

Chapter 2

Force Spectroscopy and Recognition Imaging of Cells from the Immune System

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2.1 Introduction

Several techniques have emerged in the last decade to measure biological interactions at single molecular level (Svoboda et al. 1993; Kellermayer 1997; Evans et al. 1995; Leckband and Israelachvili 1993). Among these, surface force apparatus (Leckband et al. 1992), biomembrane force probe (Merkel et al. 1999), and optical tweezers (Ashkin 1997) allow to investigate the interaction forces of systems, from small molecules to living cells. The atomic force microscope employs one of the smallest force sensors that, besides high-resolution imaging, allows to measure single molecular receptor/ligand forces down to subnanometer level.

This chapter focuses on single-molecule force spectroscopy as well as simultaneous Topography and RECOgnition imaging (TREC) applied to THP1 living cells using the atomic force microscope (Bozna et al. 2011). While the force

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spectroscopy yields information regarding the structure and the dynamics of the recognition process (Florin et al. 1994; Lee et al. 1994; Hinterdorfer et al. 1996; Hinterdorfer and Dufrene 2006), the TREC method offers the possibility to map the distribution of specific molecular recognition events on the cell surface with nanometer accuracy under physiological condition (Ahmad et al. 2011; Preiner et al. 2009; Stroh et al. 2004a, b).

By using single-molecule force spectroscopy, specific recognition events between Invariant Natural Killer T (iNKT) T cell Receptor (TCR) and hCD1d–glycosphingolipids (GSL) complexes were analyzed to reveal the interaction forces and the kinetic rate constants that govern the bond stability. In order to characterize the interaction forces between the iNKT-TCR and hCD1d complexes owing to different affinities for the iNKT TCR (i.e., loaded with either α -GalCer or OCH12), the receptor was attached to the 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– α -GalCer and CD1d–OCH12.

2.2 Sample and Tip Preparation Methods

2.2.1 iNKT TCR Tip Functionalization

To allow measuring of the binding interactions between single molecule on an AFM tip and molecules on the cell membrane, the functionalization of the AFM tips with the molecule of interests is needed.

A soluble biotinylated iNKT TCR is tethered to the AFM tip via a flexible heterobifunctional PEG [poly(ethylene glycol)]-cross-linker with an extended length of 6 nm, containing an aldehyde and an *N*-hydroxysuccinimide ester (NHS-ester) group at its ends (Fig. 2.1a). The silicon nitride AFM tips (Veeco Instruments) are first functionalized with an amine group using APTES coating (3-aminopropyl triethoxysilane) procedure. To the aminofunctionalized tip is then attached an aldehyde-PEG-NHS linker by incubation for 2 h at room temperature in 0.5 mL of chloroform containing 3.3 mg aldehyde-PEG-NHS and 0.5% triethylamine.

The tips are subsequently rinsed in chloroform and dried with nitrogen before their incubation for 50 min in a mixture of 65 μ L of streptavidin with a concentration of 0.2 mg mL⁻¹ in a PBS buffer (150 mM NaCl, 5 mM NaH₂PO₄, pH 7.5) and 2 μ L of 1 M NaCNBH₃ (freshly prepared by dissolving 32 mg of solid NaCNBH₃ in 500 μ L of 10 mM NaOH). In order to block unreacted aldehyde groups, 5 μ L of 1 M ethanolamine hydrochloride (adjusted to pH 9.6 with 20% NaOH) is added while the incubation continues for 10 min. After washing the tips in PBS, they are treated with 20 μ g mL⁻¹ biotinylated iNKT TCR in TRIS buffer (50 mM Tris, 100 mM NaCl, Glycerol 1%, pH 7.5) and the incubation at 4°C is allowed to continue overnight. The tips are finally washed and stored in TRIS buffer in cold environment.

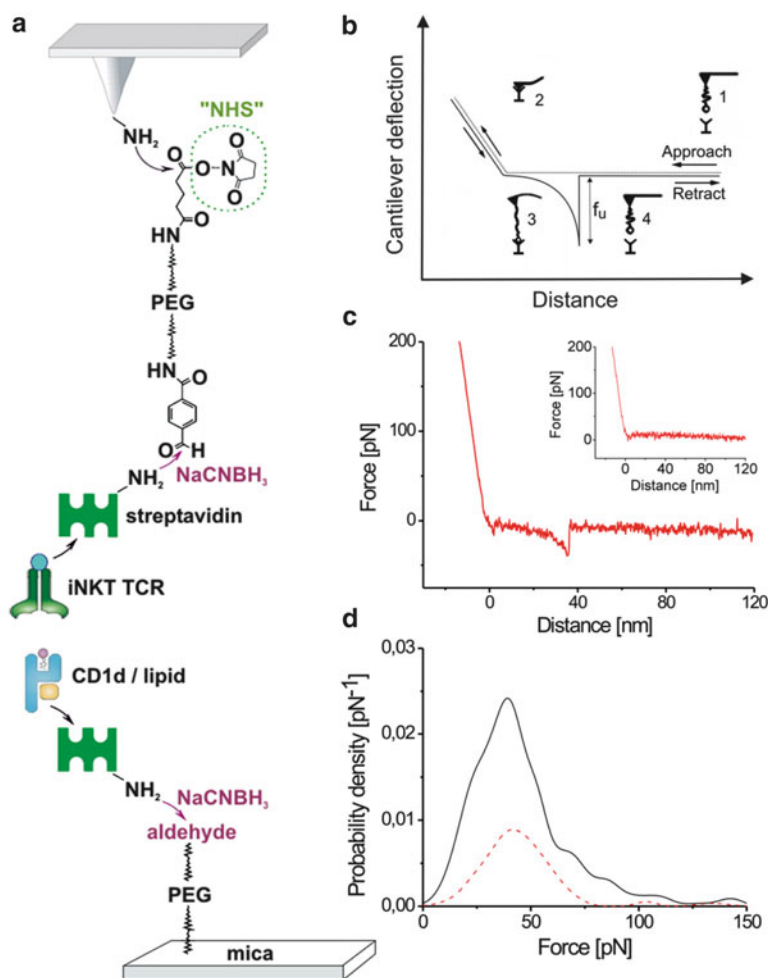


Fig. 2.1 Specific recognition of isolated CD1d-GSL molecules by an iNKT TCR coated tip. (a) Schematic representation of the immobilization strategy; (b) diagram of the force-distance cycle; (c) typical force curve showing an iNKT TCR-unbinding event from CD1d- α -GalCer upon tip retraction. *Inset:* the specific interaction blocked by injecting of free anti-CD1d mAb into the bath solution; (d) probability density function showing the distribution of unbinding force events occurring between the iNKT TCR and CD1d- α -GalCer (black line). The effect of the anti-CD1d blocking antibody on the iNKT TCR/CD1d interaction (red dashed line)

2.2.2 THP-1 Cell Culture

The THP1 cells [American Type Culture Collection (ATCC)], which were transfected with a lentiviral vector encoding CD1d/YFP fusion protein, 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

(5,000 U penicillin/mL). The cells were allowed to grow at 37°C in 5% CO₂ maintaining their concentration between 2×10^5 and 9×10^5 cells mL⁻¹.

2.2.3 CD1d Immobilization on Mica

To allow SMFS measurements of interaction between isolated iNKT TCR and biotinylated CD1d–GSL complexes, freshly cleaved mica were functionalized with 20 µg mL⁻¹ of CD1d-α-GalCer or OCH12, using the same chemical procedure as thoroughly described in Sect. 2.2.1. The surface modified mica substrates were then stored in TRIS buffer at 4°C.

Soluble iNKT TCR heterodimers and human CD1d monomers loaded with α-GalCer or OCH12 were prepared by following a procedure previously reported (McCarthy et al. 2007).

2.2.4 Immobilization of CD1d Transfected THP-1 Cells on Glass Slides

The THP1 CD1d cells were incubated overnight in a 48-well plate at 37°C with α-GalCer or OCH12 (1 µg mL⁻¹), and the next day were washed and resuspended in 500 µL RPMI 1640 w/o FBS.

After washing the glass coverslips with isopropanol and water and then dried, a Poly-L-Lysine (PLL) solution of 500 µL (0.01%) was added and allowed to incubate for 30 min at 37°C.

After rinsing the glass coverslips three times with PBS (Dulbecco), cells (500 µL) were added and incubated for 1 h at 37°C and 5% CO₂. Finally, the glass slides were washed twice with HBSS (Dulbecco) containing Ca²⁺ and Mg²⁺. The glass coverslips were rinsed three times with PBS (Dulbecco), and cells (500 µL) were subsequently added and incubated for 1 h at 37°C and 5% CO₂. Finally, the glass slides were washed twice with HBSS (Dulbecco) containing Ca²⁺ and Mg²⁺.

For TREC measurements, the cells were fixed with 2% paraformaldehyde for 20 min at room temperature and carefully washed with PBS buffer (150 mM NaCl, pH 7.4).

2.2.5 Simultaneous Topography and Recognition Imaging and Instrumentation

Both AFM topography and recognition data were recorded in the MAC (magnetic alternating current) mode by using a PicoPlus AFM (Agilent Technologies, AZ, USA). Magnetically coated Olympus cantilevers having a nominal spring constant of 80 pN nm⁻¹ with a quality factor (*Q*) of ~1 in liquid were used for bottom-MAC

(magnetic field excitation below the sample) mode. All images were taken by closed loop large-scan size scanner ($100 \times 100 \mu\text{m}^2$) in TRIS buffer solution at room temperature. During TREC measurements, integral and proportional gains were adjusted to optimize the sensitivity of the feedback loop and oscillation amplitude was chosen at the optimum driving frequencies of each cantilever (~ 3 kHz). The TREC data were acquired by scanning $\sim 1 \times 1 \mu\text{m}^2$ area of the cell surface and recorded by using a commercially available electronic unit (PicoTREC, Agilent, AZ, USA). The scan speed for imaging was 2 line/s at 256 data points per line. Specificity of iNKT-TCR modified AFM tips to glycolipids loaded CD1d molecule was proven by injecting free anti-CD1d antibody into the fluid cell of the AFM during scanning.

2.3 Single-Molecule Force Spectroscopy of iNKT TCR with hCD1d–GSL Complexes

iNKT cells are a heterogeneous population of lymphocytes that share properties of both T cells and natural killer (NK) cells with the ability to regulate the immune system in response to a broad range of diseases (Kronenberg 2005; Kinjo Yuki et al. 2006; Mattner et al. 2005). These cells express an invariant TCR by which they recognize glycolipids bound to or presented by the CD1d molecule, a nonpolymorphic major histocompatibility complex (MHC) class I-like molecule. The presence of CD1d-lipid complexes on several types of cells including antigen-presenting cells allows the engagement of iNKT TCR, leading to a rapid activation of iNKT cells and secretion of significant levels of inflammatory cytokines such as pro-inflammatory T helper type 1 (Th1) [interferon- γ (INF- γ) and tumor-necrosis factor- α (TNF- α)] and anti-inflammatory Th2 cytokines [interleukin-4 (IL-4), IL-10 and IL-13], which allows these cells to coordinate both innate and adaptive immunity and the development of autoimmune, antimicrobial, antitumor, antitransplant, and allergic immune responses (Spada et al. 1998; Kawano et al. 1997; Hermans et al. 2003; Fujii et al. 2003).

The affinity of the iNKT TCR for CD1d-glycolipid complexes plays an important role in evaluating the biological effects of iNKT cell agonists (Cerundolo et al. 2009). One of the most potent iNKT cell agonists is α -galactosylceramide (α -GalCer) that contains a galactose connected to a ceramide lipid through an α glycosidic-linkage. As revealed by the CD1d crystal structure, the ceramide lipid containing acyl and phytosphingosine chains is embedded in the groove of CD1d molecules (Koch et al. 2005; Zajonc et al. 2005). Several synthesized analogues of α -GalCer have been reported to activate iNKT cells when presented by CD1d expressing cells. Among them is OCH12, which differs from α -GalCer by a shorter phytosphingosine chain (C12 instead of C18).

The affinity of binding of the iNKT TCR to hCD1d–GSL complexes has been previously studied by ensemble-averaged methods, such as surface-plasmon resonance and flow-cytometry (FACS), suggesting that the length of the

phytosphingosine chain influences the affinity of the iNKT TCR for CD1d/lipid complexes (McCarthy et al. 2007). However, the interaction forces that govern the bonds stability have not been determined. To address this issue, binding strength measurements between the iNKT TCR and hCD1d molecules loaded with GSL were performed using the atomic force microscope (AFM). A particular advantage of the AFM, besides high-resolution imaging, is the possibility to measure the intra-molecular (i.e., unfolding and refolding patterns of complementary DNA strands and proteins) and inter-molecular forces (i.e., receptor–ligand interactions) at the molecular level, yielding information regarding the dynamics of the recognition process. The dynamic aspects of molecular recognition are addressed in force spectroscopy experiments, where the unbinding forces between ligands and receptors, either on isolated molecules or on cellular surfaces, are measured. To this end, the AFM tips and solid substrates are functionalized with relevant biomolecules or cells. The low interaction force between a molecule tethered to the tip and its target molecule immobilized to the sample surface is measured in force–distance cycle by monitoring the cantilever deflection.

2.3.1 Principle of Single-Molecule Force Spectroscopy

The molecular recognition of many biological systems can be approximated by a simple configuration consisting of only two states, bound and unbound, which are separated by a transition state characterized by a single energy barrier (Fig. 2.2).

In the thermally activated model (Bell 1978; Evans and Ritchie 1997), the rate of bond dissociation under an increasing applied force is expressed by

$$k_d(F) = k_d(0)e^{F x_\beta / k_B T}, \quad (2.1)$$

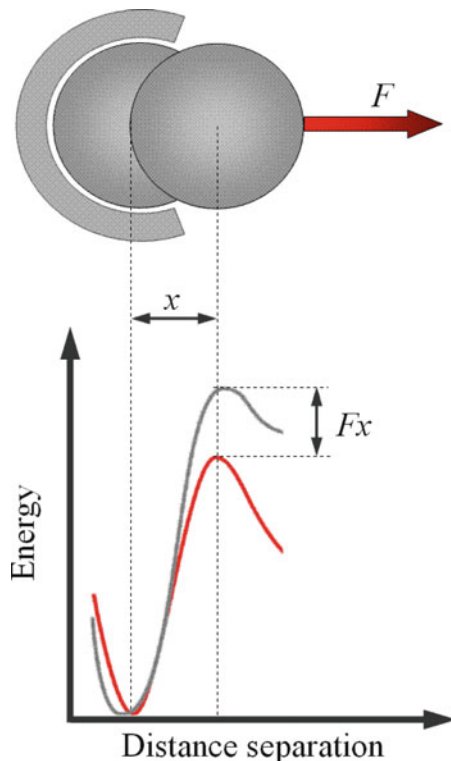
where $k_d(F)$ is the dissociation rate under applied force, and $k_d(0)$ stands for the dissociation rate for zero external force. When a constant force is applied to the bond, the energy barrier is linearly decreased, resulting in a characteristic length scale x which signifies the distance from the bound state to the transition state (Fig. 2.2).

Force-induced dissociation of receptor–ligand complexes using AFM can be regarded as an irreversible process, because following dissociation the two binding partners are further separated. Therefore, rebinding of receptor and ligand can be neglected. Due to the small size of the system, the surrounding heat bath causes significant energy fluctuation, resulting in a stochastic escape process. Thus, the probability $N(t)$ to be in the bound state under a linearly increasing force $F = rt$ can be obtained by solving the master equation:

$$dN(t) = -k_{\text{off}}(rt)N(t), \quad (2.2)$$

where k_{off} is the dissociation rate constant (Strunz et al. 2000).

Fig. 2.2 Schematic representation of a single barrier potential under applied force. The unbinding process takes place via a transition state with a characteristic energy barrier. An external force lowers the energy barrier and facilitates the dissociation caused by thermal energy fluctuations



In force spectroscopy experiments, a force is applied on a binding complex which lowers the activation energy barrier and deforms the interaction energy landscape. The lifetime of noncovalent bonds in the absence of external forces is considerably high. By varying the dynamics of pulling on the specific receptor–ligand bonds, detailed structural and kinetic information of the bond rupture can be determined. This approach is very useful to assess kinetic parameters of the unbinding process, including the length and relative heights of the energy barriers.

By extrapolation to zero forces, the kinetic off-rate constant for the dissociation of the complex in solution can be estimated (Fritz et al. 1998).

In typical force spectroscopy experiments, the cantilever is moved upward at a constant pulling speed, which results in a linear force ramp:

$$F(t) = k_c vt, \quad (2.3)$$

where k_c is the spring constant of the cantilever, and v is the retraction speed. When a linker (e.g., PEG) is used, k_c should be substituted with the resulting spring

constant of the serial combination of the cantilever and tether $(k_c^{-1} + k_{\text{tether}}^{-1})^{-1}$, where k_{tether} is the spring constant of the linker molecule. As the spring constant usually depends on the applied force, a simple but powerful approximation is to use for the resulting combined spring constant at rupture, k_{eff} (Evans and Ritchie 1999; Friedsam et al. 2003), yielding a linear force ramp:

$$F(t) = k_{\text{eff}} vt, \quad (2.4)$$

where $k_{\text{eff}} v = r$ represents the so-called *loading rate*.

From Eq. (2.2), the distribution of the unbinding forces $p(F)$ can be derived with $p(F) = -dN(F)/dF = -dN(t)/(rdt)$, which yields $p(F) = k_d/r \cdot N(F/r)$. Considering Eq. (2.1) it results in

$$p(F) = \frac{k_{\text{eff}}}{r} \exp\left(\frac{Fx_\beta}{k_B T} - \frac{k_{\text{off}} k_B T}{rx_\beta} (e^{Fx_\beta/k_B T} - 1)\right), \quad (2.5)$$

where k_{off} is the dissociation rate in the absence of an applied force, and r is the loading rate defined as $k_{\text{eff}} v$. The applied force decreases the energy barrier, facilitating the dissociation induced by thermal energy fluctuations, which results in a distribution $p(F)$ of the measured unbinding forces.

The most probable unbinding force $F^*(r)$ for the respective loading rate is expressed by

$$F^*(r) = \frac{k_B T}{x_\beta} \ln\left(\frac{x_\beta r}{k_B T k_{\text{off}}}\right). \quad (2.6)$$

The maximum of each force distribution $F^*(r)$ is found to scale linearly with the logarithm of the loading rate. Hence, considering a single energy barrier, the unbinding force versus logarithm of the loading rate is characterized by a simple, linear dependence.

To gain an estimate of the rupture force distribution, hundreds of force curves at the same loading rate are typically acquired during a dynamic force spectroscopy experiment (Baumgartner et al. 2000a). The thermal off-rate constant k_{off} and the distance of the barrier from the energy minimum along the pulling coordinate x can be obtained from a linear fit of these data.

2.3.1.1 Other Models Describing the Bond Rupture

When more barriers are involved during dissociation, the dependence follows a sequence of linear regimes, each of which marking a particular barrier (Merkel et al. 1999).

Different approaches have been used for better characterizing the energy landscape of dissociation under applied external force. In particular, analytical

expressions for $k(F)$ have resulted from calculations based on the free-energy surfaces model proposed by Hummer and Szabo (2003), in which the most probable unbinding force is dependent on the loading rate through $F^* \sim (\ln r)^{1/2}$. Here, a harmonic potential with a cusp-like feature at x_β was used:

$$U_0(F) = k_B T \cdot \Delta G_\beta \left(\frac{x}{x_\beta} \right)^2 \quad (2.7)$$

for $(x < x_\beta)$ and otherwise $-\infty$. Dudko and co-workers (Dudko et al. 2003) used a linear-cubic surface:

$$U_0(F) = \left(\frac{3}{2} \right) \Delta G_\beta \left(\frac{x}{x_\beta} \right) - 2 \Delta G_\beta \left(\frac{x}{x_\beta} \right)^3. \quad (2.8)$$

By applying Kramers' theory of escape from a potential well, the expressions for $k(F)$ were determined, allowing the distribution of rupture forces to be obtained. These models in combination with the model described by Evans et al. (1995) can be combined within a single theoretical framework (Dudko et al. 2006):

$$k(F) = k_{\text{off}} \left(1 - \frac{\mu F x_\beta}{\Delta G_\beta} \right)^{1/\mu-1} \exp \left(\frac{\Delta G_\beta}{k_B T} \left[1 - \left\{ 1 - \mu F x_\beta / \Delta G_\beta \right\}^{1/\mu} \right] \right) \quad (2.9)$$

$$p(F) = \frac{k(F)}{r} \exp(k_B T k_{\text{off}} / x_\beta r) \times \exp \left(- [k_B T k(F) / x_\beta r] \left[1 - (\mu F x_\beta / \Delta G_\beta) \right]^{1-1/\mu} \right), \quad (2.10)$$

where $\mu = 2/3$ and $1/2$ correspond to the linear-cubic and quadratic free-energy surfaces, respectively. For $\mu = 1$ and for $\Delta G_\beta \rightarrow \infty$ independent of μ , the expression reduces to the result of Evans and Ritchie (1997). When $\mu \neq 1$, permissible values of force F are limited from above by the value of the critical force $F_c = \Delta G_\beta / (\mu \cdot x_\beta)$ at which the barrier disappears, leading to inaccurate results for $k(F)$ as the Kramers' theory does not apply within this limit.

The most probable rupture force F^* and the variance of the force distributions σ_F can be estimated by

$$F^* \cong \frac{\Delta G_\beta}{\mu} \left\{ 1 - \left[\frac{k_B T}{\Delta G_\beta} \ln \frac{k_B T k_{\text{off}} e^{\Delta G_\beta / k_B T}}{x_\beta r} \right]^\mu \right\} \quad (2.11)$$

$$\sigma_F^2 \cong \frac{(k_B T \pi)^2}{6 x_\beta^2} \left[\frac{k_B T}{\Delta G_\beta} \ln \frac{k_B T k_{\text{off}} e^{\Delta G_\beta / k_B T + \tilde{\gamma}}}{x_\beta r} \right]^{2\mu-2}, \quad (2.12)$$

where $\tilde{\gamma} = \gamma^2 - 3/\pi^2\psi''(1) \approx 1.064$, with $\gamma = 0.577$ being the Euler–Mascheroni constant and $\psi''(1) = -2.404$ a particular value of the tetragamma function (Abramowitz and Stegun 1964). As concluded in Eq. (2.6), the most probable rupture force is proportional to $(\ln r)^\mu$.

2.3.2 Force Spectroscopy Measurements on Isolated Molecules and Living THP-1

All spectroscopy measurements were performed in force–distance cycle at room temperature using Pico Plus setup (Agilent Technologies, Tempe, USA) equipped with an optical microscope and a CCD camera. To probe the iNKT TCR/CD1d–GSL complex interaction, TCR-coated cantilevers (Veeco Instruments) with spring constants in the range of 0.01–0.03 N m^{−1} were utilized. The spring constants of the cantilevers were determined using the thermal noise method (Butt and Jaschke 1995; Hutter and Bechhoefer 1993), which is a critical parameter for single force spectroscopy measurements. In order to accurately determine the spring constant, it is first necessary to measure the cantilever sensitivity. Usually the cantilever sensitivity (i.e., cantilever deflection detected by the photodiode) is measured by bringing the tip into contact with a hard surface (such as cleaved mica) while the bending of the cantilever is monitored by continuously recording the photodiode signal. This was achieved by acquiring of few force–distance cycles with z-range of 100 nm and a frequency of 1 Hz. Subsequently, the cantilever was withdrawn far away from the surface and the free cantilever movement was recorded. From this complex signal and the already determined cantilever sensitivity, a power density spectrum is calculated by using a Fourier transformation. The spring constant of the cantilever was obtained by using the MATLAB program.

Force measurements on isolated molecules were achieved by recording a 1,000 force–distance cycles, at the same lateral position, varying the z-range (100–300 nm) and duration times (0.2–4 s) to attain different pulling speeds, which resulted in different loading rates. The acquired data were analyzed using a MATLAB program as described by Baumgartner and others (Hinterdorfer et al. 1996; Baumgartner et al. 2000b) to obtain the probability density function (pdf) of the unbinding force and unbinding length. That is achieved by fitting with a Gaussian function, calculated from the mean and the variance of each unbinding event, yielding the most likely unbinding force (maximum of the distribution). A Gaussian of unitary area with the width representing its measuring uncertainty is positioned for each data point of a measured unbinding force value. Subsequently, all Gaussians representing the measured data points are simply summed up to give the final pdf. Hence, pdfs are “continuous” histograms and benefit from the fact that the data accuracy is considered and binning artifacts can be excluded. The loading rates for the iNKT TCR/hCD1d–GSL interaction were calculated by multiplying the tip pulling velocity v

with the effective spring constant $k_{\text{eff}}(r = v \cdot k_{\text{eff}})$, resulting in values of 50–10,000 pNs⁻¹.

The study was initially focused on the interaction forces between the iNKT TCR and hCD1d–GSL (α -GalCer and OCH12) *in vitro* using SMFS. The soluble biotinylated iNKT TCR was linked to AFM tips via a heterobifunctional PEG cross-linker (Haselgrübler et al. 1995), which carried an aldehyde group on its free end that chemically coupled streptavidin. 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 α -GalCer 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. 2.1a.

In force spectroscopy, the unbinding force measurement is achieved through force–distance cycle by approaching and withdrawing the functionalized tip from the sample surface (Fig. 2.1b). During cantilever approaching, the signal recorded by the photo-detector is constant, as there is no bending of the cantilever (Fig. 2.1b, step 1). Once the tip reaches the surface, the cantilever bends upward and the corresponding deflection is typically represented by the ascending slope (Fig. 2.1b, step 2). When the tip is withdrawn, the cantilever first regains its initial shape. Continuing the tip retraction, if a specific interaction occurs between the receptor and glycolipid, the PEG get stretched and the cantilever bends downward (Fig. 2.1b, step 3) until the bond breaks at a critical force (Fig. 2.1b, step 4), which is a direct measure of the binding strength. This force is termed the “unbinding force” f_u which can be calculated based on Hooke’s Law ($F = k \cdot \Delta x$), in which k represents the experimentally obtained spring constant (pN/nm) and Δx is the measured cantilever deflection (nm).

Figure 2.1c shows a typical force–distance curve in which specific interaction between isolated iNKT TCR and CD1d- α -GalCer is monitored. The unbinding force traces are characterized by a particular parabolic-like shape caused by the stretching of the tip-coupled PEG linker before unbinding. To check the specificity of these interactions, 25 $\mu\text{g mL}^{-1}$ of free anti-CD1d monoclonal antibody (mAb) were by injected into solution which largely resulted in a disappearance of the unbinding events because of blockage of the CD1d- α -GalCer molecules on the surface (Fig. 2.1c, inset).

Empirical pdfs displaying the most probable unbinding forces were calculated based on unbinding force analysis. Figure 2.1d shows a representative example of a pdf for iNKT TCR/CD1d- α -GalCer interaction obtained from more than 100 unbinding events in which the maximal probable force was 39 ± 2 pN for a loading rate of 1,260 pN s⁻¹ (Fig. 2.1d, black curve). The binding probability, which represents the frequency of occurrence of specific interaction events in force distance cycles, was 17.4%. Upon injecting the blocking antibody, the binding probability decreased to 5.7% (Fig. 2.1d, red dotted curve). The same experimental procedure and analysis sequence was applied to the CD1d-OCH12 complex (data not shown) for which the binding probability decreased from 8.6 to 2.6% after injection of blocking antibodies. To further check the interaction specificity, a bare

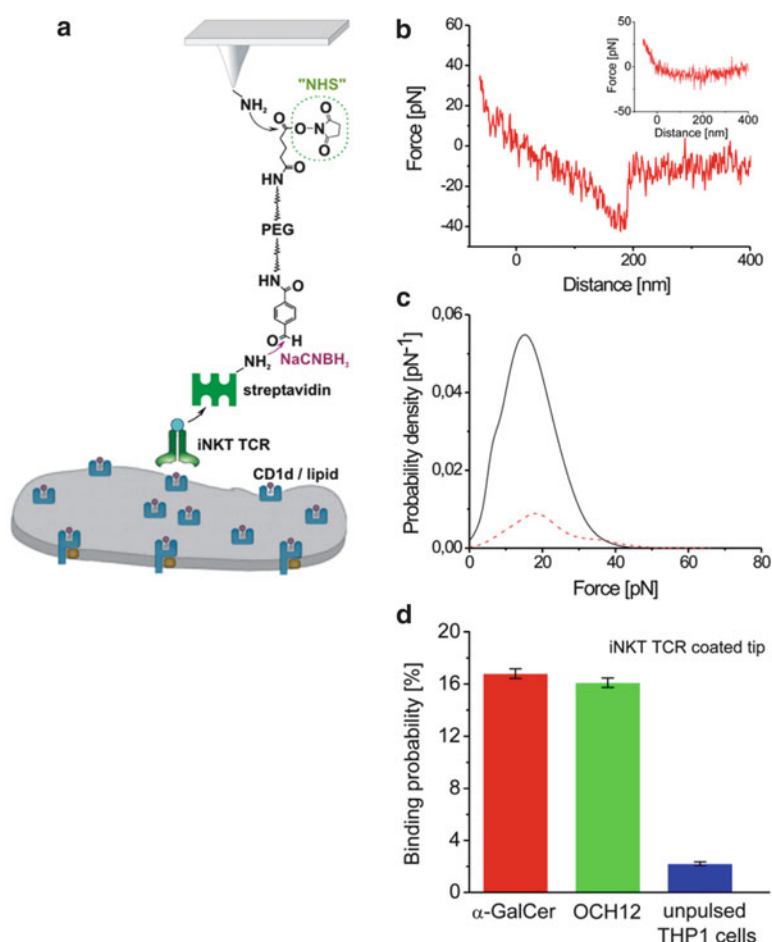


Fig. 2.3 Schematic diagram of single-molecule force measurements on live cells. The THP1 CD1d cells pulsed with iNKT agonist α -GalCer or OCH12 attached onto poly-L-lysine coated glass coverslips. **(b)** Typical force–distance cycle on living cells showing a receptor–lipid interaction. *(Inset)* Blocking of the specific interaction by free anti-CD1d monoclonal antibody. **(c)** Distribution of unbinding forces of iNKT TCR/CD1d- α -GalCer (black line) based on 150 unbinding events out of 1,000 force–distance cycles. **(d)** Comparison of binding probabilities of iNKT TCR coated tip on live CD1d-transfected THP1 cells either pulsed with α -GalCer (red) or OCH12 (green) or unpulsed (blue)

tip was used instead of a receptor-coated tip. Under these conditions, only very few rupture events were observed.

In the second part of the study, we assessed the iNKT TCR affinity for hCD1d–GSL complexes at the THP1 cell surface. SMFS measurements on living cells were carried out with iNKT TCR modified tips probing CD1d molecules loaded with α -GalCer or OCH12 (Fig. 2.3a).

A typical force curve recorded on living cells during AFM tip withdrawal exhibits a particular slope characteristic of soft substrates (Fig. 2.3b). As anticipated from previously experiments performed on isolated molecules, when the lipids were embedded in the cell membrane, significant recognition events of CD1d-lipids by the iNKT TCR modified AFM tips occurred as well (Fig. 2.3c, black line). The pdf diagram (Fig. 2.3c) reveals the maximum of the force distribution, which was found to be 15 ± 3 pN for CD1d- α -GalCer. The specificity of this interaction was proven by using CD1d mAb, which dramatically decreased the binding probability (Fig. 2.3c, red dotted line). For CD1d-OCH12 a similar behavior was found.

The specificity of the interaction was further confirmed by additional force-distance cycles performing on live CD1d transfected THP1 cells pulsed with lipids or unpulsed, using the same receptor coated tip for reference. Significant number of unbinding events was only observed with lipid pulsed CD1d transduced THP1 cells for which the binding probability values were $16.8 \pm 0.37\%$ for CD1d- α GalCer and $16.1 \pm 0.36\%$ for CD1d-OCH12, respectively. In contrast, the binding probability was only $2.2 \pm 0.15\%$ for unpulsed CD1d THP1 cells (Fig. 2.3d). These results clearly support the assumption that the iNKT TCR binds specifically to CD1d transfected cells only when loaded with lipids.

The unbinding force pdfs for living cells were compared with the ones obtained for isolated molecules, revealing a similar trend. Significantly higher mean values of the unbinding force for α -GalCer with respect to OCH12 were observed. By varying the dynamics of the force-pulling experiments, the nature of these interactions was further analyzed.

As previously discussed, the dissociation process in SMFS is driven by an external force applied to the complex that makes the bond more susceptible to reaching the unbound state by overcoming the activation barrier. Consequently, when the external force is slowly applied to the complex (slow loading rate), dissociation will occur at low forces and vice versa.

In agreement with the single-barrier model (Bell 1978; Evans and Ritchie 1997), the unbinding forces measured at different loading rates for iNKT TCR-CD1d-lipids complexes were found to increase linearly with the logarithm of the loading rate. The unbinding force dependence on the loading rate for iNKT TCR-CD1d-lipid interactions determined for both isolated molecules and for living cells is shown in Fig. 2.4. It was found that for both systems the unbinding forces for iNKT TCR-CD1d- α -GalCer were significantly higher than for iNKT TCR-CD1d-OCH12 for all loading rates.

The dynamic parameter such as kinetic off-rate, k_{off} , as well as the distance from the energy minimum to the transition state, x_{β} , was estimated by fitting the force spectroscopy plot (Fig. 2.4a, b) to Eq. (2.6), which estimates the most probable unbinding force at a certain loading rate.

The kinetic off-rate from the living cell measurements for α -GalCer and OCH12 was found to be $k_{\text{off}} = 0.50 \pm 0.52 \text{ s}^{-1}$ and $1.04 \pm 1 \text{ s}^{-1}$, respectively. These values compare very favorably with SPR data: $0.39 \pm 0.01 \text{ s}^{-1}$ for α -GalCer and $1.00 \pm 0.12 \text{ s}^{-1}$ for OCH12 (McCarthy et al. 2007).

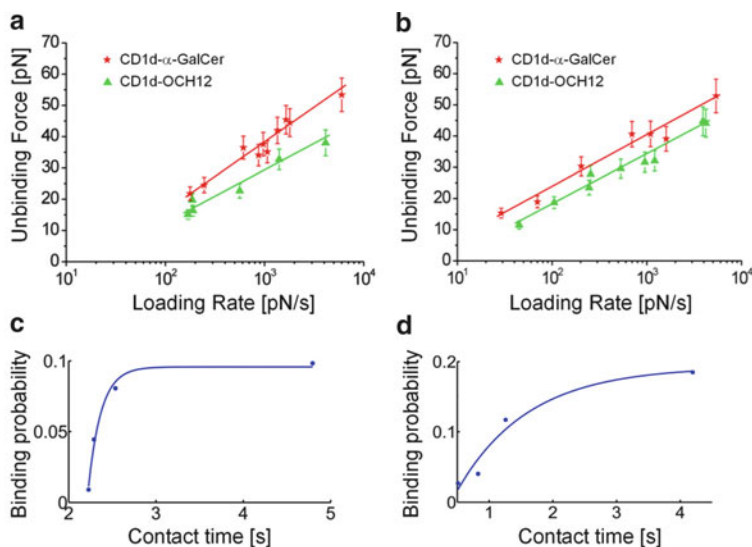


Fig. 2.4 Loading rate dependence of unbinding force. Unbinding force spectra of iNKT TCR bound to CD1d- α -GalCer (red stars) and CD1d-OCH12 (green triangles) on isolated molecules (a) and on living THP1 cells (b). The uncertainty in the determination of the unbinding force is represented by error bars. Binding probability as a function of contact time for CD1d- α -GalCer (c) and OCH12 (d) on living THP1 cells

These results indicate that the kinetic rate constant for the iNKT TCR–CD1d- α -GalCer and iNKT TCR–CD1d-OCH12 complexes, k_{off} , is in good agreement with the data previously derived from surface-plasmon resonance experiments.

2.3.3 Kinetic On-Rate Measurements

In addition to determining the kinetic off-rate constant value of these complexes, we estimated the kinetic on-rate parameter k_{on} on living cells by varying the receptor–CD1d-lipid interaction time. The experimentally obtained values for CD1d- α -GalCer (Fig. 2.4c) and CD1d-OCH12 (Fig. 2.4d) reveal an exponential increase. Longer dwell times resulted in a higher binding probability until reaching a saturation plateau.

Fitting the binding probability versus dwell time to $P = A(1 - \exp(-(t - t_0)/\tau))$ in which A represents the maximum observable binding probability and t_0 the lag time, the characteristic interaction time τ was estimated (Atkins 1998). Subsequently, the kinetic on-rate was derived from τ using the equation $k_{\text{on}}(\tau c_{\text{eff}})^{-1}$ (Baumgartner et al. 2000b; Rankl et al. 2008), where c_{eff} is the effective concentration of iNKT TCR on the AFM tip expressed by the inverse of the effective volume of a half sphere with effective radius r_{eff} , in which the tip-bound iNKT TCR molecule can move freely. By summing the cross-linker length in equilibrium

(3 nm) and the diameter of streptavidin plus iNKT TCR (6 nm), the effective radius was determined.

An estimate of the dissociation constant $K_d = k_{\text{off}}/k_{\text{on}}$ was calculated, based on the obtained values for dissociation and association rate constants. For CD1d- α GalCer, the dissociation constant was found to be about 40.32 μM , whereas for CD1d-OCH12 the value was 630 μM . These values were put into perspective by comparing them to the data obtained from surface-plasmon resonance measurements (McCarthy et al. 2007).

From data analysis, one can conclude that the dissociation rate of CD1d-OCH12 from the receptor is about twofold larger than CD1d- α -GalCer. In addition, the kinetic off-rate values from the isolated molecule measurements are relatively close when compared to 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.

Unlike the dissociation rate which was close to that obtained from surface-plasmon resonance, the values for k_{on} determined by SMFS measurements appear to be scaled down by about one order of magnitude with respect to the values reported from SPR (Table 2.1), which 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 for determining the true kinetic on-rate.

However, for both force SMFS and SPR, the k_{on} ratio between CD1d- α -GalCer and CD1d-OCH12 is comparable (~ 8) so that it appears evident that a similar trend exists for dissociation constant K_d (cf. Table 2.1). For both isolated molecules and living cells, higher unbinding forces are required to dissociate the iNKT TCR from the CD1d- α -GalCer complex than from CD1d-OCH12 at the same loading rate, suggesting that unlike OCH12, the CD1d- α -GalCer is more resistant to the external forces.

These results are consistent with previous findings (McCarthy et al. 2007) demonstrating that shortening the phytosphingosine chain reduced the affinity of binding to the iNKT TCR, leading to changes in the iNKT cell immunological synapse, polarization of the iNKT cell cytotoxic granules and iNKT cell activation.

Also, this study emphasizes the advantage of single-molecule force spectroscopy technique by which critical parameters can be determined directly on living cells.

2.4 TREC Imaging of CD1d-Glycolipid Complex on THP1 Cells

Simultaneous recognition, visualization, and quantifications of receptor binding over cell surfaces with high spatial accuracy are important tasks in the life sciences and especially in molecular cell biology. There are many different microscopy techniques, such as epi-fluorescence microscopy, photo-activated localization

Table 2.1 Kinetics measurements

	Force spectroscopy measurements on isolated molecules		Force spectroscopy measurements on live THP1 cells		SPR ⁽⁸⁾			
	x_{β} (nm)	k_{off} (s^{-1})	x_{β} (nm)	k_{off} (s^{-1})	k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	K_d (μM)	k_{off} (s^{-1})	K_d (μM)
hCD1d-GSL complex	0.43 ± 0.10	1.94 ± 1.44	0.57 ± 0.14	0.50 ± 0.52	1.24×10^4	40.32	0.39 ± 0.01	1.29 ± 0.08
hCD1d- α GalCer	0.56 ± 0.15	2.49 ± 1.47	0.59 ± 0.18	1.04 ± 1.0	1.65×10^3	630	1.00 ± 0.12	23.3 ± 1.41

Comparison of obtained kinetic parameters with respect to previous results by SPR

microscopy (PALM), stimulated emission depletion microscopy (STED), single-particle tracking, single dye tracing, or scanning electron microscopy, that might be used for these purposes. However, they have the drawbacks of limited resolution, lack of topographic information, and/or inapplicability under physiological conditions. On the other hand, AFM, which represents a nonoptical microscopy, offers a unique solution to obtain possible high-resolution topographical images at the nanometer scale and single-molecule interaction forces of biological specimens (e.g., proteins, DNA, membranes, cells, etc.) under ambient conditions and without the need for rigorous sample preparation or labeling (Horber and Miles 2003). With the recent development in AFM, a new fastest and most straightforward method, called “simultaneous Topography and RECOgnition imaging” (TREC) (Stroh et al. 2004a, b; Ebner et al. 2005), it becomes possible to quickly obtain the local distribution of receptors on cell surface with a lateral resolution of 5 nm (Chitchevlova et al. 2007). In this AFM mode, the surface of a biological specimen is scanned with a biofunctionalized tip at regular imaging speed, yielding a map of specific ligand-binding sites together with a topographic image (Hinterdorfer and Dufrene 2006; Stroh et al. 2004a, b).

2.4.1 Principle of Simultaneous Topography and Recognition Imaging

The operating principle of TREC is based on the MAC mode, in combination with a ligand attached to the AFM tip on the end of an elastic PEG linker with defined length (~6 nm). The flexible PEG linker allows the ligand molecule to easily bind the receptor on the cell surface. During scanning, the functionalized cantilever is oscillated close to its resonance frequency. When the specific recognition occurs between the ligand on the tip and the receptor site on the cell surface, oscillation amplitude of the cantilever decreases, which is evident of the binding sites. In TREC, the cantilever oscillation amplitude is divided into two parts (i.e., lower and upper parts with respect to the baseline of the oscillation) and processed in different paths by using a specially designed electronic circuit (PicoTREC, Agilent). While the lower part of the signal is used for generation of the topography image, the upper part reflects recognition events and gives the recognition image (Fig. 2.5).

The starting point for a successful TREC experiment is a cantilever with a low (~1) quality (Q)-factor. The Q -factor represents the “memory” of the cantilever. A low Q -factor (and therefore a low “memory” ability) ensures that an amplitude reduction in the lower part of the oscillation (originating from a change in the topography) is sufficiently separated in time from amplitude reductions in the upper part of the oscillation (originating from molecular recognition between the ligand and the receptor).

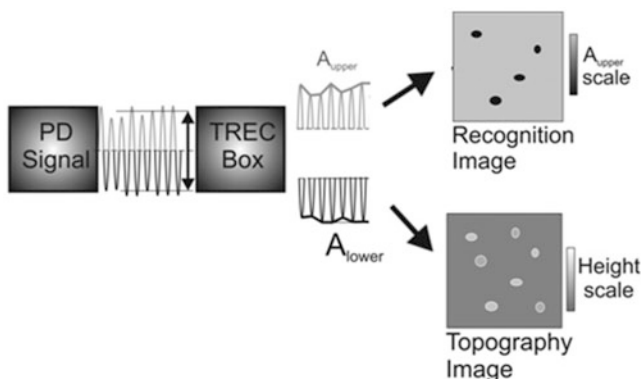


Fig. 2.5 Principle of TREC. The cantilever oscillation is divided into two parts in the TREC box. While the envelope of the *upper part* yields the recognition image, the *lower part* provides for the topography image

Consequently, only the lower part of the sinusoidal oscillation is fed into the feedback loop and is thereby held constant to obtain the unbiased surface topography. The upper part of the oscillation, solely containing information on recognition between ligand and receptor, is recorded to generate a recognition image simultaneously to the topography image.

2.4.2 Adjustment of Imaging Parameters

In order to reveal reliable recognition sites on cell membrane, imaging parameters such as feedback loop, oscillation amplitude, and driving frequency should be adjusted properly.

First, in conventional MAC mode AFM, the peak-to-peak value of oscillating amplitude of bare (i.e., without any tip functionalization) AFM tip is utilized as feedback parameter and called “full amplitude feedback.” Using feedback loop, the full amplitude (FA) is held constant during scanning by adjusting the voltage applied to the piezo actuator which controls the z distance between the tip and the surface. However, during TREC imaging in which the ligand functionalized AFM cantilever tip is used, the modified “half amplitude (HA) feedback loop” has to be used to obtain the true surface topography. When molecular recognition occurs between tip-tethered ligand and its receptor on cell surface, the flexible PEG linker complex is stretched due to the upward movement of the cantilever oscillation and the top peaks of the oscillation is reduced. Thus, both topographical features (at the bottom peaks) and molecular recognition (at the top peaks) affect the value of the FA (Preiner et al. 2009). Therefore, using the conventional feedback loop for TREC leads to errors in the height value of the topographical

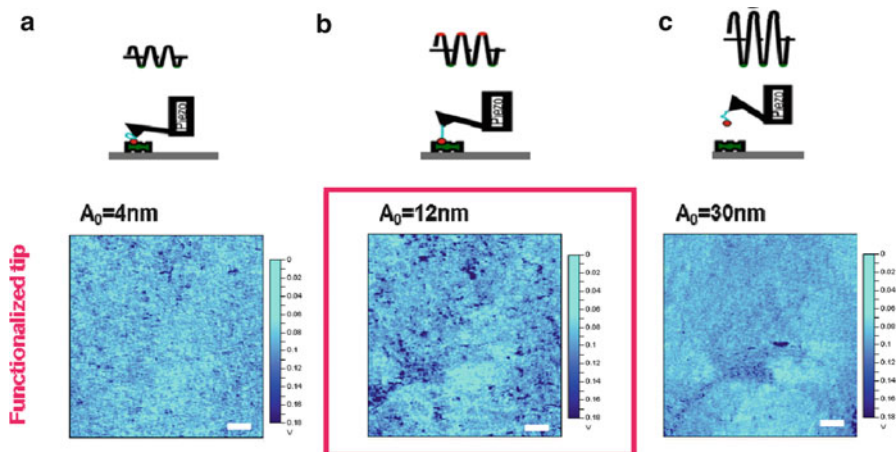


Fig. 2.6 Three different regimes of oscillation amplitudes; No recognition signal at (a) lowest (4 nm) and (c) highest (30 nm) amplitude. Pronounced recognition signal (b) was detected at efficient amplitude (12 nm)

image since the feedback itself cannot discriminate the different contributions to the amplitude reduction. The stretching of the polymer linker exhibits a strong nonlinear behavior so that for small linker extensions the force acting on the cantilever is negligible. It is, however, much higher at extensions approaching the linker's contour length. In other words, the value of the amplitude in the lower part of the oscillation is not affected by the linker stretching. This part is only affected by changes in topography, and is therefore used as feedback parameter in the so-called HA feedback loop, yielding the unbiased surface topography.

The adjustment of oscillation amplitude is the second important parameter of TREC imaging in which the proper recognition signal only occurs when the ligand–receptor complex survive until the tip has moved laterally away from the position of the receptor molecule. Therefore, the choosing of ideal amplitude, which is physically determined by the stretching behavior of the linker molecule, plays a critical role in TREC experiments. There are three different regimes of oscillation amplitudes as sketched in Fig. 2.6. For small amplitudes, the ligand (TCR) molecule has bound to the receptor (CD1d complex) on the cell surface. However, the linker does neither stretch nor exert an efficient force on the cantilever in the upward swing, generating no recognition signal (Fig. 2.6a). When the amplitude has been increased to the second regime, the linker is efficiently stretched without detaching the TCR from the CD1d complex in the upper part of the oscillation, resulting in a pronounced recognition signal and shown as dark spots in recognition image (Fig. 2.6b). When the amplitude is further increased in the last regime (III), the peak-to-peak value of the amplitude is already higher than the contour length of the linker molecule. Therefore, the TCR molecule unbinds from the CD1d complex as soon as the every top peak of the first oscillation cycle. Because continuous binding while scanning over the CD1d complex is prevented in

this case, no recognition signals are generated (Fig. 2.6c). Consequently, the oscillation amplitude of the AFM tip must properly be adjusted as in regime (II) to ensure sufficient linker stretching without rupturing the TCR molecule from the CD1d complex when scanning laterally over the cell surface.

The third important imaging parameter is a properly chosen driving frequency. As mentioned before, the cantilever has a kind of memory, which is the time it needs to “forget” information (e.g., recognition or topography information) caused by the damping of the environment. To obtain a true recognition image without any topographical information or feedback artifacts, the cantilever must have lost all of the information collected during the lower part of the oscillation, when the recognition information is measured (at the top peak of the oscillation). If this is not the case, i.e., when features from the amplitude-error image (originating from the finite feedback speed) contribute to the recognition image, the time given to the cantilever to forget this topographical information is too short. Therefore, this time has to be increased, which can be done by lowering the excitation frequency. The contribution of the amplitude error to the contrast in the recognition image and its dependence on the excitation frequency have been demonstrated experimentally and explained in detail (Preiner et al. 2009).

2.4.3 Nanomapping of CD1d-Glycolipids Complexes on THP1 Cells by Using TREC

The localization and distribution of glycolipids pulsed CD1d complexes were examined by using TREC imaging. Before identifying the binding site, magnetically coated AFM tip was modified with the soluble biotinylated iNKT TCR via a heterobifunctional PEG cross-linker (Fig. 2.3). The affinity of iNKT TCR to glycolipid loaded CD1d complexes is already shown in the literature (Bozna et al. 2011; McCarthy et al. 2007). In this study, in order to locally identify the CD1d glycolipid complexes on THP1 cell surfaces, expressed CD1d molecules, the cells were incubated with three different glycolipids (α -GalCer, C20:2, and OCH12) for 16 and 4 h.

The oscillation of functionalized AFM tips was adjusted (~ 8 nm) slightly smaller than the extended PEG linker, which has length of ~ 10 nm to allow binding iNKT-TCR molecule on the tip to the glycolipid loaded CD1d molecule complex on the cell surface during scanning.

The recognized CD1d glycolipids microdomains were acquired by scanning THP1 cells which were loaded with α -GalCer, C20:2, and OCH12, respectively, and showed in Fig. 2.7 as representative simultaneous topography and recognition images. Figure 2.7a, b is the image of the control cell group which was not loaded with any glycolipids. The statistical analysis of the area distribution of microdomains was achieved by measuring recognition sites in four different areas ($\sim 1 \times 1 \mu\text{m}^2$) of the same THP1 cell surface and is shown in the right panels of Fig. 2.7.

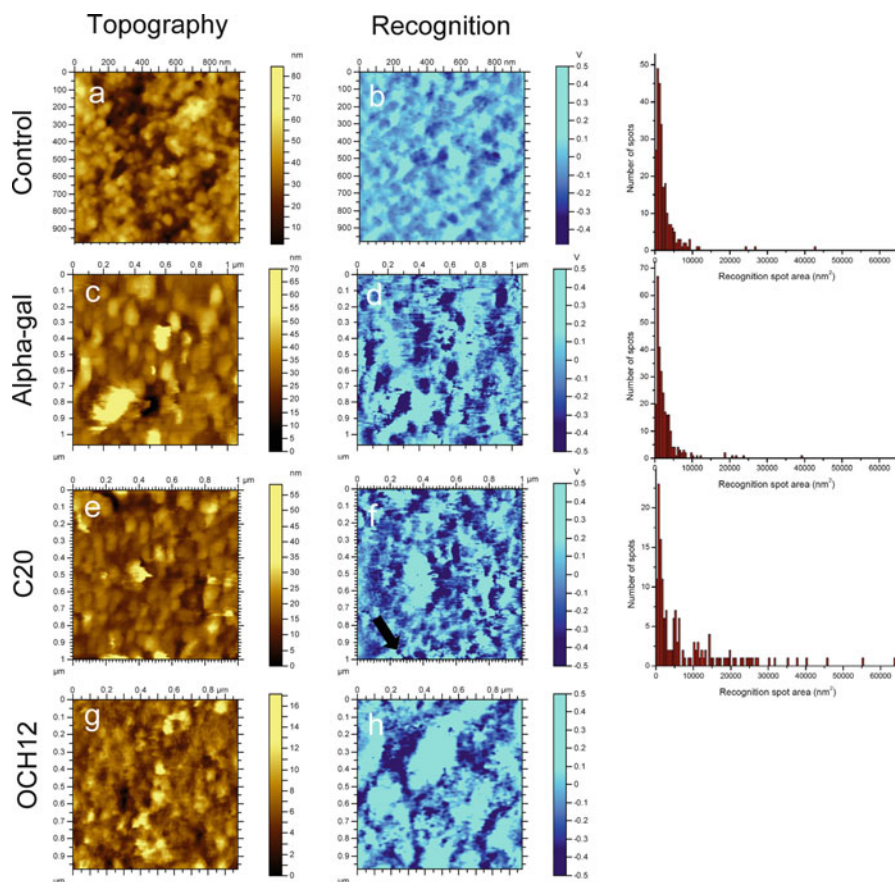


Fig. 2.7 Topography and recognition images of control (a, b), α -GalCer (c, d), C20 (e, f), and OCH12 (g, h) loaded THP1 cells. *Right panels* represent the area distribution of microdomains corresponding recognition images detected in four different areas of the same cell

In the case of the cells that were pulsed with α -GalCer and C20:2 for 16 h, the dimension (area) of the recognition spots (microdomains of the CD1d glycolipid complexes) were detected from the amplitude reduction, arising from interactions between iNKT-TCR and CD1d-glycolipid complex, and were revealed in recognition images (Fig. 2.7d, f). According to topographical images (Fig. 2.7c, e), the cellular membrane features were organized into typical spherical form with ~ 100 – 150 nm in length and heights varying from ~ 20 to ~ 70 nm. Analyzed recognition images (Fig. 2.7, right panels) showed that the α -GalCer and C20:2 loaded CD1d proteins formed microdomains with the dimension (area) from ~ 250 to $\sim 10,000$ nm² (mean $\pm$ SD, $2,219 \pm 989$, $n = 523$) and distributed nonuniformly. On the other hand, low-affinity profile of iNKT TCR to OCH12 affected the distribution and area size of the CD1d-OCH12 complexes. A closer look at recognition spots (Fig. 2.7h) reveals that they consist of larger connected microdomains.

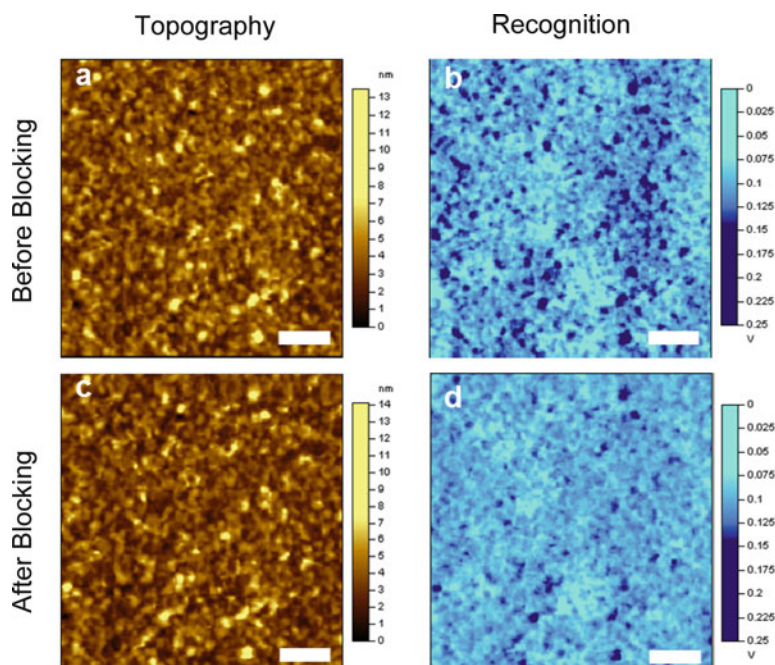


Fig. 2.8 Specificity of iNKT TCR functionalized AFM tip to CD1d- α -GalCer complexes. Topography (a, c) and recognition (b, d) images before and after addition of free anti-CD1d antibody solution. Scale bar is 100 nm.

The analyzed dimension area of the recognition spots was increased up to 30,000 nm² (mean $\pm$ SD, 8,197 $\pm$ 6,925, $n = 155$). Specificity of iNKT-TCR functionalized AFM tips to glycolipid pulsed CD1d complex was proven by using both control cell group, which was not loaded with any glycolipids, and blocking experiment. As expected and shown in Fig. 2.7b, no recognition event was detected when scanning the control cell group. Furthermore, specificity of recognition signal was also proven by adding anti-CD1d antibody to block CD1d molecules on the cell surface, while scanning the sample at the same position. The recognition clusters (Fig. 2.8b) partly disappeared (Fig. 2.8d) 1 h after addition of anti-CD1d antibody, whereas no changes in topography images (Fig. 2.8a, c) have been observed.

THP1 cells also pulsed with same glycolipids (α -GalCer, C20:2 and OCH12) for 4 h to detect the effect of short incubation time of glycolipids to distribution of CD1d-glycolipid complexes. When the cells were loaded with α -GalCer for 4 h, we could not identify pronounced recognition spots of CD1d- α -GalCer complexes (Fig. 2.9b). However, CD1d-C20:2 complexes were successfully detected even after 4 h incubation time (Fig. 2.9d). It is recently reported that while α -GalCer underwent intracellular loading and was presented on CD1d more slowly (longer internalization), C20:2 showed rapid kinetics of direct loading (without internalization) (Im et al. 2009). These findings are in agreement with our observations. In contrast to THP1 cell, incubated with OCH12 for 16 h, the cells that were pulsed

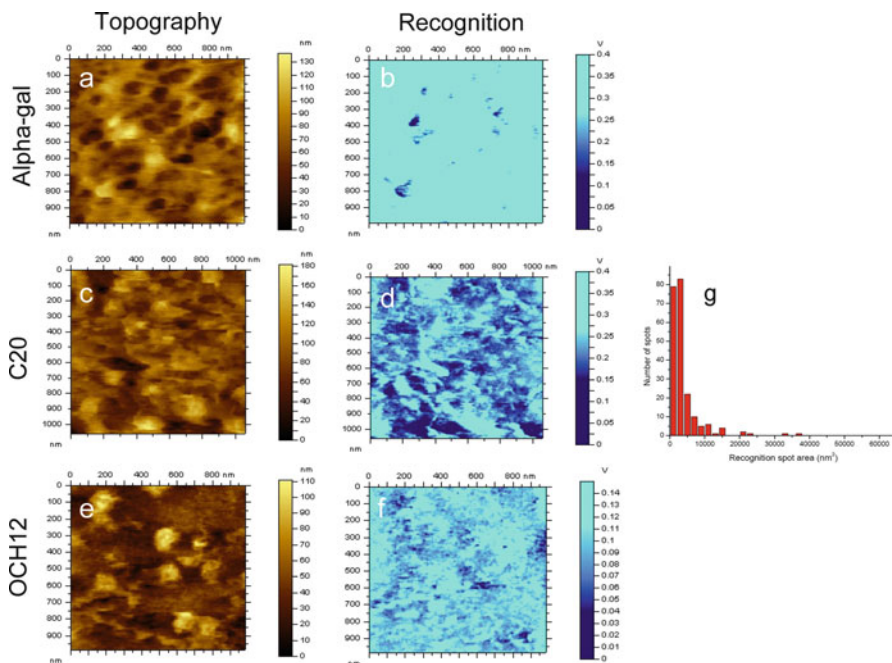


Fig. 2.9 Distribution and localization of CD1d molecules when they were loaded with α -GalCer (a, b), C20 (c, d), and OCH12 (e, f) for 4 h. The area distribution (k) of CD1d-C20 complex corresponding recognition images detected in two different areas of the same cell

with same glycolipids for 4 h did not show any pronounced recognition event (Fig. 2.9f). This is most likely caused by improper presentation of OCH12 on CD1d molecule during selected time period. Thereby the binding strength between iNKT-TCR functionalized AFM tip and CD1d-OCH12 complex was not sufficient to stretch PEG linker enough to create proper amplitude reduction for selected incubation time. After analyzing the dimension area of recognition images for 4 h C20:2 loaded cells, CD1d-C20:2 complexes again formed similar sizes (with a dimension area from ~ 250 to $\sim 10,000$ nm²) microdomains with the 16 h pulsed ones (Fig. 2.9g).

Blocking experiments were also applied to show specificity of modified AFM tip by adding anti-CD1d antibody to liquid cell while acquiring the topography (Fig. 2.10a, c) and recognition (Fig. 2.10b, d) images on the same area of 4 h C20:2 pulsed THP1 cell surfaces. Almost all dark spots (recognition spots) in Fig. 2.10b were abolished in 1 h after injection of anti-CD1d antibody (Fig. 2.10d).

2.5 Concluding Remarks

“Simultaneous Topography and REcognition” (TREC) imaging is a combination of high-resolution AFM topography imaging with single-molecule force microscopy. This powerful AFM technique not only yields fine structural details about

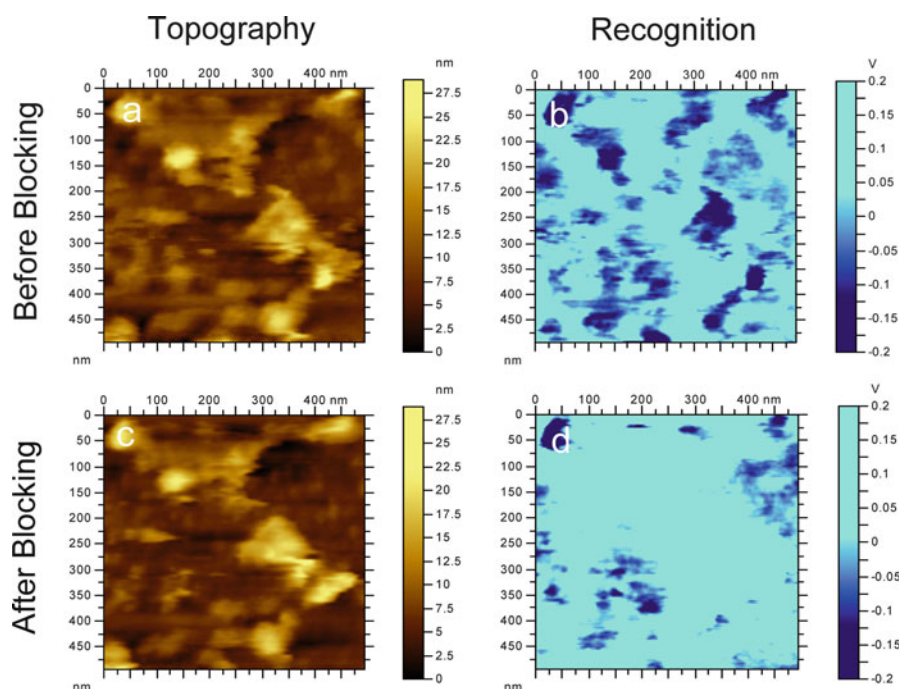


Fig. 2.10 Specificity of iNKT TCR functionalized AFM tip to 4 h pulsed C20-CD1d complex. (a–c) topographical images simultaneously recorded with recognition maps (b–d). After addition of free anti-CD1d antibody solution into the liquid, most of the recognition spots disappeared (d), while topographical images remained unchanged (c)

topography but also senses biochemical composition of native biological samples under physiological conditions. The present work shows a major advantage of TREC over optical approaches to cells with a spatial topographical and recognition resolution of ~ 5 nm. In the presented work, TREC has successfully been exploited to identify CD1d glycolipid complex sites on THP1 cells and to colocalize their position with membrane topographical features. The recognition events which were shown as dark spots in recognition image were revealed with the diameter ranging between 25 and 160 nm (Fig. 2.8f). Since the diameter of the iNKT-TCR/CD1d complex is ~ 3.5 nm (Borg et al. 2007) and the free orientation of the PEG linker allows binding 10 nm before and 10 nm after the binding sites, the expected diameter of a single-receptor recognition spot is 23.5 nm, which is the minimal patch size of recognition spots we observed in the recognition images (see the arrow in Fig. 2.7f).

Overall, TREC imaging allows to detect single-molecular interactions, and thus to visualize, identify, and quantify local receptor binding sites and assign their locations to the topographical features of cell surfaces. This study illustrates the great potential of TREC for the investigation and localization of membrane proteins

on cell surfaces with several piconewton force resolution and a positional accuracy of a few nanometers. For these reasons, TREC is a promising tool for the identification and location of receptor binding sites on cells, organelles, and other subcellular structures.

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