Supporting Information

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SI Materials and Methods

Cell Culture, Peptides, and Antibodies. L-K^b cells, derived from L929 stably transfected with K^b cDNA, were cultured in DMEM-containing 7.5% (vol/vol) FBS. DC-like cell line DC2.4 cells (H-2b) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 7.5% FBS. For exogenous peptide-loading, cells were cultured overnight at 27 °C with 5 μg/mL of human β_2-microglobulin, followed by loading of synthetic SIIN and/or SIYR peptides (5 μM for each) at 1 h at 4 °C. For establishing of stable cell lines, DNA constructs were transfected into LK^b or L929 cells with Lipofectamine 2000 (Invitrogen), followed by antibiotic screening and cell sorting with BD FACS Aria Cell Sorter.

We generated 25D1.16 Fab fragments using the Mouse IgG1 Fab preparation kit (Pierce). The 25D1.16 Fab or 2Cm67 were directly conjugated with Alexa Fluor 488/568/647 conjugation kits (Molecular Probes). Conjugated 25D1.16 Fab or 2Cm67 were separated from residual aggregates and debris with AKTA Prime Plus FPLC equipped with Superdex 75 column. All of the aliquots were stored at −80 °C. All reagents were microfuged at 14,000 rpm just before use to remove aggregates that might have formed.

VV Construction and Infection. VVs expressing (*i*) Ub-SIIN or Ub-SIYR (K^b binding peptides SIIN or SIYR liberated from Ub during or shortly after translation), (*ii*) NP-SIIN-eGFP (SIIN expressed at the C terminus of influenza nucleoprotein), (*iii*) Ub-Arg-NP-SIIN-eGFP (SIIN expressed at the C terminus of rapidly degraded influenza nucleoprotein), and (*iv*) Ub-SIYR-IRES-Ub-SIIN (both of SIYR and SIIN peptides were liberated from Ub with IRES sequence inserted in between) were generated as described using pSC11 (1). VSV expressing Ub-SIIN was generated as described (2). Cells were infected at an MOI of 1, unless otherwise specified, for 30 min at 37 °C with mixing every 5 min in HBSS containing 0.1% BSA (BSS/BSA), followed by incubation at 37 °C in growth media for the remainder of the assay.

Confocal Microscopy and TIRF. L-K^b cells (3×10^5/well) were plated overnight on 12-mm coverslips (Deckglaser). Following infection, cells were fixed with 3.2% paraformaldehyde (PFA) at ambient temperature for 15 min, followed by permeabilization with 0.05% saponin at room temperature for 10 min before intracellular staining with the indicated primary and secondary antibodies. For cells only stained on the surface, the permeabilization step was omitted. For 2Cm67 staining, the fixation step was omitted due to the high nonspecific staining issue by 2Cm67 on fixed cells. Saponin permeabilized L-K^b cells were stained by incubating with Alexa488-conjugated 2Cm67 and 25D1.16, washed, and incubated with rabbit polyclonal anti-Alexa488 Abs. Cells were then fixed with 3.2% PFA for 15 min, and further stained with Alexa488-conjugated goat anti-rabbit IgG and Alexa647-conjugated goat anti-mouse IgG. Confocal imaging was performed with a Leica TCS-SP5 DMI6000 and 63× 1.45 NA oil objective (Leica Microsystems).

For TIRF, L-K^b cells (1×10^5/well) were plated in 8-well chambers (Lab-tek) and infected as indicated. At the end of infection, cells were fixed with 3.2% PFA and visualized with Leica AF 6000 LX equipped with a 100× 1.49 NA TIRF object. Live single- or dual-color TIRF imaging was also performed using an Olympus IX71 fluorescence microscope equipped with a 150× magnification 1.45 numerical aperture (NA) TIRFM objective (Olympus) and a customized TIRF apparatus to minimize chromatic aberration (3). Images were captured using identical QuantEM electron multiplying (EM) CCD cameras (Photometrics) with a resolution of 0.1 μm per pixel. Lasers used for TIRFM included air-cooled argon (delivering 488 and 514 nm) laser (Dynamic Laser), diode pumped solid state (DPSS) (561 nm; Cobolt) and 640-nm diode laser (Blue Sky Research). Cells were first incubated with 5% casein to reduce nonspecific binding of fluorescent probes, and imaged in HBSS containing 1% BSA (BSS/BSA) for live TIRF. Exposure times of 0.5–1 s were used to acquire images. Labeled 25D1.16 Fab or 2Cm67 were present at 1 μg/mL throughout the experiment. To optimize focus in dual-color TIRF, three consecutive images with ± 0.1 μm variation in z depth were collected for each sample. The image having the maximum intensity was determined to be the one in optimal focus and was used for further analysis.

Flow Cytometry. After viral infection, L-K^b cells were labeled at 4 °C for 30 min with Alexa647-conjugated 25D1.16 or Alexa488-conjugated 2Cm67. After three washes with HBSS/BSA, the cells were analyzed by flow cytometry using a BD LSR II flow cytometer (BD Biosciences). Quantitation of the surface complexes was done as described (4) with slight modification. In brief, L-K^b cells were incubated with a saturating amount of fluorescein-25D1.16 and analyzed by flow cytometry. In parallel, a standard curve of FITC molecules vs. MFI was obtained by running FITC-coated calibration beads (Spherotech), using the identical instrument setting. With the standard curve and the specified F/P ratio of FITC-conjugated 25D1.16 (1.2 in this case), the K^b–SIIN MFI was converted into the number of molecules per cell.

CD8 T-Cell Functional Assay. OT-I T-cell lines were generated from OT-I transgenic C57BL/6 splenocytes (Taconic Farms). In brief, homogenized splenocytes were stimulated with 2×10^{-7} M SIINFEKL peptide for 2 d, followed by culture in complete IMDM media with 10 mg/mL recombinant human IL-2 (Petprotech), β-mercaptoethanol (Gibco), and gentamycin (Cellgro). Cells were harvested on day 7 poststimulation using lymphocyte separation medium (Lonza). L929 cells stably transfected with wild-type K^b (L929/K^b) or cytoplasmic tail truncated K^b (L929/ΔK^b) were infected with VV-Ub-SIIN, VV-Ub-Arg-NP-SIIN-eGFP, VV-L106P-SIIN-eGFP, or VV-NP-SIIN-eGFP (MOI = 2). Samples were taken every 20 min postinfection and coincubated with OT-I cell lines (Effector: Target = 1:1) for 1.5 h in the presence of 25 mg/mL brefeldin A. Cells were analyzed on a BD LSR II flow cytometer (BD Biosciences) after intracellular cytokine staining of OT-I with anti-mouse CD8a–pacific blue (clone 53–6.7) and anti-mouse IFN-gamma (clone XMG-1.2; ebiosciences). For peptide-loaded samples, cells were pretreated with 5 μg/mL human β_2-microglobulin overnight, infected with VV-βGal (vaccinia backbone without expression of SIINFEKL) for 2 h, followed by loading of SIINFEKL peptide at 4 °C for 1 h. After three media washes, the cells were coincubated with OT-I cells.

Image Analysis. Image analysis was performed using Leica LAS AF Lite, Metamorph, Imaris and ImageJ software. Dual-color TIRF images were preprocessed as described (3). Background subtraction was performed using the subtract background feature of Image J, which employs a rolling ball algorithm to subtract local background. A rolling ball radius of 200 was chosen for all images. Individual cells showing sufficient expression in the two channels were cropped and saved as independent image files for further processing. Colocalization analysis was performed using the Image correlation plugin, which generates a correlation co-

efficient proportional to the degree of colocalized pixels in the different color channels. An intensity threshold was applied to both the images to remove correlation arising from image noise. At least 10 cells were analyzed in each group and a correlation coefficient was determined for each cell and plotted as shown in Figs. 1 and 4. Curve fitting was done with GraphPad Prism software and statistical analysis was performed using Mann-Whitney, nonparametric two-tailed test.

1. Chakrabarti S, Brechling K, Moss B (1985) Vaccinia virus expression vector: Coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. *Mol Cell Biol* 5:3403–3409.
2. Obuchi M, Fernandez M, Barber GN (2003) Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity. *J Virol* 77:8843–8856.
3. Crites TJ, Chen L, Varma R (2012) A TIRF microscopy technique for real-time, simultaneous imaging of the TCR and its associated signaling proteins. *J Vis Exp* 61):e3892.
4. Princiotta MF, et al. (2003) Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity* 18:343–354.

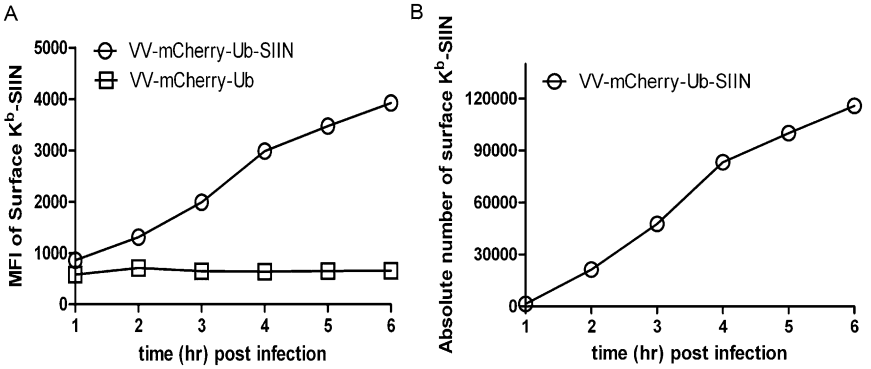

Fig. S1. Quantitating surface K^b–SIIN complexes. At the indicated times p.i. with VVs expressing either Ub-liberated SIIN (VV-mCherry-Ub-SIIN with mCherry as an infection indicator) or control VV without SIIN (VV-mCherry-Ub, MOI = 1). The MFI from each sample stained with fluorescein-25-D1.16 is shown in *A*. The absolute number of surface K^b–SIIN complexes is shown in *B*. The conversation was calculated on a standard curve obtained from beads with known numbers of fluorescein molecules.

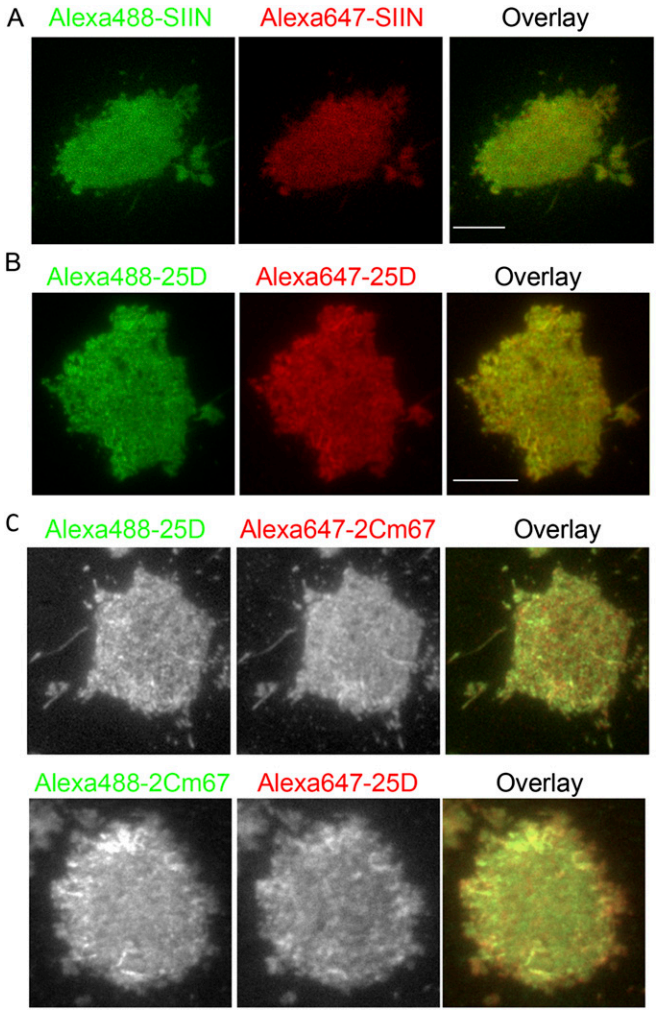

Fig. S2. K^b–SIYR and K^b–SIIN generated from synthetic peptide loading extensively colocalize regardless of fluorophores used for detection. (*A*) The hβ2m-sensitized L-K^b cells loaded with a mixture of directly conjugated Alexa488-SIIN and Alexa647-SIIN synthetic peptides were visualized by dual-color TIRF. (*B*) L-K^b cells as in *A*, but incubated with SIIN synthetic peptide and then costained with a mixture of Alexa488-25D1.16 and Alexa647-25D1.16 Fabs. (*C*) Experimental conditions were the same as in Fig. 1*E*. Peptide-loaded L-K^b cells were costained live with a mixture of Alexa488-25D1.16 Fab and Alexa647-2Cm67 (*Upper*) or Alexa488-2Cm67 and Alexa647-25D1.16 Fab (*Lower*), followed by immediate live dual-color TIRF imaging.

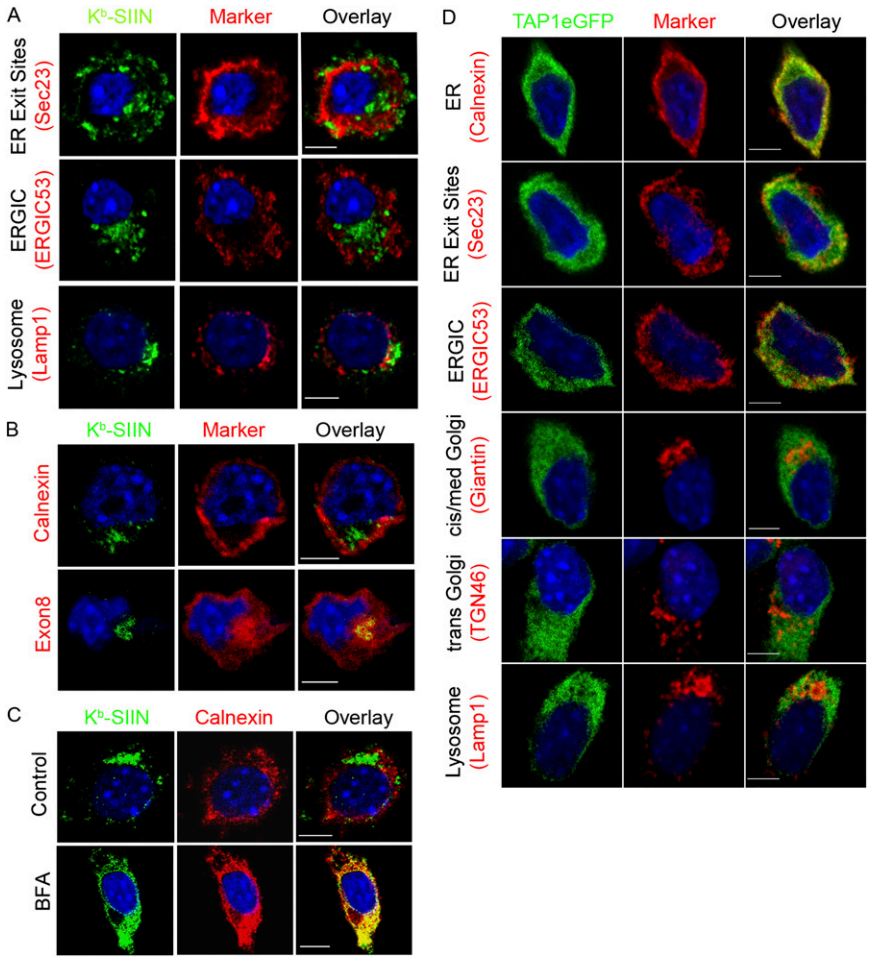

Fig. S3. K^b–SIIN localizes to post-ER compartments, but TAP localizes to pre-GC compartments. (*A*) Four hours p.i. with VV-expressing Ub-liberated SIIN (MOI = 1), cells were stained intracellularly with 25D1.16 and antibodies against the indicated intracellular markers, followed by the appropriate secondary antibody staining. (*B*) K^b–SIIN complexes do not reside in the ER upon low temperature (15 °C) incubation. L-K^b cells were infected with VV expressing Ub-liberated SIIN (MOI = 1) for 1 h, and then cultured at 15 °C overnight. Cells were stained intracellularly with 25D1.16 and anti-calnexin antibodies (*Top*) or rabbit anti-exon 8 pAbs (recognizing the cytoplasmic tail of K^b; *Bottom*) followed by the appropriate secondary staining. (*C*) As in *A*, but cells were incubated with BFA at 10 μg/mL starting at 1 h p.i. (*D*) After fixation with paraformaldehyde, TAP1-eGFP stably transfected L-K^b cells were stained with the indicated markers. Note extensive colocalization with calnexin (ER marker) and partial colocalization with Sec 23 (ER exiting vesicles) and ERGIC53 (ER GC intermediate compartment), and lack of colocalization with giantin (GC) and TGN 46 (trans GC).

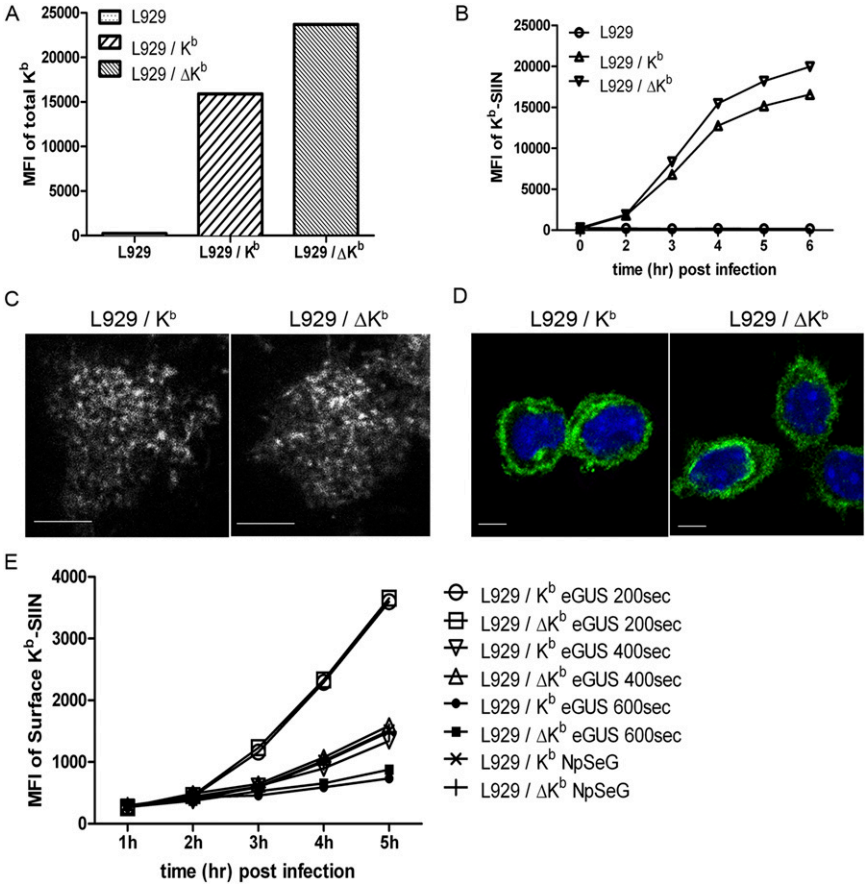

Fig. S4. Characterization of the cytoplasmic tail-deleted K^b. (*A*) L929 cells stably transfected with wild-type (L929/K^b) or cytoplasmic tail-deleted K^b (L929/ΔK^b) were stained with APC-conjugated AF6-88.5 (anti-K^b-mAb) and level of surface K^b determined with flow cytometry. (*B*) After infection with VV-Ub-SIIN (MOI = 1), samples taken at the indicated times were stained with Alexa647-25D1.16 and analyzed by flow cytometry. (*C*) Four hours p.i. with VV-Ub-SIIN, surface K^b-SIIN complexes were visualized live with TIRF in the presence of monovalent Alexa647-25D1.16 Fab. (*D*) L929 cells transfected with wild-type or tailless K^b were fixed with paraformaldehyde, followed by intracellular staining with AF6-88.5 and secondary staining with Alexa488-conjugated goat anti-mouse IgG. The nucleus was stained with Hoechst 33258. (*E*) To limit SIIN generation to subsaturating levels, VV-eGFP-Ub-SIIN (eGUS) virus was UV-irradiated for 200s, 400s, or 600s before infection of L929/K^b or L929/ΔK^b cells. In parallel, cells were infected with nonirradiated VV-NP-SIIN-eGFP (NpSeG; MOI = 2). Samples were taken at indicated times p.i. and K^b–SIIN complexes were quantitated with flow cytometry after staining with Alexa647-25D1.16.