Endogenous viral antigen processing generates peptide-specific MHC class I cell-surface clusters

Xiuju Lu\textsuperscript{a}, James S. Gibbs\textsuperscript{b}, Heather D. Hickman\textsuperscript{a}, Alexandre David\textsuperscript{d}, Brian P. Dolan\textsuperscript{c}, Yetao Jin\textsuperscript{b}, David M. Kranz\textsuperscript{c}, Jack R. Bennink\textsuperscript{k}, Jonathan W. Yewdell\textsuperscript{b,}\textsuperscript{l}, and Rajat Varma\textsuperscript{d}

\textsuperscript{a}Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; \textsuperscript{b}Laboratory of Chemistry, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892; \textsuperscript{c}Department of Biochemistry, University of Illinois, Urbana, IL 61801; and \textsuperscript{d}Laboratory of Systems Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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Sensitivity is essential in CD8\textsuperscript{+} T-cell killing of virus-infected cells and tumor cells. Although the affinity of the T-cell receptor (TCR) for antigen is relatively low, the avidity of T-cell-antigen–presenting cell interactions is greatly enhanced by increasing the valence of the interaction. It is known that TCRs cluster into protein islands after engaging their cognate antigen (peptides bound to MHC molecules). Here, we show that mouse Kb class I molecules segregate into preformed, long-lasting (hours) clusters on the antigen-presenting cell surface based on their bound viral peptide. Peptide-specific Kb clustering occurs when source antigens are expressed by vaccinia or vesicular stomatitis virus, either as proteasome-liberated precursors or free intracellular peptides. By contrast, Kb-peptide complexes generated by incubating cells with synthetic peptides are extensively intermingled on the cell surface. Peptide-specific complex sorting is first detected in the Golgi complex, and compromised by removing the Kb cytoplasmic tail. Peptide-specific clustering is associated with increased T-cell sensitivity: on a per-complex basis, endogenous SIINFEKL activates T cells more efficiently than synthetic SIINFEKL, and wild-type Kb presents endogenous SIINFEKL more efficiently than tailless Kb. We propose that endogenous processing generates peptide-specific clusters of class I molecules to maximize the sensitivity and speed of T-cell immunosurveillance.

\textbf{Results}

\textbf{Peptide-Specific Clusters Predominate on the Cell Surface.} To localize peptide-specific MHC class I complexes by immunofluorescence, we used the 25-D1.16 mAb (10) or 2C m67 TCR (11), which demonstrate high specificity for mouse H-2 Kb class I molecule complexed respectively with model antigenic peptides SIINFEKL (SIIN) or SIYRYYGL (SIYR). To facilitate detection, we infected t-K\textsuperscript{b} cells (L929 mouse fibroblasts stably transfected with K\textsuperscript{b} with recombinant vaccinia viruses (VV) expressing SIIN or SIYR as ubiquitin (Ub) fusion proteins. These peptides are immediately liberated in saturating amounts from nascent Ub by highly active cellular ubiquitin hydrolases (12, 13) [this mechanism is used for natural Ub synthesis, which is liberated from Ub-Ub or Ub-ribosomal protein fusions (14)].

We used total internal reflection fluorescent (TIRF) microscopy to selectively image pMHC complexes at, or just below, the plasma membrane (15). Imaging fixed, nonpermeabilized cells at 4 h post infection (p.i.) with VV-Ub-SIIN or VV-Ub-SIYR revealed that both 25-D1.16 and 2C m67 detected (via indirect immunofluorescence) their cognate pMHCs in highly clustered structures of 200–900 nm diameter (Fig. 1A). Imaging infected cells clearly revealed a distinct spatial separation of Kb-SIIN and Kb-SIYR complexes (Fig. 1B). Fixation immobilizes pMHCs (confirmed by photobleaching following indirect staining), preventing antibody-induced redistribution, but potentially introduces artifacts.

To avoid fixation or reagent-based cross-linking artifacts, we imaged live cells with monovalent preparations of directly conjugated 25-D1.16 (Fab) and 2C m67 (naturally monovalent), taking care to remove multivalent and aggregated species. Due to the lower affinity of monovalent agents and lack of amplification from secondary reagents, the signal-to-noise ratio suffers. Exploiting the minute focusing volume of TIRF, we enhanced the signal by imaging cells in the presence of direct conjugates. Images were collected simultaneously by illuminating with two wavelengths and collecting with dual aligned detectors (16). At 4 h post-co-infection with VV-Ub-SIIN and VV-Ub-SIYR, surface Kb–SIIN and Kb–SIYR complexes were each highly clustered and still predominantly nonoverlapping (Fig. 1C).

To determine the kinetic stability of clusters, we incubated infected cells 4 h p.i. with brefeldin A (BFA) to abrogate cell-surface delivery of new pMHC. This revealed that clusters remain spatially segregated on a peptide-specific basis for at least 2 h, and are therefore stable structures (Fig. 1D).

\textbf{MHC class I clustering | CD8 T cell recognition | dual-color TIRF imaging | antigen processing/presentation | intracellular trafficking}

CD8\textsuperscript{+} T cells recognize MHC class I molecules bearing oligopeptides derived largely from proteasome-degraded proteins. Recognition is based on the activation of the T-cell receptor (TCR) signaling complex. Sensitivity, a key feature of T-cell immunosurveillance, enables detection of low copy number peptides and accelerates recognition of virus-infected cells, where speed is of the essence, because cells must be killed before the release of progeny virus, which can occur within hours of infection.

Although the intrinsic affinity of the TCR for peptide–MHC complexes (pMHC) is low (1, 2), the functional avidity of the T-cell-antigen–presenting cell interaction is greatly enhanced by increasing the valence of the interaction. When exposed to cognate pMHC on the antigen presenting cell surface, TCRs cluster into protein islands (3, 4), increasing their sensitivity for activation. Class I molecules are delivered to the cell surface in clusters (5–8). T-cell sensitivity is increased by class I clustering (9), but the relevance of clustering to detecting viral and other endogenously generated peptides has yet to be established. Because clusters contain 50 or fewer class I molecules (5), it is difficult to see how this could enhance detecting viral peptides early after infection, when viral peptides are of low abundance and would be statistically unlikely to be present in the same cluster.

What if, however, there were a mechanism for enhancing T-cell immunosurveillance by delivering viral peptides to the cell surface in preformed clusters? Here, we use TCR-like reagents to explore the generation of peptide-specific clusters by virus-infected cells and cells exposed to synthetic cognate peptides.
Quantitative flow cytometry indicates that 4 h p.i. ~50,000 Kβ-SIIN complexes are present at the surface of cells infected with VV-mCherry-Ub-SIIN (Fig. S1), similar to our previous findings (17, 18). Because we detect ~25% of the cell surface in the TIRF imaging volume, which contains ~250 clusters, we can estimate that each cluster contains ~50 Kβ-peptide complexes. This is similar to the class I clusters described by Edidin (5), and roughly matches the number of TCRs per cluster detected by Lillemeier et al. (3).

Exogenously Loaded Synthetic Peptides Do Not Generate Highly Clustered pMHC Complexes. To examine the potential contribution of endogenous peptide loading per se to clustering, we generated pHMHC at the cell surface by incubating cells with synthetic peptides. As a control, i-Kβ cells exposed to a mixture of Alexa488-SIIN and Alexa647-SIIN synthetic peptides demonstrate the high degree of colocalization expected, and also little clustering by live-cell TIRF (Fig. S2A). A near-identical pattern was observed when SIIN-pulsed cells were incubated with a mixture of 25D1.16 Fab conjugated with either Alexa488 or Alexa647 (Fig. S2B). It is important to note that cells pulsed with equimolar SIYR and SIIN then incubated with Alexa674-conjugated 25D1.16 Fab and Alexa488-conjugated 2Cm67 demonstrated extensive colocalization and little clustering (Fig. 1E and F) [similar results were obtained switching the fluorescent labels (Fig. S2C)].

Together, these findings show that TIRF is capable of detecting colocalization, and that clustering is not induced by 25D1.16 or 2Cm67. Rather, the mutually exclusive clustering of Kβ-SIIN and Kβ-SIYR complexes requires endogenous antigen presentation.

Viral infection Generates Peptide-Selective Clusters in Distinct Intracellular Compartments. To gain mechanistic insight into peptide-specific clustering, we indirectly stained fixed and permeabilized cells with 25D1.16 4 h p.i. with VV-Ub-SIIN. Laser-scanning confocal microscope imaging with marker antibodies (Abs) revealed that Kβ-SIIN complexes are detected in the distal-GC (Giantin, TGN 46 staining) and cis-GC (Giantin staining), but not the endoplasmic reticulum (ER) (calnexin staining) (Fig. 2A), ER exit sites (Sec 23 staining), or ER-GC intermediate compartment (ERGC 53 staining) (Fig. S3A).

The absence of ER 25D1.16 staining is surprising, because the ER is well established as the principal site of class I assembly with transporter associated with antigen processing (TAP)-transported peptides (19). The absence of 25D1.16 ER staining is probably due to rapid transport of peptide-loaded MHC.
complexes from the ER, because prolonged incubation of infected cells at 15 °C, which greatly retards ER export of nascent membrane proteins (20), failed to reveal Kβ-SIIN complexes in the ER. As expected, we easily detected Kb molecules in the ER using pAbs specific in the GC and other intracellular compartments (Fig. 2B).Prising noncolocalization between 25-D1.16 and 2C m67 staining, Intracellular costaining with 25D1.16 and 2C m67 revealed surprising noncolocalization between 25-D1.16 and 2C m67 staining, implying that clusters are released from their loading sites after achieving a threshold number.

We used live-cell TIRF to examine cells coinfected with VVs expressing rapidly degraded NP fused to either SIIN or SIYR. Once again, distinct surface Kβ clusters segregated based on their peptide cargo (Fig. 3G), extending this phenomenon to proteasome-dependent–antigen processing.

**Peptide-Specific Cluster Segregation Requires the Kβ Cytoplasmic Tail.** The class I cytoplasmic domain modulates class I intracellular trafficking (25–33). Removal of the Kβ cytoplasmic domain (ΔKβ) had little effect on class I cell surface expression, kinetics of Kβ–SIIN expression, gross clustering of Kβ–SIIN complexes, intracellular distribution, or efficiency of Kβ–SIIN generation from cytosolic or defective ribosomal product (DrIrp)-liberated peptides (Fig. S4 A–E). Kβ tail deletion, however, significantly reduced the intracellular (Fig. 4A) and cell-surface segregation (Fig. 4B) of Kβ–SIIN and Kβ–SIYR clusters in coinfected cells. That maximal spatial segregation of SIIN and SIYR complexes depends on the cytoplasmic tail of Kβ provides an important functional control that clustering is not an artifact associated with detection of the complexes with 25D-1.16/2Cm67.

This observation extends the function of the class I cytoplasmic domain, previously shown to be involved with class I plasma membrane internalization and endosomal trafficking (25–33). Because the efficiency of Kβ loading with SIIN is not affected by the loss of the cytoplasmic tail, we infer that ΔKβ properly associates with TAP and functions normally in the peptide-loading complex. This implies that the cytoplasmic tail affects peptide segregation only after Kβ release from the loading complex, consistent with a role for the tail in maintaining Kβ–peptide clusters in the GC and plasma membrane.

**Peptide Clustering Enhances T-Cell Sensitivity.** Do clusters enhance T-cell sensitivity? We compared activation of OT-I transgenic T cells (specific for Kβ–SIIN) by virus-infected (cluster generating) vs. synthetic SIIN-exposed T-Kβ cells (no clusters). To compare a similar range of sublimiting numbers of Kβ–SIIN complexes, we infected cells for increasing times with VV-Ub-SIIN, abrogating antigen presentation by exposing cells to BFA during the T-cell activation assay. In parallel, we exposed cells to increasing amounts of synthetic SIIN. Cells were then assessed for their ability to activate OT-I IFN-γ synthesis by intracellular cytokine staining and for Kβ–SIIN expression by binding of Alexa 647 25D1.16. Because VV-induced changes in antigen-presenting cells that could potentially influence T-cell activation, we exposed cells infected with a non-SIIN-expressing VV-βGal to synthetic SIIN.
OT-I cells are a more sensitive measure of Kb–SIIN expression than 25D1.16 staining, as originally reported (10). We could, however, detect 25D1.16 binding to VV-Ub-SIIN infected cells at 110 min postinfection, a time when T-cell activation was not saturated. At this time point, infected cells gave a 25D1.16 signal 195 mean fluorescent intensity (MFI) units above background levels. To achieve the same level of T-cell activation (~12%), nearly six times as many Kb–SIIN complexes, 1,124 MFI units, were present on peptide-sensitized cells (Fig. 5A).

If clustering of endogenously generated peptides enhances OT-I activation, then ΔKb, which exhibits less clustering than WT Kb, should be less efficiently recognized per Kb–SIIN complex expressed (as determined by 25D1.16 binding). OT-I cells are triggered with equal efficiency per Kb–SIIN complex when synthetic SIIN is presented by ΔKb vs. WT Kb (Fig. 5B), establishing that removing the tail does not negatively impact Kb T-cell activation function. Next, we infected cells with VVs expressing SIIN in the context of an Ub-fusion protein (Venus-Ub-SIIN), rapidly degraded protein (L106P-SIIN-eGFP, Ub-R-...
Removing the cytoplasmic tail has a less marked effect on T-cell peptides in parallel with a near-complete decrease in clustering. These findings support the conclusion that the peptide-specific clustering associated with endogenous antigen processing enhances T-cell recognition.

**Discussion**

Despite the availability of class I peptide–specific reagents for 15 y (10, 34), there is little published on their detection of intracellular complexes generated by endogenous antigen processing. Makler et al. (35) exclusively detected an abundantly naturally processed CMV peptide complexed with HLA-A2 in the GC and plasma membrane. Also, we were unable to detect K\(^\alpha\) complexed with either SIIN or SIYR in the ER, detecting each complex in the ER export sites awaiting transport to the GC.

We therefore favor the idea that 25-D1.16 and 2C-m67 are unable to detect their epitopes in the ER possibly due to steric interference from proteins that participate in loading class I molecules. Or, as an alternative, class I molecules may undergo a conformational alteration upon release from the loading complex needed to create the respective epitopes. The clear staining of the ER by 25-D1.16 after BFA treatment, which merges the ER and early GC compartments, as reported (10), is consistent with either of these possibilities. We note that although we previously used 25-D1.16 to detect K\(^\alpha\)–SIIN required in each condition to achieve equivalent levels of OT1 activation. (B) L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells pretreated with human β2m were loaded with SIIN peptide and tested for their ability to activate OT-I cells as in A. (C) OT-I activation tested as in A with L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells after infection with indicated recombinant VV expressing SIIN in the form of peptide (Venus-Ub-SIIN) or full-length rapidly degraded (L106P-SIIN-eGFP, Ub-Arg-NP-SIIN-eGFP) proteins or stable (NP-SIIN-eGFP) proteins.

**Fig. 5.** K\(^\alpha\)–SIIN clustering is associated with increased T-cell sensitivity. (A) L929/K\(^\alpha\) cells infected with VV-Ub-SIIN (MOI = 2) or VV-jGal (control) were coincubated with OT1 CD8 T cells (E:T = 1:1) for 1.5 h in the presence of brefeldin A. IFN-γ expression was measured by flow cytometry for intracellular anti-IFN-γ expression, gating for CD8+ cells. In parallel, L929/K\(^\alpha\) cells were stained with 25D1.16 for surface K\(^\alpha\)–SIIN expression. For peptide loading, high-m sensitivity L929/K\(^\alpha\) cells were infected with VV-jGal for 2 h followed by peptide incubation for 1 h at 4 °C. Arrows and numbers indicate the MFI of K\(^\alpha\)–SIIN required in each condition to achieve equivalent levels of OT1 activation. (B) L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells pretreated with human β2m were loaded with SIIN peptide and tested for their ability to activate OT-I cells as in A. (C) OT-I activation tested as in A with L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells after infection with indicated recombinant VV expressing SIIN in the form of peptide (Venus-Ub-SIIN) or full-length rapidly degraded (L106P-SIIN-eGFP, Ub-Arg-NP-SIIN-eGFP) proteins or stable (NP-SIIN-eGFP) proteins.

NP-SIIN-eGFP), or stable protein (NP-SIIN-eGFP). In each case, for nearly all time points p.i., OT-I cells were better activated by WT vs. ΔK\(^\alpha\) for equivalent or even diminished levels of K\(^\alpha\)–SIINFEKL expression (Fig. 5C).

Taken together, the efficiency of OT-I activation on a per complex basis parallels the degree of clustering. Synthetic peptides demonstrate a large decrease in efficiency vs. endogenous peptides in parallel with a near-complete decrease in clustering. Removing the cytoplasmic tail has a less marked effect on T-cell activation efficiency in parallel with a partial decrease in clustering. These findings support the conclusion that the peptide-specific clustering associated with endogenous antigen processing enhances T-cell recognition.

**Fig. 5.** K\(^\alpha\)–SIIN clustering is associated with increased T-cell sensitivity. (A) L929/K\(^\alpha\) cells infected with VV-Ub-SIIN (MOI = 2) or VV-jGal (control) were coincubated with OT1 CD8 T cells (E:T = 1:1) for 1.5 h in the presence of brefeldin A. IFN-γ expression was measured by flow cytometry for intracellular anti-IFN-γ expression, gating for CD8+ cells. In parallel, L929/K\(^\alpha\) cells were stained with 25D1.16 for surface K\(^\alpha\)–SIIN expression. For peptide loading, high-m sensitivity L929/K\(^\alpha\) cells were infected with VV-jGal for 2 h followed by peptide incubation for 1 h at 4 °C. Arrows and numbers indicate the MFI of K\(^\alpha\)–SIIN required in each condition to achieve equivalent levels of OT1 activation. (B) L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells pretreated with human β2m were loaded with SIIN peptide and tested for their ability to activate OT-I cells as in A. (C) OT-I activation tested as in A with L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells after infection with indicated recombinant VV expressing SIIN in the form of peptide (Venus-Ub-SIIN) or full-length rapidly degraded (L106P-SIIN-eGFP, Ub-Arg-NP-SIIN-eGFP) proteins or stable (NP-SIIN-eGFP) proteins.

**Fig. 5.** K\(^\alpha\)–SIIN clustering is associated with increased T-cell sensitivity. (A) L929/K\(^\alpha\) cells infected with VV-Ub-SIIN (MOI = 2) or VV-jGal (control) were coincubated with OT1 CD8 T cells (E:T = 1:1) for 1.5 h in the presence of brefeldin A. IFN-γ expression was measured by flow cytometry for intracellular anti-IFN-γ expression, gating for CD8+ cells. In parallel, L929/K\(^\alpha\) cells were stained with 25D1.16 for surface K\(^\alpha\)–SIIN expression. For peptide loading, high-m sensitivity L929/K\(^\alpha\) cells were infected with VV-jGal for 2 h followed by peptide incubation for 1 h at 4 °C. Arrows and numbers indicate the MFI of K\(^\alpha\)–SIIN required in each condition to achieve equivalent levels of OT1 activation. (B) L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells pretreated with human β2m were loaded with SIIN peptide and tested for their ability to activate OT-I cells as in A. (C) OT-I activation tested as in A with L929/K\(^\alpha\) or tailless L929/ΔK\(^\alpha\) cells after infection with indicated recombinant VV expressing SIIN in the form of peptide (Venus-Ub-SIIN) or full-length rapidly degraded (L106P-SIIN-eGFP, Ub-Arg-NP-SIIN-eGFP) proteins or stable (NP-SIIN-eGFP) proteins.
wild-type class I molecules and a mutated version unable to bind TAP formed separate clusters. Could it be that the clusters they observed also segregate based on their peptide cargo?

Cluster segregation is partially dependent on the K* cytoplasmic tail. MHC class I tail truncations are known to increase the mobility of class I molecules on the cell surface (32, 40), suggesting that the K* tail helps to maintain clusters once they are formed, rather than in initiating cluster formation per se. Indeed, removing the tail did not detectably affect the efficiency of K*-SIIN from various VV-encoded SIIN-containing gene products.

Eddin and colleagues have reported that class I clustering increases the sensitivity of T-cell recognition (6, 9, 39). Our abundance peptides, and could contribute to the high sensitivity of such clustering would greatly facilitate the 2D presentation: Impact of TAP transport.

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SI Materials and Methods

Cell Culture, Peptides, and Antibodies. l-Kβ cells, derived from L929 stably transfected with Kβ 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 β2-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 LKβ 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 (Kβ binding peptides SIIN or SIYR liberated from Ub during or shortly after translation), (ii) NP-SIIN-eGFPSIN (SIIN expressed at the C terminus of influenza nucleoprotein), (iii) Ub-Arg-NP-SIIN-eGFPSIN (SIIN expressed at the C terminus of rapidly degraded influenza nucleoprotein), and (iv) Ub-SIYR-URES-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 loading of synthetic SIIN and/or SIYR peptides (5 μM for each) at 1 h at 4 °C. Cells were infected with a saturating amount of IFN-γ (clone XMG-1.2; BD Biosciences) for 4 h. Cells were then stained by incubating with Alexa488-conjugated 2Cm67 and stained by incubating with Alexa647-conjugated 25D1.16 or Alexa488-conjugated 2Cm67. Cells were further stained with 3.2% PFA for 15 min, and then fixed and permeabilized 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 by 2Cm67 on fixed cells. Saponin permeabilized l-Kβ 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 63x 1.45 NA oil objective (Leica Microsystems).

For TIRF, l-Kβ cells (1 × 10⁶/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 objective. Live single- or dual-color TIRF imaging was also performed using an Olympus IX71 fluorescence microscope equipped with a 150x magnification 1.45 numerical aperture (NA) TIRF 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 TIRF 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 (BSA/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-Kβ 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 complex was done as described (4) with slight modification. In brief, l-Kβ 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 Kβ3–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 × 10⁷ M SIINFEKL peptide for 2 d, followed by culture in complete IMDM media with 10 mg/mL recombinant human IL-2 (Peprotech), 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 Kβ (L929/Kβ) or cytoplasmic tail truncated Kβ (L929/ΔKβ) were infected with VV-Ub-SIIN, VV-Ub-Arg-NP-SIIN-eGFPSIN, VV-LI06P-SIIN-eGFPSIN, or VV-NP-SIIN-eGFPSIN (MOI = 2). Samples were taken every 20 min postinfection and co-incubated 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 CD8α–bungar毒素 (clone 53–6.7) and anti-mouse IFN-gamma (clone XMG-1.2; ebioscience). For peptide-loaded samples, cells were pretreated with 5 μg/mL human β2-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 coinubated 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-

Supporting Information

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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.


Fig. S1. Quantitating surface K\textsuperscript{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\textsuperscript{b}–SIIN complexes is shown in B. The conversion was calculated on a standard curve obtained from beads with known numbers of fluorescein molecules.
Fig. S2. \( \text{K}^b\)-SIYR and \( \text{K}^b\)-SIIN generated from synthetic peptide loading extensively colocalize regardless of fluorophores used for detection. (A) The \( \text{h}2\text{m}\)-sensitized \( \text{l-K}^b\) cells loaded with a mixture of directly conjugated Alexa488-SIIN and Alexa647-SIIN synthetic peptides were visualized by dual-color TIRF. (B) \( \text{l-K}^b\) 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. 1E. Peptide-loaded \( \text{l-K}^b\) 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.** Kb–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) Kb–SIIN complexes do not reside in the ER upon low temperature (15 °C) incubation. L-Kb 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 Kb; 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-Kb 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 Kb. (A) L929 cells stably transfected with wild-type (L929/Kb) or cytoplasmic tail-deleted Kb (L929/ΔKb) were stained with APC-conjugated AF6-88.5 (anti-Kb-mAb) and level of surface Kb 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 Kb-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 Kb 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/Kb or L929/ΔKb cells. In parallel, cells were infected with nonirradiated VV-NP-SIIN-eGFP (NpSeG; MOI = 2). Samples were taken at indicated times p.i. and Kb-SIIN complexes were quantitated with flow cytometry after staining with Alexa647-25D1.16.