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Varicella-Zoster Virus and Herpes Simplex Virus 1 Differentially Modulate NKG2D Ligand Expression during Productive Infection

Tessa M. Campbell, Brian P. McSharry, Megan Steain, Barry Slobedman, Allison Abendroth

Discipline of Infectious Diseases and Immunology, Sydney Medical School, Charles Perkins Centre, University of Sydney, Sydney, NSW, Australia

ABSTRACT

Natural killer (NK) cell-deficient patients are particularly susceptible to severe infection with herpesviruses, especially varicella-zoster virus (VZV) and herpes simplex virus 1 (HSV-1). The critical role that NK cells play in controlling these infections denotes an intricate struggle for dominance between virus and NK cell antiviral immunity; however, research in this area has remained surprisingly limited. Our study addressed this absence of knowledge and found that infection with VZV was not associated with enhanced NK cell activation, suggesting that the virus uses specific mechanisms to limit NK cell activity. Analysis of viral regulation of ligands for NKG2D, a potent activating receptor ubiquitously expressed on NK cells, revealed that VZV differentially modulates expression of the NKG2D ligands MICA, ULBP2, and ULBP3 by upregulating MICA expression while reducing ULBP2 and ULBP3 expression on the surface of infected cells. Despite being closely related to VZV, infection with HSV-1 produced a remarkably different effect on NKG2D ligand expression. A significant decrease in MICA, ULBP2, and ULBP3 was observed with HSV-1 infection at a total cellular protein level, as well as on the cell surface. We also demonstrate that HSV-1 differentially regulates expression of an additional NKG2D ligand, ULBP1, by reducing cell surface expression while total protein levels are unchanged. Our findings illustrate both a striking point of difference between two closely related alphaherpesviruses, as well as suggest a powerful capacity for VZV and HSV-1 to evade antiviral NK cell activity through novel modulation of NKG2D ligand expression.

IMPORTANCE

Patients with deficiencies in NK cell function experience an extreme susceptibility to infection with herpesviruses, in particular, VZV and HSV-1. Despite this striking correlation, research into understanding how these two alphaherpesviruses interact with NK cells is surprisingly limited. Through examination of viral regulation of ligands to the activating NK cell receptor NKG2D, we reveal patterns of modulation by VZV, which were unexpectedly varied in response to regulation by HSV-1 infection. Our study begins to unravel the undoubtedly complex interactions that occur between NK cells and alphaherpesvirus infection by providing novel insights into how VZV and HSV-1 manipulate NKG2D ligand expression to modulate NK cell activity, while also illuminating a distinct variation between two closely related alphaherpesviruses.

Varicella-zoster virus (VZV) and herpes simplex virus type 1 (HSV-1) are two medically important human alphaherpesviruses that cause widespread disease in human hosts. VZV is the causative agent of varicella (chickenpox) and herpes zoster (shingles), while HSV-1 causes recurrent orofacial herpes infection and, in severe cases, encephalitis. Despite manifestation as distinct diseases, these two viruses share a high degree of homology in the structures of their genomes and encode many similar proteins, as well as employ extensive immune evasion strategies to evade early detection and clearance during primary infection (reviewed in references 1 and 2). Control of viral infection involves a coordinated response from both the innate and adaptive arms of the immune system (reviewed in references 3 and 4). For VZV and HSV-1, this is especially dependent on efficient natural killer (NK) cell activity, as evident from clinical observations of extreme susceptibility to disseminated, life-threatening VZV and HSV-1 infections in NK cell-deficient patients (5–12).

Like other human herpesviruses, both VZV and HSV-1 downregulate surface expression of major histocompatibility complex class I (MHC-I) molecules to protect infected cells from $CD8^+$ T cell recognition (13–17). This state of "missing self," where inhibitory NK cell receptors are no longer engaged, typically renders virally infected cells more sensitive to NK cell lysis (18, 19). To counteract this, many viruses encode mechanisms to evade NK cell detection and activity. Considering the clear importance of NK cells in human alphaherpesvirus infections, it is surprising that this critical point of interaction has not been studied in significant detail. In regard to VZV, research has been limited to early studies, which suggested that NK cells are capable of lysing VZV-infected target cells (20, 21); however, to our knowledge, investigation into the direct interactions that occur is completely absent from the literature. NK cell lysis of infected cells has also been shown for HSV-1 (22), with only a small number of studies examining how HSV-1 interacts with human NK cells (23, 24), and only

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Address correspondence to Allison Abendroth, allison.abendroth@sydney.edu.au.

T.M.C. and B.P.M. contributed equally to this work. B.S. and A.A. contributed equally to this work.

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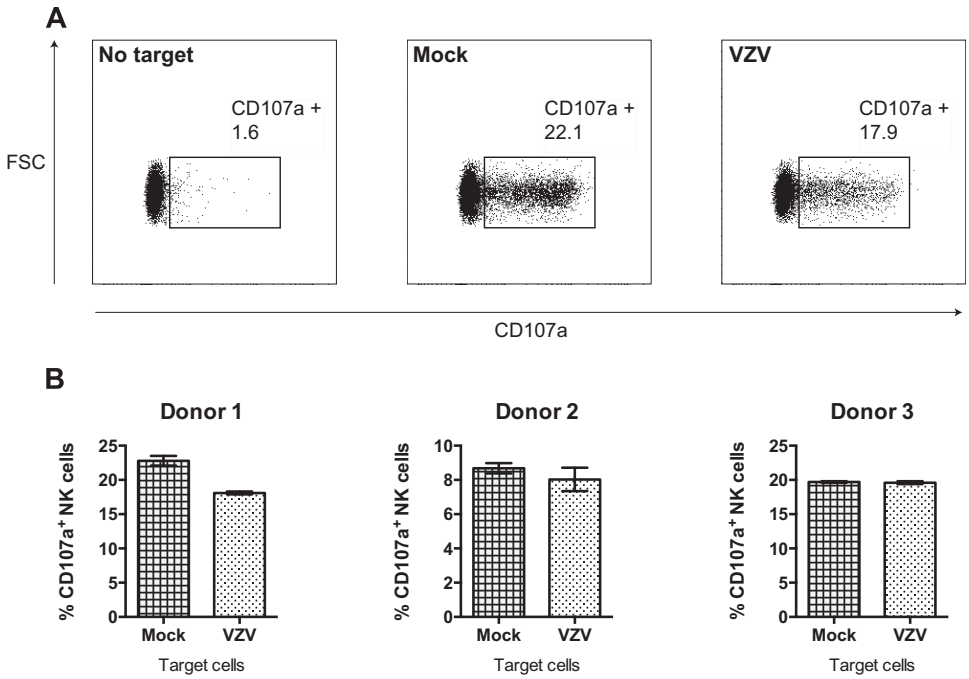

FIG 1 VZV-infected target cells do not enhance activation of NK cells in coculture. CD107a assays were performed with IFN-α-stimulated human PBMCs using ARPE-19 cells infected for 48 h as targets. Flow cytometry was used to identify NK cells ($CD56^+$ $CD3^-$) for CD107a detection. (A) Representative dot plots for donor 1 show comparison of levels of NK cell CD107a expression when coincubated with no target (negative control), mock-infected ARPE-19 cells, or VZV-infected ARPE-19 cells. (B) Three separate donors were assessed. Means ± SEM from duplicate samples are shown.

one other report examining the impact of other alphaherpesviruses on NK cells, in which it was shown that HSV-2 and pseudorabies virus (PRV) are able to suppress NK cell lysis of infected cells via gD glycoprotein-mediated downregulation of the cellular DNAM-1 ligand CD112 (63).

In contrast to the alphaherpesviruses, the impact of beta- and gammaherpesviruses on NK cell interactions is well established (reviewed in reference 25). A common theme among these herpesviruses is targeted interference with expression of ligands for NKG2D, an NK cell activating receptor. NKG2D is one of few receptors expressed ubiquitously on NK cells, as well as being present on most $CD8^+$ αβ T cells, γδ T cells, and a subset of NKT cells (26, 27). Distinctively, NKG2D recognizes several ligands upregulated on stressed cells, particularly due to viral infection (reviewed in references 28-30). Currently eight NKG2D ligands (NKG2DLs) have been identified in humans: MHC-I chain-related proteins A and B (MICA and MICB) and UL16-binding proteins 1 to 6 (ULBP1 to -6) (31, 32). With the diversity of ligands, as well as the strong NK cell-activating signal provided by NKG2D engagement, it is perhaps not surprising that disruption of NKG2DL expression is a common viral immune evasion strategy. In the herpesvirus family, mechanisms of NKG2DL modulation have been identified for human cytomegalovirus (HCMV) (32–37), human herpesvirus 7 (HHV-7) (38), Kaposi's sarcoma-associated herpesvirus (KSHV) (39, 40), and Epstein-Barr virus (EBV) (40).

Given the shared target of NKG2DLs for immune evasion among human herpesviruses, as well as the documented susceptibility to severe and persistent alphaherpesvirus infections in patients with NK cell deficiencies, we hypothesized that VZV and HSV-1 may also modulate NKG2DL expression. In the present study, we examined the interaction of alphaherpesviruses with NK cells, focusing on the regulation of NKG2DLs during VZV and HSV-1 infection. We demonstrate that VZV has a dual effect on NKG2DL expression—strongly upregulating MICA, while reducing cell surface expression of ULBP2 and ULBP3. In contrast, HSV-1 was found to potently diminish both total cellular protein and cell surface expression of MICA, ULBP2, and ULBP3. We also identify an alternative pattern of modulation by HSV-1 for ULBP1, where cell surface expression was downregulated but protein levels were unchanged. This study presents new findings on the regulation of NKG2DL expression by VZV and HSV-1 and reveals a significant difference between two closely related human alphaherpesviruses.

MATERIALS AND METHODS

Cells. Human foreskin fibroblasts (HFFs), ARPE-19 epithelial cells, human embryonic kidney 293T cells, and Vero cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS).

Viruses and treatment. Clinical strain VZV-S (41) (kindly provided by A. Arvin, Stanford University) was propagated in ARPE-19 cells. For infections, VZV-infected ARPE-19 cells at a cytopathic effect of 3+ to 4+ (approximately 75 to 100% of the cells showing altered morphology) were added to seeded ARPE-19 cells at a ratio of 1:1 or 1:10 infected cells to uninfected cells. A clinical isolate of HSV-1 (strain F) (42) (kindly provided by R. Diefenbach, Westmead Millenium Institute) was propagated in Vero cells and used at a multiplicity of infection (MOI) of 3 to infect seeded cells. UV irradiation of HSV-1 was performed as previously described (43). Successful UV inactivation was confirmed by lack of plaque formation over 12 days in a cell monolayer inoculated with UV-inactivated HSV-1 (UV-HSV).

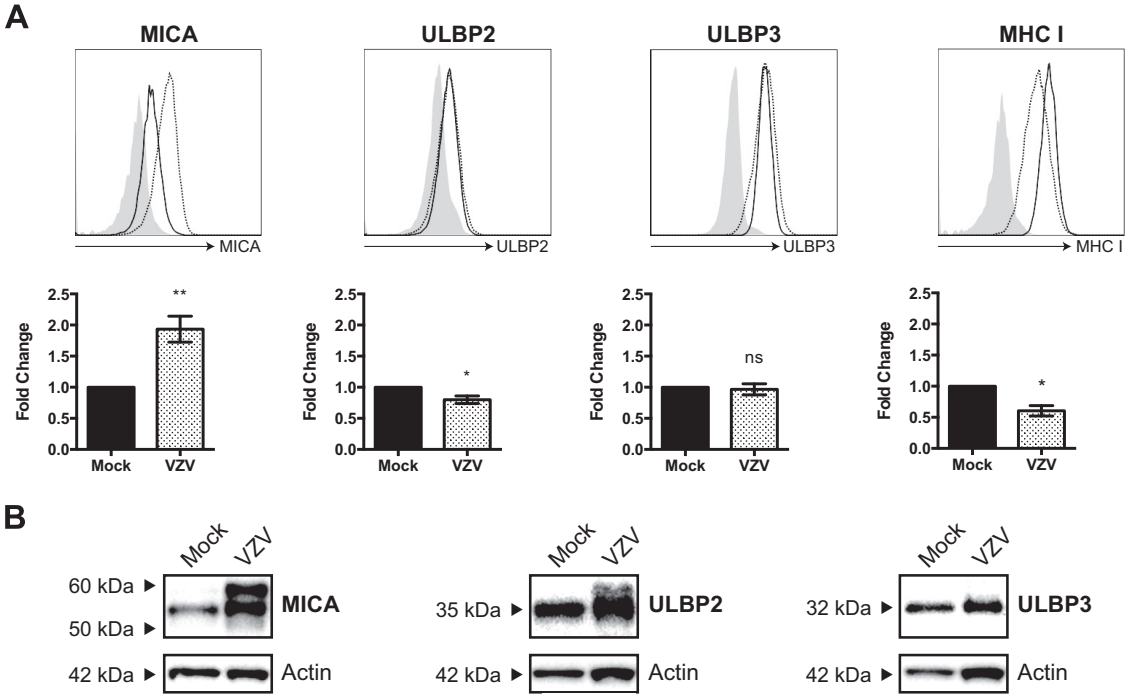

FIG 2 VZV differentially regulates NKG2D ligand expression. ARPE-19 cells were infected with VZV or mock infected at a 1:1 ratio and harvested 48 hpi. (A) Cells were stained with antibodies against MICA, ULBP2, ULBP3, and MHC-I, and levels of expression were compared between mock (continuous black lines) and VZV (dotted black lines) infections on the cell surface, using flow cytometry. Isotype control antibody staining is indicated by the gray-filled histogram. One representative histogram of at least three independent experiments is shown. The relative mean fluorescence intensity (MFI) fold change over mock in NKG2D ligand or MHC-I expression (less respective isotypes) is presented below each correlating histogram as the mean ± SEM of data from biological replicates (NKG2DLs, $n = 6$; MHC-I, $n = 4$). Statistical significance was established by two-tailed paired Student's *t* test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. (B) Mock- or VZV-infected protein lysates were harvested at 48 hpi and immunoblotted with antibodies against MICA, ULBP2, and ULBP3. Corresponding probing for a housekeeping protein (actin) is shown beneath. One representative blot of three independent experiments is shown.

NK cell CD107a mobilization assays. ARPE-19 cells were mock or virally infected with VZV at a ratio of 1:1 for 48 h for use as target cells. Peripheral blood mononuclear cells (PBMCs), isolated from human blood in accordance with University of Sydney ethics approval, were stimulated overnight with interferon alpha (IFN-α) (1,000 U/ml) prior to coincubation of 1×10^6 PBMCs with target cells for 5 h total. Assays were performed in the presence of anti-CD107a–fluorescein isothiocyanate (FITC) monoclonal antibody (MAb) (clone H4A3; BD Biosciences), with the addition of 4 μl/ml GolgiStop (BD Biosciences) for the last 4 h. PBMCs were subsequently harvested and stained for 30 min at 4°C with anti-CD56-allophycocyanin (APC) (clone B159, BD Biosciences) and anti-CD3–phycoerythrin (PE) (clone HIT3a; BD Biosciences) MAbs. A minimum of 5×10^3 $CD56^+$ $CD3^-$ NK cells were acquired using a FACSCanto flow cytometer (BD Biosciences), and data were analyzed with FlowJo software.

Flow cytometry. Cells were detached with trypsin-EDTA, washed with phosphate-buffered saline (PBS), and stained with primary antibodies in flow cytometry staining buffer (PBS with 1% FCS and 10 mM EDTA) for 30 min at 4°C. The following MAbs were utilized: anti-MICA (clone 159227), anti-ULBP1 (clone 170818), anti-ULBP2 (clone 165903), anti-ULBP3 (clone 166510; all R&D Systems), anti-MHC-I (HLA-A, -B, and -C, clone G46-2.6; BD Biosciences), anti-CD71 (clone T56/14; Life Technologies [PE conjugated]), and anti-glycoprotein E/glycoprotein I (gE:gI) (clone SG1; Meridian Life Science [FITC conjugated]). All primary antibodies were conjugated to APC unless otherwise specified. Appropriate isotype control antibodies were also used for each specific stain. For intracellular staining, cells were fixed with 1% paraformaldehyde (PFA) and permeabilized with BD Perm/Wash buffer (BD Biosciences) prior to staining. Flow cytometry was performed using a FACSCanto, and data were analyzed with FlowJo software.

Protein analysis. Total cell lysates were prepared by scraping the cell monolayer and lysing in cell lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP-40, and 1% Triton X-100) containing protease inhibitors (Sigma-Aldrich). Lysates were denatured by heating in reducing sample buffer (Bio-Rad) and resolved by SDS-PAGE on polyacrylamide gels. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes and probed with the designated primary antibodies, followed by incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (all Santa Cruz Biotechnology). The following primary antibodies were utilized: anti-MICA (clone ab150355; Abcam), anti-ULBP1 (clone ab176566; Abcam), anti-ULBP2 (clone AF1298; R&D Systems), and anti-ULBP3 (clone AF1517; R&D Systems). Subsequent probing with anti-actin (clone A2066; Sigma-Aldrich) was used to control for protein loading. Bands were visualized by enhanced chemiluminescence.

Statistical analysis. Paired Student's *t* tests were performed using GraphPad Prism software. Data are presented as means ± standard errors of the means (SEM).

RESULTS

VZV limits NK cell activation in coculture. To initiate in-depth study into the interaction of NK cells with VZV, NK cell activation during coculture with VZV-infected target cells was first assessed by a CD107a mobilization assay. CD107a protein, which lines preformed cytolytic granules, remains on the surface of activated cells following degranulation, and thus the cell surface binding of CD107a antibody can be used as a functional marker for NK cell activation (44). The assay was performed using IFN-α-stimulated human PBMCs cocultured with ARPE-19 epithelial cells that had

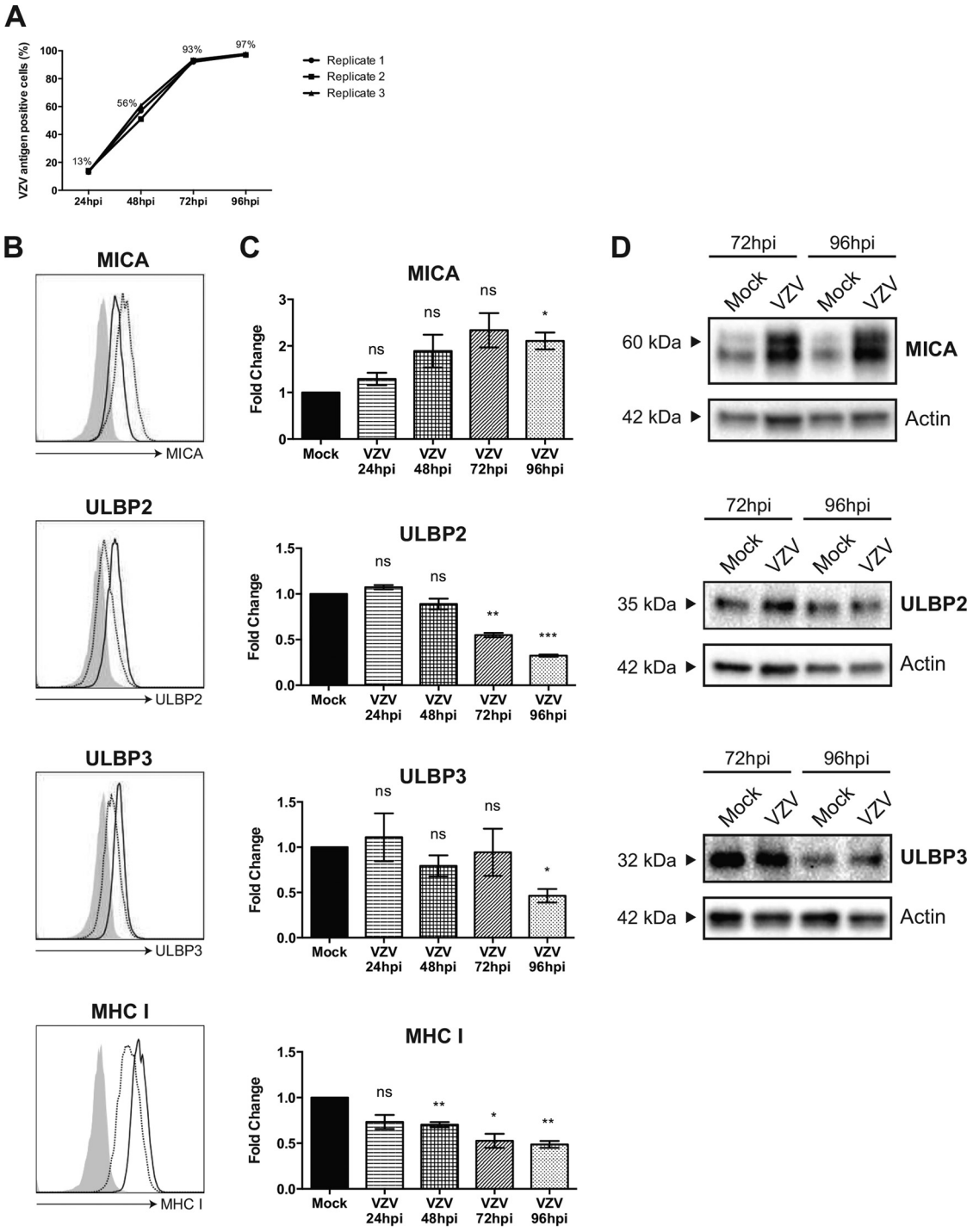

FIG 3 VZV significantly modulates cell surface NKG2D ligand expression over time. ARPE-19 cells were infected with VZV or mock infected at a 1:10 ratio and harvested at various times postinfection. (A) The percentage of total cells infected over time was monitored by flow cytometry staining for VZV antigen, gE:gI, at each time point, for three independent experiments. The mean percentage of VZV antigen-positive cells is recorded above each time point. (B and C) Mock- or VZV-infected cells were harvested at 24, 48, 72, and 96 hpi and stained with antibodies against MICA, ULBP2, ULBP3, and MHC-I for flow cytometry analysis. (B) One representative histogram at 96 hpi is shown, comparing mock (continuous black lines) and VZV (antigen-positive [dotted black lines]) infections for cell surface NKG2D ligand or MHC-I expression. Isotype control antibody staining is indicated by the gray-filled histogram. (C) The relative MFI fold change over mock infection in surface NKG2D ligand or MHC-I expression (less respective isotypes) over time is presented as the mean ± SEM of data from biological replicates ($n = 3$). Statistical significance was established by two-tailed paired Student's *t* test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) Mock- or VZV-infected protein lysates were harvested at 72 and 96 hpi for immunoblotting with antibodies against MICA, ULBP2, and ULBP3. Corresponding probing for a housekeeping protein (actin) is shown beneath. One representative blot of three independent experiments is shown.

been mock or virally infected with VZV at a 1:1 ratio of infected to uninfected cells for 48 h. As a consequence of the highly cell-associated nature of VZV infection *in vitro* (45), a cell-associated model of infection was employed, and ARPE-19 cells were used due to their high permissibility to VZV infection (46). At the time of the assay, greater than 95% of ARPE-19 target cells were confirmed as VZV infected by parallel flow cytometry staining for VZV antigen, gE:gI. As a negative control in the CD107a assay, PBMCs incubated alone without target cells were used, stimulating low-level spontaneous activation detected via CD107a expression on $CD56^+$ $CD3^-$ NK cells (Fig. 1A). Analysis of CD107a expression from three separate donors revealed that NK cell activation was not increased by coculture with VZV-infected cells compared to mock target cells (Fig. 1). This finding indicates that VZV infection does not enhance NK cell activation, as could be predicted, suggesting that the virus employs specific mechanisms to limit NK cell functional activity.

VZV differentially regulates expression of NKG2DLs. The lack of increase in NK cell activity during coculture with VZV-infected target cells prompted investigation into the potential regulation of ligands for NK cell receptors by VZV. Focusing on NKG2DLs, cell-surface expression in ARPE-19s was assessed following mock or VZV infection performed at a ratio of 1:1, with typically 96% of cells staining positive for VZV antigen, gE:gI, at 48 h postinfection (hpi). VZV-infected cells displayed a significant upregulation of MICA expression, while the levels of the other NKG2DLs, ULBP2 and ULBP3, were not distinctly changed on the cell surface (Fig. 2A). Additionally, cell surface expression of the NKG2DLs MICB and ULBP1 was examined but below the limits of detection (data not shown). In parallel, we confirmed previous reports (14–16) that cell surface MHC-I was downregulated with VZV infection (Fig. 2A).

The pattern of NKG2DL modulation observed on the cell surface was also reflected at a total cellular protein level, as assessed by Western blotting. A dramatic increase in MICA cellular protein was detected in VZV-infected cells, contrasting with the expression of ULBP2 and ULBP3, which remained comparable to mock infection (Fig. 2B). These results indicate two distinct patterns of NKG2DL modulation by VZV at the cell surface and total cellular protein level.

VZV upregulates MICA but restricts ULBP2 and ULBP3 expression on the cell surface. Analysis of the effect of VZV on infected cells is complicated by the asynchronous spread of virus that arises from a cell-associated model of infection. The highly permissive nature of ARPE-19 cells to VZV infection, however, allowed the use of a lower proportion of infected inoculating cells, which limited the influence of the inoculum on subsequent analysis. By performing these experiments at a ratio of 1:10 infected to uninfected cells, it was possible to focus the analysis of VZV modulation of NKG2DL expression on newly infected cells, as well as assess changes over time. Infection consistently spread at a steady rate from the inoculum to seeded cells in each of the three replicate experiments, reaching a peak of 97% infected by 96 hpi, as detected by flow cytometry staining for VZV antigen, gE:gI (Fig. 3A). Flow cytometry was therefore used to investigate NKG2DL cell surface expression at 24, 48, 72, and 96 hpi, while Western blot analysis, which examines the total cell population, was only employed to analyze expression at 72 and 96 hpi to ensure the majority of cells assessed were infected with VZV. These analyses revealed an upregulation of MICA cell-surface expression over time with VZV infection, which reached statistical significance at 96 hpi (Fig. 3B and C). This was accompanied by an increase in total cellular protein levels of MICA in VZV-infected cells at both 72 and 96 hpi (Fig. 3D). Of note, time course analysis of ULBP2 expression revealed a decrease in cell surface ULBP2 on infected cells, compared to the increase in MICA (Fig. 3B and C). The significant downregulation in cell surface ULBP2 at 72 and 96 hpi was in contrast to total cellular protein levels at the same time points, which did not markedly differ between mock- and VZV-infected cells (Fig. 3D). Unlike MICA and ULBP2, VZV regulation of cell surface ULBP3 displayed a less consistent modulation at 24 to 72 hpi; however, by 96 hpi, ULBP3 was significantly downregulated (Fig. 3B and C). Despite the decrease in cell surface ULBP3, total cellular ULBP3 protein was not reduced in VZV-infected cells (Fig. 3D). As expected, MHC-I expression was significantly downregulated by VZV infection over time (Fig. 3B and C). Collectively, the data demonstrate that MICA expression is stimulated by VZV, while also revealing a reduction of ULBP2 and ULBP3 protein on the cell surface. These findings, together with the lack of detection of NK cell activation (Fig. 1), provide further evidence of VZV-mediated control of NK cell function.

HSV-1 diminishes expression of MICA, ULBP2, and ULBP3 on the surface of infected cells. In addition to VZV, modulation of NKG2DLs by HSV-1 was assessed to compare immunomodulatory functions between these two closely related alphaherpesviruses. A time course analysis was employed to assess cell surface expression during HSV-1 infection of ARPE-19s, which revealed a remarkably different phenotype of NKG2DL modulation from that observed during VZV infection. In comparison to mock-infected cells, infection with HSV-1 significantly downregulated MICA, ULBP2, and ULBP3 over time (Fig. 4). ULBP2 downregulation was detected by 6 hpi and decreased further at 12 and 24 hpi (Fig. 4A and C). At 12 hpi, MICA and ULBP3 were also significantly reduced on the surface of infected cells compared to mock-infected cells (Fig. 4B and C), and all NKG2DLs were further reduced on the cell surface by 24 hpi (Fig. 4C). MHC-I was confirmed to be consistently downregulated with HSV-1, as previously described (13, 17) (Fig. 4). To validate the observed decreases in cell surface ligands as a product of selective regulation, and not a general effect on host cell proteins, expression of transferrin receptor (CD71) was also examined. As shown in Fig. 4, CD71 was not downregulated on the surface of HSV-1-infected cells at any of the time points assessed.

The ability of HSV-1 to potently downregulate expression of MICA, ULBP2, and ULBP3 was then assessed in two additional cell types, comparing infection in ARPE-19 cells to that in HFFs and 293Ts. Analysis of cell surface expression at 24 hpi revealed that for all cell types assessed, HSV-1 exerted a striking downregulation of NKG2DLs (Fig. 5). In many cases, HSV-1 infection reduced NKG2DL surface expression to near isotype control levels, especially in ARPE-19 cells, where expression of MICA was reduced 4.5-fold, expression of ULBP2 30-fold, and expression of ULBP3 9-fold, relative to mock infection (Fig. 5A). As previously demonstrated, MHC-I was consistently reduced by HSV-1 infection (Fig. 5), while CD71 was not downregulated on infected cells in any cell type assessed (data not shown). These data indicate that HSV-1 is capable of targeting MICA, ULBP2, and ULBP3, significantly reducing the expression of these ligands on the surface of various cell types during infection.

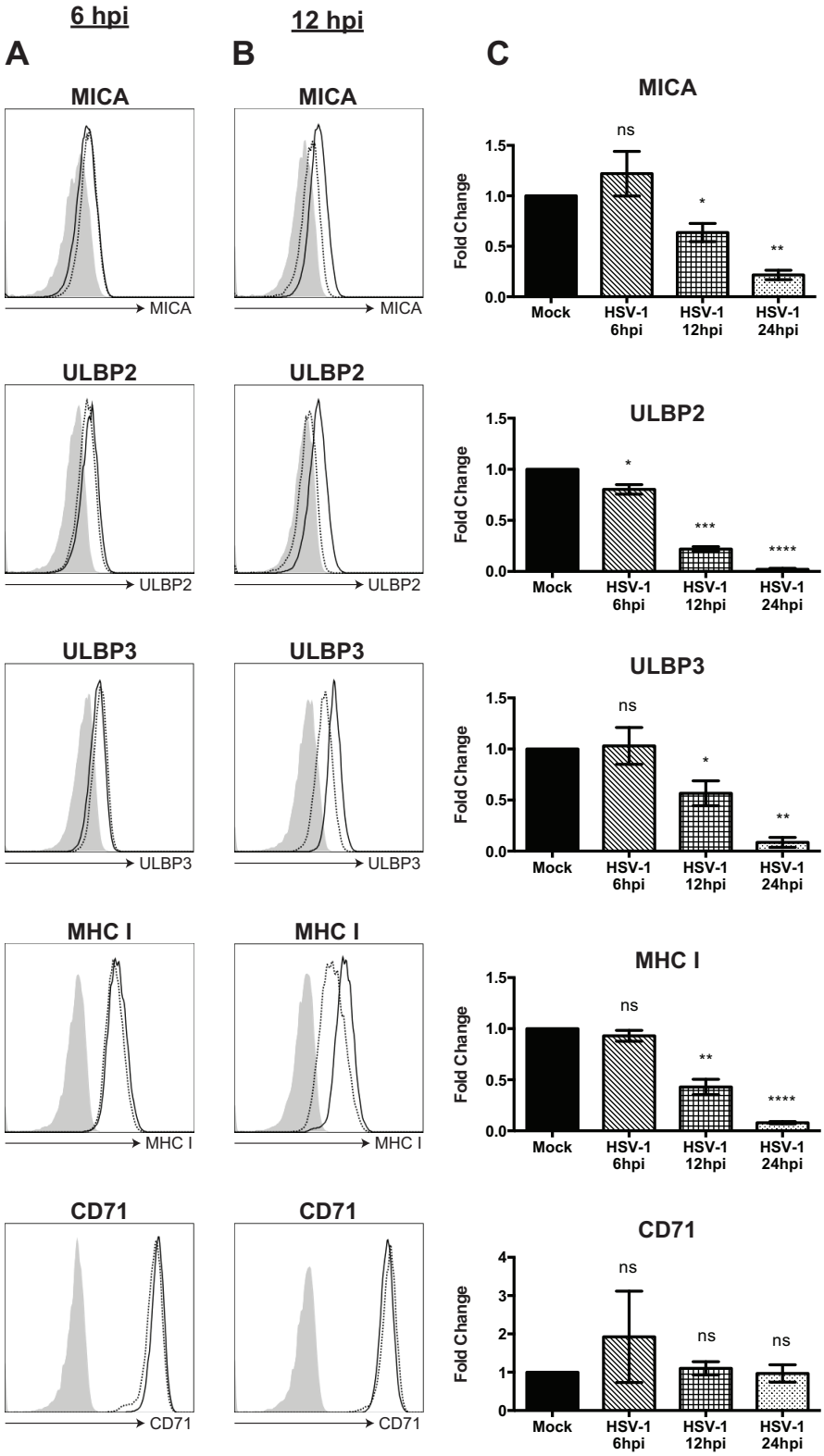

FIG 4 HSV-1 significantly downregulates cell surface NKG2D ligand expression over time. ARPE-19 cells were infected with HSV-1 (MOI of 3) or mock infected and stained at 6, 12, and 24 hpi for analysis by flow cytometry. Cells were stained with antibodies against MICA, ULBP2, ULBP3, MHC-I, and CD71, and levels of surface expression were compared between mock (continuous black line) and HSV-1 (dotted black line) infections. Isotype control antibody staining is indicated by the gray-filled histogram. Representative histograms at 6 hpi (A) and 12 hpi (B) are shown. (C) The relative MFI fold change over mock infection in surface NKG2D ligand, MHC-I, or CD71 expression (less respective isotypes) is presented as the mean ± SEM of data from biological replicates ($n = 3$). Statistical significance was established by one-tailed paired Student's t test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

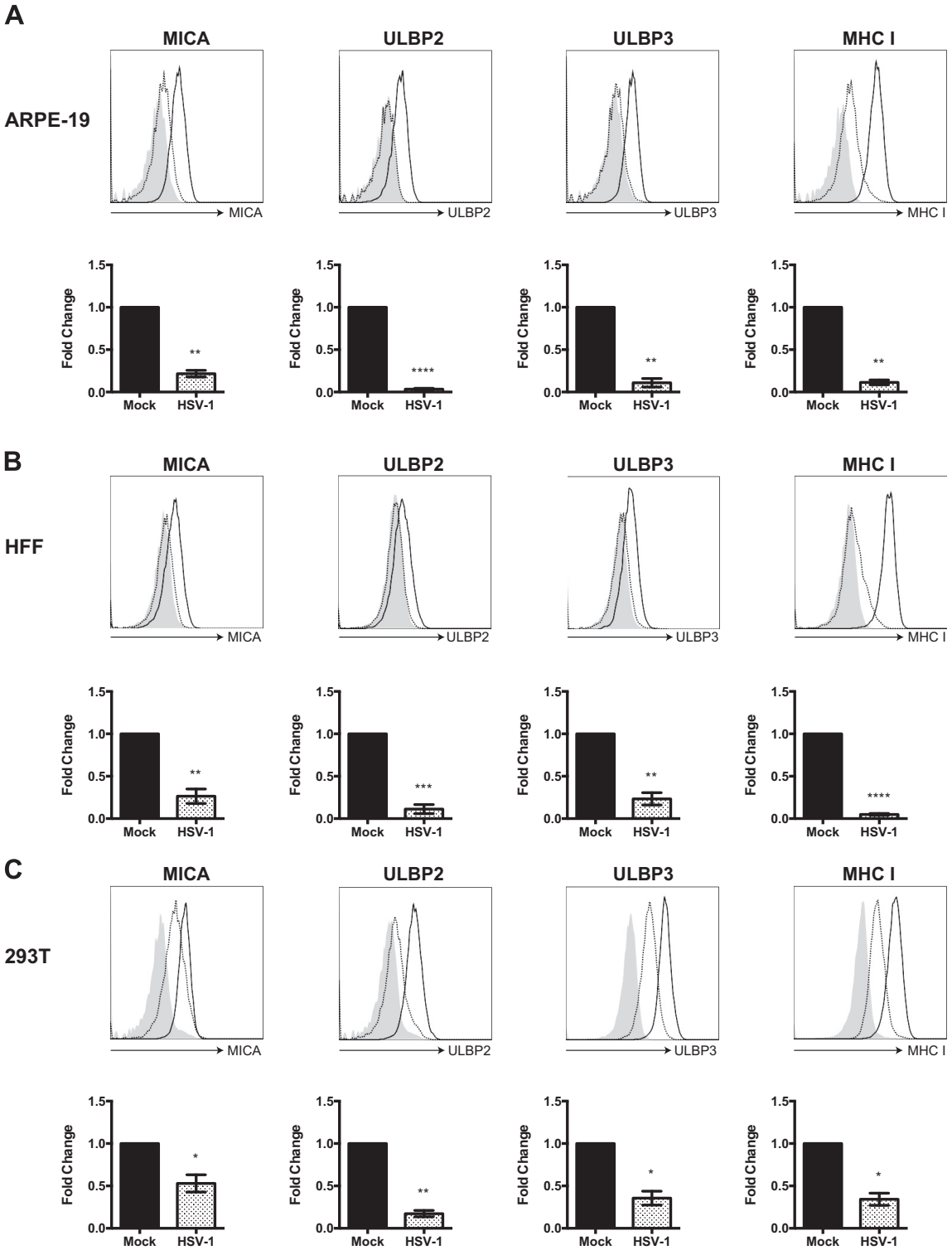

FIG 5 HSV-1 downregulation of cell surface NKG2D ligand expression occurs in various cell types. ARPE-19 (A), HFF (B), and 293T (C) cells were infected with HSV-1 (MOI of 3) or mock infected and stained 24 hpi for analysis by flow cytometry. Cells were stained with antibodies against MICA, ULBP2, ULBP3, and MHC-I, and levels of surface expression were compared between mock (continuous black lines) and HSV-1 (dotted black lines) infections. Isotype control antibody staining is indicated by the gray-filled histogram. One representative histogram of at least three independent experiments is shown. The relative MFI fold change over mock infection in surface NKG2D ligand or MHC-I expression (less respective isotypes) is presented below each correlating histogram as the mean ± SEM of data from biological replicates (A, $n = 3$; B, $n = 4$; C, $n = 3$). Statistical significance was established by two-tailed paired Student's t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

HSV-1 regulation of NKG2DL total cellular protein contrasts with VZV infection and highlights differential modulation by HSV-1. To determine whether the dramatic reduction in cell-surface NKG2DL expression with HSV-1 infection was reflected at the total cellular protein level, Western blot analyses were performed. At 24 hpi, HSV-1-infected cells showed a striking reduction in MICA, ULBP2, and ULBP3 cellular protein in both ARPE-19 cells (Fig. 6A) and HFFs (Fig. 6B), while maintaining levels of actin expression comparable to that in mock infection. This modulation by HSV-1 contrasted with VZV infection, which maintained or increased NKG2DL total cellular protein compared to mock infection. In order to establish whether *de novo* viral gene expression was necessary for this striking reduction in MICA, ULBP2, and ULBP3, UV-inactivated virus infection was utilized. Comparison of mock infection and HSV-1 infection to HFFs inoculated with UV-inactivated HSV-1 (UV-HSV), revealed unchanged MICA, ULBP2, and ULBP3 protein expression between the mock and UV-HSV infections, contrasting with the potent downregulation observed with viable HSV-1 infection (Fig. 6B). This finding validates a requirement for *de novo* HSV-1 gene expression to mediate the downregulation in MICA, ULBP2, and ULBP3 total cellular protein in infected cells.

Using Western blot analysis, total protein levels of an additional NKG2DL, ULBP1, could also be assessed. This analysis demonstrated that HSV-1 regulation of ULBP1 protein was markedly different from those of the other NKG2DLs examined. In contrast to the reduction in protein expression observed for MICA, ULBP2, and ULBP3 in HSV-1-infected cells, the total cellular protein level of ULBP1 was unaltered by HSV-1 infection, with comparable levels between mock, viable HSV-1, and UV-HSV infections (Fig. 6C).

Given that HSV-1 differentially regulated ULBP1 at the total protein level, viral regulation of this NKG2DL was more closely examined using 293T cells, which express detectable levels of cell surface ULBP1. While intracellular staining demonstrated no significant difference in total ULBP1 expression between mock- and HSV-1-infected cells (Fig. 7A), ULBP1 expression was significantly reduced on the cell surface with HSV-1 infection (Fig. 7B).

These data identify two differential patterns of NKG2DL regulation by HSV-1: while MICA, ULBP1, ULBP2, and ULBP3 are all strongly downregulated on the surface of infected cells, only ULBP1 is exempt from an equally potent downregulation in total cellular protein by HSV-1 infection.

DISCUSSION

Clinical evidence has implicated a critical role for NK cells in VZV and HSV-1 pathogenesis (5–12); however, research into the molecular interactions that occur has remained limited. Both of these viruses are highly successful pathogens, capable of significant immune evasion in order to establish disease and latency following primary infection. NK cells are a key component of the initial innate immune response, and thus evasion of antiviral NK cell activity would be beneficial to the virus in establishing infection. Here we demonstrate novel findings of immune modulation by VZV and HSV-1 that would be likely to impede detection of viral infection by NK cells. This study is the first to report an impact of VZV on NKG2DL expression and represents a comprehensive examination of HSV-1 regulation of NKG2DLs at both the cell surface and total cellular protein levels.

Evasion of $CD8^+$ T cell recognition via downregulation of cell surface MHC-I occurs during many viral infections, including VZV (14–16); however, the reduction of inhibitory receptor signaling via MHC-I downregulation would be predicted to increase susceptibility to NK cell attack (18, 19). It would therefore be expected that coincubation of NK cells with VZV-infected target cells would stimulate NK cell activity. In contrast, however, we show that NK cell activation (as measured by expression of CD107a) did not increase following exposure to VZV (Fig. 1), implying extensive immune evasion of additional NK cell ligands in order to restrict NK cell activation. This finding is similar to that of HCMV, where infected cells are resistant to NK cell-mediated killing, despite a strong reduction in cell surface MHC-I (47). Through stimulation of inhibitory receptors on NK cells and downregulation of several activating ligands, especially NKG2DLs, HCMV is able to evade NK cell activity (reviewed in reference 48).

The absence of heightened NK cell activation when exposed to VZV warranted further investigation into the possible modulation of ligands to activating NK cell receptors. NKG2D is one of the best-characterized activating NK cell receptors, and the signal provided by receptor engagement is of sufficient strength to override the inhibitory signal mediated by binding of MHC-I (26). This makes NKG2D a dominant influence on NK cell activity but also designates it as a prime target for immune evasion by viruses. In this study, we demonstrate that VZV modulates expression of NKG2DLs, identifying two distinct patterns of regulation. MICA was potently induced by VZV infection at a total cellular protein level as well as on the cell surface, from 24 to 96 hpi. At 48 hpi, the expression and cellular localization of ULBP2 and ULBP3 were not significantly modulated by VZV. By 96 hpi, VZV selectively downregulated cell surface expression of ULBP2 and ULBP3, while maintaining increased levels of MICA. Interestingly, total cellular ULBP2 and ULBP3 protein remained unchanged relative to mock infection, implying an immunomodulatory function of VZV to retain these molecules within infected cells. It is also possible that VZV is capable of intracellularly retaining MICA, despite the cell surface increases observed in this study. It has been shown for adenovirus that wild-type virus can upregulate cell surface MICA relative to mock infection in some cell types; however, this increase is greatly amplified when the E3/19K gene, responsible for retaining MICA in the endoplasmic reticulum, is deleted (49). Thus, identification of any VZV gene or genes that may encode functions to retain NKG2DLs within infected cells will be an important component of future studies aimed at further defining the mechanism of VZV-mediated NK cell evasion.

The results presented here indicate that VZV has a differential, rather than global, effect on the expression of NKG2DLs. In the cellular regulation of these ligands, several stress responses have been implicated in modulating expression, with activation of the heat shock pathway selectively upregulating MICA/B (31, 50). In particular, these previous studies identified heat shock response elements in the MIC promoter, homologous to the promoter of heat shock protein HSP70. Interestingly, VZV infection has been shown to induce transcription and protein expression of HSP70 (51), although it remains to be determined whether activation of the heat shock pathway by VZV is linked to the strong induction of MICA expression in infected cells. Our findings provide evidence of VZV regulation of NK cell-activating ligands; however, it would be interesting to determine whether VZV modulates additional ligands, including ULBP1. Within this study, regulation of cell

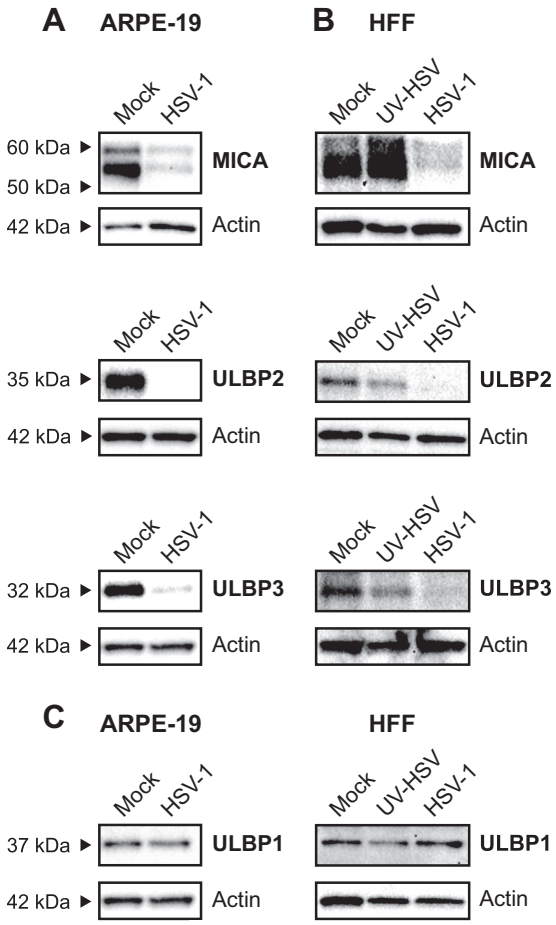

FIG 6 HSV-1 differentially regulates NKG2D ligand protein expression. ARPE-19 (A) and HFF (B) cells were infected with HSV-1 or UV-inactivated HSV-1 (UV-HSV) (MOI of 3) or mock infected. Protein lysates were harvested 24 hpi for immunoblotting with antibodies against MICA, ULBP2, and ULBP3. Corresponding probing for a housekeeping protein (actin) is shown beneath. One representative blot of at least three independent experiments is shown. (C) ARPE-19 and HFF cells were infected with HSV-1 or UV-HSV (MOI of 3) or mock infected. Protein lysates were harvested 24 hpi for immunoblotting with antibody against ULBP1. The corresponding probing for a housekeeping protein (actin) is shown beneath. One representative blot of at least three independent experiments is shown.

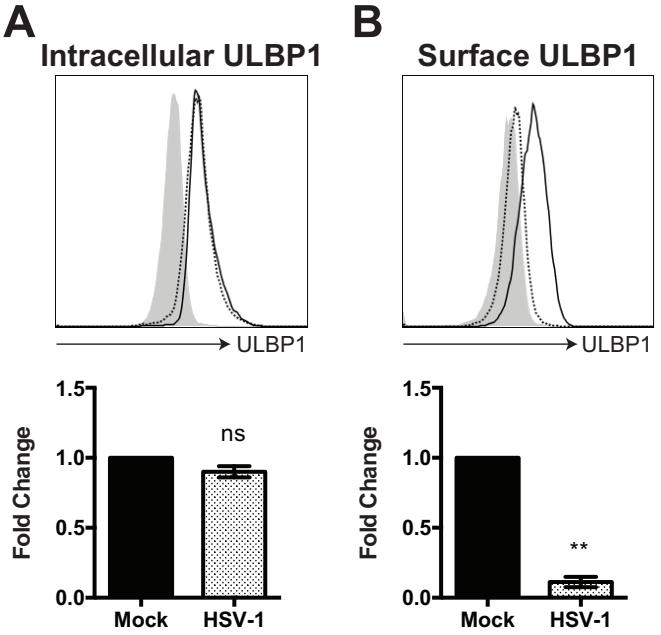

FIG 7 HSV-1 downregulates ULBP1 on the cell surface but not intracellularly. 293T cells were infected with HSV-1 (MOI of 3) or mock infected. Twenty-four hours postinfection, cells were harvested and stained with anti-ULBP1 antibody for analysis by flow cytometry. Levels of intracellular (A) and surface (B) ULBP1 expression were compared between mock (continuous black line) and HSV-1 (dotted black line) infections. Isotype control antibody staining is indicated by the gray-filled histogram. One representative histogram from at least two independent experiments is shown. The relative MFI fold change over mock infection in ULBP1 expression (less respective isotypes) is presented below each correlating histogram as the mean ± SEM of data from biological replicates (A, $n = 2$; B, $n = 3$). Statistical significance was established by two-tailed paired Student's t test. ns, not significant; **, $P < 0.01$.

surface ULBP1 by VZV could not be assessed, as basal ULBP1 expression was not readily detectable on ARPE-19 cells (data not shown). 293T cells, which were employed in HSV-1 analyses of ULBP1 expression, could not be used, as they were not permissive to productive VZV infection (data not shown).

Our analyses of HSV-1, an alphaherpesvirus closely related to VZV, revealed a remarkably different pattern of regulation of MICA, ULBP2, and ULBP3. HSV-1 appeared to target these NKG2DLs for downregulation at both the cell surface and total cellular protein levels. By 24 hpi, cell surface MICA, ULBP2, and ULBP3 expression was potently diminished (Fig. 5), and parallel Western blot analysis revealed an almost complete absence of detectable protein (Fig. 6A and B). Consistent with the data presented here, it has previously been reported that HSV-1 downregulates MICA and ULBP2 on the surface of infected HeLa cells; however, the authors could not assess additional NKG2DLs due to low-level detection on uninfected cells, and total cellular protein levels were not examined by Western blotting (24). The aforementioned study also reported that intracellular flow cytometry staining for MICA did not vary between mock- and HSV-1-infected cells. In our study, we were unable to detect basal levels of MICA intracellularly by flow cytometry analysis; however, thorough analysis of MICA total cellular protein expression in ARPE-19 cells (Fig. 6A), HFFs (Fig. 6B), and 293T cells (data not shown) by Western blotting indicated a potent downregulation of MICA protein expression following HSV-1 infection. The limited variation described is not due to allele-specific effects as both reports confirm that HSV-1 is capable of downregulating surface expression of the common MICA*008 allele in 293T and HeLa cells, respectively, and thus may reflect differences in the experimental setup or the effect of analyzing protein expression using alternative techniques.

It is interesting to note that HSV-1 is capable of targeting MICA in each of the several cell types assessed here. MICA is a highly polymorphic molecule, with over 60 human alleles identified (52, 53). Of the cells used in this study, ARPE-19 cells express the MICA*011 and -*027 alleles (G Wilkinson, Cardiff University, personal communication), while 293T cells are homozygous for the MICA*008 allele (49), the most prevalent allele in many populations (54, 55). MICA*008 specifically escapes downregulation by viral proteins known to target other MICA alleles, including UL142 and US18/20 of HCMV (33, 37, 56) and K5 of KSHV (39),

and it has been suggested that the MICA*008 allele may have evolved and increased in prevalence as a product of the host immune response developing to counter viral evasion strategies (57). A recent study has now identified that US9 of HCMV is capable of downregulating cell surface MICA*008 (58), as we have similarly observed with HSV-1 infection. The finding that HSV-1 downregulates a broad range of NKG2DLs supports a previous study that reported that the NKG2D receptor does not play a role in NK cell detection of HSV-1 infection in human fibroblasts (23). Potent reduction in NKG2DL expression on HSV-1-infected cells, as demonstrated here, would presumably protect HSV-1 from detection via NKG2D-mediated interactions, thus supporting an immunoevasive advantage of NKG2DL downregulation by HSV-1.

The near absence of detectable MICA, ULBP2, and ULBP3 in HSV-1-infected cells provides a striking phenotype suggestive of cellular degradation. This decrease did not occur when HSV-1 was UV irradiated, indicating that the effect of HSV-1 on NKG2DL protein is dependent on *de novo* viral gene expression. It has yet to be determined which viral gene or genes may mediate this or the mechanism of such pronounced protein downregulation. Preliminary experiments inhibiting proteasomal and lysosomal degradation were undertaken, but interpretation of the data was complicated by a number of factors. Addition of both proteasomal (MG132) and lysosomal (folimycin) inhibitors strongly increased basal NKG2DL expression (data not shown), and inhibition of these pathways is known to hinder HSV-1 replication (59, 60), confounding interpretation of results. Despite this, it can be concluded from the presented findings that the pronounced decrease in MICA, ULBP2, and ULBP3 cellular protein is a selective process as ULBP1 protein levels were not altered by HSV-1 infection. Intriguingly, HSV-1 was equally capable of downregulating cell surface ULBP1 expression, despite not significantly affecting total cellular protein. HSV-1 thus clearly encodes at least two mechanisms for modulating NKG2DL expression in order to effectively target multiple paths of NK cell activation. HSV-1 is not the only virus with several NKG2DL evasion strategies: HCMV (reviewed in reference 61), KSHV (39, 40), and HHV-7 (38) all encode different mechanisms of NKG2DL modulation, the latter also modulating expression via a combination of degradation and intracellular retention. It is surprising that ULBP1 is differentially modulated by HSV-1 when it shares relatively close homology with the other ULBP proteins assessed. A similar pattern of selective viral interaction is seen with HCMV, where the binding ability of viral glycoprotein UL16 is dependent on a single amino acid, allowing it to target MICB and ULBP1/2/6 but not their closely related counterparts MICA and ULBP3/4, respectively (62).

In establishing infection in human hosts, downregulation of NKG2DL expression by VZV and HSV-1 would prove beneficial against various antiviral responses. NKG2D is ubiquitously expressed on NK cells and is a potent activator; however, the NKG2D receptor is also present on $CD8^+$ αβ T cells, γδ T cells, and a subset of NKT cells (26, 27). Modulation of NKG2DLs could possibly represent a wider strategy of these viruses to interfere with both innate and adaptive immune responses to viral infection. The finding that VZV and HSV-1 modulate expression of NKG2DLs, but that they do so differentially, raises the possibility that these alphaherpesviruses have evolved independent means to evade the activity of NK cells and other NKG2D-expressing cell types.

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