CD1c-specific lipidome enriched in GM2 (36:1;2), GD1 and GD2 species; Cholesterol was enriched in the CD1d unique lipidome.

Primer #	Primer name	5'-->3' Sequence
422	S2-CD1-Rjp2c-A1	CTATAAACTGCACGTACACA(N3:03039103)(N2:03910303)S(N1:91030303)(N2)S(N3)(N3)S(N4:03030391)(N1)(N2)(N2)S(N4)(N2)SCTTTCTGGTATGTCCAATATC
423	S2-CD1-Rjp2c-A2	GTCTACAGCTCCTCCTGAAA(N3:03039103)(N2:03910303)S(N1:91030303)(N2)S(N1)(N1)(N3)(N3)(N2)S(N3)(N1)(N4:03030391)(N3)(N1)(N2)(N1)(N1)(N3)GGAAGCAACAAAGGTTTGAAG
424	S2-CD1-Rjp2c-A3	ACTCAGCGGTGTACTTCTGT(N3:03039103)(N2:03910303)S(N2)(N4:03030391)S(N4)(N2)S(N3)(N1:91030303)(N4)(N2)(N1)(N3)(N4)(N1)(N2)(N3)(N3)S(N4)(N3)(N3)(N3)S(N1)(N1)(N1)(N2)(N4)S(N2)(N1)(N3)TTTGGAGCAGGGACCCAGG
425	S2-CD1-Rjp2c-B1	CTTTCAGGTGTGATCCAATT(N4:03030391)(N2:03910303)S(N3:03039103)(N1:91030303)(N1)(N2)(N1)(N2)(N1)(N1)(N2)(N2)(N3)SCTTTATTGGTACCGACAGAC
426	S2-CD1-Rjp2c-B2	CCCAGAGTTTCTGACTTAC(N4:03030391)(N4)(N2:03910303)(N2)(N1:91030303)(N3:03039103)(N1)(N1)(N4)(N3)(N1)(N1)(N3)(N2)S(N2)(N1)(N1)CTAGAAAAATCAAGGCTGCTC
427	S2-CD1-Rjp2c-B3	ACTCGGCCATGTATCTCTGT(N3:03039103)(N2:03910303)S(N4:03030391)(N2)S(N4)(N2)S(N2)(N2)S(N2)(N3)S(N1:91030303)(N2)S(N3)(N3)S(N2)(N3)S(N3)(N3)S(N3)(N2)S(N3)(N1)(N1)(N3)(N2)S(N4)(N4)(N2)TTTGGACAAGGCACCAGACTC
428	S2-CD1-Rjp2c-A1-R	TGTGTACGTGCAGTTTATAG
429	S2-CD1-Rjp2c-A2-R	TTTCAGGAGGAGCTGTAGAC
430	S2-CD1-Rjp2c-A3-R	ACAGAAGTACACCGCTGAGT
431	S2-CD1-Rjp2c-B1-R	AATTGGATCACACCTGAAAG
432	S2-CD1-Rjp2c-B2-R	GTAAGTCAGAACTCTGGG
433	S2-CD1-Rjp2c-B3-R	ACAGAGATACATGGCCGAGT
	YOL13	tcacacaggaaacagctatg
	YOL22	cattttcagggatagcaagc

Supplementary Table 13.1 First generation affinity maturation library building primers

Lipid Class	MS mode	Structural detail level	Quantification
Chol	MS	species	quantified
GM1	MS	species	quantified
GM2	MS	species	Semi-quantified
GM3	MS	species	quantified
GM4	MS	species	Semi-quantified
GD1	MS	species	Semi-quantified
GD2	MS	species	Semi-quantified
GD3	MS	species	Semi-quantified
GT1	MS	species	Semi-quantified
GT2	MS	species	Semi-quantified
GT3	MS	species	Semi-quantified
GQ1	MS	species	Semi-quantified
Gb3	MS	species	quantified
Gb4	MS	species	Semi-quantified
Sulf	MS	species	quantified
DiHexCer	MS	species	quantified
Cer	MS	species	quantified
CL	MS	species	quantified
HexCer	MS	species	quantified
LPA	MS	species	quantified
LPC	MS	species	quantified
LPCO-	MS	species	quantified
LPE	MS	species	quantified
LPEO-	MS	species	quantified
LPG	MS	species	quantified
LPI	MS	species	quantified
LPS	MS	species	quantified
PA	MSMS	subspecies	quantified
PC	MSMS	subspecies	quantified
PCO-	MSMS	subspecies	quantified
PE	MSMS	subspecies	quantified
PEO-	MSMS	subspecies	quantified
PG	MSMS	subspecies	quantified
PI	MSMS	subspecies	quantified
PS	MSMS	subspecies	quantified
SM	MS	species	quantified
CE	MSMS	subspecies	quantified
DAG	MSMS	subspecies	quantified
TAG	MSMS	species	quantified

Supplementary Table 13.2 List of analysed lipid classes. MSMS mode (i.e. the fragmentation of the lipid molecules) delivers additional confidence of lipid identification and additional structural information.

Primer design for NNK mutagenesis of CD1c-S2-END-a2b1

GNVSTQMEGPVLTSEEAFLTINCTYATGYPSLFWVYQYPGEGQLQLLKKATKADDKGSNKGFEATYRKETTSFHLEKGSVQVSDSAVYFCA
LSGQYGWGKLQFGAGTQVVVTPDIQNPDPAVYQL

CD1c-S2-END-b1(**TRBV7-9**)(4D8-S2cB Pan4 O/N)

DTGVSQNPRHKITRGQNVTRCDPISEHNRLYWYRQTLGQGPEFLTYFQNEAQLEKSRLLSDRFS AERP KGSFSTLEIQRT EQGDSAMYLCASSPRMYRGAEAFFGQGTRLTVVEDLNKVFPP

ATGAAAAAATTATTATTGCAATTCTTTAGTTGTTCTTTTATTCTCACAGCGCGCAGGATACTGGAGTCTCCAGAACCCAGACA
CAAGATCACAAAGAGGGGACAGAATGTAATTTAGTGTGATCCAATT**TTCTGAACACAATCGG**CTTTATTGGTACCGACAGACCCT
GGGGCAGGGCCAGATTTCTGACTTAC**TTCCAGAATGAAGCGCAA**CTAGAAAAATCAAGGCTGCTCAGTGATCGGTTCTCTGCAG
AGAGGCCTAAGGGATCTTTCTCACCTTGGAGATCCAGCGCACAGAGCAGGGGGACTCGGCCATGTATCTCTGT**GCGTCGTGCCG**
CGCATGGTGCGCGGGGCGGAAGCGTTCTTTGGACAAGGCACCAGACTCACAGTTGTA

Library 1: aCDR1 NNK ATGYPS

A T G Y P S
 GAGGCCCTTCCTGACTATAAACTGCACGTACACAGCGACGGGGTACCCGTCGCTTTTCTGGTATGTCCAATATCCTGGAGAA
 G
A1-1 CTATAAACTGCACGTACACANNKNNKNNKNNKNNKTCGCTTTTCTGGTATGTCC
A1-2 TAAACTGCACGTACACAGCGNNKNNKNNKNNKNNKCTTTTCTGGTATGTCCAATATC
A1-1R TGTGTACGTGCAGTTTATAGTCAG
A1-2R CGCTGTGTACGTGCAGTTTATAGT

Library 2: aCDR2 NNK LKATKADDKGS

L K A T K A D D K G S

GTATGTCCAATATCCTGGAGAAGGTCTACAGTCTCTCCTGAAAGCCACCAAGGCGGACGACAAGGGAAGCAACAAAGGTTTTGAAG
CCAC

A2-1 GAGAAGGTCTACAGTCTCTCANNKNNKNNKNNKNNKGGGACGACAAGGGAAGCAAC

A2-2 GTCTACAGTCTCTCCTGAAANNKNNKNNKNNKNNKNGACAAGGGAAGCAACAAAGG

A2-3 AGTCTCTCCTGAAAGCCACCCNNKNNKNNKNNKNNKGGGAAGCAACAAAGGTTTTGAAG

A2-4 TCCTGAAAGCCACCAAGGCGNNKNNKNNKNNKNNKAAACAAAGGTTTTGAAGCCAC

A2-1R GAGGAGCTGTAGACCTTCTCCAGG

A2-2R TTTCAGGAGGAGCTGTAGACCTTC

A2-3R GGTGGCTTTCAGGAGGAGCTGTAG

A2-4R CGCCTTGGTGGCTTTCAGGAGGAG

Library 3: aCDR3 NNK ALSGQYGWGKLQ

A L S G Q Y G W G K L

Q

GGCTCAGTTCAAGTGTCTCAGACTCAGCGGTGACTTCTGTGCGCTCTCGGGTCAGTACGGCTGGGGCAAAC**TGCAG**TTTGGA
GCAG

A3-1 ACTCAGCGGTGACTTCTGTNNKNNNNNNNNNNNN**TACGGCTGGGGCAAAC**TGCAG****

A3-2 CGGTGACTTCTGTGCGCTC**NNNNNNNNNNNNNN****TGGGGCAAAC**TGCAG**TTTG**

A3-3

ACTTCTGTGCGCTCTCGGGTNNKNNNNNNNNNN**AACTGCAG**TTTGAGCAGG

A3-4

GTGCGCTCTCGGGTCAGTACNNKNNNNNNNNNN**CAG**TTTGAGCAGGGACCCAG

A3-5

CGCTCTCGGGTCAGTACGGCNNKNNNNNNNNNNNNTTTGAGCAGGGACCCAGGTTG

A3-1R ACAGAAGTACACCGCTGAGTCTGA

A3-2R GAGCGCACAGAAGTACACCGCTGA

A3-3R ACCCGAGAGCGCACAGAAGTACAC

A3-4R GTACTGACCCGAGAGCGCACAGAA

A3-5R GCCGTACTGACCCGAGAGCGCACA

Library 4: bCDR1 NNK SEHNR

S E H N R

AAAGAGGGGACAGAATGTAACCTTCAGGTGTGATCCAATT**TCTGAACACAATCGG**CTTTATTGGTACCGACAGACCCCTGGG
GCAG

B1-1 CTTTCAGGTGTGATCCAATTNNKNNNNNNNNNNNNCTTTATTGGTACCGACAGAC

B1-1R AATTGGATCACACCTGAAAGTTAC

Library 5: bCDR2 NNK TYFQNEAQLE

T Y F Q N E A Q L E

GGTACCGACAGACCCCTGGGGCAGGGCCCAGAGTTTCTG**ACTTAC**TTC**AGAA**TGAAGCGCAA**CTAGAAAATCAAGGCTGC**
TCAG

B2-1 GGCAGGGCCCAGAGTTTCTGNNKNNNNNNNNNNNN**GAAGCGCAA**CTAGAAAATC****

B2-2 GCCCAGAGTTTCTG**ACTTAC**NNNNNNNNNNNNNN**CAA**CTAGAAAATCAAGGCTG****

B2-3

AGTTTCTG**ACTTAC**TTC**AGNNKNNNNNNNNNNNN****GAAAATCAAGGCTGCTCAG**

B2-4

TTCTG**ACTTAC**TTC**AGAA**NNNNNNNNNNNNNN**AAATCAAGGCTGCTCAG**

B2-1R CAGAAACTCTGGGCCCTGCCCCAG

B2-2R GTAAGTCAGAAACTCTGGGCCCTG

B2-3R CTGGAAGTAAGTCAGAAACTCTGG

B2-4R ATTCTGGAAGTAAGTCAGAAACTC

Library 6: bCDR3 NNK ASSPRMVRGAEAF

A S S P R M V R G A E A F

AGCAGGGGGACTCGGCCATGTATCTCTGT**GCGTCGTCGCGCGCATGGTGCGGGGGCGGAAGCGTTCT**TTTGACAAGGCA
CCAGACTCACAGTTGTA

B3-1 ACTCGGCCATGTATCTCTGTNNKNNNNNNNNNNNN**ATGGTGCGGGGGCGGAAGC**

B3-2 CCATGTATCTCTGT**GCGTCGNNNNNNNNNNNNNN****CGCGGGGCGGAAGCGTTCTTTG**

B3-3 ATCTCTGTGCGTCGTCGCCGNNKNNKNNKNNKNNKGCAGGAAGCGTTCTTTGGAC
 B3-4 GTGCGTCGTCGCCGCGCATGNNKNNKNNKNNKNNKGCCTTTTGGACAAGGCAC
 B3-5 CGTCGCCGCGCATGGTGGCNNKNNKNNKNNKNNKTTTGGACAAGGCACCAG
 B3-1R ACAGAGATACATGGCCGAGTCCCC
 B3-2R CGACGCACAGAGATACATGGCCGA
 B3-3R CGGCGACGACGACAGAGATACAT
 B3-4R CATGCGCGGCGACGACGACAGAG
 B3-5R GCGCACCATGCGCGGCGACGACG

Library 7 (Exp1): Targeting helix 1, allowing for co-variance

Alpha
 GNSVTQMEGPVTLSEEAFLTINCTYTATGYPSLFWYVQYPGEGQLQLLKATKADDKGSNKGFEATYRKETTFSFHLEKGSVQ
 VSDSAVYFCALSDQYWGKLGQFGAGTQVVVTPDIQNPDPAVYQL
 A T G Y P S
 GAGGCCTTCCTGACTATAAACTGCACGTACACAGCGACGGGTACCGTCGCTTTTCTGGTATGTCCAATATCCTGGAGAA
 G
 Exp1-1 CTATAAACTGCACGTACACANNKNNKNNKNNKCCGTCTTTTCTGGTATG
 Exp1-1R TGTGTACGTGCAGTTTATAGTCAG
 A L S G Q Y G W G K L Q
 GGCTCAGTTCAAGTGTGAGACTCAGCGGTGTACTTCTGTGCGCTCTCGGGTCAGTACGGCTGGGGCAAACCTGCAGTTTGA
 GCAG
 Exp1-2 ACTTCTGTGCGCTCTCGGGTNNKNNKGGCTGGGGCAAACCTGCAGTTTG
 Exp1-2R ACCCGAGAGCGCACAGAAAGTACAC

Library 8 (Exp2): targeting direct contacts to helix 1, not be covered in standard NNK library

beta
 DTGVSQNPRHKITKRQNVTFRCDPISSEHNRLYWYRQTLGQGPEFLTQFQNEQLEKSRLLSDRFSAERPFGSFSTLEIQ
 TEQGD SAMYLCASSPRTGRGAEEFFGQGRLLTVVEDLNKVFP
 T Y F Q N E A Q L E
 GGTACCGACAGACCCTGGGGCAGGGCCCAGAGTTTCTGACTTACCTCCAGAATGAAGCGCACTAGAAAAATCAAGGCTGC
 TCAG
 Exp2-1 AGGGCCCAGAGTTTCTGACTNNKTTCTNNKNNKGAANNKCAANNKNNKAAATCAAGGCTGCTCAG
 Exp2-1R AGTCAGAACTCTGGGCCCTGCC

Library 9 (Exp3) - targeting helix 2, potentially stabilising cdr3b allowing for co-variance

alpha
 GNSVTQMEGPVTLSEEAFLTINCTYTATGYPSLFWYVQYPGEGQLQLLKATKADDKGSNKGFEATYRKETTFSFHLEKGSVQ
 VSDSAVYFCALSDQYWGKLGQFGAGTQVVVTPDIQNPDPAVYQL
 beta
 DTGVSQNPRHKITKRQNVTFRCDPISSEHNRLYWYRQTLGQGPEFLTQFQNEQLEKSRLLSDRFSAERPFGSFSTLEIQ
 TEQGD SAMYLCASSPRTGRGAEEFFGQGRLLTVVEDLNKVFP
 L K A T K A D D K G S

GTATGTCCAATATCCTGGAGAAGGTCTACAGCTCCTCCTGAAAGCCACCAAGGCGGACGACAAGGGAAGCAACAAAGGTTT
 TGAA
 Exp3-1 AAGGTCTACAGCTCCTCCTGNNKGCCNNKNNKCGGGACGACAAGGGAAGCAAC
 Exp3-1R CAGGAGGAGCTGTAGACCTTCTCC

A S S P R M V R G A E A F
 AGCAGGGGACTCGGCCATGTATCTCTGTGCGTCGTCGCCGCGCATGGTGCGCGGGGCGGAAGCGTTCTTTGGACAAGGCA
 CCAG
 Exp3-2 GTGCGTCGTCGCCGCGCATGNNKNNKNNKCGGGAAGCGTTCTTTGGAC
 Exp3-2R CATGCGCGGCGACGACGCACAGAG

Library 10 (Exp4): Loop extension;

1 additional:

L	K	X	X	X	X	X	D	D	K	G	S
L	K	A	X	X	X	X	X	D	K	G	S
L	K	A	T	K	X	X	X	X	X	G	S
L	K	A	T	K	A	D	D	K	G	S	

GTATGTCCAATATCCTGGAGAAGGTCTACAGCTCCTCCTGAAAGCCACCAAGGCGGACGACAAGGGAAGCAACAAAGGTTT
 TGAA

Exp4-1 L K X X X X X D D K G S
 GTCTACAGCTCCTCCTGAAANNKNNKNNKNNKNNKGACGACAAGGGAAGCAACAAAG

Exp4-1R TTTCAGGAGGAGCTGTAGACCTTC

L	K	A	X	X	X	X	X	D	K	G	S
---	---	---	---	---	---	---	---	---	---	---	---

Exp4-2 TACAGCTCCTCCTGAAAGCCNNKNNKNNKNNKNNKGACAAGGGAAGCAACAAAG
 Exp4-2R GGCTTTTCAGGAGGAGCTGTAGACC

L	K	A	T	K	X	X	X	X	X	G	S
---	---	---	---	---	---	---	---	---	---	---	---

Exp4-3 TCCTCCTGAAAGCCACCAAGNNKNNKNNKNNKNNKGGAAGCAACAAAGGTTT
 Exp4-3R CTTGGTGGCTTTTCAGGAGGAGCTG

Library 11 (Exp5): Loop extension;

2 additional:

L	K	X	X	X	X	X	A	D	D	K	G	S
L	K	A	T	X	X	X	X	X	D	K	G	S
L	K	A	T	K	A	X	X	X	X	X	G	S
L	K	A	T	K	A	D	D	K	G	S		

GTATGTCCAATATCCTGGAGAAGGTCTACAGCTCCTCCTGAAAGCCACCAAGGCGGACGACAAGGGAAGCAACAAAGGTTT
 TGAA

Exp5-1 L K X X X X X A D D K G S
 GTCTACAGCTCCTCCTGAAANNKNNKNNKNNKNNKCGGACGACAAGGGAAGCAAC

Exp5-1R TTTCAGGAGGAGCTGTAGACCTTC

L	K	A	T	X	X	X	X	X	D	K	G	S
---	---	---	---	---	---	---	---	---	---	---	---	---

Exp5-2 AGCTCCTCCTGAAAGCCACCNNKNNKNNKNNKNNKGACAAGGGAAGCAACAAAGG

Exp5-2R GGTGGCTTTTCAGGAGGAGCTGTAG

L	K	A	T	K	A	X	X	X	X	X	G	S
---	---	---	---	---	---	---	---	---	---	---	---	---

Exp5-3 TCCTGAAAGCCACCAAGGCGNNKNNKNNKNNKNNKGGAAGCAACAAAGGT
 Exp5-3R CGCCTTGGTGGCTTTTCAGGAGGAG

Library 12 (Exp6): Loop extension;

3 additional:

```

L K X X X X X K A D D K G S
L K A T X X X X X D D K G S
L K A T K A X X X X X K G S
L K A T K A D X X X X X G S
L K A T K A D D K G S
ATGTCCAATATCCTGGAGAAGGTCTACAGCTCCTCTGAAAGGCCACCAAGGCGGACGACAAGGGAAGCAACAAAGGTTTTGAAGTTTG
L K X X X X X K A D D K G S
Exp6-1 TCTACAGCTCCTCTGAAANNKNNKNNKNNKNNKAAGGCGGACGACAAGGGAAG
Exp6-1R TTTCAGGAGGAGCTGTAGACCTTC
L K A T X X X X X D D K G S
Exp6-2 AGCTCCTCTGAAAGGCCACCNKNNKNNKNNKNNKGACGACAAGGGAAGCAACAAG
Exp6-2R GGTGGCTTTCAGGAGGAGCTGTAG
L K A T K A X X X X X K G S
Exp6-3 TCCTGAAAGGCCACCAAGGCGNNKNNKNNKNNKNNKAAGGGAAGCAACAAAGTTTG
Exp6-3R CGCCTTGGTGGCTTTCAGGAGGAG
L K A T K A D X X X X X G S
Exp6-4 TGAAAGGCCACCAAGGCGGACNNKNNKNNKNNKNNKGGAAGCAACAAAGTTTG
Exp6-4R GTCCGCCTTGGTGGCTTTCAGGAG

```

Cdr2a loop extension

```

LLKXXXXXDDK 1 additional residue
LLKXXXXXDDK 2 additional residues
LLKXXXXXADDK 3 additional residues

```

```

L K A T K A D D K G S
GTATGTCCAATATCCTGGAGAAGGTCTACAGCTCCTCTGAAAGGCCACCAAGGCGGACGACAAGGGAAGCAACAAAGGTTTTGAAGCCACA
TACCGTAAAGAAACCACT
Exp2-1 GTCTACAGCTCCTCTGAAANNKNNKNNKNNKNNKGACGACAAGGGAAGCAACAAAG
Exp2-2 GTCTACAGCTCCTCTGAAANNKNNKNNKNNKNNKNNKGACGACAAGGGAAGCAACAAAG
Exp2-3 GTCTACAGCTCCTCTGAAANNKNNKNNKNNKNNKNNKGCGGACGACAAGGGAAGCAAC
Exp2-123R TTTCAGGAGGAGCTGTAGACCTTC

```

Lipid standard mix

phosphatidate 17:0/17:0 (PA)
phosphatidylcholine 17:0/17:0 (PC)
phosphatidylethanolamine 17:0/17:0 (PE)
phosphatidylglycerol 17:0/17:0 (PG)
phosphatidylinositol 16:0/16:0 (PI)
phosphatidylserine 17:0/17:0 (PS)
lyso-phosphatidate 17:0 (LPA)
lyso-phosphatidylcholine 12:0 (LPC)
lyso-phosphatidylethanolamine 17:1 (LPE)
lyso-phosphatidylglycerol 17:1 (LPG)
lyso-phosphatidylinositol 17:1 (LPI)
lyso-phosphatidylserine 17:1 (LPS)
cholesterol ester 16:0 D7 (CE)
sphingomyelin 18:1;2/12:0;0 (SM)
sulfatide d18:1;2/12:0;0 (Sulf)
cardiolipin 14:0/14:0/14:0/14:0 (CL)
ceramide 18:1;2/17:0 (Cer)
diacylglycerol 17:0/17:0 (DAG)
hexosylceramide 18:1;2/12:0 (HexCer)
dihexosylceramide 18:1;2/12:0 (DiHexCer)
Globoside 3 18:1;2/17:0 (Gb3)
GM3-D3 18:1;2/18:0 (GM3)
GM1-D3 18:1;2/18:0 (GM1)
triacylglycerol 17:0/17:0/17:0 (TAG)
cholesterol (Chol)

Supplementary Table 13.4: Lipid Standard mix for shotgun mass spectrometry.