AFM analysis of the multiple types of molecular interactions involved in rituximab lymphoma therapy on patient tumor cells and NK cells

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ABSTRACT

Rituximab is a monoclonal antibody drug approved for the treatment of patients with lymphomas. Rituximab's main killing mechanism is antibody-dependent cellular cytotoxicity (ADCC). During ADCC, rituximab's fragment antigen binding (Fab) region binds to the CD20 antigen on the tumor cell and its fragment crystallizable (Fc) region binds to the Fc receptor (FcR) on the natural killer (NK) cells. In this study, two types of molecular interactions (CD20–rituximab, FcR–rituximab) involved in ADCC were measured simultaneously on cells prepared from biopsy specimens of lymphoma patients by utilizing atomic force microscopy (AFM) with functionalized tips carrying rituximab. NK cells were detected by specific NKP46 fluorescent labeling and tumor cells were detected by specific ROR1 fluorescent labeling. The binding affinity and distribution of FcRs on NK cells, and CD20 on tumor cells, were quantitatively measured and mapped. The binding affinity and distribution of FcRs (on NK cells) and CD20 (on tumor cells) were associated with rituximab clinical efficacy. The experimental results provide a new approach to simultaneously quantify the multiple types of molecular interactions involved in rituximab ADCC mechanism on patient biopsy cells, which is of potential clinical significance to predict rituximab efficacy for personalized medicine.

1. Introduction

The advent of atomic force microscopy (AFM) [1] provides an invaluable tool that allows us to probe the activities of individual molecules on single living cells under near-physiological conditions [2]. By linking specific molecules (e.g., ligands and antibodies) onto the AFM tip, we can locate and manipulate their cognate membrane proteins (e.g., receptors and antigens) on the cell surface [3]. This technique is termed single-molecule force spectroscopy (SMFS) [4]. Recently, SMFS has been proven to be a useful method to analyze the activities of single molecules in situ on living bacteria [5] and mammalian cells [6]. However, current SMFS experiments are performed on purified biomolecules isolated from cell lines [7] or directly on cell lines [8]. Cell lines cultured in vitro are known to be quite different from cells in the human body, due to the huge difference between the in vitro and in vivo environment. Thus, the conclusions obtained using cell lines may not completely reflect the in vivo conditions in the human body [9]. Directly measuring the molecular interactions on cells obtained from patient biopsies can undoubtedly better reflect the true physiological properties of membrane proteins. Besides, current SMFS experiments are commonly designed to measure only one type of molecular interactions, while, in fact, multiple types of molecular interactions occur during many biological activities (e.g., cancer cell signaling networks often includes many different types of molecular interactions [10]). Consequently, developing SMFS methods that can simultaneously probe multiple types of molecular interactions occurring during certain biological processes directly on cells obtained from patient biopsies will significantly enrich our understanding of cell biological problems and may bring novel possibilities in the field of translational medicine.

Rituximab is a chimeric monoclonal antibody (consisting of human immunoglobulin G1 heavy-chain sequences and murine immunoglobulin variable regions) that was approved in 1997 by the United States Food and Drug Administration (USFDA) for the treatment of patients with B-cell lymphomas [11]. The target of
rituximab is the CD20 antigen on the B-cell lymphoma cells. In vitro evidence indicated that rituximab’s main killing mechanism involves antibody-dependent cellular cytotoxicity (ADCC) [12], as shown in Fig. 1(A). During the ADCC process, rituximab fragment antigen binding (Fab) region binds to the CD20 on the tumor cell and, subsequently, its fragment crystallizable (Fc) region binds to the Fc receptor (FcR) on the natural killer (NK) cells. NK cells then release perforin and granzymes that can lyse tumor cells [13]. Hence, the ADCC effect is determined not only by CD20–rituximab interactions occurring on tumor cells, but also by FcR–rituximab interactions occurring on NK cells. In fact, several new anti-CD20 antibodies with increased binding to CD20 (e.g., AME-133V) or with increased binding to FcR (e.g., Ocrelizumab, PRO131921, and GA101) have been developed, but their efficacy is yet to be determined by their performance in the ongoing clinical trials [14]. An unprecedented success has been achieved in the clinical practice using rituximab, which has become the standard treatment (either as a single agent or in combination with chemotherapy regimens) for patients with lymphoma [11]. However, 40–50% of patients with lymphoma are still insensitive or develop resistance to rituximab [15]. Providing effective therapies for those patients who are insensitive or develop resistance to rituximab has become a challenging problem that urgently needs to be solved [14].

In order to develop drugs with enhanced efficacies, we should elucidate rituximab’s exact in vivo mechanisms. However, traditional biochemical methods are essentially based on ensemble experiments and the results reflect the average behaviors of a heterogeneous cell population [16]. Thus, this approach cannot reveal the rare events in individual cells in complex and heterogeneous cell populations [3]. As a result, we possibly neglect some important information (e.g., information regarding small cell subpopulations [17]) that is critical to further understand cellular activities and drug actions. AFM-based SMFS is known to be a powerful tool for single-cell and single-molecule assay. Single cell molecular activities detected by SMFS considerably complement the results obtained by traditional methods [18]. However, the current SMFS experiments present two problems, including the use of cell lines grown in vitro and measuring only one type of molecular interaction. In order to address these two problems, in this study, we explored the possibility of using SMFS to simultaneously probe the multiple types of molecular interactions directly on cells prepared from cancer patient biopsies. Concretely, SMFS was applied to simultaneously probe the two types of rituximab molecular interactions (CD20–rituximab and FcR–rituximab) on cells from patient biopsies (tumor cells and NK cells) involved in the rituximab-induced ADCC mechanism.

2. Materials and methods

2.1. Sample preparation

Biopsy samples used here were bone marrow fluid and peripheral blood cells from patients with lymphoma. Biopsy samples were prepared by the medical personnel from the Affiliated Hospital of Military Medical Academy of Sciences according to the standard clinical biopsy procedures. Patients with lymphoma that were confirmed by clinical pathological diagnosis were selected for the study. This study was approved by the ethical committee of the Affiliated Hospital of Military Medical Academy of Sciences (Beijing, China). Bone marrow samples were collected before the clinical treatments. For these patients, tumor cells had invaded into the bone marrow. Thus, the bone marrow cells contained tumor cells. Bone marrow cells (or peripheral blood cells) were dropped onto the glass coverslips that were coated with poly-l-lysine (Solarbio Company, Beijing, China). Poly-l-lysine is positively charged and cells are negatively charged, therefore, cells can be attached onto the glass slide via electrostatic adsorption. Cells on the slides were then chemically fixed in 4% paraformaldehyde (Solarbio Company, Beijing, China) for 30 min. The prepared cell samples were then used for fluorescent labeling experiments.

2.2. Tip functionalization

Rituximab (obtained from the Affiliated Hospital of Military Medical Academy of Sciences) was linked onto the surface of the AFM tip by using the method of covalent coupling of silanes on silicon oxide [19]. The silicon nitride probe used for the functionalization had a nominal constant spring of 0.01 N/m (MLCT, Bruker, Santa Barbara, CA, USA). The heterobifunctional polyethylene glycol (PEG) molecule, NHS-PEG-MAL, (MW 3500, JenKem Technology, Beijing, China) was used as a linker molecule. The process of tip functionalization was performed as previously described [9,20]. First, the tip was coated by a layer of NH2 by putting clean tips and silanization reagents (aminopropyltriethoxysilane and N,N-diisopropylethylamine, purchased from Sigma–Aldrich, Saint Louis, MO, USA) in a glass desiccator under argon gas. Second, the NH2-modified tips were incubated with NHS-PEG-MAL molecules, which allowed the NHS groups of the PEG molecules to covalently bind to the surface of tips. Third, rituximab was treated with N-succinimidyl-3-(acetylthio) propionate (SATP) (Thermo Scientific, Rockford, IL, USA) to form SH groups. The tips were then incubated with SH-modified rituximab, which allowed the MAL groups of the PEG molecules to covalently bind to rituximab. The functionalized tips were then used for AFM experiments.

2.3. Fluorescence microscopy

ROR1 and Nkp46 fluorescent labeling experiments were performed as follows: (1) Six prepared cell sample glass coverslips

![Fig. 1. Schematic diagrams. (A) Rituximab-induced ADCC mechanism. (B) Probing of the two types of molecular recognition interactions (CD20–rituximab, FcR–rituximab) involved in rituximab-induced ADCC using SMFS.](Image 38x72 to 278x326)
(bone marrow or peripheral blood) were placed into the wells of 6-well culture plates (Jet Bio-Filtration Products Co., Ltd, Guangzhou, China). (2) Fresh phosphate buffered saline (PBS) (Hyclone Laboratories, Inc., Logan, UT, USA) was added into the wells to wash the coverslip samples, three times (10 min each). (3) Two hundred microliters of working solution of donkey blocking serum was added to each well and then incubated for 30 min at room temperature. After the incubation, the samples were washed three times (10 min each) with PBS. (4) Goat-anti-human ROR1 antibody solution (10 μg/mL; R&D Systems, Minneapolis, MN, USA) was added into three wells and goat-anti-human NKp46 antibody solution (10 μg/mL; R&D Systems) was added into the other three wells, and then incubated for 3 h at room temperature. After incubation, the samples were washed three times (10 min each) with fresh PBS. (5) FITC-conjugated donkey-anti-goat IgG solution (KangChen Company, Shanghai, China) was added into the wells and incubated for 30 min. After incubation, the samples were washed using PBS three times (10 min each). (6) The coverslip samples were placed on the stage of the fluorescence microscope (Nikon, Tokyo, Japan) and the fluorescence distribution was analyzed.

2.4. AFM imaging and measurements

AFM imaging and measurements were performed using Bioscope Catalyst AFM (Bruker, Santa Barbara, CA, USA), which is set on an inverted fluorescence microscope (Nikon, Tokyo, Japan). AFM experiments were performed in PBS at room temperature using functionalized tips. For AFM experiments, the fluorescence-labeled coverslip samples were placed in a petri dish containing PBS. The petri dish was then placed on the AFM stage. NK cells and tumor cells were recognized by FITC-fluorescence. Based on the fluorescence, AFM tips were moved to the NK cells or the tumor cells. AFM images of NK cells and tumor cells were obtained. The imaging mode was contact mode. Both the height and deflection images were obtained. The scan rate was 0.5 Hz. The sample/line was 256 and the scan line was 256. AFM images of normal cells (red blood cells) were also obtained as negative controls.

For SMFS experiments, the exact spring constant of cantilever (functionalized probe) was calibrated using the thermal noise method [21]. Force curves were first obtained in the bare areas of the coverslips to calibrate the deflection sensitivity of the probe. The functionalized tips were then moved to the NK cells and tumor cells to obtain force curves based on the fluorescence. The following were the correlative parameters used to obtain the force curves: the ramp size was 2 μm, the ramp rate was 0.5 Hz, the scanning point was 512, and the surface delay was 1 s. In order to map the distribution of specific proteins on the cell surface (CD20 on tumor cell and FcR on NK cell), 16 × 16 force curves were obtained in 500 × 500 nm² areas on the cell surface. To demonstrate the specificity of FcR–rituximab interactions, blocking experiments were performed. Force curves were obtained on NK cells to probe the FcR–rituximab interactions and free rituximab was added to block the FcRs on the NK cells. Finally, force curves were obtained on the NK cells at different time after rituximab addition. In order to test rituximab activity on the AFM tips, force curves were obtained again on NK cells that were not blocked. Similarly, blocking experiments were also performed to demonstrate the specificity of CD20–rituximab interactions on tumor cells using the protocol described above for NK cells. To quantitatively analyze the binding affinity and distribution of FcR on NK cells and CD20 on tumor cells for different patients with lymphoma, SMFS molecular experiments were performed on the samples from two patients with lymphoma. For each patient, 10–15 NK cells and 10–15 tumor cells from the bone marrow samples were examined.

2.5. Data analysis

Original force curves were imported to the Matlab software for analysis. To compute the adhesion forces, the retract curves were used. The molecular interaction forces were equal to the magnitude of the specific unbinding peak in the retract curves. The adhesion forces were fitted by Gaussian function to obtain the statistical values. To map the distribution of proteins on the cell surface, 16 × 16 force curves were first computed to obtain the adhesion forces, and then, the adhesion forces were normalized to 0–255 to construct the gray maps by using image processing software.

3. Results and discussion

The SMFS principle used to simultaneously probe the two types of molecular interactions involved in the mechanism of rituximab-induced ADCC is presented in Fig. 1(B). The bone marrow biopsy samples prepared from patients with lymphoma contained various cell types, including tumor cells, NK cells, red blood cells, and other white blood cells. Hence, in order to investigate the molecular interactions on tumor cells and NK cells, we first needed to recognize tumor cells and NK cells from the bone marrow cells. Recent evidence has shown that ROR1 is a specific cell surface marker expressed on B lymphoma cells, but not expressed on normal cells [22,23]. Hence, we were able to recognize the tumor cells in the bone marrow cells prepared from patients with lymphoma by ROR1 fluorescent labeling. Based on the fluorescence, we were then able to move the functionalized tips carrying rituximab to probe the CD20–rituximab interactions on the surface of tumor cells. NKp46 is a specific cell surface marker exclusively expressed on the surface of NK cells and is not expressed on other cells [24]. Hence, we were able to recognize NK cells in the bone marrow cells from patients with lymphoma by NKp46 fluorescent labeling. Based on the fluorescence, we were then able to control the functionalized tips carrying rituximab to probe the FcR–rituximab interactions on the surface of NK cells. The prerequisite of this experimental approach was that rituximab linked to the surface of the AFM tips can bind to both the CD20 on the tumor cell and the FcR on the NK cell. In this study, rituximab was treated with SATP to form SH groups and the SH groups could then covalently bind to the MAL end of the NHS–PEG–MAL linker. Based on the supplier’s instruction (Instructions SATP, Thermo Scientific), SATP contains a sulfhydryl and SATP can react with the primary amines on the protein (rituximab) to form an amide bond. Additionally, primary amines are abundant and distributed over the entire antibody, both on the Fab and Fc regions. Hence, in the process of tip functionalization, some SATP molecules reacted with the primary amines on the Fab region and some SATP molecules reacted with the primary amines on the Fc region. Because many rituximab molecules are linked to the AFM tips during the functionalization, the tips contained both types of modified rituximab. Using the rituximab-conjugated tips, we could then simultaneously probe the CD20–rituximab and FcR–rituximab interactions. In fact, with the same protocol of tip functionalization, the tips can be used to measure the antibody–antigen interactions [19,25,26], which validated the activity of the Fab region of the antibodies linked to the AFM tip. However, the activity of the Fc region of the antibodies linked to the tips has not been largely reported.

Fig. 2(A) shows the integrated AFM-fluorescence experimental platform used in this study. Cells attached to coverslips were placed in a petri dish containing culture medium or buffered solution and the dish was then placed on the sample stage of the integrated system. The beam of fluorescence was released under the sample stage and penetrated the petri dish to irradiate the cells. This system allowed us to simultaneously perform AFM...
experiments and fluorescence experiments. We could move the tip to the interesting cells, which were fluorescently labeled to perform AFM imaging and measurements. A critical issue in the SMFS experiments is linking rituximab to the AFM tips. In order to test whether rituximab was linked to the AFM tip, we performed secondary antibody fluorescence labeling experiments. Functionalized probes were incubated with RBITC-conjugated goat-anti-human IgG (Solarbio Company, Beijing, China) for 30 min, and fresh PBS was then used to wash the probes three times. If rituximab molecules bound to the tips, the goat-anti-human IgG molecules could then bind to rituximab and exhibit fluorescence after excitation. As a negative control, normal probes were also tested. Fig. 2B shows the fluorescence results of functionalized and normal probes. Fig. 2B (I, II) shows the optical bright field image and the corresponding fluorescence image of functionalized probes. Fig. 2B (III, IV) shows the optical bright field image and the corresponding fluorescence image of a normal probe. The functionalized probe exhibited bright fluorescence, while the normal probe was dark, indicating that rituximab was linked to the AFM tip.

We first performed the NKp46 fluorescent labeling experiments on the peripheral blood cells of patients with lymphoma. Fig. 3A and C show the bright field images and Fig. 3B and D show the corresponding fluorescence images. The fluorescence images were black and cells did not exhibit fluorescence. In the peripheral blood, the red blood cells represent the majority of the cells (approximately 99%) and white blood cells make up less than 1%. Among the white blood cells, NK cells account for less than 10% [27]. Hence, NK cells account for less than 0.1% of the peripheral blood cells. This very low level of NK cells in the peripheral blood makes it difficult to recognize NK cells directly from the peripheral blood cells. We then performed the NK fluorescence labeling experiments on the bone marrow cells from patients with lymphoma. Many cells were shown in the sample as shown by the bright field images (Fig. 4A and C). Few cells, exhibiting green fluorescence (Fig. 4B and D). Because only NK cells express NKp46, we can conclude that these fluorescence cells were the NK cells. NK cells originate, develop, and differentiate in the bone marrow [28,29]. There are higher levels of NK cells in the bone marrow than in the peripheral blood, which facilitate the detection of NK cells directly in the bone marrow. In order to recognize NK cells in the peripheral blood, we may have to lyse the red blood cells [27] and then perform NKp46 fluorescent labeling experiments.

The results presented in Fig. 4 indicate that NK cells can be recognized in the bone marrow samples prepared from patients with lymphoma. Based on the fluorescence, we then performed AFM imaging to obtain the morphology of NK cells. Fig. 5A and B show the bright field image and the corresponding fluorescence image of NKp46-labeled bone marrow cells. Fig. 5C shows the merged image of the bright field image and fluorescence image. Four cells were detected in the bright field image, among which only one cell exhibited fluorescence, indicating that it was a NK cell. The AFM probe was moved to the NK cell to obtain the AFM height image (Fig. 5D) and deflection image (Fig. 5E). The scan size was 30 μm. From the AFM images, one cell was identified as a red blood cell based on its biconcave shape, specific to red blood cells. As expected, the red blood cell, which does not express NKp46, did not exhibit any fluorescence (Fig. 5B). Compared with that of red blood cells, the AFM images showed that the NK cell exhibited a plump shape. Fig. 5F shows the section profiles taken along the dashed lines in Fig. 5E. The section curve of the red blood cell had a hollow, which was consistent with its biconcave shape. The section curves also showed that the diameter of the NK cell was about 11 μm and the diameter of the red blood cell was about 7.5 μm. Red blood cells do not express FcR and, therefore, can be used as negative controls in SMFS measurements. Due to the 200 nm resolution limit, optical microscopy often cannot reveal the detailed information of cellular ultra-structures, while AFM imaging can clearly reveal the fine morphological features of cells [30,31]. This is useful to better understand cell activities. Fig. 5G and H show the AFM height image and deflection image of the local area on the NK cell denoted by the dashed square in Fig. 5D. AFM images showed that the surface of NK cells was a little rough. The section curve (Fig. 5I) showed that the NK cell had protrusions with a 10–60 nm size. NK cells are large granular lymphocytes, which are principally responsible for the innate immune response in mammals [32]. NK cells from rats have been imaged using AFM before [33]. Here, we imaged the detailed morphology.
Fig. 3. NKp46 fluorescence labeling of peripheral blood cells from patients with lymphoma. (A, C) Bright field images. (B, D) Corresponding fluorescence images.

Fig. 4. NKp46 fluorescence labeling of bone marrow cells from patients with lymphoma. (A, C) Bright field images. (B, D) Corresponding fluorescence images.
of NK cells from patients. In recent years, the use of AFM to directly probe cells obtained from patient biopsies has been reported, allowing the measurement of cell mechanics [34] and molecular interactions [9,20]. Directly investigating the behavior of primary cells from patients can better reflect the biological situations occurring in the human body and be useful for both uncovering cellular biological functions and drug development.

Using functionalized tips, we then obtained the force curves on NK cell surface to probe the FcR–rituximab interactions. Fig. 6A–C show representative force curves with specific dissociation peaks. The left insets in Fig. 6A–C present the enlarged view of the retract curves in the range of 1.7–1.9 μm. The right insets in Fig. 6A–C present the cartoon diagrams of the forces measured using functionalized tips. During the process of obtaining a force curve, the tip carrying rituximab first approached and touched the NK cell, and then retracted from the cell surface. Hence, each force curve contained two portions, the approach curve and retract curve. The arrows in Fig. 6A indicated the moving direction of the AFM tip. During the approaching process (black curve), the tip was apart from the cell and then gradually approached the cell. Before the tip touched the cell, the force curve was flat. Once the tip touched and indented the cell, the force curve became bent. During the retracting process (red curve), the tip retracted along the track of the approaching process. If the rituximab on the AFM tip bounded to the FcRs on the cell surface during the tip-cell contact, then the FcR–rituximab were stretched during the tip retracting process. When the pulling force was larger than the strength of the FcR–rituximab bond, the FcR–rituximab bond ruptured and a specific dissociation peak occurred in the retract curve, as shown in the insets in Fig. 6A–C. The dissociation peak presented a change in the slope [35] due to the nonlinear elasticity of PEG linker molecules stretched by the tip [36]. The magnitude of the peak (denoted by the double-head arrows) corresponded to the FcR–rituximab binding force. As a negative control, we obtained force curves on red blood cells. Red blood cells were discerned by scanning the cells with AFM (Fig. 5). Fig. 6D shows a representative force curve obtained on red blood cells. The left inset in Fig. 6D shows the enlarged view of the retract curve in the range of 1.4–1.8 μm. There was no specific dissociation peaks in the retract curves. FcR are expressed on a wide range of effector cells, including macrophages, neutrophils, NK cells, and dendritic cells [37,38], but not on red blood cells. Hence, we cannot probe the specific FcR–rituximab molecular interactions (right inset of Fig. 6D). In order to demonstrate the interaction specificity, force curves were obtained.
on NK cells after free rituximab addition. Fig. 6E shows a representative force curve obtained on blocked NK cells. The enlarged view of the retract curve in the range of 1.4–1.7 μm (left inset in Fig. 6E) shows that the specific peaks are not observed since the FcRs on the NK cells are blocked by the free rituximab (right inset of Fig. 6E). The molecular binding frequency that occurred on NK cells, red blood cells, and blocked NK cells was statistically analyzed. Areas shown in Fig. 6F, the molecular binding frequency on NK cells was significantly larger than that on red blood cells and blocked NK cells. Red blood cells do not express FcR, and FcRs on the blocked NK cells were masked by free rituximab, thus the molecular binding frequency decreased considerably. The negative control and blocking experiments validated the specific FcR–rituximab interactions measured on NK cells using rituximab-conjugated tips. A range of antibodies have been linked to AFM tips to measure the antigen–antibody interaction forces such as anti-human serum albumin antibody [39], anti-histone H3 antibodies [25], anti-Band III antibodies [26], Herceptin [40], and anti-UCP1 antibodies [41]. In these studies, the interactions between the Fab region of antibodies linked to the AFM tip and the antigen were probed. We know some membrane proteins bind to the Fc region of antibody such as the FcRs. The results presented in Fig. 6 indicate that we can use the antibody-functionalized tips to probe the interactions between the Fc region of an antibody and membrane proteins, improving our understanding of the antibody functionalization and providing a feasible way to probe the interactions between FcR and antibody.

At the force volume mode using functionalized tips, we can detect the distribution of specific membrane proteins on the cell surface [42,43]. In this mode, AFM tips carrying specific molecules (e.g., antibodies and receptors) were controlled to obtain arrays of force curves in local areas on the cell surface to probe the distribution of cognate membrane proteins. Fig. 7 shows the results of mapping the FcR on the surface of NK cells using rituximab-conjugated tips. Sixteen by sixteen force curves were obtained in local areas (500 × 500 nm²) on the cell surface. For each force curve, we computed the adhesion force by analyzing the specific peak in the retract curve. If there were no specific peaks in the retract curve, then the adhesion force was 0. After analyzing the 256 force curves per 500 × 500 nm² on the cell surface, a gray map that reflects the distribution of FcR on the cell surface was constructed. Fig. 7A–E show the gray maps obtained from NK cells, indicating...
the distribution of FcR. In order to demonstrate that the gray pixels corresponded to the FcRs on the NK cells, we performed blocking experiments. Fig. 7F–J show the gray maps obtained on NK cells at different time after the free rituximab addition. The gray pixels decreased remarkably after rituximab addition and eventually disappeared approximately 2 h after the addition. Since the decrease of gray pixels may be due to the loss of rituximab activity on the AFM tips, the AFM tips were used again to obtain force curves on NK cells that were not blocked. Many gray pixels were detected in the maps (Fig. 7K–O), indicating that rituximab was still active on the AFM tips. The results presented in Fig. 7 demonstrated that the gray maps reflected the distribution of FcRs on the cell surface. Some FcRs clustered into nanodomains (denoted by the red dashed circles), while some FcRs distributed sparsely on the cell surface. Traditional methods used to investigate the distribution of FcR are based on flow cytometry [44] or fluorescence microscopy [45], which makes it impossible to understand how single molecules localize, assemble, and interact on the cell surface at the nanometer scale [6] due to the 200 nm resolution limit of optical microscopy. AFM provides exciting possibilities for mapping the distribution of single molecules on the surface of cells [46], paving the way for elucidating the highly complex activities between various molecules on the cell surface. In previous studies, we have investigated the FcR–rituximab interactions on murine RAW 264.7 macrophage cell line [47]. Here, we investigated the FcR–rituximab interactions directly on NK cells from patients with lymphoma. Studies have shown that both macrophages and NK cells are important for depleting tumor cells, but their exact contribution to the clinical effects of drugs remains unclear [37]. In the future, the established procedure will be applied to quantify the difference of FcR distribution on macrophages and NK cells at the single-molecule level, to analyze their contribution to rituximab clinical therapeutic outcome.

The results presented in Figs. 6 and 7 indicate that the binding affinity and nanoscale distribution of FcRs on the surface of NK cells can be measured using rituximab-conjugated tips. As shown in Fig. 1, ADCC involves two types of molecular interactions, CD20–rituximab interaction on tumor cells and FcR–rituximab interaction on NK cells. We then used rituximab-conjugated tips to probe the CD20–rituximab interactions on the surface of tumor cells prepared from lymphoma patient bone marrow samples. As for the FcR–rituximab interaction analysis, we needed to first recognize the tumor cells. In previous studies, we successfully recognized tumor cells in lymphoma patient bone marrow based on ROR1 fluorescent labeling, which is a specific cell surface marker expressed on lymphoma cells, but not on normal cells [9,20,42]. Here, using the rituximab-conjugated tips, which had been used to measure the FcR–rituximab interactions on NK cells, we measured the CD20–rituximab interactions on tumor cells based on ROR1 fluorescence labeling. Fig. 8A–C show the bright field image, fluorescence image, and overlay image of the ROR1-labeled bone marrow cells, respectively. Five cells were visualized, among which only one cell exhibited fluorescence. Since ROR1 is expressed only on tumor cells, we can conclude that the fluorescence-positive cell
Fig. 8. Probing of the CD20–rituximab interactions on the surface of tumor cells with functionalized tips. (A) Bright field image, (B) fluorescence image, and (C) overlay image of the ROR1-labeled bone marrow cells. (D) AFM height image and (E) deflection image of the cells. Representative force curves obtained on tumor cells (F) and blocked tumor cells (G). The left insets in F and G show the enlarged views of the retract curves and the right insets show the cartoon diagrams of the measurements. (H) Comparison of the molecular binding frequency observed on tumor cells and blocked tumor cells. (I) CD20 distribution mapping on the surface of tumor cells by obtaining 16 × 16 force curves in the 500 × 500 nm² areas on the cell surface with functionalized tips.

was a tumor cell. Fig. 8D and E show the AFM height image and deflection image of the cells. After recognizing the tumor cells, force curves were obtained on tumor cells. Fig. 8F shows a representative force curve obtained on tumor cells. The left inset in Fig. 8F shows the enlarged view of the retract curve in the range of 1.6–1.8 μm. There was a special molecular dissociation peak in the retract curve, which reflected the CD20–rituximab specific interactions (right inset of Fig. 8F). The magnitude of the peak (denoted by the double-head arrow) corresponded to the CD20–rituximab binding force. In order to demonstrate that the peak was specific to the CD20–rituximab interactions, force curves were obtained on tumor cells after free rituximab addition. Fig. 8G shows a representative force curve obtained on the blocked tumor cells. The left inset in Fig. 8G shows the enlarged view of the retract curve in the range of 1.6–1.9 μm. The specific peak disappeared, due to the blocking of CD20s on the tumor cell by free rituximab (right inset of Fig. 8G). The molecular binding frequencies observed on tumor cells and blocked tumor cells are presented in Fig. 8H. Free rituximab addition remarkably decreased molecular binding, which demonstrated the specificity of the CD20–rituximab interaction. The distribution of CD20 on tumor cells was also mapped by obtaining arrays of force curves in local areas on the surface of tumor cells, as shown in Fig. 8I. Before blocking, many gray and bright pixels were detected in the maps, while free rituximab addition induced a decrease of gray pixels in the maps, which eventually completely disappeared. The blocking experiments validated the specificity of the CD20–rituximab interaction in tumor cells. The results presented in Fig. 8 indicate that the CD20–rituximab binding force and the distribution of CD20 on tumor cells can be measured using rituximab-conjugated tips based on ROR1 fluorescent labeling. By combining the results presented in Figs. 6–8, it can be concluded that the CD20–rituximab interactions on tumor cells and FcR–rituximab interactions on NK cells can be measured simultaneously using rituximab-conjugated tips based on specific fluorescence recognition. SMFS is a powerful tool to investigate the activities of single molecules (e.g., the dynamics of dissociation between a single molecular pair, the binding affinity, the nanoscale distribution of membrane proteins on the cell surface, and the unfolding of single proteins [48]). However, current SMFS experiments are performed on isolated molecules or on cell lines, which do not represent in vivo situations. Thus, there is a large gap between laboratory studies and clinical actual requirements.
As we gradually enter the era of personalized medicine, accelerating the clinical application of basic biomedical studies has become a bottleneck hindering the development of medicine. Here, we directly investigated molecular interactions on cells (tumor cells and NK cells) obtained from lymphoma patient biopsy, using SMFS, providing an alternative method to investigate the biophysical properties of membrane proteins in conditions close to in vivo conditions.

Using the described method, the CD20–rituximab interaction on tumor cells and FcR–rituximab interactions on NK cells were measured using samples from two patients with lymphoma, with bone marrow invasion. The clinic pathological characteristics of the two patients are presented in Table 1. Fig. 9A and B show the histogram of FcR–rituximab binding forces measured on NK cells and the histogram of CD20–rituximab binding forces measured on tumor cells from patient one, respectively. The Gaussian fitting showed that the FcR–rituximab binding force was 65 ± 38pN and the CD20–rituximab binding force was 95 ± 46pN. The CD20–rituximab binding force was larger than that of the FcR–rituximab for patient one. Fig. 9D and E show the histogram of FcR–rituximab binding forces measured on NK cells and CD20–rituximab binding forces measured on tumor cells for patient two, respectively. CD20–rituximab binding force (117 ± 83pN) was also larger than that of FcR–rituximab (78 ± 48pN). Radioimmunoassays have been used to measure the binding affinity between CD20 and rituximab, showing that the binding constant value between human CD20 and rituximab was $2.0 \times 10^3$–2.8 × $10^6$ M$^{-1}$[49–51]. The binding affinity between FcR and IgG1 was also measured and the binding constant between human FcR and human IgG1 was $1.3 \times 10^{-4}$–$4 \times 10^6$ M$^{-1}$[52,53]. Rituximab is a chimeric antibody with human IgG1 constant regions and murine variant regions[11]. FcR binds to the constant regions of rituximab. Thus, the binding affinity between FcR and rituximab is equal to the binding affinity between FcR and IgG1. Based on the results of traditional radioimmunoassays, the binding constant of CD20–rituximab was remarkably larger than that of FcR–rituximab. Accordingly, in this study, the measured CD20–rituximab binding forces (95 ± 46pN for patient one and 117 ± 83pN for patient two) were significantly larger than that of FcR–rituximab (65 ± 38pN for patient one and 78 ± 48pN for patient two). Fig. 9C shows the distribution frequency of FcR on NK cells and CD20 on tumor cells for patient one. The distribution frequency was computed from the gray maps constructed by obtaining arrays of force curves on the cell surface. In order to obtain the distribution frequency of FcR on NK cells with stochastic significance, 30 gray maps were constructed using arrays of force curves on 10–15 NK cells. Each gray map corresponded to a value of distribution frequency. For each gray map, the distribution frequency was equal to the ratio of the number of gray pixels and the overall number of pixels. To obtain the distribution frequency of CD20 on tumor cells 30 gray maps were also produced using arrays of force curves on 10–15 tumor cells. CD20 frequency distribution on tumor cells (22.2%) was larger than that of FcR on NK cells (18.4%) for patient one. For patient two (Fig. 9F), CD20 distribution frequency on NK cells (22.1%) was slightly less than that of CD20 on tumor cells (23.6%). One reason behind the unprecedented success obtained with rituximab in clinical practice is that CD20 is expressed at high levels on B lymphoma cells compared with most targets (often more than 250,000 molecules per cell), allowing the dense accumulation of rituximab on the plasma membrane [37].

For FcR distribution, studies have shown that NK cells display a low level of FcR expression on the plasma membrane [54]. Here, the results obtained by SMFS showed that the FcR distribution density on NK cells was less than that of CD20 on tumor cells in patient samples, which was comparable to the results obtained by traditional biochemical methods.

Patient one was a case of diffuse large B-cell lymphoma and patient two was a case of chronic lymphocytic leukemia (Table 1). Both patients were treated by a combination of chemotherapy and rituximab therapy. After the treatment (6 cycles), patient one presented a partial remission, while patient two presented a complete remission. By comparing the FcR–rituximab interactions in samples from the two patients, we observed that the FcR–rituximab binding force on NK cells from patient one (Fig. 9A) was slightly less than that of patient two (Fig. 9D), while the distribution density of FcR on NK cells from patient one (Fig. 9C) was significantly less than that of patient two (Fig. 9F). Comparing the CD20–rituximab interactions, we observed that the CD20–rituximab binding force on tumor cells from patient one (Fig. 9B) was a little less than that of patient two (Fig. 9E), while the distribution density of CD20 on tumor cells of patient one (Fig. 9D) is similar to that of patient two (Fig. 9F). The results showed that higher binding affinity and distribution of FcR on NK cells and CD20 on tumor cells seemed to be associated with better treatment outcomes. Higher binding affinity and distribution of FcR on NK cells and CD20 on tumor cells can make the connection between NK cells and tumor cells more stable. In this situation, more NK cells can be recruited to attack the tumor cells, improving rituximab killing effects. It should be noted that samples from only two patients were used in this study; therefore, it is difficult to determine the respective contribution of FcR–rituximab interactions and CD20–rituximab interactions from the results. In order to investigate the respective role of FcR–rituximab interactions and CD20–rituximab interactions in rituximab lymphoma therapy, further studies involving more patients are warranted.

### Table 1

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor subtype</th>
<th>Clinical therapy</th>
<th>Therapy cycle</th>
<th>Therapy outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>50</td>
<td>Diffuse large B-cell lymphoma</td>
<td>Rituximab + CHOP</td>
<td>6</td>
<td>Partial remission</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>47</td>
<td>Chronic lymphocytic leukemia</td>
<td>Rituximab + Fludarabine + Cyclophosphamide</td>
<td>6</td>
<td>Complete remission</td>
</tr>
</tbody>
</table>
biology. While using SMFS to quantify the interactions between single molecular pairs has become a mature technique, to our knowledge, simultaneously measuring the multiple types of molecular interactions on patient cells has not yet been reported. In this study, we simultaneously probed the multiple types of molecular interactions (CD20–rituximab and FcR–rituximab) occurring in the rituximab-induced ADCC mechanism on tumor cells and NK cells, respectively. The experimental results demonstrated the effectiveness of the measurements. The results obtained using SMFS in this study indicate that the binding affinity and distribution of FcR on NK cells and CD20 on tumor cells are related to rituximab clinical efficacy. In the future, we plan to apply the established method (Fig. 10) to simultaneously measure the CD20–rituximab and FcR–rituximab interactions in samples from more patients with lymphoma to investigate the exact roles of CD20–rituximab and FcR–rituximab interactions during the rituximab clinical therapy. Rituximab targeted therapy is costly ($2,871 per infusion in the USA) and some serious adverse effects (hypotension, rilgors, bronchospasm, and angioedema) often occur during rituximab treatment [11]. Since many patients do not respond to rituximab, predicting rituximab efficacy using patient samples before the treatment is meaningful as it may allow us to avoid the unnecessary expense and adverse effects for rituximab-insensitive patients. Combining SMFS-obtained novel information with clinical data may inspire us to discover new biomarkers that can be used for efficacy prediction, which is of important significance for personalize medicine and drug development.

4. Conclusions

This study demonstrated the use of SMFS to simultaneously probe the two types of molecular interactions (CD20–rituximab, FcR–rituximab) occurring in rituximab-induced ADCC on biopsy cells (tumor cells and NK cells) from patients with lymphoma. NK cells were detected by NKP46 fluorescent-specific labeling and tumor cells were detected by ROR1 fluorescent-specific labeling. Using SMFS and rituximab-conjugated tips, the binding affinity and nanoscale distribution of FcRs on NK cells and CD20s on tumor cells were quantitatively measured and visualized. The measurements performed on samples from two patients with lymphoma showed that the higher binding affinity and distribution of FcRs on NK cells and CD20s on tumor cells seemed to be associated with better therapeutic outcomes, providing novel insights into rituximab variable efficacy among patients. The experimental results provide new possibilities for developing an in situ rapid detection method of target–drug interactions, which can be used to characterize the biophysical properties (binding affinity to drug molecules and nanoscale distribution on the cell surface) of target molecules and evaluate drug activities. In the future, the established method will be validated in larger scale studies using samples obtained from more patients with lymphoma. These studies will be particularly useful for us to understand rituximab resistance in the clinical practice and promote the development of new anti-CD20 drugs with enhanced efficacy.
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References