

Title: Kinetics of activation of regulatory molecules during immunological synapses in T cells

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Abstract

T cell activation through TCR stimulation leads to the formation of the immunological synapse (IS), a specialised adhesion organised between T lymphocytes and antigen presenting cells (APCs) in which a dynamic interaction among signalling molecules, the cytoskeleton and intracellular organelles achieves proper antigen-mediated stimulation and effector function. The kinetics of molecular reactions at the IS is essential to determine the quality of the response to the antigen stimulation. Herein, we describe methods based on biochemistry, flow cytometry and imaging in live and fixed cells to study the activation state and dynamics of regulatory molecules at the IS in the Jurkat T cell line CH7C17 and primary human and mouse T CD4⁺ lymphocytes stimulated by antigen presented by Raji and HOM2 B cell lines and human and mouse dendritic cells (DCs).

1. Introduction

T lymphocytes or T cells constitute one of the main components of the adaptive immunity. T cells express the T cell receptor (TCR), containing the protein complex accompanied by the signalling CD3 complex. The $\alpha\beta$ TCR recognizes antigen-derived peptides complexed with the major histocompatibility complex (pMHC) at the plasma membrane of antigen-presenting cells (APCs) [1,2]. The recognition of the pMHC by the TCR is a first signal for T cell activation. The binding of CD28 co-stimulatory receptor to its ligands, CD80 or CD86 on the APC side, constitutes a second signal, which allows T cell expansion and anergy evasion [1,2]. A third signal is provided by the binding of secreted cytokines by the APC or other immune cells, allowing cell division and differentiation of naïve T cells into memory or helper T lymphocytes (Th), including effector Th₁, Th₂, Th₁₇ or regulatory T cells (T_{reg}) [3]. It has been recently proposed that the uptake of glucose or amino acids through specific receptors constitute a fourth signal to promote the metabolic response required for sustained T cell activation and differentiation [4,5].

The TCR, together with integrins, such as lymphocyte function associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4), co-stimulatory molecules, as CD28, chemokine receptors, such as CXCR4, and scaffold proteins, such as LAT are required to diversify the initial signalling that is promoted by cell-cell contact with APCs during the nascent immune synapse (IS). The IS of CD4⁺ T cells is a transient, highly organized structure conformed by many molecules [1,2] that serves as a platform to different signalling pathways leading to T cell activation and to the effector function of T cells [6]. The molecules involved in T cell activation are accumulated and organised in concentric rings called supramolecular activation clusters (SMACs) at the so-called mature IS [1,7]. In the classic mature IS, the TCR gathers into signalling microclusters along with CD28 and other regulatory proteins, which move towards the central area of the IS, called central SMAC (cSMAC) [6,7,8]. LFA-1 and its associated proteins accumulates around this central area, in the

so-called peripheral SMAC (pSMAC) [9]. Actomyosin arcs at the pSMAC help the movement of the TCR to the central area, mainly depleted of filaments of actin (F-actin) [9, 10]. The so-called distal SMAC (dSMAC) is enriched in a network of branched actin. There, polymerisation and depolymerisation of F-actin mediates an actin retrograde flow to favour the movement of receptors towards the more internal areas [11,12,13]. Tubulin-cytoskeleton rearrangements relocate the centrosome and allow the movement of different organelles to the IS, facilitating T cell activation [14,15]. TCR signalling after binding to the pMHC enables cytoskeleton dynamics, which, in turn, is required for efficient sustained TCR signalling [16,17]. All these events are regulated in time, space and strength and this dynamics should be analysed to understand their relevance and role in T cell activation. This article summarises relevant methods to study the complexity of the IS by approaching both the dynamics and the activation status of relevant molecules during T cell activation, including biochemical techniques as immunoblot, flow cytometry and microscopy analyses.

2. Materials

2.1. Common disposables

- o Automatic pipette and serological pipettes (5 and 10 mL) (Grenier Bio-One, Spain)
- o Micropipettes (0.2–2 μ L, 1–20 μ L, 20–200 μ L, 200–1000 μ L) and tips (Grenier Bio-One, Spain)
- o Coverslips for immunofluorescence (IF) (thickness No. 1.5H; 0.170 mm \pm 0.005 mm such as 0117530 Marienfeld).
- o Neubauer hemocytometer (Hirschmann, Spain)
- o 1.5 mL microcentrifuge tubes (Nirco, Spain)
- o 24 and 96-well plates for cell culture (Grenier Bio-One, Spain)
- o 15 mL and 50 mL conical-bottom centrifuge tubes (Grenier Bio-One, Spain)
- o 25 and 75 cm² cell culture flask (Grenier Bio-One, Spain)
- o Cryovials (Grenier Bio-One, Spain)
- o 35 mm, 10 mm glass-bottom dishes, 1.5 coverslip thickness (homemade or Mat-tek corporation P35G-1.5-10-C, USA)
- o Rectangular cell dishes 127 x 85 x 42 mm³ (Nunc OmniTray 165218) for humidity chambers

2.2. Common equipment

- o Cell culture incubator (forma steri-cycle CO₂ incubator, Thermo Scientific, USA)
- o BioRad Gene-pulse Electroporator (USA)
- o Sterile cell culture laminar flow hood safety level II (Telstar AV-100, Telstar Technologies S.L, Spain)

- Flow cytometer BD FACSCanto II Analyser Cytometer (405 nm violet laser, 488 nm solid state blue laser and 633 nm He-Ne) (Becton Dickinson, USA)
- Confocal imaging: TCS SP5 confocal laser scanning unit with spectral detection and resonant scanner, attached to an inverted epifluorescence microscope (DMI6000) fitted with an HCX PL APO 63× 1.40NA –0.6 oil objective and TCS SP8 Navigator confocal laser scanning unit with spectral detection and resonant scanner, equipped with a WLL laser (range 470–670 nm), attached to an inverted epifluorescence microscope (DMI6000) fitted with an HC PL Apo CS2 63x/1.4 OIL objective
- Microscopes are mounted into microscope environmental chambers with heat (Temperature regulator TempControl-37-2 digital) and humidity and CO₂ gas controllers (CTI-Controller 3700 digital)

2.3. Analytical software

- FlowJo (BD, USA)
- Image J freeware (National Institutes of Health, USA. <http://imagej.nih.gov/ij/>; Java 1.8.0_66; 64 bit)
- Imaris software 7.2.2 (Bitplane, USA. <http://www.bitplane.com/imaris/imaris>)
- Leica accompanying software (LAS; Leica microsystems, Spain)
- PRISM 8 (GraphPad Software, USA)

2.4. Cell lines and primary cells

- Jurkat lymphoblastoid cells: CH7C17 (HA1.7 Vβ3⁺ transgenic αβTCR, specific for HA peptide) cell line [18].
- Primary T lymphocytes from human healthy donors (purified CD4⁺ or SEE/SEB-specific blasts).
- Primary CD4⁺ T lymphocytes from mouse lymph nodes and spleen.
- B cell lines as antigen presenting cells for conjugates: Raji B (Burkitt lymphoma) and HOM2 (HLA-DRB1*0101 positive, EBV-transformed) lymphoblastoid cells with MHC-II compatible for peptide presentation for Jurkat E1–6 cells and CH7C17 cells, respectively. Raji B cells can present SEB to CH7C17
- Conventional dendritic cells derived from mouse bone marrow with GM-CSF and IL-4 [15-19].

2.5. Reagents

- Complete medium: RPMI 1640 supplemented with Glutamine (100 mM), nonessential aminoacids, Hepes (25 mM), FCS (Fetal calf serum; 10%), β-mercaptoethanol (1 mM; only for mouse cells) (HyClone, USA)
- Incomplete medium: RPMI 1640, l-Glutamine (100 mM), nonessential aminoacids, HEPES (25 mM) (HyClone, USA)
- Lymphocyte separation medium: any commercial density gradient media such as Ficoll Histopaque (Sigma Aldrich, USA)
- Transfection medium: Optimem I (Thermo Fisher Scientific, USA)
- Fetal bovine serum (FBS) (HyClone, USA)
- Saline solution: NaCl (154 mM)
- Dimethyl-sulfoxide (DMSO) (Sigma Aldrich, USA)
- 0.4% Trypan blue solution (Thermo Fisher Scientific, USA)
- Paraformaldehyde (Sigma Aldrich, USA)
- Triton X-100 (Sigma Aldrich, USA)
- Cytokines: human recombinant IL-2 cytokine. Mouse or human recombinant IL-7 and GM-CSF

- Staphylococcal Enterotoxin E (SEE) (Toxin Technologies, USA)
- Staphylococcal Enterotoxin B (SEB) (Toxin Technologies, USA)
- Synthetic HA₃₀₇₋₃₁₉ peptide (sequence: PKYVKQNTLKLAT) [14, 18]
- Dynabeads™ human CD3/CD28 T cell activators (Thermo Fisher Scientific, USA)
- ProLong Gold antifade mounting medium (Thermo Fisher Scientific, USA)
- Poly-L-Lysine (high adherence) γ -irradiated for cell culture: 75,000>Mw>150,000 (Sigma Aldrich, USA)
- Fibronectin from human plasma suitable for cell culture

- Antibodies for Western Blot:
 - anti-CD3 ζ pY83 (Abcam, USA)
 - anti-LAT pY132 (Abcam, USA)
 - anti-PLC γ pY783 (Cell Signalling, USA)
 - anti-PKC θ pT538 (Cell Signalling, USA)
 - anti-ZAP70 pY615 (Cell Signalling, USA)
 - anti-tubulin (clone DM1A) (Sigma Aldrich, USA)
 - goat anti-rabbit HRP-conjugated secondary antibody (Thermofisher Scientific, USA)
 - goat anti-mouse HRP-conjugated secondary antibody (Thermofisher Scientific, USA)

- Antibodies and probes for immunofluorescence:
 - CMAC (7-Amino-4-Chloro-methylcoumarin) (Life Technologies, USA)
 - anti-EB1 (BD Biosciences, USA)
 - anti-PKC θ pT538 (Cell Signalling, USA)
 - anti-alpha Tubulin-FITC (clone DM1A) (Sigma Aldrich, USA)
 - anti-TOM20 (Santa Cruz Biotech, USA)
 - anti-Talin (Santa Cruz Biotech, USA)
 - anti-PLC γ pY783 (Cell Signalling, USA)
 - anti-CD3 ϵ produced in our laboratory
 - anti-CD3 ζ (clone 448) (Sigma Aldrich, USA)
 - fluorophore-conjugated phalloidin (Fisher Scientific, USA)
 - mCherry- β -Actin plasmid was kindly donated by M. Vicente-Manzanares (Institute of Molecular and Cellular Biology of Cancer, Salamanca, Spain)
 - goat anti-rabbit alexa568-conjugated highly cross-absorbed secondary antibody (Molecular Probes, USA)
 - goat anti-rabbit alexa488-conjugated highly cross-absorbed secondary antibody (Molecular Probes, USA)

- Antibodies for flow cytometry:
 - anti-CD3 (clone Hit3a) – Phycoerythrin (PE) (Tonbo Biosciences, USA)
 - anti-CD19 (clone 4G7) – Allophycocyanin (APC) (BioLegend, USA)
 - anti-CD69 (clone FN50) – V450 (BD Biosciences, USA)
 - anti-CD25 (clone BC96) – PE/Cy7 (BioLegend, USA)

2.6. Buffered solutions

- Tris-buffered saline (TBS): 20 mM Tris Base, 150 mM NaCl in distilled water (dH₂O).
- TBST: TBS supplemented with 0.1% (w/v) Tween-20

- Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄.
- Wash solution: Hank's Balanced Salt Medium (HBSS).
- Coating bicarbonate-carbonate buffer for stimulating surfaces: NaHCO₃ (0.1 M), Na₂CO₃ (0.032 M), pH: 9.6
- Lysis buffer for immunoblot: 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1.5 mM MgCl₂ and phosphatase and protease inhibitors
- PHEM (2×): 120 mM Pipes, 50 mM Hepes, 20 mM EGTA, 4 mM MgCl₂; pH 6.9
- Immunofluorescence (IF) Fixation Solution: PHEM (1×), 4% paraformaldehyde (PFA), 0.12 M sucrose
- IF Blocking solution: PHEM (1×), bovine serum albumin (BSA) 3%, human γ -globulin 100 μ g/ mL, sodium azide 0.2%
- IF Blocking and permeabilizing solution: PHEM (1×), BSA 3%, human γ -globulin 100 μ g/ mL, sodium azide 0.2%, 0.2% Triton Tx-100
- MACSQuant Running buffer for FACS (Miltenyi Biotec, Germany)

3. Methods

3.1. Isolation of T cells

3.1.1. CD4⁺ T-Cell Isolation from Human Peripheral Blood Lymphocytes (PBLs)

1. Isolate the PBMCs from complete blood (50–200 mL) or buffy coat preparations (50 mL from 450 mL complete blood) from healthy human donors through a Ficoll Histopaque gradient (or a similar solution). Dilute the buffy coat preparation (1:3, v/v) or the complete blood (1:1, v/v) with HBSS or saline solution and mount the gradient over a lymphocyte separation medium cushion (1:2, v/v).

2. Centrifuge for 700 x g, 20 min, R/T without brake or acceleration.

3. Recover the cells from the interphase with the Ficoll in a new tube.

4. Wash the cells with saline solution four to six times to drain the platelets (PBMCs).

5. Purify CD3⁺CD4⁺ cells by negative selection with magnetic beads-based methods. A cocktail of antibodies and streptavidin-conjugated beads for autoMACS (Miltenyi Biotec) or Stem Cell Solutions is recommended.

3.1.2. Isolation of Mouse CD4⁺ T Cells from spleen and lymph nodes.

CD4⁺ T lymphocytes may be isolated from the spleen and/or pools of peripheral lymph nodes (mesenteric, auricular, maxilar, brachial, axillar, inguinal, and others). Mouse naïve T cells are more difficult to transfect; we recommend a pre-activation step for higher transfection or nucleofection efficiency. Mouse T cells can also be transfected immediately after purification with the magnetic bead-based method without activation, with lower efficiency. Mouse T-cell activation can be performed with o/n treatment with CD3/CD28 antibodies, PMA/ionomycin, OVA₃₂₃₋₃₃₉ peptide (only for T cells isolated from OT-II transgenic mice), or ConA (48-h treatment).

1. Grind lymphoid organs through a 70 μ m cell strainer with T-cell isolation solution (500 x g, 5 min, R/T).

2. Add 1 mL of erythrocyte lysis buffer per spleen cell suspension (3–5 min, R/T).

3. Add T-cell isolation solution or complete medium to stop cell lysis.

4. Purify CD3⁺CD4⁺ cells by magnetic bead-based negative selection for subsequent transfection (see Subheading 3.3). A cocktail of antibodies and streptavidin-conjugated beads is recommended (StemCell or Miltenyi Biotech). Purification step is recommended after OVA₃₂₃₋₃₃₉ peptide treatment as APCs are needed for activation.

5. For longer culture time, count and grow them at 2 x 10⁶ per mL in complete medium + IL-2 (200–500 Units/mL). Add IL-2 every 2 days up to 6 days.

3.2. Generation of Lymphoblasts from Human PBLs: HAp or SEE/SEB-Specific

1. Count cells after recovering from adhesion step and plate them at 2 x 10⁶ per mL in complete medium.

2. Add SEB (5 µg/mL) or HAp (1 µg/mL) and incubate for 48–72 h or 24 h, respectively.

3. Wash the cells twice with HBSS (1,000 x g, 5 min, R/T).

4. Count them and grow them at 2–4 x 10⁶ per mL in complete medium + IL-2 (20–50 Units/mL). Add IL-2 every 2 days.

5. Upon 8 days, SEE/SEB-treated cells can be restimulated for 18–24 h (0.1 µg/mL SEE and 0.4 µg/mL HAp). Wash them twice and let grow as above. Upon 18–24 h, they can be used for transfection. The percentage of Vβ8⁺ cells found at this stage is approximately 40–60%, as measured by flow cytometry (anti-Vβ8-FITC, BD Biosciences).

6. Restimulation with SEE and HAp is possible every 15 days. Lymphoblasts may be frozen (10⁷ cells per mL). After cryogenization procedures, an effective transfection is less efficient, but cells can be restimulated again before transfection (**see Note 1**).

3.3. Transfection of T cells

3.3.1. Amaxa Nucleofection for mouse CD4⁺ T cells

1. Pre-warm the nucleofection solution, the cuvettes, the cell culture medium post-transfection, and cell culture plates before nucleofection.

2. Count the cells. Use 5 x 10⁶ cells maximum per nucleofection.

3. Spin the cells and discard supernatant (500 x g, 2 min, 4 °C).

4. Wash cells twice in cold HBSS (500 x g, 2 min, 4 °C).

5. Aspirate all the washing media and add 100 µL of the pre-warmed transfection mix: Opti-MEM I with the DNA or RNA included (**see Note 2**).

6. Add the cell mix to the pre-warmed cuvette and nucleofect immediately.

7. Use program X-01 Nucleofector I-Amaza (**see Note 3**).

8. Nucleofect a single cuvette, recover the cells, and plate them immediately in complete medium at 4 x 10⁶ cells per mL supplemented with recombinant mouse IL-7 (5 ng/mL). Do not keep cells in the nucleofection solution for more than 3–5 min.

3.3.2. Electroporation for cell lines and human SEE/SEB-Specific T lymphoblasts

1. Count the cells from cell culture. Use 10–20 x 10⁶ cells per sample.

2. Spin the cells (500 x g, 2 min, 4 °C) and discard supernatant.

3. Wash cells twice with cold HBSS (500 x g, 2 min, 4°C).

4. Wash cells with cold Opti-MEM I (500 x g, 2 min, 4°C).
5. Resuspend in 400 µL of Opti-MEM I with the desired DNA or RNA. A mix of DNA and RNA can be used as well. The final volume should be less than 410 µL for the mix. Cells in solution may be kept at 4°C until transfection (not more than 30 min). Use a 0.4 mL cuvette.
6. Electroporate cells with a pulse of 240 V, 975 mΩ (usual time: 27.5–29 ms in a GenePulser II (Bio-Rad)).
7. Recover the cells from the cuvette, and plate them immediately in incomplete medium for 4–6 h at 2×10^6 cells per mL. Do not leave the samples for a long time in the cuvette after electroporation. To avoid cell death, up to ten samples may be electroporated at once.
8. Add FCS at 5% for the first 18–24 h and then supplemented to 10%.
9. Supplement with IL-2 primary T lymphoblasts (10–50 U/mL).

3.4. Signalling assays by immunoblotting: stimulating antibodies and cell conjugates

3.4.1 Preparation of Human SEE/SEB-Specific T Lymphoblast Cells or CH7C17 T Cells

1. Count the cells. Use 2×10^6 cells for each sample; a minimum of 0.5×10^6 cells is required. **Figure 1** includes examples of human T lymphoblasts stimulated with monoclonal anti-CD3 + anti-CD28 coated beads (**Figure 1A**) and mouse CD4 T cells stimulated with OVA-loaded DCs (**Figure 1B**).
2. Spin cell culture; decant cell culture medium and wash cells twice with HBSS.
3. Resuspend cells at 10^7 /mL of complete medium; 200 µl will be used for each sample.

3.4.2 Preload of antigen presenting cells (APCs) with Antigen

1. Count the cells. Use 2×10^5 cells for each sample (ratio 1:10 APC:T).
2. Use 100 µl of a 2×10^6 /mL cell suspension in complete medium per condition.
3. Load APCs with antigens and super-antigens:
 1. HOM2 B cells with HA peptide for 2 h at 37 °C in complete medium
 2. Raji B cells with SEE or SEB for 30 min at 37 °C in complete medium.
 3. DCs with OVA peptide for 2 h at 37 °C in complete medium.
 4. Monoclonal anti-CD3 + anti-CD28 coated beads: the day before the assay coat the of latex beads (6.6 µm of diameter) with 1 µg/mL of anti-CD3 and 0.33 µg/mL of anti-CD28 in bicarbonate buffer at 4 C for 18 h with gentle movement. Beads are recovered and blocked with 3% BSA for 1 h at 4 °C before use.

3.4.3 Formation of Conjugates

1. Mix T cells and APCs at 1:10 ratio and centrifuge at low speed to facilitate the formation of conjugates.
2. Incubate cells for the required time conditions at 37 °C. The times normally used for kinetics assays are 0 (non-stimulated), 5, 15 and 30 minutes.
3. Stop activation by incubation of cells at 4 °C; spin the cells for 5 min at 4 °C ($1000 \times g$) to collect cells. Discard supernatants.

3.4.4 Lysis and IB

1. Gently resuspend cells in lysis buffer (50 µl/10⁶ cells).
2. Incubate for 20 min at 4 °C.
3. Spin lysates at 21,000 × g for 10 min at 4 °C to remove debris and nuclei.
4. Remove the supernatant and place it in a clean tube. Mix it with 5x Laemmli solution and β-mercaptoethanol (final concentration 0.15 M).
5. Boil samples for 15 min at 85 °C.
6. Separate proteins by SDS-PAGE and perform wet electrotransfer for IB with nitrocellulose or PVDF membranes.
7. Stain with Ponceau S solution (0.1% Ponceau S w/v in 5% acetic acid v/v) to detect the transferred proteins. Wash with water and TBS-T until blots are unstained.
8. Block membranes with TBS containing 0.2% TWEEN and 5% BSA for 1 hour at R/T.
9. Cut the membrane into pieces to incubate the area that contains the proteins of interest exclusively with the corresponding primary antibodies. Use the molecular weight standards as a guide.
10. Blot membranes with primary antibodies (o/n at 4 °C). Usually, phospho-specific antibodies require 1% BSA to increase signal to noise ratio.
11. Wash with TBS containing 0.2% Tween at least three to four times each antibody.
12. Incubate membranes with corresponding peroxidase-conjugated secondary antibodies (30 min-1 hour).
13. Wash with TBS containing 0.2% Tween at least three to four times each antibody.
14. Detection of chemiluminescence signal may be performed with different chemoluminescence developers and imaging systems (e.g.: ImageQuant880 Amersham imaging system (GE Healthcare). Loading controls must be used for each experiment (**Figure 1**).

3.5. Stimulation assays: flow cytometry dose-response assays in CH7C17/HOM2 conjugates

3.5.1. Cell preparation and staining (**Figure 2**)

Conjugate CH7C17 cells and SEB or HAp-loaded HOM2 cells at a 1:1 ratio (**Note 4**)

1. Make serial dilutions 1/2 from 10 µg/mL to 0.3 µg/mL of the SEB and serial dilutions 1/2 of HAp from 400 µg/mL to 50 µg/mL in culture medium (**Note 5**)
2. Incubate B cells with SEB dilutions at 37 °C and 5% CO₂ for 1 hour in complete medium and with HAp dilutions at 37 °C and 5% CO₂ for 2 hours in complete medium (**Note 6**). Wash twice with complete medium to remove the excess of SEB or HAp.
3. While B cells are being loaded with the SEB or HAp, count CH7C17 cells to take the required number for the assays
4. Add 10⁵ JK and 10⁵ Raji cells in a U-bottom p96 plate and incubate overnight (o/n) (around 18-24 hours) at 37 °C and 5% CO₂. Add a sample containing CH7C17 cells alone and HOM2 w/o SEB as negative controls of stimulation (**Note 7**).
5. Wash cells with FACS running buffer

6. Add the V450-labelled anti-CD69 antibody (1:200) and PE/Cy-7 labelled anti-CD25 antibody (1:200) to estimate the CH7C17 cell activation, the APC-labelled anti-CD3 (1:200) and the PE-labelled anti-CD19 (1:200), to gate out CH7C17 cells for CD69 and CD25 analysis (*see* 3.5.2 section; **Note 8**).
7. Incubate during 30 min inside a light-protected ice container.
8. Wash the unbound antibody by centrifugation in 200 μ L of PBS at 4 $^{\circ}$ C.
9. Prepare samples for the flow cytometry acquisition by resuspending the cell pellet in a volume of 200 μ L of FACS running buffer (**Note 9**).
10. Analyze the samples in the Flow Cytometer machine.

3.5.2. Flow cytometry data analysis

Flow cytometry data can be analyzed with accompanying software or by using the FlowJo software.

1. Open the compensation controls and sample files.
2. Gate the live cells population in SSC/FSC plot (**Figure 2A**).
3. Select the single cell population in SSC-A/FSC-A plot.
4. Gate CH7C17 cells by selecting CD3⁺ CD19⁻ population in the APC-CD3/PE-CD19 plot (**Figure 2A**).
5. Export the data corresponding to CD69-V450 and CD25-PE/Cy7 parameters from CH7C17 cells gate: Mean, Geometric Mean (GeoMean), SD, cell count, percentage of total.
6. Graph the GeoMean and SD of CD69 and CD25 as a function of SEB and HAp concentration (**Figure 2B**). Softwares such as GraphPad PRISM software can be used.
7. Perform statistical analysis with this software to determine significance.

3.6. Imaging of IS formation in conjugates through immunofluorescence analysis

3.6.1. Coating of Coverslips

1. Use a humidity chamber to coat the coverslips: cover with a parafilm a square or round cell culture plate.
2. Place the coverslips on the humidity chamber in a scattered mode to avoid overlapping.
3. Pipette 25–50 μ L of poly-L-lysine hydrobromide in sterile water (50 μ g/mL) per coverslip and incubate o/n at 4 $^{\circ}$ C.
4. Wash twice with sterile deionized water.
5. Store in imaging medium until used. They can be frozen at this step. Do not allow to dry.

3.6.2. Formation of conjugates and staining

3.6.3.1. Preparation of T Cells

1. Count the cells. Use 1.5×10^5 for each coverslip.

2. Spin the cell culture, decant the cell medium.
3. Resuspend in complete medium (3×10^6 /mL). Use 50 μ l for each sample.

3.6.3.2. Preparation of APCs

1. Preload APCs with a cell tracker such as CMAC (1 μ M) in incomplete medium for 30 min at 37 °C and add FCS (5%) to the samples (**see Note 10**).
2. Split APCs in two tubes: Negative controls do not contain stimulating antigens or superantigens (**Figure 3A, upper panel**).
3. Load APCs (+) with the antigens or superantigens for 2 h or 30 min, (peptides or superantigens, respectively) in complete medium:

OVA peptide: DCs (2 μ g/mL) (**Figure 3B, lower panel**)

SEE: Raji cells (0.3 μ g/mL) (**Figure 4A**)

SEB/HA peptide: HOM2 cells (5 μ g/mL or 200 μ g/mL) (**Figure 4B**)

Alternatively, loading of antigens can be performed simultaneously to the cell tracker for the required time.

4. After incubation, wash the cells twice with HBSS to exclude excess of antigen.
5. Resuspend cells in complete medium (3×10^6 /mL).
6. Use 50 μ l for each coverslip preparation.

3.6.3.3. Formation of conjugates

1. Use a humidity chamber to allow cells to conjugate in small volumes.
2. Place the coverslips in the humidity chamber numbering their location for identification.
3. Wash poly-L-Lysine-coated coverslip with 150 μ l of HBSS and aspirate.
4. Immediately, conjugate APCs with T cells (ratio 1:1): DCs, Raji or HOM2 B cells with CD4⁺ OT-II T cells, Jurkat or CH7C17 T cells, respectively. Mix 50 μ l of APCs (\pm antigens) with 50 μ l of T cells directly onto the coverslips to favor the formation of conjugates.
5. Incubate cells for 30 min at 37 °C.
6. Carefully, stop the formation of conjugates by adding 50 μ l of fixation buffer onto the coverslip; mix drop by drop to avoid the separation of the cells.
7. Let the fixative act for 2 min.
8. Discard the supernatant carefully.
9. Add 50 μ l of fixation buffer for 10 min at room temperature (RT).
10. Discard the supernatant.
11. Wash with HBSS once and then TBS (10 min).
12. Block the coverslips with blocking solution. Coverslips can be stored at 4 °C.

3.6.3.4. Immunofluorescence

1. If the staining is only for intracellular epitopes/antigens: Permeabilize and fix the cells for 5 min at RT with a mix of 50% fixation solution + 50% IF blocking solution and 0.2% Triton X-100 (see **Note 11**).
2. Block the cells with the blocking and permeabilizing solution for 30 min at RT.
3. Add the primary antibodies diluted in the blocking and permeabilizing buffer o/n at 4 °C or 1 h at 37 °C depending on the antibody affinity.
4. Wash the cells 5 × 3 min with TBS.
5. Add the secondary antibody for the corresponding species diluted in the blocking and permeabilizing buffer for 30 min at 37 °C.
6. Wash the cells 5 × 3 min with TBS.
7. Repeat the process for the subsequent antibodies to stain different proteins to localize the immune synapse and the signalling or structural proteins. Avoid species cross-reactivity of primary and secondary antibodies and fluorescence dyes overlapping.
8. Wash the cells 5 × 3 min with TBS. Proceed with a final wash with distilled water (Dh₂O).
9. Dry the water drop of the coverslip and mount the coverslip by adding 8–10 µl of mounting medium (e.g., Mowiol or Prolong) to the coverslip and putting the coverslip over the microscope slide. Be careful to avoid air bubble formation.
10. Visualize the samples under a confocal microscope to image the immune synapse.
11. Analysis of Z-stacks can be performed with different softwares, such as Image J or Imaris.

Some examples of immune synapses are shown in **Figures 3** and **4**, including staining for EB1, tubulin and F-actin for cytoskeleton related proteins, and CD3ζ, PKCθ and Talin for signalling molecules in mouse DCs and CD4 OT-II T cells (**Figure 3**) and EB1, Tubulin, mitochondria, PLCγ and CD3ζ for T cell lines (**Figure 4**), indicating active cell-cell interactions. Maximal projections are shown.

3.7. Imaging of actin dynamics during immune synapse formation in live T cells by confocal microscopy

3.6.1. Coating of cell chambers

1. The day before of the assay: Pipette 25–50 mL of fibronectin in incomplete medium (200 µg/mL) per cell chamber of 10 mm diameter and incubate o/n at 4 °C.
2. Wash twice with incomplete medium.
3. Store in imaging medium until used. They can be frozen at this step. Do not allow to dry.

3.7.2. Preparation of T cells

1. Transfect T cells with speckle actin-mCherry plasmid 24 h before imaging.

2. Collect transfected cells, spin them (500 x g, 5 min, R/T), and resuspend in 2 mL of HBSS.
3. To recover live cells, add 1 mL of lymphocyte separation medium to the bottom of the tube. Spin cells without brake (1,200 x g, 5 min, R/T). Live cells relocate at the interface with the lymphocyte separation medium, whereas dead cells accumulate at the bottom of the tube.
4. Cells can be sorted at this point to allow homogeneous expression of the fluorescent proteins. Spin the cells after sorting and keep them in the cell incubator with complete medium 2 h before imaging.
5. Count the cells. Use 5×10^5 cells for each video.
6. Wash cells twice with HBSS and resuspend in imaging medium (10^7 cells/mL). Pre-warm at 37 °C in 5% CO₂; maintain until use.
7. Pre-warm fibronectin-precoated dishes with 2 mL of imaging medium before use.

3.7.3. Preparation of APCs

1. Preload APCs with a cell tracker such as CMAC (1 mM) in incomplete medium for 30 min at 37 °C and add FCS (5%) to the samples (see **Note 10**).
2. Split APCs in two tubes: Negative controls do not contain stimulating antigens or superantigens.
3. Load APCs (+) with the antigens or superantigens for 2 h or 30 min, (peptides or superantigens, respectively) in complete medium:
 - OVA peptide: DCs (2 µg/mL)
 - SEE: Raji cells (0.3 µg/mL)
 - SEB/HA peptide: HOM2 cells (5 µg/mL or 200 µg/mL) (**Figure 5**)
4. Wash cells twice with HBSS and resuspend in imaging medium.
5. Count the cells. Prepare a 10^7 cells/mL suspension. Use 5×10^5 cells for each video.
6. Pre-warm at 37 °C in 5% CO₂; maintain until use.

3.7.4. Formation of conjugates and imaging

1. Place the fibronectin-coated chamber containing 2 mL of imaging medium on the pre-warmed, microscope stage.
2. Add 50 µL APCs, allow them to spread and focus the cells.
3. Add 50 µL of T cells.
4. Adjust the acquisition parameters for video recording. The intensity of the beam light should be enough to visualize the fluorescent proteins or trackers provide phototoxicity and photobleaching is avoided (see **Note 12**).
5. Start video acquisition. To visualize the lamella formation the usual Z-stack contain 9-10 images acquired every 10-20 s.
6. Stop acquisition once the immune synapse or the event of interest has been imaged.

4. Notes

Note 1. Cells may be nucleofected every time with the Amaxa system (human T-cell kit, program T23) or electroporated after the first restimulation in Optimem I medium (Thermo Fisher Scientific) with the Genepulser II from Bio-Rad (240 V, 975 m ω) with either DNA or RNA. CD3⁺CD4⁺ T cells (most of them V β 8⁺) can be isolated by magnetic bead-based negative selection and transfected afterward.

Note 2. The cell viability is about 50% after the transfection procedure. Viable cells will divide properly 24 h post-transfection. Some over-expressed or exogenously expressed molecules can affect the normal cell cycle. A maximum of 5 mL of DNA or RNA should be added to 100 mL of mix. RNA concentration ranges 1–4 mM. Optimized range: 1.5–2.5 mL in 100 mL of Optimem I or Amaxa mix.

Note 3. To improve cell survival, plate all cells immediately after nucleofection in a single mix with warmed medium in a cell culture dish or flask. This will improve cell survival.

Note 4. Raji B and HOM2 B cells express the MHC class II recognized by Jurkat E1–6 T cells and CH7C17 cells, respectively. E1–6 T cells express a TCR complex containing the V β 8 region bound by SEE. CH7C17 cells express a transgenic $\alpha\beta$ -TCR specific for HA peptide containing the V β 3 region bound by SEB. They were engineered from a β -deficient, E1–6 cells-derived clone (13–31) reconstituted with α - and β -TCR-containing plasmids.

Note 5. This enables us to uncover the minimal amount of SEB and HA peptide reaching the maximal CH7C17 stimulation using CD69 and CD25 increased expression at the cell surface as readout.

Note 6. HA peptide requires increased incubation times than SEB, because it competes with the already present peptide in the MHC-II of HOM2 cells, whereas the superantigen B binds externally to the MHC-II complex.

Note 7. Supernatants of conjugates can be recovered to measure secreted IL-2 by ELISA if desired, following the manufacturer's instructions or see [20].

Note 8. Include samples or beads to compensate the fluorescence detected in different channels due to overlapping emissions of fluorochromes: negative and single staining controls. FMO (fluorescence minus one) controls are recommended to detect slight changes in expression of different antigens.

Note 9. FACS running buffer includes fetal calf serum (FCS) and human γ -globulins, which are used to block any unspecific cross-reaction through lateral interactions of antibodies and specific interactions with Fc receptors expressed by the cells present in the samples. This buffer also includes EDTA to help the presence of single cells and to prevent the formation of large cell aggregates during the use of the flow cytometry equipment.

Note 10. Serum esterases present in cells and FCS will cut off the ester residue of cell trackers such as CMAC, preventing its exit from cells and a possible entry in other cells than APCs, respectively.

Note 11. Particular antibodies against proteins belonging to microtubule binding families, such as EB1, need a supplementary fixation in methanol at 20% in blocking buffer. Perform fixation at -20 °C for 2 min.

Note 12. The adjustment of parameters for image acquisition can be adjusted in chambers with isolated APCs or T cells before formation of conjugates.

5. Discussion

The use of different cell systems and methodologies to study T cell activation through the organization of the IS constitutes a holistic strategy, which facilitates the correlation of different events and parameters. The combined use of antigens and super-antigens helps determining the intracellular organization of molecules and organelles in the T cell and the required signal level to surpass the activating threshold in a given cell type or organism (human vs mouse) in different extents of TCR activation [6,8,10,12-15,18-19].

The kinetics of activation of different proteins can be studied by analyzing post-translational modification (PTM) of specific residues, such as phosphorylation of tyrosine, serine and threonine. Phospho-proteomics were initially used to describe these PTMs, which are transiently regulated by kinases and phosphatases and extremely relevant for cells [21]. In the context of IS formation after T cell activation these PTMs represent useful indicators to find active or repressed pathways [14,19]. This piece of information can be obtained through immunoblot as shown herein, and complemented by flow cytometry analyses of specific activation markers for T cells subsets, such as CD69 and CD25 [22,23]. Dose-response experiments including different amounts of antigens or super-antigens allow investigating the magnitude of the response. Flow cytometry and microscopy enable us to determine the regulation of particular components in single cells by contrast to biochemistry studies in cell extracts that render the result of the average cell population. Thus, the combination of classical biochemistry and single cell analysis based in flow cytometry and microscopy is required to understand molecular mechanisms causing the cell behavior.

The imaging of the reorganization of relevant proteins, such as CD3 ζ , PLC γ 1, tubulin or actin during IS through confocal microscopy can inform about the kind of IS, which is not provided by biochemical or flow cytometry methods. An interesting issue is the finding of differences in the reorganization of the cytoskeleton related to the dynamics and localization of organelles and proteins between more transient and stable synapses [1,2,5]. Therefore, full characterization of the process of T cell activation in terms of spatial and temporal regulation requires the combination of different techniques, some of which have been described herein.

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Figure Legends

Figure 1. T cell receptor (TCR) signalling in SEB-activated human lymphoblasts. (A) Blots showing phosphorylated (active) forms of several molecules of the TCR downstream signalling such as CD3 ζ -Y83, ZAP70-Y315, PLC γ 1-Y783, LAT-Y191 and PKC θ -T538 in human SEB + SEE-specific lymphoblasts at 0 (non-stimulation), 5 and 15 minutes after SEE/SEB-Raji stimulation. A-Tubulin was used as loading control (B) Blots showing phosphorylated (active) PKC θ -T538 and total PKC θ (loading control) after stripping from CD4⁺ OT-II T cells activated by DC (\pm OVA peptide).

Figure 2. Dose-response curve of SEB and HAp stimulation of CH7C17 cells. (A) Gating of CH7C17 and HOM2 cells with SSC and FSC channels and CD3-APC/CD19-PE plot for segregating CH7C17 cells (CD3⁺, CD19⁻) from HOM2 cells (CD3⁻, CD19⁺). (B) Graphs showing the mean \pm SD of the CD69 GeoMean (n=2). (C) Graphs showing the mean \pm SD of the CD25 GeoMean (n=2).

Figure 3. Imaging of signalling and structural proteins during immune synapse formation by mouse dendritic cells and CD4⁺ T cells. (A) Cell conjugates of control mouse conventional DCs with CD4⁺ OT-II T cells showing lack of centrosome (EB1, green) presence near the cell-cell contact and CD3 ζ (red) homogenous distribution in T cells; actin polymerized as F-actin (blue) is low in T cells, and DCs show spreading lamellas. Cells were conjugated for 30 min. (B) Cell conjugates of OVA-loaded, mouse conventional DCs with CD4⁺ OT-II T cells showing centrosome translocation to the cell-cell contact (white arrows) and CD3 ζ (red) and F-actin (blue) accumulation in T cells. (C) Cell conjugates of OVA-loaded, mouse conventional DCs with CD4⁺ OT-II T cells showing centrosome (α -tubulin, green) translocation to the cell-cell contact (white arrows) and talin (red) and F-actin (blue) accumulation in T cells. Cells were conjugated for 30 min.

Figure 4. Imaging of signalling and structural proteins during immune synapse formation by human T cells and antigen presenting cells. (A) Cell conjugates of human SEE-specific T lymphoblasts derived from peripheral blood and SEE-loaded Raji B cells. The centrosome localization is observed by EB1 and α -tubulin accumulation (green and cyan, respectively) near the cell-cell contact and PLC γ 1-Y783 (red) accumulation in T cells. Cells were conjugated for 15 min. (B) Cell conjugates of CH7C17 T cells and HAp-loaded HOM2 B cells showing centrosome (α -tubulin, cyan) and mitochondria (green) translocation to the cell-cell contact and CD3 ζ (red) accumulation in T cells. Cells were conjugated for 45 min.

Figure 5. Time-lapse video showing F-actin-based lamella formation during immune synapse. (A) CH7C17 cells transfected with mCherry- β -Actin were allowed to conjugate to HAp-loaded HOM2 cells and recorded for 20 min. A maximal projection of Z-stack is shown. (B) CH7C17 cells transfected with mCherry- β -Actin were allowed to conjugate to SEB-loaded Raji cells and recorded for 10 min. A maximal projection of Z-stack is shown.

Figure 1

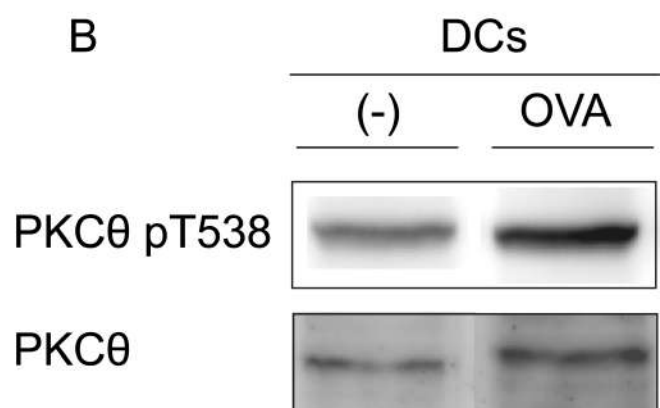
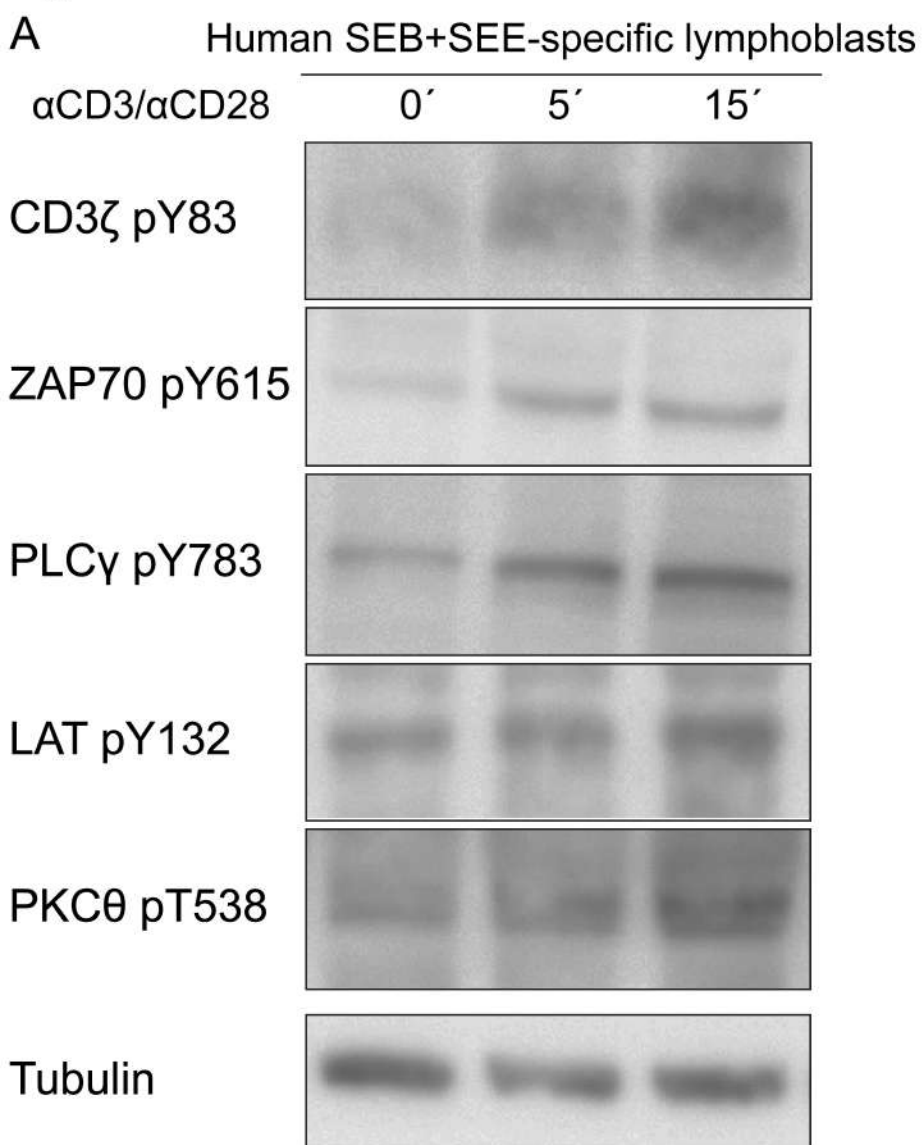
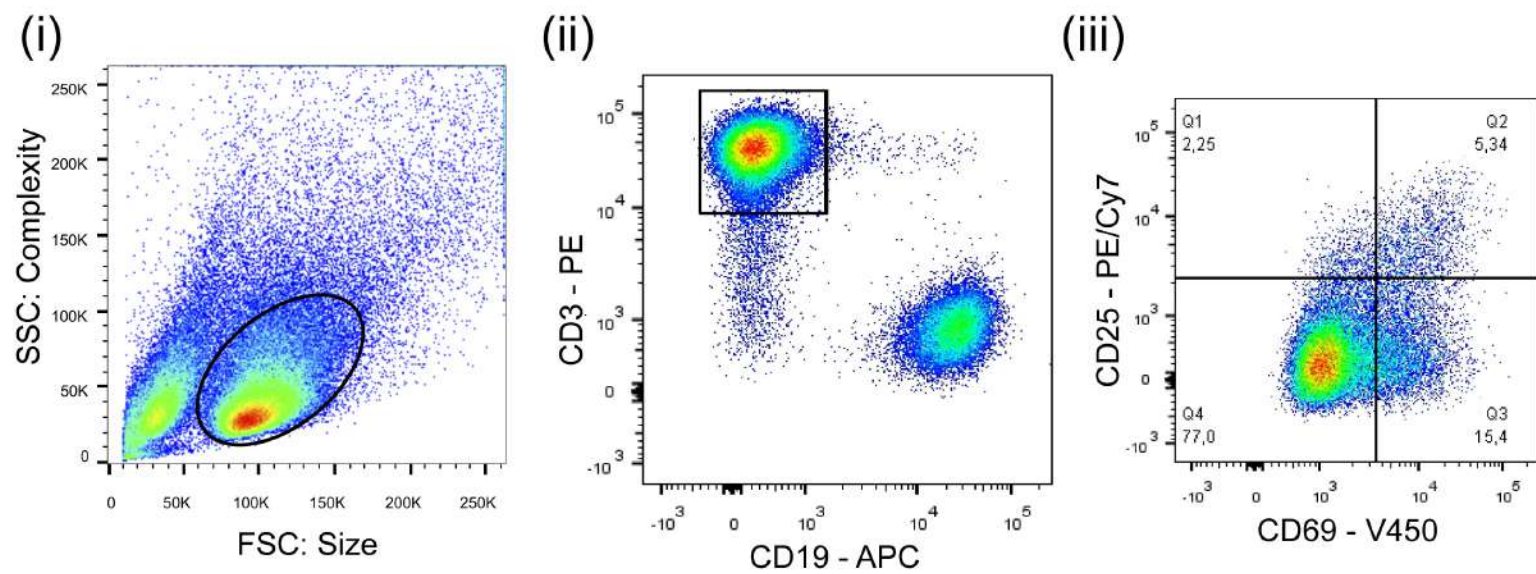
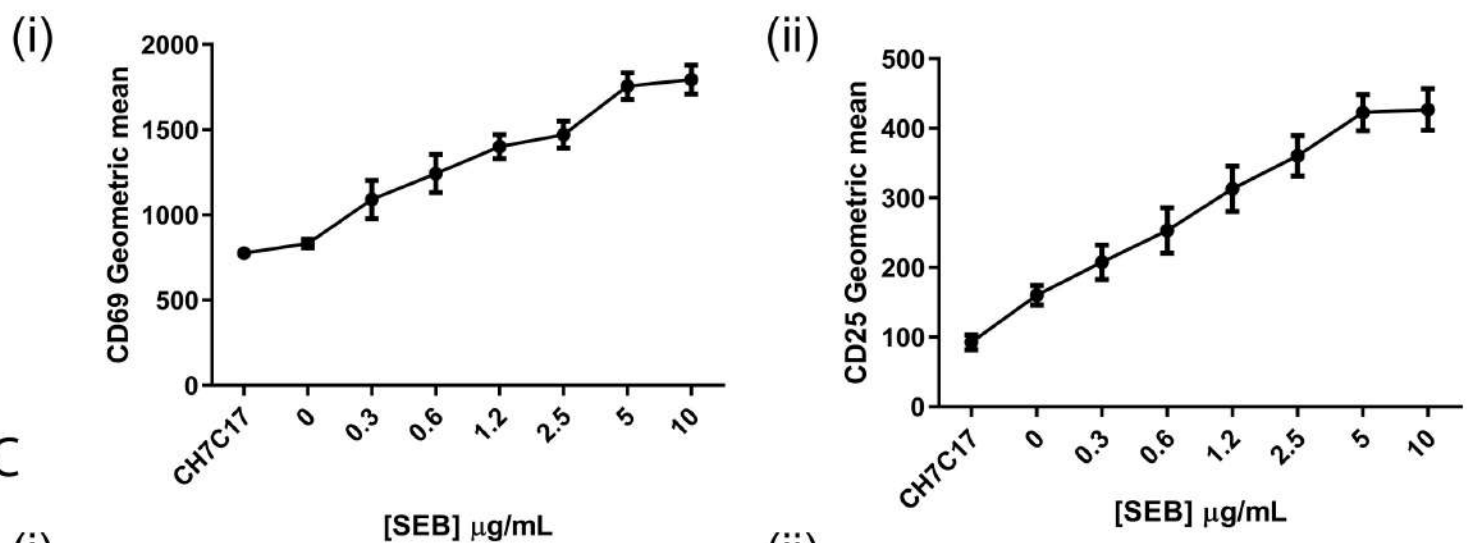


Figure 2

A



B



C

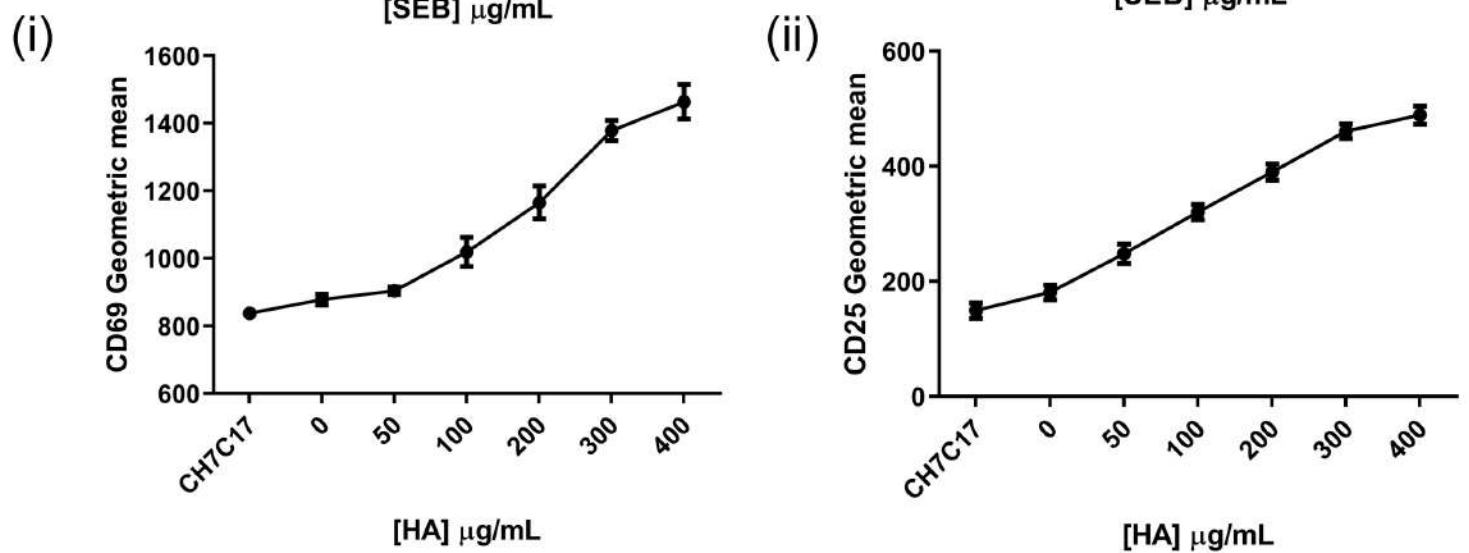


Figure 3

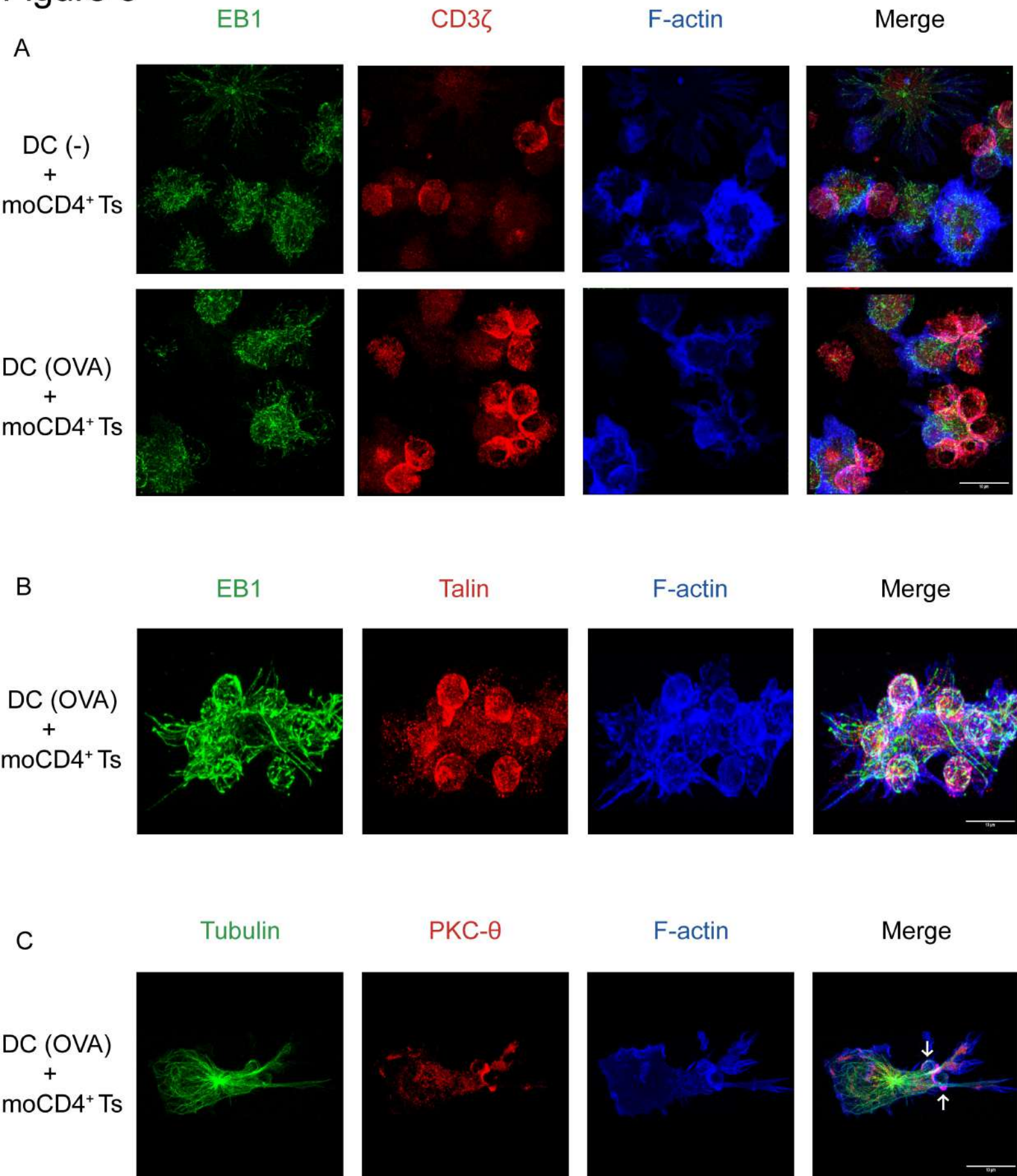


Figure 4

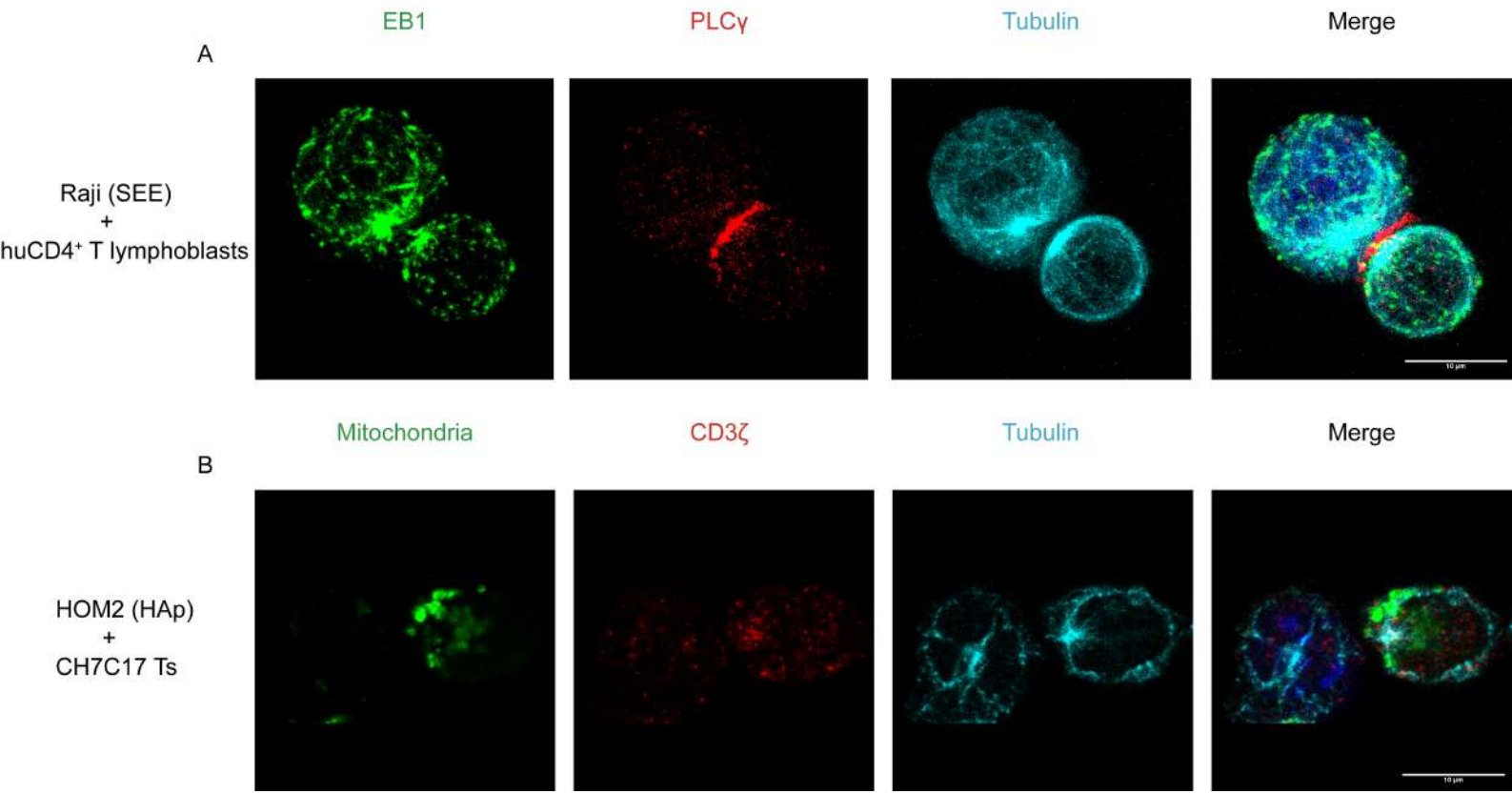


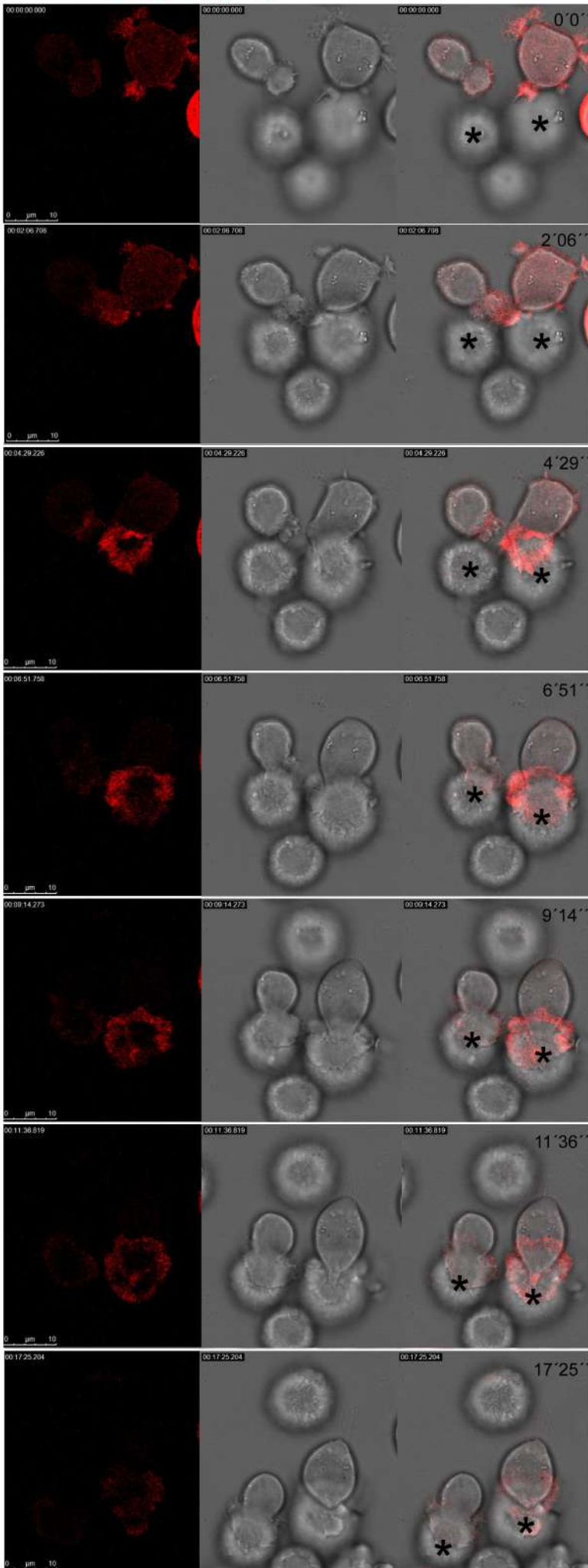
Figure 5

CH7C17 T cells + HOM2 (HAp)

A

mCherry- β -actin (T cell)

* APC: HAp-HOM2 cell



CH7C17 T cells + Raji (SEB)

B

mCherry- β -actin (T cell) + SEB-APC (Raji cell)

