Supplemental Text and Figures

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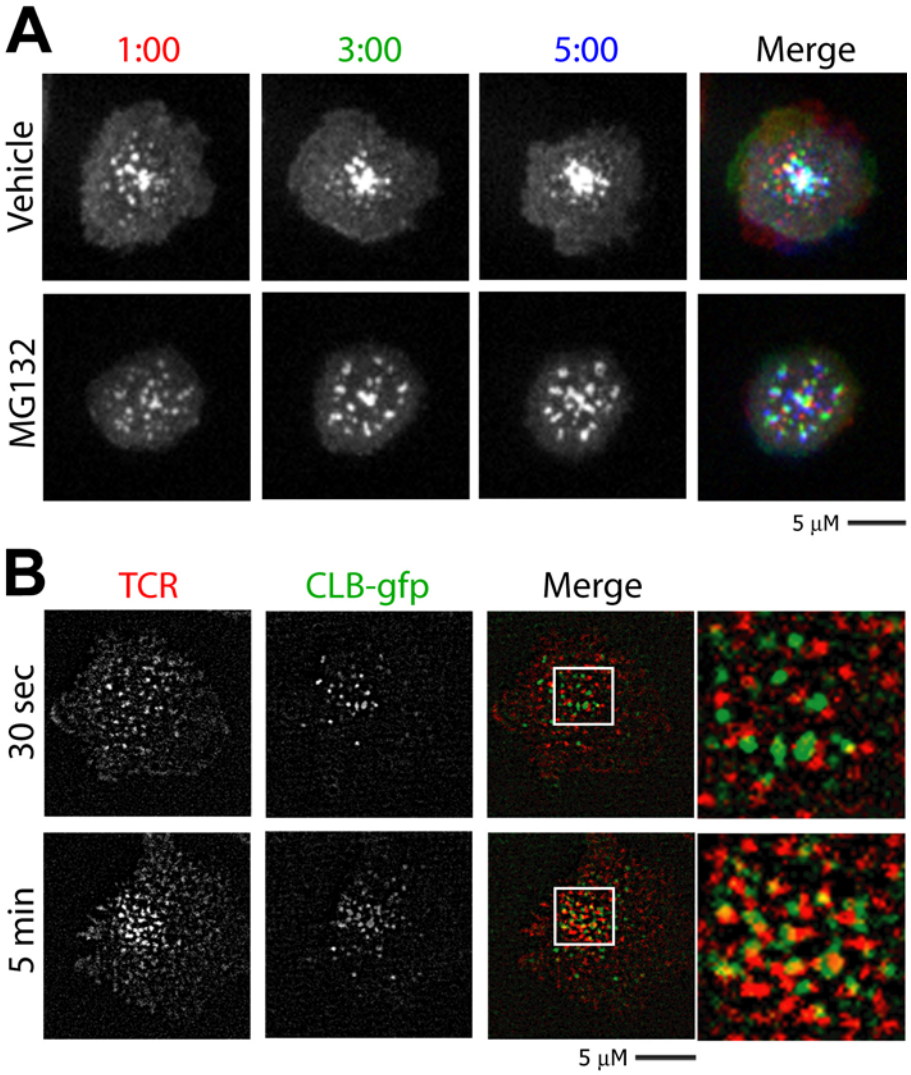
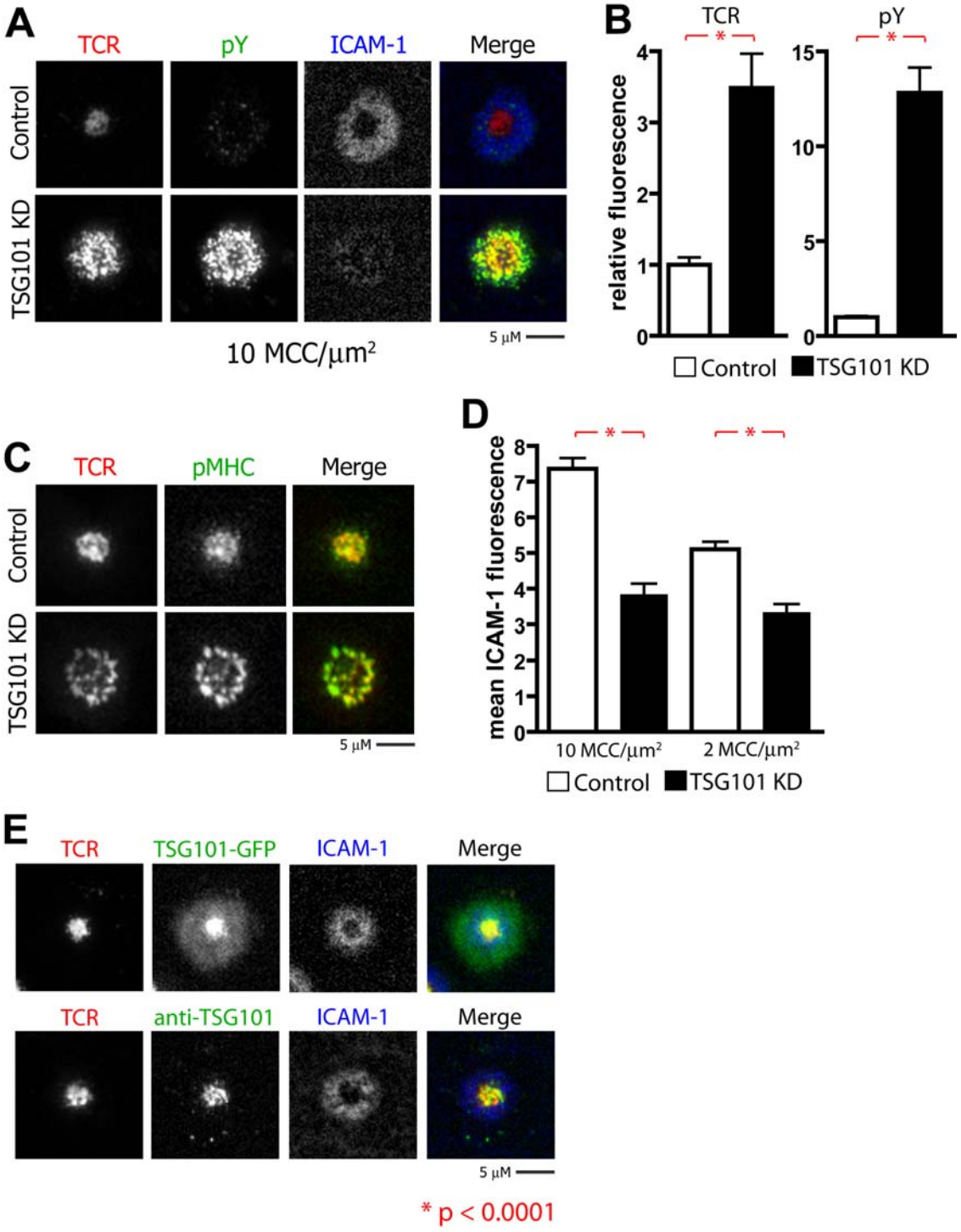
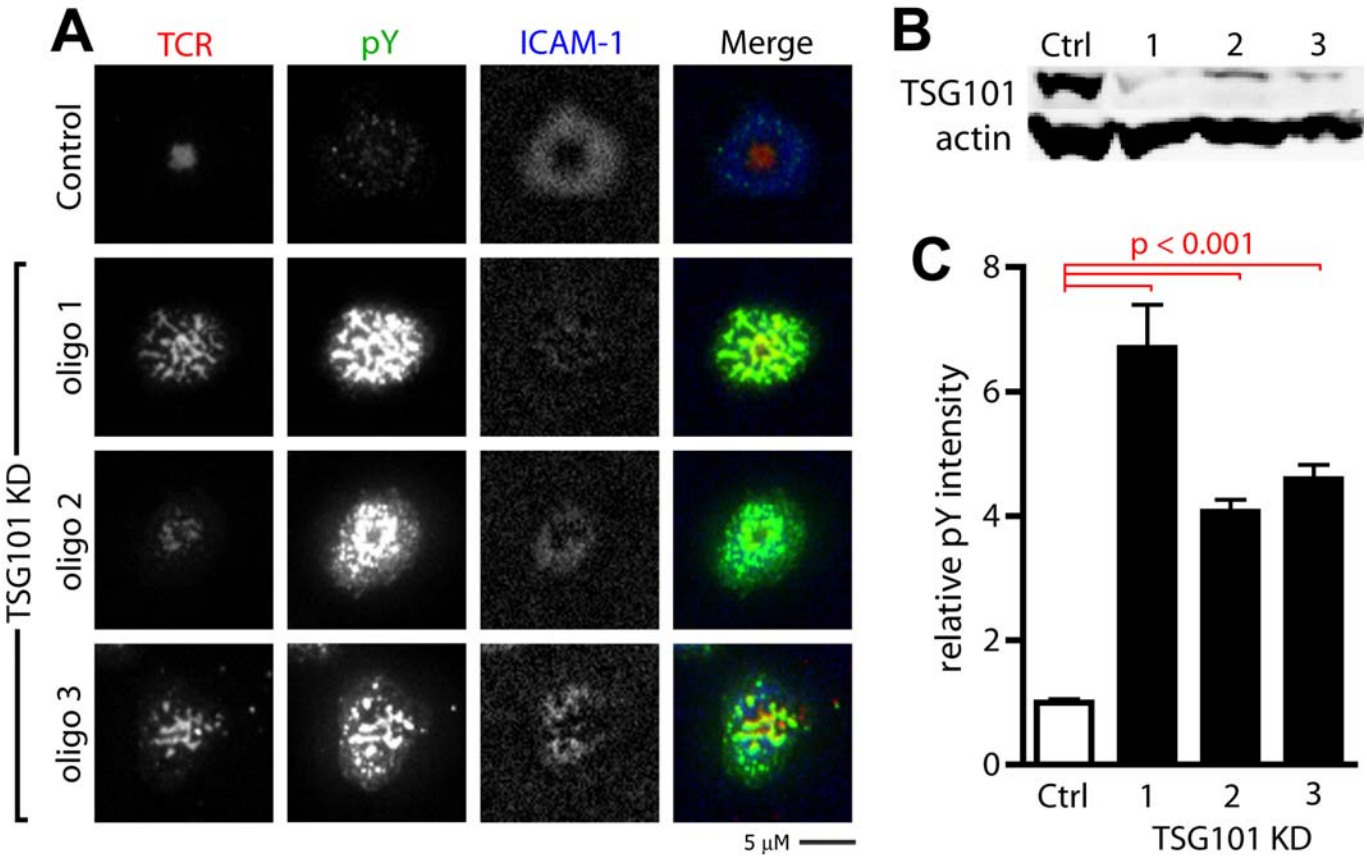


Supplemental Figure S1. Ubiquitination but not clathrin is required for central TCR accumulation. a) AND T cells were treated with DMSO or MG132 to deplete cells of free ubiquitin. They were then incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 at 37°C and imaged in TIRFM in the presence of fluorescent anti-TCR Fab. The presence of white indicates overlap, and immobilization, while any individual color (R, G, or B) indicates movement. b) Activated AND T cells were transfected with plasmids encoding clathrin light chain-GFP. They were then incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1for at 37°C for the indicated times and imaged in TIRF in the presence of fluorescent anti-TCR Fab. Images show representative distribution of clathrin light chain and TCR at early (30 sec) and late (5 min) timepoints following interaction with bilayers.

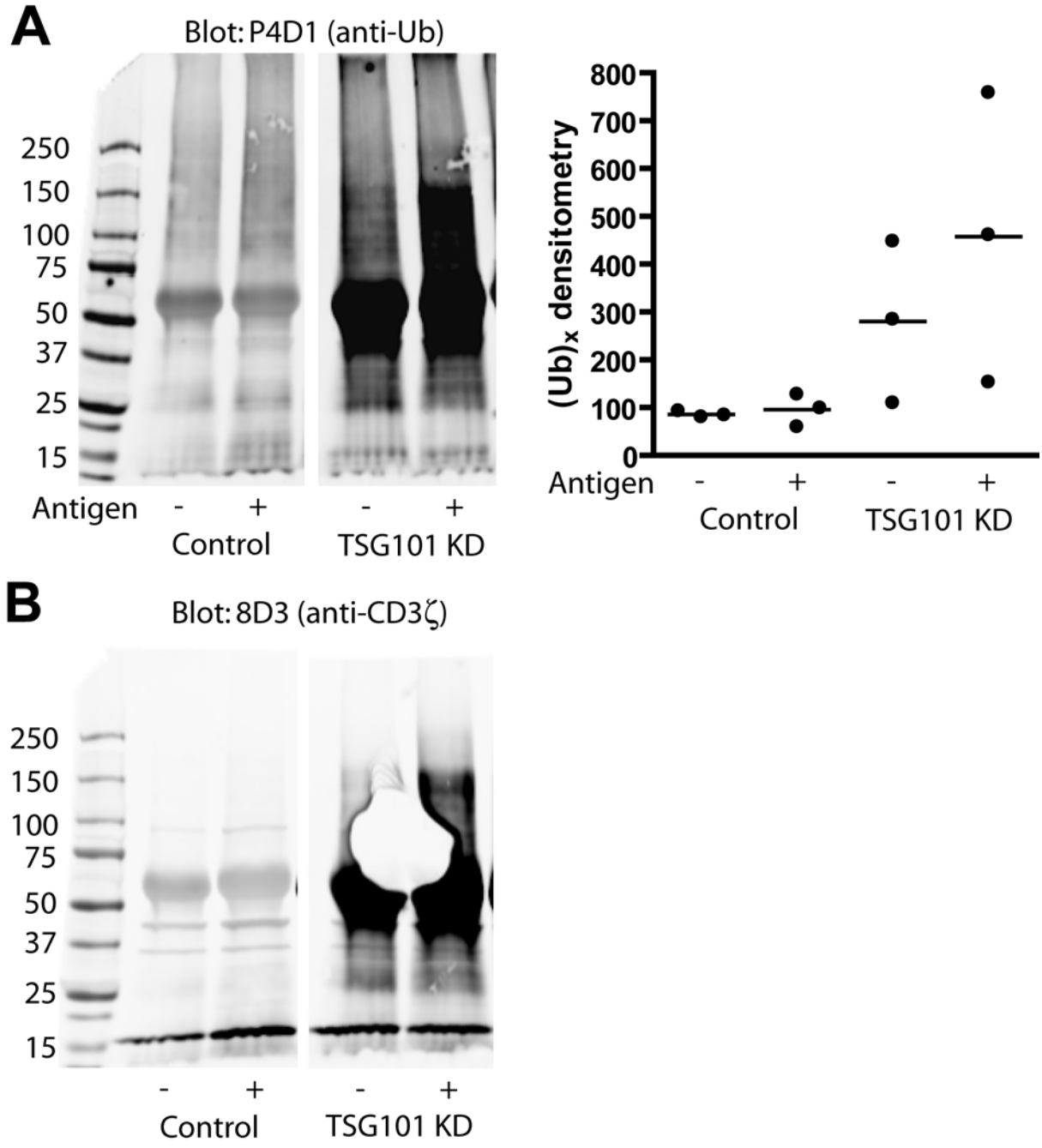


Supplemental Figure S2. TSG101 is required for central TCR accumulation and dephosphorylation. a-b) Control and TSG101 KD T cells were incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 30 minutes at 37°C, fixed, and stained for TCR and phosphotyrosine (pY). Bar graphs depict quantitation in arbitrary units (AU) of mean fluorescence at cell contact interfaces, and represent >100 cells across 2 or more experiments. c) Control and TSG101 KD T cells were incubated on bilayers containing 2 mol/μm^2 I-E^k-MCC-Alexa568 and 200 mol/μm^2 ICAM-1 for 20 minutes at 37°C, after which TCR and MHCp fluorescence was acquired in TIRFM. Images show representative distribution of TCR and MHCp in Control and TSG101 KD synapses. d) Control and TSG101 KD T cells were incubated on glass-supported planar bilayers

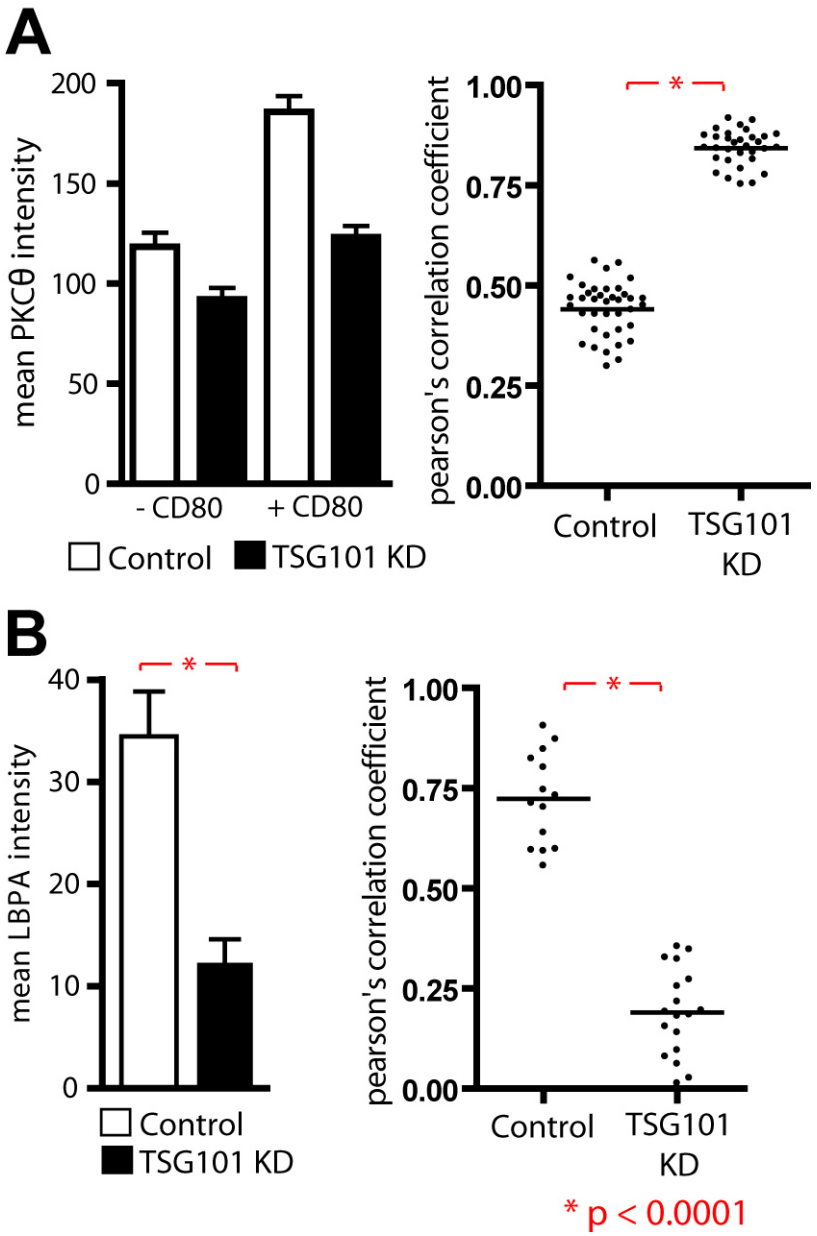
containing 2 or 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 30 minutes at 37°C. Bar graphs depict quantitation in arbitrary units (AU) of mean ICAM-1 fluorescence at cell contact interfaces, and represent >100 cells across 2 or more experiments.e) (above) TSG101-GFP expressing AND T cells were incubated on bilayers containing 2 mol/μm^2 I-E^k-MCC-Alexa568 and 200 mol/μm^2 ICAM-1 for 20 minutes at 37°C, after which TCR and TSG101-GFP fluorescence was acquired in TIRFM. (below) AND T cells were incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 30 minutes at 37°C, fixed, and stained for TCR and TSG101. Images show representative distribution of TCR and TSG101 in T cells forming IS.



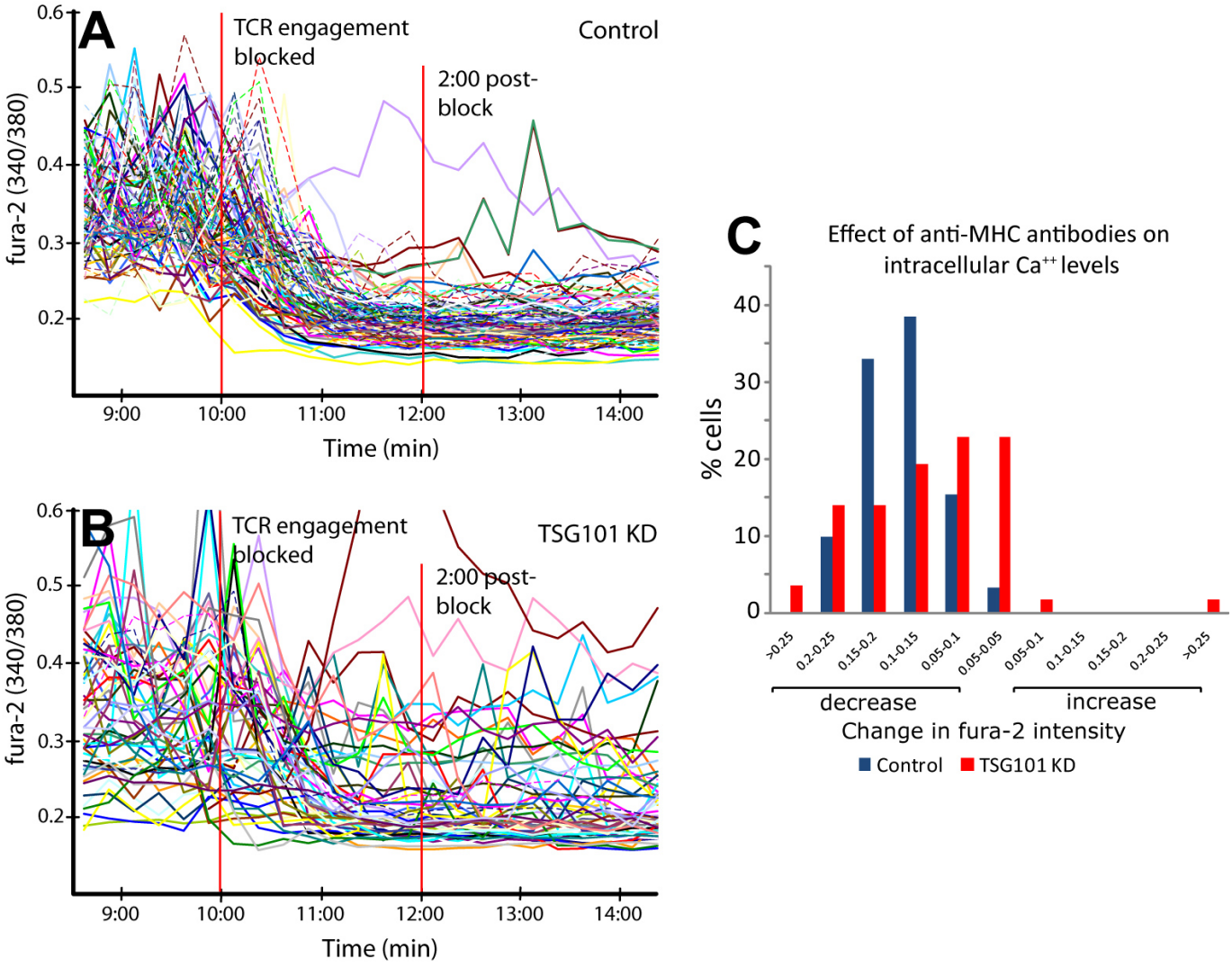
Supplemental Figure S3. Confirmation of TSG101 KD phenotypes with multiple siRNA oligomers. Activated AND Tg T cells were transduced with either control or one of 3 different TSG101-specific siRNA oligomers (labeled 1-3). Control and TSG101 KD T cells were incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 30 minutes at 37°C, fixed, and stained for TCR and phosphotyrosine (pY). Images (a) show representative TCR and pY distribution in control and TSG101 KD synapses. Cell lysates (b) from control or TSG101 KD T cells were run on 4-15% polyacrylimide gels, transferred to nitrocellulose paper, and blotted for TSG101 and actin. Bar graphs (c) depict quantitation in arbitrary units (AU) of mean pY fluorescence at cell contact interfaces, and represent >100 cells across 2 or more experiments.



Supplemental Figure S4. TSG101 KD results in accumulation of ubiquitinated substrates. 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 were tethered to supported lipid bilayers formed on 5μm silica beads for use in biochemical assays. Control and TSG101 T cells (2 million/lane) were mixed with antigen-presenting silica beads at a 1:2 ratio, and brought into contact by brief centrifugation. Following incubation at 37°C for the indicated times, cells were lysed and separated on a 4-15% polyacrylamide gel, Following transfer onto nitrocellulose , membranes were blotted for ubiquitin (a) or CD3ζ (b). Graph depicts quantitation of ubiquitin densitometry in the presence or absence of antigen in 3 separate experiments.

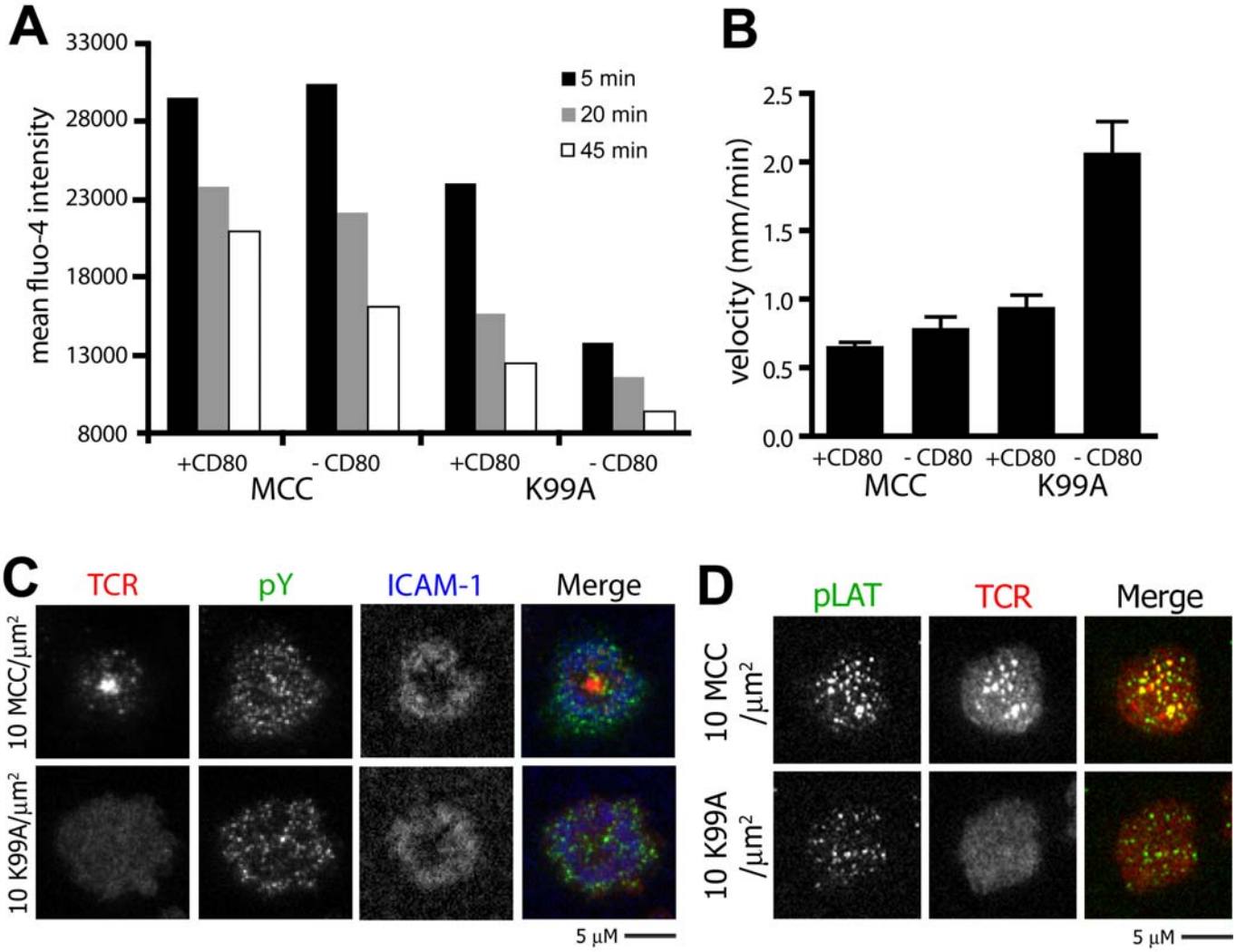


Supplemental Figure S5. TSG101 is required for sorting of TCR and CD28 at the IS center. a) Control and TSG101 KD T cells were incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 with or without 100 mol/μm^2 CD80 for 30 minutes, fixed, and stained for PKCθ. Bar graphs depict quantitation in arbitrary units (AU) of mean PKCθ fluorescence at cell contact interfaces and represent >100 cells across 2 or more experiments. b) Control and TSG101 KD T cells were incubated on glass-supported planar bilayers containing 2 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 30 minutes at 37°C, fixed, and stained for LBPA. Bar graphs depict quantitation in arbitrary units (AU) of mean LBPA fluorescence at cell contact interfaces and represent >100 cells across 2 or more experiments.

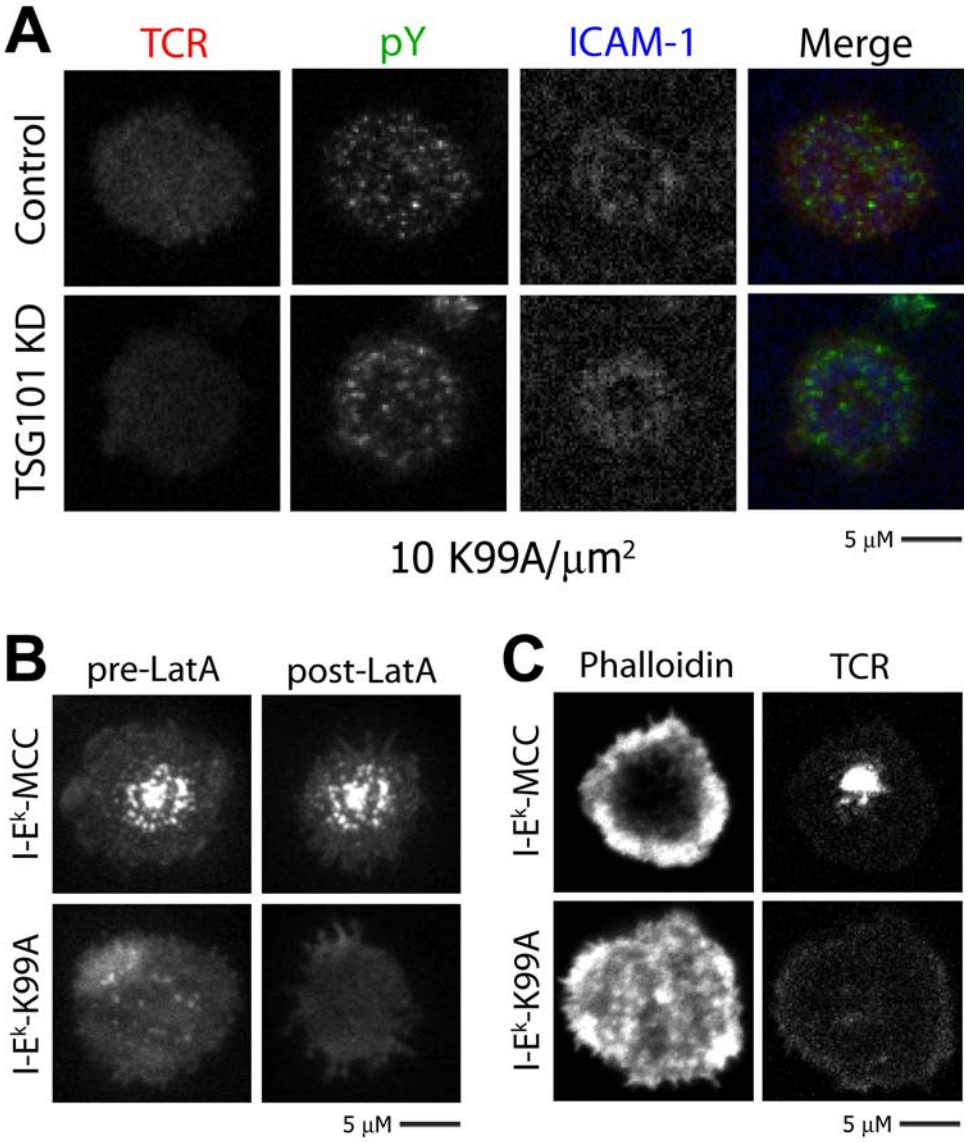


Supplemental Figure S6. TSG101 suppression leads to chronic TCR signaling.

Single-cell intracellular calcium measurements were performed on Fura-2 loaded (a) control (n= 91) and (b) TSG101 KD (n= 57) T cells following incubation on glass-supported planar bilayers containing 2 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 10 minutes at 37°C. Cells were then treated with saturating concentrations (30 μg/mL) of anti-MHC blocking antibodies. Each track depicts individual cell intracellular calcium signal presented as a ratio of fluorescence at 510 nm when excited at 340/380 nm. For each cell, the average fura-2 intensity prior to and 2 minutes after anti-MHC treatment was quantified, and the change in fura-2 intensity was determined. Examination of Ca2+ levels in individual cells revealed a greater heterogeneity in the TSG101 KD cells, perhaps due to the partial disruption of the LFA-1-ICAM-1 interactions in the pSMAC, which are important for sustained Ca2+ elevation (Wulfing C, Sjaastad MD, Davis MM. 1998. Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. Proc Natl Acad Sci U S A 95: 6302-7) (Fig. S6). The graph in (c) shows the distribution of changes in fura-2 intensity following anti-MHC treatment in Control and TSG101 KD T cells. The one cell that shows an increase following addition of anti-MHCp is likely due to a fluctuation in the Ca^{2+} level in a cells in which signal termination in stable MCs has failed



Supplemental Figure S7. Low avidity ligands signal through CD80-dependent TCR microclusters. a-b) Fluo-4 loaded AND T cells were incubated on bilayers containing 200 mol/μm^2 ICAM-1 and 10 mol/μm^2 I-E^k-MCC or I-E^k-K99A as indicated, with or without 100 mol/μm^2 CD80. At the indicated times, the average of individual intracellular calcium values determined by the fluorescence at 510 nm when excited at 488 nm is presented in (a). The X-intercept of 7500 represents baseline Fluo-4 fluorescence as determined by EGTA-treated cells in Ca++-free buffer. b) Average velocity was determined by the displacement of cells on supported planar bilayers over a 45 minute time period. c-d) AND T cells were incubated on glass-supported planar bilayers containing 200 mol/μm^2 ICAM-1, 100 mol/μm^2 CD80, and 10 mol/μm^2 I-E^k-MCC or I-E^k-K99A as indicated for 30 minutes at 37°C, fixed, and stained for TCR and pY (c) or TCR and phospho-LAT (d). Images show representative distribution of TCR and pY (c) or TCR and phospho-LAT (d) in IS formed in response to I-E^k-MCC or I-E^k-K99A.



Supplemental Figure S8. Low avidity ligands generate TCR MCs which remain actin-dependent and do not recruit TSG101. a) Control and TSG101 KD T cells were incubated on glass-supported planar bilayers containing 200 mol/μm^2 ICAM-1, 100 mol/μm^2 CD80, and 10 mol/μm^2 I-E^k-MCC or I-E^k-K99A as indicated for 30 minutes at 37°C, fixed, and stained for TCR and pY. Images show representative distribution of TCR and pY in IS formed in response to I-E^k-MCC or I-E^k-K99A-presenting bilayers. b) AND T cells were incubated on glass-supported planar bilayers containing 200 mol/μm^2 ICAM-1, 100 mol/μm^2 CD80, and 10 mol/μm^2 I-E^k-MCC or I-E^k-K99A as indicated for 2 minutes at 37°C. They were imaged by TIRFM in the presence of fluorescent anti-TCR Fab preceding and immediately following treatment with 1μM Latrunculin A. c) AND T cells were incubated on glass-supported planar bilayers containing 200 mol/μm^2 ICAM-1, 100 mol/μm^2 CD80, and 10 mol/μm^2 I-E^k-MCC or I-E^k-K99A as indicated for 30 minutes at 37°C, fixed, and stained with anti-TCR Fab and phalloidin to mark polymerized actin. Images show representative distribution of TCR and F-actin in IS formed in response to MCC and K99A.

Supplemental Movie S1. Sorting of TCR into the IS center requires ubiquitin. AND T cells were treated with vehicle (a) or MG132 (b) to deplete cells of free ubiquitin. They were then incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 at 37°C. TCR was imaged in TIRF in the presence of fluorescent anti-TCR Fab. Images were acquired every 5 seconds.

Supplemental Movie S2. Engaged TCR in the IS does not recruit clathrin. Activated AND T cells were transfected with plasmids encoding clathrin light chain-GFP. GFP+ cells were then incubated on glass-supported planar bilayers containing 10 mol/μm^2 I-E^k-MCC\ and 200 mol/μm^2 ICAM-1 at 37°C and imaged in TIRF in the presence of fluorescent anti-TCR Fab. Images were acquired every 5 seconds.

Supplemental Movie S3. Tsg101 is required for central TCR accumulation. Control (a) and TSG101 KD T cells (b) were incubated on glass-supported planar bilayers containing 2 mol/μm^2 I-E^k-MCC and 200 mol/μm^2 ICAM-1 for 20 minutes at 37°C, after which TCR was imaged in TIRFM in the presence of fluorescent anti-TCR Fab. Images were acquired every 5s.

Supplemental Movie S4. Low avidity ligands induce TCR MC formation but do not accumulate TCR at the IS center. AND T cells were incubated on glass-supported planar bilayers containing 200 mol/μm^2 ICAM-1, 100 mol/μm^2 CD80, and either 10 mol/μm^2 I-E^k-MCC (a) or 10 mol/μm^2 I-E^k-K99A (b) at 37°C. TCR was imaged in TIRFM in the presence of fluorescent anti-TCR Fab. Images were acquired every 5s.

Supplementary Methods

Mice, Cells and Cell Culture

AND TCR transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were crossed to B10.Br mice. All experiments were performed on F1 progenies between 8 and 12 weeks of age. All mice were housed in specific pathogen-free conditions and cared for in accordance with protocol approved by Institutional and Animal Care and Use Committee. AND TCR Tg spleen cells were activated in OK-DMEM (Invitrogen) with 10% FBS (Hyclone) using 2 μM MCC peptide (MCC88-103). At 48 h, the cells were washed twice in complete medium and plated at 2 x 10^6 cells/ml with 50 U/ml of recombinant IL-2. Cells were replenished with fresh media and IL-2 every 24 h for 4 days (Campi et al., 2005). B cells were obtained from B10.Br mice by negative selection of splenocyte suspensions using anti-CD43 and –CD4 magnetic beads (Miltenyi Biotech) following the manufacturer's instructions. This routinely yielded >95% CD19 positive cells as analysed by flow-cytometry. B cells were activated with 100 μg/ml LPS (Sigma) for 24 hrs in RPMI-1640 media, washed, and loaded with 50 μM MCC peptide for 5 hrs. Cells were and resuspended in HBS/HSA prior to use in T-B cell conjugate experiments.

Retroviral transduction of T cells

Phoenix packaging cells (provided by G. Norlan, Stanford University) were transiently transfected with retroviral pMX vectors encoding either WT or UEV-mutant human TSG101 using Lipofectamine (Invitrogen). Supernatants containing retrovirus were collected 48 and 72 hours later and concentrated tenfold by centrifugation at 8,000*g* for 12 h. AND T cells that had been activated for 48 h were resuspended in the retroviral supernatant and were centrifuged at 2000 RPM for 120 min in the presence of 15 μg/ml of polybrene (Sigma) and 50 U/ml of recombinant IL-2. They were subsequently replenished with fresh media and IL-2 every 24 h for 4 days.

Suppression of Protein Expression by siRNA electroporation

AND T cells were activated as previously described. 96h after activation, proliferating CD4 T cells were transfected with 4ug of siRNA duplexes either against the desired target or a negative control sequence. Electroporation reagents were purchased from Amaxa Biosystems. Custom siRNA duplexes were purchased from Dharmacon, Inc. After electroporation, the cells were rested for 4h before switching the media to supplemented T cell media containing IL2. 48h later, the cells were once again electroporated with either specific or control siRNA. Cells were used for experiments 48h after the second electroporation. For rescue experiments, activated AND T cells were retrovirally infected with WT or UEV-mutant human TSG101 2 d post-activation as described above and electroporated with control or anti-murine TSG101 siRNA at 4 and 6 d post-activation as described above.

Bilayers

Glass-supported DOPC bilayers incorporating GPI-anchored forms of I-E^k, CD80, and ICAM-1 were applied to positively charged glass coverslips treated with 70% H_2SO_4/30% H_2O_2. This treatment allows lipid bilayers to adhere to coverslips while permitting lateral mobility. These cover slips were then incorporated into flow cells (Bioptechs) as previously described (Grakoui et al., 1999; Varma et al., 2006). For incorporation of His-tagged proteins into bilayers, NTA lipids were used, and His-tagged proteins were applied to flow cells in the presence of 100 μM nickel sulfate. Bilayers were blocked with 5% casein in PBS to prevent non-specific adhesion. The buffer for flow cell preparation and synapse

formation was Hepes buffered saline supplemented with 5 mM glucose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 1% human serum albumin (HBS/HSA).

For biochemistry experiments, bilayers were incubated with 5 μM silica beads (Bangs Laboratories) for 10 minutes at room temperature. Blocking and loading of His-tagged proteins was then performed at equivalent concentrations used in preparation of flow cells. AND T cells were made to form conjugates with beads by gentle co-sedimentation with bilayer-coated beads and incubation at 37°C in HBS/HSA.

TIRFM Imaging

All microscopy was performed on an Olympus inverted IX-70 microscope equipped with a Xenon-arc lamp as light source, Ludl shutter and filter wheels equipped with appropriate excitation and emission filters, Ludl XY motorized state, piezo-electric stage for z movement of objective, Olympus TIRF module, and 2W Kr-Ar laser (Spectra Physics) launched in a single mode fiber (Oz Optics) via an AOTF (Solamere Technologies). We used 2 cameras for fluorescence detection: A Hamamatsu 12-bit ORCA-ER cooled CCD for high-resolution imaging of fluorophores at standard density, and an EM-CCD 9100-12 (Hamamatsu) for highly sensitive detection of fluorophores at low density. The ORCA-ER was used with a 60× 1.45 N.A. TIRF objective from Olympus, and the EM-CCD was used with a UAPON 150x 1.45 NA (Olympus). The hardware on the microscope was controlled by IPLAB software. TIRF alignment and imaging was performed as previously described (Varma et al., 2006).

AND T cells were suspended in HBS/HSA, and were labeled with 5 μg/ml Alexa568 conjugated H57 Fab. The labeled cells were made to interact with the bilayers at 37°C. For TCR tracking purposes, images were collected at an interval of 5 s in the constant presence of labeled anti-TCR Fab. In wide-field or TIRFM mode, images were exposed for 500-800 msec.

Confocal microscopy

Conjugates of MCC-laoded B cells and AND T cells were formed by mixing at 2:1 ratio and brief centrifugation (400g). Cells were allowed to interact at 37°C, prior to fixing in 2% PFA, and placed on clean coverslips. Following quenching with 50 mM glycine/PBS, and saponin permeabilization (0.1%), cells were sequentially stained for TCRζ and pY. Samples were mounted in Prolong Gold (Invitrogen), and cured at 25°C for 12 hrs. Sealed and mounted samples were imaged by confocal microscopy (Zeiss LSM 510) using identical acquisition settings between experiments. For en face synapse reconstructions, z-stacks of appropriately oriented cell conjugates were collected at 200 nm steps to a distance of ~8 μm from the coverslip surface. For FRET imaging by donor dequenching, TSG101-gfp transduced T cells were labeled with DiI and conjugates formed with MCC-loaded B cells. Pre-bleach images were collected in channels for GFP and DiI emission during excitation at 488 nm. DiI emission was also collected by direct excitation at 563 nm. Following photobleaching of DiI by continuous excitation with the 563 nm laser line at 100% power, post-bleach images were collected using identical settings to pre-bleach acquisition.

Image Analysis and Statistics

Images were acquired using IPLab software. Images were background subtracted using ImageJ. All data was analyzed with the Metamorph Software, with the exception of calcium measurements and tracking, which was analyzed with Volocity. Statistical analyses were done using GraphPad software. Mann-Whitney and Fisher's Exact tests were used for continuous and categorical variables, respectively.

For reconstruction of cell-cell interfaces from images acquired via confocal microscopy, image stacks were deconvolved in ImageJ software using measured PSFs from 40nm multispectral beads (Tetraspeck, Invitrogen) in conjunction with a constrained iterative deconvolution algorithm (http://www.optinav.com/Iterative-Deconvolve-3D.htm). Bead stacks for each channel were used to estimate PSFs, and single-channel image stacks deconvolved until <0.1% convergence or a maximum of 20 iterations. Merged stacks were used to identify the interface, and xz planes reconstructed in Metamorph software. Shown are maximum intensity xz projections of three xy planes corresponding to the cell-cell interface.

Plasmids

WT and UEV-mutant human TSG101 containing plasmids were a generous gift of Wes Sundquist (Pornillos et al., 2002) and were subcloned into pMX vectors for retroviral infection. Clathrin light chain-GFP fusion protein in N1 vector was a generous gift of Ernst Ungewickell (Ungewickell et al., 2004). Murine TSG101-gfp fusion protein was generated by PCR amplification of murine TSG101 from cDNA incorporating flanking BamHI sites, and insertion into pEGFP-N1 (BD Clontech). To ensure correct orientation and sequence of cloned expression constructs, all plasmids were sequenced.

Antibodies and Chemical Reagents

H57 (anti-TCRβ) and 14-4-4 (anti-I-E^k) hybridomas were obtained from ATCC, and D4 (anti-IE^k in complex with MCC) was a generous gift of M. Davis. Anti-lyso-bis-phosphatidic-acid (6C4) hybridoma supernatant was obtained from J. Gruenberg (Kobayashi et al., 1998). MG132 was purchased from Calbiochem and administered for 8h at 25 μM to deplete cells of free ubiquitin. Alexa-fluor and other dyes were from Molecular Probes. Fab fragments were prepared and labeled as previously described (Varma et al., 2006). Anti-PKCθ (C-18), anti-Hrs (M-79), and anti-ubiquitin (P4D1) antibodies were purchased from Santa Cruz Biotechnologies. Anti-PY (4G10) was purchased from Upstate Biotechnology. Anti-TSG101 (T5701) was purchased from Sigma. Anti-GFP (AB16901) was purchased from Millipore. Anti-α actin (C4) and anti-CD3ζ (8D3) were purchased from BD Biosciences. CM-DiI was purchased from Invitrogen, and cells were labeled at 25°C for 4 minutes. For intracellular staining, cells were fixed with 2% paraformaldehyde at 37°C for 15 minutes and stained with primary antibody in the presence of 0.05% saponin for permeabilization, as previously described (Varma et al., 2006).

Western Blotting

Control and KD primary T cells were lysed using freshly made lysis buffer: 1% NP40, 1mM EDTA, 10 mM iodoacetamide, 50 mM hepes, 150 mM NaCl, 1 mM pervanadate, 1x mammalian protease inhibitor cocktail (Sigma P8340), pH 7.4. Cytoplasmic extracts were then run on a 4-15% gradient SDS-PAGE gel and then transferred to nitrocellulose membranes. They were then blocked and stained with specific antibodies in Li-Cor Blocking Buffer and subsequently stained with secondary antibodies from Li-Cor, Inc. Wash buffer was 20 mM Tris-HCl, 125 mM NaCl, 0.1% Tween-20. Bands were visualized using the Odyssey Infrared Imaging system. Anti-alpha actin antibodies were used as loading controls. KD was quantified by subtracting the background intensity, then comparing KD band intensity to the Control band intensity, which was normalized to a value of 1.0.

Calcium Measurements

AND T cells were labeled with 2 μM of Fura-2 in serum-free media for 30 min at room temperature. The dye was washed with serum-containing media and further incubated at 37°C for 30 min in serum-containing media, after which they were immediately used for experimentation. Images were acquired through a 40× NA 1.3 NeoFluar objective. The ratio of emission at 510 nm excited at 340 nm and 380 nm was determined with appropriate background subtractions. At the end of each experiment, high Ca^{2+} (1 μM ionomycin + 20 mM Ca^{2+} in media) and low Ca^{2+} (1 μM ionomycin + 0 mM Ca^{2+}, 2 mM EGTA in the media) were determined.

IL2 Capture

AND T cells were pre-incubated with an IL2 capture antibody (Miltenyi) before being loaded onto bilayers containing I-E^k-MCC + ICAM-1. They were incubated at 37°C for 6 hours before fixing and staining with anti-IL2-PE antibody (Miltenyi).

TCR Downregulation

A20 transformed B cells stably expressing I-E^k were a generous gift of Mark Davis. They were loaded with MCC peptide at either 10 or 100 μM for 3 hours at 37°C. They were then washed to remove free peptide, gently co-sedimented with AND T cells, and incubated at 37°C for 30 minutes. They were then fixed and stained for flow cytometry. Surface TCR was measured by anti-Vβ3-PE (BD Biosciences). Expression levels were assessed by flow cytometry using a FacsCalibur. Compensation and analysis was performed using FlowJo software.

TSG101 and Hrs siRNA

For murine TSG101 the following target sequences were used: (1) CGCTTAGATCAAGAAGTA (2) CGTAAACAGTTCCAGCTAA (3) GTACAATCCCAGTGCGTTA. For murine Hrs the following sequence was used: CAAGATACCTCAACCGGAA. Murine and human TSG101 mRNA sequences follow with the underlined sections corresponding to the targeted murine sites, which are not found in human TSG101 (start and stop codons are also underlined).

Murine:
gattgtctggcggcctggggcggccccgtggcgcctaccctctgcctgtggggacggaggagcgcgccatggcggtgtccgagagtcagctgaaga
agatgatgtccaagtacaaatatagagatctaaccgtccgtcaaactgtcaatgtcatcgctatgtacaaagatctcaaacctgtattggattcatatgtttttaa
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Human:
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