Supporting information

Studying the mechanism of CD47-SIRPα interactions on red blood cells by single molecule force spectroscopy

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MATERIALS AND METHODS

Reagents

Mouse anti–human CD47 (clone B6H12) were from BD Biosciences. TSP-1 peptide (4N1K, amino acid sequence; KRFYVVMWKK) was synthesized from Sangon Biotech (Shanghai). The recombinant human SIRPα (expressed with sequence Glu31 Arg370 of Human SIRPA fused with a polyhistidine tag at the C-terminus) was purchased from Novoprotein Scientific Inc (Shanghai, China).

APTES Functionalized Mica.

A desiccator was purged with argon for 2 min, and 30 μL of APTES (aminopropyltriethoxysilane, 99%, Sigma-Aldrich, St. Louis, MO) was placed into a small container at the bottom of the desiccator[1]. Ten microliters of N,N-diisopropylethylamine (99%, distilled, Sigma-Aldrich) was placed into another small container, and the desiccator purged with

argon for a further 2 min. Mica sheets were stripped on one side until smooth and immediately placed into the desiccator. The desiccator was purged for another 3 min and then sealed off, leaving the mica exposed to APTES vapor for 1h. After this exposure, the APTES was removed, the desiccator was purged, and the treated mica (AP-mica) was stored in the sealed desiccator until needed.

Preparing human red blood cells

To prepare human red blood cells (hRBC), two drops of fresh human blood from healthy donor (B-type) were washed five times in 150 mM PBS buffer (pH 7.5). The diluted hRBC were deposited onto AP-mica for 15 min.

Aging human red blood cells

hRBCs isolated from whole blood were spun down and resuspended in PBS supplemented with 0.2mM $CuSO_4$ and 5mM ascorbic acid to a final concentration of 0.4×10^8 cells/mL[2,3]. After incubating the cells at 37°C for 60 minutes in a shaking heating block, the cells were washed 3 times with PBS and resuspended in HEPES. Then the cells were preincubated with 4N1K (final concentration 50μg/ml) for 30 minutes, and the aged hRBCs were deposited onto AP-mica for 15 min. The hRBCs were incubated with free Cys(final concentration 20μg/ml) for 10 min and then experimentally aged these hRBCs by the methods as described above.

Single molecule force spectroscopy

Force curve measurements were acquired using AFM 5500 (Agilent Technologies, Chandler, AZ) in PBS solution. The deflection sensitivity of the photo-detector was determined by the slope of the force-distance curves taken on the bare surface of mica. The AFM cantilevers were calibrated by a reference cantilever (CLFC, Veeco, Santa Barbara, CA) as described. Thousands of force curves were recorded at different positions on the RBCs. Blocking experiments were carried out by the addition of 100 μg/mL CD47 antibody(Novoprotein Scientific Inc, Shanghai, China) into the sample cell(inal concentration 100 ng/ml) for 30min. The data were processed with MatLab 7.9 (Math Works Inc.).The relation between the unbinding force and the loading rate can be expressed as:

$$F_u = \frac{k_B T}{x_\beta} \ln\left(\frac{r x_\beta}{k_B T k_{off}}\right) \qquad (1)$$

in which F_u is the most probable unbinding force; x_β is the distance of energy barrier to the minimum energy along the separation path; r is the loading rate, $r=k_{off}v$, k_{off} is the effective spring constant, v is the retraction velocity; k_{off} is the dissociation rate constant at zero force; T is the thermodynamic temperature; k_B is the Boltzmann constant[4].

Tip chemistry

AFM tips (Microlever, Veeco, Santa Barbara, CA) were functionalized with APTES in a manner similar to the preparation of APTES-glass slides. The cantilevers were cleaned in a UV cleaner and vapor-treated with APTES. Subsequently, PEG crosslinker (benzaldehyde-PEG76-NHS,

FW~3962, SensoPath Technologies, Bozeman, MT) was conjugated in triethylamine and trichloromethane as described[5]. After drying with argon, the tips were then immersed in a mixture of 100 μL SIRPα in PBS and 4μL 1M $NaCNBH_3$. After functionalization for 60min, 10μL 1M ethanolamine was added to the solution in order to passivate the unreacted aldehyde groups. Then the AFM tips were washed with PBS three times and stored at 4˚C.

Fluorescence imaging of the functionalized AFM tip

Images of fluorescently stained cells were obtained using a Leica TCS SP2 confocal microscope. Cy5-labeled SIRPα were excited with a 633 nm helium–neon laser. The fluorescence image was obtained by Volocity image analysis software (Improvision, Perkin Elmer).

References

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