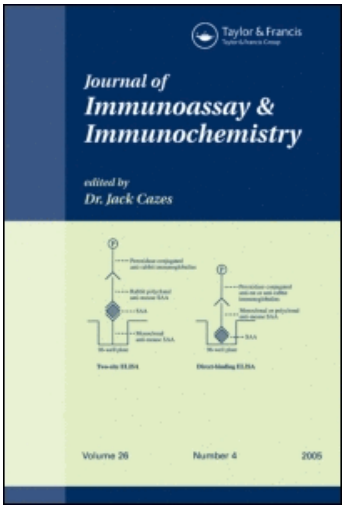


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# Selective Identification of Vα14i T Cells Using Slide-Immobilized, CD1d-Antigen Complexes

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# Selective Identification of Vα14i T Cells Using Slide-Immobilized, CD1d-Antigen Complexes

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**Abstract:** The ability to correlate changes in antigen-reactive lymphocytes with disease will provide information needed to develop strategies for combating illness. One critical group of lymphocytes are the CD1-restricted T cells. It is desirable to use CD1 molecules in an array format to query CD1-restricted lymphocytes in humans. To investigate the feasibility of this technique, we employed mCD1d and α-galactosylceramide to demonstrate that-slide immobilized, CD1d-α-GalCer complexes capture an NKT cell hybridoma in the presence of a competitor. The success of this scheme represents the first step toward the development of CD1-antigen arrays that could be used to profile biological samples.

**Keywords:** CD1, Antigen, NKT cell, Array, Glycolipid, Hybridoma

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## INTRODUCTION

CD1 molecules are $\beta$2-microglobulin associated glycoproteins that present non-peptide antigens to various T cell subsets including natural killer T cells (NKT cells).[1] Over the last decade, the number and types of antigens that can be bound by human CD1 proteins (CD1a-e) and murine CD1d has grown to include a range of pathogen-derived compounds (*e.g.*, mycolic acids, $\alpha$-glucuronosylceramide), self lipids (phosphatidylinsoitol, iGB3), synthetic glycolipids ($\alpha$-GalCer), and even aromatic small molecules.[1–7] Given the structural complexity of the compounds presented by CD1 glycoproteins, it is clear that many more CD1-restricted antigens will be identified in the context of both infectious diseases and non-infectious diseases such as cancer and autoimmune disorders.

Humans express five CD1 molecules[1] and it is important to query the entire population of CD1-restricted T cells (*i.e.*, the CD1-restricted immunosome) to fully understand how the CD1 restricted immunosome responds to disease onset, progression, and treatment. Ultimately, it would be desirable to use immobilized, ligand-bound CD1 molecules in an array-based format to rapidly quantify changes in the levels of various CD1:antigen-specific T cells from a blood sample or tissue biopsy. As a first step toward this goal, mouse CD1d and the well defined glycolipid antigen, $\alpha$-GalCer, were used to test the efficacy of CD1:antigen complexes, immobilized on slides, to selectively bind a free floating NKT cell hybridoma (DN32) that expresses a surface receptor with affinity for this antigen (V$\alpha$14i).[8] The results demonstrate that CD1d-Ig-$\alpha$-GalCer complexes can specifically immunoprecipitate the V$\alpha$14i$^+$ NKT cell hybridoma and do so even in the presence of closely related, non-V$\alpha$14i expressing competitor cells. While inherently simple in design, the success of these studies suggests that it should be possible to engineer a comprehensive CD1-antigen microarray for applications in the diagnosis and treatment of disease.



## EXPERIMENTAL

### Reagents and Hybridomas

Dimeric mouse CD1d-Ig (CD1d-Ig) and anti-CD3$\varepsilon$ antibody (145-2C11) were purchased from BD Biosciences (San Diego, CA). $\alpha$-galactosylceramide ($\alpha$-GalCer) was obtained from Axxora LLC (San Diego, CA). SNARF-1 carboxylic acid, acetate, succinimidyl ester (SNARF), and carboxyfluorescein diacetate, succinimidyl ester (CFSE) were from Molecular Probes (Eugene, OR). Permanox tissue culture slides were purchased from VWR (Indianapolis, IN). Media, supplements, and PBS were purchased from GIBCO/Invitrogen (Grand Island, NY). The mouse DN32 (V$\alpha$14i$^+$) and 431 (V$\alpha$14i$^-$) NKT cell hybridomas have been described previously.[8–10]

### Loading of Dimeric CD1d-Ig with α-GalCer and Slide Immobilization

CD1d-Ig was incubated overnight at 37°C in PBS (pH 7.4) containing a 40-fold molar excess of $\alpha$-GalCer dissolved at 192 ng/μL in 0.5% v/v Tween 20/PBS. Permanox slides were fitted with sheeted silicone rubber (McMaster-Carr, Chicago, IL) in which 3 mm wells were cut for complex deposition. CD1d-Ig-$\alpha$-GalCer (8 μg/mL), vehicle loaded CD1d-Ig (8 μg/mL), and anti-CD3$\varepsilon$ (4 μg/mL) in PBS were placed in the designated wells and incubated at 37°C, 5% $CO_2$ for 2 hours to allow the complexes to adhere. The slides were washed extensively in PBS prior to adding cells.

### Labeling of NKT Cell Hybridomas, Co-Culture with Dimeric CD1d-Ig-α-GalCer and Visualization of Bound Cells

$1 \times 10^7$ DN32, 431, or a 1 : 1 mix of both cell types were washed and resuspended in 500 μL of Iscove's Modified Dulbecco's Medium (IMDM) containing 0.5% heat-inactivated FBS. 0.5 mM SNARF and CFSE (in DMSO) were added to the cells and incubated at 37°C for 15 minutes to allow dye uptake. Labeled cells were washed three times and resuspended in 10 mL of IMDM containing 10% heat-inactivated FBS. Prepared slides were placed in 100 mm dishes, covered with the cell suspensions, and incubated for 2 hours at 37°C, 5% $CO_2$ with occasional rocking. Slides were washed extensively in PBS, fixed using 2% formaldehyde, and viewed using an MRC-1024 (BioRad, Hemel Hempstead, England) with a Diaphot 300 inverted microscope (Nikon, Tokyo, Japan) at 40 × or 60 × magnification.



## RESULTS AND DISCUSSION

To assess the practicality of utilizing CD1:antigen complexes for an array-based assay, dimeric CD1d-Ig, loaded with the well characterized glycolipid antigen, $\alpha$-GalCer, was immobilized on treated slides and tested for the ability to selectively capture DN32 cells, a V$\alpha$14i$^+$ murine NKT cell hybridoma. Wells containing PBS, unloaded CD1d-Ig, or the anti-CD3$\varepsilon$ antibody were used as controls. As shown in Figure 1A, both the TCR reactive, anti-CD3$\varepsilon$ antibody and the CD1d-Ig-$\alpha$-GalCer complex effectively pulled down SNARF-labeled DN32 cells, whereas CD1d-Ig alone or PBS did not. To assess the specificity of the slide-immobilized, CD1d-Ig-$\alpha$-GalCer, the assay was repeated with CFSE-labeled mouse 431 cells, a NKT cell hybridoma that does not express the V$\alpha$14i TCR $\alpha$-chain and should not interact with CD1d-Ig-$\alpha$-GalCer. In this case, the only positive well contained anti-CD3$\varepsilon$ (Figure 1B), demonstrating that the immobilization of CD1d-Ig-$\alpha$-GalCer preserved the specificity of the antigen. Lastly, to examine both the effectiveness and the specificity of the slide-immobilized,

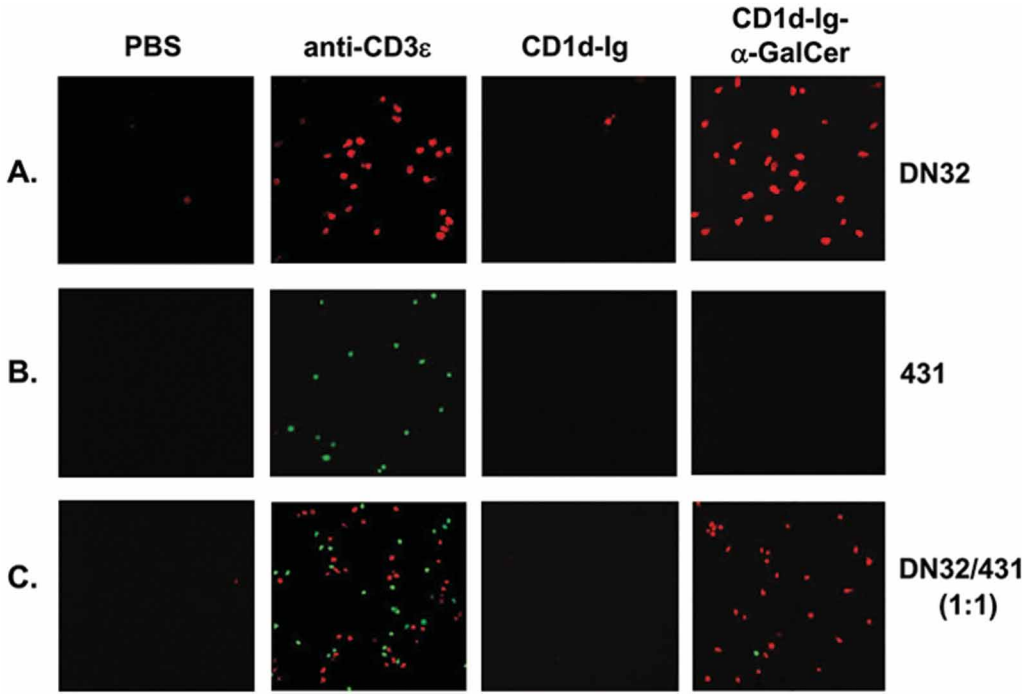


***Figure 1.*** Slide-immobilized CD1d-Ig-$\alpha$-GalCer selectively captures V$\alpha$14i$^{+}$ DN32 cells. Slides with wells containing each of the indicated capture reagents were prepared as described in Experimental and incubated with A. SNARF-labeled DN32 cells (red), B. CSFE-labeled 431 cells (green), or C. a 1 : 1 mixture of both cell types. After washing and fixing, bound cells were visualized by confocal microscopy and images captured electronically at 60X magnification (A.) or 40X magnification (B. and C.).

CD1d-Ig-$\alpha$-GalCer, co-culture experiments were performed utilizing SNARF-labeled DN32 cells and CSFE-labeled 431 cells, suspended together at a 1:1 ratio. As shown in Figure 1C, while anti-CD3$\varepsilon$ immunoprecipitated both hybridomas, CD1d-Ig-$\alpha$-GalCer selectively identified only the V$\alpha$14i$^+$ DN32 cells.

The success of this selective capture experiment supports the engineering of a high density, CD1:antigen array containing any number of defined antigens that can be efficiently loaded, either passively or with transfer proteins, onto CD1 family members *in vitro*. In addition to the CD1 lipid antigens already identified in a number of labs, a recent study has shown that human CD1d can present a non-lipid, small molecule containing an aromatic functional group to a non-invariant T cell clone.[4] This suggests that CD1-restricted T cells may be activated *in vivo* by chemotherapeutic agents that contain this chemical signature. Thus, the ability to profile the full complement of CD1-reactive T cells in a patient's blood could be applied to the diagnosis of disease, to the design of a custom disease treatment, or as a way to monitor the success or failure of a chemotherapeutic regimen.

The immobilization of specific subsets of the CD1-restricted immunosome by this method also has experimental applications. The procedure described in this report already has been combined with a biochemical assay to compare the antigen-induced, transcriptional responses of the DN32 and 431 NKT cell hybridomas (A. Zullo and E. Taparowsky, in preparation). In addition, this capture technology could be used for the *in situ* visualization of signaling events, such as immune synapse formation and marker co-localization, in rare T cell subsets. For example, while the expression of the NK1.1, CD44 and CD4 markers are used routinely to analyze populations of NKT cells by flow cytometry, the spatial organization of these markers and how their localization relates to signal transduction is virtually unknown. As a result, the usefulness of CD1-antigen array technology extends beyond the analysis of biological samples to allow for in depth study of the molecular mechanisms that make CD1-restricted T cells unique.

## CONCLUSIONS

The work presented here with slide-immobilized, CD1d-Ig-$\alpha$-GalCer complexes provides a foundation for the future development of an array-based assay that could be used to query CD1-restricted T cells in a heterogeneous cellular sample. In comparison to dimer and tetramer technology, which are currently the most effective methods for capturing individual T cell clones, CD1-antigen arrays would provide a means to simultaneously profile many CD1-restricted T cell subtypes in one biological sample. The ability to rapidly and reliably profile the CD1-restricted immunosome could have a major impact on how we diagnose and treat a variety of human diseases.

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