The Wiskott-Aldrich syndrome protein permits assembly of a focused immunological synapse enabling sustained T-cell receptor signaling

Ronan Calvez,[1,2] Fanny Lafouresse,[1,2] Julie De Meester,[1,2] Anne Galy,[3] Salvatore Valitutti,[1,2] and Loïc Dupré [1,2]

[1]INSERM, U1043, Toulouse, France; [2]Université Toulouse III Paul-Sabatier, Centre de Physiopathologie de Toulouse Purpan, Toulouse, France and [3]INSERM, U951, Généthon, Evry, France

Citation: Calvez R, Lafouresse F, De Meester J, Galy A, Valitutti S, and Dupré L. The Wiskott-Aldrich syndrome protein permits assembly of a focused immunological synapse enabling sustained T-cell receptor signaling. Haematologica 2011;96(10):1415-1423. doi:10.3324/haematol.2011.040204

Supplementary Design and Methods

Cell lines

CD4$^+$ T cells were isolated by negative depletion using Rosette Sep (StemCell Technologies). CD4$^+$Vβ2$^+$ T cells were then positively selected by incubating the CD4$^+$ T cells with an anti-T-cell receptor (TCR) Vβ$_2$ monoclonal antibody (Beckman Coulter) followed by magnetic sorting using beads coated with GAM-antibody (Miltenyi). CD4$^+$Vβ2$^+$ T cells were cultured in RPMI 1640, 5% human serum and 100 U/mL human interleukin-2. They were expanded by repeated stimulation every 19-21 days with either anti-CD3/CD28 coated beads[1] or feeder cells.[2] Cell purity was assessed by flow cytometry (FACScan, Becton Dickinson) using fluorescein isothiocyanate-labeled anti-CD4 monoclonal antibody (Becton Dickinson) and phycoerythrin-labeled anti-T-cell receptor Vβ$_2$ (Beckman Coulter) and was routinely between 95% and 99% pure. To restore WASP expression stably in WAS CD4$^+$Vβ2$^+$ T cells, a single transduction was performed as previously described[3] with the w1.6W lentiviral vector (VSVg-pseudotyped self-inactivating lentiviral vector containing human WAS promoter/cDNA in its clinical configuration: pCCL backbone and mutated WPRE sequence[4]). Epstein-Barr virus (EBV)-transformed B cells (JY) were cultured in RPMI 1640, supplemented with 10% fetal calf serum, non-essential amino acids, sodium pyruvate, penicillin, streptomycin and 50 μM 2-mercaptoethanol. For superantigen loading, JY cells were incubated with the indicated concentration of TSST-1 for 1 h at 37°/5% CO_2 in complete medium and washed twice to remove unbound TSST-1.

Intracytoplasmic cytokine staining

T cells (1×10^5) were stimulated with an equal number of CMFDA-labeled B-EBV cells (JY) preloaded with the indicated concentration of TSST-1, or with PMA (0.25 μM) plus ionomycin (2.5 μM). Brefeldin A (BD Plug) was added, at a concentration of 1 μg/mL, after 1 h of stimulation to promote cytokine accumulation, and the incubation was carried on for another 3 h. Cells were then collected, fixed in BD Perm/Fix solution, stained with anti-human interleukin-2-phycoerythrin or anti-human interferon-γ-Alexa647 monoclonal antibodies (BD Pharmingen) and analyzed on a FACSCalibur. Data were analyzed using FlowJo software.

Detection of CD3ζ phosphorylation by confocal microscopy

Healthy donors' cells and WAS CD4$^+$Vβ2$^+$ T cells were incubated for 25 min with APC pulsed with 10 ng/mL TSST-1. Cell conjugates were then fixed and stained with anti-phosphotyrosine monoclonal antibodies (clone pY99 from Santa Cruz Biotechnology) and with anti-pCD3ζ antibodies (rabbit monoclonal antibody recognizing pY142 from Epitomics, Burlingame, CA, USA).

Analysis of cell morphology and immunological synapse structure from confocal images

T-cell morphology and F-actin distribution were analyzed by examining the phalloidin-stained conjugates along the z-axis. Three-dimensional images were obtained by processing z-stacks (approximately 30 stacks per conjugate) with Imaris software (Bitplane AG, Zurich, Switzerland). For the study of immunological synapse (IS) structure, images (planes through the center of the IS and 3-dimensional reconstructions) were obtained without processing with the Zen file browser (Carl Zeiss). Using single planes through the center of the IS, IS length and markers of T-cell polarization (MTOC) distance to IS center were measured using the single line function of MetaMorph software (Universal Imaging Corporation). The relative distribution of CD45 and pTyr inside and outside the IS area was obtained from the corresponding staining intensity profile along the cell perimeter, using MetaMorph software (line scan width 6). CD45 was considered excluded from the IS when the corresponding staining intensity was higher outside than inside the IS area. Phosphotyrosine activation at the IS was assessed as the ratio between pTyr intensity at the IS *versus* pTyr intensity outside the IS.

Measurement of the rate of conjugate formation

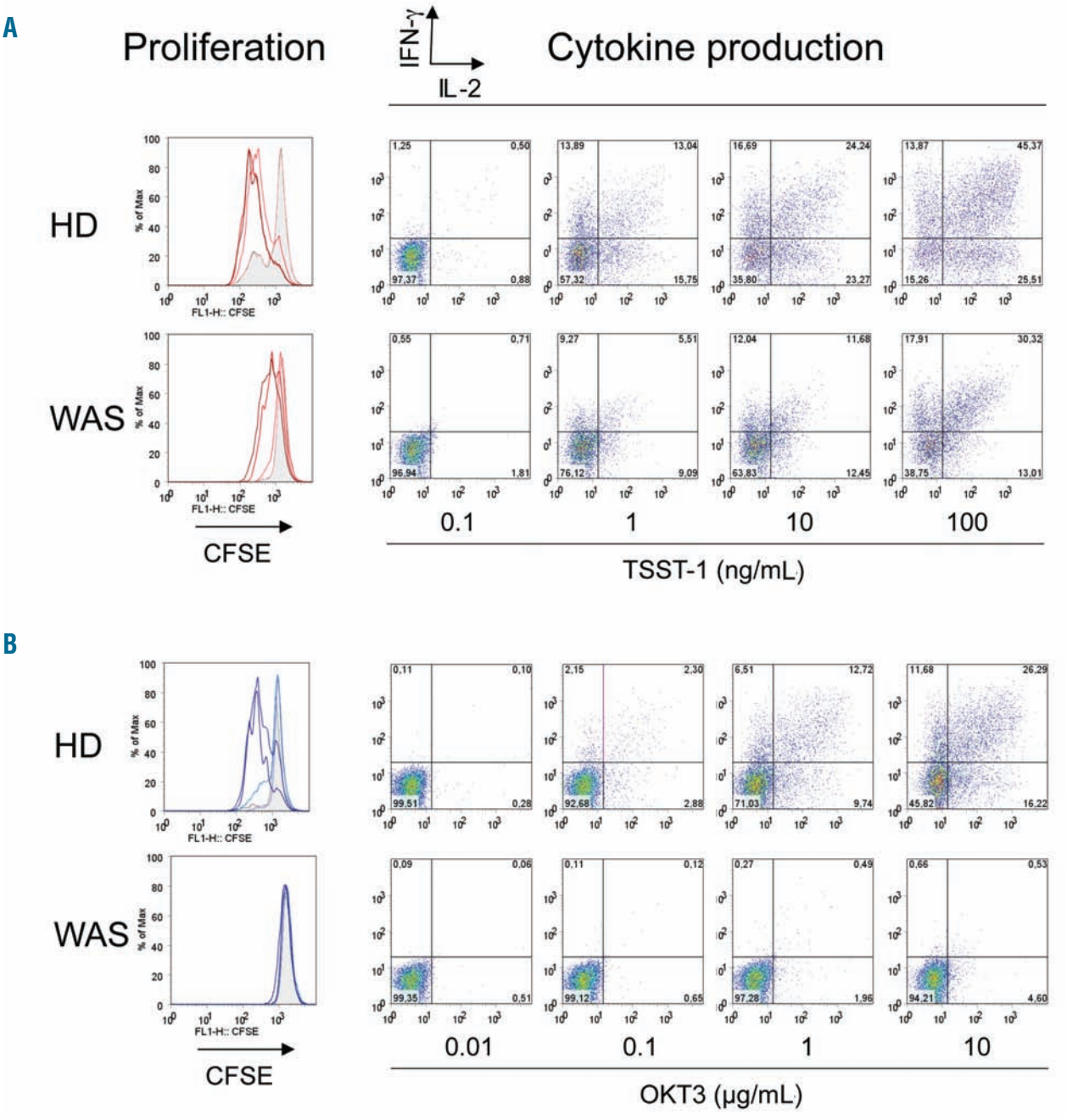
JY cells were pulsed with 10 ng/mL TSST-1, labeled with 1 μM CMTMR, washed and resuspended at a cell density of 4×10^6/mL. T cells were labeled with 0.5 μM CMFDA and resuspended at 4×10^6/mL. T cells and B cells (100 μL of each) were mixed in a 5 mL FACS tube, sedimented by centrifugation and incubated for various time at 37°C. After incubation, conjugates were fixed in 4% PFA and read on a BD FACScalibur. The percentage of T cells was defined as the proportion of CMTMR$^+$/CMFDA$^+$ events within the CMFDA$^+$ population.

T-cell receptor down-regulation

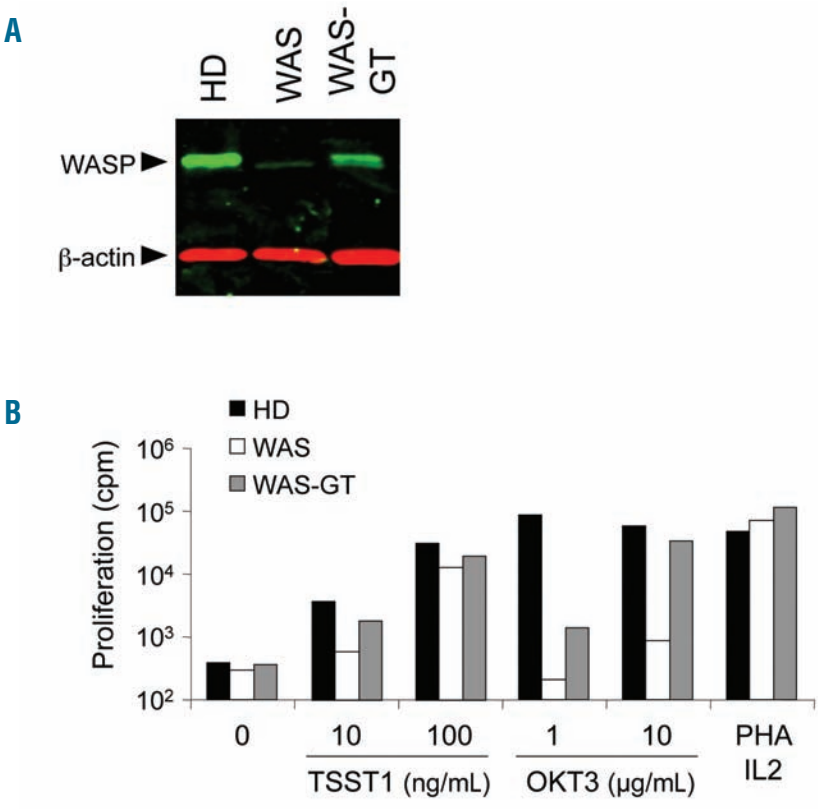
TSST-1-pulsed and unpulsed JY cells were labeled with 1 μM CMTMR. CMTMR-labeled JY cells (1×10^5) were then mixed with an equal number of T cells in U-bottomed 96-well plates. After 3 h at 37°C/5% CO_2, cells were resuspended in phosphate-buffered saline/10 mM EDTA on ice and washed in FACS buffer (phosphate-buffered saline/0.5% bovine serum albumin). TCR surface expression was assessed by staining with an anti-CD3 monoclonal antibody (OKT3, eBioscience) followed by a GAM-IgG2a-AlexaFluor-488 secondary antibody. TCR/CD3 expression level in stimulated T cells was expressed as the percentage of the level of CD3 in T cells stimulated by unpulsed B-EBV cells.

References

1. Bertrand F, Esquerre M, Petit AE, Rodrigues M, Duchez S, Delon J, et al. Activation of the ancestral polarity regulator protein kinase Czeta at the immunological synapse drives polarization of Th cell secretory machinery toward APCs. J Immunol. 2010;185(5):2887-94.
2. Dupre L, Aiuti A, Trifari S, Martino S, Saracco P, Bordignon C, et al. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. Immunity. 2002;17(2):157-66.
3. Dupre L, Trifari S, Follenzi A, Marangoni F, Lain de Lera T, Bernad A, et al. Lentiviral vector-mediated gene transfer in T cells from Wiskott-Aldrich syndrome patients leads to functional correction. Mol Ther. 2004;10(5):903-15.
4. Marangoni F, Bosticardo M, Charrier S, Draghici E, Locci M, Scaramuzza S, et al. Evidence for long-term efficacy and safety of gene therapy for Wiskott-Aldrich syndrome in preclinical models. Mol Ther. 2009;17(6):1073-82.

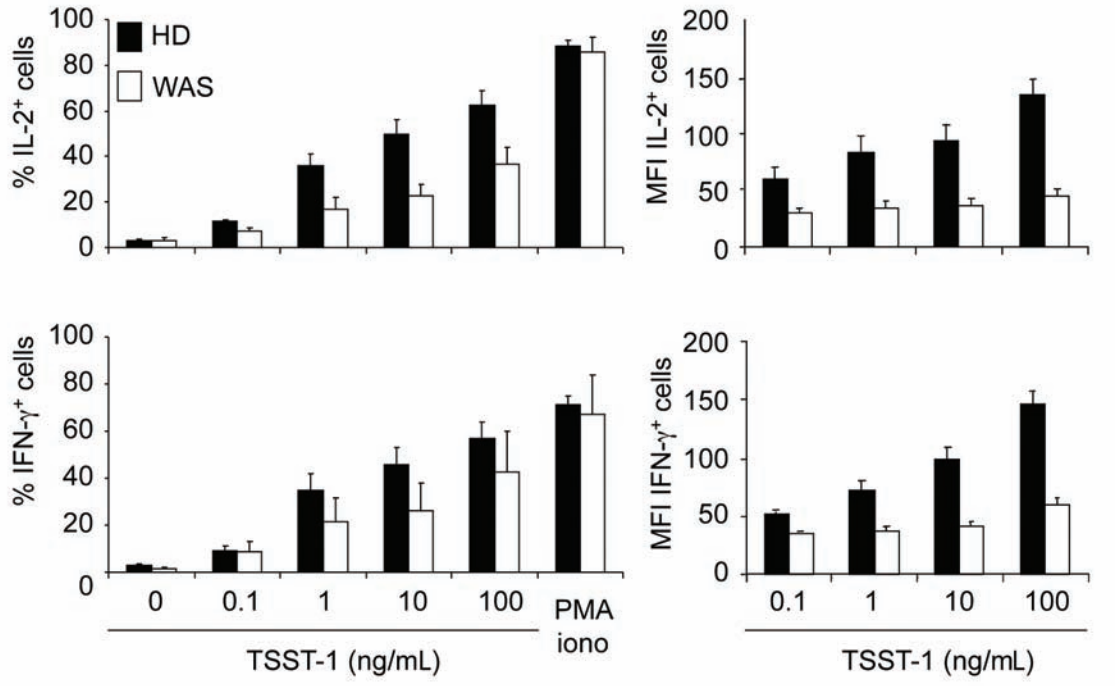


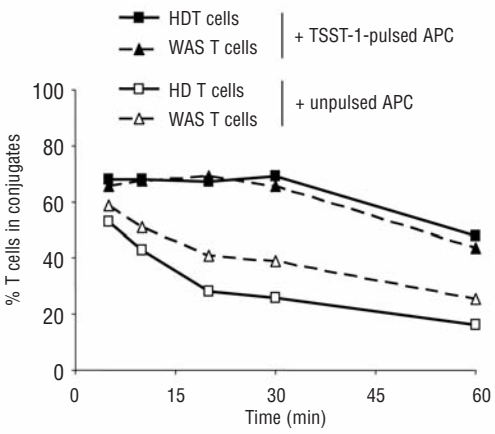
Online Supplementary Figure S1. T-cell activation by TSST-1-loaded APC and immobilized anti-CD3 antibody. (A) Flow cytometry histograms and density plots showing the proliferative and cytokine production responses of healthy donor (HD) and WAS T cells in response to the indicated concentrations of TSST-1 presented by JY cells. (B) Flow cytometry histograms and density plots showing the proliferative and cytokine production responses of HD and WAS T cells in response to the indicated concentrations of immobilized anti-CD3 antibody (OKT3).



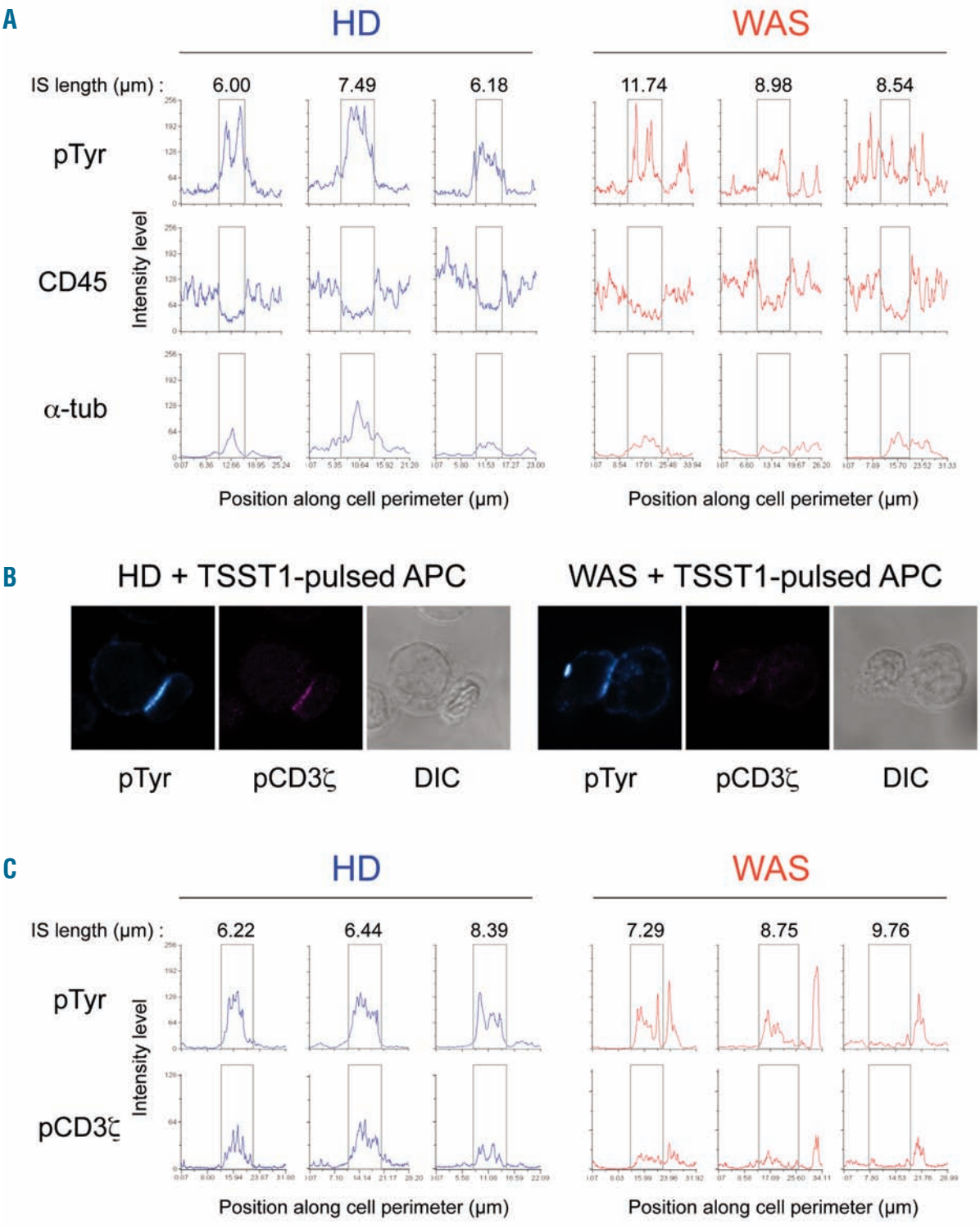
Online Supplementary Figure S2. *WAS* gene transfer restores the proliferative potential of WAS T cells. (A) Purified $CD4^+V\beta2^+$ T cells from a WAS patient were transduced with a lentiviral vector encoding WASP under the human WASP promoter (LVw1.6W) and WASP expression level was assessed by western blot. (B) TSST-1-, anti-CD3- and PHA/IL2-induced proliferation in HD, WAS and WASP reconstituted WAS T cells.

Online Supplementary Figure S3. WASP is required for cytokine production following APC stimulation. Healthy donor (HD) and WAS T cells were stimulated with EBV-B cells presenting the indicated concentrations of TSST-1 (or PMA/ionomycin, 100 nM/1 μM) for 4 h. Intracellular production of interleukin-2 (IL-2) and interferon (IFN-γ) was quantified by flow cytometry [percentage of cytokine-producing cells and mean fluorescence intensity (MFI) in cytokine-producing cells]. Data represent the average ± SEM of seven (CFSE proliferation) and six (cytokine production) independent experiments.

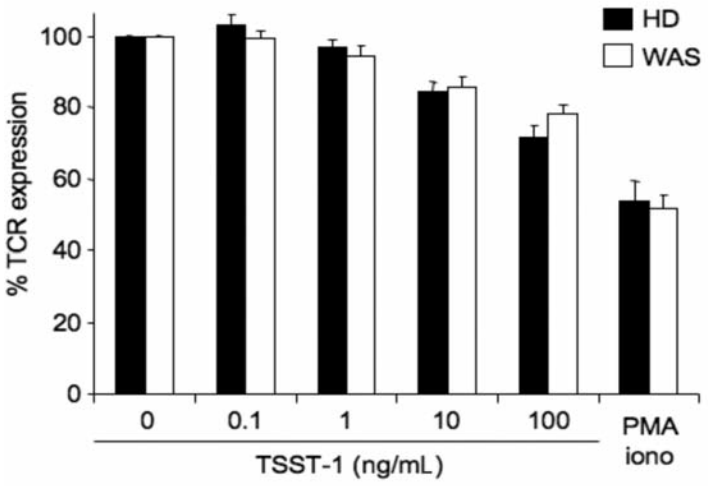




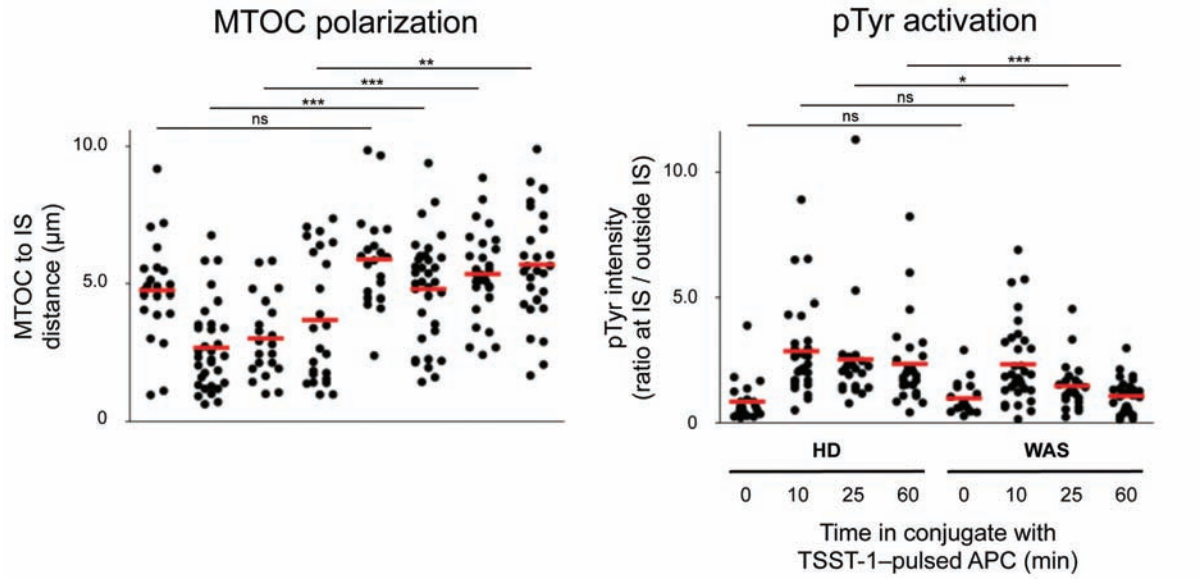
Online Supplementary Figure S4. Normal conjugate formation and TCR engagement in WAS T cells. CMFDA-labeled $CD4^+V\beta2^+$ T cells from either a healthy donor (HD) or a WAS patient were allowed to form conjugates with CMTMR-labeled EBV-B cells pulsed or not with 10 ng/mL TSST-1. The percentage of T cells in conjugates with APC was assessed at different time points by flow cytometry as the proportion of $CMFDA^+/CMTMR^+$ events (conjugates) among $CMFDA^+$ events (T cells). Data are from one experiment representative of three performed.



Online Supplementary Figure S5. Phosphotyrosine outside the confinement of the IS in WASP-deficient T cells coincides with phosphorylated CD3ζ. (A) Representative healthy donor (HD) and WAS T cells in contact with TSST-1-loaded APC were analyzed for IS geometry by representing the intensities of CD45, pTyr and α-tubulin along the complete cell perimeter. The limits of the T cell/APC interaction are reported and the corresponding IS length indicated. (B) HD and WAS $CD4^{+}V\beta2^{+}$ T-cells were incubated for 25 min with APC pulsed with 10 ng/mL TSST-1. Cells were then fixed and stained for phosphotyrosine (cyan) and for the phosphorylated form of CD3ζ (pink). One representative T-cell/APC conjugate per condition is shown. (C) Representative HD and WAS T cells in contact with 24 TSST-1-loaded APC were analyzed for the distribution of pTyr and pCD3ζ along the cell perimeter. The limits of the T-cell/APC interaction are reported and the corresponding IS length indicated.



Online Supplementary Figure S6. Normal rate of TCR engagement in WAS T cells. Healthy donor (HD) and WAS CD4$^+$Vβ2$^+$ T cells were stimulated for 4 h with EBV-B cells presenting the indicated amount of TSST-1 (or PMA/ionomycin). TCR expression levels in stimulated T cells were then assessed by flow cytometry and expressed as the percentage of the TCR levels detected on T cells stimulated with non-pulsed APC. Results are the average ± SEM of three independent experiments.



Online Supplementary Figure S7. Defective MTOC polarization in WAS T cells precedes abnormal pTyr distribution. MTOC translocation to the IS (distance to IS center) and phosphotyrosine activation (expressed as a ratio between pTyr intensity at IS and pTyr intensity outside IS) were assessed in parallel in healthy donor (HD) and WAS CD4$^+$Vβ2$^+$ T cells after conjugation with unpulsed APC ("0") or after 10, 25 or 60 min conjugation with TSST1-pulsed APC. At least 30 conjugates were considered per condition. An unpaired Student's t test was applied and significant differences between conditions are represented as follows: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Video 1. 360° animation of a 3-D reconstruction showing the contact of a healthy donor T cell with an unpulsed APC. Staining for α-tubulin appears in green, staining for CD45 appears in red and staining for phosphotyrosine appears in blue. SEE VIDEO FILE

Video 2. 360° animation of a 3-D reconstruction showing the contact of a healthy donor T cell with a TSST1-pulsed APC. Staining for α-tubulin appears in green, staining for CD45 appears in red and staining for phosphotyrosine appears in blue. SEE VIDEO FILE

Video 3. 360° animation of a 3-D reconstruction showing the contact of a WAS T cell with a TSST1-pulsed APC. Staining for α-tubulin appears in green, staining for CD45 appears in red and staining for phosphotyrosine appears in blue. SEE VIDEO FILE

Video 4. 360° animation of a 3-D reconstruction showing the contact of a WAS T cell with a TSST1-pulsed APC. Staining for α-tubulin appears in green, staining for CD45 appears in red and staining for phosphotyrosine appears in blue. SEE VIDEO FILE

Video 5. 360° animation of a 3-D reconstruction showing the contact of a WASP-reconstituted WAS T cell with a TSST1-pulsed APC. Staining for α-tubulin appears in green, staining for CD45 appears in red and staining for phosphotyrosine appears in blue. SEE VIDEO FILE

Video 6. 360° animation of a 3-D reconstruction showing the contact of a WASP-reconstituted WAS T cell with a TSST1-pulsed APC. Staining for α-tubulin appears in green, staining for CD45 appears in red and staining for phosphotyrosine appears in blue. SEE VIDEO FILE

Video 7. Time-lapse video recording of a healthy donor T cell entering in contact with a TSST-1-pulsed APC (blue). The T cell was loaded with Fura-4 to visualize rises in intracellular calcium. SEE VIDEO FILE

Video 8. Time-lapse video recording of a WAS T cell entering in contact with a TSST-1-pulsed APC (blue). The T cell was loaded with Fura-4 to visualize rises in intracellular calcium. SEE VIDEO FILE