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Review

CD1 tetramers: a powerful tool for the analysis of glycolipid-reactive T cells

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Abstract

CD1 proteins constitute a third class of antigen-presenting molecules. They bind lipids rather than peptides, and the T cells reactive to lipids presented by CD1 have been implicated in the protection against autoimmune diseases and infectious microorganisms and in the immune surveillance for tumors. Thus, the ability to identify, purify, and track the response of CD1-reactive cells is of paramount importance. Previously existing methods for identifying these T cells were not based on TCR specificity, and therefore the data obtained by these methods were in some cases difficult to interpret. The recent generation of tetramers of α-galactosyl ceramide (α-GalCer) with CD1d has already permitted significant insight into the biology of NKT cells. Tetramers constructed from other CD1 molecules also have been obtained during the previous year. Collectively, these new reagents promise to greatly expand knowledge of the functions of lipid-reactive T cells, with potential use in monitoring the response to lipid-based vaccines and other treatments and in the diagnosis of autoimmune diseases.

Keywords: CD1 tetramers; Lipid-reactive T cells; α-GalCer

1. Introduction

CD1 proteins are cell surface glycoproteins expressed as approximately 50-kDa glycosylated heavy chains that are noncovalently associated with β2-microglobulin (β2m) (Porcelli, 1995). The human CD1 gene family contains consists of five genes, *CD1A* through *CD1E*; there are only two highly similar genes in mice, called *CD1D1* and *CD1D2*. On the basis of their predicted amino acid sequence homologies, the members of the CD1 family can be divided into two groups (Calabi and Milstein, 2000). CD1a, -b, and -c form group I and human, mouse, rat, and rabbit CD1d form group II. Although CD1 proteins display some unique characteristics, they are distantly related to MHC class I and class II molecules, and they are considered to constitute a conserved third class of antigen-presenting molecules. CD1 proteins are encoded by genes outside the MHC locus. Unlike classical antigen-presenting molecules encoded in the MHC, they are nonpolymorphic, their surface expression does not require a functional transporter associated with antigen processing (TAP) (Por-

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celli et al., 1992; Hanau et al., 1994; Brutkiewicz et al., 1995; Beckman et al., 1996; Teitell et al., 1997), they localize to endosomes as well as the cell surface (Sugita et al., 1996; Prigozy et al., 1997; Brossay et al., 1998b; Jackman et al., 1998), and they are expressed by cells in a limited number of tissues, primarily bone marrow-derived cells (Porcelli and Modlin, 1999). So far, the only available crystal structure of a CD1 molecule is the one of the mouse CD1d (mCD1d) protein (Zeng et al., 1997). Although this structure confirms the overall similarity of CD1 proteins to MHC class I and class II antigen-presenting molecules, there are significant features that distinguish the antigen-binding groove of CD1. The mCD1d groove is relatively narrow, deep, and highly hydrophobic, and it has two deep binding pockets instead of the several shallow pockets described for the classical MHC-encoded antigen-presenting molecules (Zeng et al., 1997). Based upon their amino acid sequences, the binding groove of the other CD1 molecules is also predicted to be highly hydrophobic. Such a hydrophobic groove provides an ideal environment for the binding of lipid antigens. Indeed, CD1 molecules can present a variety of lipid antigens (Beckman et al., 1994; reviewed in Porcelli and Modlin, 1999). Group I CD1 molecules present microbial glycolipids derived from the mycobacterial cell wall, as well as autologous glycolipids (reviewed in Burdin and Kronenberg, 1999; Gumperz and Brenner, 2001; Kronenberg et al., 2001; Matsuda and Kronenberg, 2001). So far, no natural, immunogenic ligands for group II CD1 molecules have been unequivocally identified. Attempts to directly elute compounds from purified mCD1d have identified glycosylphosphatidylinositol (GPI) as a major CD1d-associated component (Joyce et al., 1998). While it has been proposed that GPI anchors are important for the stimulation of CD1d-reactive T cells, these data remain controversial (Schofield et al., 1999; Molano et al., 2000; Park and Bendelac, 2000; Pied et al., 2000).

Despite some difficulty in identifying natural lipid antigens presented by CD1d, several years ago mCD1d and human CD1d were shown to present alpha-galactosyl ceramide (α-GalCer), providing the first evidence for a lipid antigen presentation by group II CD1 molecules (Kawano et al., 1997; Brossay et al., 1998a; Burdin et al., 1998; Couedel et al., 1998; Spada et al., 1998; Nieda et al., 1999). α-GalCer is derived from a marine sponge (Natori et al., 1994), and it was obtained as part of a screen for compounds that could prevent the metastases of transplanted tumors to the liver. Although it is a glycosphingolipid, its structure is distinguished from other natural compounds in this class by the α linkage of the 1′ carbon of the sugar to the 1 carbon of the sphingosine. Compounds with this α-linked structure have not been found in abundance elsewhere, and therefore α-GalCer is not considered to be the natural ligand for CD1d-reactive T cells.

T cells that recognize CD1 were initially identified as a unique population of lymphocytes that did not require MHC class I or class II for their selection and activation (Porcelli et al., 1989; Bendelac et al., 1995; Cardell et al., 1995). They are also distinct from T cells reactive with MHC-encoded antigen-presenting molecules by the fact that they can express either the CD4 or CD8 co-receptors, or they can be double negative (DN) cells devoid of both CD4 and CD8 (Porcelli et al., 1989; Cardell et al., 1995; Stenger et al., 1997; Rosat et al., 1999). Even if the size and the diversity of the CD1-reactive population is not yet fully characterized, these lymphocytes can be divided in three broad categories, according to their specificity and their T cell receptor (TCR) usage (Kronenberg et al., 2001). The first category of CD1-reactive T lymphocytes is formed by cells that are reactive to microbial lipids presented by group I CD1 molecules (Beckman et al., 1996; Porcelli and Modlin, 1999; Moody et al., 2000; Matsuda and Kronenberg, 2001). These T cells express highly diverse αβ TCRs and they have been implicated in host defense against mycobacteria (Porcelli and Modlin, 1999; Moody et al., 2000). CD1-reactive T cells in the second category generally also have diverse αβ TCRs, although some can bear the alternative γδ TCR isotype. These T lymphocytes are reactive to autologous glycolipids presented by group I or group II CD1 molecules. In most cases the CD1-bound lipids recognized by these T cells are unknown, but in one set of studies, brain-derived lipids, such as sulfatides and gangliosides, have been shown to be recognized (Shamshiev et al., 1999, 2000). The function of CD1-autoreactive T cells with diverse TCRs is unknown, but they may be involved in immune regulation or in the pathogenesis of autoimmune disease. For example, it has been

shown that the frequency of T cells reactive to autologous glycolipids is increased in the blood of multiple sclerosis (MS) patients, and that these cells produce Th1 cytokines (Shamshiev et al., 1999, 2000), suggesting they could be important for MS pathogenesis. The third category of CD1-restricted T cells is constituted by the CD1d-dependent natural killer T cells (NKT) that have a limited TCR diversity. Cells in this subpopulation are activated by α-GalCer presented by CD1d molecules (Kawano et al., 1997; Brossay et al., 1998a; Burdin et al., 1998; Couedel et al., 1998; Spada et al., 1998; Nieda et al., 1999). These NKT cells represent a distinct lymphocyte sublineage (Bendelac et al., 1997; Elewaut and Kronenberg, 2000). In contrast to conventional αβ T cells, their TCR repertoire is highly restricted. In mice, they express an invariant Vα14–Jα18 rearrangement, paired with Vβ8, Vβ7, or Vβ2 (Lantz and Bendelac, 1994; Bendelac et al., 1996). A similar CD1d-dependent NKT cell population is found in humans, and its TCR is composed of an invariant Vα24-JαQ rearrangement paired with Vβ11. These V genes are highly similar to the rearrangements found in CD1d-dependent mouse NKT cells (Porcelli et al., 1993, 1996; Dellabona et al., 1994; Lantz and Bendelac, 1994; Bendelac et al., 1996). Human and mouse NKT cells are the largest population of CD1-reactive T cells identified in vivo so far (Bendelac et al., 1995). In mice, NKT cells represent up to 30% of the total lymphocytes in the liver, 20% of the αβ T cells in the bone marrow, and 3% of αβ T cells in the spleen (Benlagha and Bendelac, 2000; Godfrey et al., 2000). The activation of mouse NKT cells propagates rapidly to other cell types, among which are NK cells, dendritic cells, and subsets of B and conventional T cells. This NKT cell mediated activation is accompanied by the induction of both co-stimulatory molecules and cytokines by the responding cell types (Burdin et al., 1999; Carnaud et al., 1999; Eberl and MacDonald, 2000; Godfrey et al., 2000). Indeed, the quick and vigorous response of NKT cells, which in terms of its kinetics is more similar to an innate rather than an adaptive immune response (Eberl and MacDonald, 1998; Benlagha and Bendelac, 2000; Godfrey et al., 2000; Matsuda et al., 2000), may allow NKT cells to regulate adaptive immunity in several contexts, including the protection against autoimmune diseases (Gombert et al., 1996; Hammond et al., 1998; Wilson et al., 1998), parasites (Denkers et al., 1996; Schofield et al., 1999; Pied et al., 2000) bacteria (Emoto et al., 1997; Flesch et al., 1997), and in anti-tumor responses (Cui et al., 1997; Kawano et al., 1998).

Studies of NKT cell development and function have been significantly hampered by the lack of antibodies that can be used in flow cytometry or immunohistochemistry for the unambiguous identification of these cells. Most inbred mouse strains do not express the NKRP1.1 allele recognized by the anti-NK1.1 mAb, PK136 (Yokoyama and Seaman, 1993), the single mAb available for detecting NKRP1 in mice. Furthermore, no reliable mAb against the invariant Vα14 chain of the NKT cell TCR has been generated. In addition, in NK1.1-positive strains, such as C57BL/6, NK1.1^{+}, T cells that neither express Vα14 TCR nor are CD1d-dependent have been found in various tissues (Eberl et al., 1999). Finally, CD1d-autoreactive Vα14–Jα281-expressing hybridomas have been derived from NK1.1^{-} lymphocytes (Chiu et al., 1999), indicating that some ‘‘NKT cells,’’ defined by expression of Vα14 and CD1d reactivity, may not express NK1.1.

To overcome these difficulties, we and others took advantage of the fact that the majority of mouse NKT cells recognize α-GalCer in the context of CD1d molecules (Kawano et al., 1997; Burdin et al., 1998). Indeed, this almost universal recognition of a single antigenic target by many NKT cells suggested that a similar approach to the one employed for staining conventional T cells with tetramers of peptide–MHC complexes (Altman et al., 1996) might be employed to identify CD1d-reactive NKT cells, even in the absence of any prior immunization or stimulation. In this article, we will review the different protocols used for the production of tetramers containing CD1 molecules, we will then present some of the data generated by the use of α-GalCer-loaded CD1d tetramers, which already has provided insight into the biology of NKT cells.

2. Production of tetrameric complexes of α-GalCer with CD1d

In the original methodology for the production of tetramers of antigen-presenting molecules (Altman et

al., 1996), the construct encoding the MHC class I heavy chain was modified by adding a 15-amino acid sequence at the COOH terminus, which served as a substrate for site-specific biotinylation using the BirA enzyme (Schatz, 1993). This site-specific biotinylation correctly orients the MHC molecule in the tetramer, as the binding site for peptide antigen, and the TCR is located at the opposite NH_2 terminus of the protein. Furthermore, this method avoids the potential inactivation of the protein by modification of critical lysines, as might occur using chemical biotinylation methods. The generation of MHC class I tetramers was then achieved by refolding of the soluble recombinant forms of the MHC class I heavy chain and β2m, obtained from bacterial inclusion bodies. Refolding was carried out in the presence of a single peptide, thereby generating native class I molecules homogeneously loaded with a single peptide (Altman et al., 1996). After biotinylation, tetramers were formed by incubating biotinylated peptide–MHC complexes with phycoerythrin-labeled avidin.

This method, however, has not been successful in most cases for the generation of either peptide–MHC class II tetramers or α-GalCer-loaded CD1d tetramers (Matsuda et al., 2000). Concerning α-GalCer–CD1d tetramers, at least two hypotheses may explain this failure. First, the most probable cause is the highly hydrophobic nature of the antigen. Hydrophobic lipid ligands are likely to aggregate in aqueous solutions, and thus will be unavailable for loading into CD1. The second hypothesis is that CD1d heavy chain may have some special requirements for folding with β2m. In the case of the construction of MHC class II tetramers, to overcome difficulties in peptide loading and refolding the peptide has been genetically linked to the class II molecule via a flexible spacer (Crawford et al., 1998). This alternative obviously cannot be employed, however, for the construction of tetramers with nonpeptide antigens. Therefore, we and another research group have adopted an alternative strategy for CD1d tetramer production, using native mCD1d molecules produced in insect tissue culture cells (Benlagha et al., 2000; Matsuda et al., 2000). In those studies, two different cell lines, *Drosophila melanogaster* SC2 cells and High Five™ cells, have been used for protein production.

The *D. melanogaster* SC2 expression system has been widely used for the production of several MHC class I and class II molecules, as well as CD1d–β2m heterodimers (Jackson et al., 1992; Matsumura et al., 1992; Castaño et al., 1995; Scott et al., 1996; Garcia et al., 1997). To do this, β2m had been cloned adjacent to the inducible metallothionein promoter in the pRHMa-3 vector (Bunch et al., 1988). A soluble mCD1d protein lacking the transmembrane segment and intracytoplasmic domains was engineered with a substrate for BirA site-specific biotinylation at the COOH terminus (Benlagha et al., 2000). The modified mCD1d heavy chain was cloned into the pRHMa-3/6His expression vector, which places a 6 histidine tag at the COOH terminus. *D. melanogaster* SC2 cells were then co-transfected by the calcium phosphate precipitation method with the heavy chain and β2m expression constructs, along with pUChsneo, which confers the neomycin resistance (Benlagha et al., 2000). After 3 weeks of G-418 selection, resistant cell lines were treated for 3 days with 0.7 mM $CuSO_4$ to induce the metallothionein promoter, and then tested for expression. Individual clones were then cloned by limiting dilution and selected for a high expression level. For large-scale protein production, the selected clone is expanded in 10–12 l of culture medium and then expression is induced.

Like the *D. melanogaster* SC2 expression system, the baculovirus expression system has proven useful for the production of MHC class II tetramers (Crawford et al., 1998). To successfully make mCD1d tetramers, the heavy chain was modified to include a substrate for the BirA site-specific biotinylation, and a 6 histidine tag for purification, as described above for the *Drosophila* cell expression system (Matsuda et al., 2000). The modified mCD1d heavy chain was cloned under the control of the polyhedrin promoter of the dual promoter baculovirus transfer vector pBacp10H (Matsuda et al., 2000). β2m was cloned under the control of the p10 promoter of the same vector. The mCD1d–β2m transfer vector was co-transfected with linearized BaculoGold™ baculovirus DNA (Invitrogen) into High Five™ (BTI-TN-5B1-4) (Invitrogen) cells using the Lipofectin Reagent® (Gibco/BRL). After 5 days of infection, recombinant viruses were collected, amplified, and cloned by limiting dilution. The virus with highest expression level of mCD1d was then used for protein production. Routinely,

soluble protein was produced by infecting 40–60 175 cm^2 flasks (Nalgene) containing around 20×10^6 adherent High Five™ cells each, at a multiplicity of infection of 10.

Supernatants from induced *D. melanogaster* SC2 cells or baculovirus-infected cells were then harvested by centrifugation. The media were then concentrated by either tangential flow (Benlagha et al., 2000), or concentrated and dialyzed against 0.15 M sodium phosphate buffer (pH 7.4) using an Amicon Y1S10 Spiral Wound Membrane Cartridge (Millipore) (Matsuda et al., 2000). mCD1d–β2m heterodimers were purified by Ni-NTA-agarose chromatography. As shown in Fig. 1, after this one-step affinity purification, mCD1d–β2m heterodimers are almost pure. The remaining contaminants were then eliminated by anion exchange chromatography on a monoQ column (Amersham Pharmacia Biotech) (Fig. 1). The purified proteins were extensively dialyzed against 10 mM Tris–HCl pH 7.4 and concentrated to 1 mg/ml using a YM30 concentrator (Millipore). BirA enzymatic biotinylation of mCD1d was carried out overnight at 30 °C, according to the manufacturer's protocol (Avidity) (Benlagha et al., 2000; Matsuda et al., 2000). The degree of biotinylation was analyzed by Western blot using HRP-conjugated streptavidin (Jackson Laboratories) (Fig. 2). The free remaining biotin was then eliminated by extensive dialysis against phosphate-buffered saline (PBS), pH 7.4. An average of 1–1.5 mg of biotinylated purified protein could be obtained from forty to sixty 175-cm^2 flasks. The mCD1d–β2m heterodimers in solution in PBS pH 7.4 are stable at 4 °C for over 3 months. Indeed, we recommend production of 1-mg batches of mCD1d–β2m heterodimers at a time, because this quantity is sufficient for the preparation of large amounts of α-GalCer–

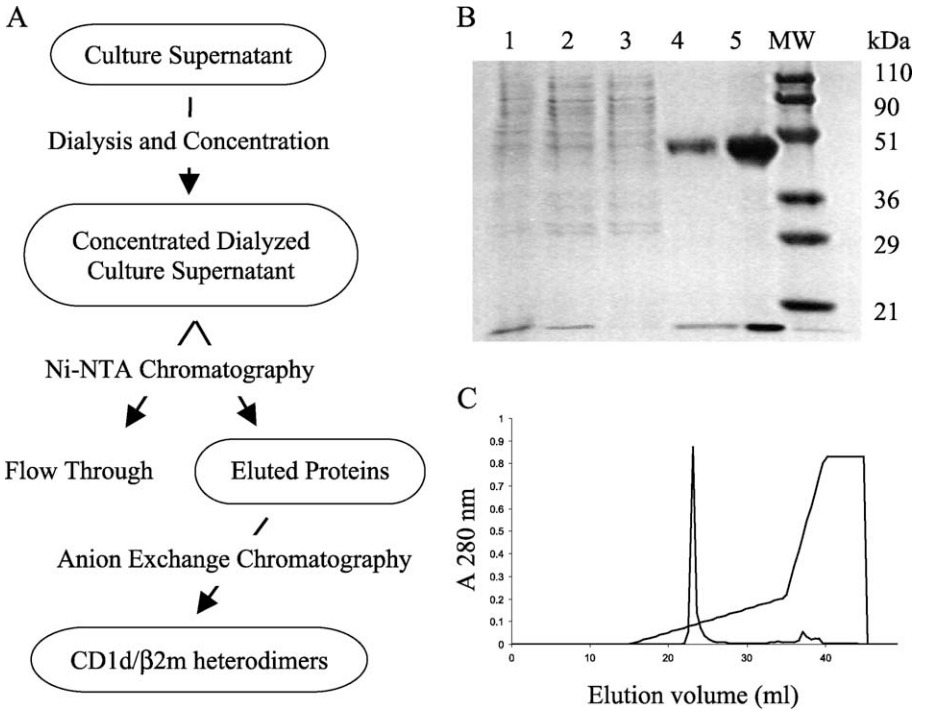

Fig. 1. Purification of native mouse CD1d molecules using insect tissue culture cells. (A) Protocol for purification of CD1d–β2m heterodimers. The encircled fractions are the ones containing CD1d–β2m heterodimers. (B) SDS-PAGE analysis of the purification of CD1d–β2m heterodimers. 1 = culture supernatant; 2 = concentrated dialyzed culture supernatant; 3 = Ni-NTA column flow through; 4 = eluted proteins; 5 = purified CD1d–β2m heterodimers after anion exchange chromatography; MW = molecular weight markers. (C) Chromatogram showing the final purification step of CD1d–β2m heterodimers on anion exchange column.

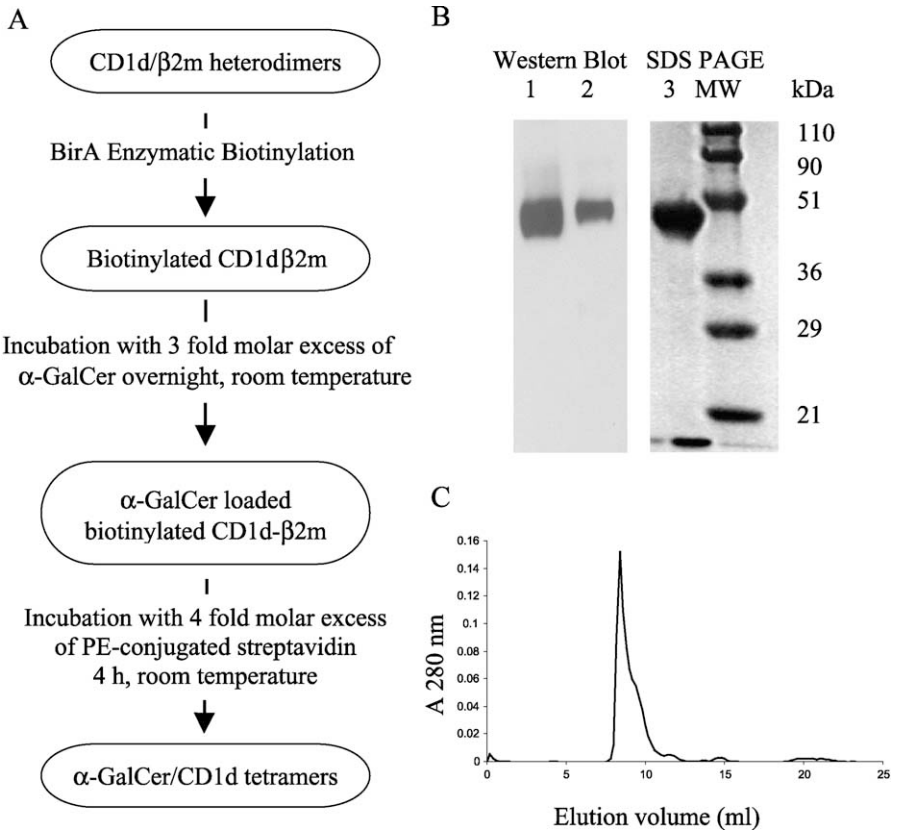

Fig. 2. Production of α-GalCer–CD1d tetramers. (A) Protocol for production of α-GalCer–CD1d tetramers. The encircled fractions are the ones containing CD1d–β2m heterodimers or α-GalCer–CD1d tetramers. (B) SDS-PAGE and Western blot analysis of the biotinylation of CD1d–β2m heterodimers. 0.4 μg (lane 1), 0.2 μg (lane 2), or 5 μg (lane 3) of biotinylated CD1d–β2m heterodimers were run on a 12% polyacrylamide gel in reducing conditions. Lane 3 was Coomassie blue stained while lanes 1 and 2 were transferred on nitrocellulose membrane and revealed using HRP-conjugated streptavidin.

mCD1d tetramers for analytical purposes. A similar method has been used to produce tetramers of human CD1d (Metelitsa et al., 2001).

Stable binding of α-GalCer to CD1d is a prerequisite for tetramer staining of α-GalCer-reactive T cells. Using chase experiments, Benlagha et al. (2000) estimated the half-life of the α-GalCer–mCD1d complex to be more than 12 h. This seems to be in disagreement with the report published by Naidenko et al. (1999), which estimated the half-life of a monomeric α-GalCer–mCD1d complex to be approximately 6 min from surface plasmon resonance studies in a BIAcore instrument. This discrepancy, however, might be explained by basic differences in the design of the experiments. Indeed, the acyl chain of the α-GalCer used in the BIAcore studies had been modified to introduce a biotin group, by which the compound can be immobilized on the sensor chip surface. It is likely, however, that this modified acyl chain may be at least partially excluded from interaction with groove of CD1d, leading to a significantly shorter half-life of the bound antigen. Another fundamental difference is that BIAcore studies uses dynamic conditions, while the chase experiments are performed at a steady state. Finally, the excess α-GalCer is not likely to have been removed completely in the chase experiment, In any event, it is clear that α-GalCer can interact with mCD1d with a

sufficiently high affinity (Naidenko et al., 1999; Benlagha et al., 2000), allowing for the formation of lipid-CD1d complexes that can detect α-GalCer-reactive T cells.

In order to load CD1d molecules with α-GalCer, soluble biotinylated CD1d–β2m heterodimers are incubated for 12–18 h at room temperature with a threefold molar excess of α-GalCer, dissolved in 0.5% Tween 20, 0.9% sodium chloride. Some of the excess of free α-GalCer can be removed by dialysis (Benlagha et al., 2000), but this constitutes an optional step (Matsuda et al., 2000), and the removal of lipid antigen in bilayers or micelles may not be efficient. The addition of α-GalCer does not alter the stability of the CD1d–β2m heterodimers. One-milligram batches of CD1d–β2m heterodimers can be loaded with α-GalCer and stored at 4 °C. Alternatively, small batches of α-GalCer-loaded CD1d can be made for each individual tetramer preparation. It has been shown that other lipids, such as β-GalCer, the ganglioside GM1 or the phospholipid dipalmitoyl phosphatidyl ethanolamine bind to CD1d with an affinity comparable to α-GalCer (Naidenko et al., 1999; Benlagha et al., 2000), this approach should be used to load CD1d–β2m heterodimers with additional ligands.

Tetramers are generated by mixing α-GalCer-loaded CD1d monomers with fluorochrome-labeled streptavidin in a molar ratio of 5:1 (Benlagha et al., 2000) or 4:1 (Matsuda et al., 2000). After incubation at room temperature for 4 h, the mixture can be used without further purification (Benlagha et al., 2000; Matsuda et al., 2000). As shown in Fig. 2, the preparation contains predominantly α-GalCer–CD1d tetramers, although lower molecular weight products still remain. Their presence, however does not interfere with the staining, and mCD1d dimers also can detect NKT cells, although less efficiently than tetramers (Naidenko et al., 1999). The quantity of tetramer per staining may be adjusted for each kind of experiment. Nevertheless, it is possible to obtain a good staining with a concentration of tetramer as low as the equivalent of 10 nM of CD1d.

A potential disadvantage of this strategy is that insect cell-derived CD1d molecules might have a lipid loaded in their antigen-binding groove. Indeed, because the binding of lipid ligands to CD1d is fairly stable (Benlagha et al., 2000; S. Sidobre unpublished), it is likely that at least a portion of the insect cell-derived CD1d monomers are loaded with a ligand they acquired during their intracellular folding and trafficking. This is consistent with the analysis of the electron density map of the crystallized mCD1d produced in the *D. melanogaster* SC2 expression system, which shows the presence of a ligand occupying the groove (Zeng et al., 1997). Thus, the presence of this hydrophobic compound might prevent uniform loading of CD1d by α-GalCer. CD1d tetramers that were not loaded with α-GalCer, however, do not stain NKT cells (Fig. 3), and α-GalCer-loaded tetramers, constructed using insect cell-derived CD1d, have proven to be a powerful tool for the identification of NKT cells (Benlagha et al., 2000; Matsuda et al., 2000). This might be due to the high affinity of the invariant NKT cell TCR for the α-GalCer–CD1d complex (Sidobre et al., in preparation). The extent to which the insect cell-derived CD1d molecules become loaded with α-GalCer is not known. However, the increased efficiency of tetramer binding, as compared to α-GalCer-loaded CD1d dimers, suggests that a significant portion of the CD1d molecules produced can be loaded with α-GalCer.

The required replacement of an insect cell-derived ligand bound to native CD1d, however, could be a serious issue when antigens other than α-GalCer are used or when group I CD1 molecules are studied. To overcome this potential problem, a new strategy called oxidative refolding chromatography has been used to refold CD1a, -b, and -d molecules from bacterial inclusion bodies that are uniformly loaded with different glycolipids (Altamirano et al., 2001; Karadimitris et al., 2001). Indeed, successful refolding was achieved for CD1a and -b loaded with ceramide galactoside 3-sulfate and monoasialoganglioside, respectively (Altamirano et al., 2001). Human CD1d was also refolded in the presence of α-GalCer, β-GalCer, α-mannosylceramide, or the ganglioside GM1 (Karadimitris et al., 2001). In this method, the refolding was facilitated by the use of a ternary refolding matrix. The matrix was composed of an equimolar mixture of agarose-gel bead immobilized prokaryotic chaperones. These include miniGroEL, DsbA, and a peptidyl-prolyl *cis–trans* isomerase (PPI). MiniGroEL, the apical domain of

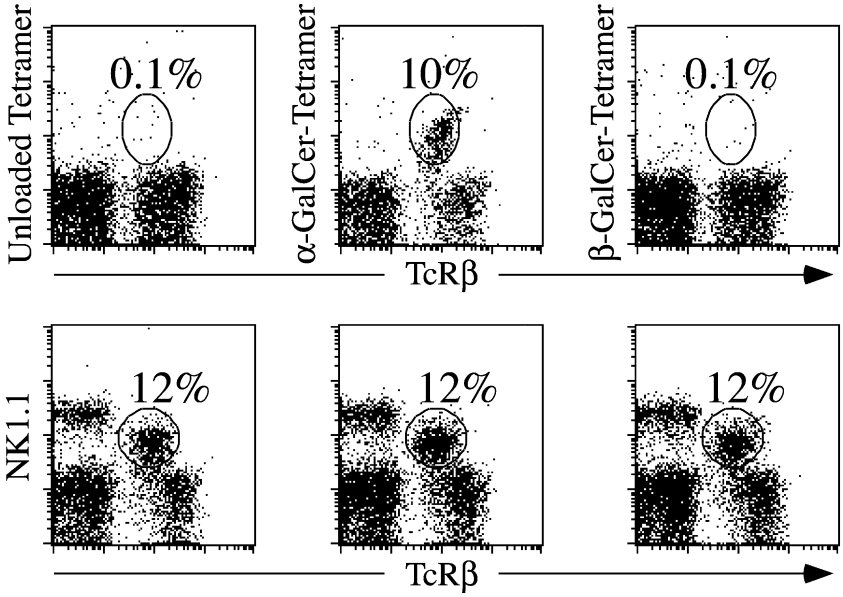

Fig. 3. α-GalCer–mCD1d tetramers, but not β-GalCer–mCD1d tetramers, stain NKT cells in the liver of C57Bl/6 mice. Representative staining of NKT cells in the liver of C57Bl/6 mice using a combination of TCR-β, NK1.1 mAb (lower panel), or TCR-β mAb and unloaded (left), α-GalCer-loaded (center), and β-GalCer-loaded (right) mCD1d tetramers (upper panel).

the bacterial chaperone GroEL, prevents hydrophobic interactions of early folding intermediates and therefore prevents protein aggregation. DsbA and PPI catalyze native disulfide bond formation and proline isomerization, respectively (Karadimitris et al., 2001). To refold CD1a, -b, and -d, heavy chains and β2m were produced in an *Escherichia coli* expression system, leading to their overexpression as inclusion bodies. The inclusion bodies inclusion were then solubilized in freshly prepared 6 M guanidinium hydrochloride, 0.1 M dithiothreitol in 0.1 M potassium phosphate buffer pH 8.0. The denatured/reduced heavy and light chains are mixed (1:3) and immediately added slowly into a stirring suspension of the ternary refolding matrix equilibrated in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.3 M L-arginine HCl, 8 mM oxidized glutathione, 1 mM ethylediaminetetraacetic acid (EDTA), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), to a final dilution of 1/100 (Altamirano et al., 2001). The experiment is designed to obtain a final 100-fold molar ratio excess of chaperones and foldases relative to the mixture of heavy and light chains. To insure the uniform loading of the ligand, a 10-fold molar excess of glycolipid is added to the stirring suspension of the ternary refolding matrix, prior to the addition of the mixture of heavy and light chains. After incubation times as brief as 6 min at 4 °C, the mixture is centrifuged ($< 1000 \times g$ for 5 min) and the supernatant is concentrated using a dialysis membrane covered with D-trehalose and Ultrafree-15 centrifugal filter devices (Millipore). Because the CD1 heavy chains were cloned in the pET-23d vector that introduces a BirA biotinylation site at the COOH terminus of the heavy-chain proteins, the concentrated proteins were directly enzymatically biotinylated (Avidity). Monomeric, biotinylated CD1–β2m complexes were then separated by gel filtration chromatography on a Superdex 75 column (Amersham Pharmacia Biotech). This procedure leads to the production of up to 1 mg of monomeric biotinylated CD1–β2m complexes from a 1-l refolding mixture (Stephan Gadola, personal communication). Those biotinylated monomers were then multimerized using fluorochrome-labeled streptavidin (Karadimitris et al., 2001). Interestingly, this procedure allows for refolding of CD1a and CD1b in the absence of added lipid

ligands (Altamirano et al., 2001), suggesting that empty CD1 molecules might be produced and available for loading on the cell surface. These empty molecules require 60 mM guanidinium hydrochloride in order to be stable (Altamirano et al., 2001), however, and therefore it remains to be determined if empty or unloaded group I CD1 molecules exist under physiologic conditions.

3. Specificity of α-GalCer-loaded CD1d tetramers

The specificity of the α-GalCer-loaded mCD1d tetramers has been evaluated by staining T cell hybridomas (Benlagha et al., 2000; Matsuda et al., 2000). Indeed, hybridomas expressing the invariant Vα14 TCR α chain paired with different Vβs were successfully stained, while staining of non-$V\alpha14^+$ hybridomas was not observed (Benlagha et al., 2000; Matsuda et al., 2000). The ability of α-GalCer–mCD1d tetramers to specifically identify NKT cells in complex mixtures of cells from liver, thymus, and spleen also has then been examined. In the thymus and liver, tetramer-positive cells have the same phenotype as the majority of $NK1.1^+$ T cells (Benlagha et al., 2000; Matsuda et al., 2000). Indeed, the tetramer-reactive cells appear to be activated, on account of their high expression levels of CD44 and CD69. They also stain for CD5 and express intermediate levels of TCR β (Matsuda et al., 2000). The tetramer binding cells are either $CD4^+$ or $CD4^-CD8^-$ DN, with very few $CD8^+$ cells (Benlagha et al., 2000; Matsuda et al., 2000). Those cells are absent in the spleen of CD1d-deficient mice while their frequency increases up to 30-fold in Vα14–Jα281 transgenic mice (Benlagha et al., 2000). In the liver, tetramer-positive cells represent 20–30% of the total mononuclear cells in C57Bl/6 and BALB/c mice. By contrast, no distinct tetramer-positive population was found in either $\beta2m^{-/-}$, $CD1^{-/-}$ or in mice deficient for the Jα used in the invariant α rearrangement ($J\alpha281^{-/-}$ or $J\alpha18^{-/-}$ mice) (Matsuda et al., 2000). Specificity controls for these stainings include the use of tetramers of native CD1d molecules not loaded with α-GalCer, or CD1d tetramers loaded with non-antigenic β-GalCer (Karadimitris et al., 2001). We also produced β-GalCer–mCD1d tetramers and as shown in Fig. 3, β-GalCer–mCD1d tetramers failed to stain NKT cells in the liver of C57Bl/6 mice. NK cells freshly isolated from the spleen or cultured in IL-2 did not stain with the CD1d tetramers (Benlagha et al., 2000; Matsuda et al., 2000). This was found despite a report indicating that CD1d can inhibit the killing oof RMA/S target cells by spleen-derived NK cells cultured in IL-2, which suggested the presence of a CD1d-reactive, inhibitory NK receptor (Chang et al., 1999). If such a CD1d-specific NK receptor does exist, post-translational modification of CD1d by the insect cells, or the absence of the proper CD1d-bound ligand, could explain the lack of tetramer staining.

The interspecies conservation of mouse and human CD1d has been extended by studies showing that α-GalCer can be recognized by NKT cells from either species, regardless of the CD1d used as antigen-presenting molecule (Brossay et al., 1998a; Spada et al., 1998; Naidenko et al., 1999). This suggests that α-GalCer–mCD1d tetramers might recognize human Vα24-JαQ NKT cells. Indeed, Benlagha et al. (2000) showed that peripheral blood lymphocytes (PBLs) expressing a Vα24Vβ11 TCR were specifically stained by α-GalCer–mCD1d tetramers. Conversely, soluble, recombinant human CD1d, constructed as described above in insect tissue culture cells, and dimerized using an anti-CD1d antibody, stains mouse α-GalCer-reactive NKT cells (Matsuda et al., 2000).

Moreover, α-GalCer–hCD1d tetramers, obtained by oxidative refolding chromatography, were reported to stain NKT cells in the spleen, thymus, and liver of C57Bl/6 mice but not in $\beta2m^{-/-}$ mice (Karadimitris et al., 2001). The specificity of these α-GalCer–human CD1d tetramers had been previously determined by staining $V\alpha24^+V\beta11^+$ T cells (Karadimitris et al., 2001). Indeed, T cell lines enriched for expression of both Vα24 and Vβ11 TCRs were generated by in vitro stimulation of PBLs from healthy donors by culture with α-GalCer and cytokines. Staining of such a culture with α-GalCer–human CD1d tetramers, anti-Vα24 and anti-Vβ11 antibodies revealed the same frequency of $V\alpha24^+V\beta11^+$ double-positive T cells and tetramer-positive cells, indicating a specific staining of Vα24Vβ11 T cells by the α-GalCer–human CD1d tetramers (Karadimitris et al., 2001).

4. Identification and purification of NKT cells subsets using α-GalCer-loaded CD1d tetramers

Approximately 20–30% of the total mononuclear cells in the liver of C57Bl/6 and BALB/c mice are positively stained by α-GalCer-loaded mCD1d tetramers. By contrast, no tetramer-positive population was found in the same organ of β2m$^{-/-}$, CD1d$^{-/-}$, or Jα281$^{-/-}$ mice, although some TCR β^{+} NK1.1^{+} cells are still present (Matsuda et al., 2000). Thus, the use of the α-GalCer–mCD1d tetramers permits the unambiguous identification and enumeration of a population of Vα14^{-} and CD1d-independent NKT cells (Benlagha et al., 2000; Matsuda et al., 2000). As shown in Fig. 3, although 80% of the NK1.1^{+} TCR-β^{+} population is tetramer positive, 20% of the population is not stained by the α-GalCer–mCD1d tetramers. In addition, a subset of tetramer-positive cells appears to be NK1.1^{-} in normal as well as Vα14–Jα281 transgenic mice (Benlagha et al., 2000). Those NK1.1^{-} tetramer-positive cells exhibit typical features of CD1d-dependent NKT cells, such as the biased expression of Vβ8, Vβ7, and Vβ2, and a CD44^{hi}CD69^{+}CD122^{+}Ly6C^{hi} phenotype. However, they differ from NK1.1^{+} tetramer-positive cells by their upregulation of CD49d. Therefore, the α-GalCer-loaded mCD1d tetramers identify a novel subset of CD1d-restricted Vα14^{+} NKT cells that had could not have been detected based on NK1.1 and TCR β staining (Benlagha et al., 2000).

α-GalCer–CD1d tetramers have proven useful for the purification of NKT cells. The method depends upon the use of beads coupled to an antibody specific for phycoerythrin (PE) (Matsuda et al., 2000; Gapin et al., 2001). In conjunction with the staining of NKT cells with tetramers constructed with streptavidin–PE, up to a 100-fold enrichment can be achieved in a single-step column separation. Additionally, mouse (Gapin et al., 2001; Matsuda et al., in press) and human (Metelitsa et al., 2001) NKT cells have been highly purified by cell sorting in the flow cytomter using the tetramer to positively select those cells, although this purification will generally activate the NKT cells. Additionally, plates coated with α-GalCer/human CD1d tetramers have been used successfully in the stimulation of human NKT cell lines (Metelitsa et al., 2001).

5. Characterization of NKT cell development and function using α-GalCer-loaded mCD1d tetramers

In a recent study, α-GalCer–mCD1d tetramers were used to explore the thymus development pathway of α-GalCer-reactive NKT cells (Gapin et al., 2001). The results indicate that both DN and CD4^{+} α-GalCer-reactive NKT cells derive from CD4^{+}CD8^{+} double-positive (DP) precursors (Gapin et al., 2001). This insight into NKT cell ontogeny required the use of tetramers, as the immature tetramer-positive cells are relatively rare and they do not express NK1.1 (Lantz et al., 1997; Gapin et al., 2001).

In adult mice, Vα14^{+} NKT cells do not express CD8 (Benlagha et al., 2000; Matsuda et al., 2000), they are either CD4^{+} or DN. As there is no evidence indicating that CD4 serves as a co-receptor for CD1d, or that the DN T cells are significantly less responsive to α-GalCer, the data suggest that the NKT cell TCR operates independently of a typical TCR co-receptor. Using different concentrations of α-GalCer–mCD1d tetramers, we showed that the specific binding of α-GalCer–mCD1d tetramers to NKT cell-derived hybridomas is characterized by apparent equilibrium dissociation constants ranging from 0.5 to 1.6 nM. Tetramer staining decay experiments also indicate that this high avidity is driven by a very long half-life ($t_{1/2}$) of 300 min at 37 °C (Sidobre et al., in preparation). The high avidity exhibited by α-GalCer–mCD1d tetramers for NKT cell-derived hybridomas, which is indicative of a high affinity of the NKT cell TCR for α-GalCer–mCD1d complex, may explain why NKT cells can function independently of a typical TCR co-receptor.

Besides the high affinity of the NKT cell TCR for α-GalCer-loaded mCD1d molecules, this αβ TCR is distinguished by its lack of diversity. Indeed, due to the usage of the invariant Vα14–Jα281 rearrangement preferentially paired with Vβ8, Vβ7, or Vβ2, structural variations in α-GalCer-reactive CD1d-dependent T cells are only permitted in the CDR3 region of the Vβ chain (Lantz and Bendelac, 1994). Two recent studies, however, showed that CD4^{+} and DN NKT cells, identified on the basis of NK1.1, TCR β, and CD4 staining, use polyclonal Vβ rearrangements (Apostolou et al., 2000; Ronet et al., 2001). Conclusions regarding the TCR β repertoire of CD1d-dependent NKT cells have to be drawn with much

caution from these studies, however, as the selected populations analyzed should be contaminated with cells that are not α-GalCer–CD1d reactive, ranging from 50% of the total in the spleen to lower percentages (10–20%) in the thymus and liver. The use of α-GalCer–mCD1d tetramers allowed Matsuda et al. (in press) to purify CD1d-dependent NKT cells based upon specificity. The results of the analysis of the TCRs expressed by these highly purified cells are consistent with a completely polyclonal set of CDR3β sequences in α-GalCer–CD1d-reactive T cells, despite a highly restricted CDR3α sequence and a limited set of Vβs expressed. Furthermore, there is almost no overlap when the sequences from two individual mice were compared, or when NKT cells from two different organs were compared (Matsuda et al., in press). This indicates almost no selection for this region of the TCR β chain, and is not consistent with the idea that NKT cells in different organs are selected for reactivity to different sets of endogenous ligands.

α-GalCer–mCD1d tetramers also were used to follow the NKT cell population ex vivo in response to antigen administration (Matsuda et al., 2000). The response of NKT cells to stimulation by α-GalCer is characterized by a rapid, massive but transient production of cytokines, followed by the disappearance of NKT cells. The stimulation propagates rapidly to NK cells (Matsuda et al., 2000). Indeed, the characteristics of the NKT cell response are more reminiscent of an innate rather than an adaptive immune response (Eberl and MacDonald, 1998; Burdin et al., 1999; Carnaud et al., 1999; Benlagha and Bendelac, 2000; Matsuda, 2000 #86; Eberl and MacDonald, 2000; Godfrey et al., 2000), as there is no evidence for antigen-induced clonal expansion or memory.

Tetramers have been used to analyze the frequency of α-GalCer–CD1d-reactive NKT cells in NOD mice, which spontaneously develop type I diabetes (Hong et al., 2001; Sharif et al., 2001). Earlier reports had suggested that NOD mice have fewer CD1d-dependent NKT cells (Gombert et al., 1996; Hammond et al., 2001), but much of this analysis was difficult because NOD mice do not express the NK1.1 allele reactive with antibody. Interestingly, treatment with α-GalCer was shown to inhibit diabetes development (Hong et al., 2001; Sharif et al., 2001; Wang et al., 2001), perhaps by promoting Th2 responses to disease-related antigens. Despite this, consistent with the results in normal animals, analysis of the α-GalCer-treated mice with tetramers showed that repeated administration of α-GalCer did not lead to a strong clonal expansion of the CD1d-dependent NKT cells. Therefore, the long-term effects of α-GalCer treatment probably are due to changes in other cell populations as a result of NKT cell activation.

6. Conclusions

It is still too early to fully appreciate all the ramifications of the development of CD1 tetramers containing lipid antigens. However, as we reviewed here, α-GalCer–mCD1d tetramers have already permitted significant insight into the biology of NKT cells. In the future, it is likely that tetramers will be used to monitor NKT cell distribution and frequency in the context of the responses to infectious agents, cancer, and the development and treatment of autoimmune diseases. It also is likely that different ligands will be analyzed, and therefore that it will be possible to detect populations of CD1d-reactive T cells present at lower frequencies than the predominant $V\alpha14^+$ group. Tetramers of group I CD1 molecules with defined antigens also can be generated, and perhaps will be used in the same contexts as those described for the CD1d tetramers. Furthermore, because group I CD1 molecules present antigens from mycobacteria, there are obvious applications in terms of monitoring the response to mycobacterial infection or vaccination with cell wall antigens. Finally, it cannot be excluded that tetramers will be used to expand CD1-reactive T cells in vitro and also to purify these cells prior to transfer for possible cell-mediated immune therapies. Therefore, while CD1 tetramers are established as an important tool for studying immune responses, they also may prove to have significant applications in the diagnosis and treatment of disease.

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