Another View of T Cell Antigen Recognition: Cooperative Engagement of Glycolipid Antigens by Va14Ja18 Natural TCR

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Va14Ja18 natural T (iNKT) cells rapidly elicit a robust effector response to different glycolipid Ags, with distinct functional outcomes. Biochemical parameters controlling iNKT cell function are partly defined. However, the impact of iNKT cell receptor β-chain repertoire and how α-galactosylceramide (α-GalCer) analogues induce distinct functional responses have remained elusive. Using altered glycolipid ligands, we discovered that the Vb repertoire of iNKT cells impacts recognition and Ag avidity, and that stimulation with suboptimal avidity Ag results in preferential expansion of high-affinity iNKT cells. iNKT cell proliferation and cytokine secretion, which correlate with iNKT cell receptor down-regulation, are induced within narrow biochemical thresholds. Multimers of CD1d-αGalCer- and αGalCer analogue-loaded complexes demonstrate cooperative engagement of the Va14Ja18 iNKT cell receptor whose structure and/or organization appear distinct from conventional αβ TCR. Our findings demonstrate that iNKT cell functions are controlled by affinity thresholds for glycolipid Ags and reveal a novel property of their Ag receptor apparatus that may have an important role in iNKT cell activation. The Journal of Immunology, 2003, 171: 4539–4551.

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1Abbreviations used in this paper: iNKT, Va14Ja18 natural T; αGalCer, α-galactosylceramide; βGalCer, β-glucosylceramide; mH, minor histocompatibility; MFI, mean fluorescence intensity; FRET, fluorescence resonance energy transfer.
intrinsic high affinity and long dwell time for activation of this T cell subset.

Ag recognition by conventional T cells entails self-nonself discrimination. Thus, T cells are tuned to extraordinarily sensitive recognition of foreign Ags (on the order of 20–100 molecules per cell) and base activation decisions on affinity and dwell time (23, 24, 28, 29). Considering that a large body of evidence indicates that iNKT cells recognize self-Ag, a paradox ensues. How is it that iNKT cells are not continually activated by very small amounts of self-Ag presented on APCs in vivo? Self-Ag recognition must be finely tuned to prevent iNKT cell activation during physiological conditions, but to rapidly respond to disturbances in cellular physiology. In other words, iNKT cells need to be very sensitive to modest changes in self-Ag concentration. In biological systems, this kind of fine-tuning is often achieved by cooperative ligand engagement. Cooperativity itself is defined as a positive or negative change in multimeric receptor affinity for ligand following primary and subsequent subunit binding events. Thus, positive cooperativity permits disproportionately sensitive ligand engagement by multimeric receptors, resulting in an almost digital on-off response (33). A form of cooperativity in conventional T cell Ag recognition is afforded by coreceptor-mediated stabilization of TCR-Ag interaction in the immunological synapse. How iNKT cells substitute for coreceptor usage and yet remain unresponsive to low levels of self-Ag remains unknown. Initial report of CD1d-αGalCer tetramers demonstrated that an iNKT cell hybridoma engages Ag with a Hill coefficient of 4.5, which was interpreted to signify the tetravalency of CD1d-αGalCer tetramers. Hill coefficient depends on valency but is always lower than the number of binding sites of the multimer (i.e., lower than four for any tetrameric molecule) (34). Because the Hill equation used to calculate the Hill coefficient was not provided in the first (35), how the value of 4.5 was obtained remains elusive.

Several questions regarding CD1d1-lipid/TCR interactions remain: Are there affinity and concentration thresholds for the induction of distinct iNKT cell responses? Does the TCR β-chain repertoire impact iNKT cell Ag reactivity in vivo? Does cooperativity play a role in iNKT cell receptor-Ag interactions? In studies relevant to these questions, we demonstrate that the avidity thresholds for iNKT cell receptor determine sensitivity for glycolipid Ag recognition. Despite the invariant nature of the TCR α-chain, TCR β-chain usage by iNKT cells critically impacts the specificity and the avidity for glycolipid Ags. Furthermore, when responding to a suboptimal affinity ligand, high relative avidity iNKT cells are selected. Interestingly, iNKT cell receptor appears to have structure and/or organization distinct from other αβ TCR and engages Ag cooperatively. Taken together, these features of iNKT cell receptor permit sensitive self-Ag recognition and determine their functional outcomes.

Materials and Methods

Mice

Experiments with B6–Jal*80 (36) (a gift from M. Taniguchi (University of Chiba, Chiba, Japan)), B6.129-CD1d1m1(Ja18) (37), and C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) were in compliance with the regulations of the Institutional Animal Care and Use Committee of Vanderbilt University.

Cell lines and hybridomas

CTL clones and hybridomas (generously provided by A. Bendelac (Princeton University, Princeton, NJ) and K. Hayakawa (Fox Chase Cancer Center, Philadelphia, PA)) have been described (38–41).

NKT cell enrichment

C57BL/6 thymocytes and splenocytes reacted with anti-CD161-PE were separated with anti-PE magnetic microbeads using an automated sorter (Milenyi Biotec, Auburn, CA). Samples were typically >95% CD161⁺.

Antigens

Glycolipids (12, 19) and peptides (38, 39) have been described. Kirin Brewery (Gumma, Japan) generously provided αGalCer. SV40 T Ag-derived epitope IV (VVYDFKL), H28 (ILENFPRL), and H60 (LT-FNYRNL) peptides were synthesized by F-moc chemistry at the Molecular Core Facility (Pennsylvania State University).

Generation of multimers

Preparation of CD1d1-glycolipid (15) and H2Kb-peptide tetramers (27, 28) has been described. Dimers of CD1d1 (custom order; BD PharMingen, San Diego, CA) and H2Kb⁻ (DimerX; BD PharMingen) are dimeric owing to their fusion to IgG1 H chains. To obviate the potential for artifacts induced by detection mediated via a fluorochrome-conjugated secondary Ab, the dimers were Alexa Fluor 647- or PE-conjugated via Fab specific for the Fe portion of IgG1 (anti-mouse IgG1 Alexa Fluor 647 and PE Zenon kits; Molecular Probes, Eugene, OR). Every batch of tetramer generated was tested for complete loading of αGalCer and its analogues by glycolipid titration loading and testing by reaction with the best characterized iNKT hybridoma N3b-2C12.

Flow cytometry

All Abs were from BD PharMingen unless otherwise stated. Tetramer-stained Val4Ja18 iNKT hybridomas (N37-1H5a, Vb8.2b.2; N38-2C12, Vb8.2b.2; N38-3C3, Vb8.2b.2; and DN32-D3, Vb8.2b.2A) were also labeled with anti-TCR Cβ-PE (H57-597), NKT cell-enriched glycosphingolipids and splenocytes with anti-NK1.1-PE (PK136), and anti-TCR Cβ-FITC. CTL clones (SV40 epo peptide IV-specific 2168T as well as minor histocompatibility (mH) Ag-specific SPH60, BH60, and SPH28) reacted with tetramers were also stained with anti-CD8α-FITC. Samples were analyzed using FACSCalibur and CellQuest, version 3.0 (BD Bioscience, Franklin Lakes, NJ) as well as FlowJo 4.2 (Tree Star, San Carlos, CA).

Determination of relative avidity (Km)

Equilibrium (≥2 h) binding experiments were performed using increasing tetramer concentrations in 100 μl of PBS containing 2% FCS (Invitrogen, Carlsbad, CA) and 0.05% NaN₃ at 4°C, to prevent capsing and internalization of the TCR. Km was calculated from specific mean fluorescence intensity (MFI; difference between total MFI at a defined tetramer concentration and background MFI derived from ligand-free tetramer binding to the same cells) using nonlinear regression analysis from classical Michaelis-Menten kinetics (Prism 3.02; GraphPad Software, San Diego, CA). MFI (% maximum) was shown in the relevant figures is based on Vmax calculated from nonlinear regression analysis of the data for adequate graphical representation. This permits easy and reliable comparison of data generated in different experiments. Nonlinear Michaelis-Menten regression analysis was preferred, because Scatchard transformation, which uses linear regression, amplifies any variation of the data from the linear curve. That notwithstanding, the results from the Michaelis-Menten kinetics were confirmed by using classical Scatchard transformations to derive the Kᵀ (42).

Determination of off-rates

T cells were labeled with 50 μg/ml H2Kb- peptide or 10 μg/ml CD1d-glycolipid tetramers, respectively, incubated at 4°C for 3 h, and washed extensively. Cells were also stained with 10 μg/ml anti-TCR Cβ-FITC to monitor TCR levels. Following initial tetramer binding, 106 cells were chased in 3 ml of buffer with rocking at 4 or 37°C for the indicated time periods and analyzed by flow cytometry.

Measurement of in vivo and in vitro cytokine response

Mice were injected i.v. with the indicated concentrations of glycolipids diluted in PBS from a 220 μg/ml stock solution in vehicle (0.5% v/v polysorbate and 0.9% w/v NaCl). Controls were injected with corresponding dose of vehicle. After 90 min, IL-2, IL-4, IL-13, CSF-2, IFN-γ, and TNF-α in control and immune sera were measured by ELISA using Abs and methods that we have described previously (43).

TCR down-regulation and in vitro expansion

Bulk C57BL/6 splenocytes were incubated for the indicated amounts of time with increasing concentrations of glycolipid Ags. Following stimulation, iNKT cell receptor level was determined by flow-cytometric analysis.
following staining with CD1d-αGalCer tetramer, anti-TCRβ Ab, within electronically gated B220 and CD8-negative lymphocytes. In other experiments, Ags were first equilibrium-loaded overnight onto B6.129-Tcrd^{h20} splenocytes, and then mixed with C57BL/6 splenocytes magnetically depleted of MHC class II-positive cells. To directly evaluate iNKT cell di-

vision during culture, splenocytes were labeled with 2 μM CFSE (Mole-

cular Probes) in PBS for 8 min at room temperature, followed by quenching with cold FCS and washing with ice-cold RPMI 1640 supplemented with 10% FCS before culture. Evaluation of iNKT cell proliferation was performed by multiplying the percentage of iNKT cells determined by flow cytometry with the total cell number.

**Cell-free Ag dissociation assay**

Soluble mouse CD1d was Ni-affinity purified, as described (44), and bound to ELISA plates at a concentration of 10 μg/ml. Following binding at 4°C for 18 h and blocking of unbound sites with 2% FCS, plate-bound soluble mouse CD1d1 was loaded with 0.1 μM lipids for 12 h at 37°C. After removing excess lipids, the Ag was allowed to dissociate for the indicated times at 37°C. The wells were washed again, and −5 × 10^4 hybridoma cells were added to each well. Controls included wells bound with 2 μg/ml anti-CD3e (positive) or with 5 μg/ml BSA (negative) loaded with 1 μg/ml αGalCer. IL-2 secreted upon activation was monitored by ELISA. Data are presented as the percentage of maximum activation.

**Determination of Hill coefficient**

Hill coefficient was determined from epitope titration experiments. Briefly, CD1d1 and H2K^b-H28 tetramers or dimers were loaded with increasing amounts of glycolipid ligand or peptide epitopes, respectively. Note that H2K^b tetramers were initially folded with H28-derived epitope, a peptide with low affinity for H2K^b, allowing rapid and efficient ligand exchange (Y. Yoshimura and S. Joyce, unpublished data). Glycolipid and peptide loading occurred at 37°C and room temperature, respectively, for 16–18 h.

Hill curve was derived from data transformation; fractional saturation (Y_s) of the receptor was determined as the ratio of specific MFI to maximum MFI (V_max) at a defined ligand concentration and plotted against the concentration of added ligand (glycolipid or peptide). Linear graph of logarithmic roots of the values for the x- and y-axes were used to determine the slope of the Hill curve revealing the Hill coefficient (34).

**Results**

The CD1d1-αGalCer/Va14Ja18 TCR interaction has high relative avidity

There are a number of different methods available to assess the kinetics and extent of ligand-receptor interactions. Biophysical methods using purified recombinant molecules have been extremely useful in the study of a variety of immunological receptors (45–47). That notwithstanding, methods that examine molecules on living cells are particularly powerful (24, 26, 28, 48, 49). To gain insight into the parameters that govern the binding interactions of the CD1d ligand to the specialized TCR of iNKT cells required for their activation, we first determined the K_a (measured affinity of tetrameric Ags for the cognate TCR) between tetrameric CD1d1-glycolipid Ag and its receptor on live cells. For comparison, the K_a of peptide Ags for the TCR expressed by recently activated CD8^+ T lymphocyte (CTL) clones specific for two H2K^b-restricted mH (H60 and H28 (27)) Ags and a viral (SV-40 T Ag-derived epitope IV (39)) Ag was measured. The comparison between class I-restricted Ags was used, because iNKT cells reflect memory/activated T lymphocyte phenotype similar to CTL clones.

CD1d1-αGalCer tetramer binds Va14Ja18^+ but not Va14-negative NKT hybridomas (Fig. 1A). Similarly, H2K^b-peptide tetramers specifically bind their cognate, but not irrelevant, CTL clones (data not shown and Refs. 38 and 39). Nonspecific binding was <5% in all cases (Fig. 1A and data not shown). From the binding isotherms, the K_a of Ag-TCR interaction was calculated (see Materials and Methods).

Va14Ja18 TCR binds CD1d1-αGalCer with a K_a ranging from 7 to 17 nM (Fig. 1B and Table I). TCR of conventional T cells bind H2K^b-peptide tetramers with a wide range of K_a, ranging from ~20 to 220 nM (Fig. 1C and Table I). Note that, in this study, the saturation binding isotherms at equilibrium were derived at 4°C. Whereas the K_a values obtained at 4°C may not be the same as those at 37°C, the relationship of the K_a between different Ag-TCR interactions remains unaltered (50). Consistent with that report (50), we also found that the K_a determined for two iNKT hybridomas at 4°C (N37-1H5a, 10.5 nM; N38-2C12, 18.8 nM) maintained their avidity relationship at 37°C (N37-1H5a, 39.3 nM; N38-2C12, 65.2 nM).

To obtain a more physiological estimate of the K_a between CD1d1-αGalCer and Va14Ja18 TCR, the above binding analysis was extended to NKT cell-enriched thymocytes and splenocytes. The K_a of CD1d1-αGalCer for Va14Ja18 TCR of live NKT cells (Fig. 1D and Table I) is similar to that observed with iNKT hybridomas (B and Table I). Taken together, the K_a of the CD1d1- αGalCer/Va14Ja18 TCR engagement is similar to or higher than that of immunodominant peptide Ag-TCR interaction.

Glycolipid Ag-Va14Ja18 TCR interaction is long-lived

Functional T cell responses following Ag recognition have been correlated with the dwell time, measured as the t_1/2 of ligand engagement by its receptor (28, 49, 51, 52). We found that the t_1/2 of glycolipid Ag/Va14Ja18 TCR is long, lasting between 10 and 40 min (Fig. 1E, top two panels; Table I), which is longer than the t_1/2 observed for peptide Ag/TCR interactions investigated (Fig. 1E, bottom panel; Table I). Hence, the off-rate of CD1d1-αGalCer/TCR interaction on the surface of intact NKT cells appears quantitatively distinct from that of conventional T lymphocytes.

Our data are consistent with previously published reports (28, 53) for studies of peptide-Ag-specific T cells. Due to the manner in which our experiments were performed and analyzed, the data may appear inconsistent with recent dwell-time measurements between CD1d1-αGalCer and iNKT cell receptors (26). Specifically, we did not use anti-CD1d1 or anti-MHC Abs in our experiments, and CD1d1-lipid or MHC-peptide levels detected postchase were not normalized to prechase reacted anti-TCRβ levels. Abs to MHC molecules added during the chase period prevent the dissociating monomers from reassociating with their receptor (49). In our experiments, t_1/2 of H60 tetramer for cognate TCR determined in the absence (9 ± 0.01 min; see Table I) or presence of an H2K^b-reactive Ab, EHI44 (2–10 min; n = 5), was similar. Likewise, t_1/2 of CD1d1-αGalCer tetramers for cognate iNKT cell receptor determined in the absence (9.8 ± 0.9 min; n = 5) or presence of 100 μg/ml CD1d1-reactive Ab IB1 (17.6 ± 7.4 min; n = 2) was similar. It should also be noted that, unlike MHC class II, which is not expressed by mouse T cells, MHC class I and CD1d1 are expressed by T lymphocytes. The use of anti-class I or anti-CD1d1 has the potential to cross-link the dissociating tetramer to the T cells, thereby skewing the data toward increased dwell time. Hence, the comparative off-rate measurements were performed in the absence of Abs.

We also found that the TCR levels on iNKT cells and CTL at time zero and at 120 min of chase were similar when they were stained with anti-TCRβ Ab postchase (data not shown). In contrast, prestaining with anti-TCRβ resulted in a significant loss of TCRβ staining during chase, most likely due to the t_1/2 of anti-TCRβ Ab and cell surface TCR interaction. Thus, we chose not to normalize the remaining CD1d1-αGalCer tetramer bound postchase to prechase reacted anti-TCRβ staining, as described in published reports (28, 53). Nevertheless, our results are consistent with the conclusion that iNKT cell receptor interaction with CD1d1-presenting glycolipid Ag exhibits longer dwell time than that of CTL receptor interaction with peptidic Ags (26).
TCR β-chain repertoire of iNKT cells impacts Ag specificity and the $K_a$ of their interaction

Altered glycolipid ligands derived from αGalCer elicit distinct functional responses from iNKT cells in vivo and in vitro (19). Recently, the TCR β-chain repertoire of iNKT cells was implicated in high-affinity dimeric CD1d1-αGalCer binding; the Vb8.2+ iNKT cells have higher affinity for Ag than those that express Vb7 (27). Differences in TCR β-chain repertoire and/or the affinity for altered glycolipid ligands could explain the differential Ag specificity and functional outcomes. Tetramers of CD1d1-αGalCer and its analogues OCH, 3,4D, and NH were generated concurrently under saturating conditions.

Table 1. Kinetic parameters of Ag-TCR interactions

| T Cell | Reactivity | $K_a$ (nM) ± SEM (n) | $t_{1/2}$ (min at 37°C) ± SEM (n) | Hill Coefficient ($h$) ± SEM (n) |
|--------|------------|----------------------|----------------------------------|-------------------------------|
|        |            | $t_{1/2}$ (min at 37°C) ± SEM (n) | Tetramer | Dimer |
| iNKT hybridomas/CD1d1-αGalCer | | | |
| N37-1H5a | CD1d1 + self lipid | 7.5 ± 0.3 (3) | 19.1 ± 1.3 (5) | 2.8 ± 0.4 (3) |
| N38-2C12 | CD1d1 + self lipid | 8.5 ± 2.0 (4) | 42.1 ± 4.3 (5) | 2.8 ± 0.3 (3) |
| DN32.D3 | CD1d1 + self lipid | 16.8 ± 0.9 (3) | 37.3 ± 2.6 (2) | 2.6 (1) |
| N38-3C3 | CD1d1 + self lipid | 17.6 ± 3.4 (3) | 16.4 ± 1.0 (2) | 2.4 (1) |
| Thymic | CD1d1 + self lipid | 10.7 ± 3.0 (4) | 9.8 ± 0.9 (5) | 2.5 ± 0.2 (3) |
| Splenic | CD1d1 + self lipid | 8.8 ± 0.7 (4) | 11.4 ± 0.6 (3) | 2.7 ± 0.2 (3) |
| Conventional CD8+ T lymphocytes/H2Kb-peptide | | | |
| 2168T | H2Kb + epitope IV | 23.9 ± 2.7 (3) | ND | 1.1 ± 0.1 (4) |
| SPH60 | H2Kb + H60 | 20.4 ± 2.9 (3) | 9.4 ± 0.3 (2) | 1.0 ± 0.1 (3) |
| BH60 | H2Kb + H60 | 33.4 ± 1.7 (3) | 3.9 ± 0.1 (2) | 0.9 ± 0.04 (3) |
| SPH28 | H2Kb + H28 | 216.7 ± 9.2 (4) | 2.1 ± 0.9 (2) | ND |

*a* The reactivities of all of the T lymphocytes have been described (see Results for references).

*b* Calculations of the kinetic parameters are described in Materials and Methods (also see Results for details).

*n* Number of experimental values.
FIGURE 2. CD1d1-OCH is recognized with lower $K_v$, but similar dwell time compared with CD1d1-αGalCer by a Vβ8.1,8.2-skewed iNKT cell repertoire. A, Equimolar quantities (30 nM) of CD1d1-glycolipid tetramers were reacted with a Val4-negative (N37-1A12) or two iNKT (N37-1H5a and N38-3C3) hybridomas, and NKT cell-enriched thymocytes. B, Expression of TCRβ, Vβ8.1,8.2, or Vβ7 on CD1d1-αGalCer and -OCH tetramer-positive, electronically gated HSA<sup>low</sup>CD8<sup>low</sup> thymocytes. C, Expression of TCRβ, Vβ8.1,8.2, or Vβ7 on CD1d1-αGalCer and -OCH tetramer-positive, magnetically sorted NK1.1<sup>+</sup> thymocytes. D and E, Saturation binding isotherms were generated using iNKT hybridomas and NKT cell-enriched thymocytes and splenocytes reacted with the indicated concentrations of CD1d1-αGalCer or -OCH tetramers. Similar binding isotherms were generated using Vβ8.1,8.2<sup>+</sup> and Vβ7<sup>+</sup> splenic iNKT cells. From the binding isotherms, $K_v$ was calculated as described in Fig. 1. F, $t_{1/2}$ of CD1d1-αGalCer and CD1d1-OCH binding to thymic iNKT cells was determined as described in Fig. 1. Binding reactions in A–E were performed at 4°C in the presence of sodium azide to prevent capping and internalization.

CD1d1-αGalCer, -OCH, and -3,4D have exquisite specificity for iNKT cells (Fig. 2A). However, CD1d1-3,4D (an analogue lacking the two hydroxyl groups at C atoms 3 and 4 of the long-chain base) and especially CD1d1-NH (C atom 2 amine-modified αGalCer) bind poorly or not at all, respectively, to iNKT hybridomas (Fig. 2A).

To determine the TCR β-chain repertoire of iNKT cells recognizing OCH, bulk (Fig. 2B) and sorted NK1.1<sup>+</sup> (C atom 2 amine-modified αGalCer) bind poorly or not at all, respectively, to iNKT hybridomas (Fig. 2A).

Table II. Kinetic parameters of glycolipid Ag analogue/TCR interactions

| T Cell                          | $K_v$ (nM) ± SEM (n) | $t_{1/2}$ (min at 37°C)<sup>a</sup> ± SEM (n) | Hill Coefficient (b) ± SEM (n) |
|---------------------------------|---------------------|---------------------------------------------|---------------------------------|
|                                | αGalCer<sup>c</sup> | OCH                                         | αGalCer<sup>c</sup>             | OCH                             |
| INKT hybridomas                |                     |                                             |                                 |
| N37-1H5a                       | 7.5 ± 0.3 (3)       | 29.9 ± 0.3 (2)                             | 10.7 ± 3.0 (4)                  | ND                              |
| N38-2C12                       | 8.5 ± 2.0 (4)       | 22.1 ± 1.7 (2)                             | 42.1 ± 4.3 (5)                  | ND                              |
| C57BL/6 iNKT cells             |                     |                                             |                                 |
| Thymic TCRβ<sup>+</sup>        | 10.7 ± 3.0 (4)      | 31.2 ± 0.4 (2)                             | 9.8 ± 0.9 (5)                   | 13.0 ± 1.9 (2)                  |
| Vβ8.1,8.2<sup>+</sup>          | 10.5 ± 0.1 (2)      | 35.7 ± 1.9 (2)                             | 2.5 ± 0.2 (3)                   | ND                              |
| Vβ7<sup>+</sup>                | 16.0 ± 0.8 (2)      | 46.6 ± 1.8 (2)                             |                                 |                                 |
| Splenic                        | 8.8 ± 0.7 (4)       | 27.4 ± 10.9 (2)                            |                                 |                                 |
| Splenic C57BL/6 iNKT cells     |                     |                                             |                                 |
| expanded with glycolipid Ag    | 9.1 ± 0.4 (2)       | 44.2 ± 2.9 (2)                             | 2.7 ± 0.2 (3)                   | ND                              |
| stimulation (96 h)             | OCH                 |                                             |                                 |                                 |
|                                | 9.1 ± 0.6 (2)       | 39.7 ± 7.2 (2)                             |                                 |                                 |
| 3,4D                           | 6.4 ± 0.5 (2)       | 25.1 ± 1.9 (2)                             |                                 |                                 |

<sup>a</sup> n, Number of experimental values.
<sup>b</sup> Calculations of the kinetic parameters are described in Materials and Methods (also see Results for details).
<sup>c</sup> αGalCer data is the same as presented in Table 1.
lipsids for their TCR. Therefore, CD1d1-αGalCer, -OCH, and -3,4D tetramers were used to determine their $K_{av}$ for the TCR. CD1d1-OCH binds iNKT TCR with about 3- to 4-fold lower $K_{av}$ compared with CD1d1-αGalCer, whereas CD1d1–3,4D had a 6-fold lower $K_{av}$ (Fig. 2D and/or Table II).

Considering that the TCR β-chain repertoire of cells recognizing OCH was Vb8.1,8.2 skewed, we hypothesized that TCR β-chain of the iNKT cell receptor impacts $K_{av}$ for Ag. We found that Vb7+ iNKT cells have 50% lower $K_{av}$ for both CD1d1-αGalCer and -OCH compared with Vb8.1,8.2+ iNKT cells (Fig. 2E and Table II). Note that $K_{av}$ determination was performed with Vb7+ cells that detectably bound CD1d1-OCH tetramer, which represented only ~50% of total CD1d1-αGalCer tetramer-reactive Vb7+ iNKT cells. Therefore, the results potentially represent a higher $K_{av}$ than that of the entire Vb7+ iNKT population. Because the dwell time of TCR and Ag interaction correlates with the capacity for T cell activation, the $t_{1/2}$ of CD1d1-αGalCer, and CD1d1-OCH from iNKT cell receptor was determined as described above. The results indicate that both glycolipid Ags have similar dwell times for their cognate receptors (Fig. 2F and Table II). Taken together, the data suggest that the TCR β-chain repertoire and the $K_{av}$ of Ag-receptor interaction, but not the dwell time, might govern distinct functional outcomes from iNKT cells.

iNKT cells recognize OCH and αGalCer in vivo with similar sensitivity

A number of in vitro studies have indicated that iNKT cells recognize CD1d1-αGalCer with nanomolar sensitivity (10–12, 14, 35). Ags with different binding affinity for their TCR activate T cells with distinct activation thresholds (54–58). To determine the sensitivity of effector responses by iNKT cells in vivo, C57BL/6 mice were injected i.v. with αGalCer and OCH, and serum cytokine response was measured after 90 min. CD1d1-restricted NKT cell (B6.129-CD1d1<sup>+/-</sup>)- and iNKT cell (B6-Ja18<sup>+/-</sup>)-deficient mice do not respond to these glycolipids (Fig. 3A), nor do C57BL/6 mice injected with the vehicle used to dissolve the glycolipid Ags (data not shown).

Mice administered 0.5 μg of OCH elicited substantial amounts of IL-2 and IL-4; TNF-α, IL-13, and CSF-2 (GM-CSF) were also detectable within 90 min (Fig. 3A). Administration of 1.0 μg of αGalCer or OCH elicited a robust cytokine response including TNF-α, IL-13, and CSF-2 (Fig. 3A). Note that the observed IFN-γ response is at the very low end of maximum at this early time point. Furthermore, the previously reported differential IFN-γ response to OCH and αGalCer are strikingly apparent only at or after 6 h (19), because that is the time point at which IFN-γ peaks (59). Thus, at early time, αGalCer and OCH are recognized with similar sensitivity in vivo.

Kinetics of CD1d1 loading with αGalCer and OCH explain similar early iNKT cell response in vivo

Activation of T cells is an effect of Ag-TCR engagement and consequent intracellular signaling. T cell activation correlates with the extent of receptor down-regulation due to signal-dependent altered intracellular TCR trafficking (60–62). Surprisingly, iNKT cells respond to αGalCer and OCH with similar early sensitivity (Fig. 3A), despite different equilibrium binding properties of TCR and specific Ag (Fig. 2, D–F, and Table II). To determine the cellular basis of αGalCer and OCH sensitivity, the kinetics and extent of TCR down-regulation following addition of increasing concentrations of αGalCer and OCH to splenocytes in vitro were evaluated. Both αGalCer and OCH down-regulated similar levels of surface TCR within 4–12 h of Ag stimulation (Fig. 3B, top three panels). However, αGalCer was ~10-fold more potent at inducing surface TCR down-regulation after 24 h of stimulation. Thus, the kinetics of TCR down-regulation reflected the early induced iNKT cell response in vivo.

Two plausible mechanisms can explain the difference observed in early and late iNKT cell responses to αGalCer and OCH. αGalCer, because of its higher $K_{av}$ for Val4Ja18 TCR compared with OCH, is a more potent iNKT cell ligand resulting in more sustained TCR down-regulation and activation. Alternatively, OCH, because of its shortened sphingosine and acyl chains, binds CD1d1 faster than αGalCer, and hence compensates for its low $K_{av}$ and elicits an early iNKT cell response. To distinguish between the two possibilities, B6.129-Tcra<sup>−/−</sup> splenocytes, which lack T and iNKT cells, were incubated with increasing quantities of αGalCer and OCH for 24 h. They were then used to stimulate C57BL/6 splenocytes depleted of MHC class II-positive cells, after which iNKT cell receptor down-regulation was evaluated. OCH was 10- to 20-fold less efficient in TCR down-regulation compared with αGalCer at all time points tested (Fig. 3C). This result is consistent with the hypothesis that αGalCer and OCH have different kinetics of CD1d1 loading, and that the similar early iNKT cell response to the two Ags in vivo reflects rapid on-rate of OCH compared with αGalCer.

To determine the concentration threshold required for the elicitation of distinct cytokines from iNKT cells by αGalCer and its analogue OCH, C57BL/6 splenocytes were stimulated with increasing concentrations of these glycolipids. The results revealed that IL-2 and IFN-γ response after 48 h (Fig. 3D) required at least 50% iNKT cell receptor down-regulation measured at 24 h (B, bottom panel) and medium Ag concentration threshold of αGalCer and OCH (D). In contrast, secretion of CSF-2 and IL-4 was more sensitive to low concentrations of glycolipid Ags and, hence, responded to low levels of TCR down-modulation (Fig. 3, B and D). In support of our previous report (19), OCH preferentially induced an IL-4 response, whereas 50-fold higher concentration of OCH was required to produce an IFN-γ response similar to that induced by αGalCer (Fig. 3D). Thus, the secretion of cytokines by iNKT cells follows a hierarchical Ag response pattern, with higher avidity and higher concentrations required for secretion of IFN-γ and IL-2 compared with both low avidity and low concentration for CSF-2 and IL-4.

To fully understand the properties of CD1d1-OCH interaction, we used a cell-free Ag presentation assay to determine its dissociation kinetics. Plate-bound soluble CD1d1 was loaded with equimolar quantities of αGalCer or OCH. After removing unbound lipid, the complexes were allowed to dissociate for varying time periods at 37°C. The $t_{1/2}$ of Ag-CD1d1 complex was monitored by its ability to activate iNKT cell hybridomas. OCH interaction with CD1d1 was more labile, because it dissociated faster than αGalCer from CD1 (Fig. 3E). Thus, similar early sensitivity of iNKT cells to αGalCer and OCH in vivo reflects the differences in the kinetics of their interaction with CD1d1 and also the differences in their equilibrium parameters of TCR engagement.

Activation of iNKT cells by 3,4D in vitro causes selective expansion of high-avidity clones

The altered lipid ligand, 3,4D, engages the iNKT cell receptor, albeit with low $K_{av}$ compared with αGalCer and OCH (Fig. 2B and Table II), and elicits a weak cytokine response in vivo (19). To elucidate the biochemical basis of this weak response, the proliferative capacity of iNKT cells to Ag engagement was determined in vitro by CFSE dye dilution assay. After stimulation of splenocytes with Ags for 96 h, iNKT cells were costained with CD1d1-αGalCer tetramer and TCRβ-specific Ab. At high concentration (575 nM), αGalCer, OCH, and 3,4D induced extensive iNKT cell
proliferation (Fig. 4A). In contrast, at a lower concentration (2.9 nM) of these same Ags, αGalCer, OCH, or vehicle. After 90 min, serum cytokines were monitored. Background cytokine level (<3%) elicited by vehicle-treated mice was subtracted from the Ag-treated response. The data represent cytokine responses (±SE) elicited by four individual mice in two identical experiments. B. Val14Ja18 TCR down-regulation was monitored at the indicated time points following addition of αGalCer or OCH to C57BL/6 splenocytes. iNKT cell receptor level was determined by flow-cytometric analysis following reaction with CD1d1-αGalCer tetramer and anti-TCRβ Ab, within electronically gated B220 and CD8-negative lymphocytes. C. Val14Ja18 TCR down-regulation was monitored following reaction of C57BL/6 splenocytes magnetically depleted of MHC class II-positive cells with B6.129-Tcra0/0 splenocytes equilibrium loaded with Ag overnight. D. Cytokines elicited by C57BL/6 splenocytes were monitored 48 h following addition of indicated quantities of αGalCer or OCH in vitro by sandwich ELISA. E. The dissociation of αGalCer and OCH from plate-bound soluble CD1d1 was monitored after removing excess glycolipids, and chasing the Ag for 4, 12, 24, and 36 h at 37°C using an iNKT cell hybridoma, N38-2C12, as a probe. Activation-induced IL-2 was determined and plotted as percentage of maximum, a value obtained at start of chase.

Ag concentrations inducing a maximum proliferative response, although a slight decrease in the percentage of Vb8-negative iNKT cells was noted (~35% of expanded iNKT (Fig. 4C) compared with ~45% for naive iNKT cells (Fig. 2B)). Additionally, very little if any difference was observed in the Vb repertoire of iNKT cells expanded with different suboptimal doses of αGalCer and OCH (data not shown). Interestingly, iNKT cell activation by 3,4D, but not αGalCer or OCH, resulted in the expansion of iNKT cells responding to Ag with higher $K_{av}$ for αGalCer and OCH (Fig. 4D and Table II). Thus, high-avidity iNKT cells preferentially expand to suboptimal TCR engagement.

Cooperative glycolipid Ag recognition by iNKT cells

Self-Ag recognition must be finely tuned to prevent iNKT cell activation during physiological conditions, but respond rapidly to disturbances in cellular physiology. In other words, iNKT cells need to be very sensitive to modest changes in Ag concentration.
In biological systems, this kind of fine-tuning is often achieved by using cooperative ligand-receptor interactions (33, 63). To determine whether cooperativity participates in sensitive glycolipid Ag recognition, this mode of interaction was determined by calculating the Hill coefficient (see Materials and Methods). The Hill coefficient of the interaction between the tetrameric Ag and the iNKT cell receptor was >2 (Fig. 5A and Table I). In stark contrast, all MHC class I-restricted TCR had a calculated Hill coefficient of ~1 (Fig. 5B and Table I), indicating a lack of cooperativity. Peptide binding to each H2Kb monomer of the tetrameric molecule is an independent event. Saturation binding of the tetramer to the TCR with increasing concentration of added peptide indicates occupancy of all four sites (Fig. 5B). Furthermore, an analysis of the stoichiometry of class I H chain, β2-microglobulin, and peptide following ligand exchange by Edman sequence determination (64) revealed a 1:1:1 ratio of the three components (data not shown). Thus, a Hill coefficient of 1 is not due to incomplete loading of the class I tetramer.

OCH is a structurally different Ag, particularly in the hydrophobic component thought to interact with CD1d1. Also, OCH interaction with CD1d1 has distinct kinetic parameters compared with αGalCer (Figs. 2F and 3E). Thus, to exclude the possibility that the biochemical or structural properties of αGalCer loading onto CD1d1 account for the observed cooperative response, Hill coefficient was measured for the binding of CD1d1-OCH to the Va14Ja18 TCR. As expected, we found that the Hill coefficient for CD1d1-OCH and CD1d1-αGalCer for the same Va14Ja18 TCR are very similar (Fig. 5C and Table II). Thus, Hill coefficient measurement does not reflect the loading properties of glycolipid Ags, but rather, it is the property of the Ag receptors with which it interacts.

To independently demonstrate cooperative Ag engagement by iNKT cells with multimeric Ags other than soluble, biotinylated monomers of CD1d1 and H2Kb prepared in-house, we determined the Hill coefficients with commercially obtained dimeric IgG1-CD1d1 and IgG1-H2Kb fusion molecules loaded with αGalCer and H60 peptide, respectively, for iNKT cells and H60-specific SPH60 CTL clone. iNKT cells demonstrated cooperative engagement of both dimeric and tetrameric Ag by the Va14Ja18 TCR (Fig. 5D and Table I). As expected, neither dimeric nor tetrameric H2Kb cooperatively engaged their cognate TCR (Fig. 5D and Table I). Thus, we conclude that, in contrast to conventional T lymphocytes, glycolipid Ag recognition by iNKT cells involves cooperativity.

iNKT cell receptor appears to have distinct structure and/or organization

A plausible model for cooperative tetrameric Ag engagement by Va14Ja18 TCR is receptor partitioning and oligomerization within lipid rafts (50). To test this model, Hill coefficients for Ag-receptor interactions were determined for two representative iNKT hybridomas (N38-2C12 and N37-1H5a), NKT cell-enriched thymocytes, and two CTL clones (SPH60 and BH60), following disruption of their lipid rafts. Lipid rafts were disrupted by cholesterol depletion with methyl-β-cyclodextrin (65) or alternatively by filipin-mediated intercalation of this membrane microdomain (66). Disruption of lipid rafts did not alter the Hill coefficient for any of the interactions tested (data not shown), suggesting that these membrane microdomains are not critical for cooperative Ag engagement by iNKT cell receptor.

To further examine the structural properties of iNKT cell Ag receptor, we used fluorescence resonance energy transfer (FRET)
FIGURE 5. Cooperative engagement of multimeric CD1d1 by the Vα14Jα18 TCR. A constant concentration of multimer (CD1d1 tetramer (A and C); H2Kb tetramer (B); CD1d1 and H2Kb dimers and tetramers (D)) was loaded with the indicated concentrations of Ag (αGalCer or OCH (A and C); peptide (B)) and reacted with freshly isolated NKT cell-enriched thymocytes (A), NKT hybridomas (A and C), or CTL clones (B). Binding was monitored as described in Fig. 1. From the binding curve, Hill plots and Hill coefficients (h), represented beneath the Ag-binding curves, were derived (see Materials and Methods). Tetramers of CD1d1 and H2Kb were generated by fluorochrome-conjugated streptavidin-mediated tetramerization of biotinylated monomers. Binding of dimers of CD1d1 and H2Kb, owing to their fusion to IgG1 H chains, were detected with Alexa Flour 647- or PE-conjugated Fab specific for the Fc portion. The high regression coefficient (h > 0.90) for all Hill plots indicates that the calculated h values are significant. All binding reactions were performed at 4°C in the presence of sodium azide to prevent capping and internalization.

measurements between CD1d1 and H2Kb multimers and Abs specific for components of the TCR complex. In the course of our studies, we observed that costaining of iNKT cells ex vivo by allophycocyanin- or PE-conjugated CD1d1 tetramers and PE- or allophycocyanin-TCRβ (clone H57-597) or anti-CD3ε (clone 145-2C11) Abs resulted in large and repeatable increase in FL3 channel fluorescence in a properly compensated flow-cytometric experiment (Fig. 6, A (iNKT cell hybridoma N38-2C12) and B (thymic iNKT cells)). Such large FRET shift was not observed with high-intensity staining Abs specific for cell surface molecules not within the TCR complex (e.g., anti-CD44, clone IM7; Fig. 6). As PE and allophycocyanin have overlapping fluorescence emission and absorption spectra, respectively, it was likely that this result was a consequence of nonradiative FRET. This hypothesis was tested by running samples on the flow cytometer with the red diode laser (emission, 635 nm) and its FL4 filter switched off. Indeed, we still observed FL3 fluorescence only when costaining with CD1d1 multimers and TCR complex-specific Abs. The large FRET observed upon tetramer-anti-TCRβ/anti-CD3ε binding to iNKT cells presented an opportunity to test the hypothesis that the structural orientation and/or organization of Vα14Jα18 TCR are distinct from αβ TCR of conventional CTL. When H60-specific CTL were costained in a manner identical with that of iNKT cells, and analysis was restricted to equivalent MFI of anti-TCRβ or anti-CD3ε and H2Kb multimer, very little FRET was detected (Fig. 6A). Similarly, we observed FRET using PE-conjugated CD1d1 tetramers and allophycocyanin-conjugated anti-TCRβ or anti-CD3ε Abs (data not shown). FRET between CD1d1-αGalCer tetramers and TCRβ on iNKT cells directly correlated with the staining intensity, even at relatively low concentrations (~2.5 nM; Fig. 6C). In contrast, no FRET between H2Kb tetramers and TCRβ or CD3ε was observed, even with saturating concentrations of H2Kb tetramers (~250 nM; Fig. 6C). FRET is exquisitely sensitive to small changes in donor and acceptor fluorochrome distances (FRET, ~r^-2). Thus, these results strongly suggest that iNKT cell receptor has a distinct structure and/or organization, resulting in shorter distance between donor and acceptor fluorochromes used.

Discussion
In summary, our findings demonstrate that iNKT cell receptors recognize glycolipid Ags with avidities similar to, if not higher than, those of immunodominant, high-affinity αβ TCR of conventional T cells. In contrast to CTL, which recognize Ag over a large avidity range (20–220 nM), iNKT cells efficiently recognize Ag within a narrow window of avidity (10–40 nM). Interestingly, although the TCR-Ag dwell time for αGalCer and OCH are very similar, TCR down-regulation as well as the proliferative and cytokine response of iNKT cells to these Ags directly correlate with the staining intensity, even at relatively low concentrations (~2.5 nM; Fig. 6C). FRET is exquisitely sensitive to small changes in donor and acceptor fluorochrome distances (FRET, ~r^-2). Thus, these results strongly suggest that iNKT cell receptor has a distinct structure and/or organization, resulting in shorter distance between donor and acceptor fluorochromes used.
recognition of αGalCer and OCH, poor recognition of 3,4D, and no recognition of NH (19), by the Va14Ja18 TCR with distinct \( K_a \) points toward a narrow kinetic window for iNKT cell activation. We demonstrate that both optimal Ag concentration and relative avidity are essential to elicit a strong proliferative response by iNKT cells. Interestingly, as observed with conventional T cell effector functions (56), iNKT cells exhibit hierarchical functional consequences to Ag quality and concentration. In support of our previous study (19), we also find a dissociation from a clear avidity-concentration dependence in IL-4 secretion following OCH compared with αGalCer stimulation. Both low Ag concentration and low \( K_a \) are sufficient for selective IL-4 secretion and iNKT cell proliferation. In contrast, higher \( K_a \) and Ag concentration are required for IFN-γ response. Consistent with this conclusion is the finding that dendritic cells presenting a high concentration of the high \( K_a \) Ag αGalCer induce sustained IFN-γ response from iNKT cells (67). In this regard, iNKT cell response closely follows the principle of Ag concentration threshold set for IFN-γ and IL-4 responses elicited by conventional T cells (68).

Due to their potent immunoregulatory properties, therapeutic modulation of iNKT cell number and functional responses has been proposed for prevention of autoimmunity as well as for the enhancement of immune responses to tumors and vaccines. In the nonobese diabetic mouse model of autoimmune type 1 diabetes, iNKT cell number and function are low (43, 69, 70). Increasing the iNKT cell number (71–73) or the αGalCer treatment-induced Th2 bias (17, 18, 74) effectively reduces the incidence of type 1 diabetes in nonobese diabetic mice. Our data demonstrate that distinct glycolipid administration regimens may be required to induce tolerizing activity compared with IFN-γ-dependent antitumor and adjuvant properties of iNKT cells.

The natural self-Ag recognized by iNKT cells and its structural relationship to αGalCer remain unknown. However, we recently discovered that a cell line deficient in β-glucosylceramide (βGlcCer) is defective in the presentation of a self-Ag to iNKT cell hybridomas (15). Together with the evidence that the defect was not due to altered folding, intracellular traffic of CD1d1, or recognition of βGlcCer itself, these results suggest that βGlcCer is either a precursor or an essential factor in the synthesis and/or loading of a natural Ag. Thus, it is possible that the elusive self-Ag may be αGlcCer or a similar compound. Further support for the hypothesis that a self-Ag similar to αGalCer is recognized is the finding that transgenic overexpression of CD1d1 results in preferential deletion of Vb8.1,8.2+ iNKT cells (75). This result is fully consistent with our finding that Vb8.1,8.2+ iNKT cells have a higher \( K_a \) for αGalCer and OCH than Vb8.1,8.2-negative iNKT cells. Furthermore, the high \( K_a \) binding of CD1d1-αGalCer with Vb8.1,8.2+ Val4Ja18 TCR is consistent with the high \( K_a \) binding of dimeric Ag to similar TCR (27). Importantly, for the first time, we demonstrate that the repertoire and Ag \( K_a \) of iNKT cell receptors are regulated during proliferation and result in selection of high-avidity iNKT cells under conditions of suboptimal stimulation. These data, taken together, suggest that the narrow kinetic window of recognition of αGalCer and its analogues is reflective of the parameters of natural self-Ag recognition.

The 2C transgenic TCR exhibits differing peptide Ag binding modes on naive and effector cells, suggesting cooperativity (50). The existence of two TCR αβ molecules within a single CD3 complex was evoked to explain this result (50). However, recent data suggest that the stoichiometry of TCR αβ assembly with CD3 complex is 1:1 (76). Whether this stoichiometry changes during CTL activation remains to be established. Using tetramers of CD1d1 and H2Kk, we demonstrate cooperative Ag engagement of glycolipid Ags by iNKT cell receptors but not that of peptidic Ags.

suggestion that the iNKT cell receptor structure and/or organization may be distinct from conventional αβ TCR.

Conventional T cells recognize peptide Ags with a wide range of avidities and dwell times (23–25, 28, 29). In contrast, strong
by conventional αβ TCR. The binding mode of 2C transgenic TCR was investigated using an IgG1-H2Kb dimer, and evidence for TCR αβ dimerization was obtained by data deconvolution. To confirm the cooperative engagement of glycolipid Ag, we also used IgG1-CD1d1 and IgG1-H2Kb dimer similar to that used to investigate the 2C TCR (50). The results supported cooperative Ag engagement by iNKT cell, but not CTL receptors. Thus, the relationship of our findings with those previously reported is unclear.

Because H2Kb and CD1d1 tetramers were built upon the same batches of streptavidin-PE/allophycocyanin, cooperativity in one and not the other precludes conformational change in streptavidin or the fluorochrome. Furthermore, because of the wide separation between monomeric subunits of tetrameric CD1d1, it is extremely unlikely that a conformational change within CD1d1 itself is responsible for the observed Hill coefficient. This is further emphasized by the fact that CD1d1 dimers made in a manner distinct from tetramers also show cooperative binding. Cooperativity is independent of the parameters of glycolipid binding to CD1d1, because OCH, which interacts with CD1d1 with differing properties than αGalCer, had essentially the same Hill coefficient as αGalCer. Thus, the change in Hill coefficient does not reflect a change in the structure of CD1d1 tetramer, but rather a different organization and/or orientation of the TCR engaging such Ags.

How iNKT cells respond to self-Ag and yet remain quiescent in physiological situations remains unclear. In this study, we demonstrate that iNKT cell receptors exhibit cooperative engagement of glycolipid Ag. Cooperativity in biological systems is a common mechanism for achieving sensitivity to relatively modest changes in the strength of the signal (33, 63). In other words, a relatively small change in ligand concentration will result in full binding/activation of an enzyme/receptor. It is possible that iNKT cells use cooperativity to induce sensitive response to a small change in the concentration of self-Ag. In support of this hypothesis, self-Ag recognition of ex vivo isolated iNKT cells is dependent on high levels of CD1d1 expression by target cells (2), and conversely, iNKT cell hybridomas recognizing physiologic levels of CD1d1 on target thymocytes or dendritic cells have high levels of Va14Ja18 TCR expression (6). Thus, the finding of cooperativity in iNKT cell Ag engagement, but not among CTL recognizing peptidic Ags may be one mechanism by which iNKT cells recognize self-Ag(s).

Our data indicate that the structure and/or organization of the iNKT cell receptor may be distinct from αβ TCR of CTL. FG loop within the Cb domain is a large, evolutionarily conserved structure, which forms a wall at the region where Cb and Vb domains of the TCR β-chain join to form a cavity (77). Ab mapping studies revealed that the FG loop is in close proximity to one of the CD3ε subunits (78). Transgenic mice expressing the TCR β-chain mutant lacking the FG loop have no gross deficiencies in the development and function of conventional CD4 and CD8 T cells (79), implying that αβ TCR pairing and surface expression are not grossly impaired. However, a careful analysis in a single specificity TCR transgenic system revealed that thymocytes lacking the FG loop had impaired negative selection (80), but TCR αβ pairing and expression were unenhanced. In contrast, however, Va14Ja18 TCR α-chain was found not to pair at all with a Vβ8.2-FG loop mutant, and hence, the mutant mice were impaired in iNKT cell development (81). Interestingly, the anti-TCRβ Ab H57-597, which exhibits strong FRET in conjunction with the CD1d1-αGalCer tetramer specifically binds the FG loop (77, 79). However, FRET was not observed in conjunction with H2Kb tetramers or dimers. FRET is observed between CD8α of 2C-transgenic CTL and H2Kb, suggesting the engagement of CD8α by monomeric H2Kb (82). Taken together, the data strongly suggest that the structure and/or the organization of the Va14Ja18 TCR complex are distinct from αβ TCR of conventional T cells, which might potentially account for the cooperative engagement of glycolipid Ags.

In conclusion, our findings demonstrate that iNKT cell functions are controlled by narrow avidity thresholds for glycolipid Ags and demonstrate novel properties of their Ag receptor that may have an important role in iNKT cell activation. These findings have important implications for the therapeutic use of iNKT cells.

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