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Generation of CD1 tetramers as a tool to monitor glycolipid-specific T cells

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CD1 molecules are β_2m-associated HLA class-I-like glycoproteins which have the unique ability to present glycolipid and phospholipid antigens to specific T lymphocytes. To study the biology of CD1 and its role in human disease we developed novel techniques for generation of recombinant CD1/lipid complexes by *in vitro* refolding. Fluorescent tetrameric complexes made from soluble recombinant CD1d/α-galactosylceramide complexes allowed highly sensitive and specific *ex vivo* and *in vitro* detection and functional characterization of novel human T-lymphocyte populations. Furthermore, protein crystals were obtained from soluble recombinant CD1b/β_2m-proteins loaded either with phosphatidylinositol or ganglioside GM2, which led to the first atomic structure determination of a CD1/lipid complex. The analysis of these crystal structures clarified how CD1b molecules can bind lipid ligands of different size, and revealed a broader spectrum of potential CD1b ligands than previously predicted.

Keywords: CD1; glycolipids; antigen presentation; T lymphocytes; α-galactosylceramide

1. INTRODUCTION

A recent major breakthrough in cellular immunology was the discovery that T lymphocytes cannot only respond to peptide antigens (Townsend *et al.* 1986), but are also capable of specifically recognizing glycolipid and phospholipid antigens (Porcelli 1995). While peptide antigens are presented to T lymphocytes by the highly polymorphic families of HLA class I and HLA class II molecules, lipid antigens are presented to T lymphocytes by the non-polymorphic HLA class-I-like CD1 molecules. *In vitro* binding studies allowed the definition of the molecular mechanism for lipid antigen presentation by CD1: while the alkyl chains of a lipid–ligand bind within a highly hydrophobic groove inside the CD1 protein, the polar head group (e.g. a glycan moiety) remains exposed on top of the extracellular domain, therefore allowing direct contact with the T-cell receptor (Beckman *et al.* 1994; Moody *et al.* 1997; Shamshiev *et al.* 1999). The five human CD1 proteins segregate into two groups according to their predicted amino-acid sequence homology: group 1 contains CD1a, CD1b, CD1c and CD1e molecules; and group 2 contains CD1d (Porcelli & Modlin 1999). Until recently it was thought that any ligand for CD1a, CD1b and CD1d molecules must contain two alkyl chains, while CD1c could bind single alkyl chain containing polyprenols.

Experimental evidence now indicates that CD1 group 1 and group 2 molecules serve different biological functions: group 1 CD1 proteins are absent in mice and rats, while CD1d is highly conserved in all mammals studied to date. Group 1 CD1 molecules have been shown to present pathogen-derived lipids (table 1), such as glucomonomycolates of mycobacteria, as well as endogenous phospholipids and gangliosides. T-lymphocytes recognizing lipids on group 1 CD1 molecules exhibit strong cytolytic functions. Conversely, the only ligands so far identified for CD1d include endogenous GPI and phosphatidylinositol, as well as αGC, a phytosphingosine derived from marine sponges (table 1). Interestingly, CD1d presents a still unknown endogenous ligand to a highly specialized T-lymphocyte subset expressing an invariant TCR variable alpha chain as well as natural killer cell receptors: NKT invariant cells (NKT^{inv} cells) (Porcelli & Modlin 1999; Kronenberg & Gapin 2002). Human NKT^{inv} cells use an invariant TCR Vα24-JαQ chain in association with variant TCR Vβ11 chains (Davodeau *et al.* 1997), while mouse NKT^{inv} cells use a homologue invariant TCR Vα14-Jα281 chain (Lantz & Bendelac 1994). Synthetic αGC acts as a potent mimetope of the CD1d-presented ligand of natural NKT^{inv} cells and is widely used for functional studies of NKT^{inv} cells. Another particularity of NKT^{inv} cells is their unique degree of cross-species specificity suggesting an important biological role conserved through evolution: human NKT^{inv} cells can specifically recognize mouse CD1d/αGC complex and mouse NKT^{inv} cells can specifically recognize human CD1d/αGC complex. Compelling

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Table 1. Lipid antigens presented by CD1 molecules

antigen	CD1 restriction	reference
glucose monomycolate	CD1b	Moody *et al.* (1997)
lipoarabinomannan	CD1b	Sieling *et al.* (1995)
phosphatidylinositol mannosides	CD1b	Apostolou *et al.* (1999)
hexosyl-1-phosphoisoprenoid	CD1c	Moody *et al.* (2000)
α-GalCer	CD1d	Naidenko *et al.* (1999)
glycosyl phosphatidylinositols	CD1d	Gumperz *et al.* (2000)
GM1	CD1b	Shamshiev *et al.* (1999)
sulphatide	CD1a, b, c	Shamshiev *et al.* (2002)

evidence now indicates that NKT^{inv} cells play a key role in immune regulation. Upon stimulation, NKT^{inv} cells rapidly secrete large amounts of cytokines (Kronenberg & Gapin 2002) resulting in the activation of other immune cells, such as natural killer cells or B lymphocytes. NKT^{inv} cells are also attributed an important role in tolerance induction. Consistent with such a role is the fact that numerical and functional NKT^{inv} cell deficiencies are associated with autoimmune phenomena in mice and humans (Sumida *et al.* 1995; Wilson *et al.* 1998).

2. GENERATION OF CD1 TETRAMERS

To understand the biological roles of CD1 restricted T cells, it would be very helpful to have tools to determine their frequency, specificity and phenotype. Up until recently, antigen-specific T lymphocytes had been identified mainly by functional assays—cytokine secretion, cytotoxic activity or proliferation—usually performed after culture of the cells *in vitro*. Over the past 5 years, the development of tetrameric MHC class-I–peptide complexes (tetramers) has changed radically the way that immunologists monitor T-cell responses (Klenerman *et al.* 2002). Typically, MHC class I tetramers are constructed by refolding the soluble recombinant form of the MHC class I heavy chain and β_2m obtained from bacterial inclusion bodies in the presence of a single peptide, yielding homogeneous loaded MHC class I molecules. While this protocol has been successful for the construction of MHC class I tetramers, attempts to refold CD1 molecules have so far failed, possibly due to the much higher content of hydrophobic amino acids in CD1 than MHC class I molecules, and the hydrophobic nature of the glycolipid antigens that bind to CD1 molecules. Over the past 2 years, two protocols have been described for the generation of CD1d tetramers: the first one is based on the use of insect cell derived CD1d molecules (Benlagha *et al.* 2000; Matsuda *et al.* 2000), while the second is based on the use of denatured CD1d molecules refolded *in vitro* using oxidative refolding chromatography (Karadimitris *et al.* 2001). Transfection of *Drosophila melanogaster* (fruitfly) cells with cDNA encoding CD1d alpha1, alpha2 and alpha3 domains and mouse β_2m cDNA resulted in the secretion of soluble folded CD1d molecules, that were biotinylated, complexed with streptavidin and loaded *in vitro* with αGC (Benlagha *et al.* 2000; Matsuda *et al.* 2000). Although this protocol has been used to detect and purify human and mouse NKT cells (Benlagha *et al.* 2000; Matsuda *et al.* 2000), a potential disadvantage to this strategy is that insect cells may have endogenous lipid loaded into their groove, which may reduce the efficiency of staining of T cells. To overcome this potential problem, we developed an alternative protocol for the generation of CD1d/glycolipid tetramers, based on the use of fully denatured CD1d molecules refolded *in vitro* using oxidative refolding chromatography and loaded with a single ligand species (Karadimitris *et al.* 2001). The refolding of CD1 heavy chains with β_2m from inclusion bodies by oxidative refolding chromatography requires the addition of three chaperones immobilized on agarose beads, which offers the great advantage of easy separation of the refolded protein. The bacterial chaperone GroEL, which prevents protein aggregation, a protein disulphide isomerase, which promotes correct disulphide bond formation, and a peptidyl–prolyl *cis–trans* isomerase were immobilized on agarose beads and used to aid refolding of denatured human CD1 molecules in the presence of human β_2m and αGC (Karadimitris *et al.* 2001). The use of CD1d tetramers provided an opportunity to analyse the ontogeny of mouse NKT cells (Benlagha *et al.* 2002) and more recently to identify a new population of T cells capable of recognizing CD1d/αGC (Gadola *et al.* 2002*a*), which unlike canonical NKT cells, used a broader range of Vα and Vβ TCR chains (Gadola *et al.* 2002*a*). These results have challenged the currently held view that recognition of CD1d/αGC is limited to NKT cells expressing the invariant TCR Vα24-JαQ^{+}Vβ11^{+} in humans and support a model of NKT development based on antigen driven selection.

More recently, we have developed an additional protocol to refold CD1 molecules, making use of short single alkyl chain detergents to protect exposed CD1 hydrophobic surfaces during early refolding stages. Soluble CD1b/β_2m monomers were refolded in the presence of either phosphatidylinositol or ganglioside GM2 plus detergent. The use of this protocol yielded stable soluble CD1b/β_2m complexes, that were confirmed by mass spectrometry to be loaded with the specified lipid ligands plus detergent. The resultant CD1b–glycolipid complexes were crystallized and the three-dimensional structures determined (Gadola *et al.* 2002*b*).

These structures have clarified the ligand-binding mechanisms of CD1b by describing the presence of a network of four interlinked hydrophobic channels (A′, C′ and F′ channels plus a tunnel T′), which provide a unique adaptable system required to present a broad range of ligands. We demonstrated that CD1b molecules can bind long lipid chains by accommodating alkyl chains of up to 70 carbons in length in a super-channel formed by the connection of A′ T′ and F′. The C′ pocket can accommo-

date shorter alkyl chains of about 16 carbons. An exit portal for the C′ pocket, located below the α2 helix, indicates that longer chains may also be accommodated.

To date, lipids containing two alkyl chains were thought to be the only ligands capable of binding to CD1b. The recently solved CD1b structures unexpectedly revealed that monoalkyl and tri-alkyl chain ligands can specifically bind to the CD1b groove, greatly extending the spectrum of CD1 ligands and providing insights on the mechanisms controlling *in vivo* refolding of CD1b molecules.

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GLOSSARY

αGC: α-galactosylceramide
GPI: glycosyl phosphatidylinositol
MHC: major histocompatibility complex
TCR: T-cell receptor