Invariant natural killer T (iNKT) cells are a population of T lymphocytes that play an important role in regulating immunity to infection and tumors by recognizing endogenous and exogenous CD1d-bound lipid molecules. Using soluble iNKT T cell receptor (TCR) complexes, we applied single molecule force spectroscopy for the investigation of the iNKT TCR affinity for human CD1d molecules loaded with glycolipids differing in the length of the phytosphingosine chain using either recombinant CD1d molecules or lipid-pulsed THP1 cells. In both settings, the dissociation of the iNKT TCR from human CD1d molecules loaded with the lipid containing the longer phytosphingosine chain required higher unbinding forces compared with the shorter phytosphingosine lipid. Our findings are discussed in the context of previous results obtained by surface plasmon resonance measurements. We present new insights into the energy landscape and the kinetic rate constants of the iNKT TCR/human CD1d-bound lipid molecules. Using soluble iNKT T cell agonists, yields information regarding the structure and dynamics of the recognition process (for review, see Ref. 14). Over the years, several groups have investigated molecular forces on cell surfaces using AFM (15–19). Among this, heat shock proteins, which are essential for cellular homeostasis and efficiently trigger cellular responses, were localized on stressed and unstressed endothelial cells (20), and the interaction of LFA-1 (leukocyte function-associated antigen-1) expressed on Jurkat T cells with ICAM-1 (intercellular adhesion molecule-1) and ICAM-2 was investigated (21). In other recent studies, ALCAM (activated leukocyte cell adhesion molecule)–mediated interactions were characterized with respect to their biochemical and biophysical properties (22), and the kinetics of synapse forma-

The affinity of the iNKT TCR for CD1d-glycolipid complexes is an important parameter in evaluating the biological effects of iNKT cell agonists (5). One of the most potent iNKT cell agonists is α-galactosylceramide (αGalCer), which contains a galactose moiety connected through an α-glycosidic linkage to a ceramide lipid containing acyl and phytosphingosine chains. The lipid is embedded in the groove of CD1d molecules, as revealed by the CD1d crystal structure (6, 7). It has been shown that several synthesized analogs of αGalCer can activate iNKT cells. Among them is OCH12, which differs from αGalCer by a shorter phytosphingosine chain (C12 instead of C18) (8). We have previously shown that the length of the phytosphingosine chain influences the affinity of the iNKT TCR for CD1d-lipid complexes (8). Although previous studies have focused on the affinity of binding of the iNKT TCR to human CD1d (hCD1d)-glycosphingolipid (GSL) complexes by ensemble-averaged methods such as surface plasmon resonance (SPR) and flow cytometry, the interaction forces that govern the bond stability have not been determined. To address this issue, measurements of the binding strength between the iNKT TCR and hCD1d molecules loaded with GSLs were performed using an atomic force microscope.


**Experimental Procedures**

**Substrate and Tip Functionalization**—For SMFS experiments, soluble biotinylated iNKT TCR was tethered to the AFM tips via a flexible heterobifunctional PEG cross-linker containing an aldehyde and an N-hydroxysuccinimide (NHS) ester group at its ends, with an extended length of 6 nm (depicted in Fig. 1A). In more detail, the silicon nitride tips (Veeco Instruments) were first functionalized with an amine group using the 3-aminopropyltriethoxysilane coating procedure reported previously (24). The aldehyde-PEG-NHS linker was then attached to the amino-functionalized tips via its NHS ester end by incubation for 2 h at room temperature in 0.5 ml of chloroform containing 3.3 mg of aldehyde-PEG-NHS and 0.5% triethylamine. Subsequently, the tips were rinsed in chloroform and dried with nitrogen before they were incubated for 50 min in a mixture of 65 μl of streptavidin at a concentration of 0.2 mg/ml in PBS buffer (150 mM NaCl and 5 mM NaH2PO4, pH 7.5) and 2 μl of 1 M NaCNBH3 (freshly prepared by dissolving 32 mg of solid NaCNBH3 in 500 μl of 10 mM NaOH). To block unreacted aldehyde groups, 5 μl of 1% ethanolamine hydrochloride (adjusted to pH 9.6 with 20% NaOH) was added, and the incubation was continued for 10 min. The tips were then washed with PBS and treated with 20 μg/ml biotinylated iNKT TCR in Tris buffer (50 mM Tris, 100 mM NaCl, and 1% glycerol, pH 7.5). The incubation was allowed to continue overnight at 4 °C. Finally, the tips were washed and stored in Tris buffer in a cold environment.

**Monomer Immobilization on Mica**—For measurements with the isolated iNKT TCR and biotinylated CD1d-GSL complexes, freshly cleaved mica was functionalized with 20 μg/ml CD1d-αGalCer or OCH12 following the same chemical procedure described above. The modified mica surfaces were stored in Tris buffer at 4 °C.

**Preparation of Soluble Biotinylated TCRs and Monomers**—The generation of soluble biotinylated iNKT TCR heterodimers and human CD1d monomers loaded with αGalCer or OCH12 was done as described previously (8).

**Cells and Culture Conditions**—THP1 cells (American Type Culture Collection) transduced with a lentivector encoding CD1d-YFP fusion protein3 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM GlutaMAX, 1% sodium pyruvate, 1% nonessential amino acids, and penicillin-streptomycin (5000 units/ml penicillin). Cells were maintained between 2 × 10^5 and 9 × 10^5 cells/ml and grown at 37 °C in 5% CO2.

3 M. Salio, unpublished data.

**RESULTS**

SMFS of Isolated hCD1d-GSL Complexes Probed by the iNKT TCR—We initially focused on studying the interaction forces between the iNKT TCR and hCD1d-GSL (αGalCer and OCH12) in vitro using SMFS. Soluble biotinylated iNKT TCR was linked to AFM tips via a heterobifunctional PEG cross-linker (29) that carried an aldehyde group on its free end that chemically coupled streptavidin (see “Experimental Procedures”). The strong streptavidin-biotin bond ensures that the iNKT TCR remains firmly attached to the tip in SMFS experiments. Similarly, the biotinylated hCD1d monomer loaded
either with αGalCer or with OCH12 was immobilized on amino-functionalized mica by the same strategy. A schematic representation of the tip and surface chemistry is depicted in Fig. 1A.

In SMFS, molecular unbinding forces are determined in force-distance cycles by approaching and subsequently withdrawing functionalized tips from surfaces containing cognate molecules (see sketch in Fig. 1B). During the initial approach of the cantilever, the cantilever deflection remains constant, indicating that there is no force acting on the cantilever (Fig. 1B, step 1). Once the tip reaches the surface, the cantilever bends upward, and the corresponding deflection is typically represented by the slope shown (Fig. 1B, step 2). When the tip is subsequently withdrawn and if a specific interaction eventually occurs between the tip-bound molecule and the cognate molecule on the surface, the cantilever is bent downward, and the PEG linker through which the molecule is connected to the tip is stretched (Fig. 1B, step 3), thereby stretching the PEG linker until the bond ruptures at a critical force ($f_u$), termed the unbinding force (step 4). A typical force curve showing an iNKT TCR-unbinding event from CD1d-αGalCer upon tip retraction. Inset, the specific interaction is blocked by injecting free anti-CD1d mAb into the bath solution.

A typical force-distance curve in which the specific interaction between the isolated iNKT TCR and CD1d-αGalCer is monitored is shown in Fig. 1C. In contrast to the sketch in Fig. 1B, only the retraction part is shown here. Unbinding force
traces are characterized by a characteristic parabola-like shape due to the stretching of the tip-coupled PEG linker before unbinding. The specificity of these interactions was verified by injecting 25 μg/ml free anti-CD1d mAb into the solution. This resulted largely in the disappearance of the unbinding events because of blockage of the CD1d-αGalCer molecules on the surface (Fig. 1C, inset).

Based on unbinding force analysis, empirical probability density functions of forces were calculated. Probability density functions are force distribution diagrams displaying the most probable unbinding force peak and its width (see “Experimental Procedures” for details). A representative example of a probability density function for the iNKT TCR/CD1d-αGalCer interaction, obtained from >100 unbinding events, is presented in Fig. 1D. The maximum probable force was 39 ± 2 pN for a loading rate of 1260 pN/s (Fig. 1D, black line). The binding probability, which represents the frequency of occurrence of specific interaction events in force-distance cycles, was 17.4%. After addition of the blocking antibody, it decreased to 5.7% (Fig. 1D, red dashed line). The same experimental procedure and analysis sequence were applied to the CD1d-OCH12 complex (data not shown). Here, the binding probability decreased from 8.6 to 2.6% upon injection of blocking antibodies. To further control the specificity of this interaction, a bare tip was used instead of a receptor-coated tip. With this tip, only a negligible number of unbinding events were noticed.

hCD1d-GSL Complex Recognition by an iNKT TCR-coated Tip on Living Cells—To assess the iNKT TCR affinity for hCD1d-GSL complexes at the cell surface, THP1 cells transfected with a lentiviral vector encoding CD1d molecules were chosen. Before performing SMFS on living cells, the expression level of the CD1d molecules on THP1 cells was confirmed by flow cytometry analysis using an anti-CD1d antibody (Fig. 2A). In addition, THP1 cells were pulsed with αGalCer or OCH12 and stained with the soluble iNKT TCR to verify the extent of lipid loading. As shown in Fig. 2B, THP1 cells pulsed with αGalCer had a higher level of cell-surface CD1d-lipid complexes compared with cells pulsed with OCH12. However, OCH12 did increase the level of TCR staining above the background (unpulsed), confirming that OCH12 is presented on the cell surface in the context of CD1d molecules.

AFM images were acquired in contact mode in PBS buffer to determine the cell morphology. A characteristic topography and deflection image are shown in Fig. 3 (A and B). The THP1 cells were fixed with 2% paraformaldehyde for 20 min at room temperature. Characteristic features such as the nucleus and membrane features were revealed, with a typical overall cell size of 20 μm in diameter and 1.5 μm in height. The cells appeared to be stably attached to the substrate, a prerequisite for performing force spectroscopy studies on their surface.

SMFS measurements on living cells were carried out with iNKT TCR-modified tips probing CD1d molecules loaded with αGalCer or OCH12 (Fig. 4A). A typical force curve on living cells during AFM tip withdrawal is shown in Fig. 4B. A significant number of unbinding events were detected (Fig. 4C, black line). As indicated by the probability density function diagram (Fig. 4C), the maximum of the force distribution was found to be 15 ± 3 pN for CD1d-αGalCer. The specificity of this interaction was proven with an anti-CD1d blocking mAb. Upon blocking, the binding probability decreased dramatically, as shown in Fig. 4C (red dashed lines). A similar behavior was found for CD1d-OCH12 (data not shown).

To further confirm the specificity of the interaction, additional force-distance cycles were performed on live CD1d-transfected THP1 cells pulsed with lipids or left unpulsed using the same receptor-coated tip for reference. A significant number of unbinding events were observed only with lipid-pulsed CD1d-transduced THP1 cells; the binding probability values were 16.8 ± 0.37% for CD1d-αGalCer and 16.1 ± 0.36% for CD1d-OCH12. In contrast, for unpulsed THP1-CD1d cells, the binding probability was only 2.2 ± 0.15% (Fig. 4D). These results clearly support the notion that the iNKT TCR binds specifically to CD1d-transfected cells only when loaded with lipids.

Kinetic Parameters of iNKT TCR/CD1d-GSL Complex Interactions—Comparison of the unbinding force probability density functions for living cells with those obtained for isolated molecules indicated a similar trend. The mean values of the unbinding force for αGalCer were significantly higher than those for OCH12. The nature of these interactions was further analyzed by varying the dynamics of the force-pulling experiments.
In SMFS, the dissociation process is driven by an external force applied to the complex that makes the bond more susceptible to overcome the activation barrier for reaching the unbound state. When the external force is slowly applied to the complex (slow loading rate; see also “Experimental Procedures”), dissociation will occur at low forces and vice versa. According to the single barrier model introduced by Bell (30) and Evans and Ritchie (31), the most probable unbinding force, \( F^* \), is logarithmically dependent on the loading rate, \( r \), according to Equation 1,

\[
F^*(r) = \frac{k_B T}{x} \ln(r \cdot x/k_{off} \cdot k_B T)
\]  

where \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( k_{off} \) represents the dissociation rate, and \( x \) is the distance from the energy minimum of the bound state to the activation barrier.

Indeed, the unbinding forces measured at different loading rates for iNKT TCR/CD1d-lipid complexes were found to increase linearly with the logarithm of the loading rate as expected from this model (30, 31). Fig. 5 shows the unbinding force dependence on the loading rate for iNKT TCR/CD1d-lipid interactions determined for isolated molecules (Fig. 5A) and for living cells (Fig. 5B). For both cases, the measured forces associated with iNKT TCR/CD1d-\( \alpha \)GalCer bond rupture were significantly higher than those associated with iNKT TCR/CD1d-OCH12 for all loading rates.

The kinetic off-rate parameter, \( k_{off} \), and the distance from the energy minimum to the transition state, \( x_{\mu} \), were estimated by...
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fitting the force spectroscopy plot (Fig. 5, A and B) to Equation 1. The values for $k_{\text{off}}$ and $x_0$ of the iNKT TCR interaction with CD1d-$\alpha$GalCer and CD1d-OCH12 as both isolated molecules and embedded in living THP1 cells are presented in Table 1.

We also estimated the kinetic on-rate parameter, $k_{\text{on}}$, on living cells by varying the receptor/CD1d-lipid dwell time of the iNKT TCR-functionalized tip on the cell surface and determining the binding probability. The experimentally obtained values for CD1d-$\alpha$GalCer (Fig. 5C) and CD1d-OCH12 (Fig. 5D) reveal an exponential increase. Longer dwell times resulted in a higher binding probability until a saturation plateau was reached.

A characteristic interaction time, $\tau$, was estimated by fitting the binding probability versus dwell time $p = A(1 - \exp(-t - t_0)/\tau)$, where $A$ is the maximum observable binding probability and $t_0$ is the lag time (32). The kinetic on-rate was then derived from $\tau$ using $k_{\text{on}} = (\tau c_{\text{eff}})^{-1}$ (33, 34), where $c_{\text{eff}}$ is the effective concentration of iNKT TCR on the AFM tip. Because we performed single molecule experiments, this concentration is the inverse of the effective volume of a half-sphere with an effective radius, $r_{\text{eff}}$ (33), in which the tip-bound iNKT TCR molecule can freely move. The effective radius was estimated as the sum of the cross-linker length in equilibrium (3 nm) and the diameter of streptavidin plus the iNKT TCR (6 nm).

The measured $k_{\text{on}}$ values were $1.24 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ for CD1d-$\alpha$GalCer and $1.65 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for CD1d-OCH12. Considering the values obtained for the dissociation and association rate constants, an estimate of the dissociation constant $K_{d} = k_{\text{off}}/k_{\text{on}}$ was calculated. Thus, the dissociation constants were $\sim 40.32 \text{ M}$ for CD1d-$\alpha$GalCer and $630 \text{ M}$ for CD1d-OCH12. The determined values were put into perspective by comparing them with data obtained from SPR experiments (8).

**DISCUSSION**

Utilizing molecule force spectroscopy, specific recognition events between the iNKT TCR and hCD1d-GSL complexes were analyzed to reveal the interaction forces and the kinetic rate constants that govern the bond stability. To characterize the interaction forces between the iNKT TCR and hCD1d complex due to different affinities for the iNKT TCR (i.e. loaded with either $\alpha$GalCer or OCH12), the receptor was attached to an AFM tip via a flexible polyethylene glycol linker, enabling force measurements to be done in vitro and in vivo. The same iNKT TCR-coated tip was used in each particular experiment for probing the specific interactions of both complexes, CD1d-$\alpha$GalCer and CD1d-OCH12.

The force spectroscopy results obtained in this work indicate that the kinetic rate constants for the iNKT
TCR/CD1d-αGalCer and iNKT TCR/CD1d-OCH12 complexes, $k_{\text{on}}$ and $k_{\text{off}}$ are in good agreement with data previously derived from SPR experiments (Table 1). We found the kinetic off-rates from the living cell measurements to be $k_{\text{off}} = 0.50 \pm 0.52 \text{ s}^{-1}$ for αGalCer and 1.04 ± 1 s\(^{-1}\) for OCH12, which compare very favorably with SPR data: 0.39 ± 0.01 s\(^{-1}\) for αGalCer and 1.00 ± 0.12 s\(^{-1}\) for OCH12 (8). We observed that the rate of dissociation of CD1d-OCH12 from the receptor is ~2-fold larger than that of CD1d-αGalCer. In addition, the kinetic off-rate values from the isolated molecule measurements are relatively close compared with those from the living cell experiments. The larger dissociation rate on isolated molecules can be attributed to complex instability in vitro, allowing us to conclude that CD1d-GSL complexes are more stable on living cells under physiological conditions than when isolated in solution.

In contrast to the $k_{\text{off}}$ values, the values for $k_{\text{on}}$ determined by SMFS measurements appear to be scaled down by about an order of magnitude with respect to the values reported from SPR (Table 1). This discrepancy can be explained on the basis of the rough estimate of the critical parameter represented by the effective volume (i.e. effective radius), leading to a source of errors in determining the true kinetic on-rate value from SMFS measurements. On the other hand, $k_{\text{on}}$ values from SPR experiments are often scrutinized by surface rebinding effects. The $k_{\text{on}}$ ratio between CD1d-αGalCer and CD1d-OCH12 is comparable (~8), however, for both SMFS and SPR. Therefore, it appears that the dissociation constant, $K_p$, follows the same trend (see Table 1).

At the same loading rate, higher unbinding forces are required to dissociate the iNKT TCR from the CD1d-αGalCer complex than from the CD1d-OCH12 complex for both isolated molecules and living cells, suggesting that CD1d-αGalCer is more resistant to the external forces compared with OCH12. This finding is consistent with previous results (8) that analyzed the role of the phytosphingosine chain length of the lipids in modulating the binding affinity of CD1d molecules for the iNKT TCR.

Furthermore, this study emphasizes the advantage of the SMFS technique by which critical parameters can be determined directly on living cells. These results with living cells were compared with our values obtained in isolated systems and with SPR as a different method for reference. In conclusion, we have shown for the first time on living cells that the interaction strength, kinetic off-rate, and affinity of the iNKT TCR depend on phytosphingosine chain length.

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