T-cell trans-synaptic vesicles are distinct and carry greater effector content than constitutive extracellular vesicles

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The immunological synapse is a molecular hub that facilitates the delivery of three activation signals, namely antigen, costimulation/corepression and cytokines, from antigen-presenting cells (APC) to T cells. T cells release a fourth class of signaling entities, trans-synaptic vesicles (tSV), to mediate bidirectional communication. Here we present bead-supported lipid bilayers (BSLB) as versatile synthetic APCs to capture, characterize and advance the understanding of tSV biogenesis. Specifically, the integration of juxtacrine signals, such as CD40 and antigen, results in the adaptive tailoring and release of tSV, which differ in size, yields and immune receptor cargo compared with steadily released extracellular vesicles (EVs). Focusing on CD40L+ tSV as model effectors, we show that PD-L1 trans-presentation together with TSG101, ADAM10 and CD81 are key in determining CD40L vesicular release. Lastly, we find greater RNA-binding protein and microRNA content in tSV compared with EVs, supporting the specialized role of tSV as intercellular messengers.
lymphocytes are key players in the regulation and promotion of adaptive immunity. Helper T cells (TH), cytotoxic T lymphocytes (CTL), and regulatory T cells (Treg) shape cellular networks through the assembly of cell-cell interfaces termed Immunological Synapses (IS) with antigen-presenting cells (APCs) and other T cells. These ISs enable three key receptor-ligand-driven signals critical to mounting an immune response or maintaining self-tolerance: (1) antigen recognition, (2) co-stimulation and co-repression, and (3) sensing of soluble cytokines released into the synaptic cleft. Here we propose a fourth type of trans-synaptic signal based on trans-synaptic supramolecular effectors and provide a general tool to study these challenging to isolate signaling entities.

In the past 10 years, several supramolecular effectors transferring information across the interface of cell-cell junctions have been identified. These include polarized exosomes (PE), supramolecular attack particles (SMAPs), released from multivesicular bodies into the synaptic cleft, synaptic ectosome (SE) budding from the T-cell plasma membrane across the synaptic cleft, and trans-endocytosis/trogocytosis membrane fragments across the synaptic cleft. PE and SE are extracellular vesicles actively formed by the donor T cells through the action of the endosomal sorting complexes for transport (ESCRT) machinery and together make up the trans-synaptic vesicles (tSV). SMAPs are proteinaceous particles similar in size to exosomes and SE but lack a phospholipid membrane and instead have a core-shell structure that enables the transfer of complex cargo.

The requirement for antigen sensing and the integration of multiple juxtacrine signals (i.e., trans-receptor-ligand interactions enabled by the nanometer proximity of the opposing cell membranes) enables a highly regulated and efficient exchange of supramolecular effectors across immunological synapses. In antigen-dependent contacts, T cells efficiently transfer extracellular vesicles containing T-cell antigen receptors (TCR) to APCs as early as 10–30 min of interaction. Similarly, CD40 ligand (CD40L), which is essential for B-cell help and the initiation of germinal center reactions, is transferred from T cells to B cells in a contact-, CD40- and antigen-dependent manner. The specialized molecular and organelle organization of the IS also enables the antigen-dependent delivery of microRNA (miRNA) to APCs, highlighting the critical role of the IS as a facilitator of a variety of intercellular messages. A more detailed characterization of trans-synaptic particles is nonetheless challenging due to the rapid internalization of trans-synaptic cargo by APCs and because cells engaged in contacts actively acquire membrane fragments via trans-endocytosis and trogocytosis, which altogether introduces confounding factors in the identification of T-cell-specific particulate effectors.

While tSV, trans-endocytosed fragments, and SMAPs are consumed by the synaptic partner, extracellular vesicles formed by the ESCRT machinery and constitutively released into the media (referred to herein as EVs) can be collected from the culture supernatant of activated T cells. Previous work has used EVs as a surrogate for tSV. While this approach has had some predictive power, a comparison of tSV and EVs is critical for progress in the field of cell-cell communication.

Here, we develop a platform to specifically collect tSV released from three major types of T cells, study their regulation and compare their immune receptor, protein, and miRNA cargo with those of EVs. Our findings reveal unique regulatory and compositional features in tSV and support the notion that regulated cell-cell contacts, such as the immunological synapse, facilitate the exchange of particulate messengers. This platform should also be helpful for capturing TSV in other biological cell-cell junctions and in response to a variety of juxtacrine signals that can be modeled with supported lipid bilayers (SLBs).

**Results**

**Trans-synaptic vesicles are larger and carry more cargo than EVs.** SLB on glass substrates have been used extensively to study cell recognition processes between live cells and SLB presents molecular components from a natural interaction partner. SLB on planar supports presenting surrogate antigen, adhesion, and co-stimulatory receptors were first used to characterize tSV and SMAPs from TH cells and cytotoxic T cells, respectively. The advantage of the SLB is that following T-cell release of tSV or SMAPs, the respective T cells can be selectively released, leaving the intact tSV or SMAPs behind for analysis by imaging or mass spectrometry. SLB can be readily formed on 5.0 ± 0.05 µm glass beads (BSLB) to improve compositional analysis. We have further improved on this first generation of BSLB to develop a general resource for the study of tSV and SMAPs in any synaptic model.

Our second-generation BSLB model uses a surrogate antigen, a 14 His-tagged α-CD3ε Fab, that can be gently eluted from the SLB to enable the release of tSV. We used fluorescence correlation spectroscopy to demonstrate that the recombinant α-CD3ε Fab and a co-incorporated Abberior Star-Red phosphatidylethanolamine had diffusion coefficients of 0.68 ± 0.39 and 3.2 ± 0.7 µm²/s, respectively, which are comparable to ranges reported for PM proteins and lipids. To analyze tSV from TH cells we used BSLB presenting a range of α-CD3ε Fab densities, and physiological densities of the adhesion molecule ICAM1 (200 mole/µm²) and costimulator receptor CD40 (20 mole/µm²), which are characteristic of natural APCs. Time-lapse imaging of T-cell-BSLB co-cultures demonstrated that BSLB instigated the transfer of CD40L from stimulated T cells, which left a “synaptic stamp” on the engaged BSLBs composed of the transferred tSV (Fig. 1a). To enable the synchronous release of BSLBs from conjugates, we gradually cooled the cell-BSLB coculture to 4°C to promote dissociation of the LFA-1-ICAM1 interaction while minimizing mechanical stress on BSLB and their tethered tSV. Following this treatment, flow cytometry demonstrated that BSLB acquired little CD2 and CD4, T cells acquired little fluorescent lipids (DOPE) indicating limited internalization of BSLB material (Fig. 1b and Supplementary Fig. 1b), contrasting with what has previously been reported for B-cell IS.

We next eluted the tSV with ice-cold EDTA from BSLB previously enriched by low-speed centrifugation (100 g 1 min) and used differential and ultracentrifugation to concentrate the eluted tSV (see Methods). We focused our analyses on tSV containing the T-cell effector molecules TCR and CD40L, the broad EV and PM marker CD81+, and on CD63+ tSV belonging to exosomes. Most of the TCR, CD40L, and CD81 present on BSLBs were eluted under these conditions (Fig. 1c, d–f). Elution of CD63+ vesicles was less efficient but still significant (Fig. 1g). Electron microscopy images of eluted tSV revealed classical EV-like profiles (Fig. 1h, arrows). We were now able to compare tSV with steadily released EVs isolated from activated TH cells of the same donors cultured in EV-free media. TSV was larger than EVs and contained a higher frequency of events larger than 113 and 155 nm in tSV compared with EVs (Supplementary Fig. 1d). tSV showed a higher frequency of events in the 68 nm size bin.
**Fig. 1** As synthetic APC, BSLBs trigger synapse formation and the release of tSV by stimulated T cells. 

a) Time-lapse confocal microscopy showing the interaction between TH and BSLB and the active transfer of CD40L+ synaptic stamps to BSLB (white arrowheads). CD40L was tracked using 1 μg/mL of anti-CD40L clone 24. 

b) FCM analyses of vesicular transfer from TH (gray histograms) to BSLB (teal histograms) presenting 2-fold titrations of α-CD3e Fab (0–1000 molec./μm²) ICAM1 (200 molec./μm²) and CD40 (20 molec./μm²). Vertical lines indicate the median of cells and BSLB in the condition with no α-CD3 Fab (null). 

c) After conjugate separation by cold, tSV are released from BSLBs with the use of 50 mM EDTA. Elution results in the release of δ TCRαβ, e CD40L+, f CD81+, and to a lesser extent g CD63+ tSV, from the surface of BSLB as measured by FCM staining comparing null BSLB (black histograms), with BSLBs prior to (green), and after elution (violet). 

h) Transmitted electron microscopy of eluates reveals the presence of tSV (white arrows) and small soluble proteins. Scale bar = 500 nm. 

i) Representative NanoFCM analyses showing the detection of TCRαβδ and CD81+ events in isolated EVs (left panel), and the eluted material from Null BSLB (middle panel) and α-CD3+ BSLB (tSV, right panel). Overlaid histograms showing TCRαβδ expression on CD81+ vesicles from EVs (yellow) and tSV (light blue) compared to double negatives (gray).

j) Immunoblot for comparison of CD40L, CD81, Alix, and TSG101 in EVs and eluted tSV deriving from 5 × 10⁶ of either cells or BSLB, respectively. Whole-cell lysate represents a total of 2.25 × 10⁵ cells. The bands shown are from the same immunoblot membrane. CD40L levels in eluted fractions (tSV) and EVs as measured by immunoblot (shown is CD40L normalized to TSG101). Normality was determined using Shapiro-Wilk test and statistical significance was determined by the two-tailed unpaired t test (d–g) and by the unpaired, two-tailed Mann-Whitney test (l). Data represents mean ± SEM of n = 2 (a, i–j) and n = 4 independent experiments (b, e–h, k–l). Uncropped and unprocessed versions of the immunoblots shown in k are available as source data.
(Supplementary Fig. 1d). A comparison of marker-specific vesicles within tSV and EVs revealed that CD83+ tSV coexisted with TCRαβ+ at higher frequencies in tSV than EVs (Fig. 1i–j) and a larger median size for TCR+ tSV than TCR+ EVs (Supplementary Fig. 1e–f), respectively. We also observed a larger median size for CD40L+ or CD81+ tSV than CD40L+ or CD81+ EVs (Supplementary Fig. 1e), whereas no significant size differences were observed for BST2+ or CD63+ tSV and EVs. TSV also expressed higher levels of TCR and CD40L than EVs (Fig. 1j–l, and Supplementary Fig. 1g–h). Since CD40L detection by antibodies is partly impaired by the presence of CD40L, we confirmed CD40L elution and compared its expression levels in tSV and EVs by immunoblotting (Fig. 1k and Supplementary Fig. 1h). Most likely, the overall larger size and immune receptor content of TSV derives from the subpopulation of SE, which contains high densities of TCR and CD40L as microclusters >80 nm². The membrane-anchored full-length CD40L was predominantly found (29.2 kDa) in the eluates of BSLBs, with negligible detection of its soluble ectodomain (Supplementary Fig. 1h). Null CD40+ BSLB captures negligible amounts of CD40L from TCR-unstimulated cells, which might relate to the CD40-dependent capture of a fraction of EVs released in response to adhesion (i.e., ICAM1:LEA-1 interaction). The imaging of TSV markers within T-cell-SLB interfaces with TIRFM and confocal imaging further revealed that beyond differences in size and immune receptor content, TSV displayed different degrees of co-localization in intact, non-permeabilized Is (Supplementary Fig. 2a–m) reflecting their different subcellular origin and the compositional and spatial heterogeneity of TSV in the synaptic cleft.

TSV is likely composed of TCRαβ+, CD40L+ SE, and CD63+ PE, which may have different release characteristics. We used the BSLB system to investigate requirements for TSV release using a panel of 12 pharmacological inhibitors of candidate steps. Inhibitors of cytokinetic dynamics and the transport of vesicular cargo from different organelles, such as multivesicular bodies (MVB) and the trans–Golgi network (TGN) were used (Supplementary Data 1). Following a pre-treatment for 30 min and then the co-culture of T cells and BSLBs for another 90 min at 37°C and 5% CO₂ (in the presence of inhibitors), we monitored the cell surface expression of TCR, CD40L, BST2, and CD63 (Supplementary Fig. 3a–d), their synaptic stability (Supplementary Fig. 3e), and their normalized trans-synaptic transfer to BSLB as the percent of the maximum observed in untreated controls (% Tmax) (Supplementary Fig. 3f–i).

Actin cytokinetic disruptors Latrunculin A and Jasplakinolide, but not Cytochalasin D (CytD), impaired cell-BSLB conjugates (Supplementary Fig. 3e). CytD and the other inhibitors didn’t impair cell-BSLB conjugate formation and thus allowed interrogation of distinct mechanistic steps in the delivery of tSV cargo. Fortuitously, we observed a differential inhibition in the transfer of TCR+, CD40L+, BST2+, and CD63+ tSV from TH cells to BSLB, suggesting distinct mechanisms of cargo delivery. The transfer of TCR+ tSV was selectively promoted by dynamin inhibition by Dynasore, and conversely, strongly reduced by inhibition of actin polymerization, ESCRT machinery, and neutral sphingomyelinases (Supplementary Fig. 3f). Reduced endocytosis or increased ubiquitination of TCR following Dynasore treatment might contribute to the observed increase in TCR+ tSV transfer. The transfer of CD40L+ tSV was strongly reduced following acute inhibition of several types of machinery in the hierarchy TGN > actin dynamics and ESCRT > ceramide synthesis > dynamin > vacuolar H+–ATPases, and class I and II PI3K (Supplementary Fig. 3g). Other members of the TNF superfamily, such as FasL, have also been reported to be affected by interference with TGN trafficking, suggesting a conserved mechanism of TNFSF delivery via TSV. In contrast, the transfer of BST2+ (Supplementary Fig. 3h) and CD63+ (Supplementary Fig. 3i) tSV was affected by inhibiting endosomal and lysosomal transport but not by inhibiting TGN transport. CD40L+ > TCR+ > BST2 showed a higher sensitivity than CD63+ tSV to the inhibition provided by the MG132-induced depletion of free-ubiquitin, suggesting that MVB-associated stores are not affected by the acute pharmacological inhibition of ESCRT-I. The significant reduction of CD40L+ and CD63+ tSV transfer following N-SMases inhibition likely results from interference with broader membrane trafficking events involving both the TGN and endosomes where N-SMases preferentially locate to regulate membrane curvature and budding. Finally, compared to other vesicle subpopulations, the production of CD40L+ tSV requires quick antigen-dependent upregulation of CD40L on the plasma membrane, suggesting that the majority of its trans-synaptic transfer occurs as part of budding synaptic ectosomes (Supplementary Fig. 4a–d). While BFA and manumycin inhibited the α-CD3-induced upregulation of CD40L, CytD impaired CD40L+ tSV release despite its effective cell surface upregulation (Supplementary Fig. 4b).

**T-cell subsets have distinct tSV cargo.** Next, we sought to evaluate whether different human T cells, including CD8+ CTL, CD4+ T helper cells (TH), and cultures enriched in CD127+CD25+Fox3+ cells (Treg), displayed distinctive tSV transfer hallmarks. All cells were isolated from peripheral blood, activated, and further expanded ex vivo as detailed in Methods (see Supplementary Fig. 5a–c). We evaluated the transfer of the TCRαβ heterodimer, CD2, the co-receptors CD4 or CD8, CD28, CD45, the TSV proteins CD63, CD81, and BST2, and effectors including regulatory enzymes (CD38, CD39, and CD73), and helper (CD40L) and cytotoxic (Perforin) proteins (Fig. 2a–p).

Analyses were focused on markers expressed specifically by these populations and the MFI of single T cells and single BSLB after gentle dissociation of conjugates (see Supplementary Fig. 1a for a representative gating strategy). A normalized synaptic transfer metric was defined as the percent enrichment of T-cell proteins on a single BSLB from the total mean signal observed in single BSLB and single cells (see Eq. (1) in Methods and ref. 5).

Because we reconstituted our BSLBs in the absence of ligands for TCR co-receptors CD4/CD8 (i.e., pHLA) and CD2 (i.e., CD58), we therefore expected the vesicular shedding of TCR to include limited co-receptors and CD2 (Fig. 2b–c). Compared with TH, CTLs transferred significantly more TCR and coreceptor in the range of α-CD3ε-Fab densities tested and consistently showed smaller α-CD3ε-Fab EC₅₀ values of 251.2 for TCR (compared with 1,251 in TH) and 50.87 for the coreceptor (compared with 56.44 in TH) (Fig. 2a–b). The higher antigen sensitivity of CTL compared with TH and increased physical proximity between TCR and CD8⁺, might promote their sorting in TSV. Similarly, using chimeric antigen-receptor expressing T cells (CART) specific for HLA-A*02: NY-ESO-1, we tested whether the T1 CAR displays similar transfer dynamics than TCR. Interestingly, we observed a higher transfer of the CAR than TCR at comparable densities of α-CD3ε-Fab and agonistic HLA-ε complexes (Supplementary Fig. 5d). CART also transferred higher amounts of CD8, suggesting that CAR triggering by agonistic pHLA, even at low densities, promotes the efficient release of CAR and CD8 (Supplementary Fig. 5e). Like CD2 and co-receptors, and despite their high level of cell surface expression, we observed a lack of CD28 and CD45 enrichment in the TSV released by different T-cell subsets (Fig. 2d–e). While CD45 is normally excluded from the cSMAC and the synaptic cleft, the co-receptors, CD2 (at exceptionally low levels) and
CD28 require binding to cognate ligands to be mobilized as microclusters to the synapse center\(^{29}\). The latter suggests that tSV heterogeneity is partly defined by APC ligands dynamically feeding information to T cells, which we explored further in the next section. The limited enrichment of CD4, CD8, and CD45 in tSV makes them suitable secondary parameters for discriminating single BSLBs in FCM analyses and sorting (Supplementary Figs. 1a, 5i and 9a).

TH, CTL, and Treg transferred comparable levels of CD63\(^+\), CD81\(^+\), and BST2\(^+\) tSV to BSLB (Fig. 2f–h), indicating conserved mechanisms delivering these components to the synaptic cleft. Consistently, CART showed a comparable transfer of CD63\(^+\) tSV when stimulated through the CAR and the TCR (Supplementary Fig. 5i). We next measured the vesicular transfer of effector ectoenzymes, namely the ADP-ribosyl cyclase CD38 and the AMP- and adenosine-producing ectonucleotidases CD39 and CD73 (Fig. 2i–k). While CD38 polarizes to the IS\(^{32}\) and participates in the generation of T-dependent humoral immunity\(^{33}\), CD39 and CD73 are known tolerogenic mediators\(^{34-36}\). TH and CTL showed a limited, conserved, and
The synaptic transfer of vesicular effectors relates to the functional properties of different T-cell subsets. Flow cytometry analyses measuring the transfer of various tSV to BSLBs presenting α-CD3ε Fab (0–1000 molec./µm²), ICAM1 (200 molec./µm²), and CD40 (20 molec./µm²) were used in all experiments (see Supplementary Fig. 1a for a representative gating strategy). a–p Percent Normalized Synaptic Transfer (NST%) to BSLB from TH (green circles), Treg (light blue circles), and CTL (violet circles). Markers included α the antigen-receptor heterodimer TCR/β; β CD2; c TCR co-receptors (CD4 or CD8); d CD28; e CD45; f CD63; g CD81; h BST2; i CD38; j CD39; k CD73, and l CD40L. m Representative histograms for most makers are shown on the right for TH (green histograms for BSLBs) and CTL (violet histograms for BSLBs). n Perforin transfer as null BSLB-corrected GMFI. o–p Representative histograms showing perforin deposition on BSLBs after co-culturing with TH (o) and CTL (p). Shown are overlaid histograms depicting BSLBs coated with different densities of anti-CD3 Fab. Data represent means ± SEM of normality was determined using Shapiro-Wilk test and statistical significance was determined by two-tailed Multiple t test with Holm–Šidák corrections (α = 0.05) for the multiple comparisons of tSV transfer across different α-CD3ε Fab densities and among different T-cell populations (a–l). Also, the comparison of α-CD3 Fab EC₅₀ and transfer maximum (Tmax) among T-cell populations was determined using F test fitted with three to four parameters (a–l). ns = not significant. Adjusted P values are shown next to each significant comparison.

Comparable transfer of CD38⁺ tSV to BSLB (Fig. 2i). Treg, on the other hand, showed an increased transfer of CD39 (Tmax = 17.83% compared with 6.5% in TH; Fig. 2j), and CD73 (Tmax = 5.73 % compared with 1.9% in TH; Fig. 2k), which associated with a higher central clustering of CD39 and CD73 in the cleft of Treg synapses (Supplementary Fig. 5j–o). The inclusion of itinerant enzymes such as CD38, CD39, and CD73 in tSV, although limited, might exert a feed-forward regulatory effect as shown for tumor-derived EV37.

We also compared the transfer dynamics of CD40L and perforin, two major effectors mediating the activation and killing of APCs, respectively. Upon TCR-triggering, we observed a conserved relative transfer of CD40L in T cells, with TH secreting the most (Fig. 2l–m). Conversely, compared to TH, CTLs showed a reduced threshold for perforin release, which was transferred to BSLB at α-CD3ε-Fab densities as low as 8 molec./µm² for CTLs (Fig. 2n–p). In CART cells, perforin release followed a different behavior than exocytic CD63⁺ tSV. More specifically, TCR-elicited perforin deposition on BSLB was higher than that resulting from CAR-triggering (Supplementary Fig. 5g), suggesting differential mechanisms influencing the release of tSV and cytotoxic SMAPs at the IS. Altogether, our data indicate that (1) T-cell subsets display differential dynamics of tSV transfer consistent with their effector functions and receptor-ligand interactions occurring at the IS and that (2) BSLB are excellent tools to study the biogenesis of both tSV and perforin-containing SMAPs.

Juxtacrine signals and the cell’s phenotype determine tSV cargo. In the dynamic exchange of information between T cells and APCs several factors might influence the effector cargo of tSV. Hence, we harnessed BSLB to study the dynamics influencing CD40L⁺ tSV release under near-physiological conditions and using absolute quantifications (see Methods). First, we measured the densities of CD40 on different human B-cell subsets (ranging between 16 and 646 molec./µm²; Fig. 3a). Using these near-physiological estimates we then measured the transfer of CD40L⁺ tSV to BSLB covering a range of 0 to 540 molec./µm² of CD40. As shown in Fig. 3b, we found that while freshly isolated and quiescent TH transferred negligible amounts of CD40L, activated and blasted TH showed high sensitivity to CD40, transferring CD40L to CD40 densities as low as 2 molec./µm². However, this high efficiency reached a maximum transfer (Tmax) at ~20 molec./µm² of CD40, which at higher densities outcompeted the anti-CD40L detection antibody3 (Fig. 3b). The latter is expected as CD40 displays a high-affinity ligand for its ligand (0.5–7.13 nM38). Therefore, we used the physiological minimum of CD40 (20 molec./µm²) to study the dynamics affecting CD40L sorting in tSV. We used a vesicle stamp area of 0.82 µm²25 to estimate and compare the effective densities of CD40L on the vesicular stamps of BSLB. Even after 4 h, no significant transfer of vesicular CD40L was observed from quiescent cells (Fig. 3c). In contrast, activated TH blasts transferred significant amounts of CD40L and led to densities up to 1000 times those found on the plasma membrane, suggesting a significant gain in CD40L binding valency resulting from its vesicular packing. Remarkably, higher CD40L⁺ tSV transfer in activated cells related to their higher expression of TSG101 (Fig. 3d–e), indicating that T-cell activation leads to the simultaneous upregulation of effectors and ESCRT-I. Further comparison of TH, Treg, and CTL revealed phenotype-specific differences in the dynamics of CD40L transfer among activated T cells, with the amounts released in the order TH > Treg>CTL (Fig. 3f; F test, p < 0.0001).

Next, to evaluate the effect of TCR sensitivity on CD40L tSV release we used 3 TH clones specific for the same influenza H3 hemagglutinin peptide HA₃₃₈–₃₅₅ (NVPEKQTRGIFGAIAGFI) but displaying different TCR:pMHC potencies (Supplementary Data 2). T-cell clones showed comparable levels of CD40L transfer at higher antigenic HLA densities (Fig. 3g) and formed morphologically similar synapses (Fig. 3h–i). However, contrary to their IFN-γ response (Supplementary Data 2), clones showed differences in the HLA-DR9:HA EC₅₀ for CD40L transfer with clone 40 displaying the lowest (P = 0.0002, EC₅₀ = 22.02, compared to 66.01 and 101.9 for clones 16 and 35, respectively), suggesting that TCR:pMHC affinity has more substantial effects on the release of CD40L⁺ tSV at lower antigen densities. The latter phenomenon might underpin the maintenance of an active CD40L-CD40 pathway in autoimmune diseases, where high densities of self-antigens and low affinities of self-reactive TCRs might suffice to trigger the assembly of non-canonical immune synapses39 and the release of CD40L⁺ tSV.

Beyond antigens (signal one), APCs present other juxtacrine signals (signal two) to fine-tune the activation of engaged T cells. To evaluate whether such signals shape the composition and release of tSV, we incorporated the co-stimulatory ligands CD80, CD86, ICOSL, the CD2 corola-inducing ligand CD8340, the TNF ligand superfamily (TNFSF) members OX40L (TNFSF4) and 4-1BBL (TNFSF9) and the co-repressors PD-L1 and PD-L2, whose interplay with the CD40-CD40L dyad remains poorly understood. To evaluate whether viral proteins also shape tSV, we included the HIV-1 gp120 protein, which is a known high-affinity ligand for CD4 and 4040,42. We then compared the NST% for TCR (Fig. 4a), CD2 (Fig. 4b), CD28 (Fig. 4c), CD63 (Fig. 4d), CD81 (Fig. 4e), BST2 (Fig. 4f), CD40L (Fig. 4g–i) and CD4 (Fig. 4j–k) between different BSLB compositions using those containing only ICAM1, CD40, and increasing α-CD3ε-Fab densities as controls. While CD58 incorporation slightly promoted the transfer of CD2 (Fig. 4b), incorporation of CD80, CD86, and ICOSL induced an increased synaptic transfer of CD28 (Fig. 4c), and to a lesser extent that of CD40L (Fig. 4g). Most remarkably, PD-L1/PD-L2 inclusion on BSLB led to a significant reduction in the transfer of CD40L⁺ tSV (Fig. 4g–i),...
and a less significant reduction of TCR$^+$, CD28$^+$, CD63$^+$, CD81$^+$, and BST2$^+$ tSV (Fig. 4a–f). Similarly, 4-1BBL/OX40L trans-presentation impaired CD4 transfer, TCR, CD81, and BST2 transfer to BSLBs (Fig. 4a,e–i). Importantly, even in the absence of TCR stimulation, gp120 promoted CD4 transfer to BSLB (Fig. 4j–k), which might result from both the physical engagement of CD4 and the TCR-independent phosphorylation of Src, Lck, and CD3$\varepsilon$ triggered by gp120$^{10}$. The gp120-induced transfer of CD4 and the CD80/86-instigated transfer of CD28 thus demonstrate that cognate and non-native juxtacrine signals shape the composition of tSV. These data suggest that the immunological synapse facilitates the delivery of specific, targeted, and adaptive trans-synaptic messages contingent upon ligands bound at the cell-cell interface.

Next, we used CRISPR/Cas9 genome editing to test the functional relevance of endogenous elements participating in tSV biogenesis and the synthetic release of CD40L. Because we used α-CD3ɛ Fab for the HLA-independent triggering of TCR, we expected negligible participation of CD4 in tSV biogenesis. Hence, we selected a guide RNA (gRNA) producing downregulation of the coreceptor ($67.83 \pm 10\%$ of baseline) as a control. We also selected gRNAs targeting membrane structural proteins (BST2, CD81) and the ESCRT-I TSG101, which mediates synaptic ectosome budding$^5$. We also targeted a disintegrin and metalloproteinase 10 (ADAM10), mediating the shedding and release of FasL and CD40L trimers from the plasma membrane of activated T cells$^{43–45}$. Knockout of the relevant targets were observed with some residual surface on cells edited for BST2 (45.08 $\pm$ 23.19% of control), CD81 (7.63 $\pm$ 4.8% of control), and ADAM10 (9.37 $\pm$ 2.83% of control; Supplementary Fig. 6a–g). Next, we compared tSV transfer in terms of the Tmax % observed in controls (CD4-gRNA; see Eq. (2) in Methods). While ADAM10 downregulation led to a significant increase in the upregulation of CD40L on the cell surface (224.2 $\pm$ 114.8%, Supplementary Fig. 6a, c, h, i) and its synaptic transfer to BSLBs (Fig. 5a), the downregulation of CD81, BST2 and TSG101 (Supplementary Fig. 6f, g, j, k) reduced the transfer of CD40L$^+$ tSV without altering the baseline expression levels of CD40L at the plasma membrane (Fig. 5a and Supplementary Fig. 6a, c). TSG101 editing led to a significant reduction on the vesicular transfer of CD40L and TCR without affecting their baseline surface expression levels, indicating a bonafide inhibition of their vesicular sorting dependent on ESCRT-I (Fig. 5a–b). TSG101 downregulation partly phenocopied the effects of ubiquitin depletion by MG132, which affected most substantially the transfer of CD40L$^+$ tSV, and to a lesser extent TCR$^+$ tSV (Supplementary Fig. 3f–g). CD63$^+$ tSV transfer was less affected by downregulation of CD81, ADAM10, BST2, and TSG101 (Fig. 5c), providing an additional piece of evidence for CD63 trans-synaptic transfer relating mostly to the exocytosis of
ADAM10 clustering of CD40L at the cell-SLB interface. Expectedly, (Supplementary Fig. 6q), consistent with the reduced total upregulation of cell surface CD40L upon TCR-triggering comparison.

and statistical means ± SEM of k gp120-presenting BSLBs. Transfer between control BSLBs and BSLBs decorated with different juxtacrine signals (Fig. 4 Juxtacrine signals in ARTICLE)

preformed vesicles stored in MVBs. As expected, CD81 and BST2-edited cells showed a significant reduction in the transfer of their respective CD81+ and BST2+ tSV (Fig. 5d–e, respectively). Imaging of the immunological synapses formed by CRISPR/Cas9-edited cells further revealed that changes in tSV transfer to BSLBs were consistent with the interfacial clustering behavior of CD40L in the synapses of ADAM10-, CD81- and TSG101-edited cells (Fig. 5f–h). CD81-, BST2-, and TSG101-edited cells also showed a reduced Tmax for CD40L (Supplementary Fig. 6l–q). TSG101-edited cells also showed reduced Tmax for TCRab (Supplementary Fig. 6l–q) and reduced upregulation of cell surface CD40L upon TCR-triggering (Supplementary Fig. 6q), consistent with the reduced total clustering of CD40L at the cell-SLB interface. Expectedly, ADAM10-edited cells showed increased Tmax and reduced α-CD3e-Fab EC₅₀ for the release of CD40L+ tSV (Supplementary Fig. 6l–m), and a marked surface cell upregulation of CD40L following α-CD3e stimulation (Supplementary Fig. 6q). The partial reduction in CD40L transfer following downregulation of CD81 and BST2, suggests that rather than being essential, these integral membrane proteins work as aiding factors in the release of CD40L+ tSV, but not TCR+ nor CD63+ tSV. Also, the differential requirements for TSG101 among TCR+, CD40L+, and CD63+ tSV, which is phenocopied by MG132 treatment, demonstrates different ESCRT-1 requirements among tSV. The increased transfer of CD40L+ tSV from ADAM10-deficient cells also suggests minimal inclusion of cleaved ectodomains in the transferred pool of CD40L, which is also supported by our immunoblot analyses (Supplementary Fig. 1h).

tSV carry greater RNA-binding protein content and distinct miRs than EVs. The assembly of immunological synapses lasting minutes to hours not only facilitates the exchange of juxtacrine signals recruited at the cell-cell interface. Previous studies have suggested a scarce to null load of miR copies in steadily released EVs, suggesting that the transfer of single miR species via EVs might be less efficient than initially thought and that other modes of communication might favor their intercellular transfer. As observed in previous studies using human T and B-cell lines, stable cell-cell contacts also enable the exchange of miR across the synaptic cleft, suggesting that T-cell immune synapses act as facilitators of miR transfer via tSV. To evaluate this hypothesis, we performed first label-free quantifications with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify potential RNA-binding proteins (RBPs) enabling miR transfer at the IS. We analyzed tSV and EVs isolated by differential centrifugation of BSLB eluates and cell-cleared culture supernatants, respectively. Since contaminants derived from the elution reagents and procedure might impact the quality of our comparisons, we first identified proteins enriched in eluates derived from activating (α-CD3 Fab+) BSLB comparing to null BSLB (coated only with ICAM1 and CD40; Supplementary Fig. 7a). With the aid of Pegasus, we then generated a list of differentially expressed proteins to compare with the EVs from the same TH and CTL cells, respectively (Supplementary Fig. 7b–c). Using average-based normalization we then identified unique and shared proteins within the enriched datasets of tSV and EVs TH (Fig. 6a) and CTL. (Fig. 6b, see also Source Data). More detailed gene function analyses of these enriched entities...
Fig. 5 ADAM10, TSG101, CD81, and BST2 are key proteins participating in the biogenesis of CD40L+ tSV. a–e Flow cytometry analyses showing the transfer of tSV markers to BSLBs expressed as percent of the maximum transfer (Tmax%) observed in BSLBs co-cultured with CD40L-edited controls. BSLBs were reconstituted with ICAM1 (200 molec./μm²), CD40 (200 molec./μm²) and anti-CD3ε Fab (0–2000 molec./μm²). Please refer to Supplementary Fig. 1a for a representative gating strategy showing the identification of single BSLBs and T cells by flow cytometry. a Heatmaps showing Tmax% for CD40L, b TCRε, c CD63, d CD81, and e BST2 in BSLB incubated with different CRISPR/Cas9-edited cells (gRNA gene targets shown in italic). f TIRFM quantification of normalized medians for the integrated fluorescence intensities of CD40L within synapses formed by CRISPR/Cas9-edited cells. Circles represent the integrated fluorescence median per donor per CRISPR/cas9 target normalized to its internal control (teal). CD81− (violet circles), ADAM10− (orange circles), BST2− (dark red circles), and TSG101−edited cells (green circles) were imaged with TIRFM. g Representative TIRFM images (g) showing the levels of centrally clustered CD40L in synapses of CRISPR/Cas9-edited cells imaged as depicted in h and compared with CD40L-edited controls. Scale bar = 5 μm. MFI = Mean Fluorescence Intensity, arb. units = arbitrary fluorescence units. IRM = internal reflection microscopy. Data are shown as means ± SEM from n = 10 (a–e), and 7 (f) biologically independent samples (donors) across five independent experiments. In a–e, normality was determined using Shapiro–Wilk test, and statistical significance was determined by parametric two-tailed Multiple t test with Holm–Sidák correction (α = 0.05) for the multiple comparisons of Tmax% across different anti-CD3ε Fab densities and between CRISPR/Cas9-edited cells and CD40L-edited controls (a–e), and by one-way ANOVA with mixed-effect analysis and Fisher’s LSD test (95% CI) to compare separately each CRISPR/Cas9-edited cells to controls (f). Values represent calculated P values for each comparison to control.

using PANTHER48 further revealed that compared with EVs, tSV contained a greater abundance of translational and RNA metabolism proteins in both TH and CTL (Fig. 6c–f). Furthermore, gene set enrichment analyses (GSEA) with PANTHER revealed an enrichment of nucleic acid-binding, including messenger RNA-binding and DNA/RNA helicase activities, in the molecular function categories of tSV compared with EVs (Fig. 6g–j). Consistent with the participation of ESCRT-I on the release of tSV, ubiquitin ligase and ubiquitin processing activities stood out as enriched gene ontology categories in tSV (Fig. 6g–j). Other differences included the negligible detection of ADAM10 and the contaminants Histone 3, LGALS3BP49, and RNase 4 in the tSV of TH and CTLs, and a greater detection of known miR targets in tSV (Supplementary Fig. 7d, e, respectively). Importantly, we identified YBX1, a known protein packing small noncoding RNAs (snRNAs) in EVs50,51 as an enriched protein in tSVs (TH > CTL; Supplementary Fig. 7f), as well as in synaptic ectosomes tethered to sorted BSLBs (with > 3-fold enrichment; see ref. 5). Other RNA-binding proteins were also commonly identified in independent LC-MS/MS runs of eluted tSV and subpopulations of SE anchored to sorted BSLB (Supplementary Fig. 8a). To validate our LC-MS/MS findings, we then performed TIRFM imaging of TH cell synapses and localized YBX1 and, in a lesser extent, SF3B3 in both the synaptic cleft and the proximal membrane of T cells interacting with α-CD3ε presenting SLB (Supplementary Fig. 8b, c, respectively, compare with isotype control shown in Supplementary Fig. 8d). Also, YBX1 localized in vesicular tracks left behind by cells spontaneously breaking synapse symmetry and resuming migration (Supplementary Fig. 8c, white arrows).

We next sought to evaluate whether the identification of RNA-binding proteins in tSV related to the release of tSV-containing RNA. We used RNAsSelect52, a probe fluorogenic upon binding to RNA, to selectively track the mobilization of RNA+ structures to the synaptic pole and its release as part of tSV. We also used BODIPY-TR Ceramide to follow membrane structures associated with RNA. Supporting the active release of RBPs, we further found the polarization of ER-like structures and release of ceramide+ and RNA+ puncta in both the synaptic cleft and in vesicular tracks of live, non-permeabilized TH IS (Supplementary Fig. 8e–f, yellow arrows). As evidenced by the super-resolution imaging with eTIRF-SIM, most RNA puncta lined the periphery of the synaptic cleft as early as 5 min during synapse assembly, indicating the rapid mobilization of RNA+ compartments to the contacting membrane of stimulated T cells (Supplementary Fig. 8f, yellow arrows).

The identification of RBP and RNA transfer across the synaptic cleft supports the notion of the IS as a hub facilitating the transfer of RNA effectors, such as miR. Therefore, we sought to evaluate
whether tSV represents an alternative mechanism of miR transfer by profiling the RNA content deposited on BSLBs. To minimize cell contamination, we isolated BSLB coated with TCR+ tSV using fluorescence-activated cell sorting of single BSLBs and single cells after 90 min of co-culture (Supplementary Fig. 9a, b), followed by small RNA purification. Small RNAs were also purified from EVs from the same donors as controls. Prior to comparing the RNA species identified in EVs and tSV, we used the less abundant RNA species found in null BSLBs as background for the identification of miR enriched in tSV (Supplementary Fig. 9c). Analyses of tSV and EVs libraries’ raw reads revealed a similar composition of small RNA species, of which miRs were overall more enriched in tSV than EVs and the parental cells (Fig. 7a). After identifying small RNA species conserved across samples, donors, and sequencing runs, GSEA further revealed public and tSV- or EVs-specific miRs in both TH and CTL. Of 200 miR species enriched in TH, 11 were uniquely enriched in tSV and 23 in EVs (Fig. 7b), whereas of 163 miR species enriched in CTL, 17 were unique to tSV and 19 to EVs (Fig. 7c and Supplementary Data 3). Although EVs showed slightly more miR species (Fig. 7b, c), the overall percent enrichment of these small RNA species was higher in tSV (Fig. 7a). Consistent with the wider variety of enriched miR in EVs, we observed a higher variance compared to tSV (Fig. 7d). Analyses using MEME further identified unique and shared miR species in tSV (Fig. 7e, g) and EVs (Fig. 7f, h), which together redundantly associated with immune cell signaling and anti-tumor immunity regulation in both TH and CTLs (Fig. 7i and heatmaps in Supplementary Fig. 9d–g). Congruently, miR-target enrichment analyses revealed a similar degree of redundancy in the targets of enriched miR (see Supplementary Data 4 for the full list of targets and Supplementary Fig. 9h–i for gene ontology categories). Importantly, from the list of predicted targets, AGO2, GRB2, GRAP2, RRM2, and YBX1 were co-identified in the proteomic datasets of tSV (Supplementary Fig. 7e and 8a), suggesting the transfer of the second layer of information in the form of ribonucleoprotein (RNP) complexes associated to tSV. Altogether, our data suggest that the chain of molecular events unfolding at stable cell-cell contacts facilitates the acute and multimodal delivery of a critical mass of transcellular signals.

**Discussion**

The first observation of T cells being successfully activated by bead-supported bilayers presenting antigenic MHC-II dates back to 1986. Others have demonstrated the versatility of BSLB as APCs in other experimental contexts, including measuring the dimensions of interfacial receptor-ligand interactions and the localization of lipid species and CD3ε in the interacting pole of T cells. Here BSLBs facilitated novel comparisons of tSV and EVs, providing evidence for highly heterogeneous yet unique populations of vesicles being released in the limited dwell-time of the IS. We corroborated quantitative differences in size, protein loads and association to RBP and miR between tSV and EVs. Furthermore, we also found that the rate of secretion is greater for tSV compared with EVs. We have previously shown that TH shed an approximate of 25–30 TCRαβ+ synapic ecotomes after 20 min of synapse formation on SLB. In contrast, the quantification of steadily released TCRαβ+ EVs by NanoFCM evidenced their rather low constitutive secretion ranging from a minimum of 0.54 to a maximum of 8.96 EVs/cell over a period of 48 h of culture (please refer to Source Data).

The acute pharmacological inhibition and CRISPR/Cas9-editing of TH strongly indicated that, mechanistically, tSV heterogeneity is explained by contributions of several cellular types of machinery recruited at the interacting pole of activated T cells. We provide evidence for an overarching hierarchy in the

**Fig. 6** tSV are enriched in proteins related to ubiquitination and RNA-binding proteins. a–b Venn-diagrams showing the overlap of enriched proteins identified in the tSV and EVs of TH (a) and CTL (b). c–f Pie charts showing the enriched protein classes identified with PANTHER in the tSV of TH (c) and CTL (d), and in their syngeneic EV counterparts (e–f). g–k GSEA using PANTHER revealed a greater enrichment of molecular function categories related to nucleic acid-binding and ubiquitination in the proteomics of tSV derived from TH (g) and CTL (h) as compared with syngeneic EVs of TH (i) and CTls (j). Data from n = 4 biologically independent samples (donors, a–j) across four independent experiments. Statistical analysis for functional and biological pathway enrichment analyses (c–f) using PANTHER was performed by the Fisher’s exact test and the Benjamini–Hochberg False Discovery Rate (FDR) correction using default Q values. In g–j, the result of GO enrichment analyses were filtered using Funnet with the Benjamini-Hochberg correction (FDR threshold to 0.05) on P values determined by the hypergeometric test.

**Top 20 Enriched GO terms**

| Fold Enrichment Score | TH tSV | CTL tSV | TH EVs | CTL EVs |
|-----------------------|--------|---------|--------|---------|
| GO:0036402            |        |         |        |         |
| GO:0033677            |        |         |        |         |
| GO:0043138            |        |         |        |         |
| GO:0044183            |        |         |        |         |
| GO:0070182            |        |         |        |         |
| GO:0097100            |        |         |        |         |
| GO:0048027            |        |         |        |         |
| GO:0008641            |        |         |        |         |
| GO:0055106            |        |         |        |         |
| GO:0055105            |        |         |        |         |
| GO:0004839            |        |         |        |         |
| GO:0051920            |        |         |        |         |
| GO:0034190            |        |         |        |         |
| GO:0003688            |        |         |        |         |
| GO:0003988            |        |         |        |         |
| GO:0047134            |        |         |        |         |
| GO:0004731            |        |         |        |         |
| GO:0005094            |        |         |        |         |
| GO:0055102            |        |         |        |         |
| GO:0051920            |        |         |        |         |
| GO:0023026            |        |         |        |         |
| GO:0023026            |        |         |        |         |
| GO:00150              |        |         |        |         |
| GO:00219              |        |         |        |         |
| GO:00077              |        |         |        |         |
| GO:00090              |        |         |        |         |
| GO:00060              |        |         |        |         |
| GO:00207              |        |         |        |         |
| GO:0008187            |        |         |        |         |
| GO:0044183            |        |         |        |         |
| GO:0097100            |        |         |        |         |
| GO:0048027            |        |         |        |         |
| GO:0008641            |        |         |        |         |
| GO:0055106            |        |         |        |         |
| GO:0055105            |        |         |        |         |
| GO:0004839            |        |         |        |         |
| GO:0051920            |        |         |        |         |
| GO:0034190            |        |         |        |         |
| GO:0003688            |        |         |        |         |
| GO:0003988            |        |         |        |         |
| GO:0047134            |        |         |        |         |
| GO:0004731            |        |         |        |         |
| GO:0005094            |        |         |        |         |
| GO:0055102            |        |         |        |         |
| GO:0051920            |        |         |        |         |
mechanisms participating in the vesicular transfer of CD40L, with dominant participation of ER/TGN transport and ESCRT machinery for its acute mobilization to the plasma membrane, and secondary involvement of MVB and lysosomes. The selective increase in TCR$^+$ tSV transfer resulting from dynamin inhibition and the reduced effect of ESCRT-inhibition/downregulation on CD63$^+$ tSV transfer provide additional evidence for different sorting mechanisms participating in the biogenesis of tSV (Supplementary Figs. 3f–i and Fig. 5). We provide evidence for a critical role of ADAM10, a protease known to be active at the plasma membrane, in regulating the pool of surface CD40L and the resulting levels of CD40L$^+$ tSV transferred dependent on CD40. Interestingly, our LC-MS/MS analyses indicate that compared to tSV, EVs incorporate a higher amount of ADAM10 (Supplementary Fig. 7d), explaining their reduced CD40L content (Fig. 1k-l).

Microvilli lead the formation of effector membranous particles referred to as synaptosomes enriched in LFA-1, CD2 and cytokines\cite{57}. Microvilli tips accumulate proteins including TCR, CD2, and CD4\cite{57,58}. Here we show that T cells transfer little vesicular CD2 and CD4 (Figs. 1b and 2b–c), which increased at limited levels only upon ligation by CD58 and HIV-1 gp120.

**Fig. 7** tSV are enriched in miRs with considerable functional equivalence to EVs miRs. a Abundance of different genome-mapped RNA species was found associated with tSV, syngeneic EVs, and parental TH or CTL cells. b–c Venn diagram showing the overlap of enriched miR species associated with tSV, EVs, and parental cells for TH (b) and CTL (c) (see also Supplementary Data 3). d miR heterogeneity is further reflected by PCA of the analyzed tSV and EVs. e–h MEME analyses revealed consensus motifs (central inserts) mapped in miRs of TH tSV (e), TH EVs (f), CTL tSV (g), and CTL EVs (h). i Number of miR species enriched uniquely in tSV (violet), uniquely in EVs (green), or shared (gray) annotated according to their GO categories. MiR targets are summarized in Supplementary Data 4. Data from $n=8$ biologically independent samples (donors, a–i) from six independent experiments. For functional and biological pathway enrichment analyses using MIENTURNET (s) $P$ values were determined by hypergeometric statistic test and FDR ($Q=0.05$).
respective (Fig. 4b, j–k). Also, TCR signaling, which is required for tSV biogenesis, is known to produce microvilli disassembly38, suggesting that synaptosomes differ in origin and structure from synaptic ectosomes and the overall output of tSV. Rather, synaptosome-like structures might mediate the negligible release of extracellular vesicle-like material to BSLBs sustaining cell adhesion but devoid of antigen/a-CD3 Fab (Fig. 1k).

TSV biogenesis is a highly dynamic process connecting the integration of signals presented by APCs with the particulate output of activated T cells. Earlier electron microscopy studies demonstrated budding of TCR+ SE with partial polarization of MVB and TGN in stimulated cells (Fig. 1c in ref. 5). In these experimental settings, SLB lacked CD40, which as we later demonstrated, promotes quick mobilization of CD40L to the plasma membrane and into budding SE as microclusters5. Here we provide evidence that shedding of CD40L+ tSV requires CD40 on presenting BSLBs and the rapid relocation of CD40L from the ER-Golgi, which is readily detected by 90 min of interaction between T cells and BSLBs (Supplementary Figures 4b and 6q). We provide evidence of other signals finely tuning TSV heterogeneity, including B7 receptors (CD80/86), PD-1 ligands, other TNF/CSF members, and non-native ligands. For instance, HIV-1 gp120 promoted the vesicular transfer of CD4 and CD40L at low physiological densities of a-CD3e Fab. Similarly, the non-native interaction between CARs and antigenic HLA complexes led to a remarkably efficient transfer of CAR as part of tSV and at the expense of its cell surface expression levels (Supplementary Fig. 5d, red arrow). The superior vesicular shedding of CAR containing CD3ε signaling tails might result from rewired TCR signaling networks and the formation of multiple short-lived synapses59. Since reduced CAR expression might desensitize CART to new encounters with target cells, more research is needed to understand the impact of CAR release on the therapeutic effectiveness of CART therapies.

BSLBs made possible the first side-by-side comparison of the protein and ncRNA composition of tSV and EVs. While shared and vesicle-specific miR species were identified in tSV and EVs, RBPs were significantly more enriched in tSV (Fig. 6g–j and Supplementary Fig. 7f–i). We speculate that the high level of target redundancy found in enriched miRs of tSV and EVs might synergize in the functional modulation of recipient cells. If true, such synergism among miR could circumvent the limitation imposed by only few copies of single miR species transferred associated with EVs46,47. Additionally, the acute delivery of tens of vesicles at the immune synapse might help promote the delivery of a critical mass of miRs and their partner proteins to effectively interfere target mRNAs. How the integration of juxtracrine signals, such as antigen density (and TCR affinity) and co-stimulation/co-repression, shapes the composition and loads of miR and RBPs transfer across cell-cell contacts requires further investigation.

Since miR targets participate in cell cycle regulation, senescence, and immune receptor signaling, the release of tSV might also promote the clearance of miR species controlling various aspects of the T-cell effector response. Interestingly, telomeric binding proteins were also found among the enriched GO terms of TH tSV (please refer to Source Data of Fig. 6g–j) and the differential expression analyses of synaptic ectosome proteins5. Whether the transfer of miRs, and to a lesser extent telomeres, in tSV contributes to the paracrine regulation of senescence, as preliminarily shown for APCs transferring telomeres56, remains to be elucidated.

The intrinsic feed-forward function of tSV-enriched immune receptors and miRs, specifically and timely released in response to the antigen is different in kind and structure to other signals integrated during early T-cell activation. Therefore, we consider that the heterogeneity and effector diversity of the particulate output of T-cell synapses, including tSV and SMAPs, deserves its functional classification as signal four. We expect BSLB will help others in the study of other key drivers of signal four-dependent communications. Here we provide numerous examples of how BSLBs can be tailored to model the effects of different juxtacrine signals on the composition and release of tSV. However, compared to real APCs, BSLBs lack the trogocytic and trans-endocytic machinery required to engulf large and small membrane fragments across the synaptic cleft5–7,11,61. While this has the advantage of specifically addressing molecules transferred as part of T-cell tSV, is limited in predicting the exchange of molecules resulting from phagocytic cells nibbling the plasma membrane of T cells during transient contacts.

Modeling different immune synapses and tSVs also requires considering the kinetics of interaction between T cells and different APCs, such as B cells and DCs. In vivo imaging studies have shown that compared to the interaction with DCs, which last up to several hours52–54, the interaction between T cells and B cells last from a few minutes to an hour65. BSLBs can be co-cultured with T cells in different time scales from 1 (Fig. 3c) to 24 h (Supplementary Fig. 10ac). In fact, in BSLB-T-cell co-cultures mimicking prolonged DC-T-cell interactions, BSLB efficiently capture T-cell membrane alongside the tSV markers TCR and CD81 (Supplementary Fig. 10ac). In contrast, mo- and polycytotically-derived DCs (moDCs) co-cultured with T cells acquire T-cell trans-synaptic material (i.e., antigen-driven) within minutes of synapse formation (Supplementary Fig. 11a–b), but also membrane fragments consistent with the engulfment of EVs and trogocytosis. After 24 h of culture, antigen-null moDCs engulfed TH membrane fragments despite failing at inducing the formation of stable and polarized contacts with TH cells (Supplementary Fig. 11c–e). In contrast, after 24 h of co-culture, antigen-dependent contacts led to a greater acquisition of T-cell membrane and significant activation of moDCs as evidenced by the upregulation of ICAM1, CD86, and CD40 (Supplementary Fig. 11f–i). Remarkably, the latter replicated the activation of moDCs using synthetic tSV carrying physiological densities of CD40L5 and correlated with significantly higher CD40L surface expression on moDCs (Supplementary Fig. 11i), suggesting that activation resulting from the acquisition of CD40L+ tSV is superior to that resulting from trogocytosis or the uptake of constitutive EVs in cell-cell culture systems. Thus, our findings suggest that tSV are superior cell-cell communication entities, highlighting the relevance of their direct analysis and modeling with BSLBs.

Undoubtedly, the comparison of EVs and tSVs in other immune cells known to secrete highly heterogeneous EVs, such as DCs66, or in cells using similar interfacial communications is needed to elucidate whether tSV are pivotal information units of cellular networks. Pathogen evolution has co-opted the cellular machineries mediating tSV release. For instance, the HIV-1 Gag protein facilitates the transfer of viral information among interacting cells by co-opting the budding machinery of synaptic ectosomes6,67,68. We expect BSLBs will also help unraveling new pathogenic determinants hijacking the cellular machinery required for tSV shedding, broadening our understanding of ‘troyan horses’67 driving infectious diseases.

**Methods**

**Ethics.** Different T-cell populations were isolated from leukapheresis reduction system (LRS) chambers from de-identified, non-clinical healthy donors. The non-clinical issue division of the National Health Service and the Inter-Divisional Research Ethics Committee of the University of Oxford (RECs 11/H07117/7 and 12 NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-31160-3 | www.nature.com/naturecommunications
Production of chimeric antigen-receptor (CAR) recombinant CD8+ T cells. Human T-cells were isolated from peripheral blood using a FACSAria III cell sorter. A nested gating strategy was performed after pre-gating of T cells. Of the total CD8+ T-cells and CD8+ T-cells were expanded using a bead-to-cell ratio of 1:1 and 100 U/mL of recombinant human IL-2 (Peprotech). Fresh IL-2 containing media was added every 48 h and Human T-activator CD3/CD28 Dynabeads were added again at day 7 and removed at day 15 of expansion. This protocol allowed us the enhanced recovery of FoxP3+ T cells (by day 15) and CD127low CD25high cells were gated such that only the brightest 3% of CD25high cells were sorted (Supplementary Figure S5a). Recovered cells were stimulated with Human T-activator CD3/CD28 Dynabeads (Thermo-Fisher) at a bead-to-cell ratio of 3:1 and 1000 U/mL of recombinant human IL-2 (Peprotech). Washing with HBS/HSA after 30 min. T cells (2.5 × 10⁵/well) were incubated with peripheral blood cells using a FACSAria III cell sorter. A nested gating strategy was performed after pre-gating of T cells. Of the total CD8+ T-cells and CD8+ T-cells were expanded using a bead-to-cell ratio of 1:1 and 100 U/mL of IL-2. After 3 days of activation, Human T-activator CD3/CD28 Dynabeads were removed and the conventional CD4+ and CD8+ T-cells kept in complete RPMI media supplemented with 100 U/mL of IL-2. All BSLB synaptic transfer experiments were performed after pre-gating of T cells. CD4+ depleted complete RPMI 1640 media containing 10% of either heat-inactivated AB human serum (Treg & controls) or fetal bovine serum, and 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.

Culture of CD4+ T-cell clones. The HLA-DRB1*0901-restricted T-cell clones 16, 35, and 40 (all specific antigen epitope influenza H3 HA133–155 peptide NPVEKQPTGIFGAAIGFI) were expanded using a ratio of 1:1:2 feeder cells (irradiated, pooled PBMCs from 2-3 healthy donors) at a total cell concentration of 3 × 10⁶ cells/mL in RPMI 1640 supplemented with 10% heat-inactivated AB human serum and 30 μg/mL of PHA for three days. Then, 100 U/mL of recombinant human IL-2 were added to fresh media, which was replaced every 2 days. Clones were used between days 8 and 12 of culture.

Calibration of flow cytometry data. T cells and BSLB were analyzed using antibodies with known fluorescent dye to Ab ratio (F/P) in parallel with the Quantum MESF beads (Bangs Laboratories, Inc. IN, USA), allowing the calculation of the absolute number of antibodies bound per T-cell and per BSLB after subtraction of unspecific signals given by isotype control antibodies. We used MESF standard beads labeled with the Alexa Fluor® dyes 488 and 647 to estimate number of dye molecules from mean fluorescence intensities (corrected and geometric, GMFI). Briefly, MESF beads provided five different populations of beads with increasing GMFI allowing the linear regression of corrected GMFI (cGMFI) over MESF. The resulting slope is then used for the interpolation of total fluorochromes bound to either BSLB or cells from cGMFI values. Since we also used antibodies with known fluorochromes per protein (F/P), we then estimated the number of bound antibodies (and hence molecules) per BSLB by dividing the estimated fluorescence number by the detection antibody F/P value (Number of molec./event = Fluorescence molec/molecule/F/P).

Trans-synaptic vesicle elution from BSLBs. Cells and BSLBs were incubated at a 1:1 ratio for 90 min at 37 °C and 5% CO₂ in Phenol Red-free FBS-free RPMI 1640 supplemented with 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. Cultures needed to be scaled up and therefore a CO₂ incubator and supplemented FBS-free, Phenol Red-free RPMI 1640 was used instead of 1%HSA/HBS. Cells and BSLBs were inspected at different time points of the elution protocol to control with FCM both the transfer of material from T cells to BSLB, and the release of tSV from BSLB upon addition of EDTA for the chelation of Ni. Briefly, elution was performed as follows; first conjugates were cooled down 15 min at RT and then 40 min on ice to gently separate cells and BSLB. Then, conjugate suspensions were resuspended by adding three volumes of ice-cold PBS pH 7.4 and centrifuged at 100 × g for 1 min and +4 °C to enrich for BSLB. After two washes with ice-cold PBS pH 7.4, BSLB were resuspended in ice-cold 50 mM EDTA in PBS pH 7.4 for 2 h. BSLB-free supernatants were then centrifuged at +4 °C in sequential steps at 300 × g for 5 min (twice), then at 2000 × g for 10 min and 10,000 × g for 10 min. Finally, the pellet was washed once more with PBS pH 7.4 and kept at +4 °C or frozen at −80 °C until analyses by either NTA, NanoFCM or immunoblotting. Stably released EVs were isolated by the same differential centrifugation procedure indicated above. To minimize serum and debris contamination, T cells were cultured in OptiMEM-I supplemented with 100 U/mL of IL-2, 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 for no longer than 48 h. All samples isolated by UC
were resuspended in 0.22-µm-filtered PBS pH 7.4 to reduce background signals in downstream particle measurement analyses with either nanoFCM or NTA.

**TEM.** Negative staining of thinly spread vesicle preparations for transmission electron microscopy (TEM) was performed as follows: briefly, carbon support film-coated 3 mm copper grids (300 mesh) were plasma treated for 20 s using a Leica EM ACE200 Vacuum Coater. Then, 10 µL of isolated vesicle populations were deposited on and incubated at RT for 5 min, followed by removal of the excess sample with a Whatman N°1 paper and staining with 2% uranyl acetate for 10 s at RT. After removal of excess uranyl acetate, the samples were dried for 10 min and analyzed using a Tecnai 12 TEM at 120 kV using a Gatan OneView CMOS camera. A final magnification of x29,000 was used for imaging isolated vesicle populations.

**NTA.** Eluted tSV and purified EV preparations were resuspended in 0.22-µm-filtered PBS pH 7.4 in a 1:100 dilution and kept on ice for NTA. The instrument used for NTA was NanoFCM (NanoFCM Inc., Xiamen, China) that allows single exosomes detection was used to determine the size distribution and granular concentration of EVs. The Nano-flow cytometry analysis was performed using the Flow NanoAnalyzer (NanoFCM Co., LTD) according to manufacturer’s instructions. The Silica Nanospheres Cocktail (S16M-Exo, NanoFCM) was employed as the size standard to construct a calibration curve to measure the absolute particle size of EVs. The concentration standard (200 nm PS QC beads, NanoFCM) was used to measure particle concentration. The laser used was a 488 nm laser at 25/40 mW, 10% ss decay. Lens filters were 525/40 (AF488) and 580/40 (PE). Before staining samples were acquired to determine particle concentration such that a total of 106 vesicles of either tSV or EVs were labeled on condition. Before use, fluorescence-conjugated antibodies were spun down at 10,000 × g for 10 min at +4 °C. Isotype control antibodies were used at the same effective concentrations (ranging from 0.5 to 1.5 µg/mL) and incubation times. Staining antibodies were conjugated to AF488 and AF647. Staining was performed for 30 min on ice. Samples were washed and centrifuged at 1 × 106 g and +4 °C. Labelled vesicles were then resuspended in 50 µL of PBS. Buffer alone (PBS), unstained vesicle, isotype controls, and auto thresholding were used to define positivity.

**Planar supported lipid bilayers (PSLB).** Liposomes mixtures were injectted into flow chambers formed by sealing acid piranha and plasma-cleaned glass coverslips to adhesive-backed plastic manifolds with 6 flow channels (SlideSlide V1.0; Ibi). After 30 min the channels were flushed with HBS-HSA without introducing air bubbles to remove excess Liposomes. After blocking for 20 min with 5% casesein supplemented with 100 µM NiCl2, to saturate NTA sites, followed by washing and then His-tagged proteins were incubated on bilayers for an additional 30 min. Protein concentrations required to achieve desired densities on bilayers were calculated from calibration curves constructed from FC measurements on BSLB and analyzed alongside MESF beads (MESF: Quantum™ Bangs Laboratories Inc.). Bilayers were continuous liquid dissorded phase as determined by fluorescence recovery after photobleaching with a 10 µm bleach spot on an FV1200 confocal microscope (Olympus).

**Immunosynephas formation on glass-SLB.** Prior to immunological synapse imaging, primary T-cell blasts were washed twice and resuspended in prewarmed HBSA buffer to a final concentration of 5 × 105 cells/mL. Then, 5 × 105 T cells were stimulated with 20 min at 37 °C and 5% CO2 over PSLB containing 300 µmol/µL anti-CD3e Fab (clone UCHT-1; Alexa Fluor® (AF) 488 or unlabelled), 200 µmol/µL of ICA1 anti-CD5, and 200 µmol/µL of CD40 (AF488 or unlabelled). The cells were stained with 1 µL of wheat germ agglutinin (WGA) conjugated to CF568 (Biotium; #29077-1) and 1 µg/ml anti-CD154 (BioLegend, #310818), anti-human CD40L AF647 clone 24 (BioLegend, #305442), and 5-µm-diameter 0.22-µm-filtered CA2+ (BioLegend, #314393), anti-human CD4 SV650 clone OKT4 (BioLegend, #317436), anti-human CD40 AF647 clone 24–31 (BioLegend, #310818), anti-human CD54 (ICAM1) BV711 clone H5A8 (BD Horizon, #564078), and anti-human YB1 (YBX1) clone EPR22682-2 (ab1525606), anti-human SF3B3 clone EPR81440 (ab209402), and Rabbit isotype control EPR25A AF647 (#ab199093). After three washes, cells were blocked with 0.2-µm-filtered 5% Donkey Serum for 1 h at RT before staining with 1 h at AF647 AffiniPure F(ab’)2, Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson Immunoresearch, #111-068-152). After two washes and then stained with anti-Vimentin clone EPR3776 AF555 (#ab203428). Cells were washed four times before acquisition. To mask the distribution of RNA and membrane containing structures within the Immune Synapse, T cells were labeled with BODIPY-TR Ceramide complexed to BSA and SYTO RNASelect on days 7 to 10 of culture. Resuspended T cells were stained with PBS containing 20 µM of SYTO RNASelect in PBS containing 10 mM HEPES and 5 µM of SYTO RNASelect (Invitrogen, #S32703) and 170 µg/ml of BODIPY-TR Ceramide complexed to BSA (Invitrogen, #B34400). After incubation for 20 min at 37 °C, cells were washed twice with 10 volumes of fully supplemented Phenol Red-free RPMI 1640 (BioLegend, #217710-2) and centrifugation steps of 300 × g for 10 min at RT. BODIPY-TR and SYTO RNASelect-labeled T cells were resuspended in PBS for 10 min at 37 °C. Cells were then washed twice with 10 volumes of fully supplemented Phenol Red-free RPMI 1640 (BioLegend, #217710-2) and centrifugation steps of 300 × g for 10 min at RT. BODIPY-TR labeled T cells were resuspended overnight before co-culturing with monocyte-derived DCs (moDCs). On day 7 of culture, half of the moDCs were incubated for 2 h at 37 °C and 5% CO2 with 100 ng/ml of Staphylococcal Super antigen B (SEB)^2. Then, moDCs and T cells were counted, washed and co-cultured at a 1:1 ratio for either 90 min or 24 h at 37 °C and 5% CO2. T cells and moDCs were cooled to RT for 15 min and then incubated on ice for another 30 min. Then, co-cultures were centrifuged at 500 × g for 5 min at +4 °C and then resuspended in 0.22-µm-filtered CA2+ and Mg2+-free PBS (BioLegend, #10010-015) containing 10% FBS (heat inactivated), 2 mM EDTA and 1:5 dilution of Human TruStain FcX Receptor blocking solution (BioLegend, #223200). After a minimum of 15 min of incubation on ice, cells were stained for a minimum of 30 min on ice with a preoptimized staining panel containing anti-human CD40 PerCP-Cy5.5 clone 5C3 (BioLegend, #334316), anti-human CD4 BV650 clone OKT4 (BioLegend, #317436), anti-human CD40L AF647 clone 24–31 (BioLegend, #310818), anti-human CD54 (ICAM1) BV711 clone H5A8 (BD Horizon, #564078), anti-CD11c AF647 clone 5D9 (BioLegend, #310818), anti-CD14 BV650 clone 15B9 (BioLegend, #310818), and anti-human CD54 (ICAM1) BV711 clone H5A8 (BD Horizon, #564078) and 5-µm-diameter 0.22-µm Filtered CA2+ and Mg2+-free PBS (BioLegend, #305442). Cells were washed three times using ice-cold PBS and acquired immediately using the Aurora Spectral Flow Cytometer and the SpectroFlo acquisition software (Cytrek Biosciences) equipped with 5-lasers (UV/V/BG/R) and an automated sample loader. Analysis of flow cytometry data was performed with FlowJo (v10.8.1).

**Airyscan confocal microscopy.** Airyscan imaging of T-cell and SLB was performed on a Zeiss Axio Observer Z1 LSM 980 confocal lens-scanning microscope equipped with an Airyscan 2 module (Zeiss, Oberkochen, Germany) consisting of 32 concentrically arranged GaAsP PMT detectors and 2 MA-PMT channels. The acquisition was performed using the Airyscan super-resolution (SR) and best signal Smart setup and a C Plan-Apochromat ×63/1.4 NA Oil DIC magnification objective. Illumination was provided by a Solid-State Light Source Colibri 7 LED lamp and by Diode lasers at 639 nm, 594 nm, and 488 nm with 0.4% laser power and 850 V detector gain for all channels. The imaging field was defined using a 6.0x scan zoom (crop area) and a Z-coverage spanning the totality of the synaptic cleft as defined by WGA staining. The final acquisition settings included a sequential acquisition in the order 647/594/488, a frame size of 528 × 528 px, a pixel time of 7.95 µs, a pixel size of 0.043 × 0.043 mm, and a doubled pixel sampling width. The immunofluorescence was captured using x20 magnification and the super-resolution SRAy mode and frames were acquired every 30 s. Analyses were performed using the ZEN 3.2 system edition (Carl Zeiss Microscopy GmbH) and Fiji v2.1.0.1353 (build 52f2140693)^3.**

**eTIRF-SIM.** A custom-built eTIRF-SIM microscope was used and detailed elsewhere. Structured illumination was obtained via a grating pattern generated

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by a ferroelectric spatial light modulator (SLM, Form Dimension Displays, QXGA3DM). After, the first diffraction orders are selected by the mask and sent to the Olympus BX-51 microscope head. The distance between diffraction zones was tuned by SLM settings ultimately defining the illumination angle and therefore the TIRF depth. Excitation wavelengths of 488 nm, 560 nm, and 640 nm were used (MPB communications, Inc., 500 mW, 2RU-VFL-P-500-488-BIR, 2RU-VFL-P-500-560-BIR). Sample illumination was performed using a high-SN TIRF objective (Olympus Plan-Apochromat 100 × 1.49 NA). The emitted fluorescence was collected by the same objective and sent onto sCMOS cameras recording the raw data (Hamamatsu, Orca Flash 4.0 v2 sCMOS). The excitation numerical aperture (NA) was adjusted for each wavelength by changing the period of the grating etched on the SLM, which allows controlling the TIRF angle and therefore, the penetration depth of the evanescent wave. To achieve TIRF-SIM illumination at the interface between the cells and SLB, both excitation lights were sent with an incident NA ranging from 1.38 to 1.41. Prior to experiment acquisition alignment was performed by imaging 100 nm Tetraspec fluorescent beads in all three excitation colors. Then, using Fiji plugin MultiStackReg – the beads images were used to adjust images and compensate for chromatic aberration. A total of 9 raw images were acquired per frame and for a single excitation wavelength before switching to the next wavelength. Then, raw images were processed and reconstructed into SIM images by custom made software or ImageJ fastSIM plugin. All experiments were performed at physiological conditions using a micro-incubator (H301, Okolabs, Italy) at 37 °C and 5% CO₂. For each frame, we used an acquisition time between 20 and 200 ms depending on the fluorescence signal levels and 2 colors, 18 frames (total) every 0.4–5 s. One color super-resolved image was reconstructed from 9 raw image frames (3 angles and 3 phases) using a reconstruction method described previously75,76.

**CRISPR/Cas9 editing of primary human T cells.** The ablation of genes of interest was achieved by transfection with pre-assembled CRISPR complexes. Prior to transfection, and after 48 h of stimulation with Human T-activator CD3/CD28+ or the ECM830 Square Wave electroporator. Immediately after transfection, cells were prepared in the Image Studio function and biological pathway analysis of identified contaminants database using tryptic specific files and integrity analyzed using PANTHER48 following Fisher using Illumina NextSeq 500/550 v2.5 Kits (75 cycles). Quality control and processing of the raw sequencing data were performed using sRNAtoolboxDB, miRQC79 and sRNAtoolboxDB, which allowed the assessment of sequencing yield, quality control and read-length dependent abundance of fragments from other RNA species. Functional and biological pathway enrichment analyses were performed on annotated miR species shared between the two independent sequencing runs and enriched in each EV category (i.e., n5v and EVs); MIENTURNET and the KEGG annotation database were used. Statistical analysis for functional enrichment by biological pathways using MIENTURNET was carried out by calculating the adjusted P value resulting from the hypergeometric statistical test (false discovery rate (FDR), Q = 0.05). Motif analyses of bulk, enriched miRNA sequences in each sample were performed using MEME (Multiple Em for Motif Elucidation 5.10)75. To dissect the miR-target gene interactions of our enriched miRNA repertoire, miRNet 2.08 was used to the miR interaction knowledgebase comprising annotations from mirbase, miTarBase, and ExoCarta together with interactions with other miR, genes, and transcription factors from TransmiR 2.0, ENCODE, JASPAR, and ChEA. The output of the ‘miRs’ module of miRNet 2.0 provided the motif miR-target gene interaction enrichment analysis.

**Mass spectrometry.** Samples isolated by differential centrifugation were concentrated using 100,000 NMWL centrifugal filters (Amicon® Ultra, # UFC510024, Merck Millipore Ltd.) and prepared in 5-Trap™ spin columns (#C02-micro-80)78 following manufacturer recommendations. Briefly, samples were reduced with 5 µL of 10 mM TCEP and alkylated with 50 mM of IAA for 30 min each, then acidified with 12% phosphoric acid 10:1 vol/vol, and then transferred to 5-Trap™ columns. Tryptic digestion was performed by the RNP solution for 20 and 300 ms depending on the 50% 900 × 95 °C for 10 min. For immunoblotting of BSLB eluted material, after sample lysis buffer containing a Protease/Phosphatase inhibitor cocktail (Cell Signaling Technology; #5872) to a final concentration of 2 × 10⁶ cells/mL. After sonication at +4 °C (10 cycles of 30 s on/30 s off), WCL were centrifuged at 10,000 × g for 10 min at +4 °C, and the supernatants collected, mixed with loading solution and denatured at +95 °C for 10 min. For immunoblotting of BSLB eluted material, after centrifugation at +4 °C for 4 h at +4 °C or overnight at +4 °C. WCL lysis buffer containing a Protease/Phosphatase inhibitor cocktail, mixed with loading solution and denatured at +95 °C for 10 min. When indicated, lysed elutes and EVs from an equivalent number of originating cells were resolved to compare different vesicle populations. As positive control we used WCL equivalent to 2.25 × 10⁵ CD8 T lymphoblasts. Similarly, for CRISPR/Cas9 edited cells, WCL equivalent to 3 × 10⁵ cells were used per lane. Samples were resolved using 4–15% Mini-PROTEAN SDS-PAGE gel (Bio-Rad; #4561084), transferred to 0.45 µm nitrocellulose membranes (Bio-Rad, #1620115), blocked with PBS containing 5% BSA and incubated with the following primary antibodies: rabbit anti-human CD20 (1 : 1000 dilution; Cell Signaling Technology; #1921), rabbit anti-ALIX clone EPR15314 (#ab186429), and rabbit anti-TSG101 clone EPR7130B (#ab125011). Then, membranes were incubated with IRDye® 800RD donkey anti-rabbit IgG (H+L; LI-COR, #926-80072) and IRDye® 800CW donkey anti-rabbit IgG (H+L; LI-COR, #925-32123) secondary antibodies following manufacturer guidelines. After washes, membranes were imaged and analyzed using the Odyssey® CLx Near-Infrared detection system equipped with the Image Studio Lite quantification software (LI-COR, Lincoln, NE). Uncropped, unprocessed and annotated immunoblots have been included as part of the Source Data. **RNA sequencing and miRNA analysis.** Total cell RNA was extracted using the miRNeasy Mini Kit/Cells Advanced Mini Kit (Qiagen, #217004). Purified RNA yields and quality were assessed prior to transfection and after 48 h of stimulation with Human T-activator CD3/CD28+ using Agilent 2000 RNA Pico Chips (Agilent Technologies, # 5067-1513). Before library preparation, the material was further quantified using RiboGreen (Invitrogen) on the FLUOSTar OPTIMA plate reader (BMG Labtech) and the size profile and integrity analyzed using a 4200 TapeStation. (Agilent, RNA Software). Input material was normalized to 200 ng or maximum mass for input volume prior to library preparation. Small RNA library preparation was completed using NEBNext Small RNA kit (NEW) following the manufacturer’s instructions and analyzing the input low input protocol modifications. Libraries were amplified (15 cycles) on a Tetrad (Bio-Rad) to house unique dual-indexing primers75. Size selection was performed using Pippin Prep instrument (Sage Science) using the 3% Agarose, dye-free gel with internal standards (size selection: 120 to 230 bp). Individual libraries were normalized using Qubit, and the size profile was analyzed on the 2200 or 4200 TapeStation. 10 nM libraries were denatured and further diluted prior to loading on the sequencer. Two runs of single-end sequencing were performed; read one was performed using Illumina NextSeq 500/550 v2.5 Kits (75 cycles). Quality control and processing of the raw sequencing data were performed using sRNAbench79 and miRQC79 and sRNAtoolboxDB, which allowed the assessment of sequencing yield, quality control and read-length dependent abundance of fragments from other RNA species. Functional and biological pathway enrichment analyses were performed on annotated miR species shared between the two independent sequencing runs and enriched in each EV category (i.e., n5v and EVs); MIENTURNET and the KEGG annotation database were used. Statistical analysis for functional enrichment by biological pathways using MIENTURNET was carried out by calculating the adjusted P value resulting from the hypergeometric statistical test (false discovery rate (FDR), Q = 0.05). Motif analyses of bulk, enriched miRNA sequences in each sample were performed using MEME (Multiple Em for Motif Elucidation 5.10)75. To dissect the miR-target gene interactions of our enriched miRNA repertoire, miRNet 2.08 was used to the miR interaction knowledgebase comprising annotations from mirbase, miTarBase, and ExoCarta together with interactions with other miR, genes, and transcription factors from TransmiR 2.0, ENCODE, JASPAR, and ChEA. The output of the ‘miRs’ module of miRNet 2.0 provided the motif miR-target gene interaction enrichment analysis.
Statistical analyses. Normality tests were performed using either Shapiro–Wilk or Kolmogorov–Smirnov tests. Statistical significance was determined by multiple comparisons performed either by one-way analysis of variance, or multiple two-tailed t tests. Non-linear regressions using three or four parameters F tests using least squares regression as a fitting method, with no weighting, and 95% confidence interval levels were used when indicated. All statistical tests were performed in GraphPad Prism v8 and are detailed in each figure legend. Means of significance are detailed in each figure legend.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The mass spectrometry proteomics data generated in this study have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD033917. The RNA sequencing data generated in this study have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE181216. Plasmids encoding recombinant proteins (12-His) and anti-CD3e-Fab are available upon request to the corresponding authors. Source data are provided in this paper.

Code availability

All bioinformatics analyses were performed with previously published tools and are referenced in the Methods section. The source codes for RNA sequencing and proteomic analyses were extracted from GitHub; miRNA-seq quality control (https://github.com/sert23/miRNAQC), gene set enrichment analyses with funset (https://github.com/mlhale/funset-enrichment-visualization) and ShinyGo (https://github.com/DEP-SDSU/idep/tree/master/shinyapps/gosel).

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**Competing interests**

Ben Peacock, Alice Law, and Dimitri Aubert are employed by NanoFCM Co., Ltd. The remaining authors declare no competing interests.

**Additional information**

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