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Kaposi's Sarcoma-Associated Herpesvirus ORF54/dUTPase Downregulates a Ligand for the NK Activating Receptor NKp44

Alexis Spain Madrid and Don Ganem

Howard Hughes Medical Institute and GW Hooper Foundation Laboratories, University of California, San Francisco California, USA, and Novartis Institutes for Biomedical Research, Emeryville, California, USA

Kaposi's sarcoma-associated herpesvirus (KSHV) establishes long-term latent infection in humans and can cause cancers in endothelial and B cells. A functioning immune system is vital for restricting viral proliferation and preventing KSHV-dependent neoplasms. While natural killer (NK) lymphocytes are known to target virus-infected cells for destruction, their importance in the anti-KSHV immune response is not currently understood. Activating receptors on NK cells recognize ligands on target cells, including the uncharacterized ligand(s) for NKp44, termed NKp44L. Here we demonstrate that several NK ligands are affected when KSHV-infected cells are induced to enter the lytic program. We performed a screen of most of the known KSHV genes and found that the product of the ORF54 gene could downregulate NKp44L. The ORF54-encoded protein is a dUTPase; however, dUTPase activity is neither necessary nor sufficient for the downregulation of NKp44L. In addition, we find that ORF54 can also target proteins of the cytokine receptor family and the mechanism of downregulation involves perturbation of membrane protein trafficking. The ORF54-related proteins of other human herpesviruses do not possess this activity, suggesting that the KSHV homolog has evolved a novel immunoregulatory function and that the NKp44-NKp44L signaling pathway contributes to antiviral immunity.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is a human pathogen in the lymphotropic gammaherpesvirus family. Although KSHV generally establishes lifelong asymptomatic infections, some individuals develop one of several malignancies in response to infection, especially in the setting of immune compromise (19). Kaposi's sarcoma, the malignancy that lends the virus its name, is characterized by proliferating KSHV-infected endothelial cells, accompanied by enhanced infiltration of inflammatory cells and abnormal neoangiogenesis. There are several forms of Kaposi's sarcoma, but the most severe forms occur in AIDS patients and immunosuppressed transplant recipients, highlighting the importance of a functional immune system in the control of KSHV infection. In addition to endothelial cells, KSHV is also found in circulating B cells in infected people (1) and is linked to two B-cell malignancies: primary effusion lymphoma and multicentric Castleman's disease (11).

Like other herpesviruses, KSHV infections exist in multiple transcriptional states. The default state in cell culture systems is latency, in which only a small number of viral proteins are expressed and the viral genome is maintained in the nucleus. There is also a lytic program in which most viral genes are expressed, the genome is replicated, and viruses are packaged and exported. However, the idea of two distinct latent and lytic states may be an oversimplification, as there are cases (in cell culture, at least) in which genes from the lytic program are expressed without engendering the full viral lytic cycle. For example, when cells are infected *de novo*, there is a transient burst of expression of a specific subset of lytic genes (25). In addition, a small number of lytic viral mRNAs are packaged into the virion and presumably can be expressed upon virus entry into the cell even during a latent infection (3).

Natural killer (NK) cells are lymphocytes that can react quickly to an infection, without need for selection or expansion, and are thus considered a part of the innate rather than adaptive immune system (41). Once activated, they can kill a target cell via secretion of perforin and granzymes, as well as stimulate further immune responses by secretion of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and other cytokines. Unlike B or T cells, NK cells do not undergo chromosomal rearrangement to generate clonal antigen receptors. Instead, they express on their surfaces a wide array of germ line-encoded inhibitory and activating receptors, which are able to bind to cellular or pathogenic ligands on target cells. The human activating receptors include NKG2D, DNAM-1, 2B4, NKp80, and three proteins that have been termed natural cytotoxicity receptors: NKp30 (NCR3), NKp44 (NCR2), and NKp46 (NCR1) (37). The cellular ligands for NKp44 and NKp46 currently remain unidentified, though the presence of these ligands on the surface of cells can be visualized by flow cytometric staining with an Fc-fusion protein made from the soluble extracellular domain of the receptor. For example, the ligand for NKp44, termed NKp44L, can be visualized by labeling cells with NKp44-Fc.

It is known that both NK and cytotoxic ($CD8^+$) T cells are important mediators of antiviral immunity. Studies of many herpesvirus proteins (30) have demonstrated several different mechanisms by which these viruses evade recognition by $CD8^+$ T cells. Most of these involve decreasing the surface expression of major histocompatibility complex class I (MHC-I) and thus preventing the presentation of antigen to the T cell receptor. In KSHV, two related proteins, K3/MIR1 and K5/MIR2, have been shown to

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Address correspondence to Don Ganem, don.ganem@novartis.com.

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downregulate MHC-I molecules, as well as several other proteins involved in immune recognition (14, 15, 23, 36). However, this decrease in surface MHC-I is expected to result in increased susceptibility to NK cells, since MHC-I acts as a ligand for many inhibitory NK receptors (38). Inhibitory receptor binding to MHC-I prevents the killing of normal cells, which generally express MHC-I on their surface, while allowing killing of cancerous and infected cells, many of which have lower levels of MHC-I. Thus, it stands to reason that viruses that decrease surface MHC-I levels might also attempt to evade NK cell killing. Indeed, human cytomegalovirus (HCMV), which expresses many proteins that downregulate MHC-I, also encodes several products that interfere with NK cell recognition (13, 16, 46).

These observations raise the question of whether KSHV has evolved mechanisms for evading NK cells. One recent study showed that the well-characterized viral ubiquitin ligase MIR2 could partially inhibit NK cell recognition by downregulating MICB and some alleles of MICA, both of which are ligands for NKG2D, as well as AICL (CLEC2B), a ligand for NKp80 (45). Another study showed that MIR2 downregulation of the adhesion molecule ICAM-1 could diminish NK recognition of a target cell (22). In addition, the KSHV microRNA (miRNA) mirK12-7 can decrease the levels of MICB mRNA (40). However, no study has examined global changes in NK activating ligand expression in response to KSHV infection or looked for novel KSHV-encoded NK modulators in a systematic fashion.

In this study, we show that several NK activating ligands are up- or downregulated during lytic KSHV infection, and one ligand that we chose to study in more detail, NKp44L, is also downregulated transiently upon *de novo* infection. By screening each of the KSHV genes individually, we identified one viral gene, ORF54, which is capable of downregulating NKp44L. ORF54 is expressed as a delayed-early gene during lytic reactivation. Interestingly, it is also highly expressed during the lytic burst of a *de novo* infection (25) and its mRNA is present in the virion (3). We find that ORF54 has substantial specificity for NKp44L, in that no other NK activating ligand is downregulated when it is expressed. However, ORF54 can also downregulate several cytokine receptors, including IFNAR1, gp130, interleukin-23 receptor (IL-23R), and IFNGR1. ORF54 appears to function by altering the subcellular localization of NKp44L molecules, relocating them from the cell surface to intracellular compartments.

ORF54 is annotated as a dUTP pyrophosphatase (dUTPase), a ubiquitous class of enzyme involved in nucleotide metabolism. Interestingly, we find that dUTPases from two other herpesviruses, Epstein-Barr virus (EBV) and herpes simplex virus 1 (HSV-1), lack immune-evasion activity, though a recent study has shown that ORF54 from the related mouse gammaherpesvirus mouse herpesvirus 68 (MHV-68) is capable of downregulating IFNAR1 (27). Thus, KSHV ORF54 appears to have evolved a novel immunoregulatory function, possibly to protect infected cells from NK cells after a *de novo* infection and before the virus has a chance to establish latency or, alternatively, to prevent lytic cells from being killed before virus can be released.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney 293 (293), human foreskin fibroblast (HFF), HFF.219, SLK, and iSLK.219 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% serum and 1% penicillin-streptomycin. iSLK.219 and HFF.219 cells were grown in the presence of 10 μg/ml puromycin (Invivogen) to select for cells containing the recombinant virus rKSHV.219. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown in EBM-2 medium supplemented with an EGM-2 bullet kit (Lonza). Transfections into 293 cells were performed in 6-well plates with 2 μg viral plasmid DNA, 0.1 μg pEGFP-C1 plasmid DNA, and 8 μl Fugene 6 (Roche) per well. HFF.219 cells were received from Jeffrey Vieira (University of Washington).

KSHV infection and reactivation. iSLK.219 cells were lytically induced by growth in the presence of 1 μg/ml doxycycline (Clontech) for 48 h. HFF.219 cells were lytically induced by growth in the presence of 9 mM sodium butyrate (EMD Chemical) plus adenovirus encoding replication and transcription activator (Ad-RTA) for 72 h. KSHV stocks were prepared from BCBL-1 cells as previously described (4). HUVECs were infected with enough BCBL-1-derived virus to give approximately 80% or more infected cells, by incubation in virus-containing medium together with 8 μg/ml Polybrene (Millipore) for 6 h. Cells were washed twice with phosphate-buffered saline (PBS) and induced to enter the lytic phase by superinfection with Ad-RTA for 40 h. For the *de novo* infection time course, HFF cells were infected by incubation in BCBL-1-derived virus-containing medium for 6 h (except the cells harvested at 2 h postinfection), and then cells were washed twice with PBS and grown in plain medium for the remainder of the time course.

Plasmids. Construction of the KSHV genomic library has been described previously (20). pEGFP-C1 was used in cotransfection experiments (Clontech). Flag-tagged dUTPases were cloned into pCDNA3 (Life Technologies) using EcoRI/NotI. ORF54 was amplified from the pLPCX-ORF54 library construct using primers 5′-GAATTCATGAACAA CCGCCCCAGG and 3′-GCGGCCGCTTACTTGTCGTCGTCGTCCTT GTAGTCAAACCCAGACGACCCCAG. EBV dUTPase was amplified from total DNA prepared from Raji cells using primers 5′-GAATTCATG GAGGCCTGTCCACAC and 3′-GCGGCCGCTTACTTGTCGTCGTCG TCCTTGTAGTCTTGACCCGAGGATCCAAA. HSV-1 was amplified from DNA purified from HSV stock 17+ (Margolis lab) using primers 5′-GAATTCATGAGTCAGTGGGGATCC and 3′-GCGGCCGCTTACT TGTCGTCGTCGTCCTTGTAGTCAATACCGGTAGAGCCAAA.

Flow cytometry. Cultured cells were washed with PBS and then lifted with PBS-based cell dissociation buffer (Invitrogen), washed in fluorescence-activated cell sorter buffer (PBS, 2% serum, 2 mM EDTA) on ice, and stained for 1 to 3 h with primary antibody and 1 to 2 h with secondary antibody. Primary antibodies were MHC-I (w6/32-allophycocyanin [APC]; Biolegend), IL-23R–APC (R&D Systems), IFNAR1 (R&D Systems), CD155 (R&D Systems), ULBP3 (R&D Systems), CD112 (BD Pharmingen), MICA-APC (R&D Systems), MICB-APC (R&D Systems), ULBP2-APC (R&D Systems), gp130-APC (R&D Systems), IFN-γR1 (eBioscience), epidermal growth factor receptor (EGFR; Abcam), beta-1 integrin (GeneTex), and NKp44-Fc (R&D Systems). Isotype control antibodies were IgG Fc fragment (Jackson ImmunoResearch) and mouse isotype controls (Biolegend). Secondary antibodies were anti-mouse APC and anti-human APC (Jackson ImmunoResearch). Intracellular flow cytometry was performed using IC fixation buffer and permeabilization buffer (eBioscience). For the screen analysis, the NKp44L mean fluorescence intensity (MFI) was calculated in FlowJo software, background isotype control staining was subtracted, and values were plotted as a percentage of the vector-alone signal.

Lysate preparation and Western blotting. Cultured cells were washed with PBS and then lifted with PBS-based cell dissociation buffer (Invitrogen) and pelleted. Pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate) containing Halt protease and phosphatase inhibitor cocktail (Pierce), incubated for 15 min on ice, and spun for 15 min at top speed at 4°C. The soluble portion was collected, and total protein concentration was determined by Bradford assay (Bio-Rad). Equal amounts of total protein were loaded onto a polyacrylamide gel and transferred onto a nitrocellulose membrane. Mem-

branes were blotted with antibodies against Flag (Sigma) and pyruvate kinase (Abcam).

Flag immunoprecipitation and dUTPase assay. 293 cells were transiently transfected with Flag-tagged dUTPase plasmids. Whole-cell lysates were prepared at 48 h posttransfection in RIPA buffer as described in the section above. Immunoprecipitations were performed with 4.2 mg total protein in clarified lysate and 175 μl of a 50% slurry of anti-Flag M2 affinity gel (Sigma) for 2 h at 4°C. The matrix was washed twice with RIPA buffer and four times with Tris-buffered saline. Proteins were eluted by incubation with 30 μg 3× Flag peptide (Sigma) for 2 h at 4°C. Glycerol was added to a final concentration of 10% for storage at −20°C. Five microliters of eluted protein was incubated with 4 μl of 400 μM dUTP (Promega) and 9 μl of 2× reaction mix (100 mM Tris, pH 7.5, 20 mM $MgCl_2$, 20 mM dithiothreitol, 200 μg bovine serum albumin [BSA]) for 0, 3, 6, or 24 h. Reactions were stopped in liquid nitrogen. PCR-based readout reactions were performed using GoTaq polymerase (Promega) by a method adapted from a previous study (27). PCRs were run without dTTP; instead, the dUTPase reaction mixtures were added directly to the PCRs so that the input dUTP would be limiting and, therefore, changes in PCR product levels would correlate with changes in dUTP levels. In a 25-μl PCR mixture, 7 μl of the dUTPase reaction mix was added. If there was no dUTP conversion, the maximum amount of dUTP in the PCR mixture would be 25 μM. In control lanes, dUTP was added at a 0, 6.25, 12.5, or 25 μM final concentration to show that band intensity depends on dUTP concentration.

Immunofluorescence. iSLK.219 cells were cultured and stained in glass chamber slides (Electron Microscopy Sciences). At 48 h postinfection, cells were washed and fixed in 2% paraformaldehyde (Thermo Fisher). Cells were washed with PBS and then incubated for 10 min in permeabilization buffer (PBS, 1% Triton X-100, 0.1% sodium citrate) and then 30 min in blocking buffer (PBS, 1% Triton X-100, 0.5% Tween 20, 3% BSA). Cells were stained in blocking buffer with NKp44-Fc (R&D Systems). The secondary antibody used was anti-human DyLight 647 (Jackson ImmunoResearch). Stained cells were mounted with coverslips in Vectashield mounting medium (Vector Labs). Stained cells were imaged at room temperature using confocal laser scanning microscopy with an inverted Leica SP5 system (DM1 6000 CS; Leica), with a ×63 1.4-numerical-aperture oil-immersion objective and argon (488-nm) and HeNe (543- and 633-nm) lasers. Images were acquired using Leica software, LAS AF SP5, and processed using Adobe Photoshop, where brightness was adjusted.

RESULTS

NKp44L surface levels are decreased during lytic and *de novo* infection. In order to determine the extent to which KSHV tries to subvert NK cell recognition, we monitored the surface expression of most of the known cellular NK activating ligands at various stages of an infection. First, we looked at the effects of viral lytic induction in the SLK cell line latently infected with the marked KSHV rKSHV.219. This recombinant virus expresses green fluorescent protein (GFP) from a constitutive elongation factor 1α promoter and red fluorescent protein (RFP) from the viral PAN promoter. PAN is a lytic noncoding RNA whose expression is activated by the expression of the viral gene RTA (for replication and transcription activator) (47). RTA is the viral transcription factor that controls induction of lytic gene expression; forced expression of this gene in latently infected cells triggers the switch to lytic replication (35, 43). This inducible cell line, termed iSLK.219, also contains an RTA transgene under the control of a doxycycline-inducible promoter (39). Thus, all infected cells are GFP positive; cells treated with doxycycline are triggered to enter the lytic cycle and as a result also express RFP, making this cell line a good tool for inducing and analyzing the KSHV lytic cycle.

iSLK.219 cells were grown in culture, and in half of the cells, RTA expression was induced with doxycycline. After 48 h of induction, cells were harvested and stained with a panel of antibodies against the following NK activating ligands: MICA, MICB, ULBP2, ULBP3, CD112 (PVRL2), CD155 (PVR), and NKp44L (cells were stained with NKp44-Fc to monitor NKp44L). Several other ligands (ULBP1, NKp30L, and NKp46L) were not expressed on these cells (data not shown) and therefore could not be assayed here. As a control, we also stained for MHC-I, which is known to be downregulated during lytic KSHV infection. The cells were subjected to flow cytometric analysis, and surface levels of each protein were compared between latently infected and RFP-positive lytic cells.

As expected, MHC-I was strongly downregulated from the surface of lytic cells, indicating that genes from the lytic program were indeed being expressed (Fig. 1A). Two ligands (ULBP3 and CD112) were unchanged upon lytic induction, while one ligand, MICB, was induced in lytic cells. We also found that in the lytic cells three NK activating ligands, ULBP2, NKp44L, and CD155, were downregulated (Fig. 1A). One ligand, MICA, displayed a unique pattern: its levels were unchanged in the RFP-positive lytic cells (Fig. 1A) but were downregulated in RFP-negative cells in the same doxycycline-treated population (data not shown). An uninfected, control cell line with RTA under the control of a doxycycline-inducible promoter but no KSHV was also stained, and it was determined that RTA expression in the absence of KSHV had no effect on any NK ligand expression (data not shown).

The results presented above were obtained from cells that were selected to stably maintain KSHV, which models the state of long-term latent infection. Next, we wanted to see how these same ligands behaved when cells were infected *de novo*. Primary HUVECs were infected with purified wild-type virus derived from BCBL1 cells, and half were superinfected with an adenovirus expressing RTA (Ad-RTA) to induce lytic expression. At 48 h post-KSHV infection, the cells were harvested, stained, and subjected to flow cytometric analysis as described above. As with the iSLK cells, we found that MHC-I was strongly downregulated during lytic infection of HUVECs compared to that of either latently infected or uninfected cells (Fig. 1B). Again, two ligands (ULBP3 and CD112) were completely unaffected by KSHV infection. Three ligands (MICA, ULBP2, and CD155) were downregulated only in lytic cells, and two ligands (NKp44L and MICB) were downregulated both in lytic cells and in HUVECs that were infected with KSHV but not induced with Ad-RTA. This nonlytic downregulation of MICB may be caused by miRK12-7, which is expressed during latency. Ad-RTA treatment in the absence of KSHV had no substantial effects on the proteins studied, with the exception of MICB, which was downregulated in response to Ad-RTA (data not shown).

Because NKp44L was downregulated during *de novo* infection, we speculated that this might be due to the lytic burst of gene expression at early times after *de novo* infection that has been observed in HMVECs and HFFs by Krishnan et al. (25). If this were the case, it would be expected that downregulation of NKp44L would be transient, as the early expression of lytic genes disappears over time. Therefore, we infected HFFs with KSHV and monitored NKp44L surface expression at various times after infection. We found that NKp44L expression did indeed go down transiently at early times after infection (Fig. 1C). The maximum decrease was seen after 24 h. As predicted, NKp44L surface expression returned to normal levels by 48 h postinfection. A similarly

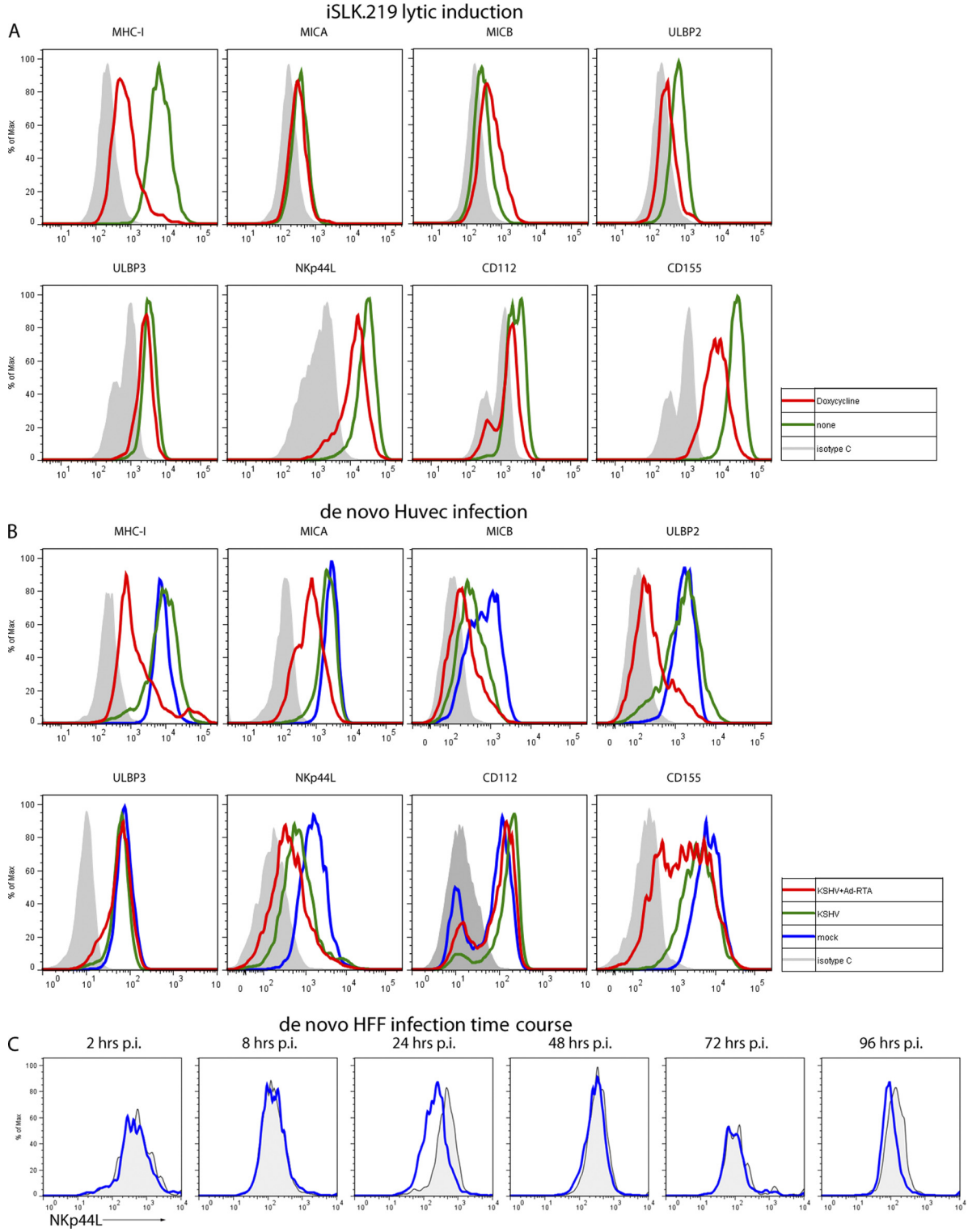

FIG 1 NK activating ligand surface expression during KSHV infection. (A) Stably infected iSLK.219 cells were either left untreated (green lines) or treated with 1 μg/ml doxycycline (red lines) for 48 h to induce ectopic expression of the KSHV lytic switch protein RTA. Cells were stained with antibodies against NK activating ligands and analyzed by flow cytometry. Doxycycline-treated lytic cells denoted by the red line have been gated on the RFP-positive population. Isotype control (isotype C) staining is shown in gray-filled histograms. Doxycycline-induced RTA in cells without KSHV had no effects on any of the proteins tested (data not shown). (B) HUVECs were infected with KSHV (green lines), and half were induced to become lytic with Ad-RTA (red lines). These cells were compared to uninfected cells (blue lines) or cells treated with Ad-RTA alone (data not shown). Ad-RTA treatment on uninfected cells had no effects on the proteins tested, with the exception of MICB (data not shown). Isotype control staining is shown in gray-filled histograms. (C) HFF cells were infected with KSHV for the indicated times. Cells at each time point were harvested and stained with NKp44-Fc. Infected cells (blue lines) were compared to mock-infected cells (gray filled histograms). p.i., postinfection.

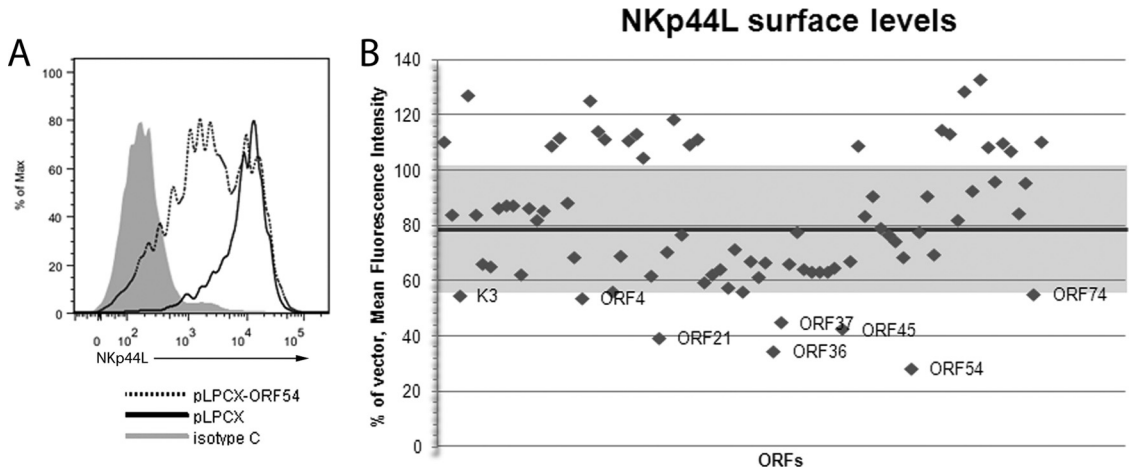

FIG 2 ORF54 was identified in a screen for NKp44L downregulation. A plasmid library containing most of the known KSHV genes was transfected individually into 293 cells together with a plasmid expressing GFP. At 48 h posttransfection, the cells were stained with NKp44-Fc and analyzed by flow cytometry. (A) Histogram comparing the NKp44L levels of vector alone (solid line) versus ORF54 (dotted line) in GFP-positive cells. Isotype control staining is shown in gray. (B) Dot plot depicting the NKp44L mean fluorescence intensity of the screened KSHV genes, including ORF54, relative to a vector control. The median value of 79 is depicted by the dark line, and the shaded area denotes 1 standard deviation above and below the median (SD = 24). The eight genes that fell below 1 SD in the primary screen are labeled.

transient (but weaker) downregulation was also seen in HUVECs infected *de novo* with KSHV derived from a bacmid, BAC16 (see Fig. S2 in the supplemental material). These results suggest that NKp44L downregulation is due to transient expression of a lytic gene product early after *de novo* infection.

A screen for NKp44L downregulation identifies ORF54. We were intrigued by the observation that the activating ligand NKp44L was downregulated during KSHV infection, being affected both after lytic induction and transiently after a *de novo* infection. Only a small percentage of viral genes are thought to be expressed during this burst of lytic gene expression, and many of those are involved in immune evasion (25). To identify which protein was responsible for this activity, we used a plasmid library that contains cDNAs for most of the known KSHV open reading frames (ORFs), as well as some viral noncoding RNAs. The following is the full list of genes screened: K1, ORF4, ORF7, ORF8, ORF10, ORF11, K2, ORF2, K3, ORF70, K4, K4.1, K4.2, K5, K6, K7, PAN, ORF16, ORF17, ORF17.5 (AP), ORF18, ORF19, ORF20, ORF21, ORF22, ORF23, ORF24, ORF26, ORF27, ORF28, ORF29b, ORF48/29b, ORF30, ORF31, ORF32, ORF33, ORF29a, ORF34, ORF35, ORF36, ORF37, ORF38, ORF39, ORF40, ORF41, ORF40/41 (PAF), ORF42, ORF43, ORF44, ORF45, ORF46, ORF47, ORF48, ORF50, ORF50 (antisense-short [as-s]), ORF49, K8, K8.1a, K8.1b, ORF52, ORF53, ORF54, ORF55, ORF56, ORF57, K9 (viral IRF1), K10/10.1 (viral IRF4), K10.5/11 (viral IRF3), K11 (viral IRF2), ORF58, ORF59, ORF60, ORF61, ORF62, ORF63, ORF65, ORF66, ORF67, ORF68, ORF69, K12, kaposin A, kaposin B, kaposin C, miRK1-5, miRK6,11,7, miR8, miR9, K13, ORF72, ORF73, K14, ORF74, ORF75, and K15. Each of these plasmids was transiently transfected together with a plasmid expressing GFP into 293 cells, and after 48 h, cells were harvested and stained with NKp44-Fc to visualize surface levels of NKp44L by flow cytometry. The resulting histograms for each viral gene were analyzed by looking for genes that substantially shifted the curve for NKp44L staining relative to empty vector in GFP-positive (i.e., transfected) cells.

Of all of the ORFs tested, ORF54 produced the most dramatic change in NKp44L surface levels (Fig. 2A and B). In an overlaid histogram, the ORF54 curve was shifted strongly to the left compared to the vector-alone curve (Fig. 2A). No other ORF displayed such a strong shift (data not shown). Next, we obtained the MFI for each sample in the screen. Since the screen was performed in batches, for each set of genes, the isotype control value was subtracted from each individual MFI and then expression of each gene was normalized to that of the vector control from that experimental batch. In Fig. 2B, each gene in the screen is represented as one point in a dot plot. We found that in our primary screen, five genes fell below 1 standard deviation from the median MFI, with ORF54 having the lowest value. We performed secondary screening where we repeated the transfection and stain with the eight plasmids having the lowest NKp44L level in the original screen (labeled genes in Fig. 2B). Only ORF54 reproduced the downregulation (data not shown). Interestingly, ORF54 was among the most highly expressed genes in the lytic burst (25), and its RNA is present in the virion (3), making it a good candidate for the transient downregulation of NKp44L after *de novo* infection. Thus, we chose to pursue ORF54 as the most likely candidate for the KSHV activity that causes NKp44L downregulation.

NKp44L downregulation is not due to the dUTPase activity of ORF54. When KSHV was originally sequenced, ORF54 was annotated as a dUTPase by virtue of its sequence homology to the dUTPases that are present in the genomes of all herpesviruses. It has also been reported to harbor a functional dUTPase activity (24). Accordingly, we addressed the possibility that the NKp44L downregulation activity was linked to the dUTPase activity. If this were the case, overexpression of other functional dUTPases would also be expected to cause downregulation of NKp44L. Therefore, we transiently transfected 293 cells with plasmids encoding Flag-tagged ORF54 homologs from two other human herpesviruses, EBV (BLLF3), and HSV-1 (UL50), both of which have been shown to possess dUTPase activity (5, 7, 44). Cells were harvested after 48 h, and surface expression of NKp44L was compared to

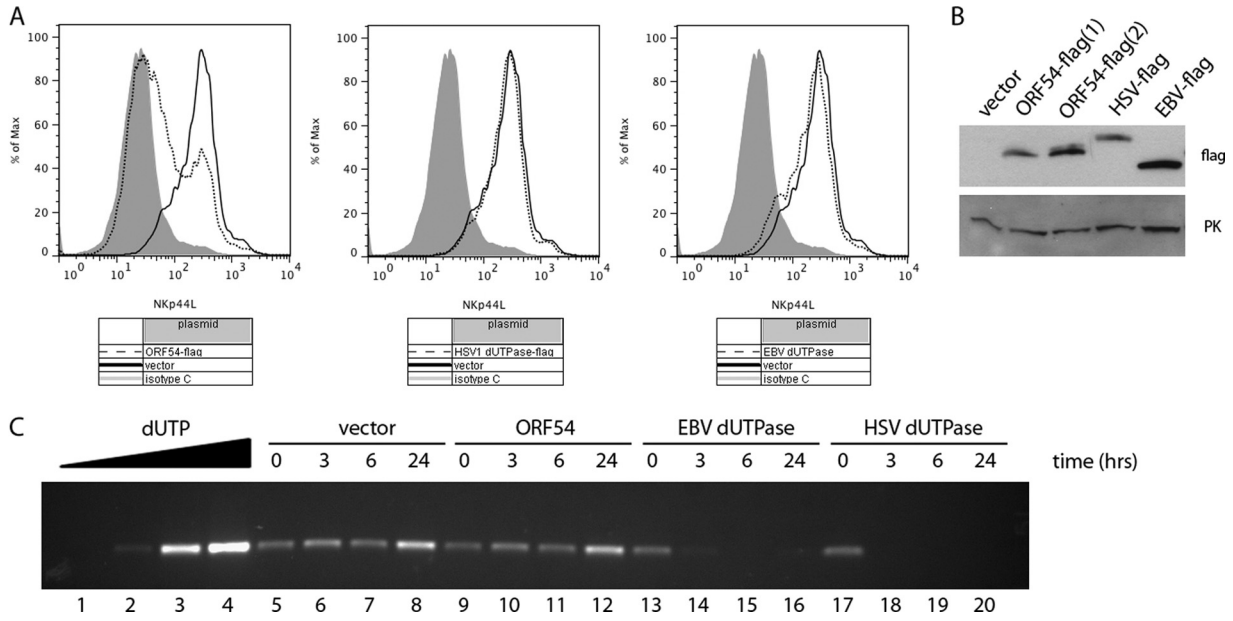

FIG 3 NKp44L downregulation is independent of dUTPase activity. Flag-tagged versions of the dUTPases from KSHV, HSV-1, and EBV were transiently transfected into 293 cells. (A) At 48 h posttransfection, cells were stained with NKp44-Fc and analyzed by flow cytometry. Cells transfected with each dUTPase (dotted lines) were compared to empty vector control (solid lines). Isotype control staining is shown in gray-filled histograms. Results are representative of three individual experiments. (B) Lysates were prepared from the same cells used for panel A, equal amounts of total protein were loaded onto a gel, and the membrane was blotted with an anti-Flag antibody, as well as an anti-pyruvate kinase (PK) antibody as a loading control. (C) Flag-tagged dUTPases were immunoprecipitated from lysates and eluted with Flag peptide. dUTPase activity was measured by incubating this purified protein with dUTP for 0, 3, 6, or 24 h at 37°C. The resulting mixture was used in place of dTTP in a PCR to measure the amount of remaining dUTP in the reaction. As a control to show dUTP concentration dependence, various amounts of pure dUTP were added to the PCR mixture: 0 (lane 1), 6.25 μM (lane 2), 12.5 μM (lane 3), or 25 μM (lane 4). Time courses of dUTPase assays for vector control (lanes 5 to 8), ORF54 (lanes 9 to 12), EBV dUTPase (lanes 13 to 16), and HSV-1 dUTPase (lanes 17 to 20).

that of cells transfected with empty vector. Interestingly, KSHV ORF54 downregulated NKp44L, while neither of the other active dUTPases had any effect (Fig. 3A). This difference in activity cannot be explained by protein expression differences because, as shown in Fig. 3B, all three proteins were consistently expressed and the downregulation activity did not correlate with protein levels.

Although the previous result suggested that NKp44L downregulation was not the result of dUTPase activity, we considered the possibility that ORF54 could have a stronger dUTPase activity than the other two homologs and that this higher activity could still be responsible for the NKp44L downregulation. In order to address this, we used the same Flag-tagged constructs transiently transfected into 293 cells to compare the dUTPase activities of the three homologs. At 48 h after transfection, dUTPases were immunoprecipitated and incubated with dUTP for various times to allow the conversion of dUTP into dUMP, and the loss of dUTP was measured indirectly using a PCR-based readout.

We found that the dUTPase activity of ORF54 was in fact much weaker than that of the orthologs from EBV and HSV-1. We were able to see strong dUTPase activity with the HSV-1 and EBV dUTPases after 3 h of dUTP incubation (Fig. 3C, lanes 13 to 20). However, we did not observe activity from the KSHV dUTPase even after 24 h of incubation with dUTP (Fig. 3C, lanes 9 to 12). Our experimental data contradict those from a study previously published by others using a different assay for the activity (24). Differences in assay sensitivity might explain this discrepancy; at a minimum, however, we can say that the dUTPase activity of KSHV ORF54 must be substantially lower than that of its HSV and EBV homologs.

To further address the role of the dUTPase activity, we mutated ORF54 residue 104 from aspartic acid to asparagine (D104N). The equivalent mutation was shown to abolish dUTPase activity in the *Escherichia coli* and human endogenous retrovirus K dUTPases (2, 21). As expected (given that we were unable to detect any activity from the wild-type protein), the D104N mutant lacked detectable dUTPase activity. Nonetheless, we found that the mutant version was still able to perform NKp44L downregulation, albeit to a slightly lesser degree (see Fig. S1A and B in the supplemental material) and with more variability than the wild-type protein (see Fig. S1B in the supplemental material). The slightly reduced activity of the mutant version is not due to underexpression or instability (see Fig. S1C in the supplemental material); perhaps its subtle phenotype reflects mild defects in proper folding of the nascent chain.

We conclude that the KSHV dUTPase ORF54 possesses an immunoregulatory activity that is distinct from its (weak or absent) dUTPase activity. In support of this conclusion, the ORF54 ortholog of MHV-68 was recently shown to have an immune-evasion activity that was independent of its dUTPase activity as well (27).

ORF54 can also downregulate cytokine receptors. Since ORF54 can downregulate NKp44L, we wondered whether this was a specific effect or whether ORF54 simply has broad effects on host membrane protein expression or trafficking. First, we stained cells transiently transfected with ORF54 for other NK activating ligands: MICA, MICB, ULBP1, ULBP2, ULBP3, CD112, and CD155. We found that none of these ligands had substantially altered surface expression as a result of ORF54 (Fig. 4A). We went on to stain cells for other immune-related molecules: MHC-I,

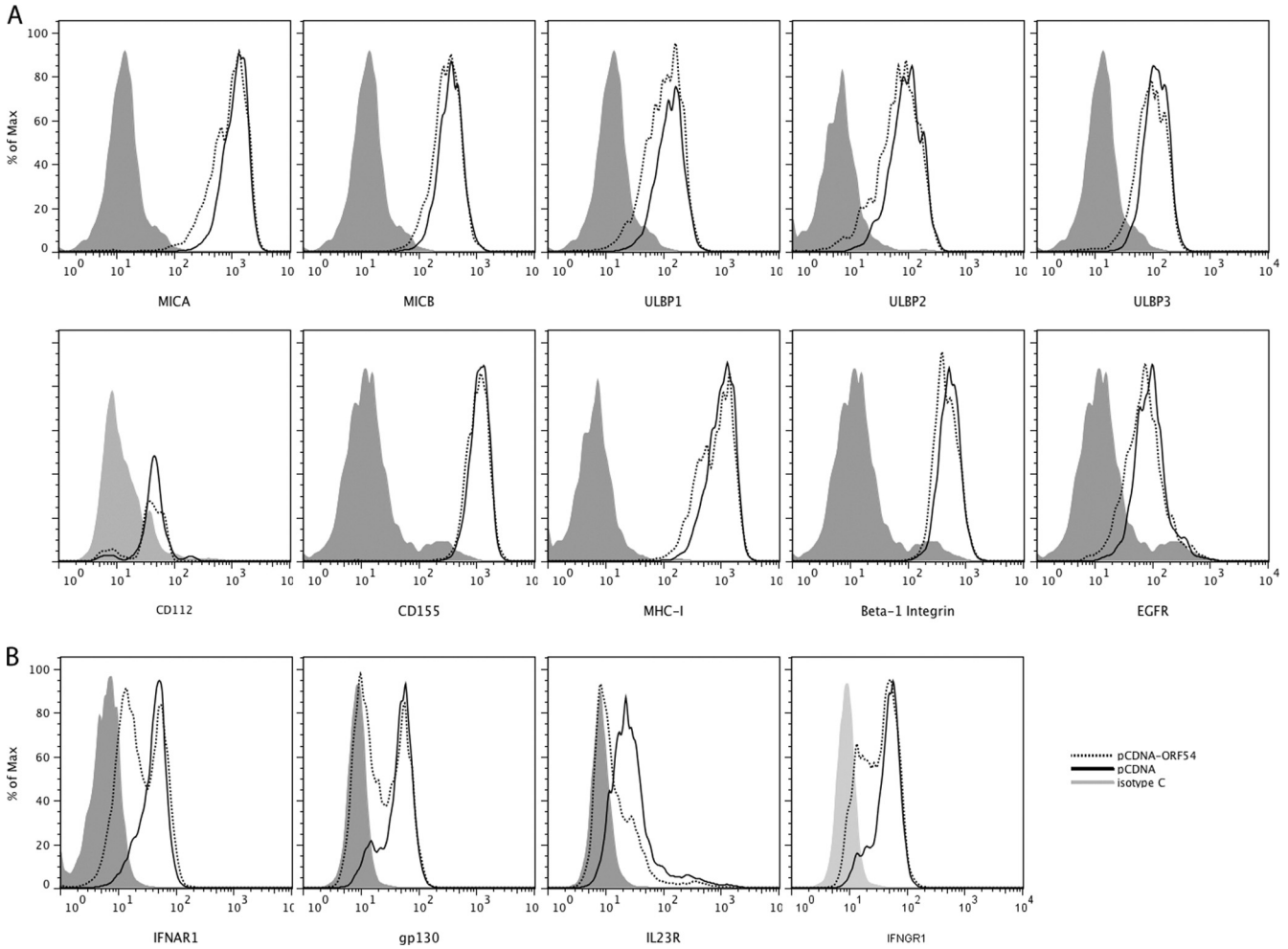

FIG 4 ORF54 specificity. 293 cells were transiently transfected with either ORF54 plasmid (dotted lines) or empty vector (solid lines) and stained with a panel of antibodies. (A) Flow cytometric cell surface staining of proteins that were unaffected by ORF54 expression (MICA, MICB, ULBP1, ULBP2, ULBP3, CD112, CD155, MHC-I, beta-1 integrin, and EGFR); (B) flow cytometric staining of proteins whose levels decreased upon ORF54 expression (IFNAR1, gp130, IL-23R, and IFNGR1).

beta-1 integrin, EGFR, and IFNAR1. For most of these proteins, we again saw no significant changes due to ORF54 (Fig. 4A). However, IFNAR1 was partially downregulated in ORF54-expressing cells (Fig. 4B). Therefore, we stained cells for other cytokine receptors that are expressed on 293 cells: gp130, IL-23R, and IFNGR1. Interestingly, all of these proteins were downregulated by ORF54 to various degrees (Fig. 4B). IL-23R was the most strongly affected, and IFNGR1 was the most weakly affected. Because the molecular identity of the NKp44 ligand(s) is not currently known, we cannot yet say whether NKp44L shares some property with this family of cytokine receptors that makes them all targets of ORF54. Recently, MHV-68 ORF54 was shown to decrease the expression of IFNAR1 as well, hinting that there could be an evolutionarily conserved activity in this protein family (27).

IL-23R is downregulated during lytic infection. During transient transfection of 293 cells, ORF54 was able to downregulate some cytokine receptors in addition to NKp44L. In order to link this observed ORF54 activity with full viral infection, we tested whether any of these proteins were also affected after lytic viral induction. We found that gp130 was very strongly downregulated in lytic infection but that many viral proteins could cause this, including viral IL-6 (data not shown), so we looked more closely at IL-23R. IL-23R is not expressed on the surface of HUVECs or SLK cells, so we used an HFF cell line latently infected with rKSHV.219. We found that when latently infected cells were lytically reactivated with Ad-RTA and valproic acid, they strongly downregulated IL-23R from the cell surface (Fig. 5A), while uninfected HFFs treated with inducers were unaffected (data not shown), thus confirming the prediction that we made based on the activity of ORF54 alone. This result implied that ORF54 is downregulating IL-23R during lytic infection, but we investigated whether this cytokine receptor was capable of being targeted by any other KSHV proteins besides ORF54.

We performed a screen for IL-23R downregulation identical to the screen for NKp44L downregulation described earlier. A set of representative genes from the screen is shown (Fig. 5B). As we observed with NKp44L, ORF54 showed the strongest IL-23R downregulation activity, and no other KSHV gene was found to significantly alter IL-23R surface expression. While circumstantial, these data support the model that ORF54 ex-

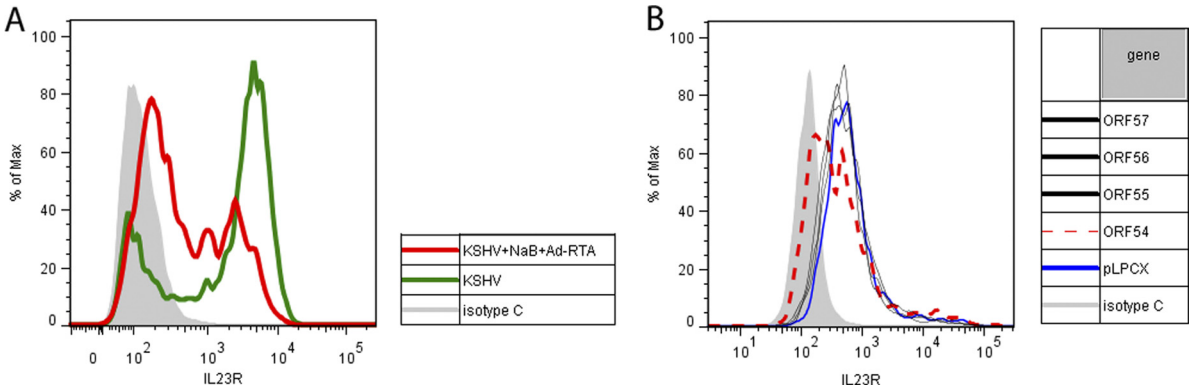

FIG 5 IL-23R is downregulated by lytic infection and ORF54. (A) HFF cells stably infected with the recombinant rKSHV.219 were either left untreated (green line) or treated with 9 mM sodium butyrate (NaB) plus Ad-RTA (red line) for 72 h to induce lytic reactivation. Cells were stained with an antibody against IL-23R and analyzed by flow cytometry. Lytic cells denoted by the red line have been gated on the RFP-positive population. Isotype control staining is shown in gray-filled histograms. Uninfected HFF cells treated with Ad-RTA and/or NaB did not have altered IL-23R levels (data not shown). (B) A plasmid library containing most of the known KSHV genes was transfected individually into 293 cells together with a plasmid expressing GFP. At 48 h posttransfection, the cells were stained for IL-23R and analyzed by flow cytometry. Histogram comparing the IL-23R levels of vector alone (blue line) versus ORF54 (red dotted line) and several other representative ORFs in GFP-positive cells. Isotype control staining is shown in gray.

hibits a novel immune-evasion activity during KSHV lytic infection.

NKp44L becomes mislocalized during lytic infection or ORF54 expression. Lytic infection and ORF54 expression both lower NKp44L levels on the cell surface. In order to understand what is happening to NKp44L, we first performed parallel stains on permeabilized and unpermeabilized infected iSLK.219 cells. Whereas our previous stains on unpermeabilized cells showed that there was downregulation at the cell surface upon lytic induction, staining of permeabilized cells should measure total cellular protein levels, both surface and intracellular. As before, we found that NKp44L was downregulated from the cell surface (Fig. 6A). However, when we measured total cellular protein levels by intracellular flow cytometry, we saw little or no change. Next, we examined 293 cells transiently transfected with ORF54 in a similar fashion. As in SLK cells, we found that NKp44L was downregulated from the cell surface but total cellular levels were unchanged (Fig. 6B). These results suggest that in response to KSHV lytic infection or ORF54 expression, total NKp44L protein levels are unaffected but that the subcellular trafficking or localization is altered in some way.

We expanded on the flow cytometric data presented above by visualizing the subcellular localization of NKp44L during lytic infection. We performed confocal fluorescence microscopy to compare latently infected and lytically infected iSLK.219 cells stained with NKp44-Fc to visualize NKp44L. Latently infected cells displayed a staining pattern in which NKp44L was found on the cell periphery as well as in a lacelike cytoplasmic network but was excluded from the nucleus (Fig. 6C). Uninfected SLK cells had an identical staining pattern (data not shown). In contrast, in lytic cells induced with doxycycline, we found that NKp44L localization was dramatically altered, becoming concentrated in perinuclear regions and with less stringent nuclear exclusion (Fig. 6C). Staining with the endoplasmic reticulum (ER) markers calreticulin (see Fig. S3 in the supplemental material) or calnexin (data not shown) showed partial overlap with NKp44L, suggesting that at least some of the ligand is localized to the ER or ER-derived structures. The localization changes were most apparent in RFP-positive cells. This suggests that NKp44L surface downregulation is due to altered trafficking of the molecule, resulting in mislocalization to both the nucleus and a perinuclear compartment, presumably derived from the ER.

DISCUSSION

The fact that KSHV reactivates and causes tumors in immunocompromised individuals underscores the importance of the immune system in KSHV control. However, the narrow host range of KSHV has made it difficult to study the regulation of the host-virus interaction by the human immune system *in vivo*. As a result, we know relatively little about the determinants of host immune control of the virus. However, from studies of putative immunoregulatory genes in cell culture systems, we have been able to make some indirect inferences about such determinants. For example, the presence of potent regulators of MHC-I display in KSHV suggests a critical role for cytotoxic T lymphocytes in immune control of KSHV. Similarly, the fact that MIR2 and a viral miRNA can also modulate NK cell recognition molecules suggests that NK cells may also be an important part of immune defense against KSHV.

Here, we have used several cell culture infection models to investigate the extent to which KSHV can evade an NK cell response. We chose to monitor NK activating ligands, as downregulation of these ligands would theoretically lessen NK recognition of an infected cell, and this strategy has been demonstrated by other viruses (13, 16, 26, 28, 32–34, 42, 46). The known *in vivo* targets of KSHV infection are B cells and endothelial cells, making these the obvious cell types in which to perform our experiments. However, it proved difficult to study NK ligands in infected B cell lines, since the histone deacetylase inhibitors and phorbol esters required to induce lytic replication in such lines also strongly induced the NK ligands. Accordingly, therefore, we have performed our study on KSHV infection of primary HUVECs, as well as the experimentally manipulable cell line iSLK.219.

Though there were some differences between our two infection models, we found that the majority of the ligands tested were affected by lytic KSHV infection. In both SLK cells and HUVECs, MICA, ULBP2, NKp44L, and CD155 were downregulated,

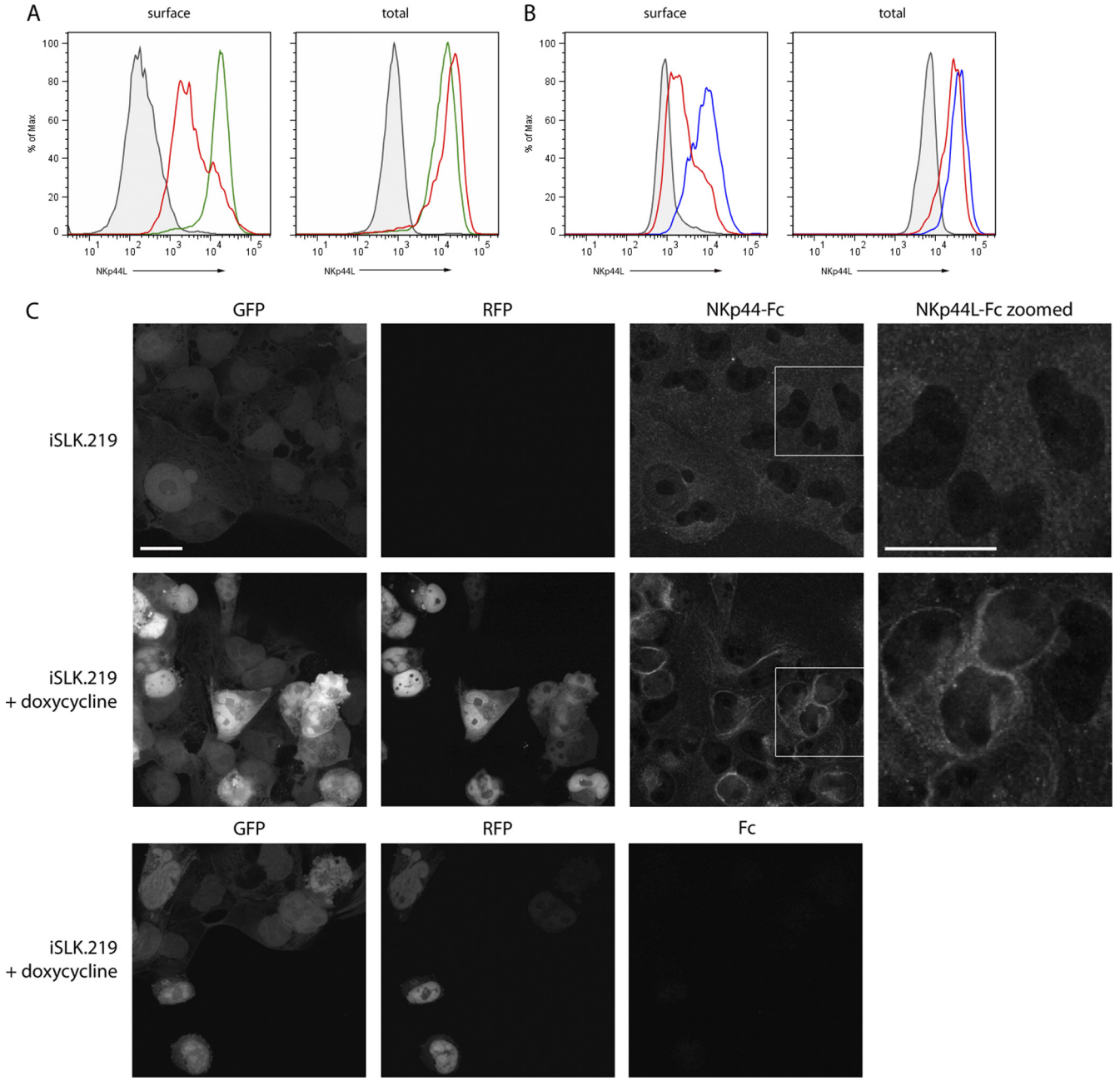

FIG 6 NKp44L subcellular localization is altered. (A) iSLK.219 cells were treated for 48 h with 1 μg/ml doxycycline to induce RTA expression and then stained with NKp44-Fc either without (left) or with (right) permeabilization. Latent cells are shown in green; lytic cells are in red. Isotype control staining is the gray-filled histogram. (B) 293 cells were transfected with vector control (blue line) or ORF54 (red line) and then stained with NKp44-Fc either without (left) or with (right) permeabilization. Isotype control staining is the gray-filled histogram. (C) iSLK.219 cells were grown without (row 1) or with (rows 2 and 3) 1 μg/ml doxycycline for 48 h and then fixed and stained with NKp44-Fc. Cells were imaged by confocal fluorescence microscopy. The boxed region of the NKp44L stain is shown blown up in the far right panel. Bars, 25 μm.

though MICA in SLK cells was an unusual case, as its levels were decreased only in RFP-negative cells in the induced population. (The basis of this peculiar phenomenon is not clear.) In HUVECs, MICB was downregulated as well, possibly reflecting cell type differences (note that the effect of MIR2 on MICB is known to be allele specific). The upregulation of MICB in the lytic SLK cell infection is in some contrast to a previous study stating that the KSHV-infected BCBL1 cell line treated with a sponge against the KSHV microRNA miRK12-7 had higher levels of MICB (40). However, that study was looking at latently infected B cells, while ours is investigating lytic infection.

In order to study the effects on NK ligands during the initial stages of a KSHV infection, we performed *de novo* infections in HUVECs. Interestingly, two ligands (NKp44L and MICB) were downregulated following KSHV infection and without lytic reactivation. This decrease in MICB could be due to the miRNA

miRK12-7, which is expressed during latency, or to MIR2, a lytic gene which might be expressed at this stage of an infection (25). The present study was undertaken to determine the nature of the viral gene(s) controlling NKp44L display.

Infection time courses performed in several cell types indicated that NKp44L downregulation after a *de novo* infection was transient. The transient change in levels at early time points is reminiscent of the burst of lytic gene expression reported by Krishnan et al. (25). Intriguingly, many of the genes that were found to be expressed during this period were immune related, leading to speculation that the virus might need to modulate the immune response immediately after infection, before the establishment of latency. Once latency is established, only a few genes are expressed and it is thought that the virus becomes hidden from the host immune system. Indeed, the gene that we identified in a screen for NKp44L downregulation, ORF54, was shown to be expressed during the lytic burst by Krishnan et al. (25). ORF54 was also identified by Bechtel et al. (3) to be one of the viral RNAs packaged into the KSHV virion and presumably available for rapid translation following viral entry. These observations taken together suggest that ORF54 is one of several viral immune-evasion proteins that function in the early stages of an infection.

In this study, we provide some insight into the mechanism of downregulation of NKp44L by ORF54. We found by flow cytometry that while surface levels decrease, total cellular levels of the ligand are unchanged, pointing toward a trafficking defect as opposed to an effect on transcriptional or translational regulation. Using confocal microscopy, we have shown that NKp44L undergoes a change in subcellular localization during lytic KSHV infection. Interestingly, NKp44L loses its normal strict nuclear exclusion, and some of the protein is found in the nucleus. This is particularly intriguing, given that we are staining cells with the NKp44-Fc fusion protein, which presumably recognizes the extracellular portion of NKp44L (assuming that NKp44L is a transmembrane protein). While the observation of an intact membrane protein becoming relocalized to the nucleus is surprising, it is not without precedent. Various receptor tyrosine kinases, with EGFR being the prime example, have been shown to localize to the nucleus under certain conditions (12, 31). The process appears to involve localization to the ER followed by extrusion through the Sec61 retrotranslocon (29). We speculate that a similar trafficking mechanism could be occurring with NKp44L, though how ORF54 might trigger this process remains unclear. We also attempted to monitor the localization of NKp44L in SLK cells transiently transfected with an ORF54-expressing plasmid; however, we were unable to attain sufficient expression of ORF54 in these cells to cause NKp44L downregulation (data not shown) and therefore did not perform imaging on these cells.

Previously categorized as a dUTPase, we find that ORF54 has immunomodulatory activity not just against NKp44L but against cytokine receptors as well. That a viral gene originally specified for one purpose might evolve a novel and distinct function is not without precedent. For example, the KSHV SOX protein possesses a host shutoff function that its homolog nuclease from HSV-1 entirely lacks (20). In the present study, we found that the dUTPase activity of ORF54 is extremely weak compared to that of other human herpesvirus orthologs and that no correlation exists between the presence of dUTPase activity and the downregulation of immunoregulatory ligands. The HSV-1 and EBV orthologs with strong dUTPase activity showed no effect on NKp44L surface levels. Conversely, a point mutation predicted to abolish ORF54 dUTPase activity retained much of its downregulation activity. Our data indicate that the dUTPase function is not sufficient for immune modulation and most likely not necessary for it. Rather, ORF54 appears to have gained a novel immunomodulatory function at some point in its evolution.

dUTPases are a ubiquitous class of enzyme that can be found in almost all species, from bacteria and viruses to humans. While eukaryotic dUTPases function as trimers, herpesvirus dUTPases seem to have undergone a gene duplication event to form a larger protein and evolved an intramolecular fold that allows them to function as monomers (44). Overall sequences can vary significantly even between human herpesvirus orthologs, but all functional dUTPases contain five conserved dUTPase domains. Interestingly, Davison and Stow (17) noted in a 2005 analysis of the sequence phylogeny of the viral dUTPase genes that there was substantial diversification and rearrangement of dUTPase domains during viral evolution. In particular, they and others noted that some herpesviruses (notably, the β-herpesvirus subfamily) had lost homology in the motifs predicted to be important for dUTPase activity (10, 17). Interestingly, the herpesvirus dUTPases also possess an additional region of sequence homology (termed motif 6) of unknown function. Motif 6 can also be found in other herpesvirus genes called dUTPase-related proteins (dURPs), such as ORFs 10 and 11 of KSHV, suggesting that these genes share an evolutionary ancestry with the dUTPase genes yet have lost the canonical dUTPase domains and activity. Based on these findings, Davison and Stow speculated that herpesvirus dUTPases and dURPs may be acquiring novel evolutionary functions (17). Our work provides striking experimental documentation of exactly such an acquisition and strongly validates their general evolutionary speculations. However, our data argue against the specific notion that motif 6 is the repository of the novel immunomodulatory function that we have discovered, since we find that other dUTPases containing motif 6, such as the dUTPases from HSV-1 and EBV, lack immune-evasion activity. Likewise we were unable to detect NKp44L downregulation activity in the KSHV dURPs ORF10 and ORF11, though we have recently reported that ORF10 can impair IFN signaling, which is consistent with the theme that diversification in this gene superfamily has evolved functions related to innate immune regulation (6).

Very recently, we have created a mutant form of KSHV BAC16 with the ORF54 gene disrupted and generated SLK cells stably (latently) infected with wild-type or mutant bacterial artificial chromosome (BAC)-derived virus. As shown in Fig. S2A in the supplemental material, induction of lytic replication in cells bearing either wild-type or ORF54 mutant viral genomes did not reveal any differences between the two in their ability to downregulate NKp44L, though downregulation is weak in this cell culture system to begin with (see Fig. S2A in the supplemental material). Virus produced from these BAC-derived viruses can also transiently downregulate NKp44L on *de novo*-infected HUVECs, and again, no difference was seen between wild-type and mutant virus (see Fig. S2A in the supplemental material).

We think it likely that this is due to the presence of redundant functions in KSHV that can also influence this ligand. We note that in MHV-68, a related murine herpesvirus, similar redundancy has been found in ORF54's role in enhancing interferon resistance (27). There, as here, mutational ablation of ORF54 does not rescue the phenotype (there, interferon sensitivity). Although

our screen did not identify any other regulators of NKp44L, we note that the screen was carried out by transfection of genes one at a time. If two or more viral polypeptides are required concurrently for this regulation, their genes would not score in this assay. We also may have missed a protein that was incorrectly expressed or modified in our transient-transfection assay. Consistent with the theme of redundancy, we note that KSHV encodes 2 genes for MHC-I downregulation, at least 3 genes for IFN modulation, and at least 4 genes capable of downregulating gp130 (A. Madrid and D. Ganem, unpublished work). A small interfering RNA screen of candidate lytic genes in cells stably infected with the ORF54 mutant to look for restoration of NKp44Lsurface expression has the potential to reveal additional viral genes involved in downregulation and may offer a way forward here.

NKp44L is the putative ligand(s) of the NK-specific activating receptor NKp44 (also called natural cytotoxicity triggering receptor 2). Much effort has gone into identifying the ligand(s) for NKp44, though so far these efforts have been unsuccessful. What is known is that NKp44L is a ubiquitously expressed cell surface molecule that is also very abundant in intracellular membrane compartments (8). The importance of this receptor and ligand(s) for the NK response to viral infections is poorly understood, as NKp44 is a human-specific receptor and thus cannot be studied in mice (9). A member of the immunoglobulin protein superfamily, NKp44 is not expressed on circulating peripheral blood NK cells but is induced on IL-2-activated NK cells and can mediate MHC-I-negative target cell lysis (9, 48). Our findings that KSHV downregulates NKp44L, together with an earlier report that the Nef protein of HIV-1, like ORF54, causes intracellular retention of NKp44L (18), strongly suggest that this ligand is important in antiviral immunity. Clearly, a better understanding of the molecular nature of NKp44L and its role in innate immune function will be necessary to further elucidate how this target of NK cell recognition is involved in the control of viral infection.

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REFERENCES

1. **Ambroziak JA, et al.** 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science **268**:582–583.
2. **Barabas O, Pongracz V, Kovari J, Wilmanns M, Vertessy BG.** 2004. Structural insights into the catalytic mechanism of phosphate ester hydrolysis by dUTPase. J. Biol. Chem. **279**:42907–42915.
3. **Bechtel J, Grundhoff A, Ganem D.** 2005. RNAs in the virion of Kaposi's sarcoma-associated herpesvirus. J. Virol. **79**:10138–10146.
4. **Bechtel JT, Liang Y, Hvidding J, Ganem D.** 2003. Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. J. Virol. **77**:6474–6481.
5. **Bergman AC, Nyman PO, Larsson G.** 1998. Kinetic properties and stereospecificity of the monomeric dUTPase from herpes simplex virus type 1. FEBS Lett. **441**:327–330.
6. **Bisson SA, Page AL, Ganem D.** 2009. A Kaposi's sarcoma-associated herpesvirus protein that forms inhibitory complexes with type I interferon receptor subunits, Jak and STAT proteins, and blocks interferon-mediated signal transduction. J. Virol. **83**:5056–5066.
7. **Bjornberg O, et al.** 1993. dUTPase from herpes simplex virus type 1; purification from infected green monkey kidney (Vero) cells and from an overproducing Escherichia coli strain. Protein Expr. Purif. **4**:149–159.
8. **Byrd A, Hoffmann SC, Jarahian M, Momburg F, Watzl C.** 2007. Expression analysis of the ligands for the natural killer cell receptors NKp30 and NKp44. PLoS One **2**:e1339. doi:10.1371/journal.pone.0001339.
9. **Cantoni C, et al.** 1999. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. J. Exp. Med. **189**:787–796.
10. **Caposio P, Riera L, Hahn G, Landolfo S, Gribaudo G.** 2004. Evidence that the human cytomegalovirus 46-kDa UL72 protein is not an active dUTPase but a late protein dispensable for replication in fibroblasts. Virology **325**:264–276.
11. **Carbone A, Gloghini A.** 2008. KSHV/HHV8-associated lymphomas. Br. J. Haematol. **140**:13–24.
12. **Carpenter G, Liao HJ.** 2009. Trafficking of receptor tyrosine kinases to the nucleus. Exp. Cell Res. **315**:1556–1566.
13. **Chalupny N, Rein-Weston A, Dosch S, Cosman D.** 2006. Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142. Biochem. Biophys. Res. Commun. **346**:175–181.
14. **Coscoy L, Ganem D.** 2000. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc. Natl. Acad. Sci. U. S. A. **97**:8051–8056.
15. **Coscoy L, Sanchez D, Ganem D.** 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. J. Cell Biol. **155**:1265–1273.
16. **Cosman D, et al.** 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity **14**:123–133.
17. **Davison AJ, Stow ND.** 2005. New genes from old: redeployment of dUTPase by herpesviruses. J. Virol. **79**:12880–12892.
18. **Fausther-Bovendo H, et al.** 2009. HIV escape from natural killer cytotoxicity: nef inhibits NKp44L expression on CD4+ T cells. AIDS **23**:1077–1087.
19. **Ganem D.** 2006. KSHV infection and the pathogenesis of Kaposi's sarcoma. Annu. Rev. Pathol. **1**:273–296.
20. **Glaunsinger B, Ganem D.** 2004. Lytic KSHV infection inhibits host gene expression by accelerating global mRNA turnover. Mol. Cell **13**:713–723.
21. **Harris JM, McIntosh EM, Muscat GE.** 1999. Structure/function analysis of a dUTPase: catalytic mechanism of a potential chemotherapeutic target. J. Mol. Biol. **288**:275–287.
22. **Ishido S, et al.** 2000. Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. Immunity **13**:365–374.
23. **Ishido S, Wang C, Lee B, Cohen G, Jung J.** 2000. Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. J. Virol. **74**:5300–5309.
24. **Kremmer E, et al.** 1999. Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) ORF54 encodes a functional dUTPase expressed in the lytic replication cycle. J. Gen. Virol. **80**(Pt 5):1305–1310.
25. **Krishnan H, et al.** 2004. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. J. Virol. **78**:3601–3620.
26. **Kubin M, et al.** 2001. ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. Eur. J. Immunol. **31**:1428–1437.
27. **Leang RS, et al.** 2011. The anti-interferon activity of conserved viral dUTPase ORF54 is essential for an effective MHV-68 infection. PLoS Pathog. **7**:e1002292. doi:10.1371/journal.ppat.1002292.
28. **Lenac T, et al.** 2006. The herpesviral Fc receptor fcr-1 down-regulates the NKG2D ligands MULT-1 and H60. J. Exp. Med. **203**:1843–1850.
29. **Liao HJ, Carpenter G.** 2007. Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. Mol. Biol. Cell **18**:1064–1072.
30. **Lilley B, Ploegh H.** 2005. Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond. Immunol. Rev. **207**:126–144.
31. **Lin SY, et al.** 2001. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat. Cell Biol. **3**:802–808.
32. **Lodoen M, Lanier L.** 2005. Viral modulation of NK cell immunity. Nat. Rev. Microbiol. **3**:59–69.
33. **Lodoen M, et al.** 2003. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J. Exp. Med. **197**:1245–1253.
34. **Lodoen MB, et al.** 2004. The cytomegalovirus m155 gene product sub-

verts natural killer cell antiviral protection by disruption of H60-NKG2D interactions. J. Exp. Med. **200**:1075–1081.
35. **Lukac DM, Renne R, Kirshner JR, Ganem D.** 1998. Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology **252**:304–312.
36. **Mansouri M, et al.** 2009. Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. J. Virol. **83**:9672–9681.
37. **Moretta L, et al.** 2006. Surface NK receptors and their ligands on tumor cells. Semin. Immunol. **18**:151–158.
38. **Moretta L, Moretta A.** 2004. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J. **23**:255–259.
39. **Myoung J, Ganem D.** 2011. Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: maintenance of tight latency with efficient reactivation upon induction. J. Virol. Methods **174**:12–21.
40. **Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O.** 2009. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe **5**:376–385.
41. **Orange J, Ballas Z.** 2006. Natural killer cells in human health and disease. Clin. Immunol. **118**:1–10.
42. **Shah AH, et al.** 2010. Degranulation of natural killer cells following interaction with HIV-1-infected cells is hindered by downmodulation of NTB-A by Vpu. Cell Host Microbe **8**:397–409.
43. **Sun R, et al.** 1998. A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. U. S. A. **95**:10866–10871.
44. **Tarbouriech N, Buisson M, Seigneurin JM, Cusack S, Burmeister WP.** 2005. The monomeric dUTPase from Epstein-Barr virus mimics trimeric dUTPases. Structure **13**:1299–1310.
45. **Thomas M, et al.** 2008. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. Proc. Natl. Acad. Sci. U. S. A. **105**:1656–1661.
46. **Tomasec P, et al.** 2005. Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat. Immunol. **6**:181–188.
47. **Vieira J, O'Hearn P.** 2004. Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. Virology **325**:225–240.
48. **Vitale M, et al.** 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in nonmajor histocompatibility complex-restricted tumor cell lysis. J. Exp. Med. **187**:2065–2072.