Single-Cell Acoustic Force Spectroscopy (scAFS)

Resolving kinetics and strength of T-cell adhesion to fibronectin

Abstract

Assessing strength and kinetics of molecular interactions of cells with the extracellular matrix is fundamental to understand cell-adhesion processes. Given the relevance of these processes, there is a strong need for physical methods to quantitatively assess the mechanism of cell adhesion at the single-cell level, allowing discrimination of cells with different behaviors. Here we introduce single-cell Acoustic Force Spectroscopy (sc-AFS), an approach that makes use of acoustic waves to exert controlled forces, up to 1 nN, to hundreds of individual cells in parallel. We demonstrate the potential of sc-AFS by measuring adhesion forces and kinetics of CD4+ T-lymphocytes (CD4) to fibronectin. We determined that CD4 adhesion is accelerated by activator cytokine interleukine-7, while CD4 binding strength remains the same. Activation of these cells likely increases their chance to bind to the vessel wall in the blood flow to locally coordinate the immune response.

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7.1 Introduction

Cell adhesion to the extracellular matrix (ECM) is not only central to tissue assembly and repair, but also to immune surveillance and response. Adhesion kinetics and strength are currently measured with atomic force microscopy (AFM), surface plasmon resonance technology (SPR), fluid-flow devices, shear-spinning disks, or centrifugation methods. Accurate measurements of cell-adhesion kinetics and strength absolutely require large and well-defined datasets, in order to grasp the heterogeneity in cell behavior of cell subsets and to capture rare events. Here we present a massively parallel approach that allows such accurate force control while continuously monitoring many cells using optical microscopy.

It has been shown that acoustic forces can be applied directly to manipulate cells or for flow-sorting purposes according to their density or their size. Recently, we presented acoustic force spectroscopy (AFS) as a new single-molecule method to apply well-controlled forces up to hundreds of pN to single (bio)molecules with standing acoustics waves, using microspheres as force transducers (as described in chapter 2). Here we show that cells have by themselves enough acoustic contrast such that forces on the order of nN can be applied to them directly, allowing accurate determination of their interactions with the ECM. We call this method single-cell acoustic force spectroscopy (scAFS).

scAFS is performed in a transparent microfluidic flow chamber, which can be combined with any optical microscope (Supplementary figure 7.1). Well-controlled high forces can be applied to hundreds of adhered cells in parallel with scAFS while tracking their positions in three dimensions in real time. Here we demonstrate the potential of scAFS by quantifying the effect of interleukin-7 (IL7) activation on the binding kinetics and strength of human primary CD4+ T-lymphocytes (CD4) to fibronectin a major component of the ECM.

7.2 Results

7.2.1 Manipulating cells with acoustic forces

In the human body, CD4 escape the blood stream upon activation, bind to the blood-vessel endothelial cells near inflamed areas, migrate through the endothelium and infiltrate inflamed or infected tissues as illustrated in (Figure 7.1). In our scAFS experiments, the flow chamber is coated
Figure 7.1 | CD4-adhesion mechanism

Three-step mechanism of leukocytes adhering to blood-vessel endothelium while infiltrating inflamed or infected tissues\(^{134,146–148}\). **Step 1**: unbound CD4 (pink) carried by the blood flow in end-vessels, rolling along the vascular endothelium (brown rectangles), while P-selectin glycoprotein ligand-1 at the CD4 surface bind transiently to endothelial cell adhesion molecules, first to L-selectins (brown sticks), later, upon inflammation to P-selectins (green sticks). **Step 2**: chemokines or cytokines (black diamonds) expressed or presented by inflamed endothelial cells, bind their cognate receptors at the surface of CD4 and trigger intracellular signals that activate different kinds of integrins (pink lollipops) targeting the ECM and ligands such as ICAM, VCAM and MADCAM (brown baskets CD4 cells slow down and crawl along the surface. **Step 3**: CD4 cells are immobilized by the formation of multiple integrin-ligand bonds. Subsequently, the CD4 cells cross the endothelium, and infiltrate the tissue.

with fibronectin (**section 7.4.2**), commonly used to mimic the ECM lining the endothelium of blood vessels. CD4, purified from healthy donor blood samples, are injected into the AFS chamber and allowed to settle down on the fibronectin-functionalized bottom surface (**Figure 7.2a,b**). Subsequently, a standing acoustic wave is generated in the flow chamber. The acoustic frequency defines the position of the nodal plane, while the amplitude determines the magnitude of the acoustic force. When acoustic force is applied, unbound cells move towards the nodal plane, while adherent cells remain attached to the surface (**Figure 7.2c,d**). Unbound and adherent cells can be discriminated on the basis of their diffraction patterns in a bright-field microscopy image, which changes when a cell moves away from the optical focal plane (**Figure 7.2d,e**). The position of each cell can be determined from its diffraction pattern in real time in three dimensions, using quadrant interpolation\(^{40}\) and a look-up table (**section 3.2.5**), with an accuracy of 2.0 nm in x and y, and 72 nm in z (**Supplementary figure 7.2**). In a typical experiment, an acoustic force of ~10 pN is applied for ~2 s, the minimal force required to
Figure 7.2 | scAFS on single CD4 cells: manipulation and high-resolution tracking
(a) Sketch showing CD4 (pink) in culture medium (light pink) in the AFS flow chamber, and sedimented on glass (blue) functionalized with fibronectin (FN, orange). (b) Digital image of CD4 cells on FN-functionalized glass in AFS flow chamber. (c) A standing acoustic wave induces a pressure force field (gray gradient) pushing the unbound cells (blue square) upward (dark, high pressure) to the acoustic node (light pink, low pressure). (d) Same cropped frame as in panel b, after the application of an acoustic wave (14.3 MHz, 4.2 Vpp, 5 s). Bound cells are immobile (orange square, the diffraction pattern is unchanged) and unbound cells have moved up towards the acoustic node, out of focus (blue square, the diffraction pattern has changed). For full fields of view see Supplementary figure 7.10. (e) Image series of a bound and an unbound cell, demonstrating the diffraction pattern of the cells upon application of acoustic force (f and g) xy- and z-position graphs as a function of time of unbound (blue) and bound (orange) CD4. Gray zones indicate the period of application of acoustic waves; black plots the amplitude of the acoustic wave (14.3 MHz, 4.2 Vpp, 5 s). The center positions of CD4 at bottom surface and acoustic node are indicated by horizontal dashed black lines.

push unbound cells upward to the nodal plane. Subsequently, the force is switched off and the cells sediment back to the bottom glass surface (Figure
7.2g). For unbound cells, substantial movements are observed in the xy-plane (Figure 7.2f), due to weak acoustic pressure deviations in this plane\(^{49}\). In contrast, adhered cells remain bound to the surface and their xy- and z-positions are unchanged. These results demonstrate that, with scAFS, force can be applied to the cells, while tracking their individual positions in the field of view (in our instrument: 1.8 mm\(^2\)), allowing detailed investigation of their adhesion to the fibronectin-functionalized surface.

**7.2.2 Three binding stages of adhering cells**

In order to quantify the kinetics of CD4 adhesion to fibronectin, we apply a minimal acoustic pulse every two minutes, 9 times in a row, while continuously imaging the cells. From the images, cells are identified and selected automatically. Clustered cells are excluded after visual inspection. Using this approach, we typically track several hundreds of cells simultaneously in three dimensions. Three populations of cells are distinguished based on their xyz-trajectories (Figure 7.3): “unbound”, “binding” and “bound” cells. Unbound cells move upward each time an acoustic force is applied, indicating that they are not bound to fibronectin. These cells move substantially in x and y when elevated by the acoustic force. Binding cells show substantial shifts in the xy-plane along the bottom surface (1-3 \(\mu\)m), without moving in the z-direction. These cells appeared to be crawling, in the process of adhering, but not completely immobilized yet. Bound cells do not move upward upon acoustic-force application and shift only minimally in the xy-plane (<1 \(\mu\)m). Based on these observations, we use xy-shifts of 1 and 3 \(\mu\)m as boundary values to classify the cells in the three categories.

**7.2.3 IL7 accelerates CD4 adhesion**

To further assess the progression from unbound to bound cells, we measure the adhesion status of cells with a minimal acoustic pulse at 5, 20, 40 and 60 minutes after cell injection in the AFS chamber. Applying only four acoustic pulses minimizes the perturbation of the cell-adhesion process. Figure 7.4a shows superimposed image pairs before and after the acoustic pulse for non-activated and IL7-activated CD4 after 20 minutes of incubation (see Supplementary figure 7.3 for 5, 40 and 60 min). Whisker plots in Figure 7.4b display the xy shifts of these cells 20 minutes after injection under different conditions. Addition of peptide inhibitors\(^{150}\) results in substantially decreased binding of cells to the fibronectin surface (Supplementary figure 7.5) and in the absence of fibronectin no adhesion of cells is observed (Figure 7.4d), confirming that adhesion is mediated by integrin-fibronectin interactions. In the presence of fibronectin, IL7 activation increases by 44% the fraction of...
Figure 7.3 | Deciphering unbound, binding and bound cells from their xy-trajectories and z-elevation

Trajectories of CD4 z- (left) and xy-positions (right) have been plotted for representative cells from free (a), binding (b) and bound (c) subsets. CD4 are subjected to a series of nine acoustic pulses (14.3 MHz, 4.2 Vpp, 2s), spaced by ~2 minutes indicated in the plot (a, left). Color coding of the trajectories is used to indicate time correspondence between left and right plots. Purple and orange zones indicate whether cells are in the binding (adhesion process) or bound (immobilization) state, respectively. Immobilized cells range from 27% to 39% (Figure 7.4b). To illustrate the progression of cell binding, the shifts in xy position of the cells are plotted as a cumulative distribution function (CDF) (Figure 7.4c). CDFs show the three cell subsets described above: bound, binding and unbound cells. The percentages of cells in the bound state are plotted at different time points, in the presence or

immobilized cells from 27% to 39% (Figure 7.4b). To illustrate the progression of cell binding, the shifts in xy position of the cells are plotted as a cumulative distribution function (CDF) (Figure 7.4c). CDFs show the three cell subsets described above: bound, binding and unbound cells. The percentages of cells in the bound state are plotted at different time points, in the presence or
absence of IL7 activation (Figure 7.4b). These results show that the fraction of bound cells increases with incubation time. While two thirds of the cells are bound after 60 minutes independently of their activation status, the adhesion process is substantially faster for IL7-activated CD4.

7.2.4 Calibration of the acoustic force acting on the cells
Determining the binding strength of CD4 to fibronectin-functionalized surface requires calibration of the acoustic forces acting on the cells. Since cells vary substantially in size, density and stiffness, it is imperative to calibrate the forces acting on a representative number of cells. To this end, we measured the Stokes’ drag force from the upward velocity (in the z direction) of each CD4 when pushed towards the acoustic nodal plane. Buoyancy and gravity acting on the cells can be calculated independently based on their size and weight. The acoustic force is in balance with these three forces and can thus be determined for each individual cell (Supplementary figure 7.6, section 3.4.4). Cell velocities are measured during application of successive acoustic pulses of increasing amplitude, spaced by ~1 minute to allow the cells to sediment back to the bottom of the AFS chamber (Figure 7.5a). The cells’ upward velocity (slope at the inflexion point) during acoustic pulses increases with the acoustic amplitude, while the cell sedimentation velocity stays the same (Figure 7.5b). From these data, we calculated the force acting on each cell as a function of the amplitude of the electric voltage ($V_{pp}$) applied to the piezoelectric element and obtained quadratic scaling in accordance with theory18. We compared the calibration for IL7-activated and non-activated cells from different donors and found that the calibration is indeed similar (Figure 7.5c).

7.2.5 Binding strength of unbound, binding and bound CD4
In order to measure the adhesion strengths to fibronectin of CD4, we applied an acoustic force ramp. In such an experiment we observe that most bound cells show an elevation in two steps (Figure 7.5d). In the first step, the cells are elevated by less than 2 $\mu$m without moving in the xy plane. In the second step, these cells are elevated to the acoustic node and also move substantially in the xy plane (Supplementary figure 7.7). We interpret the first step as either an extension of fibronectin fiber or a deformation of the cells which remain bound to the fibronectin-coated surface, and the second step as the actual rupture of fibronectin–integrin bonds (Figure 7.5d). This method to measure the force causing bond rupture is detailed in Supplementary figure 7.7. In order to demonstrate that the fibronectin is not detaching from the surface during our the application of acoustic force, we covalently attach
Figure 7.4 | CD4 cells adhere faster to fibronectin and get immobilized when activated by IL7

(a-b) CD4 are activated by IL7 (+IL7, n = 666) or not (NA, n = 463) and subsequently injected in non-functionalized (-FN) or fibronectin-functionalized (+FN) chambers. The non-activated (NA, green) and IL7-activated cells (+IL7, red) are pushed upwards with a minimal force (14.3 MHz, 4.2 Vpp, 2 s) after 5, 20, 40 and 60 minutes of incubation. (a) Images of CD4 cells before (red) and after (green) acoustic wave application are superimposed (20 minutes of incubation). Immobile cells appear in yellow (superposition of red + green). Bound fractions (xy shift < 1 μm) are indicated. Bottom images show zooms of regions indicated by squares in top images. (b) Box plot of shifts in xy of non-activated (green) and IL7-activated (red) cells after 20 min of incubation in chambers functionalized with fibronectin (+FN) and not (-FN). For other time points see Supplementary figure 7.4. Whiskers are maximum and minimum, and quartiles framing the upper and lower part of the median. Bound fractions (xy shift < 1mm, dashed orange threshold) are indicated on top of each column. (c) Cumulative probability distribution plot of the cell xy-shift, determined before and after acoustic force application, measured subsequently at 5, 20, 40 and 60 minutes incubation. Bound (orange), binding (purple) and unbound (blue) zone have a xy shift of < 1, 1-3 and > 3 m, respectively. (d) Fraction cells bound as a function of time, plotted for non-activated and IL7-activated CD4 cells on glass functionalized or not with fibronectin.
Figure 7.5 | CD4 rupture-force calibration and measurement

(a) z position of a representative unbound CD4 cell plotted with the voltage of the applied acoustic pulses (2.1, 4.2, 6.3, 8.4, 10.5 Vpp, <2 seconds). (b) Acoustic-pressure induced upward motion of the cell in panel a. Trajectories of different acoustic pulses are superimposed by adjusting time offset. Acoustic pulse is indicated by grey zone. The inset shows trajectories at different time scale (up to 30 seconds), focusing on sedimentation of cells after application of acoustic pulse (grey vertical line). (c) Force acting on the cell close to the bottom surface, as calibrated from the velocity in panel b, for cells from a representative donor (data are means ± s.d., n = 14 for not-activated and n = 37 for IL7-activated cells). Quadratic fits yield quadratic constants of 0.55 ± 0.25 and 0.54 ± 0.32 pN/Vpp² (fit value ± s.d.) for non-activated and IL7-activated cells, respectively. (d) Application of a linear (Vpp) ramp to a cell to determine rupture force (blue, 14.3 MHz, 0 to 10.5 Vpp). Corresponding complete field of view: see Supplementary figure 7.10. Most bound cells experience are slightly pushed upward (up to 3 μm) before rupture occurs. $t_0$ (vertical blue dashed line) indicates the start of acoustic force application, $t_r$ (vertical red dashed line) the time of rupture (identified here by a displacement of a cell larger than its diameter (8 μm). Amplitude of acoustic wave is indicated at bottom (black line).

microspheres to the fibronectin and observe that all microspheres remain attached after applying 500 pN (Supplementary figure 7.8). Before measuring
Figure 7.6 | CD4 rupture-force distribution, effect of IL7
(a) Rupture force distribution of the CD4 cells from the fibronectin-functionalized surface (for underlying data analyses see Supplementary figure 7.11 & Supplementary figure 7.7). Whiskers are maximum and minimum, and quartiles framing the upper and lower part of the median. Medians for unbound, binding and bound are 8.8 (n = 127), 39.8 (n = 41), 99.7 (n = 51) pN for non-activated and 14.8 (n = 160), 39.3 (n = 74), 111.7 (n = 107) pN for IL7-activated cells. Data separated for two representative blood donors are shown in Supplementary figure 7.9. (b) Bar plots of fraction of unbound, binding and bound cells out of non-activated and activated CD4 after 19 minutes of incubation (error bars are s.e.m.). (c) Cumulative probability distribution plot of the rupture forces is shown for unbound, bonding, and bound cell, IL7-activated and non-activated. The horizontal black dashed line indicates 50% of the cell subset. The vertical black dashed line indicates the change of force scale in abscise from 0-55 pN to 55-500pN.
CD4 rupture forces, we determined whether cells were unbound, binding or bound by repeatedly applying minimal force pulses. Then we applied a linear force ramp and determined at what force each cell ruptures from the fibronectin-coated surface (Supplementary figure 7.7c). Box plots of the rupture forces are clearly different for the three classes of cells (Figure 7.6a), with median values of 9, 39 and 100 pN (unbound, binding and bound, respectively) for non-activated and 15, 39 and 112 pN for IL7-activated cells. The distribution of rupture forces shown clear overlap for the three subsets (Figure 7.6c) and are similar from one blood donor to another (Supplementary figure 7.9). The binding strength is not affected by IL7-activation, while, as a result of the faster binding kinetics (Figure 7.6d), the proportion of bound cells is increased (Figure 7.6b).

7.3 Discussion
We have resolved the adhesion strength and kinetics of individual CD4 binding to fibronectin. Three classes of cells could be distinguished: unbound, binding and bound. Interaction strengths were below 30 pN for unbound cells, below 55 pN for transient binding and crawling cells, and from 55 to hundreds of pN for bound cells. In AFM experiments the average rupture force of a single integrin-fibronectin bond has been shown to be ~40 pN (at a loading rate of 20 pN/s)\(^{135}\). This single-bond rupture force suggests that the unbound, binding, or bound cells that we characterized were held by zero, one, or several integrin bonds, respectively. The adhesion strength was not affected by IL7-activation, but adhesion kinetics was. This accelerated kinetics could be necessary for the cells to resist the shear force (~150 pN) due to the blood flow challenging cell adhesion. The shear force is about four times the strength of a single integrin-fibronectin bond, which suggests that IL7 triggers the inside-out integrin activation pathway, resulting in faster binding kinetics. Nevertheless, the slower, but same-strength outside-in integrin activation pathway is still available for non-activated cells not experiencing shear stress. In capillaries 1800-2000 CD4 per microliter of blood are carried by a flow speed of 2 mm/s. CD4 rolls over endothelial cells held by selectin-ligand interactions with a rate of 10 \(\mu\)m/s. The relatively small differences in kinetics we observed here, might be enough to favor the binding of IL7-activated cells with integrins activated by inside-out pathways. On the contrary, stochastic contacts with the ECM might be too short-lived in non-activated cells for the first integrin to activate the outside-in pathway.

With these measurements, we demonstrate the advantages of scAFS: hundreds of cells can be tracked in parallel in real time, while well-controlled forces can be applied up to 1000 pN. scAFS is performed in a closed fluidics
system, allowing precise control of parameters such as temperature, flow rate and gases dissolved in the medium, which allows keeping the sample sterile and the operator safe. Given the volume of the AFS chamber (5.4 μL) measurements can be performed on small biological sample volumes, for example obtained from patients or small animals. Finally, the relative simplicity and compactness of the system allows straightforward integration in advanced fluorescence microscopes. Taken together, this illustrates that acoustic forces can be applied directly to cells in order to quantitatively assess their interactions with their environment, opening up a wide range of potential applications in research and the clinic.

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7.4 Supplementary methods

7.4.1 Blood sampling and cell preparation

Blood samples from healthy donors were provided by a blood center (Etablissement Français du Sang, Centre Necker-Cabanel, Paris). CD4 cells were separated by negative selection (RosetteSep Kit, StemCell) as described previously. D4 were equilibrated for 18 hours in RPMI 1640 medium (Lonza) supplemented with Hapes (10 mM, Sigma-Aldrich), penicillin 50 U/mL, streptomycin 50 μg/mL (Sigma) and heat-inactivated fetal bovine serum (FBS 10%, Lonza, uncomplemented at 53 °C during 45 min, containing 50 mg of proteins per mL whom 40 mg/mL of BSA) and placed in an incubator at 37 °C, 5% CO₂ in a humidified atmosphere. CD4 were activated when with 2 nM of recombinant glycosylated human IL7 expressed from HEK cells (Abcys) at 37 °C, 5% CO₂ for 15 minutes prior to injection in the scAFS flow chamber at room temperature.

7.4.2 Experimental setup, cell imaging

The AFS chip itself consists of a flow chamber and a piezoelectric element (LUMICKS b.v., AFS module). The fluid chamber surface area was 54 mm², 12 mm² under each of the two piezos (Supplementary figure 7.1). The functionalization of the flow chamber was performed by injecting 30 μL of bovine fibronectin solution (5 μg/mL diluted in PBS, Sigma-Aldrich) incubated for 30 min at room temperature as described previously. The chamber was saturated with 30 μL of RPMI medium completed with 1% FBS for 15 min then washed with 200 μL of the same medium (40 volumes of the chamber). For assessment of CD4 adhesion, 30 μL of non-activated or IL7-activated CD4 suspensions in RPMI medium, completed with hepes (10 mM final) and 1% FBS (0.5 mg of proteins /mL final), was injected in the chamber and incubated at room temperature. The piezoelectric element attached to the flow chamber was driven with a function generator (Siglent, SDG830) providing oscillating current amplified by a RF-amplifier (SCD, ARS 2_30_30, 50 Ω impedance, 10-W maximum output power). A maximum driving voltage of 42 Vpp was applied in these experiments at 14.3 MHz for not more than a few seconds, this did not lead to sample heating of more than two degrees. Part of the data was acquired using a LUMICKS AFS Stand-alone instrument. Images were acquired with a bright field inverted microscope (Nikon Eclipse Ti). Here, an LED light source (Thorlabs, M660L4) was used in combination with an air objective lens (Nikon, CFI Plan Fluor 10X/0.30 NA, field of view 1.8 mm²). To regulate the sample z position, a piezo translation stage (PI, P-517.2CL) was mounted on the
microscope and driven by a digital piezo-controller (PI, E-710.4CL). Images were taken with a CMOS camera (Thorlabs, DCC1545M, 1280x1024 pixels) with a full-frame rate of 60 Hz.

7.4.3 Cell tracking

Acquired images were processed in real time to extract the cell positions in three dimensions. To determine the $xy$ positions, a quadrant interpolation algorithm was used. A look-up table (LUT) was used to determine the $z$ position. The LUT was built from image series of cells seating on the glass surface in the absence of acoustic force for cell individually. Images of seated cells were acquired starting from the cells completely in focus up to 30 μm above the focus, with 100 nm steps. The diffraction rings of CD4 cells were used to interpolate the $xyz$-positions of the cell centers. The precision of the tracking was determined by calculating the standard deviation of a bound cell during 8 seconds at 60 Hz, resulting in an accuracy of 2.0 nm $x$ and $y$, and 72 nm in $z$ (Supplementary figure 7.2). The tracking software is available at http://figshare.com/articles/AFS_software/1195874. Additionally, a moving ROI was implemented to adjust all the ROIs, frame-to-frame, bases to the previously determined $xy$ position.

7.4.4 Validation of the integrin-fibronectin bonds

The specificity of the CD4 adhesion to fibronectin-functionalized glass through integrins has been validated using competitive peptide inhibitors: Arg-Gly-Asp and Gly-Arg-Gly-Asp-Ser (Sigma-Aldrich). Peptide solutions were added to non-activated or IL7-activated CD4$^+$ T-cell suspensions in RPMI medium completed with 1% fetal bovine serum (containing 40 mg/mL of BSA, 0.4mg/mL final) and 50mM Hepes. Peptide/cell solutions of $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$ or $10^{-2}$ M of RGD or GRGDS were prepared and measured. Peptide/cell samples were injected in the chamber and incubated at room temperature. Measurements have been performed in duplicate using CD4 isolated from three blood donors.

7.4.5 Acoustic force calibration on cells

The acoustic radiation force $F_{rad}$ has been calibrated using the method described in section 3.4.4. Here the acoustic force was determined by writing down the force balance during the uplift of unbound cells. Acceleration was neglected, because the measurements were performed in the low Reynolds number regime:
\[ F_{\text{rad}} - F_{\text{gravity}} + F_{\text{buoyancy}} - F_{\text{drag}} = 0 \] (7.1)

\( F_{\text{gravity}}, F_{\text{buoyancy}} \) and \( F_{\text{drag}} \) are the gravitation, buoyancy and drag forces, respectively. The radiation force resulted from the calculation of the other forces:

\[ F_{\text{gravity}} = \frac{4}{3} \pi r^3 \rho_{\text{cell}} g \] (7.2)

\[ F_{\text{buoyancy}} = \frac{4}{3} \pi r^3 \rho_{\text{cell}} g \] (7.3)

\[ F_{\text{drag}} = v_{\text{cell}} \gamma_{\text{faxen}} \] (7.4)

The CD4 cells are nearly perfect spheres with a radius \( r_{\text{cell}} \) of 4.01 ± 0.13 \( \mu \)m (\( n = 463 \)). Radii were computed from optical microscopy from the 2D projection surface area of individual cell using ImageJ assuming perfect spheres. The density \( \rho_{\text{cell}} \) of CD4 cells is 1030 ± 20 kg m\(^{-3}\), determined from isopycnic sedimentation. The gravitational acceleration \( g \) is 9.81 m s\(^{-2}\). The density of medium \( \rho_{\text{medium}} \) (RPMI/10% FBS) is 1008.4 kg m\(^{-3}\), as determined using a gauged flask at 23 °C. Faxen's drag coefficient \( \gamma_{\text{faxen}} \) was used to determine the drag force on the cells; this takes into account the hydrodynamic surface effect. The dynamic viscosity \( \eta_{\text{medium}} \) of the medium is 0.999 ± 0.007 mPa s, as measured with an Ostwald's viscosimeter at 23 °C. The velocity of the cell \( v_{\text{cell}} \) was calculated by taking the derivative of the cell z position versus time.

The cell sedimentation velocity, after acoustic elevation, was measured to verify the parameters used. Cells had a constant velocity of sedimentation for about 10 seconds, until they slowed down in close proximity of the glass surface due to the increase of the viscosity, as described by Faxen’s law\(^{33}\). The sedimentation velocity of cells in the absence of acoustic pressure is given by:

\[ v_{\text{sedimentation}} = \frac{4}{3} \pi r_{\text{cell}}^3 \frac{\rho_{\text{cell}} - \rho_{\text{medium}}}{6 \pi \eta r} \] (7.5)

\[ = \frac{2}{9} r_{\text{cell}}^2 g \frac{\rho_{\text{cell}} - \rho_{\text{medium}}}{\eta} \]

The sedimentation velocities of the individual CD4 cells were measured till the cell reached one diameter above the glass surface (8 \( \mu \)m), resulting \( v_{\text{sedimentation}} = 1094 \pm 33 \text{ nm s}^{-1} \) (\( n = 10, \text{ Figure 7.2g} \)). Considering that \( r_{\text{cell}} = 4.0 \text{ \( \mu \)m} \), it follows that the CD4 have a \( \rho_{\text{cell}} = 1038.7 \pm 0.9 \text{ kg m}^{-3} \) at 23 °C.

The force on the CD4 cells was calibrated using equation 7.1. The elevation velocity of the cells was used to determine the force for each time step.
A sine function (the theoretical shape of the force field) was fitted to the force versus cell height plot (Supplementary figure 7.6a and b). The force $F_{rad}$ was then extrapolated for the cells seated on the bottom surface. The curve fitting was repeated for several acquisitions on multiple cells ($n = 14$ and $37$ for non-activated and IL7-activated cells, respectively) with increasing voltage from 2.1 to 10.5V pp. The extrapolated forces $F_{rad}$ versus voltage were plotted in order to verify the quadratic dependence of the force with the amplitude.

### 7.4.6 Investigating the strength of glass-adsorbed fibronectin

We test if cell detachment is caused by breaking of the cell-fibronectin bonds and not by breaking of the interaction of fibronectin with the glass surface. The glass surface inside the AFS chamber was functionalized with fibronectin ($10 \mu$g/mL in PBS, 30 min, room temperature) then washed with PBS. Carboxylic acid groups from glass-adsorbed fibronectin were activated by adding a solution of EDC (N-3-dimethylaminopropyl-N’-ethyldiimidide, 15μL, 2mM, Sigma-Aldrich) and NHS (N-hydroxysuccinimide, 15μL, 5mM, Sigma-Aldrich) diluted in PBS and incubated for 15 min at room temperature. Polystyrene microspheres functionalized with amine groups (2-aminoethylated microspheres, 5 μm in diameter, Sigma-Aldrich) were suspended in PBS and then injected in the chamber and incubated for 2 hours. Unbound microspheres were washed away with 200 μL of PBS. Forces were calibrated as described above, but using free microspheres in the absence of fibronectin ($r_{microsphere} = 2.5 \mu$m, $\rho_{microsphere} = 1500$ kg/m$^3$).

### 7.4.7 Determination of rupture forces

To measure the rupture force of an adhered cell, a square-root amplitude ramp was applied, while the cell height is measured. The rupture force $F_r$ was calculated by determining the voltage at rupture. Using the force-(voltage)$^2$ ratio, the rupture force is given by:

$$F_r = 0.554 \cdot 10^{-12} U_r^2(t_r)$$ (7.6)

During the force ramp, some cells showed small deformations before rupture, as shown in (Supplementary figure 7.7b). These deformations affected the determination of the cell’s z-position and exact determination of the rupture event was less evident. We considered a rupture event when the elevation of the cell was larger than one diameter of the cell (8 μm). This definition resulted in a time delay between the “real” rupture event time $t_r$ and the “considered” rupture time $t_8$ (when reaching 8 μm above the glass bottom surface, at amplitude $U_8$) (Supplementary figure 7.11b). The real rupture time as a function of the $t_8$ and the amplitude $U_8$, is given by:
\[ t_r = t_0 - 0.0386 \cdot \exp (-0.372 U_0) \]  

(7.7)

The time delay \((t_0 - t_r)\) is shown in (Supplementary figure 7.11b), where the amplitude \(U_r\) driving the rupture of the CD4 cell from the fibronectin-functionalized surface is given by:

\[ U_r = U_0 \sqrt{\frac{(t_0 - t_r)}{t_{max}}} \]  

(7.8)

As shown in Supplementary figure 7.7c, cells were first subjected to a series of nine acoustic pulses (14.3 MHz, 4.2 V_{pp}, 2 s) spaced by 2 minutes (blue line) to determine whether they were unbound, binding or bound cells. Subsequently, in order to determine the cells’ adhesion strength, a square root amplitude ramp (14.3 MHz, 0-42 V_{pp}, 18 s) was applied after 19 min providing a linear force ramp (0 – 970 pN, 53.88 pN/s).
7.5 Supplementary figures

Supplementary figure 7.1 | Description of the single-cell AFS system

(a) The AFS chip consists of two glass plates (thickness 1 mm (top) and 0.17 mm (bottom)) with a fluid chamber (30 mm long, 1.8 mm wide, 0.1 mm thick, 5.4 L volume, colored in pink) in between. Two independent transparent piezoelectric elements (7x7 mm) are glued to the upper glass slide. (b) Schematic illustration of the scAFS bright-field imaging method, using a LED, condenser lens, microscope objective and CMOS camera. The z position of the AFS chip is controlled by a piezoelectric translation stage. (c) Schematic illustration of the transverse plane of the AFS chip (not to scale). An acoustic force gradient is shown (grey scale), with high force at the glass bottom plane and no force at the level of the acoustic node plane. Bound cells stay on the fibronectin, whereas unbound cells are pushed upward to the node. (d) A diagram of the hardware connected electronically to the piezo. A computer controls the function generator that feeds an alternating voltage via the amplifier to the piezoelectric element. (e) A digital camera image of the CD4 cells.
Supplementary figure 7.2 | x, y and z tracking analysis and resolution

(a, b) xyz-position trajectories of an unbound (left) and a bound (right) CD4 cells. Same data as Figure 7.2f and g, split in four panels. First, cells are seated on the surface of the flow cell (orange zone); after 10 seconds, the acoustic force is applied (14.3 MHz, 4.2 Vpp, yellow zone); finally, the force is switched off (purple zone). (c, d) xy plot of the data shown in a and b, colors correspond to zones indicated in a and b. Standard deviations of the green bound zone are 36.3 and 1.6 nm for the unbound and bound CD4 cells, respectively. (e, f) trajectories of z-position in the absence of force; standard deviations are 84 nm and 72 nm for the unbound and bound CD4 cells, respectively.
Supplementary figure 7.3 | CD4 adhesion kinetics to fibronectin induced assessed by scAFS

(a) the underlying data of Figure 7.4. Non-activated and IL7-activated CD4 cells are injected in the FN-functionalized chamber at room temperature. CD4 cells are pushed upward with a minimal force (14.3 MHz, 4.2 Vpp, 2 s) to validate the fraction of unbound, binding and bound cells, after 5, 20, 40 and 60 minutes of incubation. Images of CD4 before (red) and after (green) acoustic wave application are shown superposed. Immobile cells appear in yellow (superposition green + red). Percentage of immobile fractions (xy shift < 1 μm) are indicated in white on the upper right corner.
Supplementary figure 7.4 | CD4 adhesion to fibronectin is faster after IL7 activation
Box plot of the xy-shifts after 5, 20, 40, 60 minutes of incubation for non-activated (green) and IL7-activated (red) CD4 cells on FN-functionalized glass. Whiskers are maximum and minimum values, and quartiles framing the upper and lower part of the median. The horizontal blue bars indicate the average of xy shifts amongst the cell subset with less than 1 m of xy shift (bound subset) and the percentage of these bound subsets are indicated above the corresponding columns.

Supplementary figure 7.5 | The CD4 adhesion inhibited by RGD and GRGDS peptides
Adhesion of CD4 cells to the fibronectin-functionalized glass, challenged with increasing concentrations of peptide inhibitor Arg-Gly-Asp (RGD; plain line) and Gly-Arg-Gly-Asp-Ser (GRGDS; dashed line), after 20 minutes at 23 °C. This experiment has been performed on three different blood donors (error bars are s.e.m.).
Supplementary figure 7.6 | Force exerted by acoustic waves on the CD4 as a function of z position

(a) z-position trajectory of a single cell during application of sound wave (14.3 MHz, 4.2 Vpp, 2 s).
(b) The acoustic radiation force $F_{rad}$ is computed for every data point from the uplift velocity of the cell ($v_{cell}$), solving equation 7.1 (section 7.4.5) and plotted as a function time. Raw data (black dots) and moving average of 5 data points (red line) are displayed. (c) $F_{rad}$ as a function of z position with a sine function fitted to the data (green line). Red squares indicate the time points used for the fit: from immediately after force application up to the moment the cell reaches the acoustic node.
Supplementary figure 7.7 | Effect of acoustic forces on unbound and bound CD4 cells

(a, b) Unbound (left) and bound (right) CD4 cells are pushed up from the glass surface (horizontal green dashed line, $z_0$) to the node plane (horizontal black dashed line) as shown in the plot of $z$ position as function of time (lower panels). Vertical cyan and red dashed lines indicate the start of the elevation ($t_0$) time of reaching a height of 8 μm ($t_8$), respectively. The dark blue line indicates the linear ramp of the acoustic amplitude. Most bound cells show mild elevation (2-3 μm) after $t_0$ under the acoustic pressure and only minimal xy shifts. After bond rupture ($t_r$), cells move swiftly up to the node, while large xy shifts are observed. The black dashed line indicates the threshold height above which a cell is considered unbound ($z_0 + 8$ μm).

(c) $z$-position trajectories of an unbound, a binding and a bound CD4 cell during a typical bond-rupture measurement, with applied voltage indicated. The first 19 minutes are used to identify the cell state, also shown in Figure 7.3. Subsequently a square-root amplitude ramp (14.3 MHz, 0–42 Vp-p, 18 s) is performed, resulting in a linear force ramp (0–970 pN, 53.88 pN/s). The right panel is a zoom in on the force ramp (indicated by the grey zone on the left), providing insight in the elevation of the cells, their residence in the acoustic node and their sedimentation to the bottom glass surface after switching of the acoustic waves.
Supplementary figure 7.8 | Investigating the strength of glass-adsorbed fibronectin

Polystyrene microspheres (5 μm in diameter) are covalently bound to the fibronectin-functionalized glass (section 7.4.4). An upward acoustic force is applied from 0 to 500 pN in 2 seconds, at room temperature. A digital camera image is made before (a), during (b, green) and after (c, red) the force ramp. (d) A superimposed image, created from b and c, clearly shows that no microsphere detached during force application. (e) The z-position of a representative microsphere is plotted versus time. The force ramp is indicated by a gray zone. Maximal elevation is 150 nm from the starting position.
Supplementary figure 7.9 | CD4 rupture-force measurements shown in Figure 3e, for both donors separately

As shown in Figure 7.6d, an amplitude ramp of the acoustic wave (0–42Vpp) has been applied for 18 s to non-activated and IL7-activated cells after 19 min of incubation in the chamber. Pie charts give the proportions of unbound (blue), binding (purple) and bound (orange) cells for non-activated (a, c) and IL7-activated cells (b, d) for two representative blood donors. Box plots show the bond–rupture forces, Whiskers are maximum and minimum, and quartiles framing the upper and lower part of the median. (e, f) The bar graphs show the corresponding spectra of rupture forces of the representative donors. Cell percentages are given per slice of 20 pN of rupture force from 0 to 260 pN excluding the few high rupture force values (max 447 pN). The unbound, binding and bound zones are indicated by blue, purple and orange, respectively.
Supplementary figure 7.10 | Application of acoustic force ramp to assess cell-adhesion strength

Digital camera images of CD4 cells injected in the FN-functionalized fluid chamber of the AFS chip (left) and schematic illustration (right). The objective is focused on the cells seated on the glass bottom surface: (a) no acoustic force is applied. (b, c) A linear acoustic amplitude ramp applies a quadratic force ramp to the cells (14.3 MHz, 0–10.5 Vpp, 20 s), lifting up first unbound cells (g, ~4.2 Vpp) and next binding cells (d, ~10.5 Vpp). Cells cluster together in lines, due to weak acoustic pressure deviations within the xy plane11. (i) When the acoustic forces are off, all the cells sediment (~20 s), back to the focal plane.
Supplementary figure 7.11 | Extrapolation of the rupture force from the amplitude abscise value $U_8$ when the cell center is elevated 8 $\mu$m above the base line

(a) Force calibration was performed by applying increasing acoustic pulses (2.1, 4.2, 6.3, 8.4, 10.5 $V_{pp}$ as indicated for each curve) for ~1 second to CD4 cells as shown in Figure 7.5a,b. Trajectories of the $z$-position of a representative cell are plotted. The horizontal green and red dash lines represent 0 and 8 $\mu$m elevation, respectively. The time $t_0$ is the moment of acoustic force application (vertical blue dashed line) and the time $t_8$ is the moment when the elevation of the cell is 8 $\mu$m elevated (vertical red dashed line). (b) The time delay $t_8 - t_0$ is plotted versus the amplitude $U_8$ and fitted with the equation 7.7. The five data points obtained from panel a are plotted as open circles. Standard deviation of $U_8$ and time delay are indicated as error bars ($n = 8$). (c) The amplitude $U_8$ is plotted as a function of $U_0$ for non-activated and IL7-activated CD4 cells from two donors. The extrapolation of $U_8$ is plotted as a black line according to the equation 7.8. Three of the data points from panel a are displayed as open circles, colors are used for the different subsets of cells (not-activated and IL7-activated cells of two different donors). The interpretation of the contributions of cell deformation and bond rupture to the $z$ elevation are indicated as dashed arrows as an example for a single cell.