Supporting Information

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SI Materials and Methods

Cells. The 293T cells were routinely maintained in DMEM as described previously (1), with occasional culture in G418-selective medium. HPB-ALL cells were cultured in complete RPMI, and CD45 retransfectants were grown as described previously (2). Jurkat and SKW-3 mutants Juβ2.7 and SKW-3β2.7 lacking CD11a and CD18, respectively, have been described previously (3, 4). Human NK cell lines NKL and NK-92 and the IL-2–expressing derivative NK-92C1, as well as the NK cell-enriched line B.3NK, were maintained in complete RPMI supplemented with 100 U/mL IL-2 or were grown as described previously (5–7).

MAbs and Antisera. The following antibodies were used: polyclonal rabbit antisera R25050 raised against the C-terminal 15 amino acids of E3/49K; W6/32 (ATCC HB95) against HLA-ABC; anti–pan-CD45 specific mAb GAP8.3 (8), kindly provided by Dr. Peter Cresswell, Yale University, New Haven, CT; HI30 (BD Pharmingen); MEM-28 (9) and AICD45.2 (10), kindly provided by Reinhard Schwinzer, Hannover Medical School, Hannover, Germany; anti-CD45RO and UCHL1 (BD Pharmingen); anti-CD45RA, MEM-56 (11); and anti-CD45RB, MEM-143 and MEM-55 (9). All MEM mAbs were generously provided by Vaclav Horejsi, Institute of Molecular Genetics, Prague, Czech Republic.

Purification and Refolding of the His-Tagged 49K Ectodomain (N49K-His) Expressed in *Escherichia coli*. For expression of the 49K His-tag fusion protein (N49K-His), 49K DNA-encoding residues 20–382 were PCR-amplified using primers 49K5′NCO 5′gagcgccatgggatttcatactatcaatgctac 3′ and 49K3′BAM 5′ cgcgggatccgggaatttttgaatcataatttc 3′. After digestion with restriction endonucleases NcoI and BamHI, the fragment was cloned into the pQE-60 expression vector (Qiagen), and the sequence was verified. N49K-His was expressed in M15 [pREP4] *E. coli* cells by induction with isopropyl β–D-1 thiogalactopyranoside and was found exclusively in inclusion bodies (IBs).

For refolding, IBs were resuspended in 5 mL of IB homogenization (IBH) buffer [6 M guanidine hydrochloride, 0.1 M sodium phosphate, 10 mM Tris (pH 8), 5 mM β-mercaptoethanol] using a Dounce tissue homogenizer. After incubation for 2 h at 25 °C, debris was removed by centrifugation at 10,000 × *g* for 30 min at 4 °C. Then a 1-mL Ni-agarose bead slurry was mixed with 0.5–1 mg protein in 5 mL of IBH buffer, followed by incubation at room temperature for 1 h. The suspension was transferred to a column, washed twice with 5 mL of IBH buffer, and then resuspended rapidly in 30 mL of degassed refolding buffer [0.1 M sodium phosphate, 0.01 M Tris (pH 7.6), 0.3 mM GSSG, 3 mM GSH, 50 mM NaCl including protease inhibitors] and incubated overnight under rotation at 4 °C. Subsequently, the suspension was transferred to a column and washed twice with 5 mL of IB wash buffer [0.1 M sodium phosphate, 0.01 M Tris (pH 7.6), 150 mM NaCl, 0.2 mM β-mercaptoethanol], after which N49K-His was eluted with IB wash buffer containing 250 mM imidazol. Refolding was assessed by SDS/PAGE in the presence and absence of DTT (Fig. S1).

Production of Polyclonal Abs and Monoclonal Abs Directed Against the N-Terminal Domain of E3/49K. Recombinant N49K-His was used to generate polyclonal rabbit antiserum R48 and rat mAbs. Rabbits were immunized with ∼250 μg of His-tagged N49K-His protein in 500 μL of PBS mixed with 500 μL of complete Freund's adjuvant by s.c. injection. The specific antibody level was assessed by immunoprecipitation of E3/49K from lysates of Ad19a-infected A549 cells or by Western blot analysis.

Rat mAbs were generated essentially as described previously (12). Lou/C rats were immunized three times with 50 μg of refolded N49K-His (Fig. S1) at 3-wk intervals using first complete Freund's adjuvant and then incomplete Freund's adjuvant both i.p. and s.c. Fusion of rat immune spleen cells with the myeloma cell line P3 × 63Ag8.653 was performed at 3 d after the final boost. Supernatants from hybridoma cells were screened for the presence of anti-49K antibodies initially by ELISA. Microtiter plates were coated with ∼0.5 μg/well of N49K-His by incubation in 0.2 M sodium carbonate buffer (pH 9.5) overnight. Hybridoma supernatants were added, followed by a 30-min incubation with constant shaking, then a 30-min incubation with peroxidase-coupled goat anti-rat IgG (Dianova). Finally, bound antibody was detected using *o*-phenylenediamine. ELISA-positive supernatants were further characterized by immunofluorescence, flow cytometry, immunoprecipitation, and Western blot analysis to verify mAab recognition of the highly glycosylated native and denatured protein, respectively.

Preparation of Affinity Matrices for Purification of sec49K. Two types of affinity columns were used to purify sec49K to homogeneity. In the first type, based on protein G-Sepharose, 5 mL of 1 M Tris (pH 7.4) was added to 50 mL of hybridoma supernatant containing ∼2 mg mAb 4D1. Then 1 mL of protein G-Sepharose beads were washed three times with 10 mL of 0.1 M Tris (pH 7.4) and added to the hybridoma supernatant. Alternatively, 2 mg of purified 4D1 was coupled. After incubation for 2 h at room temperature and gentle mixing, the beads were washed three times with 0.2 M sodium borate (pH 9.0), followed by centrifugation at 3,000 × *g* for 5 min and then resuspension in 10 mL of 0.2 M sodium borate. Then 50 mg of dimethyl pimelimidate was added to give a final concentration of ∼20 mM. The beads were incubated at room temperature with gentle mixing for 45 min.

The reaction was stopped by washing the beads once in 0.2 M ethanolamine (pH 8), followed by incubation for 2 h at room temperature in 0.2 M ethanolamine (pH 8) with gentle mixing. The beads were then washed three times with PBS and stored in PBS/0.01% merthiolate (thimerosal) at 4 °C. Alternatively, 6 mg of protein G-purified 4D1 mAb was coupled to NHS-linked Sepharose beads (GE Healthcare) according to the manufacturer's instructions. Two separate columns were prepared for processing supernatants of untransfected A549 and 49K-transfected A549K27S cells. In some cases, the medium was concentrated before being applied to the affinity column by diafiltration using the Vivaflow 50 System (Sartorius Stedim Biotech).

Purification of sec49K. SecE3/49K was purified from supernatants of cell lines A549K27S and 293K35 stably expressing the E3/49K protein. As negative control, supernatants of untransfected A549 and 293 cells were processed in the same way using separate affinity columns. Typically, cells were grown in 8–10 175-cm^2 plastic flasks to 80–90% confluency and washed once with DMEM without FCS. Then 35 mL of DMEM without FCS was added, and the cells were incubated for another 7–9 d. After removing detached cells and debris by centrifugation at 500 × *g* for 5 min, the medium was stored at 4 °C until further use. After the addition of 1/10× volume of 10× phosphate buffered saline (PBS) PBS and 1/100× volume of 2% NaN_3, as well as the protease inhibitors PMSF (8 μg/mL), trypsin inhibitor (10 μg/mL), and leupeptin (0.5 μg/mL), the medium was precleared

with a protein G-Sepharose column to remove potential residual antibodies derived from FCS and other proteins that could bind unspecifically to protein G-Sepharose.

The 4D1 affinity column was washed with PBS, and the flow rate was adapted to ~5 mL/h using a peristaltic pump. After loading, the column was washed sequentially with 20 mL of PBS and 20 mL of 10 mM sodium phosphate buffer (pH 6.8), and bound sec49K was eluted with 0.1 M glycine buffer (pH 3.0) and collected in 450-μL fractions containing 75 μL of 1 M Tris (pH 8.0). The fractions were evaluated by Western blot analysis using rabbit antiserum R48 and by SDS/PAGE and silver staining as described previously (13) or using a commercial kit (Bio-Rad). Fractions were also tested quantitatively with the FACS binding assay using Jurkat cells. A549 supernatant was mock-purified as a negative control. For reconstitution, the column was washed with 20 mL of PBS and then stored in PBS/0.01% merthiolate at 4 °C.

Western Blot Analysis. Proteins were blotted onto nitrocellulose membranes using the Trans-Blot SD Semidry Transfer Cell (Bio-Rad) following the manufacturer's protocol. For detection of E3/49K, membranes were incubated for 1 h with either rabbit antiserum R48 diluted 1:200 in PBS, 0.05% Tween 20 (PBS-T) or mAb 4D1 at 5 μg/mL PBS-T. After extensive washing in PBS-T, 49K was visualized with peroxidase-conjugated goat anti-rabbit IgG and goat anti-rat IgG (1:10,000), respectively, using ECL detection reagent (GE Healthcare) according to the manufacturer's instructions, followed by exposure to Bio-Max MR film (Kodak).

For detection of ZAP-70 phosphorylation, 2×10^7 Jurkat cells were pretreated on ice for 2 h with purified sec49K (1 μg/mL) and CD3 antibody (OKT3, 5 μg/mL; eBiosciences). Cells were washed, incubated for 30 min with mAb 4D1 (5 μg/mL) and for another 30 min with goat anti-mouse IgG (20 μg/mL; Dianova) and incubated at 37 °C for the indicated times before being lysed in 1% Nonidet P-40, 140 mM NaCl, 5 mM EDTA, 5 mM iodoacetamide, 5 mM NaF, and 1 mM Na_3VO_4. For immunoprecipitation, 5 μg of ZAP-70 antibody (G-4; Santa Cruz Biotechnology) was immobilized on proteinA/G-Sepharose beads (Thermo Fischer Scientific) and then incubated with lysates at 4 °C for 2 h. After SDS/PAGE and Western blot analysis, membranes were probed with phosphotyrosine-specific (PY99; Santa Cruz Biotechnology) or ZAP-70–specific antibodies.

For monitoring of ERK activation, Jurkat cells (10^6 cells/mL) were incubated overnight at 37 °C with or without sec49K. Then 10^6 cells per sample were harvested, resuspended in 100 μL of medium, and incubated with or without 0.2 μg/mL anti-CD3 (OKT3) for 10 min at room temperature. Cells were then washed, resuspended in 50 μL of medium, and stimulated by adding 50 μL of cross-linking antibody (goat anti-mouse, 10 μg/mL) at 37 °C. After 5 min, stimulation was stopped by washing with cold PBS before lysis. Then 10 μL was loaded onto 10% or 4–12% NuPage gel (Invitrogen), and the PVDF membrane (Millipore) was incubated with anti-ppERK (1:2,000; Sigma-Aldrich) and anti-ERK (1:1,250; Upstate Biologicals) antibodies for 1 h at room temperature or overnight at 4 °C in PBS-T and 5% BSA. After extensive washing with PBS-T and 0.5 M NaCl, the membrane was developed with the appropriate HRP-conjugated secondary antibody and either SuperSignal West Pico or Dura and X-ray films.

Staining of Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells (PBMCs) isolated from heparin-treated blood or buffy coats were purified using Ficoll 400 (PAA Laboratories) according to the manufacturer's instructions. PBMCs were incubated with sec49K as described in *Materials and Methods*, but at 2×10^7 cells/mL. After washing, cells were incubated with mAb 4D1, followed by multiply absorbed R-PE and goat anti-rat IgG. Abs conjugated directly to various lymphocyte markers were used to stain the various PBMC subpopulations. All Abs used for staining these PBMC subpopulations, including the appropriate Ig isotype controls, were purchased from BD Biosciences or BD Pharmingen. Cells were analyzed with CellQuest on a FACSCalibur flow cytometer (BD Biosciences).

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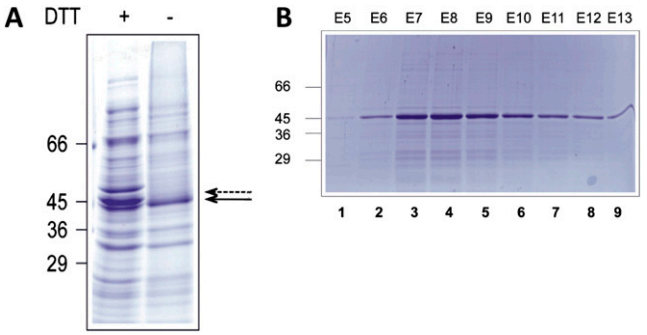

Fig. S1. (*A*) The E3/49K HisTag fusion protein expressed in *E. coli* contains disulfide bonds. Induced *E. coli* lysates were separated by SDS-PAGE in the presence (+) and absence (–) of DTT and then stained with Coomassie blue. The observed shift on reduction of the nonreduced species (solid arrow) to a species with higher apparent molecular weight (m_r; dashed arrow) indicates that the 49K protein expressed in *E. coli* contains intramolecular disulfide bonds. Of note, these reduced species were not visualized in the absence of DTT. The shift in m_r to the species indicated by the dashed arrow is essentially identical to that seen in the native E3/49K protein expressed on Ad19a infection of A549 cells metabolically labeled in the presence of tunicamycin (1). This suggests that the *E. coli*-expressed protein has formed similar disulfide bonds as the virus-derived 49K protein and is at least partly correctly refolded. (*B*) On refolding, the protein was eluted from the Ni-chelate column and collected as 0.5-mL fractions. SDS-PAGE and Coomassie blue staining reveals essentially a single protein species that was used for immunization.

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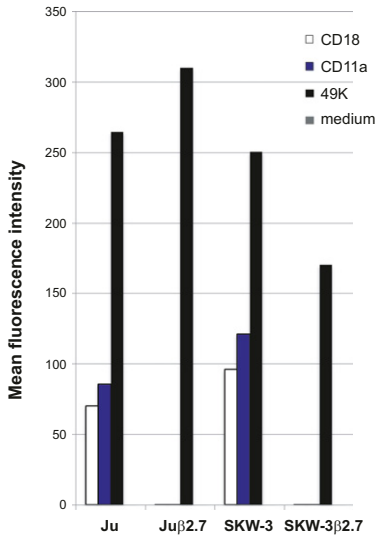

Fig. S2. Loss of CD11/CD18 (LFA-1) integrin expression on the cell surface does not affect sec49K binding. Jurkat and SKW3 lymphoblastoid cell lines defective in the production of CD11a and CD18 (3), respectively, the alpha and beta subunits of the LFA-1 integrin, were compared with their parental cell lines for cell surface expression of CD11a/CD18 (LFA-1 integrin) using FACS analysis with respective antibodies (blue and white bars). Both mutant cell lines lack LFA-1 expression; however, binding of sec49K (concentrated supernatant) did not correlate with CD11a or CD18 expression (black bars). The data are from one of two similar experiments.

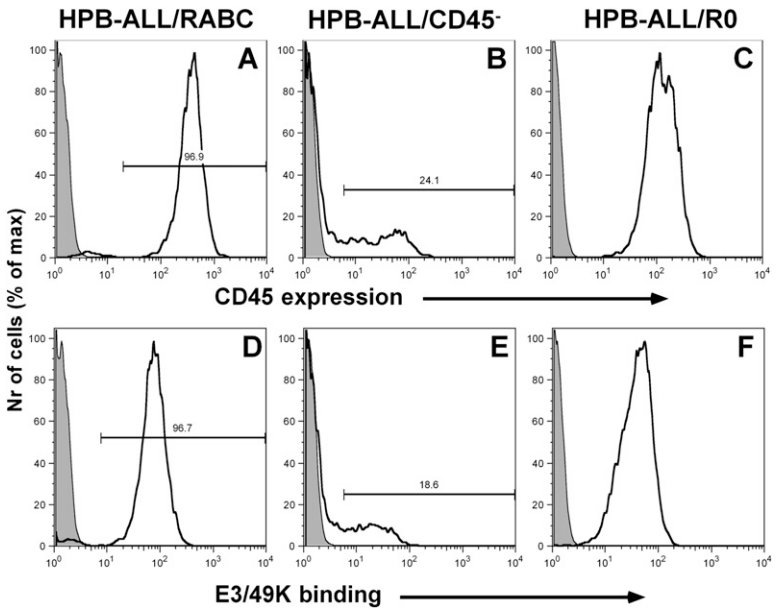

Fig. S3. Binding of sec49K to HPB-ALL cells is strictly correlated with the number of $CD45^+$ cells. We sought to confirm the identity of the sec49K receptor by also examining HPB-ALL cells for which $CD45^-$ variants had been sorted and retransfected with certain CD45 isoforms (2). Cells were stained with monoclonal antibodies toward CD45 or E3/49K. As reported previously, there is a tendency for $CD45^+$ cells to reemerge from these $CD45^-$ cells. This is shown in our CD45-HPB-ALL sample population, in which 24% of the cells express low levels of CD45 (*B*). Strikingly, a similar percentage of HPB-ALL cells (19%) exhibited sec49K binding (*E*). As seen for Jurkat cells, sec49K binding of HPB-ALL RABC and RO transfectants parallels their CD45 isoform expression (*A*, *C*, *D*, and *F*). Thus, there appears to be a strict correlation between CD45 expression and sec49K binding. Moreover, although the expression level of these isoforms varies significantly, all isoforms tested clearly have the capacity to bind sec49K. The data are from one representative experiment out of three experiments.

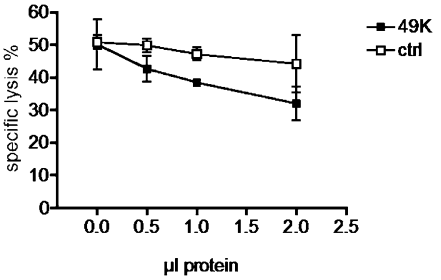

Fig. S4. Sec49K impairs the cytotoxicity of primary NK cells. Fresh NK cells from buffy coat were exposed to ^{51}Cr-labeled K562 target cells at a fixed effector/target ratio of 12:1 in the presence of different amounts of sec49K-containing supernatant or supernatant from control 293 cells (ctrl). Analyses were performed in triplicate, and percent lysis is expressed as mean ± SD.

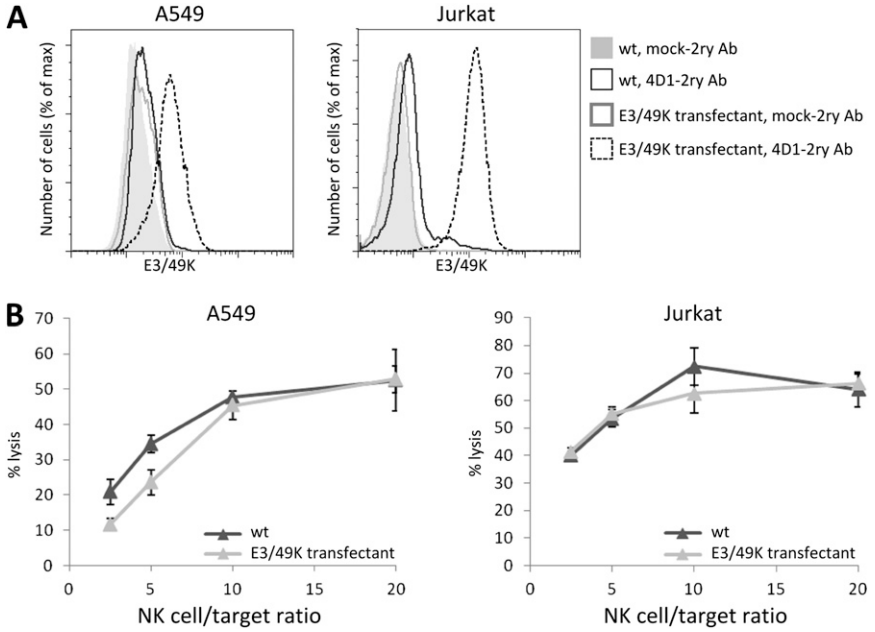

Fig. S5. The membrane form of E3/49K does not inhibit killing by primary NK cells. (*A*) WT (wt) A549 and Jurkat cells (E6.1) and the corresponding E3/49K$^+$ transfectants (K27S and Ju49K-21) were stained with 2ry antibody alone or with mAb 4D1 and 2ry antibody. (*B*) Cytokine-activated primary NK cells (vericyte medium) were exposed with the WT A549 and Jurkat cells (E6.1) or with the corresponding E3/49K transfected target cells at various NK cell:target ratios. Analyses were performed in triplicate, and percent lysis is expressed as mean ± SD. Data are from one representative donor out of three donors.

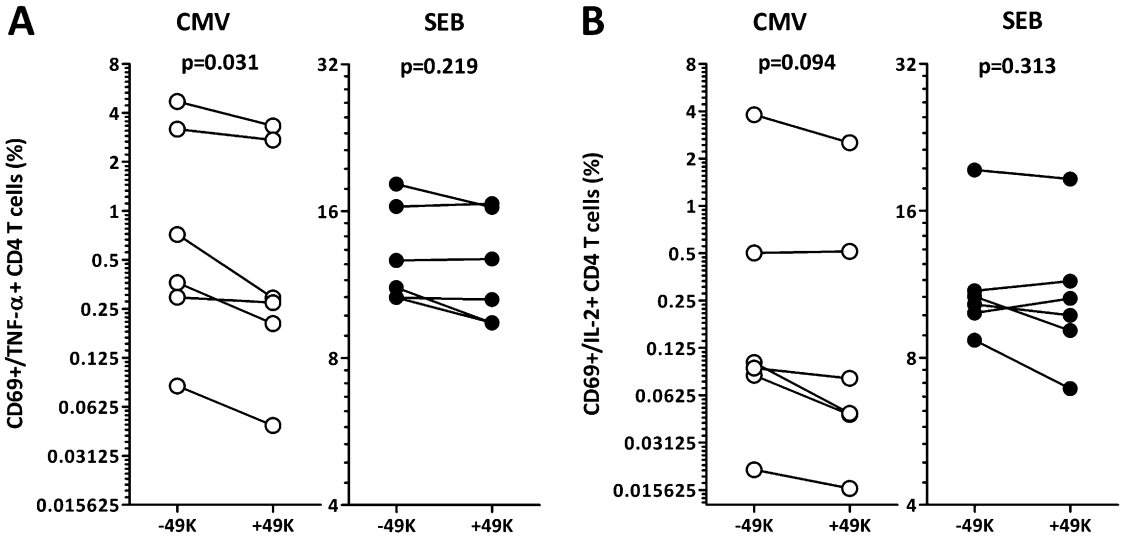

Fig. S6. Sec49K differentially affects cytokine production after antigen-specific stimulation of CD4 T cells. PMBCs of six CMV-seropositive controls were preincubated for 30 min in the presence or absence of sec49K, and then stimulated with a whole CMV antigen lysate, a control lysate, or *Staphylococcus aureus* enterotoxin B (SEB). Specific CD4 T cells were identified based on coexpression of CD69 and TNF-α (*A*) or IL-2 (*B*). The presence of 49K led to a significant reduction in the percentage of TNF-α CMV-specific CD4 T cells (by 36.0%; interquartile range 12.5–47.8%). Statistical significance of the difference in the absence and presence of sec49K was assessed by the two-tailed Wilcoxon matched-pairs test; *P* values are indicated.