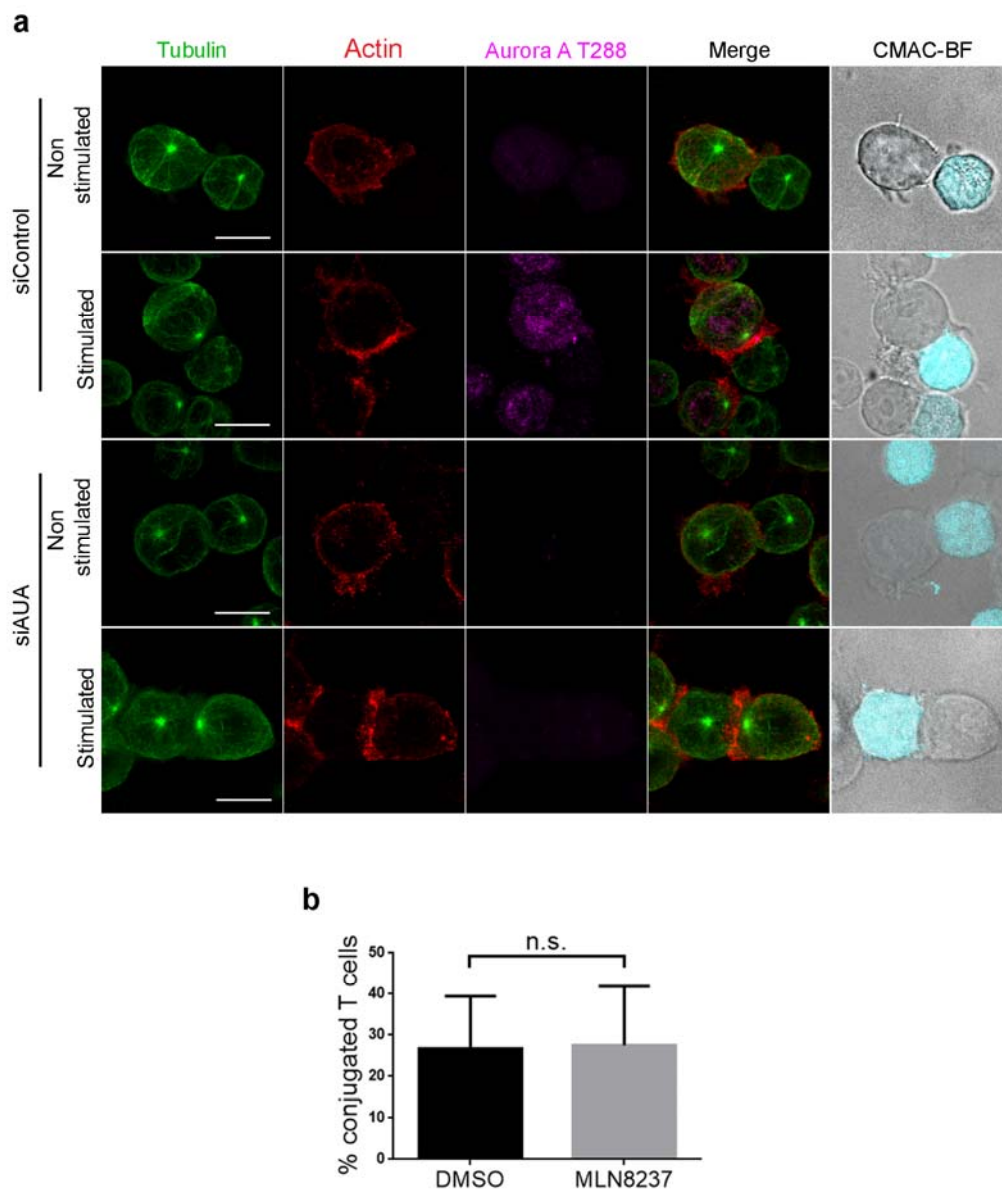
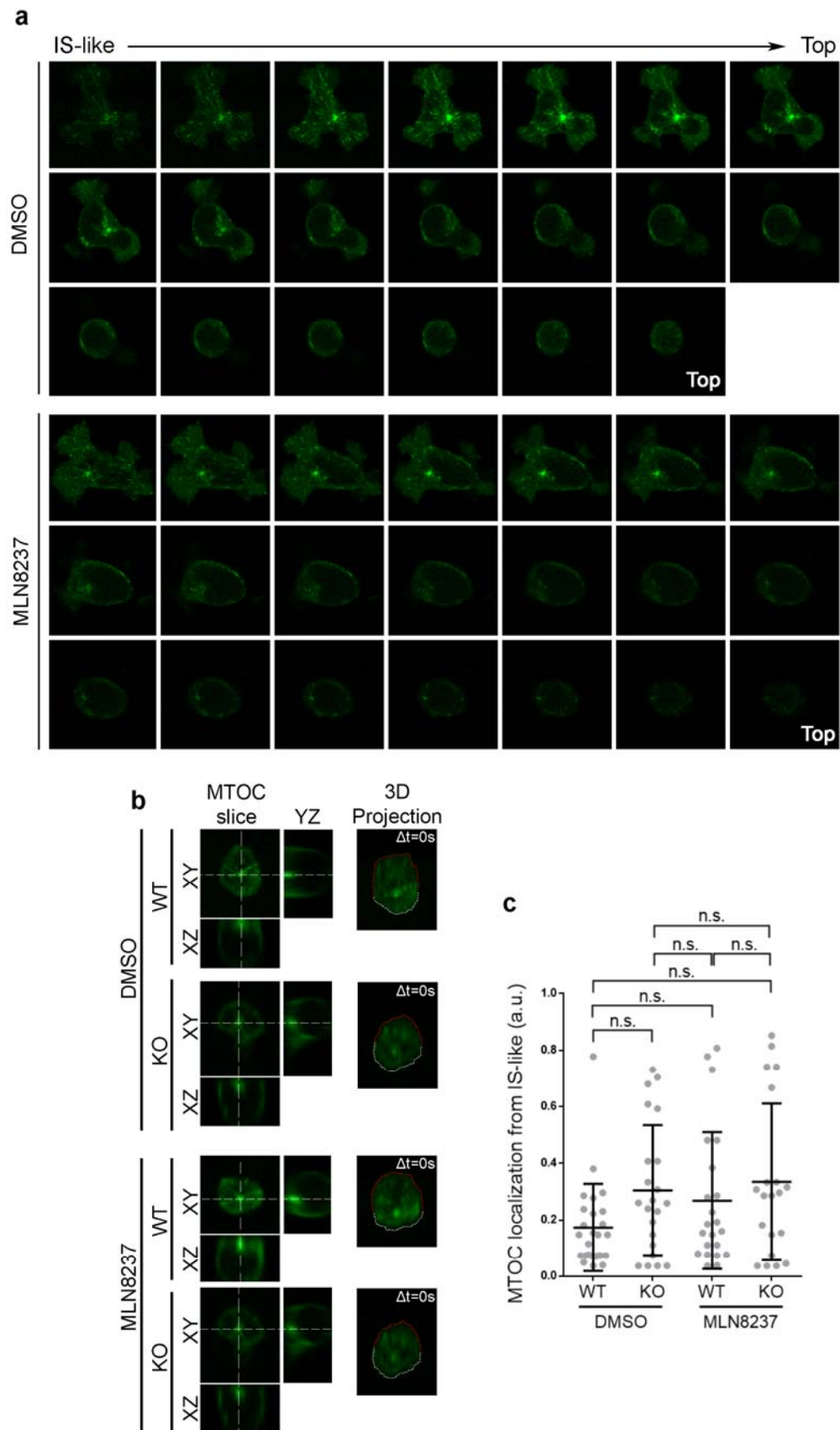


Supplementary Figure 1

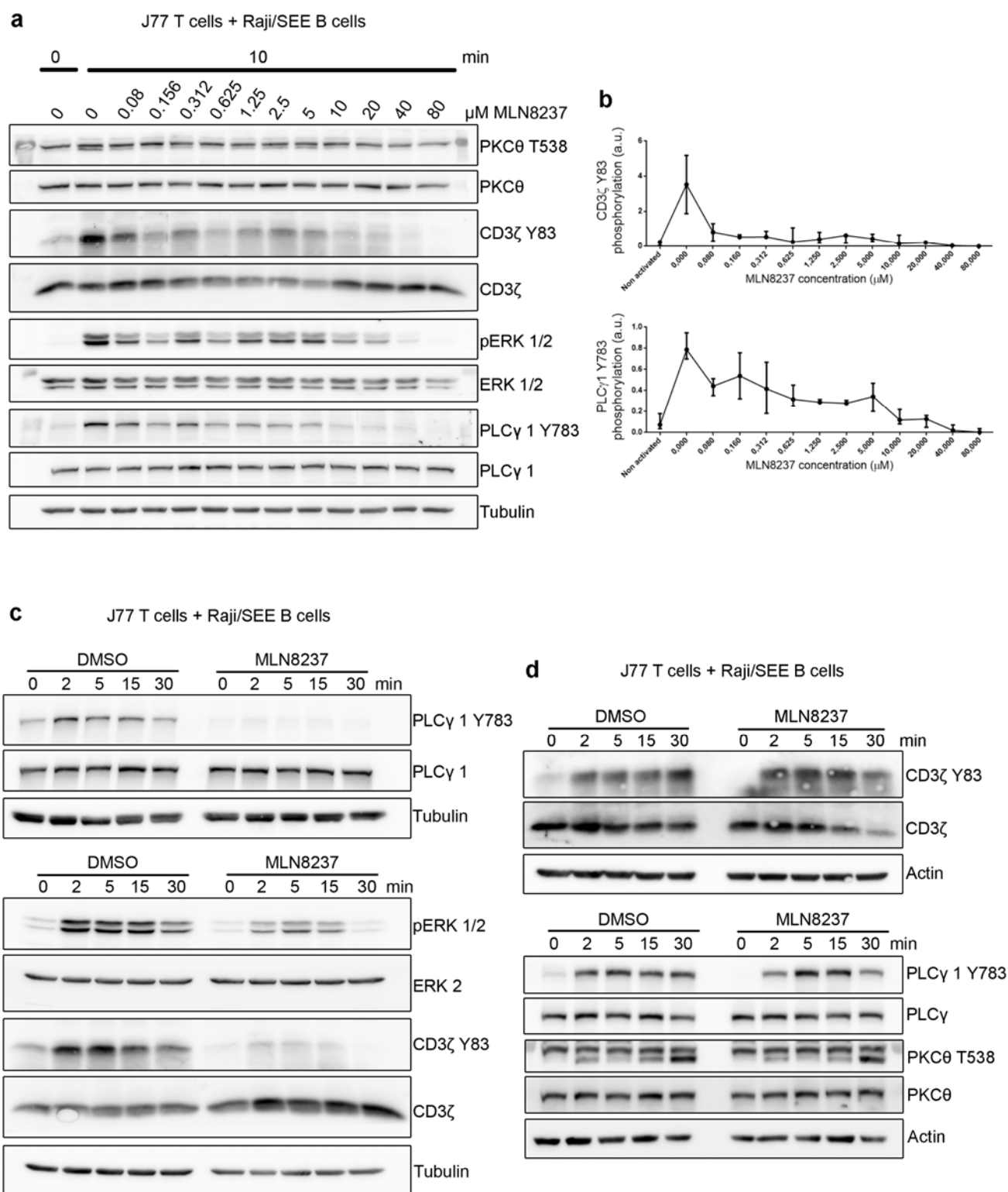


Supplementary Figure 1. Specific silencing of Aurora A. (a) Maximum intensity Z-projection of a confocal XYZ-stack of Jurkat T cells transfected with a specific siRNA against Aurora A (siAUA) or a scrambled negative control (siControl) and conjugated with SEE-pulsed Raji B cells. Cells were incubated for 30 min, fixed and stained for α -tubulin (green), T288-phosphorylated Aurora A (magenta) and actin (red). A merged fluorescence image is shown. The right-hand image shows Raji cells labeled with CMAC cell tracker (cyan) and bright field. Bar, 10 μ m. (b) Quantification of the percentage of conjugates formed between J77 T cells, pre-treated with DMSO or MLN8237, and SEE-pulsed Raji B cells (ratio 1:1).



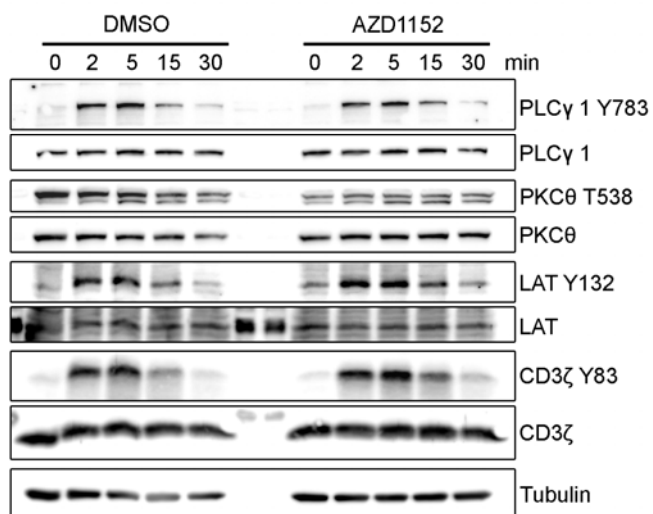
Supplementary Figure 2. Imaging of EB3-GFP decorated TIPs. (a) Single slices from XYZ-stacks of 4D imaging of EB3-GFP-transfected human CH7C17 T cells treated with DMSO or MLN8237 and plated onto anti-CD3/CD28 stimulating antibodies (time 0s). (b) Orthogonal and 3D projections from XYZ-stacks of WT and KO mouse cells, pre-treated with DMSO or MLN8237 (time 0s). Dotted white and red lines indicate contact with substrate and media in the 3D projection, respectively. (c) Ratio of the MTOC location from the IS-like (n=26 in WT, 22 in KO and WT+MLN8237 and n=20 in KO+MLN8237).

Supplementary Figure 3



Supplementary Figure 3. Dose-response effect of the Aurora A inhibitor MLN8237 on T cell activation. (a) Immunoblots showing phosphorylation of the indicated molecules in lysates of J77 Jurkat T cells pretreated with vehicle (DMSO) or Aurora A inhibitor (MLN8237) at the concentrations indicated and conjugated for 10 minutes with SEE-pulsed Raji B cells. **(b)** Quantification of blots as in **a** from three independent experiments. Medians are shown. Error bars represent interquartile range. **(c)** Immunoblots showing phosphorylation of the indicated molecules in lysates of J77 Jurkat T cells conjugated for the indicated times with SEE-pulsed Raji B cells in presence of DMSO or MLN8237. **(d)** Immunoblots showing phosphorylation of the indicated molecules in lysates of J77 Jurkat T cells pretreated with DMSO or MLN8237 for 30 min, extensively washed and conjugated for the indicated times with SEE-pulsed Raji B cells.

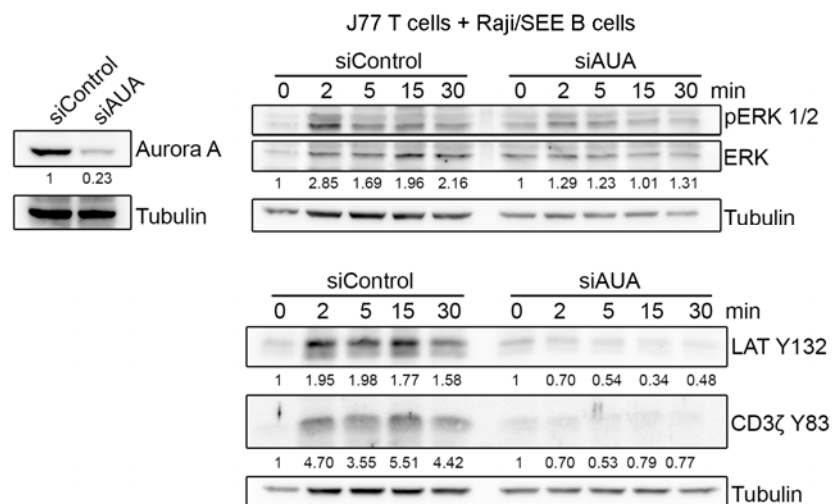
Supplementary Figure 4



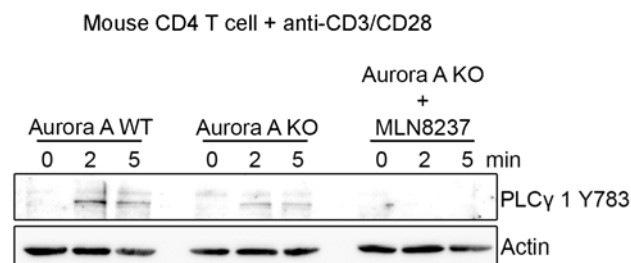
Supplementary Figure 4. TCR signaling is not affected by Aurora B inhibition. Immunoblots showing phosphorylation of the indicated molecules in lysates of J77 Jurkat T cells pretreated with vehicle (DMSO) or Aurora B inhibitor (AZD1152, 100 nM) and conjugated for the indicated times with SEE-pulsed Raji B cells.

Supplementary Figure 5

a

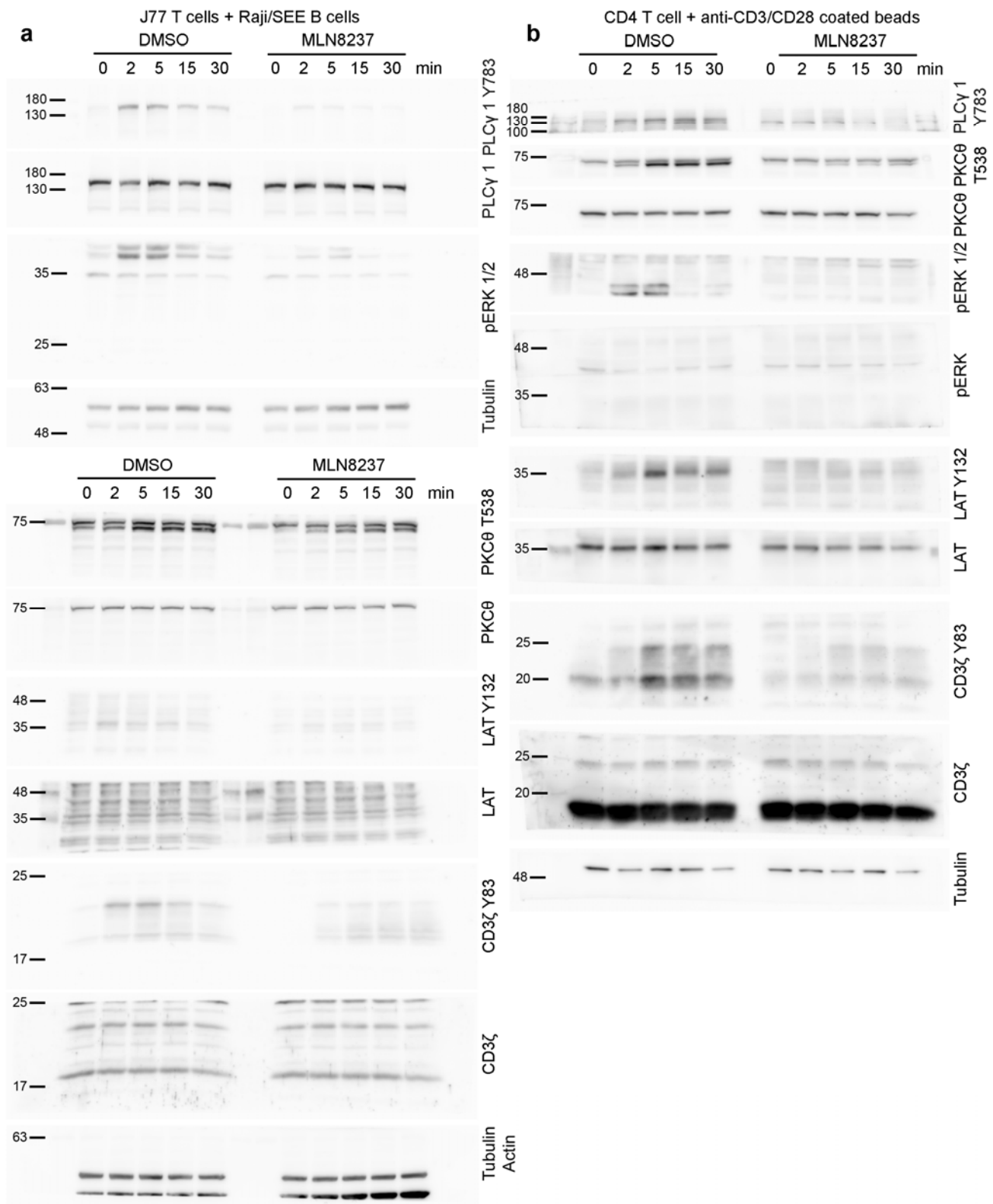


b



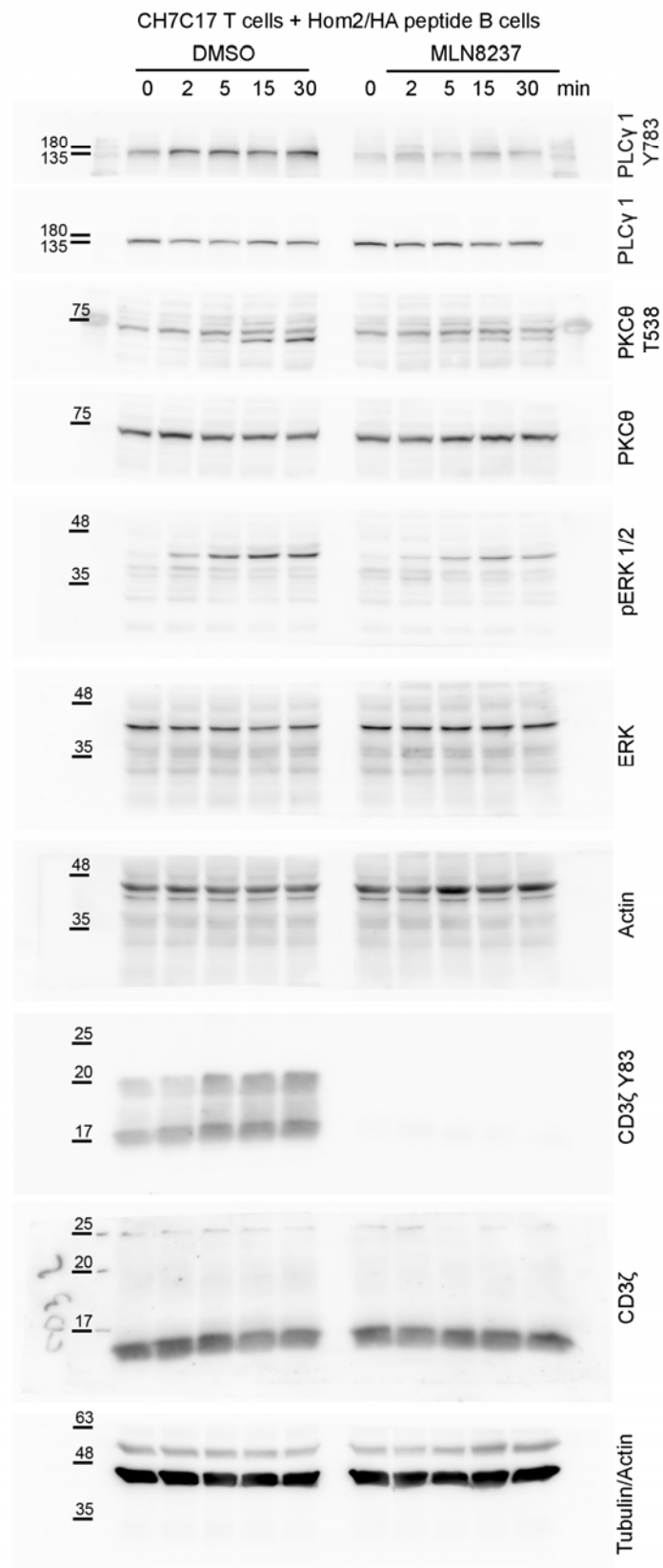
Supplementary Figure 5. TCR signaling is impaired by Aurora A silencing. (a) Immunoblots showing phosphorylation of the indicated molecules in lysates of J77 Jurkat T cells transfected with a specific siRNA against Aurora A (siAUA) or a scrambled negative control (siControl) and conjugated for the indicated times with SEE-pulsed Raji B cells. (b) Immunoblots of PLCγ1 phosphorylation (pY783) in lysates of Aurora KO pretreated with vehicle (DMSO) or the Aurora A inhibitor (MLN8237) and control mouse CD4⁺ T cells conjugated for the indicated times with anti-CD3/CD28 antibodies.

Supplementary Figure 6



Supplementary Figure 6. Uncropped Western blots. (a) Western blots correspond to Fig. 6a. (b) Western blots correspond to Fig. 6c. Molecular weights are indicated.

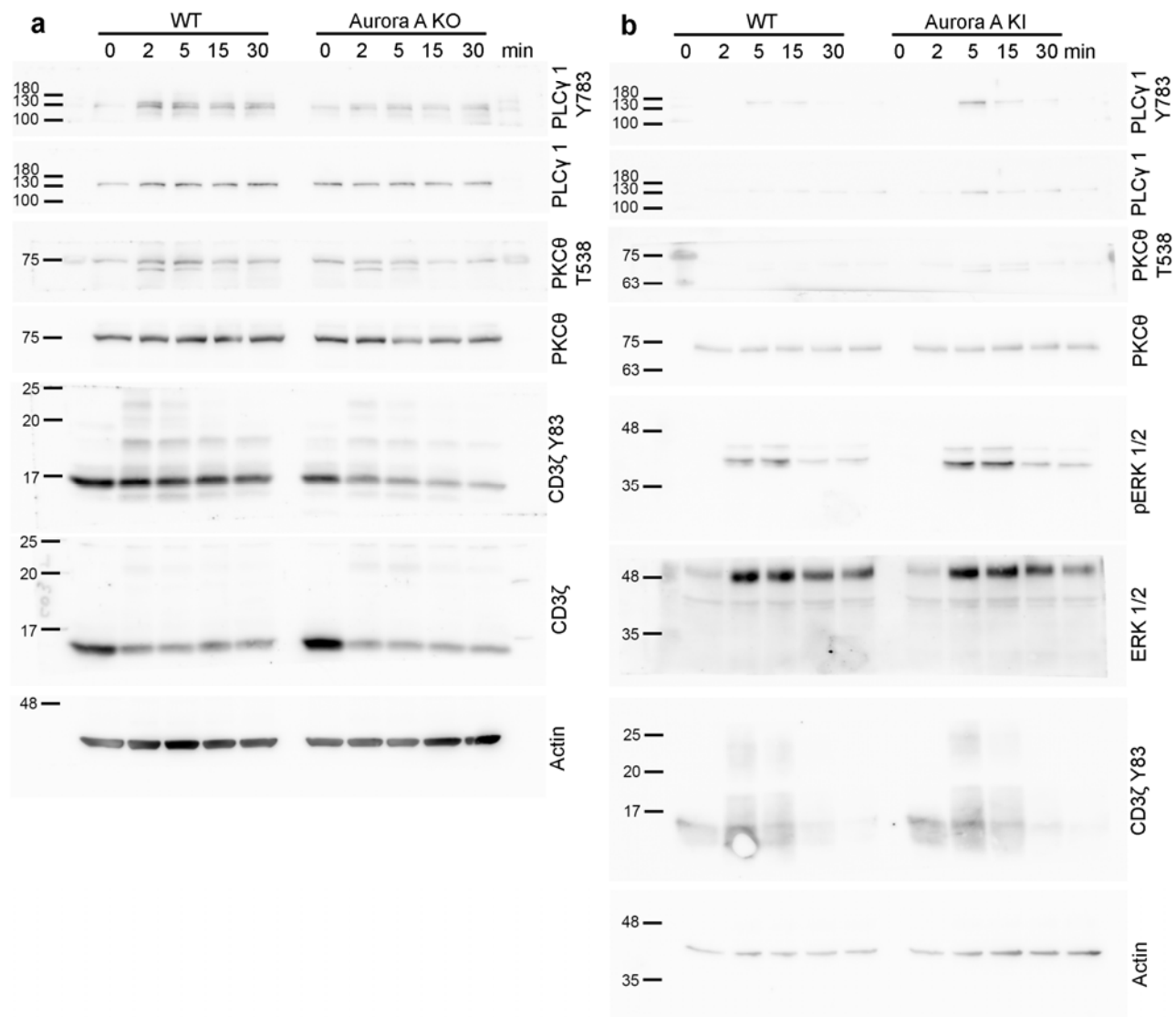
Supplementary Figure 7



Supplementary Figure 7. Uncropped Western blots. Western blots correspond to Fig. 7a. Molecular weights are indicated.

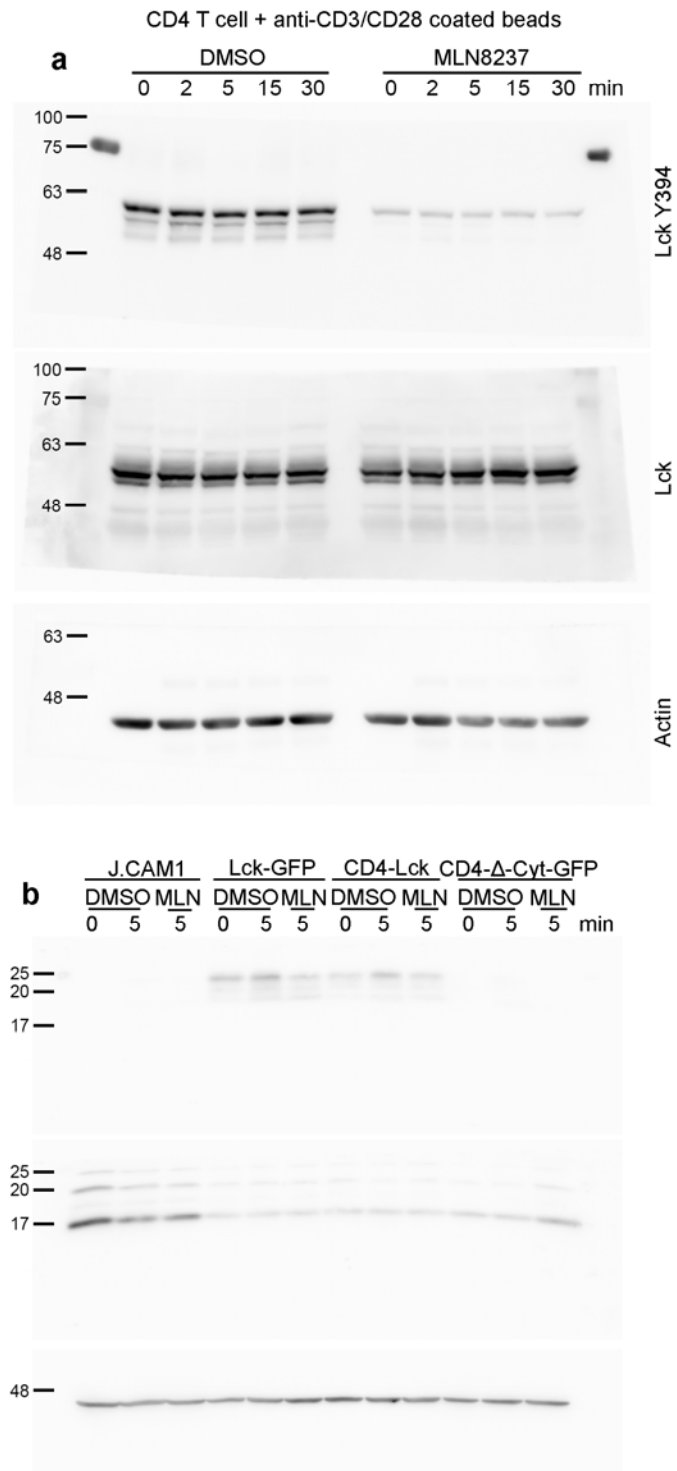
Supplementary Figure 8

Mouse CD4 T cell + anti-CD3/CD28



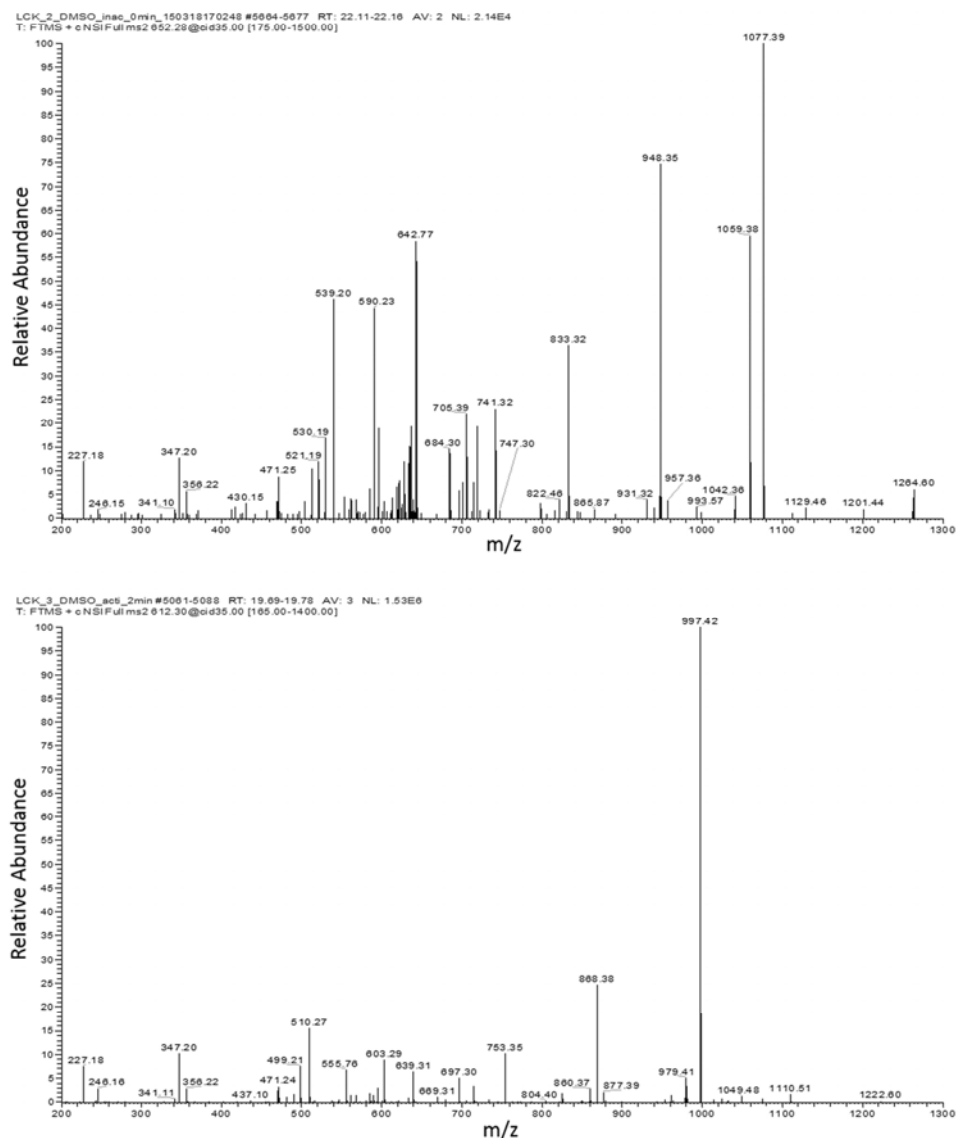
Supplementary Figure 8. Uncropped Western blots. (a) Western blots correspond to Fig. 8b. (b) Western blots correspond to Fig. 8e. Molecular weights are indicated.

Supplementary Figure 9



Supplementary Figure 9. Uncropped Western blots. (a) Western blots correspond to Fig. 9c. (b) Western blots correspond to Fig 9e. Molecular weights are indicated.

Supplementary Table 1



Supplementary Table 1. Examples of MS/MS fragmentation spectra from the Y394-phosphorylated (upper panel) and non-modified (lower panel) forms of Lck peptide LIEDNEYTAR. Ions ascribed to the main y - (containing the peptide C-terminus) and b - (containing the peptide N-terminus) fragmentation series are indicated. The Y394-phosphorylated and non-modified forms of Lck peptide LIEDNEYTAR were quantified using the MS/MS extracted ion chromatograms of the y_8 fragmentation ions.

Supplementary Table 2

Gene	Forward	Reverse
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
IL-2	AAGTTTTACATGCCCAAGAAGG	AAGTGAAAGTTTTTGCTTTGAGCTA
CD69	CAAGTTCCTGTCCTGTGTGC	GAGAATGTGTATTGGCCTGGA
CD25	CAGCCCCAGCTCATATGCA	TGAGGCTTCTCTTCACCTGGAA

Supplementary Table 2. Sequences of primers used for RT-PCR.