

SHORT THESIS 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



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by Rita Szoke-Kovacs, MSc

Supervisor: Dr. Péter Gogolák, PhD Dr. Mariolina Salio, PhD

Doctoral School of Molecular Cellular and Immune Biology, University of Debrecen

Head of the **Defense Committee**: Prof. Dr Gábor Szabó, PhD, DSc

Reviewers: Dr Árpád Szőőr, PhD
Dr Barbara Molnár-Érsek, PhD

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1. INTRODUCTION

1.1 T cell mediated immune responses

The immune system consists of the innate and adaptive systems. The innate system provides rapid, non-specific responses through physical barriers, immune cells, and inflammatory mediators. The adaptive system offers antigen specific responses via T and B lymphocytes, resulting in immunological memory. T lymphocytes play a crucial role in health and disease by recognizing peptide-major histocompatibility complex (pMHC) molecules through T cell receptors (TCRs) on infected and malignant cells. The major histocompatibility complex (MHC) molecules are highly polymorphic due to the large number of alleles present in the population, and present various peptide antigens. The immense diversity of TCRs arises from the random assembly of gene segments encoding the variable domains of their constituent chains, followed by combinational pairing of the resulting chains during thymic development. Thymocytes undergo positive and negative selection in the thymus, differentiating into CD4⁺ or CD8⁺ cells. In general, CD4⁺ T cells help regulate immune responses, while CD8⁺ T cells eliminate infected or cancerous cells. Naive T cells circulate between the bloodstream and lymphoid organs, getting activated by pMHC complexes, leading to clonal expansion and differentiation into effector and memory T cells.

1.2 Unconventional MHC molecules recognized by unconventional T cell populations

Ever since the MHC system was discovered in the 1940s, the MHC molecules have been recognized as the main regulators of T cell activity. Over time, the field has expanded with the identification of unconventional $\alpha\beta$ and $\gamma\delta$ T lymphocytes, which can recognize non-polymorphic antigen-presenting molecules. Genes encoding the CD1 family and MR1 antigen-presenting molecules are located outside the MHC locus, while HLA-E, HFE, or the mouse H2-M3, and H2-Q9 are found within it. These non-conventional antigen-presenting molecules typically capture metabolites or lipids, rather than conventional peptide antigens (except for HLA-E which presents a limited range of peptides) and are recognized by unconventional T cells. These unconventional T cells are quick to respond and are widely distributed in the peripheral tissues and circulation as memory cells. They are not donor restricted due to their recognition of non-polymorphic MHCs. Unlike conventional T cells, antigen-specific unconventional T cells are primarily found in non-lymphoid organs and have a smaller diversity of TCRs. Research in T cell biology and immunotherapy mainly focuses on peptide antigens presented by MHC class

I and II molecules. Utilizing these monomorphic antigen-presenting molecules offers a promising universal immunotherapy strategy.

1.3 Cluster of differentiation 1 (CD1) molecules

The Cluster of Differentiation 1 (CD1) family is made up of a group of antigen-presenting molecules that share structural similarities with MHC class I proteins. However, unlike MHC molecules that present peptides, CD1 proteins bind and present lipid-based molecules to T cells. These molecules first appeared in cartilaginous fish and have evolved alongside MHC proteins, B cell receptors, and T cell receptors. In mammals, the CD1 family includes five isoforms—CD1a, CD1b, CD1c, CD1d, and CD1e—all of which are encoded on chromosome 1. Structurally, CD1 molecules consist of a heavy chain and a non-covalently linked to β 2-microglobulin (β 2m). The heavy chain has three extracellular domains (α 1, α 2, and α 3), of which the α 1 and α 2 domains form the lipid-binding pocket. The CD1 Group 1 isoforms (CD1a, CD1b, and CD1c) activate adaptive T cells, while CD1d in group 2 activates subsets of T cells that exhibit innate-like functions. CD1e is not present on the cell surface, instead it has a role in lipid loading to CD1b. Unlike MHC class Ia molecules, which are found in nearly all nucleated cells, CD1 molecules mainly present on antigen-presenting cells (APCs) and double-positive (CD4+CD8+) thymocytes. CD1a, CD1b, and CD1c expression can be induced by stimuli like GM-CSF or IL -1 β . High levels of CD1a can be found on skin resident dermal dendritic cells and Langerhans cells. CD1b is primarily expressed on dendritic cells migrating to the lymph nodes and on myeloid-derived dendritic cells. CD1c is expressed on monocyte-derived dendritic cells (moDCs), Langerhans cells, and B cells under normal conditions, exhibiting the broadest expression pattern of the CD1 family. CD1d is widely expressed in many tissue and cell types, including monocytes, dendritic cells (DCs), B cells, thymocytes, innate lymphoid cells (ILCs), intestinal epithelial cells (IECs) hepatocytes and keratinocytes. Apart from a few studies that reported minor polymorphisms in the promoter region of CD1a, no other CD1 polymorphism have been described. Since there are no genetic variations in humans - apart from CD1a that has two different alleles that differ by two amino acids – these molecules are non-polymorphic. T cell responses towards CD1 molecules, therefore, are not restricted to the donor's genetic background. Moreover, targeting lipid antigens provides a lower risk of tumour escape, due to the low mutation rate of lipids compared with peptide-HLA.

1.3.1 CD1 recycling and antigen presentation

The CD1 isoforms are produced in the endoplasmic reticulum (ER) and move through the Golgi apparatus before being transferred to the cell membrane. Once they reach the surface, they go through a process of internalization and recycling across various endosomal compartments. CD1a molecules can capture external lipids at the cell surface, while CD1b, CD1c, and CD1d are less efficient at lipid loading on the surface and recycle into endosomes for lipid exchange. CD1 molecules bind to Adaptor Protein (AP) complexes on the plasma membrane facing the cytoplasm. The APs recognize the isoform-specific motif on the cytoplasmic tail, allowing the CD1 isoforms to be sorted and directed to different sub-compartments within the endosomal network. The cytosolic tail of CD1a lacks the recycling motif, so these molecules mainly remain at the surface and undergo ARF-6 dependent superficial recycling only in early endosomes. CD1b travels deeply into late endosomes and lysosomes using both AP-2 and AP-3. CD1c recycles among early and late endosomal compartments using AP-2. CD1d moves to late endosomes and lysosomes using AP-2 and possibly AP-3.

1.3.2 CD1 structure drives antigen selectivity

The antigen-binding grooves of CD1 isoforms show unique structural characteristics. Differences in pocket depth, volume, and channel design enable each isoform to specialize in binding different types of lipids, which reflects their specific cellular trafficking patterns and endosomal sampling methods. CD1a features the smallest groove (1,350 Å), which includes two main chambers known as the A' and F' pockets. The A' pocket is shaped by electrostatic interactions among its roof residues, while Valine28 at its base limits the length of the alkyl chains it can accommodate, making it more suited for lipids with shorter or unbranched hydrocarbon chains. CD1a does not have an endosomal targeting motif, which underlines its role in capturing antigens at the cell surface. CD1b has the largest binding groove (2,200 Å) that made of four interconnected pockets (A', F', C', and T'). This structure allows the accommodation of large lipids (up to C80–C86 glucose mycolates) in its ATF super channel, where alkyl chains weave through the C' portal. Crystal structures revealed, that CD1b can bind two lipids in the binding grove, with smaller molecules acting as scaffolds and aid the presentation of larger lipids. Its deep, acidic endosomal localization also aids in loading long-chain lipids, which need a low pH for optimal binding. CD1c is in an intermediate position, with a groove volume of 1,780 Å and a continuous A'/F' pocket system connected by a unique D accessory portal. This structure allows it to present branched mycoketides, like mannosyl-phosphomycoketide, where the alkyl chains spread outward within the A'

pocket. CD1c can also of accommodate lipopeptides. Structural studies indicate its ability for dual-lipid presentation, with mycoketide backbones anchored in the A' pocket and shorter hydrocarbons (C12) in the F' pocket. CD1d (1,400 Å) contains only A' and F' pockets. It was crystallized first in complex with α -galactosylceramide. The positioning of the alkyl chain is driven by conserved polar contacts between specific amino acids of the heavy chain (Asp80, Asp153, Thr156) and the polar residues of the 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.

1.3.3 The CD1c-associated lipid-antigen repertoire

Only a few studies investigated the range of lipids presented by the CD1 molecules, which is important for identifying cancer-specific lipids. Currently, the only CD1c-restricted lipid antigen, associated with cancer is methyl-lysophosphatidic acid (mLPA), which has been found in leukemic cells but is absent in healthy monocytes and B cells. While CD1c-mLPA shows promise as a therapeutic target for leukemia, we still have many unanswered questions about expression variability across patients and within tumors. This highlights the need for a more comprehensive profiling of the CD1c-bound lipidome, which could help us distinguish between tumor cells and healthy cells, potentially uncovering new lipid targets. Progress in this area has been hampered by inconsistent results of the lipid repertoire of CD1c. Earlier studies pointed to limited self-lipids, like glycosphingolipids, sulfatides, and gangliosides (GM1, GD1a, GD1b, GT1b, GQ1b) that are presented by CD1a, CD1b, and CD1c to T cells. However, more recent research by Huang et al.(1) has revealed a much broader range of self-lipids, including phospho- and glycolipids, that are captured by soluble CD1 molecules. These differences highlight the challenges we face with current methods for isolating CD1 complexes and analyzing their lipid content. To move forward and make clinically relevant discoveries, we need to develop standardized, high-resolution techniques to better characterize CD1-associated antigens.

1.3.4 TCR recognition of CD1-lipid complexes

T cells interact with CD1-lipid complexes through their TCRs in two distinct ways. The first method is known as the Head Group Discrimination Model, where TCRs recognize both the CD1 molecule and the polar head group of the lipid being presented, similar to

how $\alpha\beta$ TCRs identify peptide-MHC complexes. The second approach, called the Absence of Interference Model, involves TCRs binding to CD1 without directly engaging with the lipid antigen's head group.

It has been shown that CD1a-specific T cells often use this second strategy, with their TCRs contacting solely CD1a itself, binding over the A' roof domain, while not interacting with the lipid at all. Certain lipids, like sulfatide and sphingomyelin, can disrupt these interactions by causing conformational changes in the A' pocket, effectively preventing TCR engagement. In human blood, there's a notable population (about 0.02-0.4% of memory T cells) of autoreactive $\alpha\beta$ T cells that recognize CD1a. These cells play a role in maintaining skin homeostasis and immune balance by producing IL-22, which encourages keratinocytes to release antimicrobial peptides.

CD1b has an important role in presenting mycobacterial lipid antigens. CD1b tetramer-based studies have identified specific T cell populations that recognize these microbial lipids in a CD1b-restricted way. While autoreactive CD1b-specific T cells exist, they are less common than their CD1a-reactive counterparts. Similarly, CD1c plays a role in stimulating autoreactive T cell responses, with notable populations found in both adult and cord blood. Roy and his team(2) identified several CD1c-reactive T cell clones that can recognize a variety of mycobacterial glycolipids. Interestingly, some of these CD1c-reactive T cell receptors (TCRs) show polyspecificity, meaning they can bind to multiple self-lipid antigens presented by CD1c by interacting with the surface of the CD1c protein rather than the lipid components themselves. This was clearly illustrated by Wun and colleagues in 2018(3), who demonstrated that the 3C8 $\alpha\beta$ TCR binds directly to CD1c without engaging the lipid headgroups, marking a significant shift from traditional models of antigen recognition.

The CD1d system is particularly well understood, with invariant NKT (iNKT) cells being the classic example of a CD1d-restricted population. New findings are uncovering additional T cell subsets, such as $\gamma\delta$ T cells, that also respond to CD1d-lipid complexes. For example, there are considerable numbers of circulating $\gamma\delta$ T cells that recognize CD1d-sulfatide complexes, which further broadens the range of immune responses restricted by CD1.

1.4 CD1 restricted T cells in disease

Recent studies have shed light on the important role of CD1-restricted autoreactive T cells in various diseases. These T cells are able to recognize self-lipids presented by CD1 molecules. Research by De Lalla(4) and his team uncovered a surprisingly high prevalence of circulating T cells with CD1-autoreactivity, ranging from 1 in 10 to 1 in 300, indicating

that these cells are a significant part of the human immune system. CD1-autoreactive T cells have been linked to several autoimmune diseases. In thyroid conditions like Graves' disease and Hashimoto's thyroiditis, these cells infiltrate affected tissues alongside CD1-expressing B cells and dendritic cells, causing inflammation and tissue damage. CD1a- and CD1c-restricted T cells show cytotoxic effects against CD1a and CD1c positive thyroid cells. Beyond endocrine autoimmunity, CD1a-reactive T cells are involved in skin inflammation seen in psoriasis and allergic skin disorders, while CD1c-restricted responses have been associated with the development of systemic lupus erythematosus (SLE) by aiding autoreactive B cells in producing harmful IgG antibodies. The CD1 system is also vital for fighting infections and combating tumors. CD1c-mediated responses help protect against *Mycobacterium tuberculosis*, and CD1-restricted T cells play a role in cancer immunosurveillance. Lepore and his colleagues(5) discovered leukemia-reactive T cell clones that can recognize CD1c-presented methyl-lysophosphatidic acid (mLPA), a tumor-associated self-lipid that is abundant in leukemic cells but not found in healthy monocytes and B cells. These discoveries have sparked the development of adoptive T cell therapies aimed at targeting CD1c-mLPA complexes for treating acute myeloid leukemia. There's a growing body of evidence linking changes in lipid metabolism to the progression of autoimmune diseases. Conditions such as systemic lupus erythematosus (SLE), psoriasis, diabetes, and rheumatoid arthritis often linked with dyslipidemia. In these cases, CD1-restricted T cells can either worsen or help reduce inflammation when they encounter altered self-lipids. For instance, in psoriasis, abnormal lipid levels might trigger harmful responses from CD1-restricted T cells, while in rheumatoid arthritis, these same cells seem to play a role in joint inflammation. This points to the possibility that therapies aimed at lipid metabolism or that adjust the activity of CD1-restricted T cells could pave the way for new treatments for autoimmune disorders linked to lipids. However, despite these promising developments, there are still significant gaps in our understanding of how self-lipid specific T cells affect disease progression, especially in lipid metabolism disorders. More research is essential to determine whether these cells mainly promote inflammation or if they also have a regulatory role. The ability of some CD1c-restricted T cells to recognize both self and microbial lipids raises fascinating questions about their function in maintaining immune balance versus triggering autoimmune responses. As we deepen our understanding of these intricate interactions, we also open the door to developing targeted immunotherapies that can modulate CD1-restricted T cell responses across a variety of diseases.

1.5 Harnessing Soluble T Cell Receptors for Therapeutic Use

TCRs are the body's natural receptors for peptide-HLA (pHLA) complexes and CD1 molecules. It consists of transmembrane α and β chains (or γ and δ chains), each featuring constant (C) and variable (V) domains. Structural studies have revealed that the variable domains of the TCR α and β chains directly interact with the α -helices of pHLA complexes, as well as the peptide antigen. The specificity of the TCR is determined by three complementary determining regions (CDRs)—CDR1, CDR2, and CDR3. While CDR1 and CDR2 are encoded by germline V gene segments, the hyper-variability of CDR3 comes from nucleotide additions or deletions at the V(D)J junction. Generally, the CDR3 loop makes contact with the peptide surface, while CDR1 and CDR2 engage with the HLA helices. TCRs have great potential for therapeutic applications, whether as cellular therapies or as soluble bispecific engagers. However, turning natural TCRs into treatments comes with challenges, such as poor stability in soluble forms, low affinity for pHLA complexes, and problems like low expression yields, misfolding, and aggregation. Immunocore Ltd. employed innovative engineering strategies, including the introduction of an additional intermolecular disulfide bond between TCR constant domains to increase stability and phage display-based affinity maturation to enhance binding affinity, creating soluble TCR-based therapies that specifically target pHLA complexes. A prime example of this is Immunocore's ImmTAC (Immune-mobilizing monoclonal TCRs Against Cancer) platform. ImmTAC molecules are designed with a targeting arm, a monovalent, affinity-enhanced TCR that has a picomolar-range binding affinity, paired with an effector domain, a monovalent anti-CD3 single-chain Fc antibody fragment derived from the UCHT1 monoclonal antibody. The targeting arm binds to the pHLA complexes found on tumor cells, effectively marking them for an immune response. The effector domain recruits and activate T cells, regardless of their TCR specificity, allowing for targeted killing of cancer cells, even when the expression of target pHLA is low. This innovative strategy culminated in the first FDA-approved ImmTAC in 2022 for treating uveal melanoma, presenting the promising future of engineered TCR therapies.

1.6 Targeting HLA molecules limits patient reach

HLA molecules are valuable therapeutic targets because they present intracellular peptides on the cell surface, offering insights into cellular health and disease states such as infections and cancer. TCRs can recognize pHLA complexes and trigger strong immune responses against abnormal cells. This has led to the development of TCR-based therapies that redirect T cells to eliminate diseased tissues. However, the treatments is limited to patients expressing HLA-A*02:01, the most common HLA allele in human

population. CD1c offers a promising alternative due to its monomorphic nature and restricted expression, primarily on thymocytes, B cells, and monocyte-derived antigen-presenting cells, makes it well-suited for targeting blood cancers with reduced toxicity towards healthy tissues. Recent studies have shown that CD1c-restricted T cells can recognize and attack leukemic cells. Since CD1c is not found on hematopoietic stem cells, therapies that target CD1c-positive cancer cells would allow healthy precursor cells in the bone marrow to replenish the depleted blood cell populations. These characteristics make CD1c an ideal candidate for next-generation TCR-based bispecific T cell engagers, offering a path to overcome HLA restrictions while minimizing off-target effects in precision immunotherapy.

2. AIMS

The primary aim of this PhD is to develop CD1c-targeting bispecific engagers for hematological malignancies. This involves characterizing the CD1 lipidome and understanding the molecular recognition of CD1c by T cell receptors.

1. Characterize the Lipid Antigen Repertoire of CD1 Complexes

Objective: Identify and quantify lipids associated with CD1 isoforms in cancer cells.

Approach: Produce soluble CD1 molecules in Expi293F cells, generate CD1c-expressing cancer cell lines, and perform mass spectrometry analysis to study the CD1-associated lipidome.

2. Understand Molecular Recognition of CD1c

Objective: Investigate how TCRs recognize CD1c-lipid complexes.

Approach: Generate soluble CD1c molecules with specific lipids, isolate CD1c-lipid specific TCRs, and study the biophysical properties of TCR-CD1c interactions through crystal structures.

3. Develop High-Affinity CD1c-Lipid Specific TCRs

Objective: Enhance the affinity of CD1c-lipid specific TCRs for therapeutic purposes.

Approach: Use random and structurally guided phage panning libraries to mutate and enhance the affinity of CD1c-specific TCRs and assess their lipid-specificity by mass spectrometry and crystallography.

The ultimate goal is to translate this knowledge into the development of bispecific TCR-based reagents that can distinguish between healthy and cancer cells by targeting cancer-specific CD1c-lipid antigens. 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. MATERIALS AND METHODS

3.1 Molecular cloning and expression of soluble CD1 molecules

Human CD1a, b, c, and d $\alpha 1$ – $\alpha 3$ domains were N-terminally fused to $\beta 2$ -microglobulin via a glycine-serine linker. The single-chain construct included a C-terminal AviTag™ and 6xHis tag, synthesized by GeneArt (Invitrogen) and cloned into pCDNA3.1, which co-expresses BirA for biotinylation. Expi293F cells were transfected with 1 μ g/ml plasmid, and CD1c molecules with endogenous lipids, “CD1c-endo” monomers were expressed over five days. Proteins were purified using affinity and size exclusion chromatography. For lipid-loading studies, CD1c and CD1d were expressed in GnTI-deficient Expi293FGnTI- cells, producing glycan-deficient CD1 molecules. The crystallization construct had a C-terminal His tag removed post-purification via TEV protease. Kifunensine was added during expression to limit glycosylation to high-mannose types. Residual glycans were removed using PNGaseF.

3.2 Lipid mass spectrometry of the CD1 lipidome

Soluble CD1 monomers were expressed in Expi293F cells to study lipid-ligand interactions. HLA-A2 molecules, structurally similar but peptide-binding, served as controls to identify background lipids. Lipidomic analysis was performed by Lipotype GmbH (Dresden, Germany) using mass spectrometry. Lipids were extracted via a two-step chloroform/methanol method, with internal standards added before extraction. Samples were analyzed in both positive and negative ion modes. Lipid identification was done using LipidXplorer software, and quantification was normalized to lipid class-specific internal standards. Data analysis was conducted using an in-house R pipeline (R version 4.3.2).

3.3 In-vitro ligand exchange and validation methods

To load CD1 molecules with lipids, 50-fold molar excess of lipid was dried under nitrogen in glass vials, then re-solubilized in ligand exchange buffer (0.5% CHAPS, 50 nM sodium citrate, pH 6.0) and sonicated at 37°C for 45 minutes. CD1c-endo or CD1d-endo monomers were added and incubated overnight at 37°C. Samples were concentrated using Vivaspin 500 columns and purified via Zebra Desalting spin columns. Optimal exchange occurred at pH 6.0. Ligand loading was assessed by isoelectric focusing (IEF) gel (pH 3–7). CD1 proteins were mixed 1:1 with loading buffer, run at increasing voltages (100 V, 250 V, 500 V), fixed with 12% trichloroacetic acid, and stained with Coomassie. Shifts in isoelectric point indicated successful lipid loading. Native mass spectrometry was used to confirm exchange. Five micrograms of protein

were loaded onto a size exclusion chromatography (SEC) column; fractions from 4–14 ml was analyzed. Raw data was deconvoluted using PMI-Byos and processed with Chromeleon. Fragmentation data identified lipid species.

Fluorescent CD1c and CD1d tetramers were generated by incubating biotinylated monomers with PE- or APC-conjugated streptavidin, using a 25% molar excess of monomers. CD1 dextramers were produced using APC-labelled SA-Dextramer backbones, also oversaturated with monomers. These lipid-loaded tetramers and dextramers were used to stain CD1d-restricted T cells, validating the ligand exchange protocol.

3.4 TCR discovery by Phage display

To isolate CD1c-specific TCRs, phage display technology was used. This method generates random TCR α - and β -chain libraries displayed on bacteriophage surfaces, which are screened against 100 nM of biotinylated CD1c-endo molecules captured on streptavidin-coated magnetic beads. Phage particles, pre-blocked in PBS containing 3% milk powder to reduce non-specific binding, were incubated with the beads. After washing, bound phages were eluted with trypsin and used to infect early log-phase TGI *E. coli*. Infected cells were plated on YTEag plates and incubated at 30°C for 16 hours. Titration plates were used to estimate library size, while large square plates maximized clone diversity. This selection process was repeated for three rounds, with sequencing and titre calculation after each round. To assess TCR specificity and cross-reactivity, the MAGPIX assay was employed. TCRs were exposed to a broad panel of HLA-peptide and CD1-lipid complexes, enabling detailed evaluation of their selectivity. This approach ensured identification of TCRs with high specificity for CD1c-lipid antigens.

3.5 TCR and ImmTAC Construct design and expression

The TCR chains were cloned into the pLAS0729 and pLAS0730 vectors. TCR constructs contain the variable and constant domains of the α and β chains and the chains are stabilized with a disulphide bridge. TCR constructs for functional experiments were reformatted into ImmTAC format, where the TCR β - chain is fused with an anti-CD3 scFv using pLAS536 vector (ImmTAC molecules, total size 75kDa). The TCR chains were expressed in the BL21(DE3)RosettaLysS *E. coli* strain. Starter cultures were grown for three hours in Erlenmeyer flasks until approximately OD600 = 0.5. Then 250 ml Auto induction Media was inoculated. The culture was 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 by centrifugation at 4000 rpm for 30 minutes. The IBs were washed and re-suspended in re-suspension Buffer. The quality and purity of the IBs were assessed on SDS-PAGE gel.

3.6 TCR and ImmTAC refold and purification procedure

α and β inclusion bodies were denatured in Denaturation Buffer to unfold and dissociate the aggregated polypeptides from one another at a molar ratio of 1.5:1 (α : β) in order to limit β homodimer formation. ImmTAC α and β chains were mixed in an equimolar chain ratio (α : β 1.0:1.0). 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. The proteins fold into their native state during dialysis. 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.

3.7 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). Streptavidin molecules (5000 response units (RU)) were then linked covalently to the chip surface and the remaining reactive groups were deactivated by 1 M ethanol-amine hydrochloride. Approximately 100-1000 RU of CD1c-endo molecules were bound to the CM5 sensor chip at 10 μ L/min flow rate. One flow cell was left blank and used as a negative control.

Steady state affinity measurement of low affinity TCRs

Wild-type, low-affinity TCRs exhibit rapid binding kinetics, making kinetic measurements unreliable. Therefore, their affinities (KD) were determined using steady-state binding analysis across a concentration range covering 20–80% saturation of CD1c-endo molecules immobilized on the chip. TCRs and ImmTACs were concentrated to 100 μ M, and eight 2-fold serial dilutions were injected at 30 μ L/min at 25°C. Binding data were analyzed using Biacore T-200 Evaluation, Biacore Insight (GE Healthcare), and GraphPad Prism. KD values were calculated assuming a 1:1 binding model ($A + B \leftrightarrow$

AB). Equilibrium responses were plotted against protein concentrations, and non-linear least squares fitting was performed using the Langmuir binding equation: $AB = (B \times AB_{max}) / (K_D + B)$.

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 measurement 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. 100 RU CD1c-endo was captured on the chip. TCR dilutions of 10 nM, 5 nM, 2.5 nM, 1.25 nM and 0.625 nM were injected. The results were analyzed using Biacore T-200 Evaluation software.

3.8 Affinity Maturation of TCR chains

To enhance the affinity of the wild-type S2 TCR for CD1c, an affinity maturation process was performed using phage display. Libraries were generated by introducing random mutations into the complementarity-determining regions (CDRs) of the TCR α or β chains. A 3% randomization rate was applied to each CDR, ensuring a high retention of the wild-type sequence while introducing minimal but functionally relevant diversity. Two first-generation libraries were constructed—one for each chain—using NNN codon templates to randomize CDR1, CDR2, and CDR3, while keeping the partner chain constant. Phage particles displaying TCR variants were panned against 50 nM CD1c-endo, a concentration below the wild-type K_D , to enrich for higher-affinity binders. Panning was conducted in detergent-free buffers to preserve lipid integrity, with blocking in 2% BSA/PBS. After three rounds of selection, inhibition phage ELISA was used to rank clones. In this assay, binding of phage to immobilized CD1c-endo was competed with soluble CD1c-endo (100 nM), and inhibition of signal indicated higher affinity. Top clones were sequenced and refolded as ImmTACs for functional screening.

A TCR with nanomolar affinity, good yield, and specificity was selected as the template for second-generation libraries. These libraries introduced a higher mutational load, targeting one CDR at a time (five consecutive amino acids) while keeping the remaining five CDRs unchanged. Six libraries were generated in total. Additionally, structure-guided libraries were designed based on the CD1c-TCR crystal structure to enhance interactions with the CD1c heavy chain. Second-generation panning was performed using decreasing concentrations of CD1c-endo (100 pM, 10 pM, 1 pM) to further enrich for high-affinity variants. Inhibition ELISA was again used to rank candidates. The best-performing TCRs were cloned into mammalian expression vectors in ImmTAC format

and expressed as soluble proteins for downstream validation.

3.9 X-ray crystallography of TCR-CD1c complexes

To prepare CD1c-TCR complexes for crystallography, soluble CD1c monomers were expressed in Expi293FGnTI⁻ cells to ensure uniform glycosylation, then deglycosylated with PNGaseF and purified via Superdex 75 size exclusion chromatography. TCRs (S2c^{WT} and S2c^{a5b6}) were expressed in *E. coli*, refolded, and purified by anion exchange and size exclusion chromatography. CD1c-endo and TCRs were mixed at a 1:1.1 molar ratio, purified on a Superdex 200 column, and concentrated to ~10 mg/ml. X-ray diffraction data were collected at Diamond Light Source (UK), beamlines I03 and I04. Structures were solved by molecular replacement using PDB entries 3OV6, 4P4K, and 5FK9 for S2c^{WT}-CD1c, and the S2c^{WT}-CD1c complex as a model for S2c^{a5b6}-CD1c. Structures were visualized in PyMOL and deposited in the PDB (accession codes 9QWJ and 9QWK).

3.10 Lipidomic analysis of ImmTAC-CD1c complexes

To identify lipid ligands bound by wild-type and high-affinity ImmTACs, soluble CD1c monomers were produced in Expi293F cells, and ImmTACs (S2c^{WT} and S2c^{a5b6}) were expressed in CHO cells. After purification, ImmTACs were mixed with CD1c-endo at a 1:1 molar ratio and separated on a Superdex200 10/300 Increase column. Bound and unbound fractions were collected for lipidomic analysis by Lipotype GmbH (Dresden, Germany). Lipids were extracted using a two-step chloroform/methanol method, with internal standards spiked in prior to extraction. Samples were analyzed in both positive and negative ion modes in a single acquisition. Lipid identification was performed using LipidXplorer software, and quantification was normalized to lipid class-specific internal standards. Final data analysis was conducted using an in-house R workflow (R version 4.3.2).

3.11 Generating antibody-based CD1c-specific T cell engagers

13 in-house identified clones of anti-CD1c antibodies of various origin (llama, chicken and human) were reformatted as Bispecific T cell Engagers (BiTE). BiTE molecules are fusion proteins of two single-chain variable fragments (scFvs) connected in tandem by a flexible linker (GS). The variable light (VL) and variable heavy (VH) chains were fused in two possible orientations. One of the scFvs binds to T cells via the CD3 receptor (UCHT1), and the other to a CD1c molecule on the cell surface. The BiTE molecules were expressed in CHO cells and purified by protein L affinity chromatography.

3.12 Generation of CD1c expressing cell lines and effector cell lines

CD1c constructs were cloned into the pSF Lenti-vector and lentiviral particles were generated via transient co-transfection of HEK 293T cells using a third-generation system with packaging and envelope plasmids. Transfection was mediated by PEI. THP1(CTIIAB2mKO), K562, and MOLT4(B2mKO) cells were transduced with single-chain CD1c using concentrated lentivirus and protamine sulphate via spinfection. Transduction efficiency was assessed by flow cytometry. To generate CD1c-restricted effector cell lines, NFAT.Luc.Jurkat.TCRKO and JRT3 Jurkat cells were transduced with TCR α and β chains using the same method.

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

Jurkat.NFAT.Luc effector cells (25,000) were co-cultured with 12,500 target cells in Jurkat Assay Media in 384-well plates for ~22 hours. A dilution series ($1-1 \times 10^{-6}$ nM) of bispecific engagers (BiTE or ImmTAC) was added. The next day, BioGlo reagent was added to each well. Upon activation, Jurkat cells expressed firefly luciferase under NFAT control. Luminescence was measured at 574 nm using a Clariostar plate reader. Data were analyzed using Microsoft Excel and GraphPad Prism.

3.14 T cell activation assay

50,000 target cells were co cultured with 50,000 effector cells (Jurkat.NFAT.Luc) in a flat bottom 96 well plate in thermostat for overnight (~16h) in Jurkat Assay Media. 10 μ g/ml antiCD1c blocking antibody (L161) was added to the control wells. The next day the cells were stained first with LIVE/DEAD™ Fixable Aqua Dead Cell Stain, blocked with Human TruStain FcX™ (Fc Receptor Blocking Solution) and stained for activation markers CD69 and CD25. Flow cytometry was performed on BD LSRFortessa™ Cell Analyzer. Data were analysed using Flowjo V10.

3.15 T cell cloning

Peripheral blood mononuclear cells (PBMCs) and CD2⁺ cells were isolated from healthy internal donors at Immunocore Ltd. under a Health Technology Assessment (HTA)-approved protocol. PBMCs were separated using Lymphoprep gradient centrifugation, and CD2⁺ cells were purified with Miltenyi CD2 MicroBeads. For expansion of CD1c- and CD1d-restricted ganglioside-specific T cell clones, CD2⁺ cells were labelled with CFSE. Irradiated THP1.DKO, THP1-CD1c, and THP1-CD1d cells (66 Gy) were pulsed with lipid ligands (GD3, GM3, Neu5GM3) at 25 μ g/ml and incubated for 3 h at 37°C. CD1d-expressing cells were blocked with anti-HLA-A, B, C and HLA-DR specific L243.2 antibodies. Labelled T cells were co-cultured with target

cells for 3 days. Cell culture media with 500 IU IL-2 was added daily, and cells were split upon confluence. Proliferating cells were identified by CFSE dilution and sorted using a Sony cell sorter.

4. RESULTS

4.1 Mammalian expression of CD1 molecules

To generate functional soluble CD1 complexes, we optimized expression using ExpiCHO and Expi293F mammalian cell lines, chosen for their ability to correctly fold and glycosylate complex proteins. Cells were transfected with a dual-expression pcDNA3.1 vector encoding single-chain CD1 variants (CD1a, b, c, d) and the BirA biotin ligase. A C-terminal KDEL sequence retained BirA in the endoplasmic reticulum, enabling *in vivo* biotinylation of secreted CD1 monomers. Expression analysis showed optimal yields five days post-transfection in Expi293F cells. CD1 monomers were purified via affinity and size-exclusion chromatography. Structural integrity was confirmed by surface plasmon resonance (SPR) through ILT2 binding, which requires proper folding of CD1 and β 2-microglobulin. Functional validation was achieved via SPR with CD1c-specific TCRs. SDS-PAGE and isoelectric focusing revealed multiple CD1c bands, reflecting glycosylation at four predicted N-linked sites. Attempts to generate non-glycosylated variants via mutagenesis resulted in unstable, low-yield proteins, underscoring glycosylation's role in CD1 stability. Therefore, all downstream studies used fully glycosylated CD1, with enzymatic deglycosylation applied for structural analysis. This optimized protocol reliably produced biotinylated, properly folded CD1 monomers suitable for structural and functional studies of CD1-mediated lipid antigen presentation.

4.2 Lipid-ligand exchange method development

To enable TCR characterization against self-lipids, we developed a robust lipid-exchange protocol for CD1c. CD1c monomers produced in mammalian cells carry endogenous lipids, which are difficult to replace due to lipid insolubility and the stability of CD1-lipid complexes. We used CHAPS detergent in pH 6.0 buffer to induce partial unfolding of the CD1c binding groove while preserving structural integrity. Target lipids, sonicated for solubility, were added at 50-fold molar excess and incubated overnight at 37°C. Exchange success was validated using IEF, native mass spectrometry, TCR binding assays, tetramer staining, and ILT2 binding as a structural integrity control.

4.3 Comprehensive Validation of Lipid Exchange

4.3.1 Isoelectric Shift Analysis

Isoelectric focusing (IEF) provided initial evidence of successful lipid exchange. CD1c-endo and CD1d-endo appeared as multiple bands due to heterogeneous glycosylation. Upon loading with charged lipids, predictable shifts in migration were observed: negatively charged gangliosides (GD2, GD3, GM3, Gb4) caused acidic shifts, while neutral lipids (PC, KRN7000) maintained original pI values. Although semi-quantitative, this method confirmed lipid loading and guided further analysis.

4.3.2 Native Mass Spectrometry

Native MS enabled direct analysis of intact CD1-lipid complexes without lipid extraction. CD1c and CD1d monomers were produced in Expi293FGnTI⁻ cells to reduce glycosylation complexity. Native ESI-MS revealed CD1c-endo (55.3–56.4 kDa) and CD1d-endo (58.8–59.9 kDa) masses exceeding theoretical values (~48 kDa), confirming endogenous lipid retention. Post-exchange mass shifts indicated successful loading, but spectral complexity from residual lipids and glycosylation variants limited precise quantification.

4.3.3 TCR Binding Validation

TCR binding assays using the lipid-polyspecific NM4 clone provided functional validation. SPR revealed that CHAPS treatment alone caused non-specific “sticky” CD1c behavior, while GD1a loading disrupted binding due to its bulky headgroup. Homogeneous glycosylation improved binding consistency but reduced protein stability after extended exchange, highlighting the balance between glycan engineering and structural integrity.

4.3.4 Functional Tetramer Staining

Functional validation was achieved using CD1d-KRN7000 tetramers. Specific staining of NKT cells confirmed successful loading, while CD1d-endo tetramers showed no binding. In vitro refolded CD1d- α GalCer complexes served as positive controls. This approach confirmed both loading efficiency and biological functionality.

Together, these methods established a robust framework for evaluating lipid exchange, emphasizing the interplay between glycosylation, stability, and antigen presentation, key factors for therapeutic development targeting CD1-lipid complexes.

4.4 The CD1-associated lipid repertoire

4.4.1 Analyzing the lipid repertoire of CD1 isoforms

Our investigation into the lipid-presenting capabilities of CD1 molecules began with an optimized production system using Expi293F cells to generate soluble CD1a-d isoforms, building on Huang et al.'s (1) validation of this approach for studying transmembrane CD1 molecules. The mass spectrometry analysis pipeline we developed employed HLA-A2 as a critical negative control to distinguish true CD1-bound lipids from background noise. Through careful optimization, we determined that 500 µg of CD1 protein provided optimal sensitivity for lipid detection while minimizing abundance-related artifacts, though we analysed parallel 100 µg replicates to capture lower-abundance species. Our in-house R-based bioinformatics pipeline processed the resulting data, ultimately identifying 81 high-confidence lipid features that represented less than 6% of the background signal detected in HLA-A2 controls, ensuring the specificity of our findings.

4.4.2 Most cellular lipids captured by the CD1 isoforms

The most striking finding from our study was the remarkable breadth of lipid sampling exhibited by all CD1 isoforms. While each isoform demonstrated subtle preferences, they collectively captured a substantial portion of the cellular lipidome, with phospholipids and sphingolipids emerging as dominant classes across all variants. CD1a and CD1c showed the closest alignment with the overall cellular lipid profile, though CD1a exhibited a distinct skew toward mid-length carbon chains (C34-C38). In contrast, CD1b and CD1d displayed more pronounced deviations. CD1b enriched for both unusually short-chain (C16-C20) lipids and specific sphingolipids, while CD1d demonstrated particular affinity for C42 lipids and gangliosides. These observations suggested that while CD1 molecules broadly survey the lipid environment, their unique structural architectures impart subtle but potentially functionally significant sampling biases.

4.4.3 The shared and unique lipid profiles of CD1 isoforms

Principal component analysis of our complete dataset comprising 1,040 identified lipid species revealed substantial overlap between isoforms, with 293 lipids shared universally across all CD1 variants. These commonly presented lipids were predominantly phospholipids (PC, PE) and sphingolipids (Cer, SM), forming what might be considered a "core" CD1 lipidome. Interestingly, CD1a and CD1d exhibited the greatest degree of overlap (474 shared lipids), while each isoform maintained a distinctive lipid signature. CD1a stood out for its enrichment of phosphatidylserine (PS) and phosphatidylinositol (PI) species (128 unique lipids), while CD1b specialized in sulfatides and short-chain lysophospholipids (25 unique species). CD1c demonstrated particular affinity for gangliosides

(GM2, GD1-3) and short-chain sphingomyelins (33 unique lipids), and CD1d showed remarkable selectivity for cholesterol and GM3 (57 unique species).

4.4.4 Lipid chain lengths captured by CD1 isoforms

The structural basis for lipid sampling preferences among CD1 isoforms became clear when chain length distributions were analyzed in the context of each isoform's binding groove. CD1a favored mid-length lipids (C34–C38), consistent with its moderately sized pocket, though its ability to bind large cardiolipins (C60) suggested unexpected flexibility. CD1b displayed a dual preference for very short (C16–C20) and long (C40–C42) chains, supporting the “spacer-lipid” observations, where small lipids stabilize the groove to accommodate larger antigens. CD1c exhibited the broadest range (C30–C46), reflecting its structural plasticity, and showed evidence of short lysolipids (C14–C18) occupying the F' pocket. CD1d preferred shorter chains (C34–C36) and showed a 26-fold enrichment of GM3, suggesting a specialized role in ganglioside presentation. Headless lipids like ceramides were consistently enriched, 2–9 fold across isoforms, while headed sphingolipids—known to inhibit autoreactive CD1 restricted T cells—were more variably represented. This pattern suggests a regulatory mechanism in which CD1 molecules sample both activating and inhibitory lipids. Notably, phosphatidylethanolamines (PEs) were under-represented, while ether-linked phosphatidylcholines (PCs) were enriched, indicating structural constraints in lipid loading that may shape immune recognition. Together, these findings provide a comprehensive view of how CD1 molecules interact with the cellular lipidome. Despite overlapping sampling capabilities, each isoform maintains a distinct lipid “fingerprint”, shaped by structural features and functional roles. This has important implications for understanding lipid antigen presentation and for designing CD1-targeted immunotherapies in cancer, autoimmunity, and infectious disease.

4.5 Binding mode of CD1c-specific TCRs

4.5.1 Generating CD1c-specific TCRs

A CD1c-specific TCR (S2c^{WT} TCR) was isolated from in-house naïve TCR phage libraries. Surface plasmon resonance (SPR) revealed a KD of 16.7 μ M for CD1c-endo, aligning with typical TCR-pHLA affinities. In SPR experiments, the S2c^{WT} TCR achieved around 75% binding of the theoretical R_{max} (calculated by dividing the observed R_{max} by the theoretical R_{max} based on the amount of CD1c loaded), indicating a largely lipid-independent binding mode and consistent with the data of previously reported CD1c-reactive TCRs.

4.5.2 Affinity enhanced CD1c restricted TCRs

Unlike HLA-peptide complexes, CD1-lipid complexes are presented at low levels on the cell surface, making them challenging targets for bispecific engagers, which typically require high-affinity interactions to be effective. To address this, affinity-enhanced variants of the S2c^{WT} TCR were developed. First-generation libraries were created with a 3% randomization rate across the three CDRs of either the α or β chain. Three rounds of phage panning were performed using decreasing concentrations of CD1c-endo to enrich for higher-affinity binders, followed by a fourth round of off-rate selection. Phage inhibition ELISA was used to rank clones, identifying CD1c-S2-END-a2b1-U (KD = 1.05 nM, $t_{1/2}$ = 37 min) as the top candidate. This clone served as the template for second-generation affinity maturation. Six hard mutagenesis libraries and six structure-guided libraries were constructed, targeting specific CDRs and their interactions with CD1c helices. Panning was performed with 100 pM, 10 pM, and 1 pM CD1c-endo. Loop extension libraries further explored CDR2 α flexibility. Phage inhibition ELISA at 0.5 nM CD1c identified clone S2^{a5b1} with 60% inhibition. Twenty-eight chain combinations were expressed as ImmTACs and screened for cross-reactivity using SPR against CD1a, b, d, and various HLA-peptide complexes. Clones with cross-reactivity or reduced half-life were excluded. The optimal combination—S2-a5 (from the second-generation α CDR1 library) and S2-b6 (from the first-generation output)—achieved 100% binding to CD1c-endo and improved affinity from 16.7 μ M to 15 pM, with a half-life of 9.6 hours. Structural modelling revealed that mutations were positioned within CDR loops or at the CD1c interface, supporting enhanced binding. These results demonstrate that affinity maturation enabled high-affinity, lipid-agnostic recognition of CD1c, offering a promising platform for targeting CD1c in therapeutic applications.

4.5.3 Structural basis of CD1c lipid independent recognition

To better understand CD1-lipid recognition by TCRs, we solved the crystal structures of the S2c^{WT}-CD1c-endo complex at 2.04 Å resolution and the affinity-enhanced S2c^{a5b6}-CD1c complex at 2.27 Å. These structures revealed key insights into TCR docking geometry, contact footprints, and lipid accommodation. Overlaying the S2c^{WT}, S2c^{a5b6}, and previously reported 3C8 TCR-CD1c complexes showed a conserved diagonal docking angle (~43.3°) and central positioning over CD1c. While S2c^{WT} and 3C8 shared similar docking orientations and contacted overlapping CD1c residues, S2c^{a5b6} formed additional interactions with helix 1, whereas 3C8 engaged more with helix 2. Both S2c^{WT} and 3C8 contacted CD1c residues Asp65, Glu80, and Glu157 through van der Waals and electrostatic interactions. Unique contacts included Arg71 and Leu162 for S2c^{WT}, and Gln151, Glu153, and Ser169 for 3C8. Most TCR contacts were

concentrated above the CD1c A'-roof, particularly along helix 1, resulting in a buried surface area of 1015 Å²—within the typical range for TCR–pHLA and TCR–CD1 interactions. Interface analysis showed that the TCR α -chain contributed 63% of the contacts, with CDR1 α and CDR2 α positioned over the CD1c helices, and CDR3 β located above the F' portal. The β -chain contributed 35%, primarily through CDR2 β and CDR3 β , while CDR1 β made no contact. No clear electron density was observed for the lipid head group, likely due to the heterogeneous lipid mixture in CD1c-endo. Lipid contacts were not resolved in the S2c^{WT} structure. Key interactions included S2c^{WT} residues Tyr30 (CDR1 α), Gln95 and Tyr96 (CDR3 α), Glu52 (CDR2 β), and Arg97 and Arg100 (CDR3 β), which contacted CD1c residues Asp65, Leu68, Arg79, and Glu80. These residues are analogous to the “restriction triad” in pHLA recognition. Both S2c^{WT} and 3C8 formed multiple salt bridges (five and six, respectively), stabilizing the TCR–CD1c interface. Despite similar footprints, S2c^{WT} and 3C8 rely on distinct interaction networks. The S2c^{WT} binding mode most closely resembles CD1b-restricted TCRs, while CD1a- and CD1d-restricted TCRs exhibit different docking angles. These findings highlight conserved and divergent features of CD1c recognition and provide a structural framework for engineering TCRs with enhanced affinity and specificity for CD1c-lipid complexes.

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

Among seven introduced mutations, three hydrophobic residues (Y28 α , L30 α , and M98 β) primarily enhanced S2c^{a5b6}'s affinity for CD1c by forming additional van der Waals contacts with Glu61, Glu157, and Tyr152. D94 α reduced interaction with Asp65. Increased hydrophobic contacts, especially via S30 β and M98 β , improved both CD1c affinity and lipid selectivity. These findings highlight how targeted hydrophobic mutations can enhance TCR binding and functional recognition of CD1c-lipid complexes.

4.5.5 Lipid Accommodation and CD1c F67 Conformational Flexibility

Due to the diverse lipid species bound by CD1c-endo, clear electron density for lipid head groups was not observed in the S2c^{WT}–CD1c structure. Although structural modeling suggested potential TCR-lipid contacts, these were not directly resolved. Interestingly, the asymmetric unit contained two S2c^{WT}–CD1c complexes with differing lipid densities, indicating variability in lipid binding. CD1c residue F67 adopted two distinct conformations, influencing lipid accommodation. Larger lipid head groups likely require the outward-facing conformation of F67. The S2c^{WT} TCR adapted to this by altering side-chain conformations near the F' portal—Q95 α and Y48 β flipped to accommodate F67, shifting R31 β , Q50 β , and N30 β to support interactions with bulkier lipids. Two density fragments within the CD1c groove resembled previously described spacer lipids.

Computational modeling with decanes fit these densities well. In the S2c^{a5b6}–CD1c structure, four complexes were observed, all showing consistent lipid density protruding from the F' portal. CD1c F67 adopted the outward-facing conformation, and TCR β residues Y48 β , R31 β , and Q50 β mirrored the alternate conformation seen in S2c^{WT}. Affinity-enhancing mutations S30 β and M98 β created additional space and wrapped around the lipid density, suggesting a preference for larger lipid head groups. These findings were supported by lipid mass spectrometry of eluted lipids. Together, these structural insights reveal how TCRs adapt to CD1c-lipid diversity. While S2c^{WT} and 3C8 share some binding features, S2c^{a5b6} demonstrates how targeted mutations enhance hydrophobic interactions, refine lipid selectivity, and strengthen CD1c binding. These results deepen our understanding of CD1c-restricted TCR recognition and inform future therapeutic strategies.

4.5.6 TCR affinity purification (TCR-tap) experiments to study CD1c restricted

TCR specificity

To determine whether affinity maturation altered the lipid recognition profile of the S2c^{WT} TCR, we performed lipid mass spectrometry on CD1c-bound ligands co-purified with both wild-type and affinity-enhanced TCRs. Soluble CD1c monomers were expressed in Expi293F cells, with endogenous lipids (CD1c-endo). As validated by Huang et al. (2023)(1), secreted CD1 proteins serve as reliable surrogates for lipidomic analysis of transmembrane CD1 molecules. This soluble format also enables detergent-free purification, preserving native lipid content. Wild-type (S2c^{WT}) and affinity-enhanced (S2c^{a5b6}) TCRs were expressed in ExpiCHO cells in ImmTAC format and purified. Each TCR was mixed with CD1c-endo at a 1:1 molar ratio, and the resulting complexes were separated from unbound CD1c via size exclusion chromatography. Both ImmTAC–CD1c complexes and unbound fractions were submitted for lipidomic analysis. To control for non-specific lipid detection, HLA-A2 samples—processed identically to CD1c—were analyzed in parallel. Additionally, lipid content from TCR-only samples was subtracted as background. A total of 813 lipid features were identified, with 101 also present in HLA-A2 controls. These accounted for 184 pmol of lipids, including four GM1/GM3 species absent from TCR-only samples, suggesting specific enrichment. Lipids from TCR-only samples contributed just 2–4% of the total signal, and the most abundant lipid (GD2 42:1;2) was not detected in the ImmTAC–CD1c complexes, confirming minimal background. After subtracting shared features, background interference was estimated at ~0.4%. The S2c^{WT} and S2c^{a5b6} complexes shared 402 lipid features, including a broad range of lipid classes: ceramides,

glycosphingolipids (GM1, GM2, GM3), phospholipids (PC, PE, PI, PS), and lyso-lipids. The overall lipid profiles were highly similar, indicating that affinity maturation primarily enhanced interactions with the CD1c heavy chain rather than altering lipid specificity. However, subtle differences emerged. The high-affinity S2c^{a5b6} complex showed a slight shift toward longer-chain lipids and lacked PI and PS species, which possess negatively charged headgroups. This suggests that lipid headgroup chemistry, rather than acyl chain length, may influence TCR binding. Several large lipids—TAG 54:1;0, TAG 54:2;0, TAG 56:2;0, and GM2 42:2;2—were uniquely detected in the S2c^{a5b6} complex, suggesting enhanced accommodation of bulkier ligands. Short-chain lyso-lipids (C16–C28) were also present in both complexes and may act as spacer lipids, stabilizing the CD1c binding groove and facilitating presentation of larger antigens in the F' pocket. In summary, lipidomic profiling revealed that affinity maturation of the S2c^{WT} TCR did not drastically alter lipid specificity but subtly refined lipid preferences—particularly favouring larger, neutral headgroup lipids. These findings support a model in which enhanced CD1c binding is achieved through improved interactions with the CD1c heavy chain, while maintaining broad lipid recognition.

4.6 Re-directed T-cell activation against CD1c positive cells using a TCR-CD3 bispecific molecule

The high-affinity CD1c-specific TCR, S2c^{a5b6}, was reformatted into ImmTAC format by fusing its β chain to an anti-CD3 scFv (KD = 10 nM, half-life = 2 minutes). A dilution series (10 to 1×10^{-6} nM) of the ImmTAC was used to assess potency by redirecting T cells against CD1c-positive and -negative cell lines. To eliminate endogenous CD1 and MHC expression, $\beta 2m$ and CIITA were knocked out in THP1 cells using CRISPR/Cas9 (THP1-DKO). These cells were then transduced with single-chain CD1c- $\beta 2m$ or CD1b- $\beta 2m$ constructs to generate THP1-CD1c and THP1-CD1b lines. Additional cell lines, including C1R and K562, were transduced with CD1c- $\beta 2m$ or CD1d- $\beta 2m$. CD1c expression was confirmed by FACS using the L161 antibody. High CD1c expression was observed on THP1-CD1c, K562-CD1c, and C1R-CD1c, while parental and DKO lines showed no expression. CD1c levels were also confirmed on hematologic cancer cell lines (CCRFSB, NALM6, HPB-ALL, SKW3, OCIM1). T cell activation was measured by CD25 upregulation. S2c^{a5b6} ImmTAC potently activated CD4⁺ and CD8⁺ T cells against CD1c⁺ targets, and this activation was partially blocked by the L161 antibody. When tested across a panel of hematologic cell lines, ImmTACs triggered strong responses against CCRFSB, NALM6, HPB-ALL, and SKW3, but not OCIM1. CD1c-dependence was confirmed by L161-mediated blockade. To assess off-tumor effects, ImmTACs were

tested against primary monocytes and B cells from two donors. Reactivity was observed against B cells and, to a lesser extent, monocytes—both blocked by L161. These findings support the therapeutic potential of CD1c-targeting ImmTACs in hematologic malignancies, while highlighting the need for further engineering to minimize on-target, off-tumor effects on healthy immune cells.

4.7 T cell redirection via CD1c-specific T cell engagers

The CD1c affinity of CD1c and CD3 bispecific T-cell engagers (BiTEs) was assessed using surface plasmon resonance (SPR), revealing affinities ranging from low nanomolar to high picomolar. Human antibody-based BiTEs showed the strongest binding, with the highest percentage of CD1c-endo engagement. Minor cross-reactivity with CD1b-endo was observed across all BiTEs. To evaluate functional potency, BiTEs were tested in a T cell redirection assay alongside CD1c-specific ImmTACs. Jurkat.NFAT.Luc cells were co-cultured overnight with CD1c⁺ or CD1b⁺ THP1 cells in the presence of a dilution series (1–1×10⁻⁶ nM) of BiTEs or ImmTACs. T cell activation was measured by NFAT mediated luciferase expression. Both formats showed CD1c-dependent activation, with no response against CD1c⁻ THP1.DKO cells. BiTEs induced stronger Jurkat activation than the affinity-matured ImmTAC (S2c^{a5b6}), while the wild-type ImmTAC showed the weakest response. Low-level cross-reactivity to CD1b was observed. A background signal (~500 RLU) was detected across all conditions, likely due to CD1 expression in Jurkat cells. These results highlight the potential of CD1c-specific BiTEs as potent and specific immunotherapeutics. Their strong binding, minimal off-target activation, and superior T cell redirection compared to ImmTACs support further development and in vivo validation.

4.8 Antigen presentation by CD1 molecules

4.8.1 mLPA, a novel CD1c-associated antigen

The lipid antigen methyl-lysophosphatidic acid ester (mLPA), identified by Lepore et al.(5) from acute myeloid leukemia (AML) samples, is recognized by CD1c-restricted T cells. While its TCR specificity and anti-leukemic potential were demonstrated, the origin and loading mechanism of mLPA onto CD1c remained unexplored. mLPA is a modified phospholipid with an ether-linked alkyl chain and a methylated phosphate group—features associated with peroxisomal lipid synthesis. To better understand this antigen, I aimed to investigate the cellular pathway responsible for mLPA loading onto CD1c.

4.8.2 Modelling the CD1c lipid loading pathways

To investigate where the mLPA lipid antigen is loaded onto CD1c, leukemia cell lines expressing CD1c variants with distinct recycling properties were engineered. Wild-type CD1c (scCD1c-WT) assembles in the ER with self-lipids and traffics to the cell surface, where it recycles through endosomal compartments via AP2-mediated sorting. Based on Jackman et al.'s (6) findings on CD1d, a tail-deleted CD1c variant (scCD1c-TD) was created to prevent intracellular recycling. Additionally, a GPI-anchored CD1c variant (scCD1c-GPI) was engineered using a decay-accelerating factor (DAF, CD55) sequence, targeting CD1c to early and sorting endosomes. THP1 and K562 cells were transduced with these CD1c constructs and sorted for equal surface expression. GPI anchoring was confirmed by PI-PLC cleavage. Jurkat cells expressing mLPA-specific TCRs (DN4.99, DN4.2, PZ-CD8) recognized CD1c on THP1 and K562 cells, with a two-fold increase in activation against CD1c-GPI variants. This response was blocked by anti-CD1c antibody, confirming specificity. To explore the role of intracellular trafficking, lysosomal acidification was inhibited using Bafilomycin A1 (BafA1). CD1c surface levels remained stable in THP1 cells but increased in K562 cells, likely due to differences in endosomal sorting and Na^+/K^+ -ATPase regulation. BafA1 treatment reduced Jurkat activation across all CD1c formats, though residual activation persisted with CD1c-GPI cells. These findings suggest mLPA loading may occur in the secretory pathway or early endosomes, rather than lysosomes. BafA1's impact on peroxisomal gene expression and lipid metabolism may also influence antigen availability. Overall, this study highlights the importance of CD1c trafficking in lipid antigen presentation and suggests that mLPA may be loaded outside of classical lysosomal compartments.

4.9 Generating lipid specific T cell clones

To identify lipid-specific TCRs, a T cell cloning approach was used to selectively expand CD1-restricted T cells. Methods included antigen-presenting cell (APC) stimulation, tetramer/dextramer-based sorting, and activation marker-based isolation. For CD1d-restricted GD3 and αGalCer -specific clones, artificial APCs loaded with CD1d-lipid complexes and autologous DCs were used. PBMCs were isolated from 200 mL of donor blood, with monocytes and pan T cells purified using CD14 specific MicroBeads and negative selection, respectively.

4.9.1 Stimulation and Expansion of Lipid-Specific T Cell Clones Using Artificial APCs

Artificial antigen-presenting cells (aAPCs) were generated by coating streptavidin magnetic beads with biotinylated CD1d-lipid complexes and biotinylated anti-CD28 antibodies. Pan T cells were co-cultured with aAPCs at a 2:1 ratio in the presence of IL-

2, IL-7, and IL-15 for 10 days. After expansion, cells were stained with CD1d-lipid dextramers and antibodies against CD3, CD4, CD8, and CD16.

4.9.2 Stimulation Using Monocyte-Derived Dendritic Cells (moDCs)

Monocytes were differentiated into moDCs using GM-CSF and IL-4. On day 5, moDCs were pulsed overnight with GD3 or α GalCer. Pan T cells were then co-cultured with moDCs (ratios 3:1 and 5:1) in cytokine-supplemented media for 10 days. Cells were stained as above.

Baseline CD1d-restricted T cell frequencies were assessed prior to stimulation. Viability staining gated live cells, revealing 4.9% CD3⁺CD16⁺ NKT cells, 15.8% CD8⁺ T cells, and 74.5% CD4⁺ T cells. Dextramer staining showed minimal CD1d-endo and CD1d-GD3 reactivity, while α GalCer-specific cells were more frequent (1.7% CD4⁺, 0.12% CD8⁺, 0.79% NKT). After stimulation, no increase in GD3-reactive T cells was observed with either method. However, α GalCer-pulsed moDCs expanded α GalCer-reactive CD4⁺ T cells sixfold (6.83%), while aAPCs had no effect. These results suggest that moDCs are more effective than aAPCs in expanding lipid-specific T cells, likely due to their physiological antigen presentation, co-stimulatory capacity, and ability to form immunological synapses.

5. DISCUSSION

5.1 Lipid-Loaded CD1 Complexes Enable the Development of Bispecific Molecules

This study demonstrated successful expression of functional, soluble single-chain CD1a, CD1b, CD1c, and CD1d molecules in mammalian cells. CD1c monomers were validated through ILT2 and TCR binding assays, confirming correct folding. Glycosylation heterogeneity observed on SDS-PAGE and IEF gels affected stability, as mutating glycosylation sites led to poor expression. CD1 molecules naturally bind diverse endogenous lipids, so a ligand exchange protocol was developed to load defined lipids. CD1c was expressed with reduced glycosylation to allow partial unfolding using CHAPS detergent in pH 6.0 buffer, enabling lipid replacement. Exchange efficiency was assessed via IEF, native mass spectrometry, tetramer staining, and TCR binding assays. Tetramer staining with CD1d- α GalCer was most effective, confirming successful lipid presentation, though limited to known antigen-specific T cells. While the protocol enabled partial lipid exchange, quantifying loaded molecules remains challenging. Further optimization is needed to improve reproducibility and establish a standardized method for lipid loading and antigen discovery.

5.2 CD1-Associated Lipid Repertoire

This study investigated the lipid ligands eluted from CD1 isoforms expressed in mammalian cells, revealing that CD1 molecules broadly sample the cellular lipidome. While there is substantial overlap among isoforms, each exhibits distinct preferences. CD1a and CD1c captured lipids that closely reflect the phospholipid-rich cellular background, whereas CD1b and CD1d favored sphingolipids. Notably, CD1d over-captured cholesterol, aligning with its role in presenting oxidized cholesterol and activating PPAR- γ . Structural differences among CD1 isoforms influence lipid-binding preferences, particularly regarding chain length: CD1a binds mid-sized lipids (C34–C38), CD1b accommodates both short (C16–C20) and long (C40–C42) chains, CD1c spans a broad range (C30–C46), and CD1d prefers shorter lipids but can bind long chains. These findings support the role of CD1 binding grooves in shaping lipid selectivity. Phosphatidylcholine (PC) was abundant across all isoforms, while PE and ether-PE were underrepresented, suggesting selective capture. Gangliosides, particularly GD1, GD2, GD3, and GM2, were highly enriched in CD1c, supporting emerging evidence that gangliosides may bind around CD1c's α -helices and modulate antigen presentation. GM3, known to inhibit NKT-cell activation, was over-captured by CD1b, CD1c, and CD1d. To study CD1c in cancer, overexpression systems were developed due to its low natural expression. Affinity purification using His-tags was unsuccessful, while StrepTactinXT-based methods enabled isolation of soluble CD1c, albeit at low yields. Immunoprecipitation (IP) using Protein G magnetic beads and a panel of 32 antibodies proved more effective. Antibody AB012 showed the highest efficacy in capturing CD1c from lysates. This work highlights the technical challenges in isolating CD1c and the importance of optimizing purification strategies. Future efforts should focus on improving yields and validating antibodies across diverse cell lines to enable comprehensive lipidomic profiling and identification of cancer-associated lipid antigens.

5.3 Antigen Presentation by CD1c

The identification of mLPA as a novel CD1c-bound lipid antigen marked a key advance in targeting unconventional T cells in cancer. This study explored how mLPA is loaded onto CD1c and its role in immune activation. Leukemia cell lines expressing CD1c variants with distinct trafficking properties—wild-type (recycling), tail-deleted (surface-retained), and GPI-anchored (early endosome-retained)—were co-cultured with Jurkat cells expressing mLPA-specific TCRs. Jurkat activation was highest with CD1c-GPI, suggesting mLPA loading occurs in early endosomes or the secretory pathway. Blocking

CD1c reduced activation, confirming CD1c restriction. Treatment with Bafilomycin A1, a lysosomal acidification inhibitor, reduced Jurkat activation across all cell lines, implicating lysosomal and peroxisomal pathways in mLPA processing. These findings suggest that mLPA loading occurs early in the endocytic pathway and is influenced by intracellular lipid metabolism. Future research should dissect the molecular mechanisms of mLPA synthesis and trafficking, potentially revealing new therapeutic targets in leukemia and other malignancies.

5.4 Targeting CD1c via Bispecific Engagers

This study highlights the therapeutic potential of targeting CD1c using bispecific engagers, particularly through the S2c^{a5b6} TCR, which recognizes CD1c-lipid complexes in a largely lipid-agnostic manner. Structural and biophysical analyses revealed that the wild-type S2c^{WT} TCR binds CD1c-endo with moderate affinity (KD = 16.7 μ M), similar to classical TCR-pHLA interactions. Crystallography showed that S2c^{WT} docks in a lipid-independent fashion, engaging CD1c's α -helices and A' roof, with the TCR α -chain contributing 63% of the interaction. Key residues such as Asp65, Leu68, Arg79, and Glu80 mediate binding, while the absence of lipid headgroup density supports a heavy-chain-focused interaction. Affinity maturation via phage display enhanced the TCR's affinity to 15 pM and extended its half-life to 9.6 hours, critical for therapeutic use. Structural analysis of the high-affinity S2c^{a5b6}-CD1c complex revealed consistent lipid densities and a single F67 conformation, allowing accommodation of larger lipid headgroups. Mutations introduced during maturation increased flexibility, enabling broader lipid recognition. Lipidomic profiling of ImmTAC-CD1c complexes showed substantial overlap between wild-type and high-affinity variants, confirming that affinity maturation did not alter lipid specificity but enhanced CD1c interactions. Larger lipids (e.g., TAG 54:1;0, GM2 42:2;2) were enriched in high-affinity complexes, while negatively charged lipids were underrepresented. Spacer lipids (C16–C18 lyso-lipids) were detected in both complexes, supporting structural stability and antigen presentation. The high-affinity TCR was reformatted into an ImmTAC bispecific engager with an anti-CD3 arm, redirecting T cells to CD1c+ targets. While potent activation was observed in CD1c+ cell lines, off-tumor reactivity to CD1c+ primary cells (e.g., B cells, monocytes) was noted, limiting the feasibility of lipid-agnostic targeting. Future strategies should focus on identifying tumor-specific lipid antigens for selective targeting. To explore alternative approaches, 13 anti-CD1c antibodies were reformatted into BiTEs and compared with ImmTACs. Both formats triggered CD1c-dependent T cell activation, with BiTEs showing enhanced potency and minimal cross-reactivity to CD1b. Functional

assays revealed no significant off-target activation, and while no format proved superior, both offer promise for therapeutic applications. The development of high-affinity CD1c-targeting bispecifics presents a versatile immunotherapy platform. These molecules can identify CD1c+ cells without requiring specific lipid antigens, and their extended binding half-life supports sustained immune responses. However, specificity and safety remain critical. Further functional assays are needed to assess cross-reactivity and off-target risks. Additionally, T cell cloning methods were employed to discover lipid-specific TCRs. While artificial APCs (aAPCs) failed to expand antigen-specific populations, monocyte-derived dendritic cells (moDCs) effectively expanded α GalCer-reactive CD4+ T cells. This suggests moDCs are superior for lipid antigen presentation. However, neither approach expanded CD1d-GD3 or CD1d-endo reactive T cells, indicating limitations in antigen recognition or stimulation protocols. Future research should refine antigen-loading and culture conditions to enhance expansion of diverse lipid-reactive T cells. Understanding the molecular basis of differential responses to aAPC and moDC stimulation will further advance unconventional T cell cloning and therapeutic development.

6. NEW SCIENTIFIC RESULTS

1. Characterized the CD1 lipidome, revealing broad lipid sampling and isoform-specific profiles.
2. Isolated a CD1c-restricted TCR using phage-display libraries.
3. Affinity maturation of a CD1c-restricted TCR to picomolar affinity while maintaining lipid-agnostic recognition of CD1c.
4. Resolution of two crystal structures and published the crystal structure of the TCR–CD1c–lipid complexes, only the second and third CD1c-specific TCR structure reported to date, providing a valuable resource for understanding CD1c-mediated immunity.
5. Crystallography and mass spectrometry studies confirmed the structural flexibility and lipid-agnostic binding mode of the high-affinity CD1c-specific ImmTAC molecule, providing new insights into the mechanism of TCR recognition of CD1c–lipid complexes.
6. Developed a high-affinity TCR-based bispecific engager specifically targeting CD1c, with no cross-reactivity to other CD1 isoforms or HLA molecules.

7. Demonstrated effective CD1c targeting with potent, on-target activity against CD1c+ cell lines.

7. SUMMARY

This PhD thesis focused on developing bispecific engagers targeting CD1c for the treatment of hematological malignancies. The research was structured around two main objectives: (1) characterizing the lipid antigen repertoire presented by CD1 molecules in mammalian and cancer cells, and (2) investigating how T cell receptors (TCRs) recognize CD1c-lipid complexes at the molecular level.

To address the first objective, CD1 isoforms were expressed in mammalian systems to capture endogenous lipids. Lipid mass spectrometry revealed that CD1 molecules broadly sample the cellular lipidome, with CD1a and CD1c favoring phospholipids, while CD1b and CD1d preferred sphingolipids. CD1d also showed a strong association with cholesterol. Chain length analysis indicated isoform-specific preferences, with CD1a binding mid-sized lipids, CD1b accommodating a wide range, CD1c showing broad specificity, and CD1d favoring shorter chains. Notably, CD1c exhibited a strong affinity for gangliosides, suggesting a role in immune modulation. To explore cancer-specific lipid signatures, CD1c-expressing cancer cell lines were developed, and various enrichment strategies were tested. Although soluble CD1c was successfully purified from THP1 cancer cells, yields were insufficient for mass spectrometry. The study also examined importance of CD1c recycling in antigen presentation through mLPA, the only known cancer-associated lipid antigen presented by CD1c. Using leukemia cell lines with distinct CD1c trafficking properties, it was shown that mLPA is likely loaded in early endosomes or the secretory pathway. Inhibition of lysosomal function further implicated lysosomal and peroxisomal pathways in mLPA processing.

The second objective focused on understanding TCR recognition of CD1c-lipid complexes. Soluble CD1c-lipid complexes were used to isolate TCRs from phage display libraries. These TCRs were reformatted into bispecific engagers capable of redirecting T cells to CD1c-expressing cancer cells. Structural analysis revealed that the wild-type TCR primarily interacted with the CD1c heavy chain, rather than the lipid antigen. Affinity maturation improved binding affinity to the picomolar range and extended the half-life to 9.6 hours, making the TCR suitable for therapeutic use. Structural and lipidomic analyses showed that the high-affinity TCR could accommodate larger lipid headgroups, supporting broader antigen recognition. In parallel, anti-CD1c antibody-based bispecific T cell engagers

(BiTEs) were developed. Thirteen antibody clones were reformatted and tested. Both BiTEs and ImmTACs (TCR-based bispecifics) effectively activated T cells in a CD1c-dependent manner. While the affinity-matured ImmTAC showed potent activity, it also exhibited on-target off-tumor reactivity to healthy CD1c+ cells, such as B cells and monocytes, limiting its clinical applicability in a lipid-agnostic format. To identify lipid-specific TCRs, CD1c and CD1d molecules were loaded with defined lipid antigens using a detergent-based ligand exchange protocol. Although α GalCer-reactive T cells expanded significantly using monocyte-derived dendritic cells (moDCs), no expansion was observed for CD1d-GD3 or CD1d-endo reactive T cells. These findings highlight the superiority of moDCs over artificial APCs and the need for further optimization of antigen-loading protocols.

In conclusion, this thesis demonstrates the feasibility of targeting CD1c with bispecific engagers and underlines the importance of identifying tumor-specific lipid antigens to enhance therapeutic specificity and safety.

8. LIST OF KEYWORDS

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

9. PUBLICATIONS



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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., Karuppiyah, V.: A CD1c lipid agnostic T cell receptor bispecific engager redirects T cells against CD1c+ cells.
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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.
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