A unique secreted adenovirus E3 protein binds to the leukocyte common antigen CD45 and modulates leukocyte functions

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The E3 transcription unit of human adenoviruses (Ads) encodes immunomodulatory proteins. Interestingly, the size and composition of the E3 region differs considerably among Ad species, suggesting that distinct sets of immunomodulatory E3 proteins may influence their interaction with the human host and the disease pattern. However, to date, only common immune evasion functions of species C E3 proteins have been described. Here we report on the immunomodulatory activity of a species D-specific E3 protein, E3/49K. Unlike all other E3 proteins that act on infected cells, E3/49K seems to target uninfected cells. Initially synthesized as an 80- to 100-kDa type I transmembrane protein, E3/49K is subsequently cleaved, with the large ectodomain (sec49K) secreted. We found that purified sec49K exhibits specific binding to lymphoid cell lines and all primary leukocytes, but not to fibroblasts or epithelial cells. Consistent with this binding profile and the molecular mass, the sec49K receptor was identified as the cell surface protein tyrosine phosphatase CD45. Antibody-blocking studies suggested that sec49K binds to the membrane proximal domains present in all CD45 isoforms. Functional studies showed that sec49K can suppress the activation and cytotoxicity of natural killer cells as well as the activation, signaling, and cytokine production of T cells. Thus, we have discovered an adenovirus protein that is actively secreted and describe immunomodulatory activities of an E3 protein uniquely expressed by a single Ad species.

Significance

Human adenoviruses encode Early region 3 (E3) proteins that manipulate the host immune response to establish an infection or to persist longer. To date, only a few E3 functions from a single adenovirus species (C) have been characterized, all of which act directly on infected cells. Here we describe a secreted E3 protein that is uniquely expressed by species D adenoviruses. This protein targets noninfected leukocytes using a cell surface phosphatase as a receptor. We provide evidence that this interaction suppresses leukocyte activation and effector functions, implying that species D adenoviruses can affect the host distant from the site of infection.


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other species (25). The sequence of the extracellular/luminal domain suggested three internal repeats, named conserved region 1–3 (CR1–3), which may form an Ig-like fold. The corresponding protein was expressed by all species D Ads tested (26) and thus may be implicated in their pathogenesis. E3/49K is a highly glycosylated type I transmembrane protein migrating with an apparent molecular weight (mW) of 80–100 kDa and as such is by far the largest E3 protein. It is localized in the Golgi/ trans-Golgi network, in early endosomes, and in the late phase of infection in lysosomes as well (24).

Here we demonstrate that E3/49K is cleaved, and that its large ectodomain is secreted. This is a unique processing pathway for E3 proteins and to date E3/49K represents the only actively secreted E3 protein known. Rather than infected cells, secreted E3/49K targets selectively noninfected leukocytes via binding to the protein phosphatase CD45, and can suppress functions of both NK cells and T cells.

Results
Proteolytic Processing and Secretion of E3/49K. While monitoring the processing of E3/49K, we previously noted the emergence of 10- to 13-kDa C-terminal fragments (24). Whether the protein is further degraded or the putative large N-terminal cleavage fragment is secreted remained unclear, however. To address this question, we raised a rabbit antiserum, R48, against the bacterially expressed, refolded N-terminal (ecto)domain of E3/49K (Fig. S1). In pulse-chase experiments, this antiserum detected similar high molecular weight species of E3/49K as the antiserum against the C terminus of E3/49K (compare the bracketed areas in the left panels of Fig. 1 A and B), but not the low molecular weight fragments (C; 10–13 kDa) (see Fig. 1 A and C). This finding confirms the previous hypothesis that E3/49K is cleaved into a large N-terminal fragment of ~90 kDa and 10- to 13-kDa fragments derived from the C terminus.

Strikingly, when the supernatant was probed with R48 directed to the N-terminal domain, 49K-specific material was visualized after 120 min of chase, demonstrating that the N-terminal fragment is indeed secreted (Fig. 1B, lanes 11–14; sec). Proteolytic processing and secretion also were observed in 49K-transfected cells (Fig. 1C), demonstrating that proteolytic processing of E3/49K is independent of viral infection and can be executed by cellular proteases.

Several lines of evidence suggest that the secreted E3/49K (sec49K) is very stable. First, sec49K was detectable in pulse-chase experiments using extended chase times of up to 24 h with both infected and transfected cells (Fig. 2 A and B). Second, sec49K accumulated in the supernatant of transfected cells over a period of 9 d, as shown by Western blot analysis (Fig. 2C). Third, functional active protein (see below) could be detected in sterile supernatants even after years of storage at 4 °C. This high stability of sec49K is consistent with a potentially important function in the extracellular environment.

Monoclonal Antibodies Recognizing the E3/49K Ectodomain and Purification of sec49K. To enable purification of sec49K and its detailed functional analysis, we generated mAbs against the E3/49K ectodomain by immunizing rats with a purified and refolded N-terminal fragment of E3/49K (Fig. S1 A and B). Two mAbs, 4D1 and 1E6, were selected for further study. The use of mAb 4D1 in FACS analysis clearly showed that 49K is present not only in the endoplasmic reticulum, the Golgi apparatus, and endosomes (24), but also on the cell surface of infected cells (12) and transfected cells.

To purify sec49K for functional studies, we coupled mAb 4D1 to protein G- or N-hydroxysuccinimide (NHS)-Sepharose columns. As a reliable, noninfectious source for sec49K, we used culture supernatant from E3/49K-expressing cell clones derived from A549 cells (24) and 293 cells. Supernatant from these cells was applied to the 4D1 affinity column, and bound sec49K was eluted. Western blot analysis of eluted fractions using rabbit antiserum R48 showed that the majority of sec49K migrated as a major species of ~78–90 kDa and usually a minor species of 50–60 kDa (Fig. 2D) that may represent a degradation product.
for 1 h with [35S]-methionine and then chased with medium containing nonradioactive methionine for the indicated times. E3/49K was precipitated with serum R48 against the N-terminal domain. (C) Supernatants of A549 cells stably expressing E3/49K (B) were metabolically labeled for 1 h with [35S]-methionine and then chased with medium containing nonradioactive methionine for the indicated times. E3/49K was precipitated from lysates using the antisera directed to the C terminus, whereas sec49K was precipitated with serum R48 against the N-terminal domain. (C) Supernatants of A549 cells stably transfected with the E3/49K gene were collected over a period of 1–9 d after placement in medium without FCS. Cellular debris was removed by centrifugation, and 15 μL of supernatant was directly analyzed by Western blot analysis using antiseraum R48. (D and E) Sec49K was purified using affinity chromatography with mAb 4D1 coupled to protein G Sepharose beads. Sec49K was eluted from the columns collecting 500-μL fractions. Then 15 μL of each fraction was analyzed by SDS-PAGE, followed by Western blot analysis with antiseraum R48 (D) or silver staining (E).

Remarkably, silver staining of the same fractions (Fig. 2E) revealed only protein species of identical molecular weight as those detected by 49K-specific Western blot analysis (Fig. 2D); thus, we concluded that sec49K was apparently purified to homogeneity and so could be used for functional tests. In similar way, A549 supernatant was mock-purified as a negative control. To exclude the possibility that the purification scheme might have affected functional activity, we also used crude or concentrated supernatant in some experiments.

**Sec49K Binds Selectively to Lymphoid Cell Lines and All Primary Leukocytes.** Considering that most E3 proteins are involved in immune evasion and E3/49K is secreted, we hypothesized that unlike all other E3 gene products characterized to date that directly modulate functions of infected cells, sec49K may target surrounding cells, most likely cells associated with the immune system. This hypothesis is supported by the presence of an unusual putative Ig domain in E3/49K (24, 27). Thus, we tested a panel of lymphoid and nonlymphoid cell lines for their capacity to bind purified sec49K. Sec49K binding was observed for all human lymphoid cell lines tested, irrespective of whether they were derived from NK cells (NKL, B.3NK; NK-92C1), T lymphocytes [Jurkat, B.3CD4, 234 (CTL)], or B lymphocytes (Daudi) or represented other types of EBV-transformed lymphoblastoid cell lines (L721.221, L721.112, BW LCL, DS LCL; T2) (Fig. 3A). In contrast, no significant binding was detected for human cell lines derived from various other tissues, including A549 (lung epithelial carcinoma), 293 (embryonal kidney cell line), HeLa (cervix carcinoma), K562 (erythroleukemia), and SeBu (primary human fibroblasts) (19, 24). Thus, sec49K seems to selectively bind to lymphoid cell lines.

To ensure that this binding pattern is not an artifact of transformation of the cells, we tested primary human peripheral blood mononuclear cells (PBMCs) for 49K binding activity (Fig. 3B). As shown in the figure, significant 49K binding was detectable only after previous incubation with purified sec49K (black line), but not with mock-purified material from A549 (dotted line) or medium alone (gray histogram). Most importantly, sec49K bound to all leukocyte subpopulations examined, albeit with a hierarchical pattern. Staining was strongest for T cells, followed by B cells, NK cells, and then monocytes. Separate analysis of CD4 and CD8 T-cell populations revealed greater 49K binding for CD4 T cells compared with CD8 T cells. This binding pattern was reproduced by direct incubation of sec49K and control with whole blood, with sec49K demonstrating significantly different binding to the various leukocyte subpopulations (P < 0.0001) (Fig. 3C). Moreover, whole-blood analysis provided evidence that sec49K binds to granulocytes as well, but to the weakest degree of all leukocyte subpopulations (Fig. 3C). Taken together, these data suggest the presence of a common receptor for sec49K that seems differentially expressed on all leukocytes.

**E3/49K Coprecipitates High Molecular Weight Proteins in the Jurkat T-Cell Line.** To identify proteins interacting with sec49K in lymphocytes, we exposed various sources of 49K to lysates from Jurkat T cells expressing the sec49K receptor (Fig. 3D), to possibly allow coprecipitation. Lysate of A549 cells that did not bind sec49K in the FACS assay served as a negative control. In our first approach, A549 and Jurkat cells were mock-infected or infected with WT Ad19a or the 49K-deficient Ad19a virus (49K KO) (12) and then labeled with [35S]-methionine. As expected, E3/49K was precipitated only from lysates of A549 and Jurkat cells after infection with WT Ad19a (Fig. 4, WT; compare lanes 1–3 and 7–9). Close inspection revealed three apparently specific coprecipitating protein species of 172, 181, and 196 kDa (arrowheads) in 49K receptor-positive Jurkat cells on infection with WT Ad19a (Fig. 4, lane 9), but not in mock-infected cells (−) or 49K KO infected cells.

Consistent with the low efficiency of Ad infection in lymphocytes (28) E3/49K was poorly expressed in Jurkat cells (Fig. 4, compare lanes 3 and 9). Thus, we took two other approaches to enhance coprecipitation of potential E3/49K interaction partners. First, lysates of WT Ad19a-infected A549 cells (containing high amounts of E3/49K; see lane 3 or lane 6) or mock-infected A549 cells were mixed with metabolically labeled Jurkat cell lysates containing the putative receptor (Fig. 4, lanes 4–6). Second, purified sec49K was added to lysates of mock-infected Jurkat cells, or A549 cells as a control (Fig. 4, lanes 11 and 13). Strikingly, in the presence of receptor-positive Jurkat cell lysates, protein species with the same high apparent molecular mass (mₐ) as seen on infection of Jurkat cells were specifically coprecipi-
tated with E3/49K (Fig. 4, lanes 6 and 13). Corresponding bands were not detected in the absence of E3/49K (lanes 4 and 5 or lanes 10 and 12) or in receptor-negative A549 cell lysates on the addition of purified sec49K (Fig. 4, lane 11). We conclude that E3/49K specifically interacts with high molecular weight proteins in sec49K receptor-positive Jurkat cells.

Identification of the Leukocyte Common Antigen CD45 as the Cellular E3/49K Receptor. Based on the binding profile of sec49K and the m<sub>1</sub> of the sec49K receptor, we hypothesized that the receptor might be the leukocyte common antigen CD45 or a leukocyte-specific integrin (CD11a/CD18). Analysis of CD11<sup>+</sup> Jurkat and SKW leukemia cells revealed no significant effect on sec49K binding (Fig. S2). In contrast, J45.01 Jurkat cells that had lost most CD45 expression after negative selection (29) exhibited only minimal binding of purified sec49K, providing the first evidence that CD45 might be the sec49K receptor.

To verify these findings, we tested the independently derived Jurkat cell line J-AS-1 (30), which lacked all detectable CD45 isoforms on expression of an antisense construct (Fig. 5A, Upper). Indeed, these cells were unable to bind sec49K, whereas transfectants of J-AS-1 expressing either the CD45RO or CD45RABC isoform (Fig. 5A, Upper) regained 49K-binding capacity (Fig. 5A, Lower). This confirmed CD45 as the sec49K receptor. Further confirmation was obtained with HPB-acute lymphoblastic leukemia (HPB-ALL) cells for which CD45-negative variants were sorted and retransfected with certain CD45 isoforms. Again a strict correlation between CD45 expression and sec49K binding was observed (Fig. S3). Moreover, although the expression levels of the CD45RO and CD45RABC isoforms varied substantially, both isoforms clearly have the capacity to bind sec49K.

To unequivocally demonstrate that CD45 alone is sufficient for sec49K binding, we transiently transfected 293T cells that otherwise showed no binding activity for sec49K (Fig. 5B, control) with cDNA expression vectors encoding CD45RO and CD45RABC isoforms (31) and then performed binding tests with sec49K. As shown in Fig. 5B (Middle and Bottom), after transfection and incubation with sec49K, but not with control BSA, a large proportion of 293T cells comparable to those expressing CD45RO and CD45RABC (column 2) stained for E3/49K (column 3). Moreover, dual-color FACS staining with anti-E3/49K and CD45PE-Cy5 (∼49K+CD45) clearly demonstrated that only CD45<sup>+</sup> cells bound E3/49K (diagonal population in Fig. 5B, Right). Sec49K binding also was quantitatively correlated with CD45 expression, with cells with higher CD45 expression binding more sec49K.
Evidence that CD45 and E3/49K Interact Physically. To visualize a physical interaction between CD45 and E3/49K, we mixed lysates of CD45+ Jurkat cells with lysates of the E3/49K+ cell line 293K35 (K35) and either immunoprecipitated (IP) or immunoprecipitated and assessed the presence of E3/49K in the precipitate by Western blot analysis using E3/49K-specific Abs (Fig. 5 C, lanes 1 and 2), or immunoprecipitated E3/49K and then used CD45-specific Abs for Western blot analysis (Fig. 5 C, lanes 3 and 4). Coprecipitation of 49K was observed only when CD45 was precipitated from mixed lysates of Jurkat and 293K35 containing both CD45 and E3/49K, not when Jurkat lysates were mixed with lysates from E3/49K+ 293 cells (Fig. 5 C, compare lanes 1 and 2). Similarly, CD45 was detectable in immunoprecipitates of E3/49K only when Jurkat and K35 lysates were mixed (Fig. 5 C, compare lanes 4 and 3). Thus, we conclude that E3/49K physically interacts with CD45.

E3/49K Interacts with the Membrane Proximal Domain of CD45 Present in All Isoforms. We next investigated whether we could identify the domain of CD45 with which sec49K binds. For this, we incubated Jurkat cells with purified sec49K before FACS analysis with a panel of CD45-specific mAbs directed to different parts of CD45 (Fig. 6A). The pan-CD45–specific mAbs GAP8.3, HI30, MEM28, and AICD45.2 recognize all isoforms of CD45 and thus are likely to bind the membrane proximal cysteine-rich domain or the fibronectin type III (FNIII) domains that are common to all isoforms, whereas UCHL1 recognizes specifically the CD45RO isoform represented by amino acids N-terminal from the four common core domains. MEM56 recognizes CD45RA, and MEM55 and MEM143 recognize CD45RB-containing isoforms, including the longest isoform (Fig. 6B). Previous binding of purified sec49K, as demonstrated by corresponding CD1 staining in each experiment, consistently affected all pan-CD45–specific antibodies (Fig. 6A). We found only a modest 15–20% inhibition for HI30 and MEM28, but GAP8.3 and AICD45.2 binding was compromised more severely, showing a consistent ~30–50% reduction. In contrast, sec49K binding did not substantially inhibit the recognition of Abs directed to the more distal domains present in RO, RA, and RB isoforms. Rather, their binding actually increased (relative to the negative control mAb W6/32 directed to HLA), which is particularly obvious for mAb MEM55, indicating conformational changes on complex formation with sec49K. The results of these blocking studies are consistent with the foregoing transfection data and binding studies, indicating that sec49K binds to the membrane-proximal domains present in all CD45 isoforms.

Sec49K Inhibits NK Cell-Mediated Lysis and Expression of Activation Markers. In the 49K-binding assays (Fig. 3A), the NK cell line NKL exhibited the highest mean fluorescence intensity values, suggesting high expression of the putative sec49K receptor. To test whether the binding of sec49K might interfere with NK cell functions, we measured NK cell-mediated lysis of the MHC class I-negative K562 cells in the presence and absence of purified sec49K (Fig. 7A). Sec49K inhibited NK cell-mediated cytolysis over a range of effector-to-target (E:T) ratios compared with mock-purified material from A549 cells or PBS. A similar inhibition was seen when concentrated supernatant from 49K-expressing transfectants and A549 cells were compared, excluding the possibility that the inhibition is related to the purification process. Another NK cell line, NK-92, was also specifically affected by purified or concentrated sec49K, and a substantial, dose-dependent inhibition of cytotoxicity was noted with freshly isolated primary NK cells (Fig. S4). Interestingly, exclusive expression of E3/49K in its membrane-integrated form on Jurkat target cells (which are unable to cleave the molecule and do not produce sec49K) or on A549 cells had no effect on NK cell-mediated killing (Fig. S5).

We also examined the effect of sec49K on primary NK cells by monitoring the activation-induced expression of CD25 (IL-2 receptor α chain) and CD69 in the presence and absence of sec49K using different stimulation protocols. Sec49K significantly reduced the number of cells displaying these activation markers when stimulated with IL-2 alone (Fig. 7B, Center) or with IL-2 plus antibodies against the major activating NK receptor NK2G2D and the coreceptor 2B4 (Fig. 7B, Right). A significant reduction in the immediate activation marker CD69 was noted even without stimulation. Taken together, these findings suggest that only the secreted form of E3/49K reduces NK cell activation and target cell killing, and thus has an immunomodulatory function.

Sec49K Suppresses CD69 Expression and Disturbs Signaling and Cytokine Production in T Lymphocytes. To investigate potential mechanisms of 49K action on T cells, we stimulated Jurkat cells by CD3 cross-linking and monitored the expression of the activation marker CD69 and phosphorylation events. Similar to the effect seen in NK cells, sec49K inhibited up-regulation of the activation marker CD69 after CD3 stimulation of Jurkat T cells (Fig. 7C). Moreover, the addition of purified sec49K markedly suppressed or delayed phosphorylation of the TCR target protein ZAP-70 (Fig. 7D), as well as phosphorylation of the downstream target ERK-1/2 (Fig. 7E). In the absence of CD3 stimulation (Fig. 7E, lanes 1 and 2), sec49K prevented ERK phosphorylation, whereas triggering with anti-CD3 Abs (OKT3) in the presence of 49K (49K+OKT3) drastically re-
duced phosphorylation of ERK (Fig. 7E, lanes 3 and 4). These data demonstrate that sec49K can interfere with activating signaling pathways in NK and T cells and thereby possibly inhibit the function of these cells.

Fig. 5. Sec49K binds to the cell surface phosphatase CD45. (A) Jurkat and its CD45− derivative J-AS-1, as well as two J-AS-1 transfectants expressing the CD45R0 and CD45RABC isoform, respectively, were incubated with medium (Upper) or sec49K (Lower). After washing, cells were stained for CD45 using mAb HI30 (black histograms) followed by Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen). In parallel, cell-bound sec49K was detected by mAb 4D1, followed by Alexa Fluor 488-labeled goat anti-rat IgG (gray histograms). The background staining obtained with the secondary reagents only is shown in white. The results represent one of three similar experiments. (B) 293T cells were transiently transfected with expression constructs for CD45R0, CD45RABC, or control Ad2 E3 DNA as indicated on the right. At 40 h later, cells were incubated with either BSA or sec49K, after which CD45 expression and sec49K binding were monitored using single staining (α49K; 4D1) or double staining with Tricolor-labeled anti-CD45 mAb HI30 (α49K+CD45) and mAb 4D1. The data represent one of three similar experiments. (C) Ad19a E3/49K immunoprecipitates CD45 expressed in Jurkat cells. Jurkat cell lysates (J) were incubated with cell lysates of 293 cells (293) and 293 cells stably expressing E3/49K (K35; 0.5 mg each). Immunoprecipitations (IP) were performed using 1 μg of mAb MEM28 recognizing CD45 (CD45) or 4 μg mAb 4D1 recognizing E3/49K (49K). The precipitated proteins were evaluated by Western blot analysis (WB) with the same antibodies. The data are from one of two representative experiments.
To investigate potential effects of sec49K on antigen-specific T-cell function, PMBCs from CMV-seropositive controls were stimulated with a whole CMV antigen lysate or Staphylococcus aureus enterotoxin B (SEB), and CD69+ cytokine-producing CD4 T cells were analyzed by intracellular cytokine staining. As shown for a typical sample in Fig. 7F and by analysis of all samples in Fig. 7G, preincubation of PBMCs with sec49K led to a significant decrease in the percentage of both CMV- and SEB-reactive CD4 T cells producing IFN-γ. This also held true for CMV-specific CD4 T cells producing TNF-α, whereas only a trend toward a decrease was observed for TNF-α-positive CD4 T cells after SEB stimulation, and no effect was noted for T cells producing IL-2 (Fig. S6). Taken together, these findings indicate that binding of sec49K directly inhibits T-cell functionality, which is most pronounced for cells producing IFN-γ.

Discussion

In this study, we have uncovered a unique processing pathway and function for Ad E3 proteins. E3/49K of Ad19a, which is initially synthesized as a type I transmembrane protein, was cleaved, resulting in a ~90-kDa soluble N-terminal fragment that is secreted. Apart from Ad proteins that are released on death of the infected cells [e.g., fiber (32)], this is to our knowledge the only adenovirus protein known to be actively secreted. Considering the size of the smaller nonglycosylated C-terminal fragments (24), cleavage may occur some 20- to 50-aa N terminal of the transmembrane domain (TMD), but the precise site and the compartment in which cleavage occurs remain unclear, although the timing and cell surface exposure suggest that it occurs on the cell surface or in an endosomal compartment.

The abundantly glycosylated, secreted form of E3/49K, sec49K, is highly stable in the supernatant of infected or transfected cells, which is compatible with a role for this protein in body fluids in vivo. Unlike the other E3 proteins described thus far that all act on the infected cell, E3/49K seems to be directed primarily at surrounding/uninfected cells. This implies that E3 proteins can have long-range effects very distant from the site of infection. We cannot rule out the possibility that the transmembrane form of E3/49K or the C-terminal fragment may have additional functional activities acting on the infected cell, although we did not observe any inhibition of killing when cells expressing only the membrane form were used as target cells for primary NK cells (Fig. S5). In line with a role distant from the site of infection, we showed that the secreted form, sec49K, binds selectively to leukocytes using the leukocyte common antigen CD45 as a sec49K receptor. CD45 is a large cell surface-exposed protein tyrosine phosphatase that removes an inhibitory phosphate group from Src-family kinases. Because Src-family kinases are critically involved in immunoreceptor signaling in all leukocytes, CD45 plays an important role in signaling pathways, leading to activation and differentiation of T cells, B cells, NK cells, and macrophages (33–36). Differential splicing of at least three exons encoding the A, B, and C parts of the CD45 extracellular domain gives rise to different isoforms with m, ranging between 180 and 220 kDa and between 391 and 552 amino acids in the extracellular domain. This extracellular part assumes a rod-like structure consisting of three membrane-proximal FNIII domains followed N-terminal by a cysteine-rich domain and amino acids encoding the isoforms RO, RA, RB, RC, RABC, or other combinations (Fig. 6B) (31, 33, 37). We demonstrate here that sec49K has the capacity to bind the R0 and the RABC isoforms, suggesting that binding is independent of amino acids encoded by exons A, B, and C. This was confirmed by showing that previous binding of sec49K to CD45 significantly affected binding only of mAbs directed to the common domains. Thus, we conclude that sec49K binds to all CD45 isoforms, most likely by attaching to the membrane-proximal FNIII and/or cysteine-rich domains.

Binding of sec49K to CD45 impaired the activation and cytotoxic activity of NK cells. Sec49K also interfered with activation and cytokine production of primary T cells. The foregoing effects were correlated with impaired signal transduction in Jurkat T cells affecting tyrosine phosphorylation of the target proteins ZAP-70 and ERK known to be involved in NK- and T-cell activation. A similar suppression of ERK signaling was seen in murine CD45-deficient NK cells, whereas cytotoxicity was less affected (38). The exact mechanism of sec49K activity remains to be determined. Sec49K binding might modulate the phosphatase activity of CD45, either by inducing dimerization (33) or by interfering with its organization in lipid rafts (39).

Fig. 6. (A) Previous incubation of CD45+ Jurkat cells with sec49K selectively reduces binding of pan-CD45-specific mAbs. Jurkat cells were incubated with purified sec49K or BSA for 1 h on ice. After washing, cells were stained with a panel of mAbs directed to various isoforms of CD45. As a negative control, the impact of sec49K on the binding of mAb W6/32 against HLA was measured. The mean binding ± SEM calculated from at least four independent experiments is depicted relative to the mean value obtained on incubation with BSA. Binding of sec49K was confirmed by parallel staining with mAb 4D1. (B) Schematic view of the structure of CD45 isoforms R0, RB, and RABC, adapted from (37).
To date, a physiological ligand for the large CD45 ectodomain remains elusive. Although a number of molecules were proposed (e.g., CD22 on B cells or the apoptosis-inducing lectin, galectin 1), the interactions turned out to not be CD45-specific (33, 34, 37). It was recently reported that the human cytomegalovirus (HCMV) protein pUL11 of the RL11 family fused to the Fc domain of human Ig also is able to attach to CD45, causing impaired T-cell proliferation and cytokine production and overall T-cell paralysis (40). Based on sequence comparisons, a structural relationship between E3/49K and the HCMV RL11 family was suggested by Davison et al. (27). By altering the boundaries of the E3/49K CR1 repeat domain (25), these authors identified a homologous domain in other E3 proteins as well as in the HCMV RL11 family. Here we report a striking functional relationship between pUL11 and E3/49K as well, in that they both target the same protein, CD45. However, although we found an influence of sec49K on T-cell receptor signaling and production of IFN-γ and TNF-α after antigen-specific stimulation of CD4 T cells, we did not detect any significant effect on CTL lysis. In this respect, our findings are reminiscent of effects demonstrated by certain anti-CD45 mAbs showing a differential inhibition of NK cell lysis but not CTL lysis (41). We cannot rule out the possibility that effects

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on T cells as seen for pUL-11 might require cross-linking of sec49K; however, it should be noted that pUL6, another member of the CMV RL11 family, does not bind CD45 and does not show these effects on T cells (40), and other family members have demonstrated Fc receptor activity (42, 43). Thus, differential functions are to be expected from members containing the RL11/CR1 domain.

There is strong evidence that the extracellular portion of CD45, particularly the cysteine-rich and FNIII domains, proposed here as sec49K attachment sites, are under strong positive selection during evolution, and it has been hypothesized that CD45 evolution may be driven by pathogens (37, 44, 45). Taken together, the HCMV data and our studies on adenovirus 19a E3/49K lend further weight to this hypothesis.

It is intriguing that Ad19a initially generates a membrane-anchored-protein to eventually produce a cleaved secreted molecule. Thus, the smaller 12- to 13-kDa fragment, comprising the cytoplasmic tail, the TMD, and some 30 amino acids adjacent to the TMD, could have an additional function. In support of this idea, the small E3/49K-derived fragments exhibit a relatively long half-life of 5–6 h and may actually accumulate during the course of infection. We have evidence that the Yxxφ and LL motifs in the cytoplasmic tail are important for trafficking of E3/49K. Thus, these motifs and membrane integration may be required to direct E3/49K to the relevant processing compartment, or may point to an additional role of the C-terminal fragment in an intracellular compartment. Therefore, a dual function of E3/49K cannot be ruled out. It is not uncommon for cellular secreted proteins to be initially synthesized as membrane-anchored precursor forms, such as growth factors, cytokines, and amyloid precursor proteins. However, this is not a widely documented property of viruses. Instead, a number of viruses produce proteins in two alternative reading frames; for example, Ebola synthesizes both a secreted and a membrane-anchored form of E3/49K, the TMD, and some 30 amino acids adjacent to the TMD.

By demonstrating sec49K binding to CD45 and its functional effects on NK cells as well as T-cell signaling and cytokine production, we have discovered an immunomodulatory function for a species-specific E3 protein that is not present in the common species C Ads. It will be very interesting to determine whether this unique E3 function is common in species D Ads or limited to those species D Ads that cause EKC, a severe eye disease that is highly contagious and presents with typical subepithelial corneal infiltrates. This would implicate sec49K in EKC pathogenesis. All species D Ads have a 49K gene and also express a corresponding protein (26); however, the protein sequences and motifs of these 49K-like proteins differ considerably, particularly in the extracellular domain comprising sec49K (26). Thus, it is possible that not all E3/49K proteins are cleaved and produce a secreted product. Future studies requiring the generation of new antibodies against external domains of various E3/49K molecules of non-EKC Ads should clarify this important question.

Materials and Methods

Cloning and Molecular Biology Methods. Cloning of the Ad19a E3/49K gene into the pSG5 expression vector and generation of As49 cells constitutively expressing E3/49K (AS49K275) have been described previously (24). E3/49K transfectants of 293 cells were generated as described previously (47). Cells, Viruses, and Infection. The lung epithelial carcinoma cell lines A549 (ATCC CCL-185), 293 cells (ATCC CRL-1573), 293T cells (ATCC CRL-11268) and HeLa cells were maintained in DMEM as described previously (24). Unless noted otherwise, all lymphoid cell lines, Jurkat E6-1 and the CD45 “negative” variant J45.01 (29), J77 (SVT35), and K562 were maintained in RPMI, 10% (vol/vol) FCS, and penicillin/streptomycin (complete RPMI). The Jurkat derivative J-A5 render CD45 negative by expression of an antisense construct and CD45+ transfectants, CD45RO and CD45RABC, were cultured as described previously (30). Culture conditions of human NK cell lines are provided in SI Materials and Methods. Ad19aAd64, referred to here as Ad19a for consistency with earlier work, was grown and titrated essentially as described previously (26). Typically, cells were infected with 5–10 plaque forming units per cell for 1.5 h.

Transient Transfection. The 293T cells were transfected using polyethyleneimine (PEI) with 4 μg of LCA.1 and LCA.6 expression vectors encoding CD45RO and CD45RABC, respectively (31), or as a negative control with pBS-EcoRv, encoding the Ad2 E3 region (48). Cells were seeded at 24 h before transfection, incubated with the PEI-DNA mixture for 40 h, washed with PBS, harvested using EDTA, and then resuspended in FACS buffer. Each set of transfected cells was incubated with either BSA or sec49K as described below for the FACS binding assay and stained for sec49K and/or mAb 4D1 followed by Alexa Fluor 488-coupled goat anti-rat IgG (Invitrogen) and Tricolor (PE-Cy5)-coupled pan-CD45-specific mAb H130 (Invitrogen). Dual color plots from 10,000 cells were recorded.

MAbs and Antisera. The antisera R48 directed against the N-terminal domain of E3/49K was produced by immunizing rabbits with the 49K His-tag fusion protein (N49K-His) essentially as described previously (26, 47). N49K-His was also used as immunogen to raise rat mAbs. Two hybridoma clones (4D1 and 16E) that produce IgG2a Ig isotypes recognizing native and denatured E3/49K were cloned at least twice by limiting dilution. Unless stated otherwise, mAb 4D1 was used. Details of N49K-His production and purification and the generation of mAbs, along with a list of the other antibodies used, are provided in SI Materials and Methods.

Purification of SecE3/49K. Sec49K was purified from supernatants of cell lines A549K275 and 293K35 stably expressing the E3/49K protein. As negative controls, supernatants of untransfected A549 and 293 cells were processed in the same way using separate affinity columns (SI Materials and Methods).

FACS Binding Assay for sec49K. Typically 600,000 cells were incubated for 1 h at 4°C with purified sec49K (100–200 ng), 1–3 μL of 50-fold concentrated supernatant (Virusin with a molecular weight cutoff of 30K or 50K) or sec49K-containing supernatant from transfected cells in 200 μL. As negative controls, cells were incubated with medium alone, medium containing an equivalent amount of BSA and/or accordingly processed supernatant from untransfected A549 or 293 cells. After washing, cells were stained with 1 μg of 4D1 followed by Alexa Fluor 488-coupled goat anti-rat IgG (Invitrogen) essentially as described previously (17, 19, 47). Staining of PBMCs is described in SI Materials and Methods.

Metabolic Labeling, Immunoprecipitation, SDS/PAGE, Silver Staining, and Western Blot Analysis. Metabolic labeling of cells, immunoprecipitation, and SDS-PAGE were performed as described previously (13, 24). For silver staining of polyacrylamide gels a commercial kit (Biorad) was used. Proteins were blotted as described previously (49). E3/49K was detected with either R48 or mAb 4D1. Detection of phosphorylated ZAP-70 and ERK is described in detail in SI Materials and Methods.

NK Cell-Mediated Lysis. NK cell-mediated lysis was quantitated using NKL and K562 target cells in a standard 4-h chromium-51 (51Cr; GE Healthcare) release assay essentially as described previously (50). NKL cells (clone 234) were preincubated with purified sec49K or 10× concentrated supernatants of E3/49K+ cells or control supernatant (derived from A549, or BSA) for 30 min at room temperature and then added to 1Cr-labeled K562 target cells at varying E/T ratios.

Activation Markers on Fresh NK Cells. Human NK cells were purified from PBMCs using the Dynabeads Untouched Human NK Cell kit (Invitrogen) and rested overnight (2 × 106 cells/mL) in medium. Subsequently, NK cells were incubated overnight in 96-well flat-bottom plates coated with 10 μg/mL of anti-MHCII (W6/32) as IgG control; anti-2B4 (C1.7; Beckman Coulter) or anti-NKG2D (149810, R&D Systems) with or without sec49K (~100 ng/mL) as described previously (50, 51). Recombinant IL-2 (100 U/mL) was added to the indicated samples. After overnight incubation, cells were harvested and stained with anti-CD69PE (1:50; Biolegend) or anti-CD25FITC (1:25; BD Biosciences) and analyzed by FACS.

Antigen-Specific Stimulation of CD4 T Cells. First, 5 × 105/mL PBMCs from six CMV-seropositive healthy controls were preincubated for 30 min with or without purified sec49K (~250 ng/mL). Then 2 × 105 PBMCs were stimulated for 6 h in a total volume of 400 μL of RPMI-1% glucose-1% antibiotics-0.5% human serum albumin with 32 μL/mL CMV lyse and control lystate (Viron) or with 2.5 μg/mL s. aureus Enterotoxin B (Sigma-Alrich) in the presence of 1 μg/mL costimulatory antibodies anti-CD28 and anti-CD49d as described previously (52). After 2 h, 10 μg/mL brefeldin A was added to accumulate cytokines intracellularly. After 6 h, PBMCs were treated with 2 mM EDTA for 15 min, fixed
using 4% paraformaldehyde and stained as described previously (52) using antibodies against CD4, CD69, IFN-γ, TNF-α, and IL-2 (all from BD Biosciences). Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences) using FlowJo software (Tree Star). Antigen-specific T-cell frequencies were calculated after subtraction of reactive T cells observed after control stimulation.

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18. Filip LC, Mundy NI (2004) Evidence of molecular evolution driven by recombination events influencing tropism in a novel human adenovirus that causes epidemic keratocon-
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. HBP-ALL cells were cultured in complete RPMI, and CD45 retrotransfectants were grown as described previously (2). Jurkat and SKW-3 mutants Jup2.7 and SKW-3J2.7 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-92CI, as well as the NK cell-enriched line B3NK, 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 UCHLI (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 49K′NCO 5′ gaggcctagg-gatttcatactatcaatgctac 3′ and 49K′BAM 5′ ctagggatccggaattttagtttggaatcataatttc 3′. After digestion with restriction endonucleases Ncol 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-thiogalactopyranoside and was found exclusively in inclusion bodies (IBs).

For refolding, IBs were resuspended in 5 mL of IB homogenization (IBH) buffer [6 mM 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 imidazole. 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 antisera 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 mAb 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 μM. 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 Vivavoll 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-cm² plastic flasks to 80–90% confluence 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 1/10x volume of 10x phosphate buffered saline (PBS) PBS and 1/100x volume of 2% NaN3, as well as the protease inhibitors PMSF (8 μg/mL), trypsin inhibitor (10 μg/mL), and leupeptin (0.5 μg/mL), the medium was preclarified.
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; Di-anova) 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 Na3VO4. For immunoprecipitation, 5 μg of ZAP-70 antibody (G-4; Santa Cruz Biotechnology) was immobilized on protein A/G-Sepharose beads (Thermo Fisher 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-phosphoERK (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.


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 51Cr-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.