A SLP76-YFP   pTyr   merge

siCon

5 μ   2 μ

B

siE

5 μ   2 μ

C SiCont   SiEz

TCR ζ-GFP

5 μ   5 μ
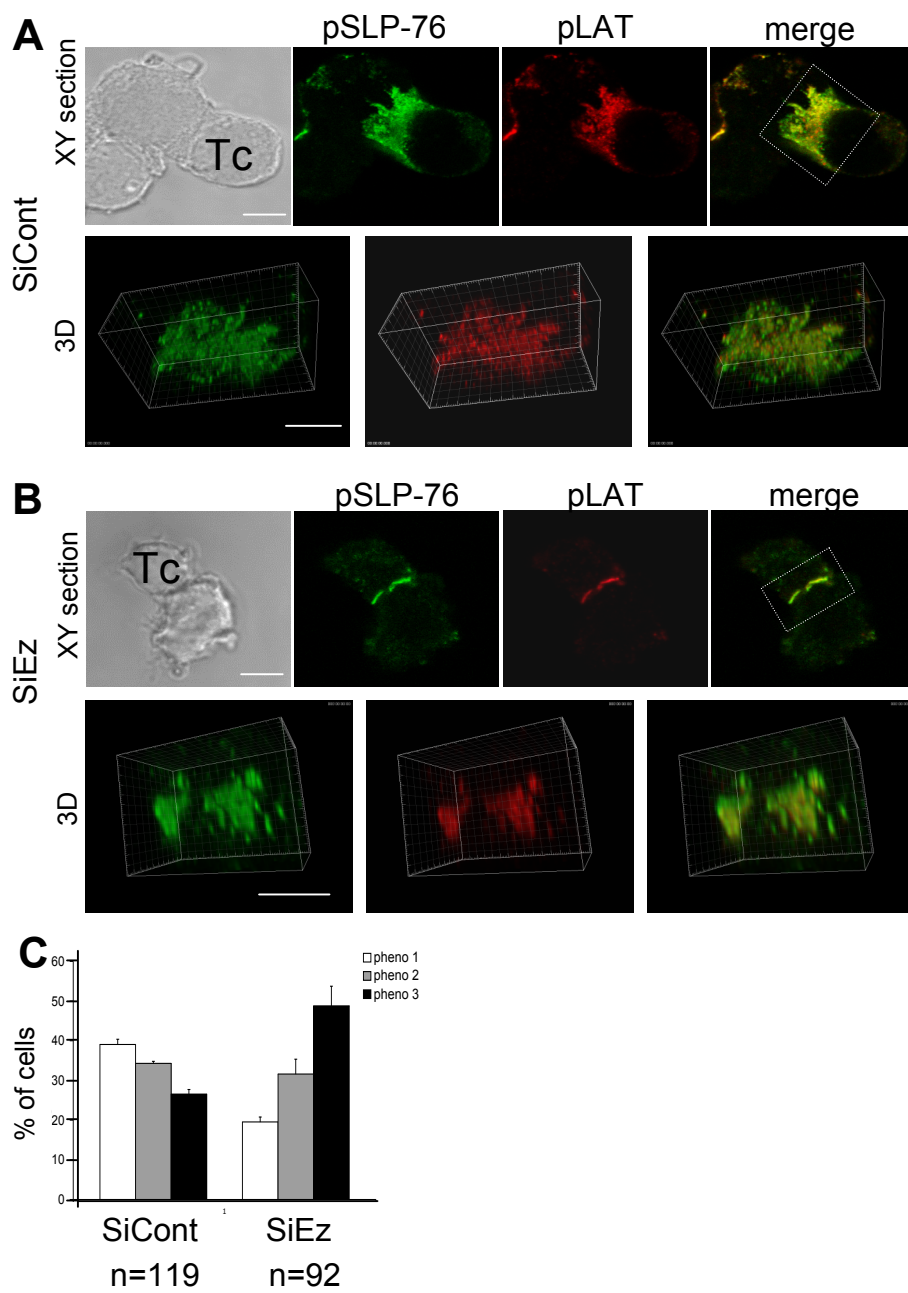
D SLP-76YFP   Ezrin   merge

5 μm

Lasserre et al Supplementary Fig. 1

Supplementary Figure 1. Ezrin silencing alters the patterns of pTyr- or TCRζ-containing microclusters. Ezrin does not concentrate in microclusters
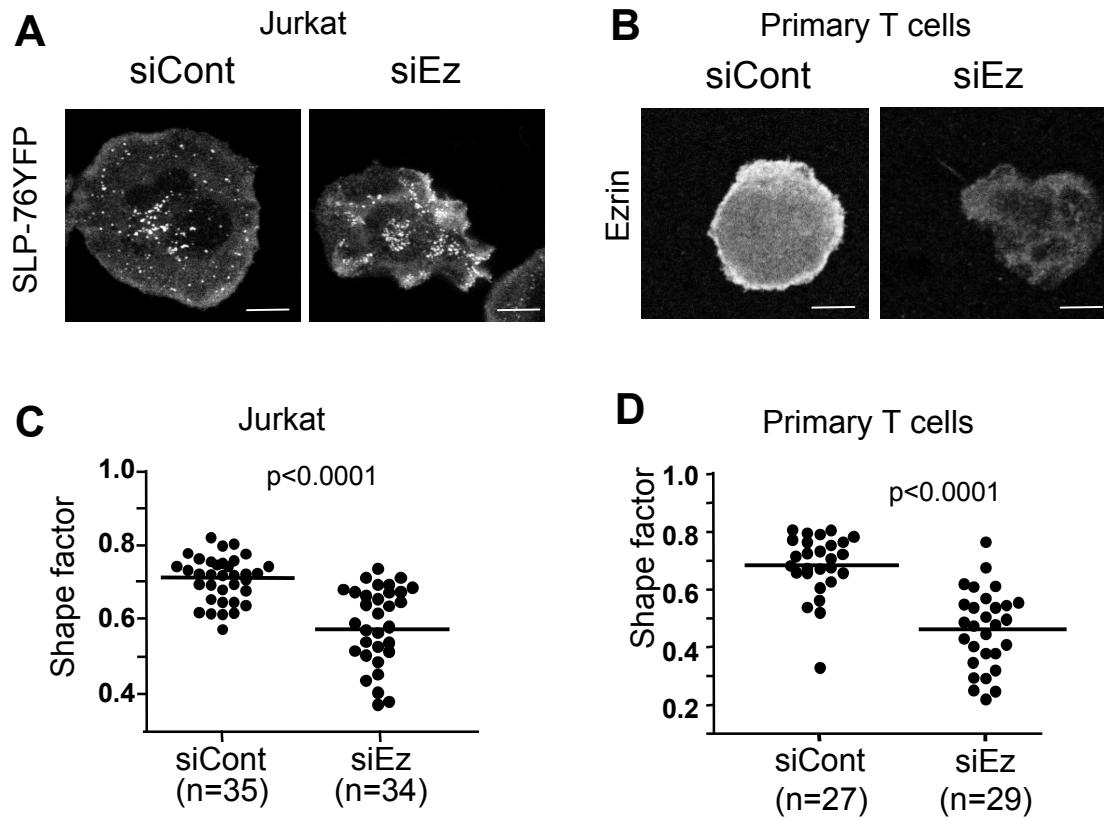
A, B: Jurkat J14 cells expressing YFP-SLP-76 treated with siRNA control (A), or siRNA ezrin-1 (B), were activated 3 min on anti-CD3-coated coverslips, fixed, stained with anti-pTyr Ab and analyzed by confocal microscopy. Right panels zoom the framed area in merge panels, which was set on a random area containing SLP-76 microclusters (A), or on a microcluster aggregate (B). Arrowheads point to SLP-76 microclusters either single or within an aggregate. One confocal optical section of the contact surface is shown. **C:** Jurkat J77 cells stably expressing TCRζ-GFP treated with siRNA control, or siRNA ezrin-1 were activated on anti-CD3-coated coverslips for 3 min, fixed and analyzed by TIRF microscopy. **D:** Jurkat J14 cells stably expressing YFP-SLP-76 were activated on anti-CD3-coated coverslips for 3 min, fixed, stained with anti-ezrin Ab. Confocal image stacks were treated by deconvolution. A projection of two XY confocal sections of the contact surface is shown. A representative experiment is shown out of three independent experiments.

A

SiCont

XY section — pSLP-76 | pLAT | merge

Tc

3D

B

SiEz

XY section — pSLP-76 | pLAT | merge

Tc

3D

C

□ pheno 1
■ pheno 2 (grey)
■ pheno 3

% of cells

SiCont
n=119

SiEz
n=92

Lasserre et al Supplementary Fig. 2

Supplementary Figure 2. Ezrin silencing alters signaling microcluster patterns in immunological synapses formed between T cells and APCs
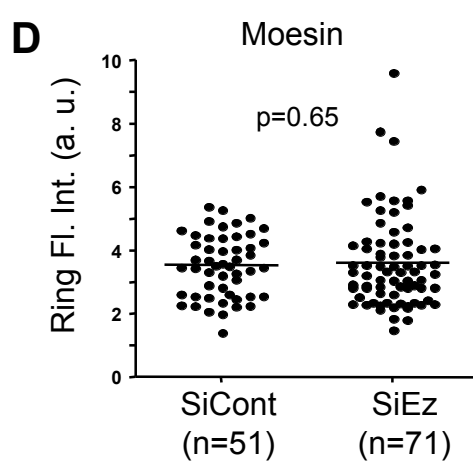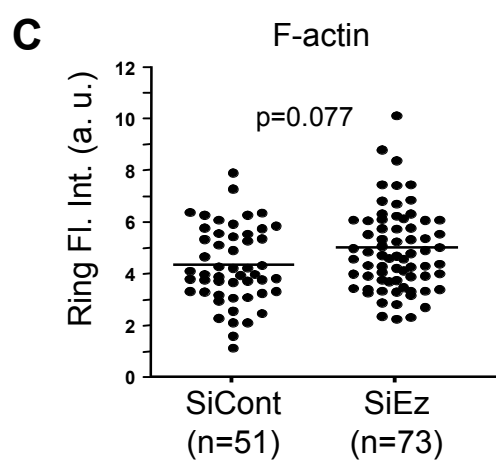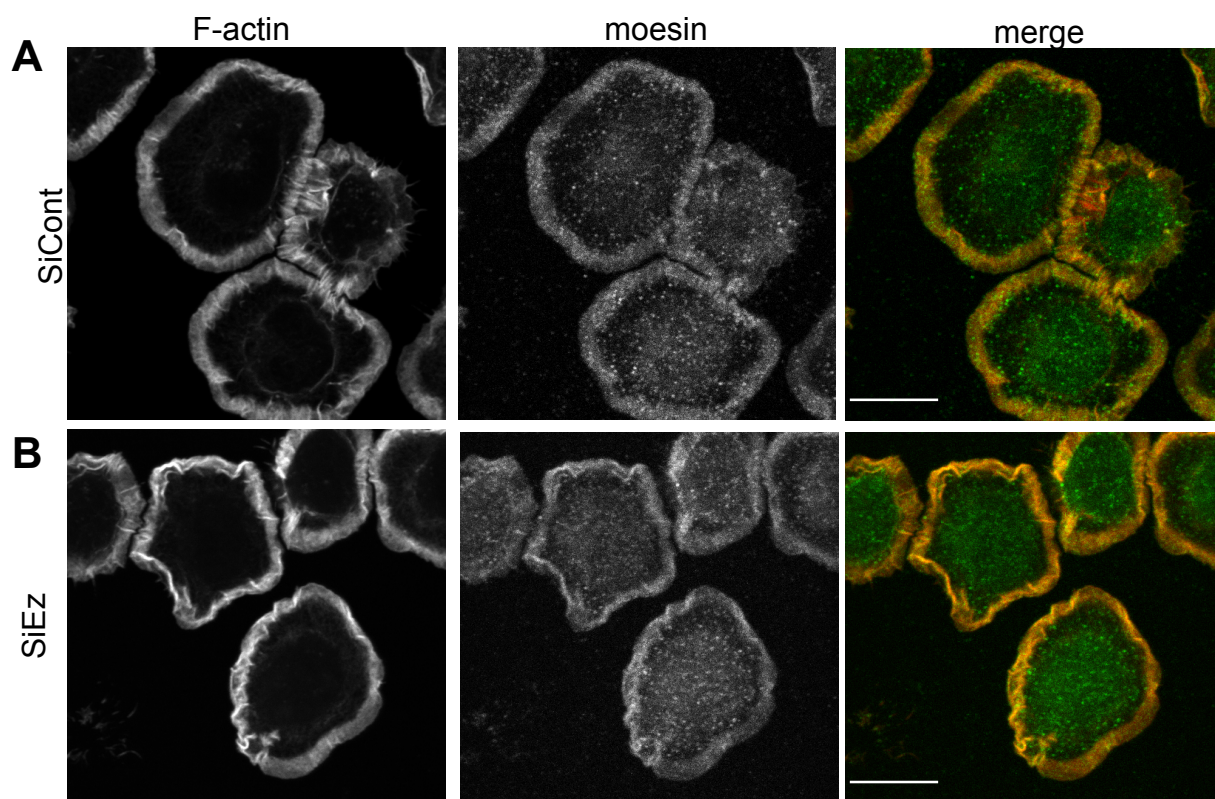
A, B: Jurkat J77 cells transfected with siRNA control (A), or siRNA ezrin-1 (B) were incubated with sAg-pulsed Raji APCs for 15 min, plated on poly-L-lysine coverslips, fixed, stained with anti-pSLP-76 and anti-pLAT mAbs and analyzed by confocal microscopy. Z-stacks of confocal images were treated by deconvolution and 3D reconstructions were carried out. Top panels show a representative single optical section of the cell conjugate. Lower panels show the 3D reconstruction of the cropped area corresponding to the synapse viewed from the APC. **C:** Cells were classified in three categories: phenotype 1 corresponds to immunological synapses displaying dispersed pSLP76 and pLAT microclusters (A). Phenotype 3 corresponds to cells displaying aggregated fluorescence microclusters (B). Phenotype 2 corresponds to intermediate patterns. Plots show data from three independent experiments ± SEM. Scale bar = 5 μm.

A Jurkat

siCont   siEz

SLP-76YFP

B Primary T cells

siCont   siEz

Ezrin

C Jurkat

p<0.0001

Shape factor

1.0
0.8
0.6
0.4

siCont
(n=35)

siEz
(n=34)

D Primary T cells

p<0.0001

Shape factor

1.0
0.8
0.6
0.4
0.2

siCont
(n=27)

siEz
(n=29)

Lasserre et al Supplementary Fig. 3

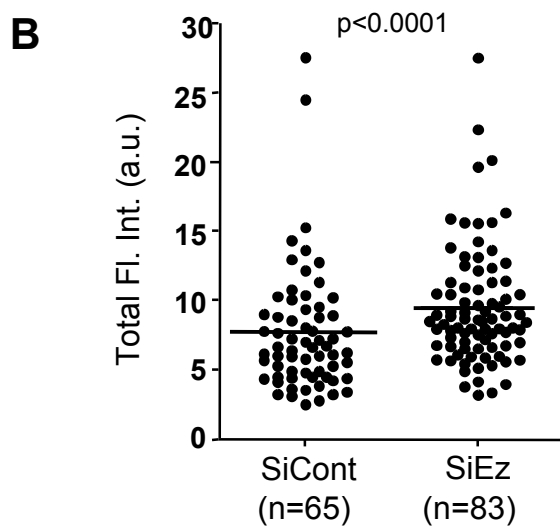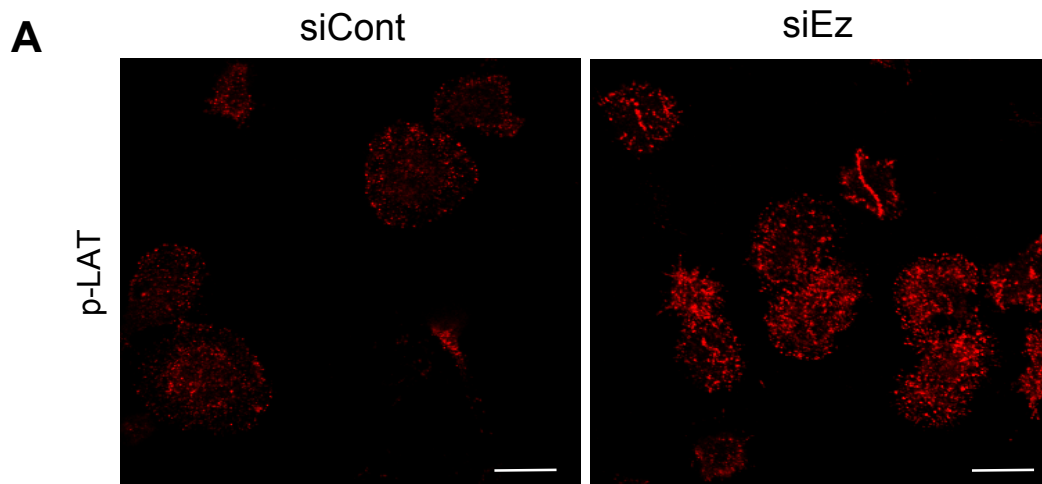**Supplementary Figure 3. Ezrin silencing alters cell shape**

A, B: Jurkat J14 cells expressing YFP-SLP-76, or primary CD4 T cells, transfected with siRNA control, or siRNA ezrin were activated on anti-CD3-coated coverslips for 3 min (A), or on anti-CD3 + anti-CD28-coated coverslips for 5 min (B). Cells were fixed and left unstained (A) or stained for ezrin (B). A confocal section of the contact surface is shown. **C, D:** Cell shape was analyzed and cell shape factor calculated as described in Supplementary Methods. The shape factor is comprised between 0 and 1. Its value tends to 1 when the shape approximates to a circle. Plots represent pooled data from three (C) or two (D) independent experiments. Bar = 5 μm.

Lasserre et al Supplementary Fig.4

Supplementary Figure 4. Ezrin silencing does not alter F-actin and moesin patterns at the immunological synapse.

A, B: Jurkat J77 cells transfected with siRNA control (A), or siRNA ezrin-1 (B) were activated on anti-CD3-coated coverslips for 3 min, fixed and stained with phalloidin and anti-moesin Ab. **C:** The fluorescence intensity due to F-actin and moesin presence at the periphery of the synapse was quantified using Acapella software as described in Methods and expressed in arbitrary units. No significant differences between control and ezrin-silenced cells were found. Representative experiment out of two carried out. Bar = 10 μm.

A

siCont   siEz

p-LAT

B

p<0.0001

Total Fl. Int. (a.u.)

SiCont
(n=65)

SiEz
(n=83)

Lasserre et al Supplementary Fig.5

Supplementary Figure 5. LAT phosphorylation at the immunological synapse is enhanced in ezrin-silenced cells

A: Jurkat J77 cells transfected with siRNA control, or siRNA ezrin-1 were activated on anti-CD3-coated cover slips for 3 min, fixed, stained with anti-pLAT Abs and analyzed by confocal microscopy (upper panel). A confocal section of the contact surface is shown.

B: Total fluorescence intensity at the contact site was quantified with Metamorph software and expressed in arbitrary units.

A representative experiment is shown out of three independent experiments carried out. Bar = 10 μm.
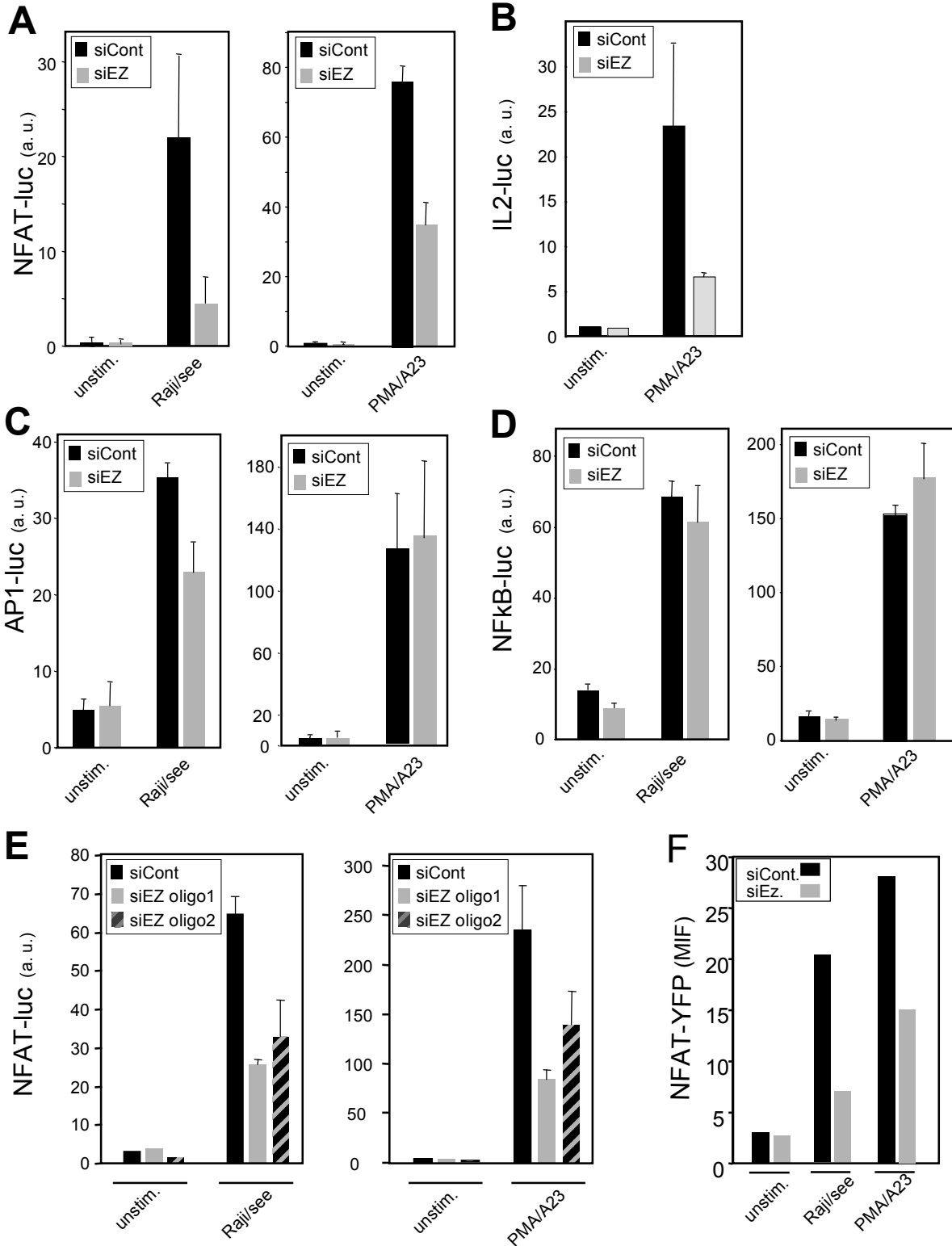
Lasserre et al Supplementary Fig.6

Supplementary Figure 6. Ezrin silencing inhibits NFAT activation

A-D: Jurkat J77 cells transfected with siRNA control or siRNA ezrin were co-transfected with thymidine kinase (TK)-Renilla luciferase constructs and luciferase reporter plasmids NF-AT (A), IL2 promoter (B), AP-1 (C), or NF-κB (D). Then, cells were left unstimulated (unstim.) or stimulated with sAg-pulsed Raji cells, or with PMA (20 ng/ml) and Ca ionophore (A23187, 1 μM) (PMA/A23) for 8 h. Results are reported as the activity, in arbitrary units, of firefly luciferase normalized to that of Renilla luciferase. **E:** Similar experiments performed by transfecting two siRNA oligos targeting distinct ezrin sequences (Supplementary Methods and Fig 1 A). **F:** Jurkat J77 cells stably transfected with a reporter gene expression vector driving YFP expression under the control of NF-AT were activated as above. YFP fluorescence intensity in cells was measured by FACS. The data represent the mean ± SD, from triplicate measurements of a representative experiment out of three performed.

Supplementary Movie 1.

Jurkat J14 cells stably expressing YFP-SLP-76 transfected with siRNA control oligonucleotides were activated on anti-CD3-coated cover slips and imaged by spinning disc confocal microscopy. Films were taken at 12 frames per min. Total time 5 min. A representative experiment is shown out of five independent experiments.

Supplementary Movie 2.

Jurkat J14 cells stably expressing YFP-SLP-76 transfected with siRNA ezrin were activated on anti-CD3-coated cover slips and imaged by spinning disc confocal microscopy. Films were taken at 12 frames per min. Total time 5 min. A representative experiment is shown out of five independent experiments.

Supplementary Movie 3.

Jurkat J14 cells stably expressing YFP-SLP-76 transfected were treated with 5 μM colchicine, activated on anti-CD3-coated cover slips and imaged by spinning disc confocal microscopy. Films were taken at 6 frames per min. Total time 5 min. A representative experiment is shown out of two independent experiments.

Supplementary Materials and Methods

Reagents. *Staphylococcus* enterotoxin E (SEE) was from Toxin Technology. Phorbol myristate acetate (PMA), calcium ionophore A23187, protease inhibitor cocktail and other chemicals, were from Sigma. Glutathione Sepharose beads, protein G sepharose, PVDF membranes and enhanced chemiluminescence (ECL) reagents were from Amersham-Pharmacia. Fluo-3 calcium probes were from Molecular Probes. Colchicine was from Sigma.

Antibodies. The rabbit anti-ezrin Ab has been described (Algrain et al., 1993). Mouse mAb anti-ezrin (clone 18) was from BD-Transduction Laboratories. Anti-phospho-PLCγ1 (Tyr^{783}), anti-phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}) (Phospho-Erk1/2), were from Cell Signaling Technology. Anti-Vav-1, anti-Erk 1/2, anti- PLCγ 1 and anti-phosphoTyr 4G10 were from Upstate Biotechnology. Anti-SLP-76 and anti-CD28 mAbs were from Serotec. Anti-phospho-SLP-76 (Tyr^{128}), Anti-IKKγ (NEMO), anti-ZAP-70, anti-moesin and anti-rabbit Ig were from BD-Biosciences. Anti-phospho-LAT (pTyr^{191}) was from Biosource. Horse-radish perodixadase-coupled anti-mouse IgG Ab was from Beckman Coulter. Anti-beta tubulin mAb was from Chemicon, anti-CD3 UCHT-1 was from Serotec and anti-CD3 MEM92 was from Exbio. Anti-centrin Ab was a kind gift of Dr M. Bornens, Institut Curie, Paris. Rabbit anti-Dlg1 Ab was from Santa Cruz. Rabbit anti-phospho-p38 recognizing the activatory phosphorylated residues pThr^{180}, pTyr^{182} was from Cell Signaling. Abs for Odyssey Infrared Imaging system were from Molecular Probes (goat anti-mouse IgG Alexa^{680}) and from Pierce (goat anti-rabbit IgG-DyLight 800).

Tranfection with siRNA oligonucleotides.

Small double stranded RNA oligonucleotides targeting human ezrin were from Eurogentec: oligo 1: 5'- AAG TTG AAG AGT GGC AGC ACA - 3'; oligo 2: 5'- GGT GGT AAA GAC TAT CGG C-3'. A siRNA targeting beta globin (5'-GGU GAA UGU GGA AGA AGU UTT –5') was used as a negative control in ezrin experiments. Dlg1 oligo used was from Dharmacon: 5'- GAA GUG ACC UUA CUG GAU.A –3'. A non targeting control oligo was used in Dlg1 experiments: 5'- UAG CGA CUA AAC ACA UCA.AUU- 3'. Jurkat cells (5-10 x 10^6 cells in 400 µl RPMI) were transfected twice with 200 pmol of siRNA by electroporation at 300 volts, 500 µF using the Gene Pulser apparatus (Bio-Rad), on day 0 and

day 1. Experiments were performed on day 3. Primary CD4 T cells were transfected with siRNA oligonucelotides using Amaxa nucleofection, according to the manufacturer's protocol and experiments were carried out on day 4.

Cell shape measurements

Shape factor was calculated using metamorph software according to the following formula: Shape factor $= 4\pi A/P^2$, where A is the area and P the perimeter. Values tending to 1 represent shapes approximating a circle, 1.0 being a perfect circle. Values lower than 1 are object shapes less close to a circle.

T cell activation, immunoprecipitation and Western Blotting.

T cells were incubated with Raji B cells either unpulsed, or pulsed with SEE (10 μg/ml) for the indicated times. Cells were lysed ($2x10^7$cells/ml) at 4°C for 30 min in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.02 % NaN_3, protease inhibitors, 1 mM Na_3VO_4, 10 mM NaF and 1% NP-40. Then, the insoluble material was removed by centrifugation at 10,000 x g and the supernatant was either completed with half volume of three times concentrated Laemli loading buffer and analyzed by Western blot, or kept for further analysis. Prior to immunoprecipitation, the cell lysate was precleared for 2 h by addition 1/1000 volume heat inactivated goat serum and 20 μl protein G sepharose beads. Proteins were then immunoprecipitated by adding 10 μg/ml 4G10 mAb and 10 μl protein G-sepharose beads to 200-400 μl lysate. After 2 h incubation at 4°C under constant agitation, the beads were washed 5 times in lysis buffer. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to a PVDF membrane. Polypeptides were detected using appropriate Ab and HRP-labeled goat anti-mouse Ig secondary Abs. Blots were revealed by enhanced chemiluminescence. Band densitometry was carried out on films (Fig 4), or membranes using Odyssey Infrared Imaging system (Fig 7).

Luciferase reporter gene assays

Jurkat cells (5×10^5 cells) transfected with siCont or siEz oligonucleotides were co-transfected with 0.1 μg thymidine kinase (TK)-Renilla luciferase constructs (Promega, Madison, WI) and 2 μg of luciferase reporter plasmids AP1-luc (a gift of Dr R. A. Hipskind, Institut de Génétique Moléculaire de Montpellier), NFκB-luc (a gift of Drs R. Weil and A. Israel, Institut Pasteur, Paris), NFAT-luc or IL2-luc (Di Bartolo et al, 2007; Roumier et al, 2001) were transfected using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) as indicated by the manufacturer. After 24 h, cells were left unstimulated or stimulated with SEE-pulsed Raji cells, or with PMA (20 ng/ml) plus A23187 (1 μM) for 8 h. Cells were then washed with PBS, and samples were prepared using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Results are reported as the activity, in arbitrary units, of firefly luciferase normalized to that of Renilla luciferase (to correct for the efficiency of transfection) in the lysates.

NF-AT-YFP reporter gene assays

Jurkat T cells stably transfected with a YFP reporter gene expression vector under the control of NF-AT trascription factor (gift of C. Hivroz, Institut Curie, Paris), were transfected with siRNA oligonucleotides and activated as above. YFP expression was analyzed by FACS.

Bootstrap statistical test

We wish to test the hypotheses

$$H_0 : F_{\text{cont}}(x) = F_{\text{treat}}(x - \delta) \text{ vs } H_1 : F_{\text{cont}}(x) \neq F_{\text{treat}}(x - \delta)$$

where F_{cont} is the distribution function for the control sample and F_{treat} is the distribution of the treatment sample. These hypotheses test if the difference between F_{cont} and F_{treat} is due only to a shift of δ in the location but have the same shape. Classical methods like the chi-squared test or the Kolmogorov-Smirnov test for this 2-sample comparison are not well-suited to flow cytometry data which have large sample sizes. Instead, we use a bootstrap resampling procedure.

Let the control sample be $X_1, X_2, \ldots, X_{n_1}$ and the treatment sample be $Y_1, Y_2, \ldots, X_{n_2}$. We transform our data samples by subtracting the sample median and continue the bootstrap testing with these transformed zero median samples. The test statistic T is the integrated squared error

$$T = \int [\hat{f}_{\text{cont}}(x; h) - \hat{f}_{\text{treat}}(x; h)]^2 \, dx$$

where $\hat{f}_{\text{cont}}(x; h) = (1/n_1) \sum_{i=1}^{n_1} K_h(x - X_i)$ and $\hat{f}_{\text{treat}}(x; h) = (1/n_2) \sum_{i=1}^{n_2} K_h(x - Y_i)$ are the corresponding kernel density estimates with common bandwidth h.

Let a bootstrap resample of size m_1 of the control sample be $X_1^*, X_2^*, \ldots, X_{m_1}^*$, and a bootstrap resample of size m_2 of the treatment sample be $Y_1^*, Y_2^*, \ldots, Y_{m_2}^*$. The bootstrap test statistic is

$$T^* = \int [\hat{f}_{\text{cont}}^*(x; h^*) - \hat{f}_{\text{treat}}^*(x; h^*)]^2 \, dx$$

where $\hat{f}_{\text{cont}}^*(x; h^*) = (1/m_1) \sum_{i=1}^{m_1} K_{h^*}(x - X_i^*)$ and $\hat{f}_{\text{treat}}^*(x; h^*) = (1/m_2) \sum_{i=1}^{m_2} K_{h^*}(x - Y_i^*)$. We repeat this bootstrap resampling to produce B replicates $T_1^*, T_2^*, \ldots, T_B^*$. From this we can compute a kernel estimate of the distribution of the bootstrap test statistic $\hat{T}^*$. The p-value for this bootstrap test is $\text{Prob}(\hat{T}^* \geq T)$.

For our data we have sample sizes $n_1 = n_2 = 10000$, and we use bootstrap resample sizes $m_1 = m_2 = 500$ or 1000 with the number of bootstrap replicates $B = 1000$.