Capturing resting T-cells: perils of PLL

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Understanding lymphocyte activation will require thorough characterization of the 'resting' state, and how it changes. Surfaces coated with the cationic homopolymer poly *L*-lysine (PLL) are widely used for total internal reflection fluorescence (TIRF) imaging of the resting organization of lymphocyte surface proteins (1-5), because PLL is assumed to be inert. Here we show that PLL initiates T-cell signaling and profoundly alters the behavior of membrane proteins such as the T-cell receptor (TCR). The emerging notion that receptors and signaling proteins cluster by default (1-5), based mostly upon studies of lymphocytes interacting with PLL-coated surfaces, therefore needs reconsideration.

We examined whether PLL-coated surfaces are actually inert by testing their capacity to induce calcium signaling in primary CD4⁺ and Jurkat T-cells. Cells were labelled with Fluo-4AM and transferred to PLL- or OKT3- (anti-CD3 antibody) coated coverslips. Signaling occurred in ~80% of primary and Jurkat T-cells after 30s on PLL (total N=247 cells in n=3 experiments; **Fig. 1a**), and in 90% of cells after 30s on OKT3 (N=631, n=3). Similar results were obtained for Jurkats with the ratiometric reporter, Fura-2 (N=270, n=3; **Supplementary Fig. 1a**). The dynamics of Jurkat responses to each surface were also comparable (**Fig. 1b**). Signaling was largely insensitive to PLL coating-concentration, coating time and polymer size (**Fig. 1c**; **Supplementary Fig. 1b**). J.CaM1.6 and J.RT3-T3.5 cells exhibited diminished responses (**Fig. 1a**) that were restorable with Lck and TCR β re-expression, respectively (**Supplementary Fig. 1c**), implying that PLL induces *bona fide* TCR signaling. PLL induced TCR-proximal signaling detected as ZAP70 phosphorylation, albeit weakly (**Supplementary Fig. 1d**), and TCR-distal CD69 upregulation (**Fig. 1d**). Although they were somewhat more refractory, calcium signaling was induced by PLL in murine primary CD4⁺ (C57BL/6) T-cells, and two of three T-cell hybridomas tested (e.g. Yae5b3k; **Supplementary Fig. 1e**). PLL also induced B-cell receptor-dependent signaling in a B-cell line (A20; **Supplementary Fig. 1f**).

Jurkats formed much larger contacts with PLL- versus OKT3-coated or uncoated glass (**Movies S1-S3**; **Supplementary Fig. 1g**). To test whether PLL perturbs receptor behavior, we used single-molecule tracking of fluorescently-labelled TCRs. On PLL-coated coverslips, TCR mobility was immediately reduced (diffusion coefficient $D=0.018\pm0.01\ \mu\text{m}^2/\text{s}$, versus $D=0.06\ \mu\text{m}^2/\text{s}$ measured at the apical surface of a murine T-cell hybridoma (6); **Fig. 1e left**; **Movie S4**; **Fig. 1f**). To determine whether this required hard surfaces, we spin-coated agarose onto coverslips, producing a softer surface still suitable for TIRF imaging. Under these conditions, the TCR exhibited high mobility ($D=0.18\pm0.07\ \mu\text{m}^2/\text{s}$; **Fig. 1e right**; **Movie S4**; **Fig. 1f**), which was reduced to near static levels when PLL was added to the surface ($D=0.04\pm0.03\ \mu\text{m}^2/\text{s}$; **Movie S4**; **Fig. 1f**). PLL therefore directly perturbed TCR dynamics, presumably *via* electrostatic polymer/receptor interactions.

But how might PLL initiate signaling, and how could such effects be avoided? CD45 is depleted at PLL-mediated contacts by ~50% (**Supplementary Fig. 1h,i**), which could favor signaling by increasing the local kinase/phosphatase ratio. Similar levels of phosphatase segregation suffice to initiate TCR phosphorylation and downstream signaling on SLBs (7). The immobilization of CD45 and TCRs in PLL-mediated contacts could potentiate signaling also by constraining receptor dephosphorylation. Overall, for imaging truly resting cells, surfaces might be best avoided altogether. ICAM-1 coated surfaces allow T cells to be captured without triggering strong calcium signaling (8), but integrin 'out-to-in' signaling will likely shift these cells away from the resting state nevertheless (9). Although TCR diffusion was apparently unimpeded on agarose surfaces, for imaging it was necessary to hold the cells in place using agarose pads, which induced signaling ($79 \pm 10\%$ responding cells, $N=80$, $n=3$). In $>70\%$ of experiments, however, signaling was undetectable when we suspended Jurkats in hydrogels straight from culture ((10); **Supplementary Fig. 2a**).

Super-resolution, stimulated emission depletion imaging revealed that TCR organization in Jurkats suspended in hydrogel and on PLL-coated surfaces was profoundly different (**Fig. 1g**). Intensity-based analysis revealed that the degree of TCR clustering was significantly higher for PLL-coated glass-contacting cells than for cells in hydrogel ($p < 0.001$, $n=5-14$ images; **Supplementary Fig. 2b**). This suggests that the high levels of receptor and signaling-protein clustering reported previously for lymphocytes interacting with PLL-coated surfaces (1-5) is probably not reflective of resting-cell behavior. The extent to which the deformable surfaces that non-activated T-cells encounter *in vivo* affect signaling-protein organization, if at all, is a separate matter.

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Figure 1

PLL induces strong T-cell signaling and profoundly alters TCR diffusional behavior and organization.

(a) Fraction of primary (CD4^+) and wild-type and mutant Jurkat T-cells eliciting calcium responses (detected with Fluo-4AM) upon contact with OKT3- (grey)- and PLL- (red) coated surfaces. Mean $\pm$ s.d. are shown for three experiments. (b) Fluo-4AM fluorescence intensity changes versus contact time for Jurkat T-cells on OKT3- (grey) or PLL- (red) coated surfaces (mean, solid lines; s.d., shaded area) were calculated for $N=100$ randomly chosen cells per condition. (c) Fraction of responding Jurkat T-cells as PLL coating-concentration is varied, for high (red, *i.e.* 150 kDa) and low (grey, *i.e.* 70 kDa) molecular weight (MW) PLL. Mean $\pm$ s.d. are shown for three experiments. (d) Surface expression of CD69 on cultured Jurkat T-cells (black), and after overnight incubation on PLL- (red) or OKT3- (grey) coated surfaces. Data are representative of three experiments. (e) Single-molecule, TIRF-based tracking of fluorescent Fab-labelled TCRs at basal surfaces of Jurkat T-cells contacting PLL- (left) or agarose- (right) coated surfaces. Tracks are coloured by diffusion coefficient (MSD analysis; colour bar). (f) TCR diffusion coefficients from single-molecule tracking for each cell calculated from ensemble average MSD curves; means $\pm$ s.d. for cell-to-cell variation are shown; *, $p < 0.001$; **, $p < 1 \times 10^{-10}$. (g) STED images of TMR-Halo labelled TCRs at basal surfaces of Jurkat T-cells contacting a PLL-coated glass surface (left) or suspended in hydrogel (right). Insets: diagrams of the experimental setup (upper right corners), and fluorescence intensity variation in the marked-up areas (lower right corners).

Supplementary materials

1. Online methods

Cell culture

Jurkat T-cells, A20 mouse B-cells and mouse T-cell hybridomas were cultured in sterile RPMI (Sigma Aldrich) supplemented with 10% FCS (PAA), 2 mM L-glutamine (Sigma Aldrich), 1 mM sodium pyruvate (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich), and 1% Penicillin-Streptomycin-Neomycin solution (Sigma Aldrich). Cells were maintained at 37°C and 5% CO₂ during culturing and, typically, kept at a density between 5-9 x 10⁵ cells/ml. Plasmid transfection and lentivirus infections were used to generate cell lines expressing the constructs described above (Lck, TCR β , TCR β -Halo, HyHEL10).

Glass surface preparation

Glass cell culture dishes (FD3510-100 FluoroDish, 35 mm) were coated with OX7 (anti-rat Thy1.1) and OKT3 (anti-hCD3 ϵ) antibodies for Jurkat T-cells, or MOPC21 (Mouse IgG1, kappa monoclonal) and KT3 (anti-mCD3 ϵ) antibodies for mouse T-cell hybridomas at a 9:1 ratio (total antibody concentration of 10 μ g/mL), and with IgG or IgM at a concentration of 10 μ g/mL, in HBS (Sigma-Aldrich) at 4°C overnight. For PLL coating, a 0.1% stock solution of high molecular weight PLL (150-300 kDa; Sigma-Aldrich) was used to coat culture dishes for 20 minutes at room temperature (Movie S3), or 0.01% stock solutions of low (70-150 kDa) or high (150-300 kDa) molecular weight PLL, or 3-fold serial dilutions thereof, were used to coat dishes overnight at 4°C (all other experiments). Prior to use, the dishes were washed three times with 1 mL HBS.

Cell labeling

For single-molecule imaging and tracking, TCRs expressed by Jurkat T-cells were labelled using antigen-binding fragments (Fabs). Approximately 10⁶ cells were incubated with UCHT1 (CD3 ϵ)-labelled Fabs diluted in 1 mL of PBS (Sigma-Aldrich) at a final concentration of 1 nM at 4°C for 30 minutes. After incubation, the cells were washed three times with PBS. For STED imaging, Jurkat TCR β -Halo T-cells were labelled with a Halo-ligand tagged Tetramethylrhodamine (TMR-Halo, a kind gift from Dr. Andreas Vargas Jentzsch, Department of Chemistry, University of Oxford) at 5 μ M final concentration for 1 hour at 37°C and afterwards washed three times with Leibovitz's L-15 Medium (Life Technologies).

Agarose spin coating

Agarose spin coating was performed using a modified version of an established procedure (1). A plasma-cleaned glass coverslip was mounted onto a silicon wafer resting on a hot plate set to 60°C. A 2% low gelling-temperature agarose (Sigma Aldrich) in PBS was maintained above its setting point on the same hot plate. The wafer was placed onto a spin coater and 200 μ L of the agarose solution was pipetted onto the cover slip. Spin coating was performed for 7 s at 500 rpm followed by 35 s at 3000 rpm. This created an ~50 nm thick layer of agarose on the coverslip (1). Agarose-coated coverslips were coated with PLL by incubation with the 0.01% PLL solution for 20 minutes followed by washing three times with 1 mL of PBS.

TIRF imaging

TIRF imaging was performed on a Nikon (Eclipse Ti-U) inverted microscope. The output of a 488 nm fiber laser (Qioptic, 15 mW iFLEX-2000) was filtered with an excitation filter (Semrock, FF01-488/6-25) and expanded to a diameter of 12 mm using a Keplerian beam expander. A Köhler lens was used to focus the beam onto the back focal plane of a 100x, 1.49 NA oil-immersion objective lens (Nikon). By focusing the beam at the edge of the aperture of the objective, the beam approached the coverslip at an angle below the critical angle for total internal reflection, creating an evanescent field with a penetration depth of ~200 nm. The light collected by the same objective lens passed through a dichroic filter (Semrock, Di01-R405/488/532/635-25x36) and an emission filter (Edmund Optics), and

was then focused onto an EMCCD camera (Photometrics, Evolve 512 Delta) *via* a tube lens. Images were captured at a frame rate of 20 Hz. An additional 1.5 magnification relay lens in the optical path resulted in a final pixel size of 106.7 nm (confirmed by a Ronchi Ruling, Edmund Optics). Labelled cells in PBS were placed onto PLL- or agarose-coated coverslips. All imaging was performed within the first 8 minutes after cell deposition. Because the cells moved around freely on the agarose-coated coverslips and did not spread or become stationary, ~1 mm 1% agarose/PBS pads were placed on top of the cells to prevent movement.

Single-molecule tracking

The single-molecule data were analyzed using a custom-made Matlab code (3). Briefly, data were filtered using a non-linear Chung Kennedy filter (4), after which intensity peaks with a signal-to-noise ratio larger than 3 were identified. Localizations were linked together to form tracks and only tracks lasting for 8 frames or longer were kept. The ensemble mean square displacement (MSD) was calculated on a cell-by-cell basis to determine the mean diffusion coefficient for each cell.

Hydrogels

Three-layered CyGEL Sustain™ hydrogels (Abcam) were used to suspend Jurkat T-cells in glass-bottomed WillCo-dishes® (GWSB). The first layer comprised 200 µL of neat hydrogel (as supplied in PBS), which was set by 5 minutes incubation at 65°C; the second and third layers comprised 50% hydrogel and 25% hydrogel, respectively, each diluted in L-15 Medium, and set by 1 minute incubations at 65°C. The cells were added to the hydrogel at 37°C, left for 10 minutes to settle, and then imaged at 37°C in 5% CO₂.

STED microscopy

STED microscopy imaging was performed on a Leica TCS SP8 microscope (Leica, Mannheim). All live-cell experiments were performed at 37°C and 5% CO₂. The microscope was equipped with a pulsed super-continuum white-light laser (WLL, Koheras SuperK, pulsing at 80 MHz) run at 70% of total power. TMR fluorescence was excited by selecting 651 nm from the WLL (using 65% of the 70% WLL output). The fluorescence was depleted using 65% of the continuous wave 660 nm STED laser line. All images were acquired on the Leica HyD detectors gated between 0.5 and 7.5 ns. The image zoom was chosen so that the resulting pixel size was roughly 30 nm (about half of the expected resolution) and every line of the image was averaged eight times to improve signal-to-noise. The STED laser beam was split into two paths each including a phase plate for creating donut-like intensity focal patterns along lateral (xy) and axial (z) directions, respectively. By setting the relative allocation of the STED laser power between these two phase-plates by use of a variable beam splitter, it was possible to tune the spatial resolution of the microscope along the xy and z directions individually. Using the Huygens STED-Deconvolution-Wizard (Huygens Software, Netherlands), only a moderate degree of deconvolution was applied to the recorded STED images to avoid deconvolution artefacts. The microscope's point-spread-function was directly calculated from the Leica imaging files, following standardized Huygens software guidelines.

Intensity-based analysis of relative levels of TCR clustering

Images acquired using STED microscopy were analysed by dividing the mean intensity of the 20% brightest pixels with the overall mean pixel intensity. For a homogenous sample, this value is 1. As features form, the image becomes more heterogeneous, therefore the fraction of high intensity pixels increases and the computed value is >1. We used this metric to quantify the extent of spatial (re)organisation versus a homogeneous sample. We confirmed that the value is low for homogeneous samples using membrane (CellMask)-stained T-cells on PLL-coated coverslips, imaged using TIRF microscopy. Such images are especially homogenous because these contacts are relatively flat.

Calcium response measurements

Human and mouse primary T-cells, Jurkat T-cells and mouse T-cell and B-cell hybridomas were labelled with either 4 μ M Fluo-4 AM (Molecular Probes™, Invitrogen) or 5 μ M Fura-2 AM (Molecular Probes™, Invitrogen).

For Fluo-4 AM labeling, cells were incubated with the dye for 30 minutes at room temperature in RPMI (Sigma-Aldrich) without supplements but containing 2.5 mM probenecid. Cells were then washed three times with HBS and finally resuspended in HBS containing 2.5 mM probenecid before addition to the microscope sample container with the prepared microscope coverslip. Cells were imaged at 37°C and 5% CO₂ using a 10× air objective on a spinning disk confocal microscope (Zeiss Cell Observer Spinning Disc Confocal), with 488 nm laser excitation and fluorescence detection at ~530 nm, and with an exposure time of 350 ms and a time between frames of 500 ms for 840 frames. We used a modified version of CalQuo (5), to detect single-cell landing events on the prepared glass surfaces, and to record fluorescence intensities over time at the coverslip surface. Briefly, for each cell, stable changes in fluorescence intensity above background levels were indicative of the cell 'landing', and calcium responses associated with cell signaling indicated by >2.5 fold increases in fluorescence intensity above the 'landing' intensity. For each cell, the time lags between 'landing' and 'signaling' was also determined. All cells were individually analyzed and the fraction of signaling cells was determined from the total number of cells detected after landing, provided that cells landed before frame 480, in order to allow three minutes for signaling to occur.

For Fura-2 AM labeling, cells were loaded for 30 minutes at room temperature with the dye in 10% FCS-supplemented RPMI with 1 mM CaCl₂ and 0.5 mM MgCl₂. Cells were then washed three times with HBS containing 2% FBS, 1 mM CaCl₂ and 0.5 mM MgCl₂, and finally resuspended in the wash buffer and kept at 37°C until imaging. A custom-written Matlab script was used to determine the fraction of responding cells. The 340 nm channel intensity was used to track cells. A bandpass filter was then used to remove the background and tracking was performed using pkfnd.m and track.m functions (<http://site.physics.georgetown.edu/matlab/code.html>). All tracks longer than 100 s (100 frames) for cells moving slower than 100 nm/s on average were analyzed. The intensity from both channels was bandpass filtered to remove the background. The intensity for each cell analyzed was integrated and the ratio between the two channels calculated. A cell was considered to have responded if the 340/380 ratio exceeded a threshold of five (resting cells had a ratio of 1-1.3).

CD69 quantification

The wells of a 96-well tissue culture plate were left untreated (incubated with PBS) or coated overnight at 4°C with 50 μ L OKT3 at 10 μ g/ml or 50 μ L of PLL at 0.01% (w/v) after which the plates were washed three times with PBS. Jurkat T-cells, at 0.5×10^6 /ml were washed in RPMI and resuspended at 1×10^6 /ml before adding 100 μ L of the suspension to the coated or untreated wells and left at 37°C for 16 hours. Cells were then stained with mouse anti-human CD69-APC antibody (clone FN50, Ebioscience) in PBS with 0.1% (w/v) BSA for 30 minutes on ice. After two washing steps with PBS, CD69 up-regulation was quantified by flow cytometry. Data from three independent experiments was analyzed in FlowJo and Prism (Graphpad).

Zap70 phosphorylation

Jurkat T-cells were starved overnight in RPMI with 1% FCS. The cells were then washed once with PBS and added to OKT3- and PLL- coated glass plates. After a 30 s spin at 100xg the plates were incubated at 37°C for 10 minutes. The cell suspension was then carefully aspirated from the plate to remove cells that had not formed contacts with the coated surfaces. SDS sample buffer with protease inhibitors (cOmplete™ Protease Inhibitor

Cocktail, Sigma-Aldrich), DNaseI (5ug/ml, Roche) and sodium orthovanadate (1mM, New England Biolabs), was then added to the plate, which was incubated for 30 minutes on ice, after which the samples were boiled and frozen. SDS-PAGE gels were run and Western blot analyses undertaken, detecting both total Zap70 (clone 99F2, Cell Signaling) and phosphorylated Zap70 (Y319, clone 65E4). Data from four experiments were analysed using ImageJ and Prism (Graphpad).

Interference reflection microscopy (IRM)

IRM experiments were performed at 37°C with a 1.4 NA 63x oil-immersion objective on a scanning laser confocal microscope (Zeiss780, Carl Zeiss AG). We used a 647 nm laser, a NT80/20 neutral beam splitter (20% excitation reflected, 80% emission transmitted), and a GaAsP detector operated in reflection mode. IRM images were recorded at a frame repetition-rate of 6.2 s and simultaneously combined with fluorescence imaging of calcium responses detected using Fluo-4AM (with excitation at 488 nm and detection at ~530 nm) along with differential-interference-contrast (DIC) imaging using the microscope's TPMT module.

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Supplementary Figure 1. Responses of T and B cells to PLL. (a) Fraction of responding Jurkat T cells, detected with Fura-2AM, on OKT3- (grey) or PLL- (red) coated surfaces. Mean $\pm$ s.d. are shown (three experiments with n = 270-407 cells per condition). (b) Fractions of Jurkat T cells responding to the indicated dilutions of PLL coated for 10 minutes (red, 150 kDa PLL; grey, 70 kDa PLL); the blue symbol indicates the response to 150 kDa PLL left to dry overnight. Mean $\pm$ s.d. are shown (three experiments with n = 164-293 cells per condition, or five experiments with n = 543 cells (high MW, drying overnight)). (c) Fraction of responding mutant Jurkat T cells (J.RT3 and J.CaM1.6) following TCR and Lck re-expression, on OKT3- (grey) and PLL- (red) coated surfaces. Mean $\pm$ s.d. are shown (three experiments with n = 289-361 cells per condition). (d) ZAP70 phosphorylation in Jurkat T cells detected on OKT3- (grey) and PLL- (red) coated surfaces, versus resting Jurkat T cells taken straight from culture (means $\pm$ s.d. are shown; n = 4 experiments; * p < 0.05). (e) Fraction of primary CD4⁺ mouse T cells and three murine T cell hybridomas responding to anti-CD3 antibody (KT3; grey)- and PLL- (red) coated surfaces. Mean $\pm$ s.d. are shown (three experiments with n = 190-283 cells per condition). (f) Fraction of wild-type, BCR-deficient (A20/BCR⁻), and HyHEL10 BCR re-expressing A20 B cells responding to IgG- (grey)- and PLL- (red) coated surfaces. Mean $\pm$ s.d. are shown (four experiments with n = 506-796 cells per condition). (g) Change in contact area with time imaged using IRM (mean $\pm$ SEM; n = 14 cells). (h) Distribution of CD45 labeled with Alexa 488-tagged Gap 8.3 Fab (green) versus that of the TCR labeled with TMR-tagged UCHT1 Fab (red), and a membrane dye (CellMask; red, bottom), on Jurkat T cells interacting with PLL-coated glass coverslips. (i) Ratio of CD45 and TCR inside (i) and outside (o) PLL-induced contacts (means $\pm$ s.d. are shown; n = 16 cells; * p < 0.001, Student's t -test).

Supplementary Figure 2. TCR organization and calcium signaling differ for cells suspended in hydrogel versus cells contacting PLL-coated glass. (a) Temporal projections of calcium signaling detected with Fluo-4AM for: (*top left*) Jurkat T cells suspended in hydrogel; (*top right*) cells leaving the hydrogel layer and interacting with OKT3-coated glass; and Jurkat T cells contacting (*bottom left*) OKT3- and (*bottom right*) PLL-coated glass. The frame-rate was 500 ms per frame; total time was 10 min. The scale bar is 100 μ m. **(b)** Intensity-based analysis of relative levels of TCR clustering. Images acquired with stimulated emission depletion microscopy were analyzed by calculating the mean intensity of the 20% brightest pixels divided by the overall mean pixel intensity. Comparisons are made between Jurkat T cells interacting with PLL-coated coverslips or suspended in hydrogel, and CellMask (membrane)-labeled cells contacting PLL-coated coverslips, which form flat homogeneous contacts ($n = 5-14$ images; $*p < 0.001$, Student's t -test). T cells suspended in hydrogel produce a larger ratio than CellMask-labeled cells, presumably due to sectioning through their ruffled surfaces.