Expanded View Figures

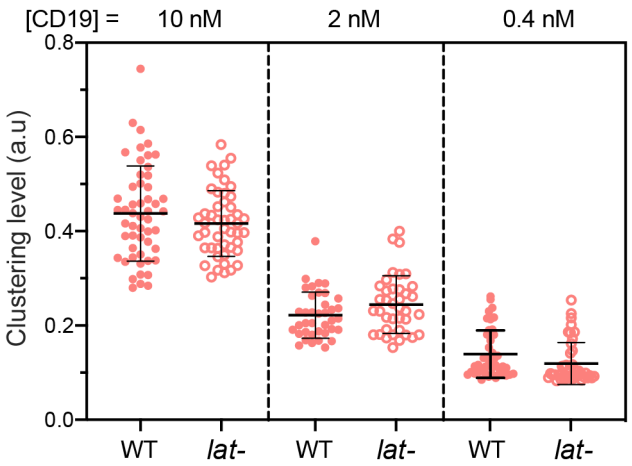

Figure EV1. Titration analysis of the LAT-independent CAR microcluster formation.

WT or LAT-deficient (J.LAT) Jurkat T cells expressing CAR-GFP were stimulated on SLB coated with CD19 of indicated concentrations. The clustering of Alexa Fluor 647-labeled streptavidin in conjugation with biotin-CD19 was imaged by TIRF microscopy and quantified as normalized variance in the same way as in Fig 3B. Shown are the means ± SD. n = 52, 38, and 44 cells for WT at 10, 2 and 0.4 nM, respectively, and 47, 38, and 44 cells for *lat*- cells at 10, 2 and 0.4 nM, respectively.

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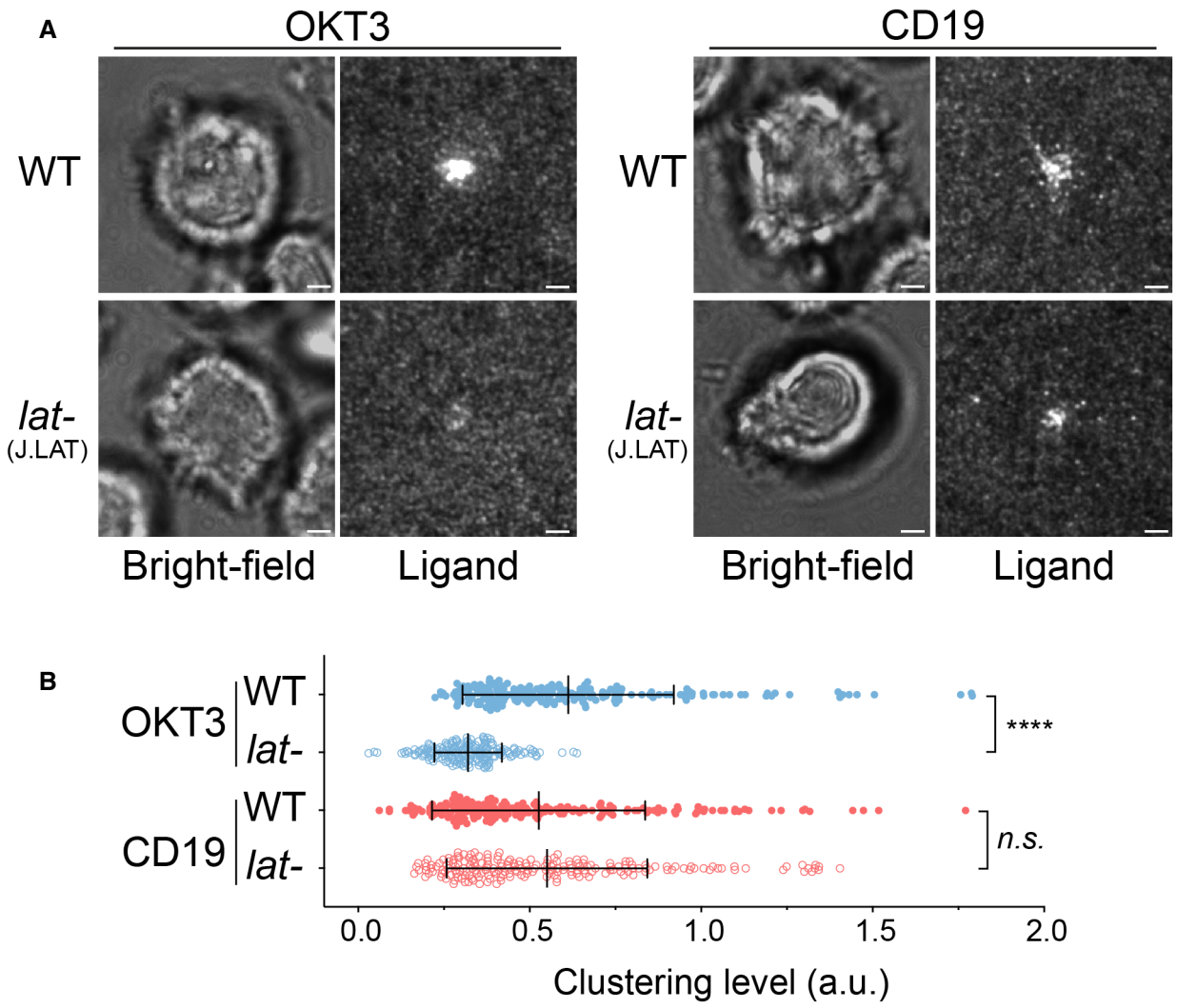


Figure EV2. LAT-independent CAR microcluster formation in J.LAT cells.

A WT or LAT-deficient (J.LAT) Jurkat cells expressing CAR were stimulated on supported lipid bilayers coated with OKT3 (anti-TCR antibody) or CD19 (CAR antigen) supplemented with ICAM-1, respectively. TIRF microscopy revealed clustering of Ax647-labeled streptavidin–biotin-OKT3 or CD19, which serves as a probe for TCR or CAR, respectively. Scale bar, 2 μm.

B Quantification of clustering as normalized variance. n = 200 cells for each condition. Shown are the mean ± SD. Statistical test: unpaired two-tailed t-test. ****$P < 0.0001$, *n.s.*, $P = 0.4208$.

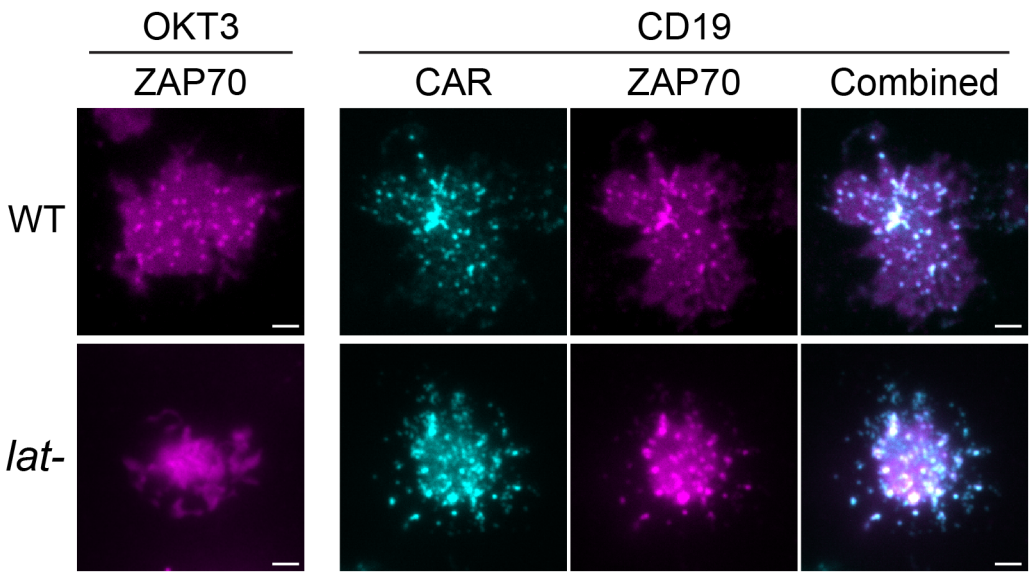


Figure EV3. ZAP70 is recruited to CAR microclusters in LAT-deficient cells.

Supported lipid bilayers were coated with OKT3 (left) or CD19 (right) supplemented with ICAM-1. Wild-type or LAT-deficient (Jcam2.5) T cells expressing ZAP70-mCherry and CAR-GFP were plated on the SLB and imaged by TIRF microscopy. Scale bar: 2 μm.

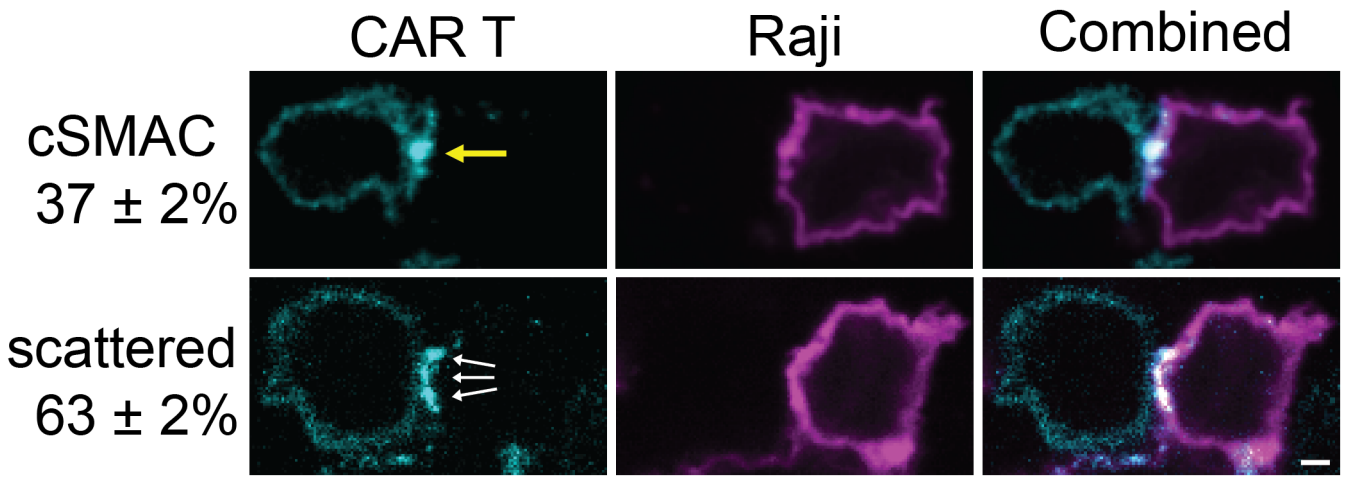
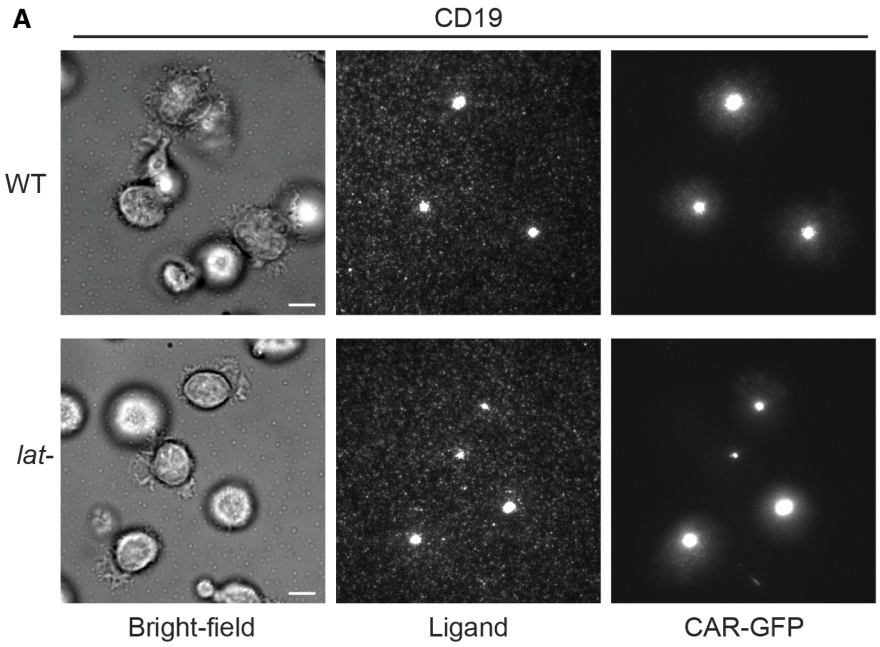


Figure EV4. Synapses between CAR T cells and Raji B cells.

CAR-GFP forms a cSMAC-like (yellow arrow) or scattered pattern (white arrows) in the immunological synapse between CAR T cells and Raji B cells. mCherry-CAAX serves as a marker for the plasma membrane of Raji B cells. T cells and B cells were mixed for 20 min before being fixed and imaged by confocal microscopy. Quantification showed the percentage of cells that display a cSMAC or scattered pattern. $N = 3$ independent experiments. Shown are means ± SD. Scale bar: 2 μm.



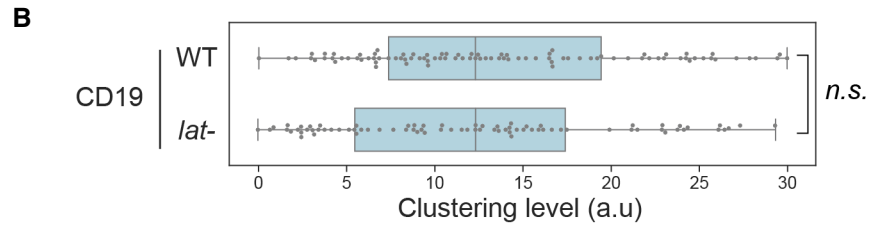


Figure EV5. 1st-generation CAR forms microclusters and immunological synapses in LAT-knockout primary T cells.

A WT or LAT-knockout (*lat*-) primary T cells expressing the 1st-generation CAR were stimulated on SLBs coated with biotin-CD19 and his-ICAM-1. TIRF microscopy revealed clustering of Ax647-labeled streptavidin–biotin-CD19 and CAR. Scale bar, 5 μm.

B Quantification of clustering level of Ax647-labeled streptavidin–biotin-CD19. Central band: mean; box: quartiles; whisker: rest of the distribution. n = 123 cells for WT, 106 cells for *lat*-. Clustering is quantified as normalized variance. *n.s.* P = 0.67. Statistical test: unpaired two-tailed t-test.