

Supplementary Materials for

Dynamics of Subsynaptic Vesicles and Surface Microclusters at the Immunological Synapse

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Other Supplementary Material for this manuscript includes the following:

(available at www.sciencesignaling.org/cgi/content/full/3/121/ra36/DC1)

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Movie S7. Dynamics of vesicular LAT in relation to clusters of ZAP-70 (.avi format).

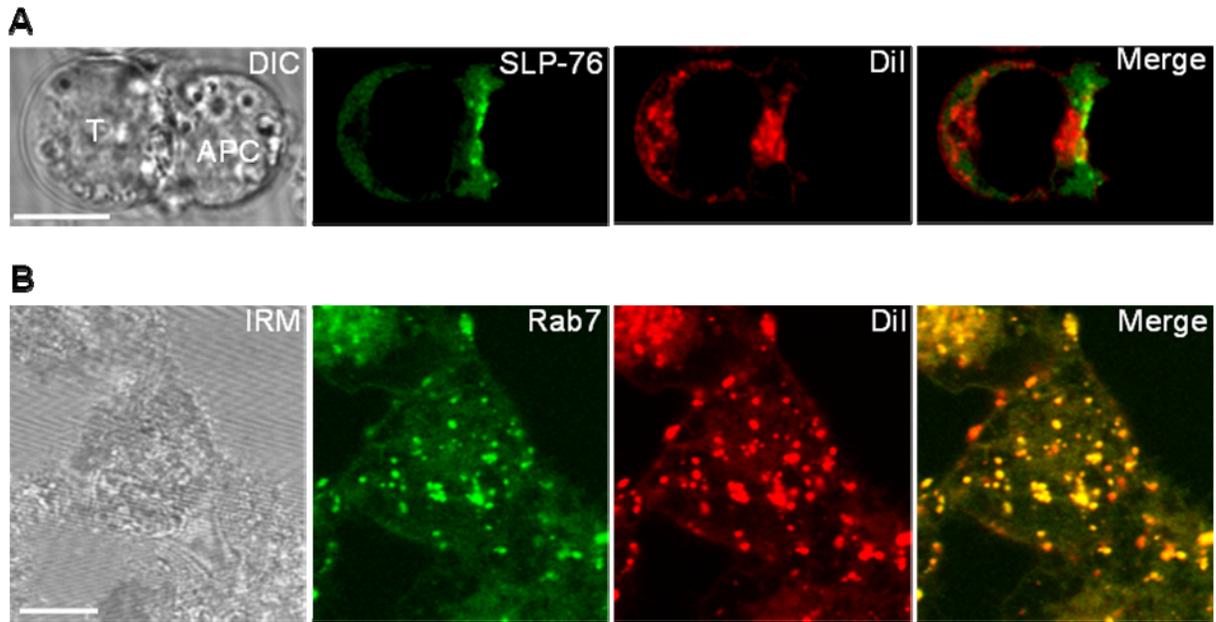


Fig. S1. DiI-stained intracellular vesicles are positioned proximal to the IS. **(A)** Image shows a Jurkat cell (T), transfected with a plasmid encoding SLP-76-YFP (green), forming an IS with an SEE-pulsed Raji cell (APC). The Jurkat cell was stained with the fluorescent lipid DiI (red). Most of the DiI was internalized during the incubation overnight, highlighting intracellular vesicles, with little dye remaining at the plasma membrane. **(B)** To further demonstrate that internalized DiI localized into intracellular vesicles, Jurkat cells transfected with a plasmid encoding mYFP-tagged Rab7, a marker of late endosomes, were incubated with DiI overnight and were then activated on OKT-3-coated coverslips. The distribution of DiI (red) and mYFP-Rab7 (green) was imaged at the activating interface. The vast majority of bright DiI patches ($90.4 \pm 5.5\%$, $n = 21$ cells) colocalized with Rab7, demonstrating that DiI stained intracellular vesicles. Data shown are representative of at least 10 cells for each experiment. Scale bars, 10 μm .

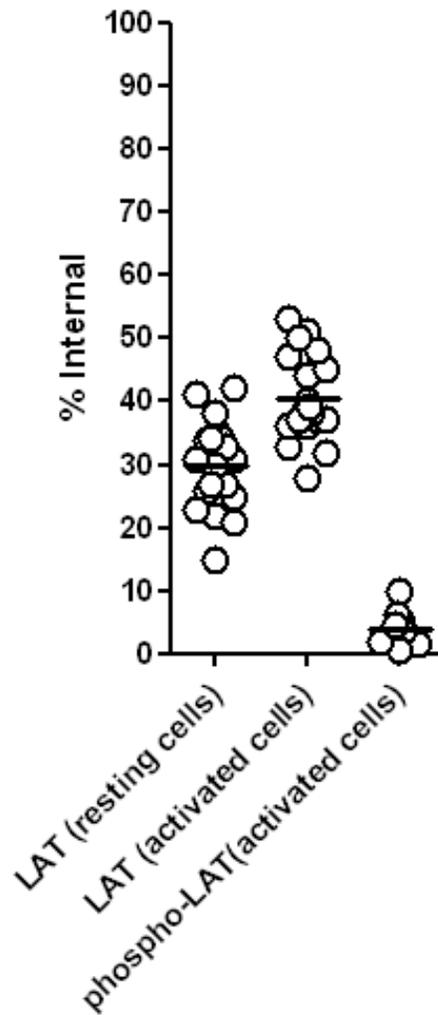


Fig. S2. The intracellular distribution of LAT. The distribution of LAT-mCherry was imaged in transfected Jurkat cells. The entire volume of each cell was imaged in 35 z-planes. Image data were used to determine the proportions of total LAT-mCherry that were either intracellular or localized to the surface. Data was compared for resting transfected cells and for transfected cells that were activated for 20 min on OKT-3-coated coverslips. To image pLAT, LAT-mCherry expressing Jurkat T cells were activated on OKT-3-coated coverslips, fixed and permeabilized, and incubated with antibody against pLAT (Tyr¹⁹¹) followed by Alexa-488 conjugated secondary antibody. The distribution of pLAT was determined from images as above. Data shown are pooled from two experiments.

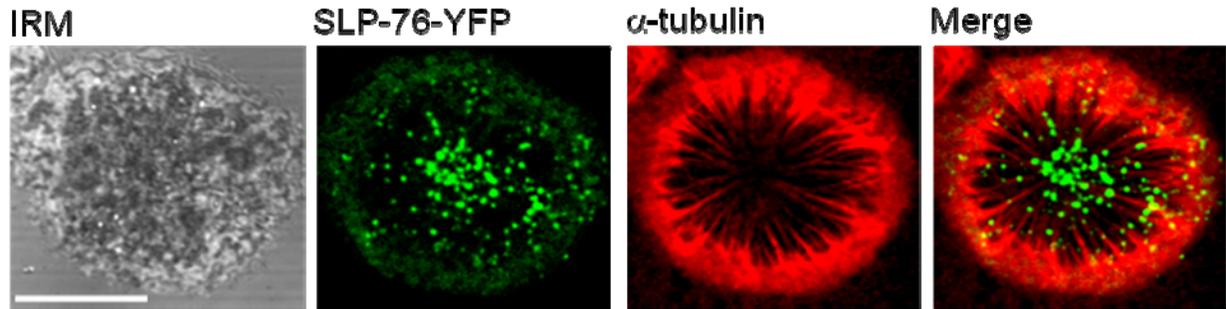


Fig. S3. Clusters of SLP-76 align with the microtubule cytoskeleton. Jurkat cells transfected with plasmid encoding SLP-76-YFP were placed on OKT-3-coated coverslips for 10 min, after which they were fixed and stained with antibody against α -tubulin. The image shows the distribution at the interface between the Jurkat cell and the OKT-3-coated coverslip of SLP-76-YFP (green) and α -tubulin (red). The majority of clusters of SLP-76 in the region between the periphery and centre of the contact area appeared to align with the radial configuration of microtubules. Data shown are representative of 20 cells analyzed.

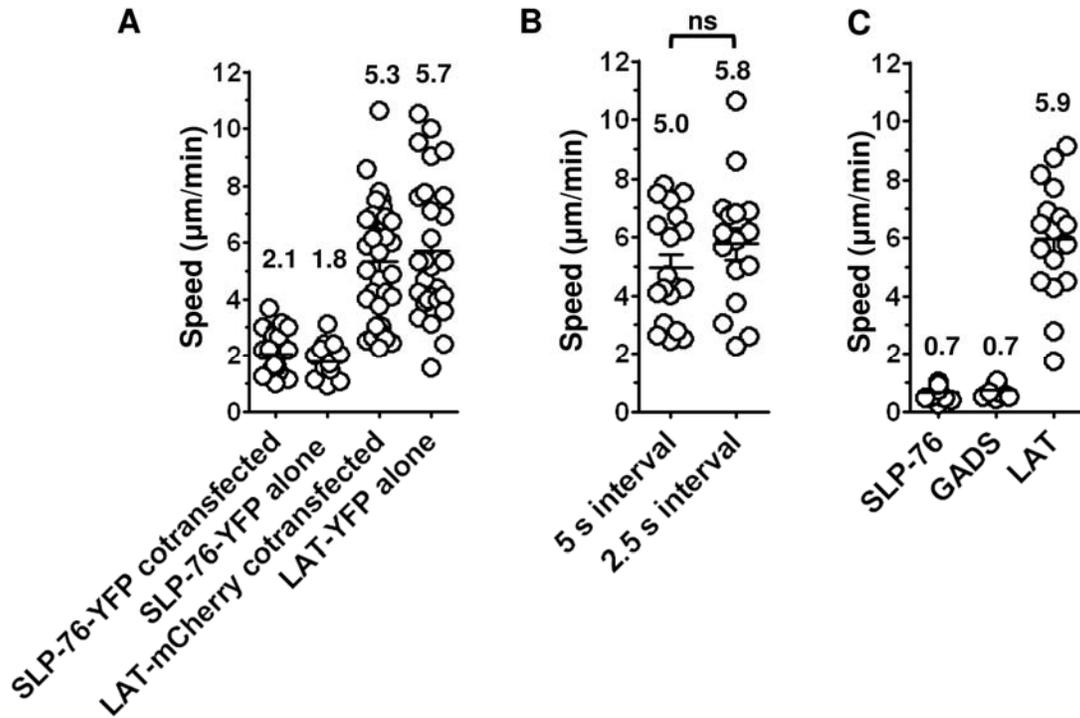


Fig. S4. Analysis of the speed of protein cluster at the interface between Jurkat cells and OKT-3-coated coverslips. **(A)** Speeds of individual SLP-76 clusters and LAT-containing vesicles measured in Jurkat cells transfected, singly or in combination, with plasmids encoding SLP-76-YFP and LAT-mCherry. No differences in cluster dynamics were observed between cells that were singly or doubly transfected. Data shown are pooled from at least six cells for each condition. **(B)** Speeds of LAT-mCherry-containing vesicles in Jurkat cell transfected with plasmids encoding SLP-76-YFP and LAT-mCherry imaged every 5 or 2.5 s. Data shown are pooled from at least four cells for each condition. **(C)** Speeds of individual SLP-76-YFP clusters, GADS-mCherry clusters, and LAT-mCherry-containing vesicles during the spreading phase of the Jurkat cell immediately following contact with the OKT-3-coated surface. SLP-76 and GADS clusters were almost completely stationary during this phase. The speed of the LAT-containing vesicles was not significantly different from those observed when the cell had spread maximally. Data shown are pooled from three cells for each condition. Pearson's correlation coefficients (R_r) for the colocalization of SLP-76 and GADS and for the colocalization of SLP-76 and LAT during the spreading phase of antigen recognition were 0.80 and 0.10 respectively.

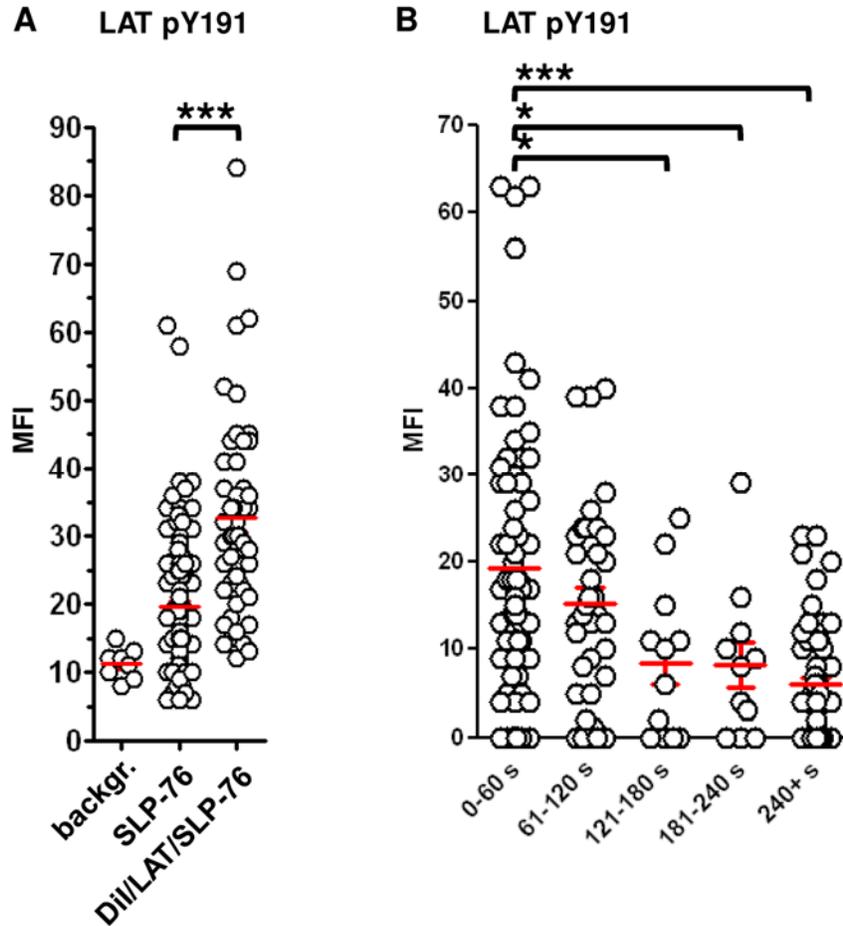


Fig. S5. LAT is phosphorylated at the sites of contact between SLP-76 microclusters and LAT-containing vesicles. **(A)** Jurkat cells cotransfected with plasmids encoding SLP-76-mYFP and LAT-mCherry were stained with DiI to mark intracellular vesicles. Cells were activated on OKT-3-coated coverslips, fixed, and incubated with antibodies against pLAT (pTyr¹⁹¹). The plot shows the mean fluorescence intensity (MFI) of pLAT (pY191) staining. The extent of pLAT staining within clusters of SLP-76 alone was compared to that in which SLP-76 clusters were in contact with vesicular LAT (that is, with LAT that colocalized with DiI). A region that lacked clusters was analyzed to determine the background (backgr.) intensity for each cell. **(B)** Jurkat cells cotransfected with plasmids encoding SLP-76-mYFP and LAT-mCherry were stained with DiI to mark intracellular vesicles. Cells were activated on OKT-3-coated coverslips. A single cell was selected and imaged every 3 s for 240 s. Cells were fixed and incubated with antibody against pLAT (pY191), and the same cell that was imaged live was then imaged again after fixation and staining. The plot shows the MFI above background of pLAT staining within SLP-76 clusters. The live cell data were used to determine the time that had elapsed for each SLP-76 cluster since its last contact with vesicular LAT (that is with LAT that colocalized with DiI). N = 6 experiments. Bars indicate the mean \pm SEM.

Descriptions of movies

Movie S1. Dynamics of SLP-76 and GADS clusters. The dynamics of clusters at the interface between a Jurkat cell transfected with plasmids encoding SLP-76-YFP and GADS-mCherry and activated on an OKT-3-coated coverslip were monitored. SLP-76 is shown in green and GADS is shown in red. Each frame represents 5 s.

Movie S2. Dynamics of SLP-76 and LAT clusters. The dynamics of clusters at the interface between a Jurkat cell transfected with plasmids encoding SLP-76-YFP and LAT-mCherry and activated on an OKT-3-coated coverslip were monitored. SLP-76 is shown in green and LAT is shown in red. Each frame represents 5 s.

Movie S3. Dynamics of a single LAT cluster. The movement of an individual LAT cluster (red) between separate clusters of SLP-76 (green) was monitored. Each frame represents 3 s.

Movie S4. Dynamics of SLP-76, LAT, and intracellular vesicles. The dynamics of clusters at the interface between a Jurkat cell cotransfected with plasmids encoding SLP-76-YFP and LAT-mCherry, stained with DiI, and activated on an OKT-3-coated coverslip were monitored. SLP-76 is shown in blue, LAT is in red, and DiI is in green. Yellow indicates the overlap of LAT and DiI. Each frame represents 2.5 s.

Movie S5. Dynamics of SLP-76 clusters imaged by TIRF. Time-lapse TIRF microscopy was used to monitor the surface dynamics of SLP-76 at the interface between a Jurkat cell transfected with plasmid encoding SLP-76-YFP and incubated on an OKT-3-coated coverslip. Each frame represents 5 s.

Movie S6. Dynamics of subsynaptic vesicles compared to those of clusters of ZAP-70. The dynamics of vesicles at the interface between a Jurkat cell transfected with plasmid encoding ZAP-70-mCFP, stained with DiI, and incubated on an OKT-3-coated coverslip were monitored. ZAP-70 is shown in green and DiI is in red. To visualize faint clusters of ZAP-70, all of all ZAP-70 images taken for the time series were added together and background ZAP-70 was removed from the image. The DiI movie is superimposed on the ZAP-70 image. Each frame represents 2.5 s.

Movie S7. Dynamics of vesicular LAT in relation to clusters of ZAP-70. The dynamics of vesicles at the interface between a Jurkat cell cotransfected with plasmids encoding ZAP-70-mCFP and LAT-mCherry and incubated on an OKT-3-coated coverslip were monitored. ZAP-70 is shown in green and LAT is in red. To visualize faint clusters of ZAP-70, all of the ZAP-70 images taken for the time-series were added together and background ZAP-70 was removed from the image. Each frame represents 3 s.