Marshall et al., http://www.jcb.org/cgi/content/full/jcb.201411093/DC1

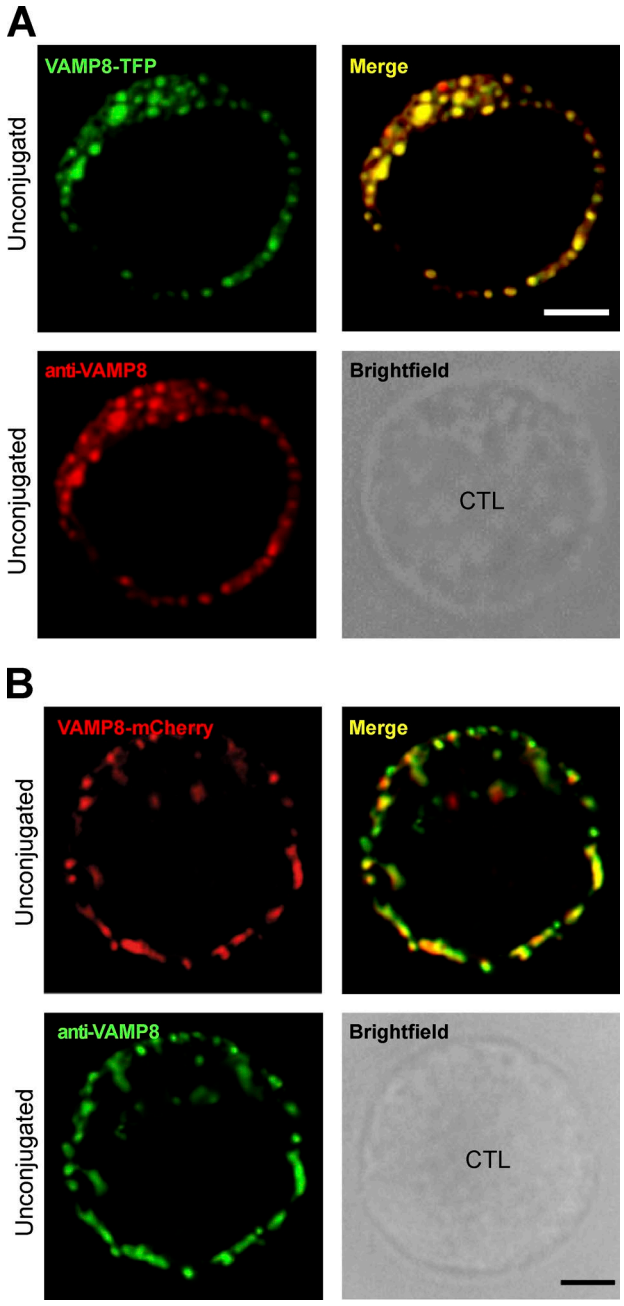


Figure S1. **Colocalization of endogenous and ectopically expressed VAMP8.** (A) SIM images of ectopically expressed VAMP8-TFP (top) costained with anti-VAMP8 antibody (bottom) in unconjugated SEA-stimulated CTLs, as indicated. (B) SIM images of ectopically expressed VAMP8-mCherry (top) costained with anti-VAMP8 antibody (bottom) in unconjugated SEA stimulated CTLs as indicated. Bars, 2.5 µm.

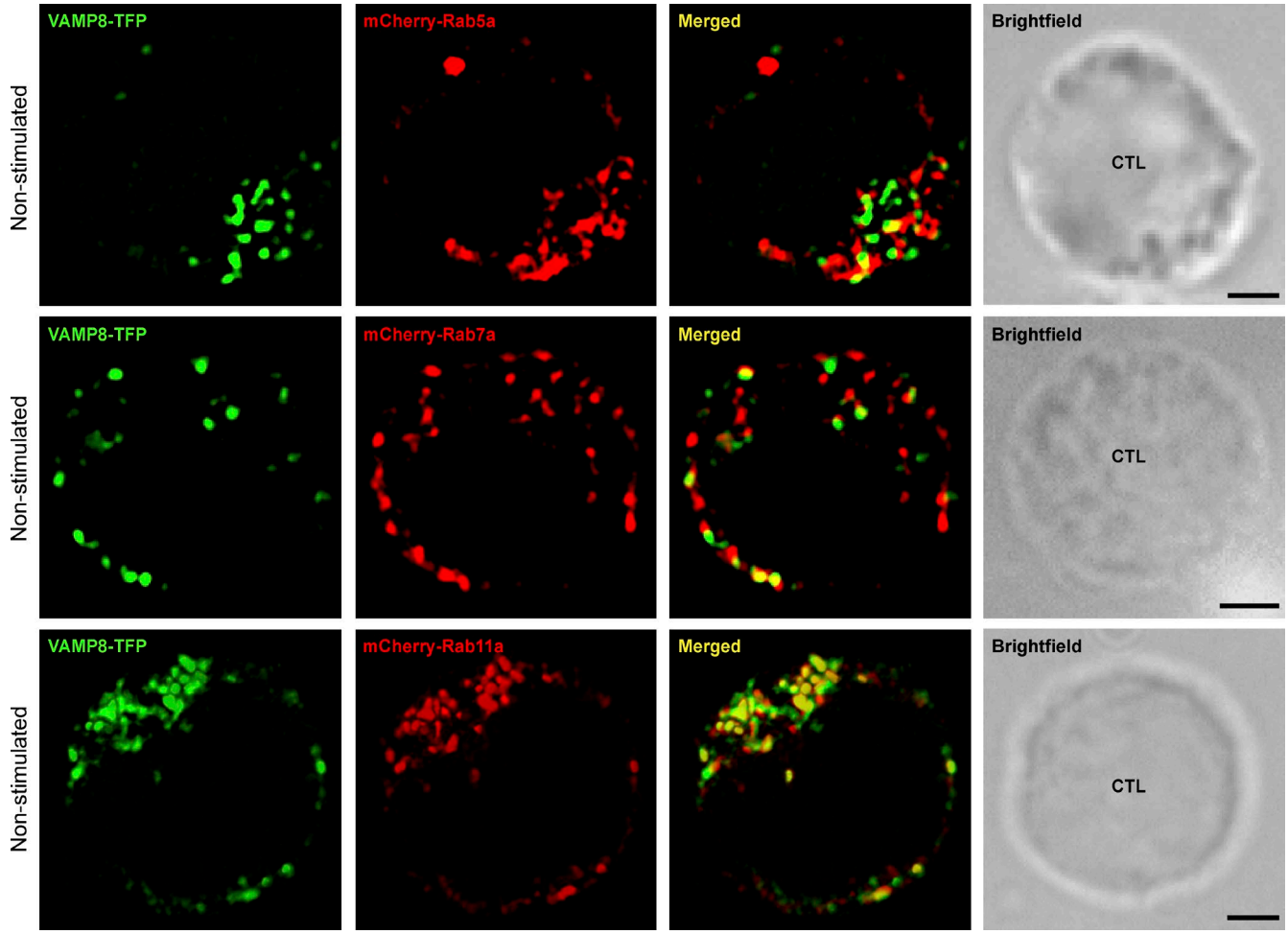


Figure S2. **VAMP8 vesicles localize with Rab11a on recycling endosomal structures.** SIM images of ectopically expressed VAMP8-TFP and mCherry -Rab5a, mCherry-Rab7a, or mCherry-Rab11a localization in unconjugated, SEA-stimulated CTLs as indicated. Bars, 2.5 µm.

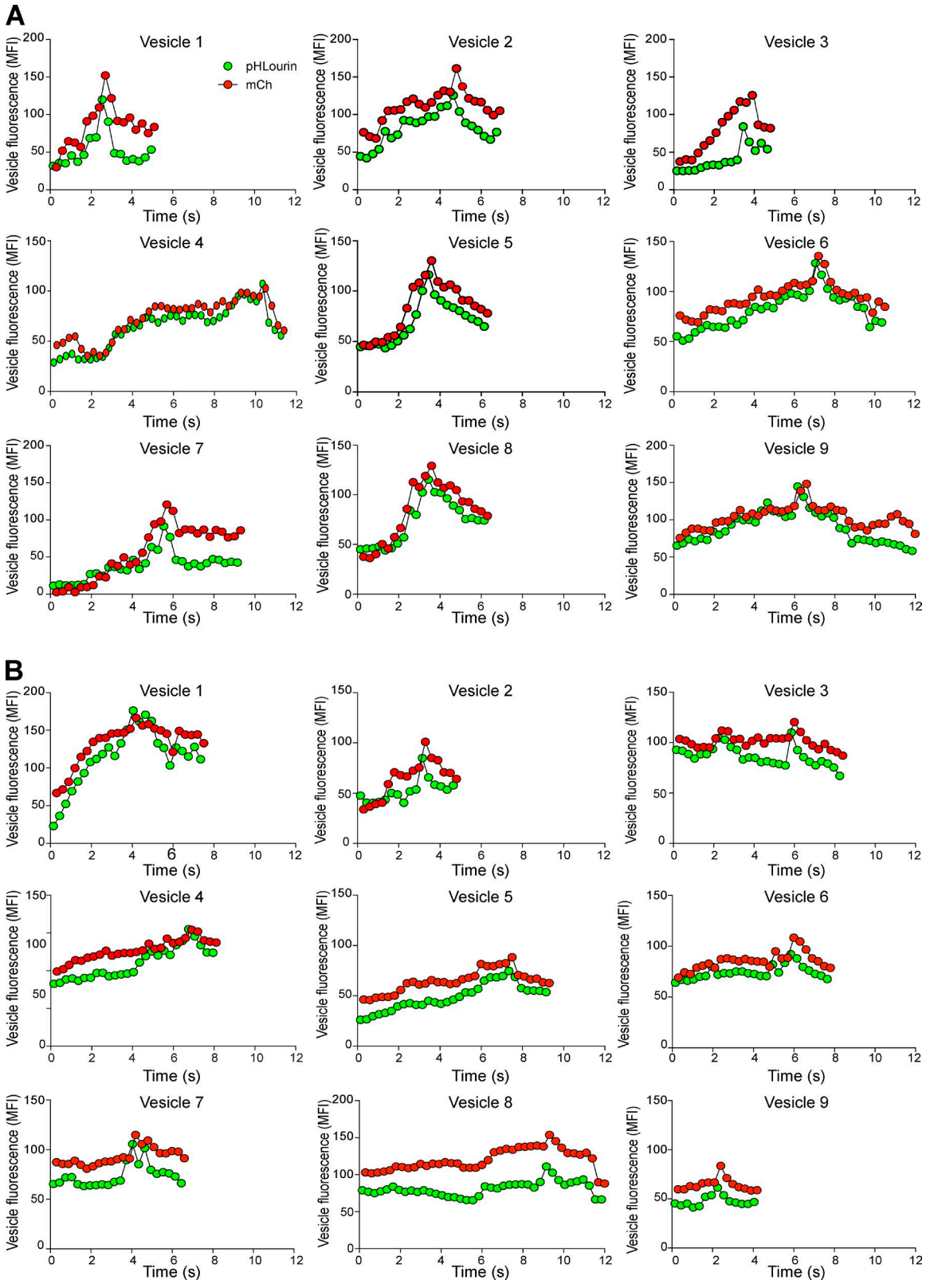


Figure S3. **VAMP8-carrying vesicles are rapidly recruited to and fuse with the plasma membrane at cytotoxic lymphocyte immune synapses.** (A and B) Bead-stimulated human CD8$^+$ T cells were transfected with VAMP8-pHluorin-mCherry encoding constructs and imaged 24 h after transfection. Graphs depict respective pHluorin and mCherry vesicle MFI from nine vesicles over time for CTLs number 1 (A) and 2 (B).

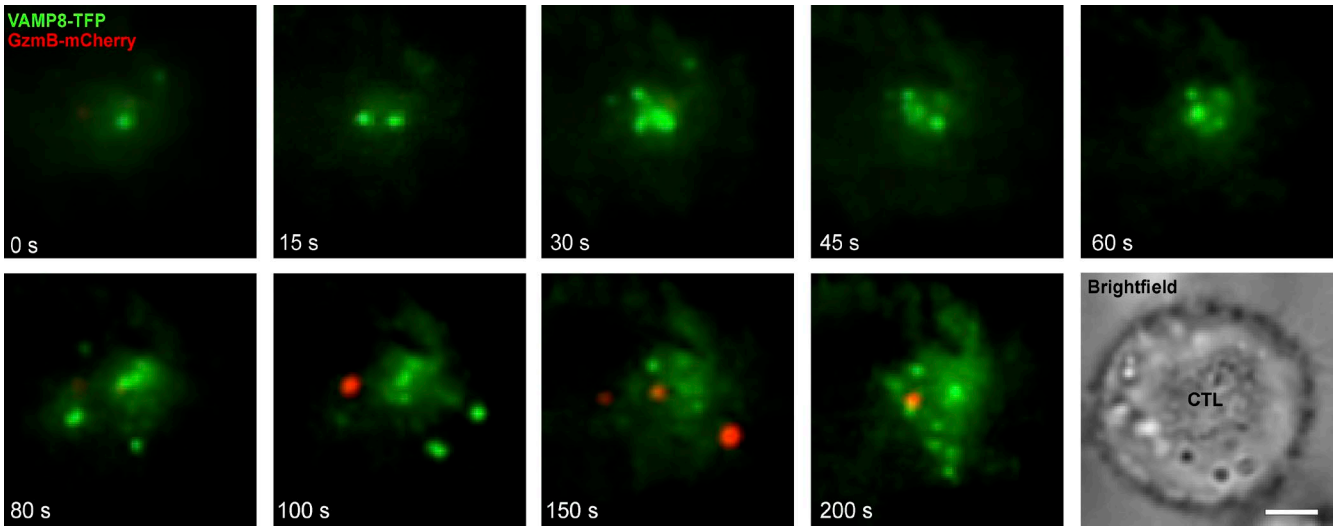


Figure S4. **VAMP8 is not colocalized with granzyme B–containing granules.** Bead-stimulated human CD8$^+$ T cells were transfected with VAMP8-TFP and granzyme B (GzmB)–mCherry encoding constructs and imaged 24 h after transfection. (A) Selected live-cell TIRF microscopy images of VAMP8-TFP and granzyme B–mCherry in a transfected CTL in contact with an anti-CD3– and anti-CD28–coated coverslips. Bar, 2.5 µm.

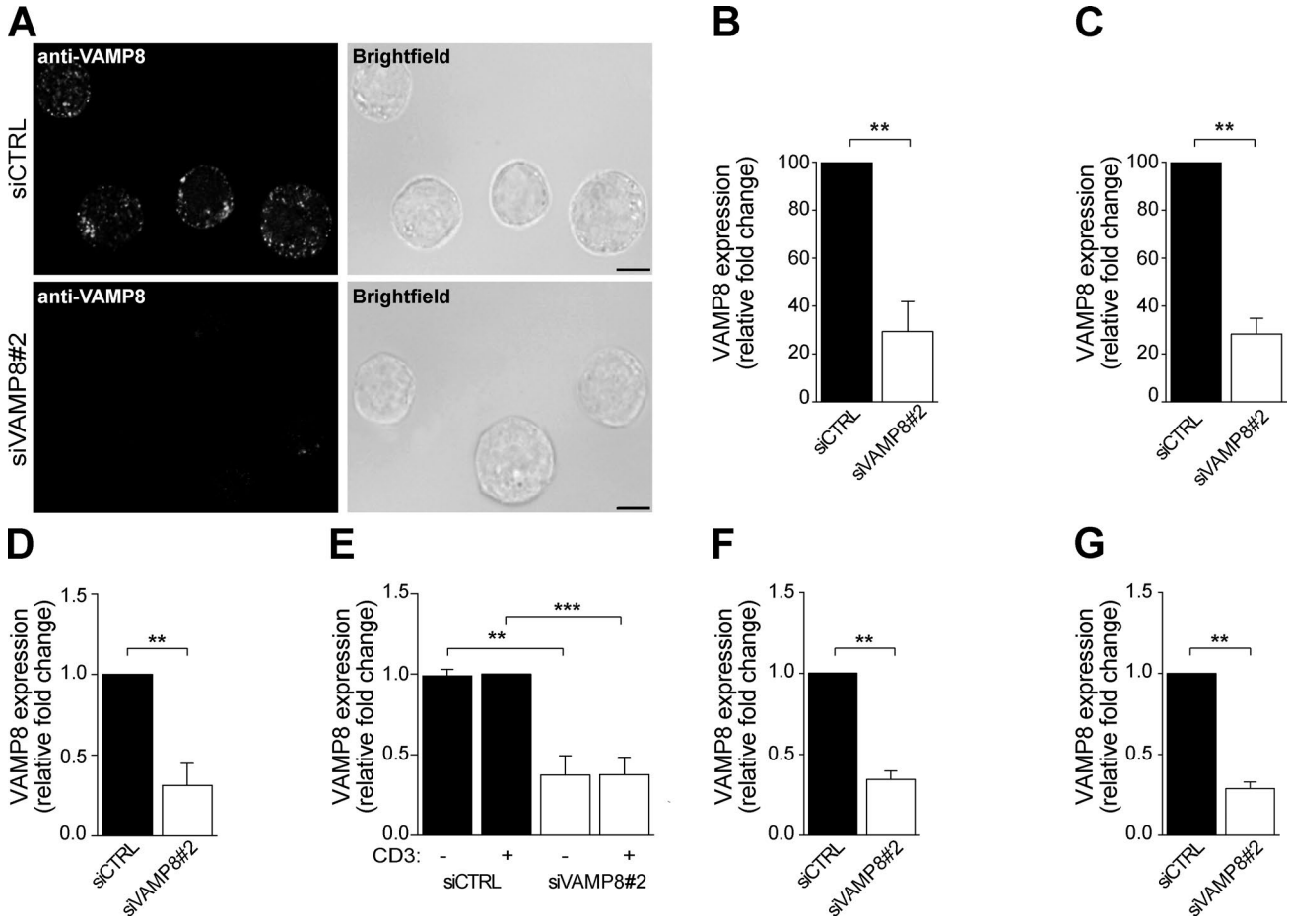
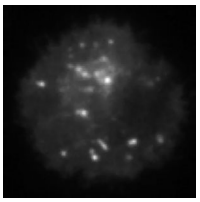
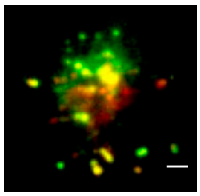


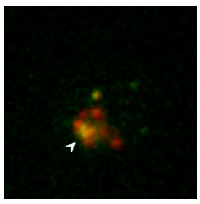
Figure S5. **VAMP8 expression is strongly down-regulated and proximal TCR signaling is normal when VAMP8 expression in down-regulated by siRNA.** (A–G) Bead-stimulated human CD8+ T cells were transfected with siRNA, as indicated. (A) Selected images of CTL transfected with control or VAMP8 siRNA, fixed, and stained for endogenous VAMP8 and imaged using SIM. Bars, 2.5 µm. (B) Graphs represent mean, normalized expression of VAMP8 in transfected CTLs, as indicated, determined by epifluorescence microscopy (Wilcoxon test, **, $P < 0.005$, $n = 8$). (C) VAMP8 expression in transfected CTL used for degranulation experiments in Fig. 6 (H and I). Graphs represent mean, normalized expression of VAMP8 relative to GAPDH in transfected CTLs, as indicated, in six representative donors, as determined by Western blotting. (D) Graphs represent mean, normalized expression of VAMP8 relative to GAPDH in transfected CTLs used for proximal TCR-induced Ca^{2+} mobilization; bars indicate means ± SD ($n = 4$). (E) Graphs represent mean, normalized expression of VAMP8 relative to GAPDH in transfected CTL after TCR stimulation and phosphorylation of the MAPK ERK; bars indicate means ± SD ($n = 5$). Tukey's multiple comparisons test, mean difference of 0.6226, ***, $P = 0.0007$. (F) Densitometry analysis of mean, normalized expression of VAMP8 relative to GAPDH in transfected CTLs for F-actin distribution analysis ($n = 6$). (G) Graphs represent mean, normalized expression of VAMP8 relative to GAPDH in transfected CTLs analyzed for Stx11 dynamics ($n = 8$). Bars indicate means ± SD; **, $P < 0.005$. siCTRL, control siRNA.



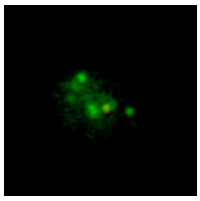
Video 1. **VAMP8 accumulates and disperses at the immune synapse upon antigen receptor engagement.** TIRF microscopy time-lapse imaging (200 s) of a human CTLs transfected with VAMP8-TFP (green) after contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using an inverted Olympus Optical IX 70 microscope with an acquisition frequency of 10 Hz (100 ms per frame) and a display rate of six frames per second.



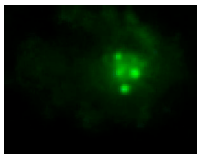
Video 2. **VAMP8 vesicles traffic and accumulate at the immune synapse with recycling endosomes markers upon antigen receptor engagement.** TIRF microscopy time-lapse imaging (80 s) of a human CTLs transfected with VAMP8-TFP (green) and Rab11a-mCherry (red) after contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using an inverted Olympus Optical IX 70 microscope with an acquisition frequency of 10 Hz and a display rate of six frames per second. Reference figure: Fig. 4 A. Bar, 2.5 µm.



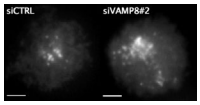
Video 3. **VAMP8-carrying recycling endosomes are rapidly recruited to and fuse with the plasma membrane at immune synapses.** TIRF microscopy time-lapse imaging (171.3 s) of a human CTLs transfected with VAMP8-pHluorin-mCherry upon contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. mCherry (red) denotes VAMP8-carrying vesicles, whereas pHluorin (green) fluorescence appears upon fusion of VAMP8-carrying vesicles with the plasma membrane. Accumulation and fusion of VAMP8-carrying vesicles at the immune synapse was visualized using a Nikon A1R multiphoton confocal microscope with a 63× Plan Apochromat objective (NA 1.49). The acquisition frequency was 150 ms per frame, and the display rate was four frames per second. Reference figures: Figs. 4 H and S3. Bar, 2.5 µm.



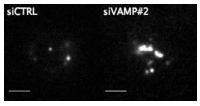
Video 4. **VAMP8-carrying and perforin-containing vesicles have different spatial and temporal characteristics.** TIRF microscopy time-lapse imaging (98 s) of a human CTLs transfected with VAMP8-TFP (green) and perforin-mCherry (red) upon contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using an inverted Olympus Optical IX 70 microscope with an acquisition frequency of 10 Hz (100 ms per frame) and a display rate of four frames per second. Reference figure: Fig. 5. Arrowheads are VAMP8-TFP fusion events. Bar, 2.5 µm.



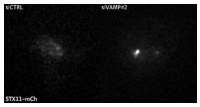
Video 5. **VAMP8 and granzyme B have different spatial and temporal characteristics.** TIRF microscopy time-lapse imaging of a human CTL transfected with VAMP8-TFP (green) and granzyme B–mCherry (red) upon contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using an inverted Olympus Optical IX 70 microscope with an acquisition frequency of 10 Hz (100 ms per frame) and a display rate of four frames per second. Reference figure: Fig. S4. Bar, 2.5 µm.



Video 6. **Recycling endosome fusion is inhibited upon knockdown of endogenous VAMP8 expression.** (A and B) TIRF microscopy time-lapse imaging (99 s) of human CTL transfected with Rab11a-mCherry (white) and control siRNA (siCTRL; A, right) or VAMP8-targeting siRNA (siVAMP8; B, left), as indicated, upon contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using an inverted Olympus Optical IX 70 microscope with an acquisition frequency of 10 Hz (100 ms per frame) and a display rate of four frames per second. Reference figure: Fig. 6.



Video 7. **Cytotoxic granule fusion is inhibited upon knockdown of endogenous VAMP8 expression.** (A and B) TIRF microscopy time-lapse imaging (99 s) of human CTL transfected with granzyme B–mCherry (white) and control siRNA (siCTRL; A, right) or VAMP8-targeting siRNA (siVAMP8; B, left), as indicated, upon contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using an inverted Olympus Optical IX 70 microscope with an acquisition frequency of 10 Hz (100 ms per frame) and a display rate of four frames per second. Reference figure: Fig. 6. Bar, 2.5 µm.



Video 8. **Deposition of Stx11 and the immune synapse upon knockdown of endogenous VAMP8 expression.** (A and B) TIRF microscopy time-lapse imaging (95–100 s) of human CTLs transfected with Stx11-mCherry (white) and control siRNA (siCTRL; A, right) or VAMP8-targeting siRNA (siVAMP8; B, left), as indicated, upon contact with glass coverslip coated with anti-CD3 and anti-CD28 antibodies. Images were analyzed using a Nikon A1R multiphoton confocal microscope with a 63× Plan Apochromat objective (NA 1.49), an acquisition frequency of 150 ms per frame, and a display rate of four frames per second. Reference figure: Fig. 7.