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Preparation of Bead-supported Lipid Bilayers to Study the Particulate Output of T Cell Immune Synapses

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Abstract

Antigen-presenting cells (APCs) present three activating signals to T cells engaged in physical contact: 1) antigen, 2) costimulation/corepression, and 3) soluble cytokines. T cells release two kinds of effector particles in response to activation: trans-synaptic vesicles (tSVs) and supramolecular attack particles, which transfer intercellular messengers and mediate cytotoxicity, respectively. These entities are quickly internalized by APCs engaged in physical contact with T cells, making their characterization daunting. This paper presents the protocol to fabricate and use Bead-Supported Lipid Bilayers (BSLBs) as antigen-presenting cell (APC) mimetics to capture and analyze these trans-synaptic particles. Also described are the protocols for the absolute measurements of protein densities on cell surfaces, the reconstitution of BSLBs with such physiological levels, and the flow cytometry procedure for tracking synaptic particle release by T cells. This protocol can be adapted to study the effects of individual proteins, complex ligand mixtures, pathogen virulence determinants, and drugs on the effector output of T cells, including helper T cells, cytotoxic T lymphocytes, regulatory T cells, and chimeric antigen receptor-expressing T cells (CART).

Introduction

The immunological synapse (IS) is a pivotal molecular structure formed at the interface of cells engaged in physical contact that facilitates the regulated exchange of juxtracrine information. Different ISs have been described in the literature, and a growing body of evidence suggests these molecular hubs are a conserved feature of cellular networks. Various immune cells, including B cells, natural killer cells, dendritic cells, macrophages, and T cells, exchange information via the assembly of short-lived contacts[1]. Multiomic studies are advancing the understanding of novel subsets of leukocytes and stromal cells driving pathogenic cellular networks and expressing surface proteins with unknown functions. As synthetic APCs, BSLBs allow the direct investigation of the functional role of individual proteins in the integration of activating signals, namely antigens

and costimulation/corepression, by T cells and the resulting release of effector particles referred to as signal four.

This paper describes the protocols and critical technical points to consider while using BSLBs to mimic the surface composition of model APCs. The protocols for the quantitative measurement of immune receptors and other surface proteins on APCs are presented along with the protocol for the reconstitution of synthetic APCs containing these measured quantities. Then, the steps required for coculturing T cells and BSLB are presented along with the protocol for the quantitative measurement of trans-synaptic particle transfer using flow cytometry. Most remarkably, BSLBs facilitate studying a plasma membrane-derived population of tSVs termed synaptic ectosomes (SEs). T-cell antigen receptor-enriched (TCR^+) SEs are shed in response to TCR triggering[2] and efficiently captured by BSLBs[3], representing an excellent readout to assess the agonistic properties of antigens and the modelled membrane composition. $CD63^+$ exosomes and supramolecular attack particles (SMAPs) are also released by stimulated T cells and captured by BSLBs. They can be used as additional readouts of activation and the resulting exocytic and lytic granule secretion by T cells. The mobilization of exocytic vesicles to the interacting pole of the T cell also facilitates the directional release of cytokines, such as IL-2, IFN-γ, and IL-10 in response to activation[4,5,6,7,8]. Although T-cell released cytokines can also be detected on BSLBs, a more dedicated study is currently under development to validate the quantitative analysis of cytokine release at the immunological synapse.

To interrogate how specific membrane compositions influence T cells' synaptic output requires defining the physiological density of the target membrane component. Flow cytometry-based quantifications of cell surface proteins are an essential step in this protocol and require: 1) the use of antibodies with known numbers of fluorochromes per antibody (F/P), and 2) benchmark beads providing a standard reference for interpolating fluorochrome molecules from measured mean fluorescence intensities (MFIs).

These benchmark standards consist of five bead populations, each containing an increasing number of equivalent soluble fluorochromes (MESFs), which span the dynamic range of arbitrary fluorescence detection. These standard populations yield discrete fluorescence peaks, facilitating the conversion of arbitrary fluorescence units into MESFs by simple linear regression. The resulting MESFs are then used alongside antibody F/P values to calculate the average number of bound molecules per cell (or BSLB in later steps). The application of estimated cell surface areas to the average number of detected molecules then enables the calculation of physiological densities as molecules/μm^2. This quantification protocol can also be adapted to the measurement of protein densities on T cells and the biochemical reconstitution of membrane compositions mediating the formation of homotypic T cell synapses (i.e., T-T synapses[9]). If needed, the valency of antibody binding can be further estimated by using recombinant targets labeled with known numbers of fluorochromes per molecule. Then, the antibody-binding valency can be calculated for the same BSLB population by simultaneously comparing the number of bound fluorescent proteins and quantification antibodies (using two different quantification fluorochromes and MESF standards).

The reconstitution of APC membranes requires the assembly of supported lipid bilayers (SLBs) on silica beads[1]. Liposome stocks containing different phospholipid species can be harnessed to form a versatile lipid-bilayer matrix, enabling the anchoring of recombinant proteins with different binding

chemistry (the preparation of liposomes is detailed in [10]). Once the physiological density (or densities) of the relevant ligand "on cells" is defined, the same flow cytometry protocol is adapted to estimate the concentration of recombinant protein needed to coat BSLBs with the target physiological density. Two different anchoring systems can be used either in combination or separately.

First, SLB containing a final 12.5 mol% of Ni^{2+}-containing phospholipids is sufficient to provide approximately 10,000 His-tag binding sites per square micron[10] and works well to decorate BSLBs with most commercially available proteins whose physiological densities do not exceed this maximum loading capacity. The second loading system harnesses biotin-containing phospholipids (as mol%) to load biotinylated anti-CD3e Fab (or HLA/MHC monomers) via streptavidin bridges. The combination of these two BSLB decoration methods then enables the flexible tailoring of BSLBs as synthetic APCs. For highly complex APC surface compositions, the mol% of phospholipids and proteins can be increased to load as many proteins as the question at hand requires. Once the working concentrations of proteins and mol% of biotinylated phospholipids are defined, BSLBs can be assembled to interrogate the synaptic output of T cells with multiparametric flow cytometry.

Protocol

1. Measurement of cell surface protein densities with quantitative flow cytometry

1. Prepare 0.22 µm-filtered human flow cytometry buffer (hFCB) by adding EDTA (to a final 2 mM concentration) and human AB serum (to a final 10%) to sterile phosphate-buffered saline (PBS), pH 7.4 (see **Table 1**). Filter the solution using a 0.22 µm pore filter unit to remove serum impurities and store at 4 °C.
2. Recover the cells and sediment them by centrifugation at 300 × *g* for 5 min at room temperature (RT).
3. Wash the cells twice with PBS. In each washing step, resuspend the cells in PBS to the original volume (before centrifugation) and spin down at 300 × *g* for 5 min at RT.
4. Count trypan blue-stained cell suspensions in a hemocytometer[11]. Alternatively, count cells using electric current exclusion. For the latter, follow the CASY-TT manufacturer's instructions (see the **Table of Materials**).
   NOTE: The CASY-TT cell counter allows the determination of percent viable cells, cell size, and cell volume.
5. Calculate the volume of PBS containing 1:1,000 dilution of Fixable Viability Dye eFluor 780 or similar (see the **Table of Materials**) required to resuspend the cells to a staining concentration of 10^7 cells/mL.
6. Resuspend the cells using the viability dye-PBS solution and incubate on ice for 30 min.
7. Remove the viability dye by adding one volume of ice-cold hFCB. Spin down at 300 × *g* for 5 min at 4 °C.
   NOTE: From now onwards, keep the cold chain unbroken.
8. Wash the cells with hFCB containing 1:50 dilution of Fc Receptor Blocking Solution (see the **Table of Materials**). Bring the volume to a final concentration of 10^7 cells/mL and incubate for an additional 15 min to achieve efficient FcgR blocking.
9. Distribute 100 µL of the cell suspension (i.e., 10^6 cells) per well of a U-bottom or V-bottom 96-well plate. Keep

the cells on ice and protected from light (cover with aluminum Foil).

10. Prepare an antibody master mix in hFCB by defining the optimal antibody concentrations for each fluorochrome-conjugated antibody, and most importantly, for those antibodies used to determine surface protein densities. For example, for quantification of ICAM-1 expression on tonsillar cell populations, as shown in **Figure 1**, prepare a mix containing 1:200 dilutions of anti-CD4, anti-CD19, and anti-CXCR5 together with saturating concentrations of an anti-ICAM-1 antibody with known AF647 fluorochromes per antibody (i.e., 10 µg/mL, which was defined by independent antibody titration experiments).

11. As additional controls, prepare an antibody master mix containing the relevant **antibody isotype controls (at the same effective concentrations as their counterparts conjugated with the same fluorochromes** for background subtraction), i.e., use 10 µg/mL of a relevant AF647 isotype control.
    NOTE: In the example above, such an isotype control is used to subtract background fluorescence from the true ICAM-1 signal on cells.

12. When quantifying protein densities on cell subsets present at low frequencies within tissues, prepare fluorescence minus one (FMO) controls containing all staining antibodies except for the markers of interest (see further details in [12]).

13. Spin down the 96-well plate containing the cells at 300 × *g* for 5 min at 4 °C, discard the supernatant, and resuspend the cells in 50 µL of either the quantification antibody master mix or the isotype antibody master mix.

14. Incubate the cells for at least 30 min at 4 °C and 400 rpm using a plate shaker. Protect the plates from light (cover with aluminum foil).

15. Wash the cells three times using hFCB and centrifuge at 300 × *g* for 5 min at 4 °C.

16. Resuspend the cell pellet using 200 µL of PBS (i.e., to a final concentration of 5 × 10^6 cells/mL).

17. For acquisition:
    1. If using a standard BD FACS Loader, transfer the samples to 5 mL polystyrene round-bottom tubes (see the **Table of Materials**).
    2. If using High-throughput Samplers (HTS, also referred to as plate readers), proceed immediately to step 1.19.

18. Before proceeding with the data acquisition for the MESF standards, check the fluorescence intensity linearity maximum and minimum limits for the quantification channels.

19. **Before compensation**, acquire data for the MESF standards, ensuring both the dimmest and brightest populations fall in the linear range of measurement.

20. Acquire the compensation samples. Keep the photomultiplier tube (PMT) voltage values for the quantification channels unchanged to preserve the dynamic range of detection. Perform slight adjustments in the PMT voltage of other channels before calculating the compensation matrixes.

21. Calculate and apply compensation.

22. Acquire and save a minimum of 2 × 10^4 total MESF beads for each of the quantification channels.

23. Select the population of single cells based on their side and forward light scattering areas (SSC-A and FSC-A, respectively, as shown in **Figure 1A** (i)), followed by the selection of events inside the time continuum (**Figure 1A** (ii)) and low for time of flight (W) in both FSC and SSC as compared to their heights (i.e., FSC-W/FSC-H, followed by SSC-W/SSC-H gating as shown in **Figure 1A** (iii) and (iv), respectively). Define a final single-event gate containing events with proportional FSC-A versus FSC-H distribution (**Figure 1A** (v)).

24. Acquire control samples (Isotype-labeled and FMO controls).

25. Acquire samples and record until a minimum of 10,000 target cells have been acquired.
    NOTE: the robust determination of average molecular densities requires the analysis of several donors across independent experiments. This is crucial when analyzing either cell subsets found in reduced frequencies or derived from scarce biological material (e.g., from human tissue biopsies).

26. Wash the cytometer running for 5 min with a FACS cleaning solution followed by 5 min of ultrapure water before shutting down the instrument. If using the HTS, follow the options under the tab **HTS and Clean Plate program**.
    NOTE: To reduce sample-to-sample carryover, activate **sit (sampler) flush** or **high-throughput sampler wash** options, which will automatically wash the cytometer between tubes or wells. Before starting the acquisition, run ultrapure water for 10 min at a high flow rate to remove any unwashed biological contaminants from the cytometer sample line.

27. Export the Flow Cytometry Standard (FCS) files.

2. MESF overcorrected mean (or median) fluorescence intensity (MFI) regression analyses

1. Open the flow cytometry analysis software and load the experiment FCS files. Select the population of beads based on their side and forward light scattering areas (SSC-A versus FSC-A), as shown in **Figure 1A** (i).

2. Control data quality by checking the distribution of single events over time, as indicated in step 1.24.
   NOTE: Bubbles create gaps in the distribution of events over time; this typically appears at the beginning of acquisition using the HTS. Avoid selecting events flanking gaps in the acquisition time as these add measurement errors due to optical aberrations.

3. Focus on the single events.
   1. Cells
      1. Identify single cells first based on their SSC-A and FSC-A distribution (**Figure 1A** (i)), followed by events low for W in the sequential gates FSC-W/FSC-H (singlets-1, **Figure 1A** panel (iii)) and SSC-W/SSC-H (singlets-2; **Figure 1A** panel (iv)). Define an additional singlets-3 gate by selecting events with proportional FSC-A and FSC-H (**Figure 1A**, panel (v)). Finally, identify live cells as those negative for the fixable viability dye.
   2. MESF beads
      1. Follow the same singlets-1 to singlets-3 discrimination as in step 2.3.1.1 (**Figure 1B**, panels (i) to (v)). Identify each of the MESF populations (blank, 1, 2, 3, and 4) based on their fluorescence intensity levels (see **Figure 1B**, panel (vi)).

4. Extract the MFI of MESF fractions blank and 1 to 4.
5. Generate corrected MFI (cMFI) values for MESF fractions 1 to 4 by subtracting the MFIs of the blank bead population from each fraction.
6. Calculate the line of best fit for the relationship between cMFIs and the MESF values provided by the vendor (independent variable, being fraction blank equal to zero).
7. Extract the slope (**b** in the equation below) of the linear regression of MESF over cMFI (**Figure 1B** panel (viii) shows a regression in which y = a + bx; with a = 0).
8. Extract the median (for bimodal fluorescence distributions) or mean (for normal or log-normal fluorescence distributions) fluorescence intensities from the populations of interest (TFH and B cells in **Figure 1A panel (vii)**).
9. As in steps 2.5-2.7, extract the MFI values from isotype-labeled control cells.
10. If the F/P of isotype controls is the same as the quantification antibodies, correct the MFIs of stained cells by subtracting the MFIs from Isotype-labeled cells.
11. If the F/P of the isotypes differs from those of quantification antibodies, extract the MESF from the isotypes by dividing the MFIs of isotype-labeled cells with the slope calculated in step 2.7. Subtract Isotype MESF from quantification MESF before estimating bound quantification antibodies.
12. Divide the MESF by the F/P of the quantification antibodies to estimate the number of bound molecules per cell ($Molec._{cell}$ as shown in the flow diagram of **Figure 1C**).
13. Divide the number of bound molecules with the estimated cell surface area (CSA (μm^2)) to extract the density of proteins as molec./μm^2 (**Figure 1C**). Refer to the representative results section for more details.

3. Functional phospholipid species to use in the calibration of proteins coating BSLBs

1. Use 0.4 mM **DOPC** (1,2-dioleoyl-sn-glycero-3-phosphocholine) as the major component of the lipid matrix forming the supported lipid bilayer. Dilute all other lipids species in this DOPC solution.
2. Use between 0.2 and 1 mol% of 0.4 mM **DOPE** (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) conjugated to dyes, including ATTO 390, 488, or 565 (see the **Table of Materials**), to generate BSLBs with intrinsic fluorescence.
   NOTE: The intrinsic fluorescence of BSLBs facilitates the identification of single BSLBs and single cells in synaptic transfer experiments.
3. Use 12.5 mol% of 0.4 mM **DGS-NTA(Ni)** (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]) to anchor His-tagged proteins. Keep the mol% of DGS-NTA(Ni) constant and perform 2-fold titrations of the His-tagged proteins starting with 100 nM as the highest concentration. Leave one condition with no protein as a negative control for absolute quantifications.
   NOTE: Accessory signals and adhesion molecules, such as ICAM-1, are designed with a 12-His tag to increase the affinity of the protein for DGS-NTA(Ni).
4. Use **Biotinyl Cap PE** (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl)) to anchor biotinylated proteins via biotin-streptavidin-biotin bridges.

Use a 5-fold serial titration of 0.4 mM Biotinyl Cap PE covering between 10 mol% and 0 mol% (as negative control). Keep the concentration of streptavidin and biotinylated proteins constant (200 nM) in both calibration experiments and the reconstitution of synthetic APCs for synaptic transfer experiments.

NOTE: The regression analyses of empirical densities of biotinylated proteins over the final mol% of Biotinyl Cap PE in the lipid matrix (also containing DGS-NTA(Ni) to anchor His-tagged proteins) will define the mol% to be used to reconstitute target densities of antigen.

4. Preparation of supplemented HEPES buffered saline containing 1% serum albumin

NOTE: Supplemented HEPES-buffered saline containing 1% human serum albumin (HBS/HSA) or 1% bovine serum albumin (HBS/BSA) is required in the washing and protein loading steps of BSLBs (**Table 1**). Prepare a 10x HBS stock solution and the working buffer as fresh as possible; keep refrigerated and use within one month. While BSA is a cheaper alternative to HSA, it provides efficient blockage of Ni-chelating lipids[13] and is recommended for high-throughput experiments.

1. Prepare a 10x stock buffer solution of supplemented HBS containing 200 mM HEPES, 7 mM Na_2HPO_4, 1400 mM NaCl, 50 mM KCl, 60 mM glucose, 10 mM $CaCl_2$, and 20 mM $MgCl_2$.

   NOTE: Dissolving $MgCl_2$ is highly exothermic and a burn hazard. Add this salt slowly and on a large volume of solvent. Avoid preparing concentrated solutions (i.e., 1 M) of these salts as they tend to precipitate over time, making their precise addition to the working buffer difficult.

2. Filter the 10x solution using a 0.22 µm filter unit and keep it sterile at 4 °C.

3. For 500 mL of HBS/has, take 50 mL of the supplemented 10x HBS solution, adjust the pH to 7.4 if needed, and make up the volume to 483.4 mL with ultrapure water.

4. Add 16.6 mL of 30% HSA solution to the 483.4 mL of HBS, pH 7.4.

5. For 500 mL of HBS/BSA, dissolve 5 g of BSA in HBS and incubate at 37 °C for 30 min. Then, mix gently at RT by inverting the bottle periodically until there are no visible protein crystals or clumps.

6. Filter the resulting solution from step 4.5 using a 0.22 µm filter unit (see the **Table of Materials**) and store it at 4 °C.

5. Protein density calibrations on BSLBs

1. Before taking the 5.00 ±0.05 µm diameter non-functionalized silica beads, mix the stock solution well and resuspend any big clumps of beads sedimented on the bottom of the flasks.

   NOTE: Silica beads tend to sediment quickly, which might lead to counting errors. Mix vigorously by pipetting up and down half of the maximum volume of a P1000 micropipette.

2. Dilute 1 µL of bead solution in 1,000 µL of PBS, count the beads using a hemocytometer chamber, and calculate their concentration per mL.

   NOTE: Trypan blue staining is not needed for visualizing silica beads.

3. Calculate the volume of silica beads needed for 5×10^5 final BSLBs per point of the titration.

4. Transfer the required volume of silica beads to a sterile 1.5 mL microcentrifuge tube.

5. Wash the silica beads three times with 1 mL of sterile PBS, centrifuge the beads for 15 s on a benchtop microcentrifuge at RT (at fixed rpm).
   NOTE: When removing the washing solution, avoid disturbing the bead pellet. A small buffer column will not affect the spreading of the liposomes composing the liposome master mix as these are also in PBS.
6. Prepare three volumes of the liposome master mix to assemble the BSLB on the washed silica beads (e.g., if the initial total volume of silica beads is 20 µL, prepare a minimum of 60 µL of the liposome master mix).
7. **For Biotinyl Cap PE mol% titrations**
   1. Prepare the lipid master mixes containing 5-fold dilutions of Biotinyl Cap PE.
      1. Dilute the 0.4 mM Biotinyl Cap PE mol% in a 100% DOPC matrix.
      2. Mix each Biotinyl Cap PE mol% titration point at a 1:1 (vol:vol) ratio with a solution of 0.4 mM 25% DGS-NTA(Ni) such that a final 12.5 mol% of Ni-containing lipids is present in all titrations.
         NOTE: The 12.5 mol% (vol:vol%) of Ni-containing lipids represent the mixed lipid composition of BSLBs on which His-tagged proteins can also be tested in parallel calibrations. For example, since all liposome stocks are prepared at the same molar concentration, to reach the target mol% mixture in 200 µL of final liposome mix, simply mix 100 µL of 25 mol% of Ni-containing DGS-NTA with 100 µL of 100 mol% DOPC.
   2. Transfer 5 × 10^5 washed silica beads to 1.5 mL microcentrifuge tubes, such that one Biotinyl Cap PE mol% titration point is assembled per tube.
   3. Add the Biotinyl Cap PE mol% titration master mixes to the washed silica beads and gently mix by pipetting up and down half of the total volume. Avoid forming bubbles, which in excess destroy the lipid bilayer.
   4. Add Argon (or Nitrogen) gas on the tube containing the now-forming BSLBs to displace air and protect the lipids from oxidation during mixing.
   5. Add Argon to the 0.4 mM lipid stocks before storage and manipulate using a sterile technique.
      NOTE: Connect a small tubing to the Argon/Nitrogen gas cylinder. Before adding gas to the tubes, adjust the gas cylinder regulator so that the pressure is set no higher than 2 psi. Connect a sterile pipette tip to the outlet tubing to direct the gas stream inside the liposome stock for 5 s and quickly close the lid. In the case of lipid stocks, seal the tube's lid with paraffin film before storing it at 4 °C.
   6. Move the BSLBs to a vertical, variable-angle laboratory mixer (see the **Table of Materials**) and mix for 30 min at RT using an orbital mixing of 10 rpm.
      NOTE: This step will prevent the sedimentation of beads during the formation of the supported lipid bilayer.
   7. Spin down the beads by centrifuging for 15 s at RT on a benchtop minicentrifuge, and then wash three times with 1 mL of HBS/HSA (BSA) to remove excess liposomes.
   8. Block the formed BSLBs by adding 1 mL of 5% casein or 5% BSA containing 100 µM of $NiSO_4$ to saturate NTA sites and 200 nM streptavidin to coat all biotin-anchoring sites on the BSLBs uniformly.

Mix gently by pipetting up and down half of the total volume and incubate in the vertical mixer for **no longer than 20 min at RT** and 10 rpm.

9. Spin down the BSLBs by centrifuging for 15 s at RT on a benchtop minicentrifuge, and then wash three times with 1 mL of HBS/HSA (BSA) buffer.
   NOTE: Keep washed beads vertically with a small volume of wash buffer covering the BSLBs. Avoid the dehydration of the BSLBs as air will destroy the lipid bilayer.

8. **For the titration of His-tagged proteins on 12.5 mol% of DGS-(Ni) NTA-containing BSLBs**

   1. Prepare three volumes of liposome master mix containing a final 12.5 mol% of DGS-NTA(Ni).
   2. Use the liposome master mix to resuspend the washed silica beads and gently mix by pipetting up and down half of the total volume. Avoid forming bubbles, which in excess damage the lipid bilayer.
   3. Add Argon (or Nitrogen) gas on the tube containing the now-forming BSLBs to displace air and protect the lipids from oxidation during mixing.
   4. Add Argon to the 0.4 mM lipid stocks before storage and manipulate using a sterile technique.
   5. Move the BSLBs to the vertical mixer and mix for 30 min at RT using orbital mixing at 10 rpm.
   6. Spin down the beads by centrifuging for 15 s at RT on a benchtop minicentrifuge, and then wash three times with 1 mL of HBS/HSA (BSA) to remove excess liposomes.
   7. Block the formed BSLBs by adding 1 mL of 5% casein (or 5% BSA) containing 100 µM of $NiSO_4$ to saturate NTA sites on the BSLBs. Mix gently and incubate in the vertical mixer for **no longer than 20 min at RT** and 10 rpm.
   8. Wash three times using HBS/HSA (BSA) to remove the excess blocking solution.
   9. In a new U-bottom 96-well plate prepare 2-fold serial dilutions of the proteins.
      1. Prepare a starting concentration of 100 nM for the protein of interest in a total volume of 200 µL of HBS/HSA (BSA) buffer, distribute 100 µL of this solution in the first column, and the remaining 100 µL on top of column #2 containing 100 µL of HBS/HSA (BSA) buffer.
      2. Continue by serially transferring 100 µL from column #2 to column #3 and repeat as necessary to cover all titration points. Leave the last column of the series with no protein, as this will be used as the blank reference for quantification.
   10. Resuspend the prepared BSLBs in a volume such that 5×10^5 BSLB are contained in 100 µL of HBS/HSA (BSA) buffer.
   11. Transfer 100 µL of the BSLB suspension to wells of a second U-bottom 96-well plate, such that each well receives 5×10^5 BSLBs.
   12. Spin down the second plate containing BSLBs for 2 min at 300 × *g* and RT and discard the supernatant.
   13. Transfer the 100 µL volumes from the protein titration plate to the plate containing the sedimented BSLBs. Mix gently, avoid excess bubble generation while pipetting, and incubate for 30 min at RT and 1,000 rpm using a plate shaker. Protect from light with aluminum foil.

14. Wash the plate three times with HBS/HSA (BSA) buffer using sedimentation steps of 300 × *g* for 2 min at RT.

15. If the recombinant protein used in the calibration is directly conjugated to fluorochromes and has known F/P values, proceed to step 5.8.20.

16. If the recombinant protein used in the calibration is unlabeled or conjugated to fluorochromes or no MESF bead standard is available, use Alexa Fluor 488 or 647-conjugated antibodies with known F/P values to stain the protein-coated BSLB.

17. Stain with saturating concentrations of quantification antibodies.
    NOTE: Depending on the target expression level, these range between 5 and 10 µg/mL.

18. Stain for 30 min at RT and 1,000 rpm using a plate shaker. Protect from light using aluminum foil.

19. Wash twice with HBS/HSA (BSA) buffer and once with PBS using sedimentation steps of 300 × *g* for 2 min at RT.
    NOTE: Use PBS, pH 7.4 to resuspend the washed BSLBs before acquisition. Do not use buffers containing protein, as this leads to the formation of bubbles during the automatic mixing of samples with high-throughput samplers.

20. Acquire the MESF standards, making sure the brightest peaks remain in the linear detection range for the quantification detector (channel), as shown in **Figure 1B** (vii).

21. Acquire the samples manually or using HTS. If using the latter, resuspend BSLBs in 100 µL of PBS and acquire 80 µL using a flow rate between 2.5 and 3.0 µL/s, a mixing volume of 100 µL (or 50% of the total volume if the resuspension volume is less), a mixing speed of 150 µL/s, and five mixes to ensure BSLBs are monodispersed.

22. Export the FCS files.

23. Focus on single events for the analyses (**Figure 2A**), as doublets or triplets will introduce error in the determination. Use nested identification of single events as indicated in protocol step 1.2.3.

24. Measure the MFI of each MESF fraction (1-4) and subtract the MFI of blank beads to extract corrected MFIs (cMFI).

25. Perform a linear regression analysis to extract the slope (**b**) of MESF over the cMFI calculated for MESF standards, which will be used in step 5.33.

26. Extract the MFI of each titration point and subtract the MFI of beads without protein to obtain cMFIs.

27. Divide the cMFIs with the slope calculated in step 5.31 to extract the MESF bound to BSLBs for each titration point.

28. Divide MESF bound to BSLBs by the F/P value of the quantification antibody to extract the average number of molecules bound per BSLB.

29. Using the diameter of the BSLBs (5.00 ± 0.05 µm), extract the bead surface area (SA = $4pr^2$) to calculate the final densities of protein (molec./μm^2) per titration point (protein concentration).

30. Perform a new regression analysis of **protein concentration** over **protein density** to calculate the slope (b) of the line of best fit.
    NOTE: The concentration of 12.5 mol% of DGS-NTA(Ni) confers a maximum anchoring capacity

of approximately 10,000 molec./μm^2 [10] without inducing the nonspecific activation of T cells or affecting the lateral mobility of the SLBs.

6. Performing synaptic transfer experiments between T cells and BSLBs

1. Before running the synaptic transfer experiment
    1. Acquire non-fluorescent BSLBs, BSLBs with fluorescent lipids, unstained cells (or compensation beads; see the **Table of Materials**), and single-color-stained cells (or compensation beads) to identify the instrument's fluorescence spectrum interactions. Focus on those detectors with high spillover spreading to redesign the polychromatic panel, increase sensitivity, and reduce the measurement error on critical detectors (see [14]).
    2. Titrate the detection antibodies to find the optimal concentration, enabling positive events detection without compromising the detection of negatives.
    NOTE: Repeat this step whenever there is an antibody lot change as the F/P values and brightness vary from batch to batch.
    3. Optional: Optimize the PMT voltages by acquiring the sample at different voltage ranges (i.e., a voltage walks) to find the PMTs leading to optimal signal over noise (i.e., separation of negatives and positives while ensuring the signal of the brightest population remains in the linear range).
2. Measurement of T-cell output transfer to BSLBs
    1. Prepare supplemented RPMI 1640 (herein R10 medium) containing 10% of heat-inactivated fetal bovine serum (FBS), 100 μM non-essential amino acids, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 μg/mL of streptomycin. Use R10 medium to culture and expand T cells.
    2. On **day 1**, isolate T cells from peripheral blood or leukoreduction system (LRS) chambers. Use immunodensity cell isolation and separation kits (see the **Table of Materials**) for enrichment of human $CD4^+$ and $CD8^+$ T cells.
    3. Seed the cells at a final concentration ranging between 1.5 and 2.0 × 10^6 cells/mL, using 6-well plates with no more than 5 mL total per well.
    4. Activate T cells using a 1:1 ratio of human T cell activation (anti-CD3/anti-CD28) magnetic beads (see the **Table of Materials**) and add 100 IU of recombinant human IL-2 to support cell proliferation and survival.
    5. On **day 3**, remove the activating magnetic beads using magnetic columns (see the **Table of Materials**). Ensure that magnetic beads remain attached to the sides of the tube before recovering the cells for additional washing steps.
    6. Wash the magnetic beads once more with 5 mL of fresh R10 medium, mix well, and put them back in the magnet . Recover this volume and mix with the cells recovered in step 6.2.5.
    7. Resuspend the cells to 1.5-2 × 10^6 cells/mL in fresh R10 containing 100 U/mL of IL-2. Replenish medium after 48 h, making sure that the last addition of IL-2 is 48 h before the day of experimentation (days 7 to 14 of culture).
    8. **On the day of the synaptic transfer experiment**, prepare the **Synaptic Transfer Assay medium**

(see **Table 1**) by supplementing Phenol Red-free RPMI 1640 medium with 10% FBS, 100 µM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 µg/mL of streptomycin. Do not include recombinant human IL-2.

9. Count cells using either trypan blue staining or electric current exclusion and resuspend them to a final concentration of 2.5 × 10^6 cells/mL in medium. If excessive cell death is observed (>10%), remove dead/dying cells using a mixture of polysaccharide and sodium diatrizoate (see the **Table of Materials**) as follows:
    1. Layer 15 mL of cell culture on top of 13 mL of the polysaccharide-sodium diatrizoate solution.
    2. Centrifuge for 1,250 × *g* for 20 min at RT with minimum acceleration and deacceleration. Collect the cell layer (cloud) in the interface between the medium and the polysaccharide-sodium diatrizoate solution.
    3. Wash the cells at least twice with prewarmed Synaptic Transfer Assay medium (prepared in step 6.2.7).
    4. Count and resuspend the cells to a final concentration of 2.5 × 10^6/mL using Synaptic Transfer Assay medium. Coculture 100 µL of this cell suspension with BSLBs (see step 6.2.15).
10. Calculate the number of BSLBs needed for the experiment; consider all the different protein master mixes and antigen titrations to be tested (either biotinylated HLA/MHC-peptide monomers or monobiotinylated anti-CD3ε Fab).
11. Assemble the BSLBs by following the same steps from protocol section 5 but this time, combine all the titrations of proteins and lipids required to reconstitute a complex APC membrane (**Figure 3A**). Keep the same vol: vol relationships between the initial silica beads volume and the volume of protein mix used during calibrations, as well as times and temperatures used in the loading of BSLBs. For example, if 0.5 µL of silica beads/well and 100 µL/well of protein mix were used for the initial calibration, maintain 5:1,000 vol:vol ratios to prepare the BSLBs to be cocultured with T cells.
12. Once BSLBs have been loaded with the protein mix of interest, wash the BSLBs twice with HBS/HSA (BSA) to remove excess unbound proteins. Use sedimentation speeds of 300 × *g* for 2 min at RT in each washing step and discard the supernatants.
13. Resuspend the 5 × 10^5 BSLBs per well in 200 µL.
14. Transfer 100 µL per well to a new U-bottom 96-well plate to make a duplicate, such that the final amount of BSLB per well is 2.5 × 10^5.
15. Spin down the BSLBs at 300 × *g* for 2 min and RT and discard the supernatant.
16. Resuspend the BSLBs using 100 µL of T cell suspension; mix gently to prevent the formation of bubbles.
17. Incubate the cocultures for 90 min at 37 °C.

    NOTE: Alternatively, cells and beads can be resuspended in HBS/HSA (BSA) buffer instead of Phenol-Red free RPMI for the coculture. In this case, the incubation must be performed in a non-CO_2

incubator as this gas will rapidly acidify the buffer in the absence of bicarbonate.

18. Cool down the BSLB-T cell cocultures by first incubating the cells at RT for a minimum of 15 min. Protect them from light.

19. Centrifuge the cells for 5 min at 500 × *g* and RT; discard the supernatant.

20. Resuspend the cells in RT 2% BSA-PBS (Ca^{2+} and Mg^{2+}-free) for blocking. Place the cells on ice for 45 min. Protect from light.

21. While incubating the cells, prepare the antibody master mix using ice-cold 0.22 µm-filtered 2% BSA in PBS as a staining buffer, which will provide extra blocking.

    NOTE: Some batches of antibodies conjugated to Brilliant Violet dyes tend to bind to BSLBs nonspecifically. Blocking with 5% BSA-PBS helps to reduce this noise. **From now on, make sure to keep the cold chain unbroken**.

22. Spin down the cocultures at 500 × *g* for 5 min and 4 °C. Before discarding the supernatants, briefly make sure the pellet is present by inspecting the bottom of the 96-well plate using a backlight.

23. Using a multichannel pipette, resuspend the cells in the staining master mix containing optimized antibody concentrations.

    NOTE: Use pipette tips with no filter to prevent the generation of bubbles and errors in the distribution of staining volumes.

24. Include isotype-labeled cells and BSLBs, fluorescent and non-fluorescent BSLBs, and cells and BSLBs stained alone. Respect the total number of events per well for all controls (i.e., only BSLBs containing 5×10^5 BSLB/well, and only cell controls containing 5×10^5 cells/well to avoid a relative increase of antibodies per stained event).

25. Mix gently by pipetting up and down half of the volume and incubate for 30 min on ice. Protect from light.

26. Wash the cells and BSLBs twice using ice-cold 2% BSA-PBS, pH 7.4, and sedimentation steps of 500 × *g* for 5 min at 4 °C. Resuspend the washed cocultures in 100 µL of PBS and acquire immediately.

27. **If fixation is needed**, fix using 0.5% w/v of PFA in PBS for 10 min, wash once, and keep in PBS until acquisition. Protect from light.

28. **Before compensation**, acquire MESF standards, ensuring both the dimmest and brightest populations fall in the linear range of measurement.

29. **Acquire compensation samples, calculate compensation, and apply the compensation matrix (link) to the experiment.**

30. Acquire and save a minimum of 2×10^4 total MESF standards for each of the quantification channels.

31. **For acquisition using high-throughput samplers**, set instrument acquisition to standard, set sample acquisition to 80 µL (or 80% of total volume), sample flow rate between 2.0 and 3.0 µL/s, sample mixing volume of 50 µL (or 50% of the total volume to avoid bubble formation during mixing), sample mixing of 150 µL/s, and mixing per well between 3 and 5.

32. Acquire a minimum of 1 × 10^4 single BSLBs per sample (refer to **Figure 3B** panels (i)-(vi) for the reference gating strategy).
33. Wash the cytometer running for 5 min a cleaning solution followed by 5 min of ultrapure water before shutting down the instrument. If using the HTS, follow the options under the tab **HTS and the Clean** option.
34. Export FCS files.

7. Measuring the synaptic transfer of particles to BSLB

1. Open the experiment FCS files. Select the population of cells and BSLBs based on their side and forward light scattering areas (SSC-A versus FSC-A), as shown in **Figure 3B** (i).
2. Select the events within the continuous acquisition window (**Figure 3B** (ii)).
3. Focus on the single events of both cells and BSLB; identify single cells first based on low W in the sequential gates FSC-W/FSC-H (singlets-1, **Figure 3B** panel (iii)) and SSC-W/SSC-H (singlets-2; **Figure 3B** panel (iv)). Define an additional singlets-3 gate by selecting events with proportional FSC-A and FSC-H (**Figure 3B**, panel (v)).
4. Extract the MFI of MESF fractions blank and 1 to 4 and from single cells and MESF for each experiment sample.
5. Generate corrected MFI (cMFI) values for MESF fractions 1 to 4 by subtracting the MFI of the blank bead population from each fraction.
6. Generate cMFIs for single BSLBs and cells (refer to **Figure 3C** panel (i)).
7. Use the signal from BSLB stained with isotype control antibodies to correct the MFI of BSLB stained with antibodies against the relevant T cell markers. Use cMFI to calculate the normalized synaptic transfer percent (NST%) by using the equation shown in **Figure 3C** panel (ii).
8. If interested instead in the particles specifically transferred in response to TCR triggering, subtract the signal from null BSLBs from the MFI of agonistic BSLBs. Use this cMFI to calculate the **TCR-driven NST%** by using the equation shown in **Figure 3C** panel (ii).
9. **If interested in determining the total number of molecules transferred** as particle cargo across the T cell-BSLB interface, acquire MESF benchmark beads using the same instrument settings and acquisition session for T cell-BSLB cocultures.
10. Analyze MESF bead populations and extract their cMFIs as indicated in protocol steps 5.8.15 to 5.8.17. Calculate the slope of the line of best fit for the regression analysis of MESF over cMFI.
11. Use the calculated slope to extract the number of MESF deposited on BSLBs. Use cMFIs calculated using either isotype controls or null BSLB as blanks to extract the number of MESF transferred specifically to stimulating BSLBs.
12. Calculate the number of molecules of markers transferred to BSLBs by dividing the calculated (average) MESFs per BSLB by the F/P value of the quantification antibody.

Representative Results

FCM for absolute protein quantification on the cell surface

The reconstitution of BSLBs presenting physiological densities of ligands requires the estimation of total protein densities on the modeled cell subset. To reconstitute BSLBs, include any relevant ligand expected to play a role in the signaling axis of interest alongside proteins supporting the adhesion and functional interaction between BSLB and cells, such as ICAM-1 and costimulatory molecules, e.g., CD40, CD58, and B7 receptors (CD80 and CD86). Additional proteins can be added depending on the question at hand, including costimulatory molecules such as ICOSL[3], PD-L1, and PD-L2[15]. For any other molecules, reconstitute BSLBs using molecular densities determined by directly analyzing cells with quantitative flow cytometry. For directly conjugated antibodies, BioLegend provides F/P values for each antibody lot number. Antibodies can also be labeled in-house and the F/P ratios determined by spectrophotometry, providing an alternative when there are no commercial antibodies conjugated to the desired fluorochrome. Since we use the same antibodies to calibrate the number of recombinant proteins on the surface of cells and BSLBs, there is no need to correct the antibody-binding valency as this remains constant. If the antibody-binding valency is required, use both recombinant proteins and antibodies with known F/P to decorate BSLBs and compare molecules of loaded recombinant proteins with the number of bound antibodies following staining under saturating conditions.

The bound antibody molecules per cell can be estimated using a dedicated monochromatic flow cytometric measurement or a polychromatic panel of antibodies intended to estimate absolute protein densities on a relatively infrequent subset of cells within a tissue of interest, such as palatine tonsils. **Figure 1** shows representative measurements of densities of ICAM-1 in CXCR5+ B cells and follicular T cells (TFH) as an example. The same staining protocol and flow cytometry analysis principles shown in **Figure 1A** can be used to measure protein densities on epithelial cells, stromal cells, monocytes, monocyte-derived dendritic cells (moDCs), or on B and T lymphocytes in other human and mouse tissues. For tissues different from blood and tonsils, caution must be taken when isolating cells using protease cocktails, as the prolonged exposure of cells to digesting enzymes reduces cell surface expression levels.

Focus the acquisition and analyses on single, live cells within the continuous acquisition window (**Figure 1A** ii), as events outside the time continuum aberrantly scatter light, compromising quantification. To increase the accuracy of determinations, reduce the nonspecific staining of APCs by efficient blocking of FcγRs. The human serum and EDTA present in hFCB enable efficient FcγR blocking while chelating free Ca^{2+} to reduce the spontaneous aggregation of cells during their manipulation and staining (black arrows in **Figure 1A** (iii) and (iv) show remaining doublets in suspensions of tonsillar cells).

Keeping track of the instrument performance by using the setup and tracking beads and the software (or similar; see the **Table of Materials**) is critical for the reproducibility of quantifications over time, especially in later steps when the synaptic transfer of particles to BSLB is measured using only MFIs (i.e., for fluorochromes for which there are no MESF standards). Similarly, check the linear range (i.e., **linearity minimum and linearity maximum**) of arbitrary fluorescence units for the quantification detector to be used alongside the MESF standards such that each calibration point keeps the linear relationship between fluorescence and the number of fluorochromes.

The preparation of samples "blank" for fluorescence, or samples providing an idea of the background staining

noise, are essential to subtract the nonspecific fluorescent signal. Isotype controls and/or biologically null samples (e.g., knockouts) are essential to correct for the background signal of cells and extract the true signal derived from the quantification antibodies (**Figure 1A** panel (viii)). Similarly, standard MESF beads use a dedicated blank population to subtract the background signal from each truly positive bead population (**Figure 1B** panels (vii) and (viii)). Once the regression analyses are performed and the slope defining the relationship between MESF and corrected MFIs is extracted, the conversion to absolute molecular densities follows simple mathematical operations (**Figure 1C**).

To estimate CSA in **Figure 1C**, electrical current exclusion (CASY-TT) was used to extract measurements of cell volume and diameter from thousands of cells. The resulting CSA estimated from the calculation of surface area for spheres ($4pr^2$) varies with the activation state of the cells, with observed values of 170.37 ± 4.91 μm^2 for nonactivated B cells and 234.52 ± 1.53 μm^2 and 318 ± 24.45 μm^2 for nonactivated and activated T cells, respectively. These CSAs are comparable to those estimated by imaging techniques such as three-dimensional refractive index tomography of nonactivated lymphocytes[16].

Once the range of physiological densities has been defined (e.g., by comparing surface densities on cells undergoing different activation programs), BSLBs can be used to model those surfaces. A titration of biotinylated antigenic HLA-peptide monomers provided by the NIH tetramer facility (or monobiotinylated monomeric anti-CD3ε-Fab) can be used alongside ICAM-1 12-His to reconstitute a canonical APC membrane. Commercial proteins tagged with 6, 9, 12, and 14 His can be used to decorate the surface of BSLB (see **Figure 2A** for examples with ICAM-1 12-His). Protein titrations together with quantitative FCM analyses provide a robust methodology to reconstitute physiological APC surfaces and test their effect on the synaptic output of different T cell subsets.

For studying the output of T cell synapses, use a 1:1 BSLB-to-T cell ratio to ensure that, on average, one cell will interact with one BSLB over the studied period. We have observed that the material transfer is proportional to the incubation time, providing a versatile platform for detecting molecules transferred in low quantities across the cell:BSLB interface. An appropriate panel design is thus critical to increasing the sensitivity and reliability of the detection of trans-synaptic material, as in the case of tSVs, the output varies between 25 and 36 vesicles/cell/20 min[2,3]. Test first the spectral spillover of each fluorochrome-labeled antibody and lipid. When high spillover is observed, we recommend the titration of staining antibodies and the percent of fluorescent lipids composing the BSLB, as well as PMT voltage walks to reduce the spreading error on compensated samples and enhance the signal over noise ratio, respectively (refer to[12,14,17] for a dedicated introduction to the subject).

The use of quantification controls, including null BSLBs (lacking antigen or anti-CD3 Fab) and either knockout cells or isotype-labeled samples[18], is essential to accurately measure the transfer of effector tSV, such as SEs, as well as supramolecular attack particles released within the synaptic cleft. Use highly abundant cell-surface proteins such as CD4, CD2, or CD45 alongside synthetic fluorescent lipids (DOPE Atto conjugates) to identify the population of single cells and BSLB upon cold-based dissociation of conjugates. Focus the analyses on the geometric mean or median of fluorescence intensities in single BSLB and cells (CD4 is used in **Figure 3B**). SEs are a specialized type of tSV derived

from the plasma membrane (PM), and their transfer to BSLB is evidenced by the gain of marker signal on BSLBs with the consistent loss of signal on the surface of the interacting cells (refer to TCR on BSLBs as shown in **Figure 2B**, violet arrows). Null BSLBs lacking either antigen or anti-CD3 are an excellent reference to keep track of the specific gaining of TCR (and other T cell markers) on BSLB resulting from stimulating interacting cells via their TCR complex.

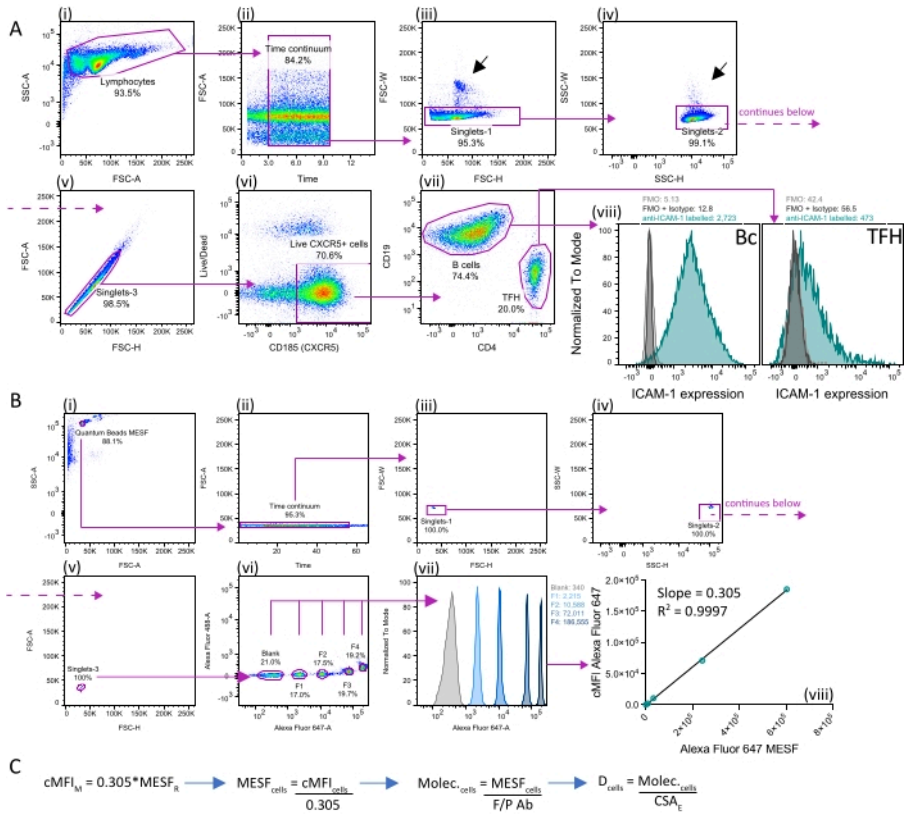

Figure 1: Absolute quantification of proteins on the surface of APCs. (**A**) Example of quantitative flow cytometry measurements of ICAM-1 on the surface of tonsillar B cells (Foll. Bc) and helper T cells (TFH). (**i-vii**) Gating strategy for analyzing single CXCR5^{+} Bc and TFH isolated from human palatine tonsils. Shown is the sequential gating strategy for identifying single, live events contained within the continuous window of acquisition. **(iii-iv)** black arrows indicate doublets. (**viii**) overlaid histograms showing the cell surface expression of ICAM-1 (teal histograms) compared to FMO controls (grey histograms) and FMO controls labeled with relevant isotypes (black histograms, which overlap with the grey histograms) of the populations shown in (vii). Arrows indicate the direction for the nested gating strategy used to identify CXCR5^{+} B cells (Bc; CD19^{+}) and TFH (CD4^{+}). (**B**) Extraction of absolute molecules on the surface of tonsillar cells from MFI requires regression analyses of MESF benchmark beads acquired using the same instrument setting as the cells shown in **A**. (**i-v**) Shown is the sequential gating strategy for identifying single, live events contained within the continuous window of acquisition. (**vi**) Gating and measurement of MFIs from different standard MESF populations. (**vii**) shown are overlaid

histograms of the MESF populations identified in (**vi**). The values displayed on the top right represent the MFIs for each of the 5 MESF populations (blank, 1, 2, 3, and 4). (**viii**) Linear regression of MESF over cMFI for the MESF populations shown in (**vii**). Shown is the slope (**b**) for extracting MESF bound to cells from data in **A**. (**C**) In the extraction of the number of molecules, follow simple mathematical operations starting with the application of the slope calculated in (**viii**) from measured MESF cMFI ($cMFI_M$) and reference MESF values ($MESF_R$). To extract the MESF bound to cells ($MESF_{cells}$), divide the corrected MFI of cells ($cMFI_{cells}$) by the calculated slope. Then, to calculate the number of molecules bound to cells ($Molec._{cells}$), divide $MESF_{cells}$ by the F/P of the detection (quantification) antibody. Finally, to calculate the molecular density on the surface of cells (D_{cells}), divide $Molec._{cells}$ by the estimated cell surface area (CSA_E). Abbreviations: X = independent variable; Y = dependent variable (measured fluorescence), $cMFI_M$ = measured corrected MFI; $MESF_R$ = reference MESF values; $MESF_{cells}$ = estimated MESF per cell; $cMFI_{cells}$ = corrected MFI cells; $Molec._{cells}$ = estimated molecules per cell. D_{cells} = estimated density on cells; CSA_E = estimated Cell Surface Area. Please click here to view a larger version of this figure.

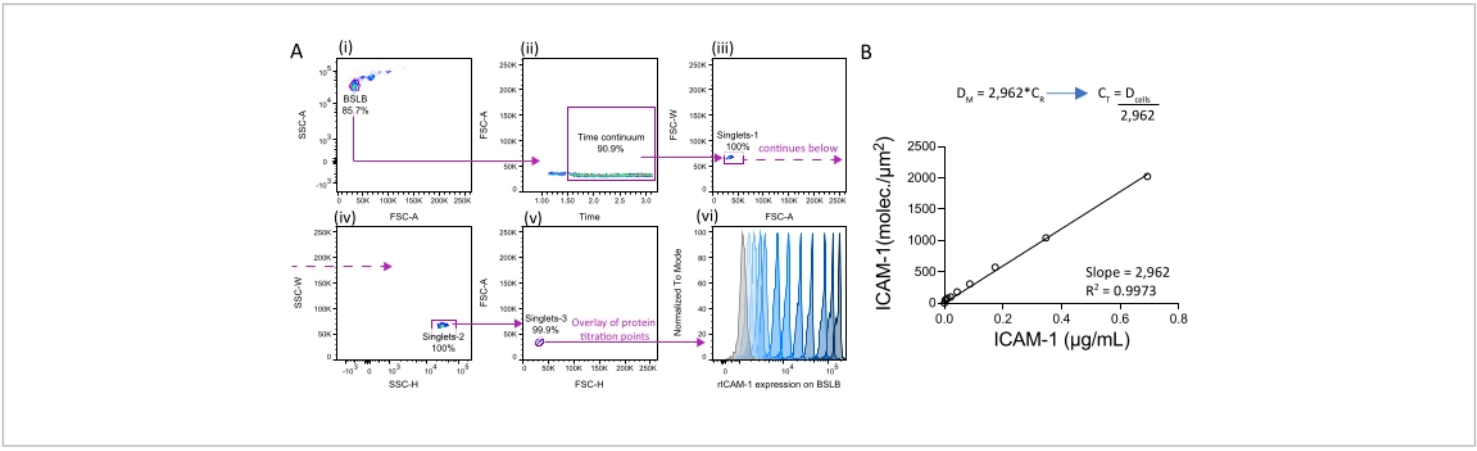

Figure 2: Reconstitution of BSLB with recombinant ICAM-1 and the measurement of particulate transfer to BSLBs. (**A, i-vi**) Flow cytometry analysis of BSLBs reconstituted with increased densities of recombinant monomeric ICAM-1 12-His (rICAM-1). **(i-v)** As in **Figure 1**, focus the gating strategy on single BSLBs within the continuous window of acquisition. Note the gap immediately before the *time continuum* gate, which was excluded to prevent errors of measurement. **(vi)** Good protein quality often results in the homogeneous coating of BSLB at high concentrations, with the observation of narrow fluorescence distributions (low Coefficient of Variation, see histograms in **vi**). (**B**) Regression analyses of ICAM-1 reference concentration (C_R) over measured density (D_M). Use the slope to calculate target concentrations of protein (C_T) to achieve the density of cells (D_{cells}) measured in the experiments in **Figure 1**. Abbreviations: 12-His = 12-histidines tag; D_M = measured molecular densities; C_R = reference concentrations of the rICAM-1; C_T = target concentration (to be interpolated); D_{cells} = densities measured in cells (see also Fig. 1C). Please click here to view a larger version of this figure.

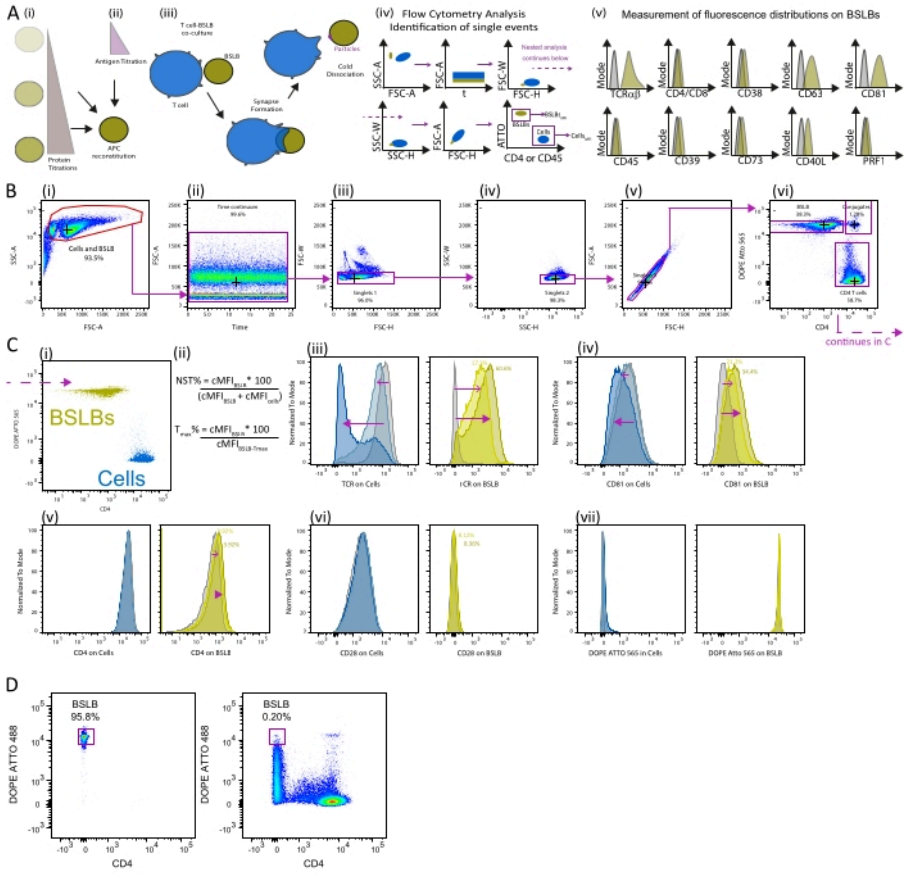

Figure 3: Measurement of T cell synaptic particles transferred to BSLBs. (**A; i-v**) Flow diagram showing the critical steps for the co-culturing of T cells with BSLBs reconstituting model membranes and the subsequent measurementof particle transfer with flow cytometry. (**iv**) Blue and dark yellow diagrams show the relative distribution and location of cells and BSLBs in biparametric flow cytometry plots. **(v)** Fluorescence distribution histograms displaying the relative gain of fluorescence of agonistic BSLBs (dark yellow) compared to null BSLBs (grey). (**B**) Exemplary synaptic transfer experiment. (**i-vi**) Shown is the gating strategy to identify single BSLBs and cells within the continuous acquisition window. Violet arrows indicate the direction of analysis, which continues in C. (**C**) (**i**) Focus the analyses on the MFI of single cells (blue) and single BSLBs (yellow). (**ii**) Equations to calculate the normalized synaptic transfer (NST%, top) and T_{max}% (bottom) from the cMFI calculated for BSLB and cells. (**iii-vi**) Overlaid histograms showing the change of fluorescence intensity distributions for cells (blue shades) and BSLBs (yellow shades) across different densities of the T-cell activating anti-CD3ε-Fab, including non-activating (grey) and activating with either 250 (soft color value) or 1,000 (high color value) molec./μm^2. Numbers in different color values represent the NST% measured for the BSLB histograms shown in yellow. The overlaid histograms show the overarching hierarchy in the synaptic transfer of T cell vesicles positive for different markers. For this composition of BSLBs (200 molec./μm^2 of ICAM-1 and increasing densities of anti-CD3ε-Fab), tSVs are transferred to BSLB with TCR$^+$ (**iii**) >

CD81$^+$ (**iv**) > CD4$^+$ (**v**) > CD28$^+$ (**vi**). As demonstrated in previous articles, TCR and CD81 are components of SEs and are transferred with comparatively higher efficiencies to CD4, despite the latter being expressed at comparatively higher surface levels. SE shedding results in the loss of cell surface CD81 and TCR and the gain of these signals on BSLBs (open purple arrows for 250 molec./μm^2, and closed purple arrows for 1,000 molec./μm^2 in yellow histograms). (**D**) Improper cooling down of conjugates leads to cells ripping off the SLB from silica beads as seen from comparing input beads (left biparametric plot) and conjugates subjected to rapid cooling down to 4 °C from 37 °C (right biparametric plot). Compare also with **Figure 3B** panel (**vi**). Abbreviations: PRF1 = perforin 1; NST% = normalized synaptic transfer; T_{max}% = percent of maximum observed transfer (in control or reference condition); tSVs: trans-synaptic vesicles; SEs: synaptic ectosomes. Please click here to view a larger version of this figure.

Table 1: Buffers used in this protocol. Please click here to download this Table.

Discussion

BSLBs are versatile tools for studying the particulate output of T cells stimulated with model APC membranes. The flexibility of the method allows the reconstitution of complex and reductionist membrane compositions to study the effects of ligands and their signals on the secretion of tSVs and supramolecular attack particles and their components. We have tested this technology on various T cells, including preactivated TH, CTL, Tregs, and CART[15]. This protocol also works for the measurement of synaptic particle release of freshly isolated and quiescent T cells. One limitation of using freshly isolated T cells is that these quiescent populations produce a different profile of trans-synaptic particles, which correlates with their cell surface composition (see [15] for more details).

As a simple flow cytometry panel, we recommend the use of anti-TCR clone IP26, anti-CD81 clone 5A6, anti-perforin (PRF1) clone B-D48 or anti-CD40L clone 24-31, and ATTO 390 or ATTO 565-containing lipids (to confer BSLBs an intrinsic fluorescence). To help discriminate single cells from single BSLBs and conjugates, we recommend the use of anti-CD4 clone OKT4 and/or anti-CD45 clone HI30, which are transferred to BSLBs at limited levels despite being expressed at very high levels at the cell surface (see **Table of Materials** for further details on fluorochromes and other validated antibodies). Panels with a higher number of fluorochromes can be designed but require a systematic evaluation of the fluorescence spectrum spillover of each fluorochrome analyzed. To increase sensitivity, try different titrations of quantification antibodies ranging from 0.5 μg to 20 μg/mL final, and repeat whenever new stocks of antibodies are used. To ensure reproducibility of the absolute and relative measurements of particle transfer, titrate and calculate the binding capacity of each new lot of biotinylated and Ni-containing phospholipids, as they might differ significantly from lot to lot. The gradual cool-down of T cell-BSLB conjugates is critical to increasing the sensitivity of detection of particles with low abundance. The rapid cool-down of cocultures leads to the destruction of BSLBs, as evidenced by the significant loss of lipid fluorescence (**Figure 3D**).

Different metrics can be used to measure the particulate output of T cell immune synapses depending on the experimental question at hand. For instance, when minor differences are expected in the baseline expression levels of surface proteins sorted into budding SEs, a normalized

synaptic transfer (NST%) metric can be used (**Figure 3C** panel (ii) top equation). The latter quantifies the percent MFI signal on BSLBs as a function of the total, combined MFI of cells and beads. One caveat from this approach is the analysis of transferred markers not expressed on the PM, such as components of supramolecular attack particles[19]. As these elements reach BSLBs by pathways independent of PM transit, such as intracellular store exocytosis, the calculation of NST% is not recommended as the result will be inflated because the numerator will be divided by a comparatively small denominator (cell surface expression level). Instead, use corrected MFIs to compare the deposition of perforin and granzymes between null BSLBs and BSLBs presenting increasing densities of antigen or anti-CD3 Fab. Alternatively, to track intracellular elements transferred to BSLBs, use for comparison either the estimated absolute molecules transferred or the percent of signal with regard to the maximum signal transferred to BSLBs (T_{max}%) (**Figure 3C** panel (ii) bottom equation). For T_{max}%, use either cMFI or molecules measured on the BSLB sample (or condition) presenting the highest antigen density as the reference T_{max}. When analyzing different donors, use donor-specific T_{max} for comparisons. When studying the material transferred in response to the specific triggering of the TCR, MFIs can also be corrected to the level of background transfer observed on antigen-negative (null) BSLBs.

Estimating the absolute number of molecules transferred to BSLBs is a more robust method, as this involves MESF calibration with the measurement of molecules as the endpoint and a better comparison of independent experiments. T_{max}% offers a similar normalization across independent experiments and is particularly useful when using polychromatic FCM for intracellular markers, such as perforin and granzymes, or for markers for which no MESF standard is available. T_{max}% is simply the percentage of signal in any given condition to the condition with the highest transfer in the control condition (e.g., vehicle/untreated controls for drug studies, control guide RNAs for CRISPR/Cas9 libraries). Further, T_{max}% can be used for both cMFIs and an absolute number of molecules and has been particularly useful for the side-by-side comparisons of the effects of gene deletions on the synaptic output of T cells. The latter is evident when gene-editing leads to high variability in the dynamic range of immune receptor expression among independent donors and experiments, which might impact absolute and NST% measurements.

BSLBs have facilitated the capture and characterization of the synaptic output of different T cell types, which otherwise are difficult to isolate due to their rapid internalization by APCs[2]. The physical stability of BSLBs also provides a platform for the fluorescence-activated cell sorting of BSLBs that have been surveyed by T cells, thus enabling the biochemical characterization of highly pure particle preparations by mass spectrometry and nucleic acid sequencing technologies. The latter facilitates the detailed characterization of intercellular messengers shed by T cells under a broad range of experimental conditions. Different questions can be addressed, including how these particulate messengers change among different T cell types and activation states, how canonical and noncanonical antigen receptors (i.e., chimeric antigen receptors), and how agonistic and antagonistic membrane signals influence particle composition. We are currently developing this technology further for the quantitative characterization of cytokines secreted at the immune synapse of stimulated T cells. The latter requires the careful study of combinations of recombinant cytokine receptors and antibodies, providing efficient capture of interleukins, interferons, and chemokines

deposited on BSLBs following T cell activation. BSLBs can be adapted to model the surface composition of other APCs suspected to trigger tSV release by T cells, such as stromal and innate immune cells. BSLBs can also be adapted to screen new pharmacological compounds seeking the positive and negative modulation of the exocytic and tSV secretion machinery for the treatment of cancer and other pathologies. Finally, BSLBs can also be used to discover virulence determinants modulating T cell function in infectious diseases[20].

Disclosures

The authors have no conflict of interest to declare.

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References

1. Cespedes, P. F., Beckers, D., Dustin, M. L., Sezgin, E. Model membrane systems to reconstitute immune cell signaling. *FEBS Journal.* **288** (4), 1070-1090 (2021).
2. Choudhuri, K. et al. Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature.* **507** (7490), 118-123 (2014).
3. Saliba, D. G. et al. Composition and structure of synaptic ectosomes exporting antigen receptor linked to functional CD40 ligand from helper T cells. *elife.* **8**, e47528 (2019).
4. Barcia, C. et al. In vivo polarization of IFN-gamma at Kupfer and non-Kupfer immunological synapses during the clearance of virally infected brain cells. *Journal of Immunology.* **180** (3), 1344-1352 (2008).
5. Huse, M., Lillemeier, B. F., Kuhns, M. S., Chen, D. S., Davis, M. M. T cells use two directionally distinct pathways for cytokine secretion. *Nature Immunology.* **7** (3), 247-255 (2006).
6. Kupfer, A., Mosmann, T. R., Kupfer, H. Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells. *Proceedings of the National Academy of Sciences of the United States of America.* **88** (3), 775-779 (1991).
7. Kupfer, H., Monks, C. R., Kupfer, A. Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. *Journal of Experimental Medicine.* **179** (5), 1507-1515 (1994).
8. Reichert, P., Reinhardt, R. L., Ingulli, E., Jenkins, M. K. Cutting edge: in vivo identification of TCR redistribution and polarized IL-2 production by naive CD4 T cells. *Journal of Immunology.* **166** (7), 4278-4281 (2001).
9. Gerard, A. et al. Secondary T cell-T cell synaptic interactions drive the differentiation of protective CD8+ T cells. *Nature Immunology.* **14** (4), 356-363 (2013).

10. Dustin, M. L., Starr, T., Varma, R., Thomas, V. K. Supported planar bilayers for study of the immunological synapse. *Current Protocols in Immunology.* **Chapter 18** Unit 18.13 (2007).
11. Abcam. *Counting cells using a hemocytometer.*, https://www.abcam.com/protocols/counting-cells-using-a-haemocytometer. (2022).
12. Roederer, M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry.* **45** (3), 194-205 (2001).
13. Dam, T., Junghans, V., Humphrey, J., Chouliara, M., Jonsson, P. Calcium signaling in T cells is induced by binding to nickel-chelating lipids in supported lipid bilayers. *Frontiers in Physiology.* **11**, 613367 (2020).
14. Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P., Roederer, M. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry A.* **83** (3), 306-315 (2013).
15. Céspedes, P. F. et al. Synthetic antigen-presenting cells reveal the diversity and functional specialisation of extracellular vesicles composing the fourth signal of T cell immunological synapses. *bioRxiv.* (2021).
16. Yoon, J. et al. Identification of non-activated lymphocytes using three-dimensional refractive index tomography and machine learning. *Scientific Reports.* **7** (1), 6654 (2017).
17. Roederer, M. Distributions of autofluorescence after compensation: Be panglossian, fret not. *Cytometry A.* **89** (4), 398-402 (2016).
18. Maecker, H. T., Trotter, J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A.* **69** (9), 1037-1042 (2006).
19. Balint, S. et al. Supramolecular attack particles are autonomous killing entities released from cytotoxic T cells. *Science.* **368** (6493), 897-901 (2020).
20. Cespedes, P. F. et al. Surface expression of the hRSV nucleoprotein impairs immunological synapse formation with T cells. *Proceedings of the National Academy of Sciences of the United States of America.* **111** (31), E3214-3223 (2014).