Article

Application of Optical Tweezers in the Research of Molecular Interaction between Lymphocyte Function Associated Antigen-1 and Its Monoclonal Antibody

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Lymphocyte function associated antigen-1 (CD11a/CD18, LFA-1) plays an important role in the structure of the immunological synapse and is required for efficient lysis of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. To study the activation mode of LFA-1 on the NK cell surface, optical tweezers were used in the work. As an emerging technology, optical tweezers are widely used to manipulate microscopic objects and measure the forces of molecular interactions in the field of biological research. In our study, a new platform was constructed to study the single molecular behavior of receptor on cell surface using optical tweezers. Based on the platform, the interaction between an NK cell and a polystyrene microsphere coated with anti-LFA-1 antibody was observed. The result confirmed that the adhesion forces between an NK cell and a polystyrene bead were time-dependent. According to our findings, we propose that anti-LFA-1 antibody may cause the clustering of LFA-1 on NK cell surface. *Cellular & Molecular Immunology*. 2007;4(3):221-225.

Key Words: NK cell, LFA-1, optical tweezers, polystyrene microsphere

Introduction

Lymphocyte function associated antigen-1 (CD11a/CD18, LFA-1) is a member of the integrin family of cell surface receptors. LFA-1 plays an important role in the structure of the immunological synapse and is also likely to be involved in signaling (1). Adhesion to target cells through LFA-1 is required for efficient lysis by CTLs and NK cells (2, 3). Although the role of LFA-1 in the activation of NK cells has attracted much attention (4, 5), the activation mode of LFA-1 on NK cells is still unclear.

As an emerging technology in the field of biological research, optical tweezers use a single-beam laser directed to provide an attractive or repulsive force to trap, image, and manipulate micron-sized particles in three dimensions. Early in 1970, Ashkin reported the detection of optical scattering and showed that the gradient forces of radiation pressure from focused laser beams could be used to significantly affect the dynamics of small transparent micrometer sized neutral particles (6). Years later, Ashkin and colleagues reported that a tightly focused beam of light was capable of holding microscopic particles stable in three dimensions (7), which means they setup the first apparatus of single-beam optical gradient force trap. Optical tweezers have been proved useful in the field of biological science. The earliest application of optical tweezers technology in biological research was in the late 1980s when Ashkin and his colleagues used it to trap viruses and bacteria (8), although bacteria were killed due to the high energy of the laser. With the application of a less damaging infrared (IR) laser, it is now possible to trap and manipulate a single yeast, bacterium and organelle without damage. The optical tweezers technology is a revolutionizing micromanipulation of biological objects (9). An optical-trap-based strategy to measure the force between macromolecules in piconewton (pN) range has been established (10, 11), which provides an alternative method to quantify the molecular behavior of cell surface receptors.

The function of LFA-1 in facilitating T cell antigen receptor (TCR)-mediated killing has been well researched.

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The activation of LFA-1 in T cell is TCR dependent. Without TCR signaling, the granule redistribution and recruitment of microtubule organizing center toward the CTL contact caused by LFA-1-ICAM-1 will be impaired (12). However, the activation of LFA-1 alone is sufficient to initiate NK cells. What is more, the anti-LFA-1 mAb can activate NK cytotoxicity (13). To study the activation mode of LFA-1 on the NK cell surface, a platform based on optical tweezers was constructed to observe the molecular behavior of LFA-1 expressed on NK cells. According to the results, the adhesion forces between an NK cell and a polystyrene microsphere coated with anti-LFA-1 antibody were confirmed to be time-dependent.

Materials and Methods

Reagents

Fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD56 antibody, phycoerythrin (PE)-labeled mouse anti-human CD11a (LFA-1) antibody, PE-Cy5-labeled CD3 and PE-labeled mouse IgG_1 antibody were obtained from BD Pharmingen. The 3-μm unibead uniform carboxyl polystyrene microspheres were purchased from Tianjin BaseLine ChromTech Research Center. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide Hydrochloride (EDC·HCl) was purchased from Bio Basic Inc. N-Hydroxysuccinimide (NHS) and 2-(N-Morpholino) ethanesulfonic acid (MES) were purchased from Sigma. Ficoll-Hypaque was obtained from Tianjin Hao Yang Biological Manufacture Co. Human CD56 MicroBeads were obtained from Miltenyi Biotec Inc.

Optical tweezers

The main components of the optical tweezers system were an ion laser-pumped titanium-sapphire laser with an average peak power of 1.0 W, and an inverted microscope (model IX 70; Olympus). The microscope was combined with a CCD camera for video microscopy. This system was provided by the Laser Biology Laboratory in the University of Science and Technology of China.

Purification of NK cells from peripheral blood mononuclear cells

PBMCs were isolated by Ficoll density gradient centrifugation from healthy human peripheral blood. Magnetic cell sorting was then applied to purify NK cells from PBMCs. After washing in buffer for one time, PBMCs were resuspended in 80 μl of buffer per 10^7 cells and added 20 μl CD56 Microbeads. The mixture was incubated for 15 min at 4°C, then washed in buffer and applied to magnetic separation with MS Column. $CD56^+$ NK cells were collected and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), penicillin (100 U/ml), and interleukin-2 (50 U/ml).

Flow cytometric analysis

PBMCs and NK cells were analyzed by flow cytometric analysis with fluorescence-conjuncted mAbs including FITC-labeled mouse anti-human CD56, PE-Cy5-conjuncted mouse anti-human CD3, PE-conjuncted mouse anti-human CD11a. First, cells were blocked with normal mouse immunoglobulin to saturate mouse Fc receptor for 30 min at 4°C. Then, the cells were stained with indicated mAb or the control antibody at 4°C for 30 min, and then washed three times. Polystyrene beads were stained with PE-conjuncted mouse anti-human CD11a without blocking. The data wwere acquired by FACSCalibur (Becton Dickinson) and analyzed with WinMDI software.

Covalent coupling of carboxyl-modified microspheres

Antibody was covalently linked to microspheres according to the two-step protocol supplied by Bangs Laboratories Inc and Duke Scientific Corporation (14). Briefly, 100 μl 5% (w/v) beads were washed in deionized water and MES buffer. After second wash, pellet was resuspended in 500 μl of activation buffer (pH = 6) containing 5 mg NHS, 10 μmol EDC, 25 μmol MES, ensuring that the microspheres were well suspended, and the beads were incubated at room temperature for 3 h. They were then washed in deionized water to remove unreacted NHS and EDC. Fluorescence-conjuncted mAbs (20 μl) were added to resuspended the microspheres, and the mixture was allowed to react at room temperature for 2 h with constant mixing. Finally, the beads were washed in quench buffer (40 mM glycine, 1% BSA) and resuspended in 500 μl quench buffer to block the uncoupled sites of beads.

Calibration of optical trap

To convert displacement signals provided by CCD camera into forces, the stiffness of the optical trap had to be determined. This was done using the standard hydrodynamics method (15, 16). Calibration for force was performed by flowing solution past a trapped bead at a known velocity and calculating the force from Stokes' law:

$$F = -6\pi\eta r v$$

where v is the velocity, r is the radius of the bead, and η is the viscosity of the solution. When bead stayed in a new balance position, the distance x between the center of the optical trap and the balance position was recorded by CCD camera and digitized so that the signals could be used for tracking the trapped particle position *via* video tracking. The force applied to the particle is linear with respect to its displacement from the center of the trap as long as the displacement is small. In this way, an optical trap can be compared to a simple spring, which follows Hooke's law:

$$F = -k_x \cdot x$$

Since the viscosity force equaled to the force of optical trap, the stiffness of optical trap k_x could be calculated,

$$k_x = \frac{6\pi\eta r v}{x}$$

Measurement of the adhesion force between a bead and a cell

The adhesion force between a bead and a cell for each

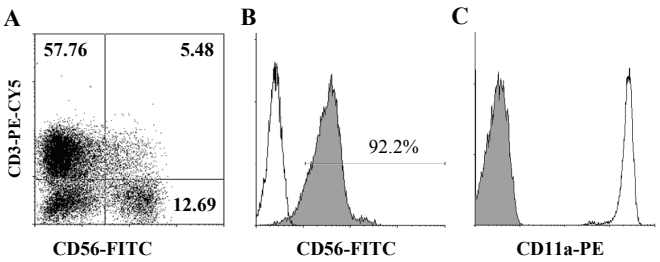

Figure 1. Purification of NK cells from PBMCs and identification of LFA-1 expressed on NK cells. Cell phenotypes were represented by using CD56-FITC and CD3-PE-Cy5 (A). $CD56^+$ NK cells were purified from PBMCs using MACS (B). The expression of LFA-1 on NK cells was identified by FACS (C).

detected binding event was calculated from the maximum displacement of the bead away from the trap center just before bond breakage (14). A micropipette was applied as a straw to absorb an IgG-coated bead as manipulable handle to hold an NK cell. Then the NK cell was fixed by the "handle" and was pushed toward another IgG-coated bead that was trapped by the optical tweezers. After contacting for a few seconds, the NK cell was pulled against the trapped bead and the trapped bead began to depart from the center of optical trap. The maximum displacement of the bead away from the trap center was recorded when the bead broke up with the cell. Finally the adhesion force was calculated as described above in the "calibration of optical trap" part.

Results

Isolation of NK cells from PBMCs and identification of LFA-1 expressed on NK cells

PBMCs were isolated by Ficoll density gradient centrifugation. Cell phenotypes were analyzed by flow cytometry (Figure 1A). Then $CD56^+$ NK cells were purified from PBMCs by magnetic cell sorting. After analysis by flow cytometry, the purity of NK cells was 92.2% and the expression level of LFA-1 on NK cells was very high (Figures 1B and 1C).

Identification of LFA-1 monoclonal antibody coated polystyrene beads

PE-labeled mouse anti-human CD11a was coated on the surface of carboxyl polystyrene microspheres. The beads coupled with antibody were observed by fluorescence microscope and the result showed that almost all polystyrene beads could be stimulated to eradiate fluorescence (Figures 2A and 2B). The result of flow cytometric analysis demonstrated that the microspheres were coupled with antibody uniformly (Figure 2C).

Measurement of adhesion forces between an NK cell and a polystyrene bead conjugated by anti-LFA-1 antibody

Optical traps are uniquely suited for measuring forces in the pN range under a variety of biochemical conditions. One case of adhesion-detachment between a polystyrene bead and an

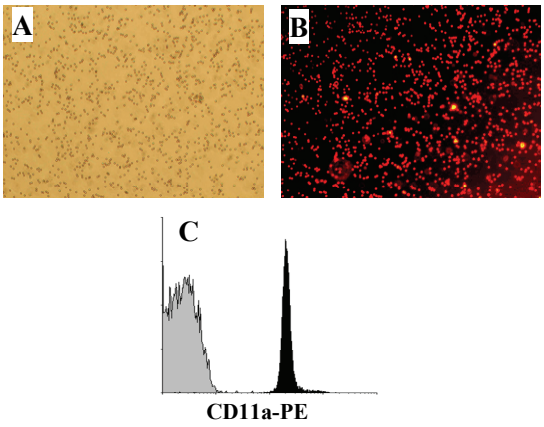

Figure 2. Identification of the LFA-1 mAbs coated on polystyrene beads. The beads coupled with antibody were observed by fluorescence microscopy (A, bright field; B, dark field) and FACS (C) respectively.

NK cell was shown in Figure 3. The adhesion time and the maximum displacement of bead were recorded by camera and the video was analyzed by the software Tiff Image Analyser.

The net force on the bead caused by the optical trap as a function of position was calibrated by determining the trap stiffness. The trap stiffness of the optical tweezers was 50.49 pN/μm. One case of experiment data analyzed by software was shown in Figure 4A. The adhesion force of each case was calculated as described in "Materials and Methods". After plotting the adhesion force (as Y bar) and adhesion time (as X bar) in a graph (Figure 4B), it was clearly seen that the adhesion forces between an anti-human CD11a mAb

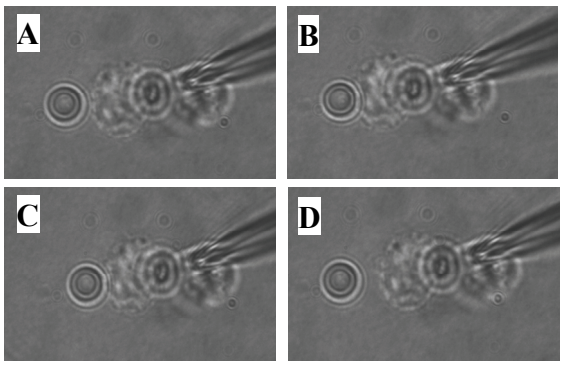

Figure 3. Measurement of the adhesion forces between a polystyrene bead coated with anti-LFA-1 monoclonal antibody and an NK cell. Light microscopy images of adhesion and detachment between a polystyrene bead and an NK cell at various time after binding. The NK cell was pushed toward the polystyrene bead trapped by optical tweezers (A), and was held against the bead for a few seconds (B). Then the NK cell was pulled away from the bead and the bead began to depart from optical trap (C). Finally the cell detached from the bead (D). The largest distance between the bead and the center of optical trap was measured by software.

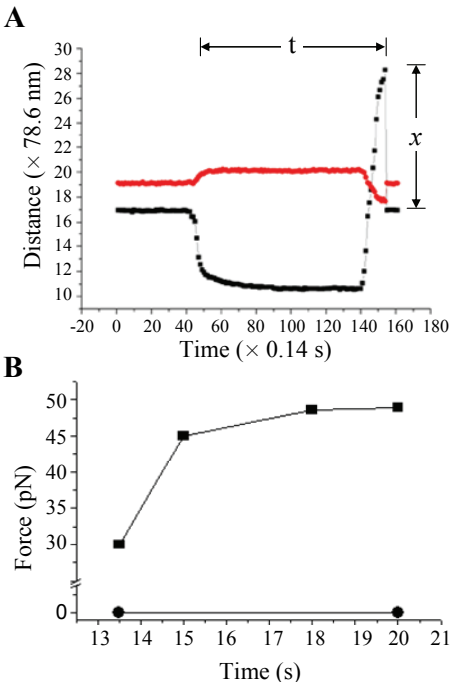

Figure 4. Determination of detachment force between a single NK cell and a bead coated with LFA-1 mAb at different contact time. (A) One example of data record showing the maximum distance (*x*) of the bead away from the trap center and the contacting time (t). (B) The relationship between adhesion force and contacting time (■beads coated with LFA-1 mAb, ●beads coated with mouse isotype IgG_1 as control).

coated bead and an NK cell were increased over time after they contacted with each other. The adhesion forces between a bead coated with mouse IgG_1 isotype control antibody and an NK cell were too small to be detected based on the platform, which eliminated the possibility of unspecific binding between the bead and the cell (Figure 4B). Besides, we also found some cases that the adhesion was too tight to be broken for the reason that the time of contact was too long.

Discussion

Optical tweezers use laser to manipulate microscopic objects. In biological research, these instruments have been used to apply forces in the pN-range and to measure displacements in the nm range of objects ranging in size from 10 nm to over 100 mm. Optical tweezers technology is a very useful tool for cell manipulation. They are non-invasive, do not damage cells, and are quite easy to use. Optical tweezers have been used to manipulate chromosomes (17), human gametes (18), micronuclei (19). Recently, optical tweezers are capable of manipulating particles at the subnanometer level, approaching the fundamental limit set by Brownian fluctuations. Because of this high accuracy of manipulation, it is convenient to establish a contact between two cells (20, 21) to observe the real-time behavior of cells.

Besides manipulating particles in the microscale, optical tweezers are also suitable for measuring the biological force of molecular interactions. Biological macromolecules, such as antibodies, can be coupled to polystyrene or silica beads. Trapped by optical tweezers, a bead can be steered into a desired experimental geometry to bind with a partner molecule attached to a coverslip. The forces and movements can be measured (22). Similar experiments can be performed with atomic force microscope (AFM) and surface force apparatus (SFA). However, it is difficult to study in liquid environment using AFM, and the force resolution of SFA is lower (10 nN) than that of the other instruments (23).

Based on the advantages described above, our platform can be used in the quantitative and real-time research of the interaction between biological macromolecules, especially the dynamic behavior of cell surface receptors before and after the stimulation of ligands or antibodies. In this study, the adhesion forces between an NK cell and a polystyrene bead conjugated with anti-LFA-1 antibody are used as a quantitative parameter to reflect the molecular behavior of LFA-1 receptors. Moreover, because the platform is constructed on a fluorescence microscopy, the fluorescent signals of cell receptors or cytoplastic proteins fused with fluorescent protein can be used as another parameter to inspect the single molecular behavior.

Our results indicate that the adhesion forces between an anti-human CD11a mAb coated bead and an NK cell are time-dependent. Two possible explanations are proposed to explain the time-dependence. One possible explanation is that it takes time for antibodies and antigens (LFA-1) to form stable bonds. Another explanation is that under the stimulation of mAbs coated on the bead, cell surface receptor LFA-1 begins to cluster at the site of interaction, which increases the density of molecule at local site. To determinate the two explanations, paraformaldehyde fixed NK cells were used as control. If the first explanation is true, then the time-dependence will still exist no matter whether the cells are fixed or not. However, the adhesion forces between coated beads and fixed cells were very small and the time-dependence disappeared (data not shown), suggesting that the first explanation may not reflect the reality. Paraformaldehyde fixation is an important step of intracellular staining in flow cytometric analysis of cytokine production, and may cause changes in cell surface phenotype (24). The result of flow cytometric analysis showed that high level of LFA-1 expression on fixed NK cell surface could be detected by staining with PE-labeled mouse anti-human CD11a antibody, excluding the possibility that fixation affects the binding ability of LFA-1 to its mAbs (data not shown). Hence, LFA-1 may be recruited and congregate at the local site of the interaction with antibodies.

Additionally, we investigated other membrane proteins expressed on NK cells, such as NKG2D and ICAM-1. However, we did not observe the same result as LFA-1, suggesting that the clustering of LFA-1 induced by antibody may be a specific character of LFA-1.

In summary, this study provides an alternative method to

monitor molecular behavior of receptors before and after the stimulation of ligands or antibodies. Meanwhile, based on our platform, we have found that anti-LFA-1 antibody may cause the clustering of LFA-1 on NK cell surface. Compared with T cells, the engagement of LFA-1 alone is sufficient to initiate activation signals in NK cells (25, 26). Our results may provide a clue to explore the activation mechanism of LFA-1 on NK cells.

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