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Human Herpesvirus 6B Downregulates Expression of Activating Ligands during Lytic Infection To Escape Elimination by Natural Killer Cells

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ABSTRACT

The *Herpesviridae* family consists of eight viruses, most of which infect a majority of the human population. One of the less-studied members is human herpesvirus 6 (HHV-6) (*Roseolovirus*), which causes a mild, well-characterized childhood disease. Primary HHV-6 infection is followed by lifelong latency. Reactivation frequently occurs in immunocompromised patients, such as those suffering from HIV infection or cancer or following transplantation, and causes potentially life-threatening complications. In this study, we investigated the mechanisms that HHV-6 utilizes to remain undetected by natural killer (NK) cells, which are key participants in the innate immune response to infections. We revealed viral mechanisms which downregulate ligands for two powerful activating NK cell receptors: ULBP1, ULBP3, and MICB, which trigger NKG2D, and B7-H6, which activates NKp30. Accordingly, this downregulation impaired the ability of NK cells to recognize HHV-6-infected cells. Thus, we describe for the first time immune evasion mechanisms of HHV-6 that protect lytically infected cells from NK elimination.

IMPORTANCE

Human herpesvirus 6 (HHV-6) latently infects a large portion of the human population and can reactivate in humans lacking a functional immune system, such as cancer or AIDS patients. Under these conditions, it can cause life-threatening diseases. To date, the actions and interplay of immune cells, and particularly cells of the innate immune system, during HHV-6 infection are poorly defined. In this study, we aimed to understand how cells undergoing lytic HHV-6 infection interact with natural killer (NK) cells, innate lymphocytes constituting the first line of defense against viral intruders. We show that HHV-6 suppresses the expression of surface proteins that alert the immune cells by triggering two major receptors on NK cells, NKG2D and NKp30. As a consequence, HHV-6 can replicate undetected by the innate immune system and potentially spread infection throughout the body. This study advances the understanding of HHV-6 biology and the measures it uses to successfully escape immune elimination.

Human herpesvirus 6 (HHV-6) (*Roseolovirus*) was long neglected in research and medical diagnostics; however, in the past few years, it gained increasing attention. A variety of clinical complications were found to be associated with HHV-6 infection, especially concerning immunocompromised patients, in whom it has the ability to cause severe morbidity and mortality (1, 2). HHV-6 is subclassified into two distinct variants, HHV-6A and HHV-6B, which are considered independent viruses but share major genome sequence homologies and epidemiologies (3, 4). Similarly to other members of the herpesvirus family, HHV-6 causes a relatively short and mild primary disease called roseola infantum, usually in children up to the age of 2 years, with a sudden rise of fever and rash as major symptoms (5). Following this initial infection, HHV-6 establishes lifelong latency in about 90% of all individuals examined (2). Reactivation frequently occurs in patients suffering from HIV infection or cancer or following transplantation and has severe consequences, such as myocarditis, liver failure, and encephalitis (2). HHV-6 reactivation facilitates transactivation of other herpesviruses, such as HHV-7 or human cytomegalovirus (HCMV) (HHV-5), and the spread of fungal infections (6–10). Interestingly, for several chronic neurological disorders such as multiple sclerosis (MS), Alzheimer's disease (AD), or chronic fatigue syndrome (CFS), markedly elevated HHV-6 titers were determined (11–13). Yet, whether HHV-6 has a causative role in these diseases or whether reactivation is a consequence of a deregulated and malfunctioning immune system requires elucidation.

The interaction of the immune system with HHV-6-infected cells is incompletely understood. The ability to establish latency and to reactivate into lytic infection strongly suggests the existence of immune evasion mechanisms employed by this virus. However, only a few general immune evasion mechanisms have been described, such as the suppression of an antiviral interferon response (14). Several specific viral mechanisms were developed by the vi-

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rus to avoid recognition by cytotoxic T cells (CTLs) that are part of the adaptive immune system. CTLs recognize HLA class I-restricted viral peptides, and long-living memory cells are found in individuals who have encountered a herpesvirus infection (15, 16), suggesting that this prevents reactivation of herpesviruses in healthy individuals. Indeed, reactivation usually does not occur in an immunocompetent host but rather occurs under immunosuppressed conditions, such as with severe coinfections or transplantation (2). HHV-6 has been shown previously to inhibit T cell function: the virus propagates in preferentially in CD4-positive T cells and lyses them at the end of the replication cycle (17). T cell receptor (TCR) activity is impaired by transcriptional downregulation of CD247 (CD3ζ, the signaling domain of the TCR) and endocytosis of the TCR complex mediated by the viral protein U24 (17, 18). HHV-6 is also capable of manipulating the function of dendritic cells (DCs) (19) and the cytokine response during an active infection, further suppressing proper T cell activation against virus-infected cells (20). Furthermore, the expression of HLA class I on infected cells is downregulated, yielding limited recognition of infected cells by T cells (21). Importantly, reduced surface expression of major histocompatibility complex (MHC) class I on a cell is known to activate NK cells, a cell type of the innate branch of the immune system (21, 22), a phenomenon known as "missing self" recognition (23). NK cells constitute the first line of defense against pathogens such as viruses, fungi, and bacteria but also against transformed cells (24–28). Their activity is controlled by a balance of signals mediated by activating and inhibitory receptors (29). The inhibitory signal is mostly dependent on the recognition of MHC class I on target cells by appropriate NK inhibitory receptors (29, 30). NK cells also possess numerous activating receptors that are able to recognize stress-induced self-ligands via the receptor NKG2D (31), immune regulatory self-molecules (for instance, the recognition of B7-H6 by the NKp30 receptor [32] or of CD48 by the 2B4 receptor [33]), or viral ligands such as the influenza virus-derived hemagglutinin that is recognized by both the NKp46 and NKp44 receptors (34, 35). We and other groups have described several diverse viral mechanisms that manipulate cellular ligands for NK cells to escape the immune system (34, 36–44); however, knowledge about the interaction of HHV-6-infected cells with NK cells is currently missing.

In our present study, we focused on HHV-6B. We demonstrate for the first time that HHV-6B downregulates the expression of several ligands for activating NK cell receptors during lytic infection to escape recognition by NK cells. In addition to ligands for the receptor NKG2D that are frequently manipulated during viral infections, we interestingly also identified B7-H6 as a novel target for viral immune modulation.

MATERIALS AND METHODS

Cell lines, HHV-6B culture, and infection. All cells were cultivated at 37°C and >95% humidity with 5% CO_2 in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich) and nonessential amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin (all from Biological Industries) according to the manufacturer's instructions. The SupT1 cell line (ATCC CRL-1942), a T cell lymphoma cell line, and MOLT-3 (ATCC CRL1552), a cell line derived from acute lymphoblastic T cell leukemia, were obtained from the NIH AIDS reagent program. SupT1 cells were infected with the HHV-6B strain Z29. The infected cells were fed with uninfected SupT1 at a 1:10 ratio every 3 or 4 days. The Jurkat cell line (ATCC TIB-152) was established from an acute T cell leukemia and obtained from the ATCC. For the performance of assays, SupT1 and MOLT-3 cells were infected by cocultivation with late-stage infected SupT1 cells at a ratio of 40:1 (uninfected to infected) and Jurkat cells at 10:1, resulting in synchronized infection of at least 70% of the cells.

Cord blood mononuclear cells (CBMCs) were purified from cord blood of a single donor using Lymphoprep (Stem Cell Technologies). A total of 2×10^5 cells were seeded in 96-F-well plates and activated for 48 h using 500 U/ml recombinant human interleukin-2 (rhIL-2) (Peprotech) and 10 μg/ml phytohemagglutinin (PHA) (Roche). Subsequently, 2×10^4 SupT1 cells with late-stage Z29 infection were added. At 24 h postinfection (hpi), cells were collected and analyzed by fluorescence-activated cell sorting (FACS).

IgG fusion proteins containing the extracellular domain of NKG2D, NKp30, NKp44, or NKp46 were prepared and used for FACS staining of infected or uninfected SupT1 cells as previously described (35).

Western blotting. Lysates of uninfected or 12-, 24-, 48-, or 72-h-infected SupT1 cells were prepared using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 0.1% SDS, 1% NP-40 substitute, 0.5% deoxycholate, EDTA-free proteinase inhibitor cocktail [Sigma]), and SDS gel electrophoresis was performed. Proteins were transferred onto a nitrocellulose membrane, and specific protein bands were detected using antibodies detecting viral DR6/7 (1:1,000 in 5% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]). The DR6/7 antibody (clone 33B12) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, via the HHV-6 Foundation. Alternatively, membranes were incubated with antivinculin (Abcam; 1:1,000 in 5% BSA in PBS) as a loading control. Chemiluminescence caused by detection antibody-linked horseradish peroxidase (HRP) (Jackson ImmunoResearch) was detected.

FACS analysis. For FACS staining of NK ligands on SupT1 and MOLT-3 cells, 1×10^6 cells were infected by cocultivation with late-stage Z29-infected SupT1 cells at a ratio of 40:1 and Jurkat cells at a ratio of 10:1. At the indicated time points, cells were stained with anti-ULBP1, anti-ULBP2, anti-ULBP3, anti-MICA, anti-MICB, and anti-B7-H6 antibodies (all obtained from R&D Systems) as well as the mouse isotype IgG2b antibody (BioLegend). Anti-CD48 was obtained from Santa Cruz. For detection of HLA, W6/32 antibody that was produced in-house was used. For detection, a goat anti-mouse IgG antibody coupled to Alexa Fluor 647 (Jackson ImmunoResearch) was used. Analysis was performed with a FACSCalibur flow cytometer (BD Biosciences). Intracellular staining for the viral protein gp102 was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. The gp102 antibody (clone 7A2) was obtained from the NIH AIDS reagent program (45).

The surface staining of uninfected or HHV-6B-infected CBMCs was performed as described above for cell lines. Following surface staining, the cells were fixed and permeabilized using the Cytofix/Cytoperm kit and stained with an anti-p41 antibody that was coupled to DyLight-488 using the Lightning-Link Rapid kit (Innova Biosciences). The anti-p41 antibody was obtained through the NIH AIDS reagent program (45).

Receptors on activated bulk NK cells were stained using anti-NKG2D–phycoerythrin (PE), anti-NKp30–allophycocyanin (APC), or anti-2B4–APC (all from BioLegend). Emitted fluorescence was analyzed in a FACSCalibur flow cytometer.

RNA extraction and cDNA preparation. RNA extracts were prepared using the QuickRNA kit (Zymo Research). For the generation of cDNA, Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) was used in the presence of anchored oligo(dT) primers (Thermo Scientific/Fermentas). Both procedures were performed according to the manufacturers' protocols.

qRT-PCR. For quantitative real-time PCR (qRT-PCR), freshly prepared cDNAs were used for SYBR green-based detection in a QuantStudio 12k Flex real-time PCR cycler (Life Technologies) with primers targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine

phosphoribosyltransferase (HPRT), MICA, MICB, ULBP1, ULBP2, ULBP3, CD48, and B7-H6, as follows: GAPDH forward, GAGTCAACG GATTTGGTCGT; GAPDH reverse, GATCTCGCTCCTGGAAGATG; HPRT forward, TGACACTGGCAAAACAATGCA; HPRT reverse, GGT CCTTTTCACCAGCAAGCT; MICA forward, ATCTTCCCTTTTGCAC CTCC; MICA reverse, AACCCTGACTGCACAGATCC; MICB forward, CTGCTGTTTCTGGCCGTC; MICB reverse, ACAGATCCATCCTGGG ACAG; ULBP1 forward, GCGTTCCTTCTGTGCCTC; ULBP1 reverse, GGCCTTGAACTTCACACCAC; ULBP2 forward, CCCTGGGGAAGAA ACTAAATGTC; ULBP2 reverse, ACTGAACTGCCAAGATCCACTGC; ULBP3 forward, AGATGCCTGGGGAAAACAACTG; ULBP3 reverse, GTATCCATCGGCTTCACACTCAC; CD48 forward, GGAATTGCTAC TGCTGCCTC; CD48 reverse, AGTTCTCAGGCAGGCTCTCA; B7-H6 forward, TCACCAAGAGGCATTCCGACCT; and B7-H6 reverse, ACCA CCTCACATCGGTACTCTC.

IF. Immunofluorescence (IF) staining was performed as previously described (46). For ULBP2 and ULBP3 IF staining, the stress ligands were overexpressed with an N-terminal His tag in SupT1 cells. As the primary antibody, anti-His antibody (R&D Systems), anti-MICB (R&D Systems), or anti-ULBP1 (Santa Cruz; clone H-46) was used with anti-mouse or anti-rabbit IgG coupled to Alexa Fluor 647 (Jackson ImmunoResearch) as the secondary antibody. Control stainings were performed without the application of a primary antibody. The nuclei of the cells were counterstained using DAPI (4′,6′-diamidino-2-phenylindole). Quantification of the immunofluorescence signal was performed using Fluoview-10 software (Olympus). On average, about 65 cells per condition were quantified. To quantify HHV-6-infected cells, we assessed fluorescence intensity in cells that were themselves mildly enlarged or adjacent to cells that were strongly enlarged due to cell-to-cell fusion during viral infection.

Proteasome inhibition. Three different proteasome inhibitors were used: bortezomib (10 nM final concentration), MG132 (20 nM), and epoxomicin (50 ng/ml). Inhibitors were applied to the infected cells or to uninfected controls at 12 h following infection, and the cells were incubated for an additional 8 h. As controls, cells were left untreated or treated with the lysosomal inhibitor leupeptin (10 μg/ml) (all inhibitors were supplied by Calbiochem).

Cycloheximide-mediated translation repression. SupT1 cells that were infected with HHV-6B or uninfected control cells were treated with 50 ng/ml cycloheximide to suppress translation from 3, 6, or 12 h from the experimental setup up to the harvest of the assay at 18 hpi. Cells were collected, washed, and stained for surface markers for FACS analysis as described above.

CD107 degranulation assay. NK cells were purified and activated as previously described (47). For FACS-based determination of NK cell degranulation, 1×10^5 NK cells were incubated without target cells or with 5×10^4 SupT1 cells infected with Z29 for 12, 14, 16, or 22 h. For the blocking of activating receptors on NK cells, 0.25 μg per well of antibody targeting NKG2D (R&D Systems) or NKp30 (Biolegend), or both antibodies simultaneously, was added prior to the experimental setup and incubated for 60 min on ice. Control NK cells were incubated with an antibody directed against CD99. The antibodies CD56-PE (0.625 μl/well; BD Biosciences) and CD107a-APC (0.625 μl/well; Biolegend) were added immediately after intermixture of NK and target cells. The assay was performed for 2 h at 37°C, and CD107a-APC levels on CD56-PE-positive cells were analyzed in a FACSCalibur flow cytometer.

RESULTS

Expression of ligands for several activating NK receptors is altered during HHV-6 infection. To test whether changes in NK cell ligands are observed following HHV-6 infection, we infected the T cell lymphoma cell line SupT1 with the HHV-6B strain Z29. To confirm infection, we used Western blot analysis to assess expression of the viral proteins DR6/7 at 12, 24, 48, and 72 hpi (Fig. 1A) and intracellular flow cytometry staining for the viral protein gp102 (Fig. 1B). On average, 70% of cells showed a synchronized expression of this marker gene. We first investigated whether MICA, MICB, ULBP1, ULBP2, and ULBP3 (ligands for the powerful activating receptor NKG2D [48]), B7-H6 (an activating ligand for NKp30), and CD48 (an activator of the costimulatory receptor 2B4 receptor) are influenced by HHV-6 infection. As a positive control, we also stained for HLA class I molecules, since downregulation of HLA class I during the course of HHV-6 infection has been described previously.

Notably, we confirmed that HLA class I expression is reduced following HHV-6 infection from 18 hpi onwards (Fig. 1C). Interestingly, changes in NK ligands were also observed during the course of infection. The levels of three ligands for NKG2D, MICB, ULBP1, and ULBP3, were drastically reduced compared to those in uninfected control cells (Fig. 1C). The decrease became apparent at 18 hpi. In contrast, MICA and ULBP2 surface expression increased over time.

The ligand for NKp30, B7-H6, also showed reduced surface expression in infected cells (Fig. 1C). However, in contrast to the NKG2D ligands, which were found to be affected earlier during infection, the decrease of B7-H6 started at 24 hpi. The ligand for the 2B4 receptor, CD48, was massively upregulated, with a quick response starting already at 8 hpi and resulting in about a 10-fold induction of this surface marker at late time points postinfection (Fig. 1C). We also stained for the inhibitory ligand CEACAM1, which interacts homophilically (49), and for PVR, which is recognized both by the activating receptor DNAM-1 and the inhibitory receptor TIGIT (50, 51). However, in our hands, SupT1 cells did not express these molecules either prior to or following infection (data not shown).

Since some of the NKG2D ligands were increasing and other decreasing during the course of infection, we attempted to fully estimate the overall amount of NKG2D ligands on the surface. To this end, we prepared a fusion protein combining the extracellular portion of the NKG2D receptor with the Fc domain of a human IgG1 and stained uninfected and HHV-6-infected cells. Reduced NKG2D-Ig binding to the infected cells was observed (Fig. 2A). Similarly, we confirmed the decreased surface expression of B7-H6 (Fig. 1C), using an NKp30-Ig fusion protein (Fig. 2A). Because the identities of the cellular ligands of the activating receptors NKp44 and NKp46 are largely unknown, we also stained the cells with Ig fusion proteins composed of the extracellular portions of NKp44 and NKp46 fused to human IgG1. No staining was observed prior and following infection (Fig. 2A).

NK ligand reduction is functional in additional T cell lines and primary T cells. To further confirm our findings, we infected two additional T cell lines: MOLT-3 cells, which are considered a "classical" HHV-6B target cell line, and Jurkat cells, which can be infected and show initial gene expression but do not show cytopathic effects and infectivity.

Notably, both cell lines showed very similar changes in their NK ligand expression compared to SupT1 cells. MICB, ULBP1, ULBP3 (the latter not expressed by Jurkat cells), and B7-H6 were downregulated during HHV-6 infection (Fig. 2B; see Fig. S1 in the supplemental material). For MOLT-3 cells, we also checked for the upregulation of MICA, ULBP2, and CD48 and saw an increase in all ligands (Fig. 2B).

However, since all cell lines underwent cellular transformation and since the responses especially of stress ligands might differ compared to those in primary cells, we decided to also test the HHV-6 effect on NK ligands by infecting primary cells. Since

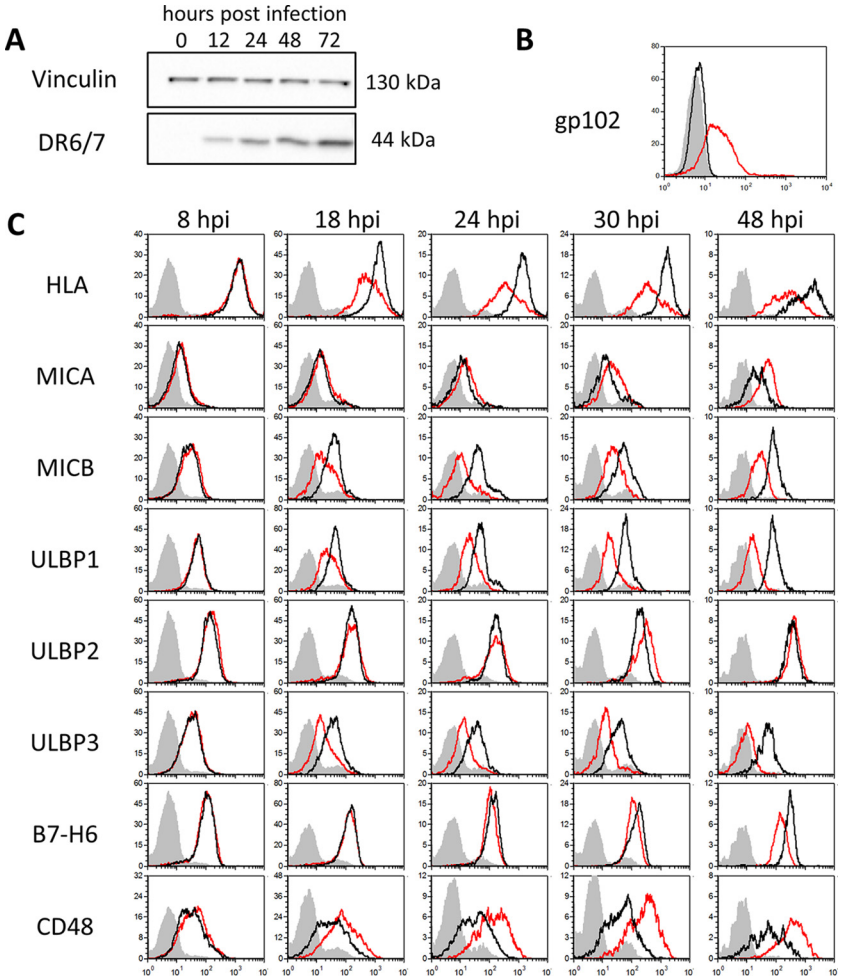

FIG 1 The expression of several NK ligands changes during lytic HHV-6B infection. (A) Detection of the viral protein DR6/7 over the course of infection (lower panel) using Western blotting with the host protein vinculin as loading control (upper panel). Sections were cropped for clarity. Results from one representative experiment out of two performed are shown. (B) Intracellular FACS staining for the viral protein gp102 at 18 h postinfection. The gray histogram indicates staining with an isotype antibody in infected cells, and the black histogram indicates staining for gp102 in uninfected cells. The red histogram shows the expression of gp102 in HHV-6-infected cells. Results from one representative experiment out of two performed are shown. (C) Staining for NK ligands on SupT1 cells that were uninfected (black histograms) or infected with HHV-6 (red histograms). The gray shaded histogram indicates staining of an isotype control. Staining for HLA class I, MICA, MICB, ULBP1, ULBP2, ULBP3, B7-H6, and CD48 was performed at 8, 18, 24, 30, and 48 h postinfection. Results from one representative experiment out of four performed are shown.

strain Z29 is capable of infecting *in vitro*-activated primary cells only, we activated cord blood mononuclear cells (CBMCs) using phytohemagglutinin (PHA) and IL-2 for 48 h and then infected them by coincubation with HHV-6B-infected SupT1 cells. At 24 hpi, we performed a double staining for expression of the relevant NK ligand on the activated CBMCs together with intracellular staining for viral gene expression by using p41 as marker.

Strikingly, we observed a strong reduction of MICB and ULBP3 also in primary cells (Fig. 2C). ULBP1 and B7-H6 were barely expressed on the activated CBMCs prior to and following infection (Fig. 2C). We did not observe the above-mentioned upregulation of CD48, MICA, and ULBP2 following infection.

Ligand changes occur via transcription-dependent and -independent mechanisms. Protein expression can be regulated on multiple levels: transcriptional, posttranscriptional, translational, and posttranslational. To identify the possible mechanisms used by HHV-6B to affect NK ligand expression, we isolated total RNAs from SupT1 cells and quantified mRNA levels of the various NK ligands during the course of the infection using qRT-PCR ($\log_2$ ratios are shown in Fig. 3, and the identical plots with fold changes are shown in Fig. S2 in the supplemental material). We normalized the mRNA levels to GAPDH. We compared these to protein surface expression on SupT1 cells, as assessed by the FACS staining shown in Fig. 1C (Fig. 3). For MICA, ULBP2, and CD48, the increase of protein expression correlated with augmented mRNA levels. For B7-H6, we observed a concurrent decrease of protein and mRNA levels starting at 24 hpi, suggesting that the expression of this activating ligand is controlled mainly at the transcriptional level.

Strikingly, for all NKG2D ligands that showed markedly reduced surface expression (MICB, ULBP1, and ULBP3), an increase in mRNA levels was observed (Fig. 3), indicating that the infected cells try to upregulate these stress ligands to be recognized by NKG2D but the virus suppresses their expression posttranslationally.

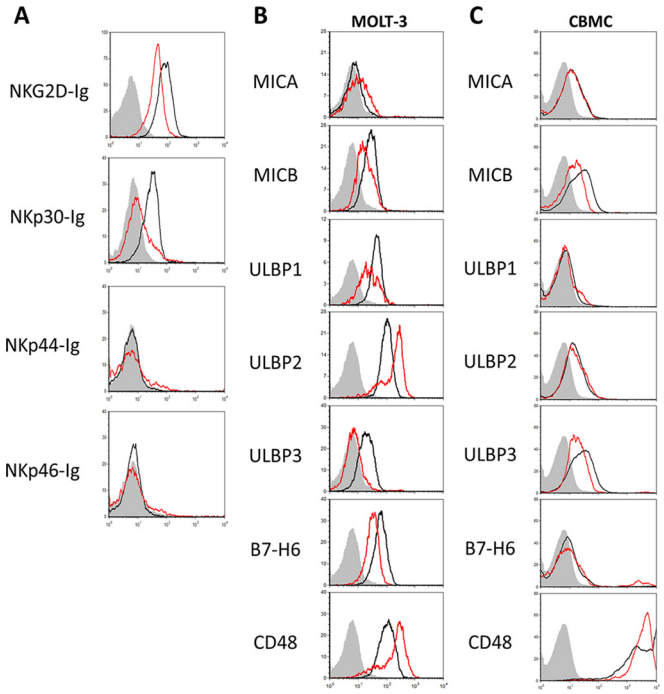

FIG 2 Staining of various cell lines and primary cells. (A) Staining for NK ligands on SupT1 cells that were uninfected (black histograms) or infected with HHV-6 (red histograms). Cells were stained with Ig fusion proteins containing extracellular portions of NKG2D, NKp30, NKp44, or NKp46 or an isotype fusion protein (gray shaded histogram). Detection was performed using an anti-human IgG–APC conjugate. For NKG2D-Ig, a staining at 24 hpi is shown, and for NKp30-Ig, NKp44-Ig, and NKp46-Ig, stainings performed at 48 hpi are presented. All experiments were executed at least three times. (B) Staining for NK ligands on MOLT-3 cells that were uninfected (black histograms) or infected with HHV-6 (red histograms). The gray shaded histogram indicates staining of an isotype control. Staining for MICA, MICB, ULBP1, ULBP2, ULBP3, B7-H6, and CD48 was performed at 24 h postinfection. Results from one representative experiment out of four performed are shown. (C) Staining for NK ligands on primary activated CBMCs that were uninfected (black histograms) or infected with HHV-6 (red histograms). The gray shaded histogram indicates staining of an isotype control. Staining was performed at 24 h postinfection. Infected cells were discriminated by intracellular expression of the viral p41 gene, and only p41-positive cells were gated. Results from one representative experiment out of two performed are shown.

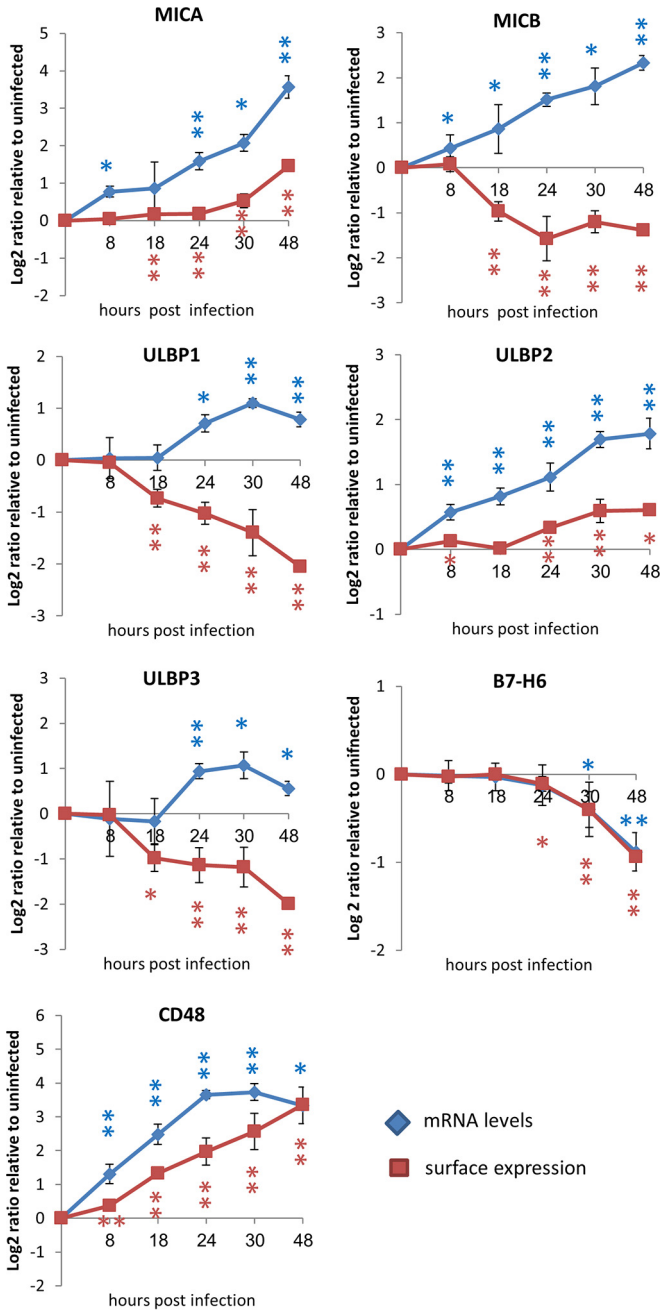

FIG 3 Comparison of surface protein and mRNA changes of NK ligands during lytic HHV-6B infection. RNA levels in infected cells were determined at 8, 18, 24, 30, and 48 h postinfection, and the $\log_2$ ratio compared to uninfected cells is shown (blue line). All values were normalized to GAPDH levels during the course of infection. Changes in the surface protein levels (also $\log_2$ ratio relative to uninfected control cells) from Fig. 1C were plotted as well (red line). Both experiments were performed at least four times, and standard errors of means were calculated. *, $P < 0.05$; **, $P < 0.005$.

NKG2D ligands are not intracellularly retained during HHV-6B infection. To further investigate the mechanisms behind the changes in NKG2D ligand expression following HHV-6B infection, we used immunofluorescence (IF) staining and confocal microscopy. We performed IF staining of endogenous ULBP1 and MICB in SupT1 cells; however, we did not have antibodies suitable for detecting ULBP2 and ULBP3 using IF. Thus, we overexpressed ULBP2 and ULBP3 with an N-terminal His tag in SupT1 cells and performed IF staining using an anti-His tag antibody. Cell nuclei were counterstained using DAPI dye.

Similar to the loss rate on the surface, we also observed a significant reduction of the whole-cell signal for ULBP1, ULBP3, and MICB within 24 h of HHV-6B infection (Fig. 4), indicating that the stress ligands are not intracellularly retained but rather degraded. As a control, we stained for ULBP2, whose levels did not change in FACS analysis at this time point (Fig. 1C), and did not observe significant changes in whole-cell expression of ULBP2.

Inhibition of proteasomal degradation restores expression of NKG2D ligands. Two major cellular foci for degrading and recycling proteins are the proteasome and lysosomes. To study the mechanisms that lead to stress ligand degradation, we infected SupT1 cells with HHV-6 and applied inhibitors of proteasomal degradation (bortezomib, MG132, or epoxomicin) or of lyso-

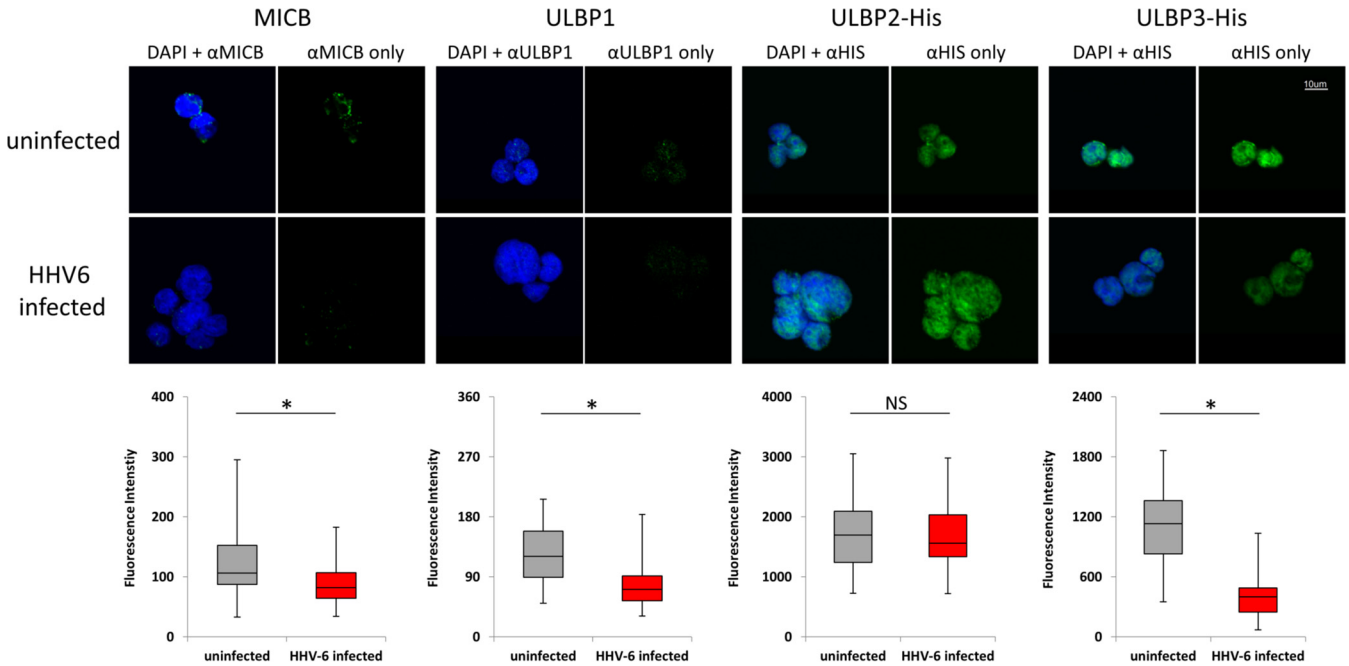

FIG 4 Intracellular staining of NKG2D ligands using immunofluorescence. Cells were infected for 24 h. Subsequently, infected or uninfected cells were fixed, permeabilized, and stained. Parental SupT1 cells were stained for MICB or ULBP1. For ULBP2 and ULBP3, transfectants for the respective proteins with an N-terminal His tag were generated and detected with an anti-His antibody. Anti-rabbit IgG (for ULBP1) and anti-mouse IgG coupled to Alexa Fluor 647 were used as secondary antibodies. The secondary antibody alone did not show any staining (not shown). Nuclei of the cells were counterstained using DAPI. Quantification of the immunofluorescence signal is shown below the respective, representative pictures and was performed using Fluoview-10 software (Olympus). On average, about 65 cells per condition were quantified. For HHV-6-infected cells, we preferably quantified cells that were themselves mildly enlarged or adjacent to cells that were strongly enlarged due to cell-to-cell fusion during viral infection. All experiments were performed twice with identical tendencies. Statistical analysis was performed using Student's *t* test. *, $P = 2.2E-7$ (MICB), $P = 4.1E-9$ (ULBP1), $P = 0.36$ (ULBP2-His), or $P = 7.7E-16$ (ULBP3-His). NS, not significant.

somal degradation (leupeptin) at 12 hpi (surface expression is still intact at this stage but is known to decrease drastically within the following hours due to viral infection [Fig. 1C]). We then incubated the treated and untreated cells for another 8 h. Subsequently, we analyzed surface expression of the NKG2D ligands by FACS. Notably, all proteasomal inhibitors, applied at 12 hpi, could completely restore the expression of MICB, ULBP1, and ULBP3 (Fig. 5A). In contrast, blocking of the lysosomal pathway did not impact the stress ligand expression and could not overcome viral measures to reduce these immune-activating molecules (Fig. 5A, right column).

Translation repression during infection suggests the existence of at least two different viral proteins responsible for the downregulation of NKG2D ligands. Having deciphered the mode of degradation, we aimed to characterize the responsible viral factors in more detail. To this end, we infected SupT1 cells, treated them with the translation inhibitor cycloheximide at 3, 6, or 12 hpi, and analyzed the cells at 18 hpi by staining the ligands in flow cytometry (Fig. 5B).

Interestingly, the cycloheximide treatment influenced ULBP3 differently from ULBP1 and MICB. If the treatment was applied at 3 or 6 hpi, all viral effects on these three stress ligands were negated. However, if the treatment was applied at 12 hpi, the impact on ULBP1 and MICB was abrogated but the ULBP3 downregulation occurred fully as in untreated controls, meaning that the viral protein relevant for ULBP3 downregulation is translated as an early but not an immediate early gene.

Additionally, cycloheximide treatment revealed the kinetics of the cellular response to HHV-6B infection; the translation of CD48 starts after 6 h and receives a significant boost between 12 and 18 h. This finding is in complete accordance with the mRNA kinetics shown in Fig. 2. Additionally, the induction of MICA is a gradual process starting early after infection. Surface expression of ULBP2 is not affected in this time frame.

Downregulation of activating ligands impairs recognition of HHV-6B-infected cells by NK cells. To investigate whether the changes in the expression of activating NK ligands are translated to impaired NK cell recognition, we coincubated activated bulk human NK cells that express the relevant receptors, i.e., NKG2D, NKp30, and 2B4 (Fig. 6A), with SupT1 cells that were infected for 12, 14, 16, and 22 h with HHV-6B. The degranulation of NK cells was determined by staining for the exocytosis marker CD107a in FACS. We preferred a degranulation determination assay over a "classical" killing assay since latter requires exchange of medium to radiolabel the cells during the course of infection. Since HHV-6B requires cell-to-cell attachment for infection, we decided not to interrupt the infection process by repeated centrifugation steps and medium exchange.

Importantly, the recognition of infected SupT1 cells by NK cells decreased significantly with advancing infection (Fig. 6B, black line). We confirmed the relevance of NKG2D for the decreasing ability of NK cells to recognize the infected cells by using a blocking antibody. Blocking NKG2D annulled the differences of NK cell degranulation between uninfected and infected SupT1

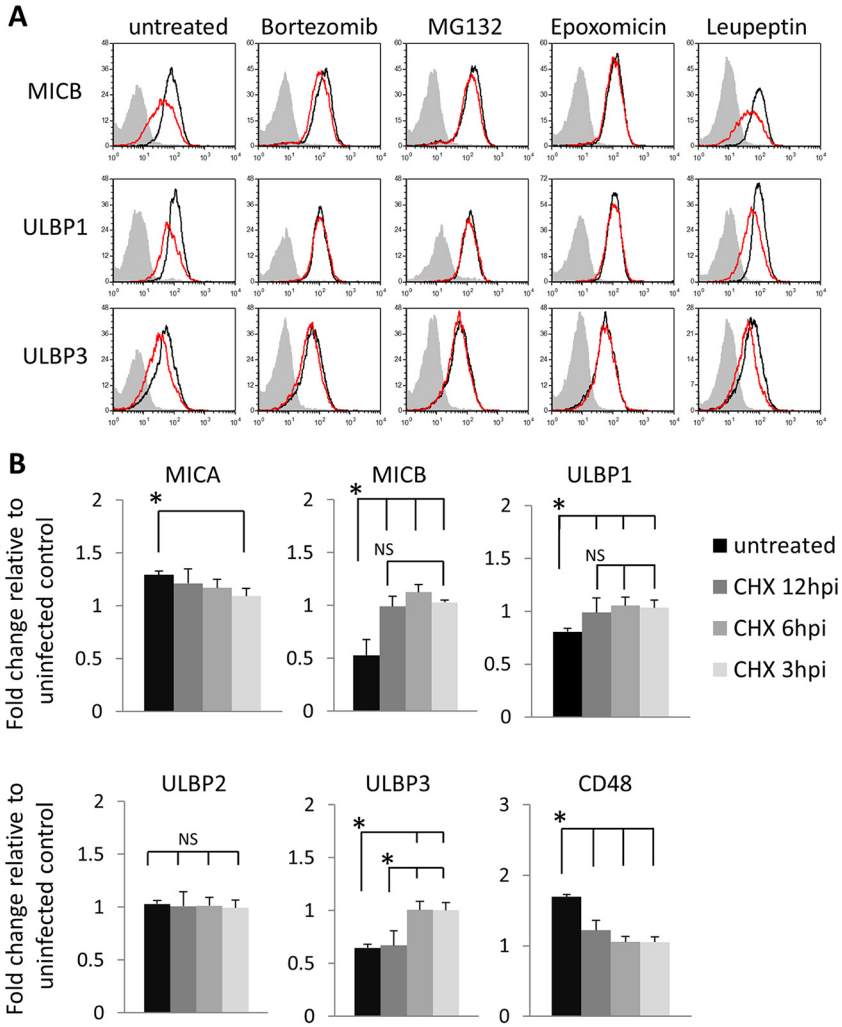

FIG 5 Proteasome inhibition and cycloheximide treatment reveal viral mode of action. (A) FACS staining of stress ligands in the course of HHV-6B infection with inhibition of protein degradation pathways. Cells were infected for 12 h with HHV-6B and treated for another 8 h with the proteasome inhibitor bortezomib, MG132, or epoxomicin or the lysosome protease inhibitor leupeptin. Control cells were left untreated. At 20 hpi, cells were washed and stained for MICB, ULBP1, and ULBP3 in FACS. The gray shaded histogram indicates the staining of an isotype antibody. The black histogram shows staining in uninfected cells, and the red histogram shows staining in HHV-6-infected cells. Three replicates of the experiment were performed, and results from one representative experiment are shown. (B) Cells were infected with HHV-6B and treated with cycloheximide at 3, 6, or 12 hpi. Controls were left untreated. At 18 hpi, cells were washed and stained for the NK ligands MICA, MICB, ULBP1, ULBP2, ULBP3, and CD48 for analysis by FACS. The detected mean fluorescence intensity (MFI) for infected cells was normalized by dividing by the MFI for uninfected cells with the same cycloheximide treatment. Data from at least four experiments per ligand are shown, and standard errors of mean were calculated. Statistics were calculated using analysis of variance (ANOVA) *t* test, and results of <0.01 (denoted by an asterisk) were considered significant. NS, not significant.

cells (red line). At 22 hpi, the HHV-6-mediated suppression of NKG2D ligands was so effective that we were unable to observe significant differences in NK cell degranulation in the presence or absence of the NKG2D-blocking antibody. NKp30 seemed to play a more dominant role in recognition of the cells, as blocking this receptor reduced NK cell degranulation to a greater extent than NKG2D blocking (green line). Interestingly, neither the downregulation of HLA class I molecules nor the strong induction of CD48 seems to play a major role at these time points, because NK cell degranulation was completely inhibited by simultaneous blocking of both NKp30 and NKG2D (blue line).

DISCUSSION

During HHV-6B virus replication, the cell cycle is halted, intracellular antiviral systems are shut off, and the transcription and translation machineries are coopted (52). Additionally, the lytically infected cells have to remain undetected by the immune system until newly formed virions are ready to spread the infection throughout the body.

NK cells that belong to the innate branch are specialized in the recognition and annihilation of virus-infected cells, as they express an array of germ line-encoded receptors to distinguish infected from healthy cells (29). In contrast to the case for T cells, the interaction of NK cells with HHV-6B-infected cells is less understood, and hitherto it was still unknown if and how HHV-6 evades NK cell recognition.

In this study, we found that HHV-6B also specifically inhibits the NK cell response. While HHV-6B-infected cells sense the viral intruder and rapidly increase mRNA levels of all NKG2D ligands

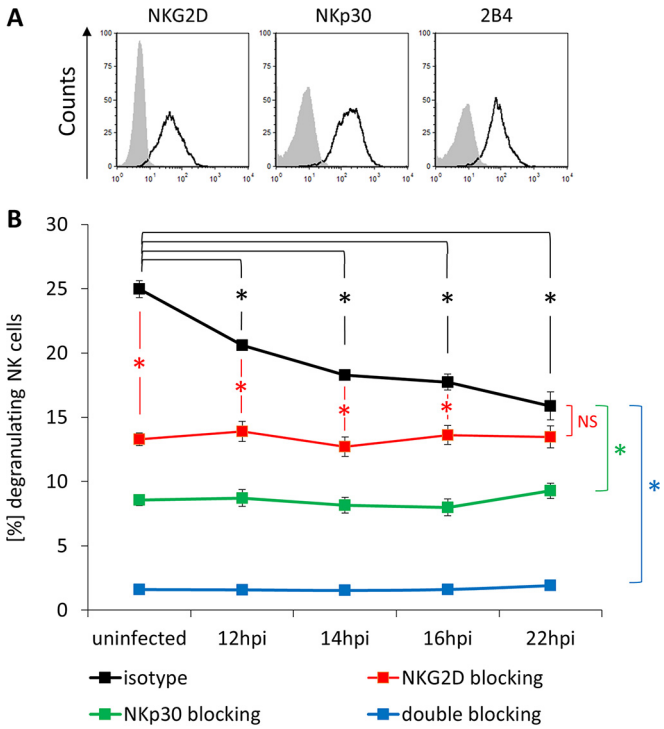

FIG 6 Reduction of ligands impairs the recognition of virally infected cells by NK cells. (A) Human bulk NK cell cultures were stained for the surface expression of the activating receptors NKG2D, NKp30, and 2B4 (empty black histograms). The gray shaded histogram shows staining of an isotype antibody on these cells. (B) NK cells were preblocked with an isotype control antibody or blocking antibodies targeting NKG2D, NKp30, or both receptors simultaneously. Subsequently, the preblocked NK cells were cocultivated at a ratio of 2:1 with SupT1 cells that were either uninfected or infected for the indicated number of hours. CD107a surface expression was assessed by FACS 2 h later. Control NK cells that were not coincubated with any target cells were defined as background degranulation. The percentage of CD107a-positive cells within the CD56-positive population was assessed and plotted. Merged data from four independent experiments are shown, with standard errors and statistics calculated by using Student's *t* test. *, $P < 0.05$; NS, not significant.

to alert the immune system, HHV-6B responds by inhibiting the surface expression of NK ligands. Two major types of ligands are targeted: the stress-induced ligands MICB, ULBP1, and ULBP3, which trigger the receptor NKG2D, and B7-H6, which triggers NKp30. Interestingly, our results suggest that the NKG2D ligands are regulated posttranslationally, while B7-H6 is targeted at the transcriptional level. The power of these viral mechanisms to downregulate the stress-induced ligand ULBP3 was particularly evident when we infected SupT1 cells that overexpressed this surface marker. Despite the presence of expression levels about 10-fold greater than physiological levels, we observed a marked (70%) reduction during infection in the IF experiments. Our data suggest that the affected NKG2D ligands are forced to proteasomal degradation, as we showed that proteasome inhibition can completely restore surface expression.

Cycloheximide treatments revealed that the expression of the viral proteins that downregulate cellular stress ligands occurs less than 12 h postinfection; therefore, they probably can be considered products of early genes and comprise some of the initial viral proteins expressed, demonstrating the importance of avoiding NK cell recognition.

Also, the cycloheximide treatment differentially affected the expression of ULBP1 and MICB on the one hand and ULBP3 on the other hand. When the treatment was applied at 3 or 6 hpi, all viral effects on these three stress ligands were negated, meaning that the translation process that is inhibited by cycloheximide treatment takes place at later time points. However, when the treatment was applied at 12 hpi, the effect on ULBP1 and MICB was still abrogated, while ULBP3 downregulation was unaffected, meaning that the viral protein relevant for ULBP3 regulation has already been translated at 12 hpi.

It remains unclear why the expression of CD48, MICA, and ULBP2 is not suppressed by this virus in both cell lines studied. One possible explanation is that the induction of these ligands is relatively low when balanced against the major downregulation of MICB, ULBP1, and ULBP3, so the net result leads to inhibition and there is no need to impair additional ligands. We confirmed that assumption, as we showed the staining with an NKG2D fusion was reduced upon infection. Another, less likely, explanation is that because MICA is highly polymorphic and since it was shown that HCMV possesses different mechanisms for different alleles (37), it may be possible that HHV-6 cannot target the specific MICA alleles expressed by SupT1 and MOLT-3 cells. Additionally, it is possible that the reactions of a transformed T cell and primary T cells following viral sensing vary tremendously. To test this hypothesis, we used activated CBMCs and infected them with HHV-6B. Importantly, we observed a decrease in the aforementioned NKG2D ligands MICB and ULBP3. ULBP1 and B7-H6 were not expressed or were only barely expressed on these primary cells. Interestingly, in our setting, we did not observe an increase of CD48, MICA, and ULBP2.

Regarding the NKp30 ligand B7-H6, our studies show that it is downregulated at the mRNA level. Future research will be required to determine if this effect is achieved by inhibiting transcription or by mRNA inhibition, potentially mediated by a viral microRNA or an RNA binding protein. Either way, our results suggest the existence of at least two independent viral proteins that are involved in the regulation of NKG2D ligands and at least one additional viral mechanism that targets B7-H6 at the mRNA level. Further studies are required to identify the responsible viral mechanisms by utilizing an expression or knockout library for HHV-6B strain Z29.

Strikingly, the degranulation of NK cells in the presence of HHV-6B-infected SupT1 cells depended solely on expression of NKG2D and NKp30 ligands, since it could be completely abolished by simultaneous blocking of both receptors. Interestingly, neither the induction of CD48, the ligand for the 2B4 receptor, nor the loss of HLA class I seemed to impact the activation of NK cells in the chosen setting. These findings might explain why the ligands which trigger NKG2D and NKp30 are exactly the ones that are suppressed during infection.

Our study shows for the first time that HHV-6 manipulates activating NK cell ligands during lytic infection. Additionally, we show for the first time that B7-H6, a ligand for NKp30, is involved in immune escape during lytic infection by a virus. The impairment of the NK cell immune surveillance enables viral immune evasion and completion of the viral replication cycle. This finding again illustrates the sophistication of herpesviruses, which evade both T cell and NK cell responses by unique virus-specific mechanisms to promote lifelong infection.

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D.S., S.D., and O.M conceptualized the study; D.S. developed the methodology and performed formal analysis; D.S., J.T., and F.L.-S. performed the experiments; D.S. and O.M. wrote the draft and administered the project; D.S., J.T., S.D., O.M., and F.L.-S. reviewed and edited the manuscript; O.M. supervised the project.

We declare no conflicts of interest.

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