Granzyme B Cleaves Multiple Herpes Simplex Virus 1 and Varicella-Zoster Virus (VZV) Gene Products, and VZV ORF4 Inhibits Natural Killer Cell Cytotoxicity

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ABSTRACT Immune regulation of alphaherpesvirus latency and reactivation is critical for the control of virus pathogenesis. This is evident for herpes simplex virus 1 (HSV-1), where cytotoxic T lymphocytes (CTLs) prevent viral reactivation independent of apoptosis induction. This inhibition of HSV-1 reactivation has been attributed to granzyme B cleavage of HSV infected cell protein 4 (ICP4); however, it is unknown whether granzyme B cleavage of ICP4 can directly protect cells from CTL cytotoxicity. Varicella zoster virus (VZV) is closely related to HSV-1; however, it is unknown whether VZV proteins contain granzyme B cleavage sites. Natural killer (NK) cells play a central role in VZV and HSV-1 pathogenesis and, like CTLs, utilize granzyme B to kill virally infected target cells. However, whether alphaherpesvirus granzyme B cleavage sites could modulate NK cell-mediated cytotoxicity has yet to be established. This study aimed to identify novel HSV-1 and VZV gene products with granzyme B cleavage sites and assess whether they could protect cells from NK cell-mediated cytotoxicity. We have demonstrated that HSV ICP27, VZV open reading frame 62 (ORF62), and VZV ORF4 are cleaved by granzyme B. However, in an NK cell cytotoxicity assay, only VZV ORF4 conferred protection from NK cell-mediated cytotoxicity. The granzyme B cleavage site in ORF4 was identified via site-directed mutagenesis and, surprisingly, the mutation of this cleavage site did not alter the ability of ORF4 to modulate NK cell cytotoxicity, suggesting that ORF4 has a novel immunoevasive function that is independent from the granzyme B cleavage site.

IMPORTANCE HSV-1 causes oral and genital herpes and establishes life-long latency in sensory ganglia. HSV-1 reactivates multiple times in a person's life and can cause life-threatening disease in immunocompromised patients. VZV is closely related to HSV-1, causes chickenpox during primary infection, and establishes life-long latency in ganglia, from where it can reactivate to cause herpes zoster (shingles). Unlike HSV-1, VZV only infects humans, and there are limited model systems; thus, little is known concerning how VZV maintains latency and why VZV reactivates. Through studying the link between immune cell cytotoxic functions, granzyme B, and viral gene products, an increased understanding of viral pathogenesis will be achieved.

KEYWORDS *Varicella zoster virus (VZV), Herpes simplex virus (HSV), granzyme B, natural killer (NK) cells*

Human alphaherpesviruses such as herpes simplex virus 1 (HSV-1) and varicella zoster virus (VZV) are characterized by their ability to establish life-long latency in sensory nerves during primary infection (1). Primary infection with HSV-1 can result in oral or genital herpes, whereas primary infection with VZV results in chickenpox (2). During primary infection, these viruses establish life-long latency in either the dorsal root (DRG) or trigeminal ganglia (TG) (2). For both VZV and HSV-1, reactivation and clinical severity is heightened in immunocompromised hosts, highlighting the impor-

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tance of the immune system in controlling alphaherpesvirus pathogenesis (3). Understanding how these viruses maintain life-long latency and reactivate is key to developing therapeutic strategies to prevent the potentially severe consequences of alphaherpesvirus reactivation.

HSV-1 latency has been studied in mouse models, in which cytotoxic T lymphocytes (CTLs) lie in close proximity to latently infected neurons (4). These CTLs have been shown to inhibit HSV-1 reactivation through the delivery of granzyme B and the subsequent cleavage of HSV infected cell protein 4 (ICP4) (5). Typically, granzyme B would induce apoptosis in target cells; however, this is not observed in HSV-1-infected neurons. Viral inhibition of granzyme B-induced apoptosis has been explored in the context of adenovirus, where the viral protein L4-100K has been shown to inhibit both granzyme B activity and CTL cytotoxicity (6). This function was linked to a granzyme B consensus motif in L4-100K. To date, it is unknown whether there are HSV-1 gene products other than HSV ICP4 that can be cleaved by granzyme B. As HSV ICP4 has a granzyme B consensus motif, it is pertinent to investigate whether HSV ICP4 can inhibit granzyme B function and CTL cytotoxicity, as this could explain the lack of CTL-induced apoptosis in the context of HSV-1 latency.

HSV-1 literature has focused on the role of CTLs in the prevention of HSV-1 reactivation; however, both CTLs and NK cells can utilize granzyme B to kill target cells. Typically, when CTLs or NK cells recognize a virally infected target cell, they create an immunological synapse with the target cell and directly secrete granules containing perforin and granzyme B along with other constituents. Perforin forms a pore in the target cell, allowing for the delivery of granzyme B. Granzyme B cleaves multiple apoptotic pathway components that converge on the cleavage of caspase 3, the executioner caspase. This ultimately results in the induction of apoptosis in the target cell.

VZV is genetically similar to HSV-1 (7); however, less is known about VZV latency and reactivation. In examination of postmortem latently infected TG samples, resident CTLs were shown to be directed against HSV-1 rather than against VZV (8). However, in postmortem DRG samples from individuals with active herpes zoster at the time of death, CTLs that are granzyme B positive were in close proximity to VZV-infected DRG neurons (9). This may highlight that, while ganglia resident CTLs are not directed toward VZV during latency, CTLs infiltrate into the ganglia in response to VZV reactivation. Interestingly, no neuronal cell death is observed with CTL infiltration during VZV reactivation, and the role of granzyme B remains unclear. NK cells have been found to infiltrate the DRG in samples that were collected 1 to 4.5 months after herpes zoster reactivation, and therefore it would be relevant to examine NK cells in the context of the relationship between VZV and granzyme B (10).

Therefore, this study sought to identify whether VZV encodes proteins that can be cleaved by granzyme B, as well as to identify additional granzyme B cleavage sites in HSV-1 proteins. In this study, HSV gene products and VZV gene products were selected for investigation on the basis of having predicted granzyme B cleavage sites. These gene products were expressed in human cells *in vitro* to examine the relationship between granzyme B, NK cells, and human alphaherpesviruses. In this report, we show that HSV ICP27, VZV open reading frame 4 (ORF4), and VZV ORF62 can be cleaved by granzyme B. We addressed the biological relevance of granzyme B cleavage of HSV-1 and VZV proteins in the context of NK cell cytotoxicity. We demonstrate that VZV ORF4 alone was able to inhibit NK cell-mediated cytotoxicity, indicating that possession of a granzyme B cleavage site is not sufficient to limit NK cell-mediated cytotoxicity. Surprisingly, mutation of the granzyme B cleavage site in VZV ORF4 did not alter the ability of ORF4 to inhibit NK cell-mediated cytotoxicity, demonstrating that this function is independent of the granzyme B cleavage site. This study increases our knowledge concerning HSV-1 and VZV proteins that may be targeted by granzyme B to limit viral spread, as well as characterizing a novel immunoevasive function for VZV ORF4 in the context of NK cell-mediated cytotoxicity. Overall, this broadens our knowledge

concerning the control of HSV-1 and VZV latency and VZV immunoevasive mechanisms during lytic infection and reactivation.

RESULTS

Granzyme B cleaves VZV ORF4, VZV ORF62, and HSV ICP27. The ability of CTLs to inhibit HSV-1 reactivation has been attributed to the cleavage of ICP4 by granzyme B (5). However, it is unclear whether other HSV-1 gene products also contain granzyme B cleavage sites and thus may be targeted by the immune response to inhibit viral reactivation. Furthermore, it is yet to be determined whether the closely related herpesvirus VZV encodes gene products that contain granzyme B cleavage sites. To determine whether there were novel granzyme B cleavage sites in VZV and HSV gene products, HSV-1 and VZV gene products were examined for potential granzyme B cleavage sites via the program GraBCas (11).

VZV ORF4, VZV ORF62, and HSV ICP27 all contained predicted multiple granzyme B cleavage sites. HSV ICP27 is an immediate-early gene that regulates mRNA synthesis and processing (12, 13). VZV ORF4 is the homolog of HSV ICP27 and an immediate-early gene that is essential for viral replication and the establishment of latency (14). VZV ORF62 is also an immediate-early protein and is a transactivator and has immunomodulatory roles (15, 16). Of note, all of these viral gene products have important roles in viral replication.

To test whether these viral proteins could be cleaved by granzyme B, plasmids were designed to express these viral proteins tagged to green fluorescent protein (GFP). Lysates from 293Ts transfected with the viral protein-GFP plasmids, as well as the parental plasmid (pGFP-C1), were generated (Fig. S1). These lysates were incubated with or without granzyme B (50 μ M for VZV ORF4, VZV ORF62, HSV ICP4, and 100 μ M for HSV ICP27; Millipore) and were analyzed for cleavage products via Western blot analysis. Granzyme B was able to effectively cleave VZV ORF4 (Fig. 1A) and VZV ORF62 (Fig. 1B), producing cleavage products of 65 kDa and 45 kDa, respectively. HSV ICP27 (Fig. 1C) was also cleaved, yielding a cleavage product of 40 kDa; however, the concentration of granzyme B required to demonstrate this phenotype was double that of the other gene products. No cleavage was observed when GFP was expressed alone (Fig. 1D). As expected from previous reports, HSV-1 ICP4 was cleaved by granzyme B, yielding a cleavage product of 40 kDa (Fig. 1E). These results were observed in at least 3 biological replicates. Using cleavage product sizes for HSV ICP27, VZV ORF4, and VZV ORF62, it was possible to further narrow down the predicted granzyme B cleavage site (Fig. 1F). These cleavage sites differed from previously predicted cleavage sites in HSV ICP4 (5) and adenovirus L4-100K (6).

To further demonstrate that granzyme B was able to specifically cleave VZV ORF4, VZV ORF62, and HSV ICP27 proteins, increasing concentrations of granzyme B were added to lysates to determine whether there was a dose-dependent response. Such a response was evident for VZV ORF4 (Fig. 1G) and VZV ORF62 (Fig. 1H), with the full-sized proteins diminishing and cleavage products increasing with increasing concentrations of granzyme B. This pattern was also observed for HSV ICP27 (Fig. 1I); however, double the amount of granzyme B was required to see this effect. As HSV ICP27 required double the amount of granzyme B to observe a cleavage product, it is possible that its granzyme B cleavage site may not be as efficiently recognized by granzyme B in comparison to the other gene products studied. These results were observed in 3 biological replicates. These experiments demonstrate for the first time that VZV ORF62, VZV ORF4, and HSV ICP27 are cleaved by granzyme B.

VZV ORF4 can restrict cell death by NK cell-mediated cytotoxicity at a low ratio. L4-100K in adenovirus can inhibit granzyme B function and CTL-induced cell death due to its granzyme B consensus motif (6). HSV ICP4 also contains a granzyme B consensus motif (5); however, this has not been linked to inhibition of immune cell-mediated cytotoxicity. NK cells also utilize granzyme B to kill virally infected target cells and are critical in the control of HSV-1 and VZV infection. Therefore, we sought to determine

FIG 1 VZV ORF4, VZV ORF62, HSV ICP27, and HSV ICP4 are cleaved by granzyme B. 293Ts were transfected with VZV or HSV ORF-containing plasmids. Day 2 posttransfection cells were harvested and protein lysates generated for immunoblotting. ORF4 (A), ORF62 (B), and ICP4 (E) lysates were treated with 50 μ M (1 $\times$) granzyme B (Merck), ICP27 (C) and green fluorescent protein (GFP) (D) lysates were treated with 100 μ M (2 $\times$) granzyme B. Blots were probed for GFP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are representative of 3 biological replicates. Purple and blue boxes indicate the full-sized protein and the protein cleavage product, respectively. –, untreated; GrzB, granzyme B treated. Previously predicted granzyme B cleavage sites of HSV ICP4 and adenovirus L4-100K are displayed in conjunction with predicted granzyme B cleavage sites for VZV ORF4, VZVORF62, and HSV ICP27, based on the cleavage product size (F). VZV ORF4 (G), VZV ORF62 (H), and HSV ICP27 (I) containing lysates were either untreated (UT) or treated with increasing concentrations of granzyme B, as indicated. Blots were probed for GFP and GAPDH and are representative of 3 biological replicates. Solid arrows indicate protein size ladder.

whether the HSV-1 and VZV gene products that we identified to be cleaved by granzyme B would affect cell death by NK cell-mediated cytotoxicity. To assess this in a human cell setting, human NK cells were isolated by CD56⁺ magnetically activated cell sorting (MACS) selection and incubated overnight with interleukin-2 (IL-2) (Fig. 2A). We then assessed NK cell activation (CD69) and cytotoxicity against 293Ts that had been transfected with pGFP, pORF4, pORF62, pICP27, or pICP4,

FIG 2 NK cell cytotoxicity assay setup and gating strategy. Peripheral blood mononuclear cells (PBMCs) were extracted from buffy coats using a Ficoll gradient and cryopreserved (A). NK cells were isolated from resuscitated PBMCs via MACS bead selection of CD56⁺ cells and were treated overnight with IL-2. NK cells were cocultured with cell trace violet (CTV)-labeled 293Ts expressing the viral protein of interest or with GFP alone or were untransfected. Cells were incubated for 4 h and subsequently collected and stained for flow cytometry analysis. Cells were first gated on size and granularity (FSC-A versus FSC-H) (B). Single cells were gated for CTV. CTV⁺ cells were classified as live (CC3⁻, live/dead negative), early apoptotic (CC3⁺, live/dead negative), late apoptotic (CC3⁺, live/dead positive), or other cell death (CC3⁻, live/dead positive) based on CC3 and live/dead staining. Within the live cell gate, GFP⁺ cells were identified using GFP versus FSC-A. Median fluorescence intensity (MFI) of CC3 for CTV⁺ cells was determined. NK cells were identified in CTV⁻ population by CD3 versus CD56 staining (CD3⁻, CD56⁺). In the NK cell population, CD69 MFI was measured.

or were left untransfected. 293Ts have previously been shown to efficiently activate NK cells (17). 293Ts were cell trace violet (CTV) labeled to allow for identification in subsequent flow cytometry analysis (Fig. 2B). NK cells were incubated with 293Ts at a 5:1 (high) or 1.5:1 (low) ratio for 4 h, and cells were analyzed by flow cytometry. 293Ts incubated without NK cells were used to determine whether any of the viral gene products affected 293T viability.

To assess the cytotoxicity of the NK cells, cell death in 293Ts was measured via CC3 and Zombie live/dead staining, as we have previously characterized (18). Live cells were defined as CC3⁻ and live/dead negative, early apoptotic cells as CC3⁺ and live/dead negative, late apoptotic as CC3⁺ and live/dead positive, and nonapoptotic cell death was defined as CC3⁻ and live/dead positive. Transfection of different viral gene products into cells can affect cell viability. To account for this, NK cell cytotoxicity was measured as the fold change in percentage of live cells after NK cell incubation relative to untreated transfected cells for each viral gene product (Fig. 3A and B). At a high NK cell ratio, we found no significant difference with NK cell coculture between 293Ts expressing any of the viral proteins compared to GFP-expressing or untransfected 293Ts (Fig. 3B). Furthermore, when cultured with NK cells, there was no significant difference in CC3 MFI between 293Ts expressing viral proteins, GFP-expressing 293Ts, or untransfected 293Ts (Fig. 3C and D). Together, these data demonstrate that at a 5:1 ratio, expression of VZV or HSV-1 viral proteins that are cleaved by granzyme B does not alter target cell apoptosis mediated by NK cell-mediated cytotoxicity.

In our assay, 293T cells were not 100% GFP positive. Therefore, the frequency of GFP⁺ cells in the live gate was examined (Fig. 3E), with the expectation that GFP⁺ cells would be enriched if the viral gene product was protective (Fig. 3E). To account for the differences in transfection efficiencies of the viral gene products, GFP changes are displayed as fold change of GFP⁺ cells that have been incubated with NK cells relative to untreated transfected cells for each viral gene product.

Analyzing fold change of GFP⁺ cells cultured with NK cells over GFP⁺ cells cultured alone, we found no significant differences between 293Ts expressing ORF4, ORF62, ICP27, or ICP4 compared to 293Ts expressing GFP (Fig. 3F). However, there was a trend toward reduced live GFP⁺ ICP4-expressing 293Ts, suggesting that cells expressing ICP4 were more vulnerable to NK cell-mediated cytotoxicity. Together, these data suggest that at a high NK cell to target ratio the HSV and VZV proteins examined could not protect cells against NK cell-mediated cytotoxicity.

It is possible that at high NK cell to target ratios, the NK cell response could overwhelm any potential protective effects of the viral gene products. Therefore, a lower NK cell to target cell ratio (1.5:1) was examined (Fig. 4). Strikingly, at a lower NK cell ratio, VZV ORF4-expressing 293Ts trended toward a higher frequency of live cells compared to those of 293Ts expressing other viral proteins and to control cells, which was significant compared to that of ORF62-expressing 293Ts (Fig. 4A and B). VZV ORF4-expressing cells had, on average, 10% more live cells in the NK cell cytotoxicity assay than did control cells and cells expressing other viral genes. VZV ORF4-expressing cells also had a significant reduction in CC3 MFI in comparison to VZV ORF62-expressing cells and trended toward having a lower MFI in comparison to all other cells (Fig. 4C and D). This suggests that VZV ORF4 was able to protect against NK cell-mediated cytotoxicity at a low NK cell to target cell ratio. There were no significant differences in fold change of GFP⁺ cells when cultured with NK cells for ORF4, ORF62, ICP27, or ICP4 in comparison to control cells (Fig. 4E and F). Overall, these data suggest that VZV ORF4 may be able to protect cells from NK cell-mediated cytotoxicity. This may not be due to the granzyme B cleavage site, however, as other granzyme B cleavage site-containing viral proteins did not affect NK cell-mediated cytotoxicity.

Herpesviruses encode proteins that can alter NK cell activation through interfering with target cell activating and inhibitory ligands (19). To ensure that the ability of VZV ORF4 to protect cells from NK cell-mediated cytotoxicity was not due to an inhibition of NK cell activation, CD69 expression was assessed via flow cytometry (Fig. 5B). NK cells were identified as CD3⁻ and CD56⁺ (Fig. 5A). Expression of viral proteins or of GFP

FIG 3 HSV and VZV gene products which can be cleaved by granzyme B cannot protect 293Ts against NK cell cytotoxicity at a high NK cell to target ratio. 293Ts were untransfected or were transfected to express GFP alone, HSV ICP27-GFP, HSV ICP4-YFP, VZV ORF4-GFP, or VZV ORF62-GFP. Two days posttransfection, 293Ts were incubated with interleukin 2 (IL-2)-treated NK cells isolated from PBMCs in an NK cell to target cell ratio of 5:1 or were cultured without NK cells. Cells were collected and analyzed via flow cytometry. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (A). Bar graph depicting fold change in live cells $\pm$ standard error of the mean (SEM) is representative of 3 biological replicates (B). Representative histograms of cleaved caspase 3 (CC3) MFI are displayed (C) and collated in a bar graph ($n = 3$, $\pm$ SEM) (D). GFP expression was measured in CTV⁺ live cells. Representative flow plots for both untreated and NK cell-incubated 293Ts are displayed. GFP expression was measured in CTV⁺ live cells. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (E). Bar graph depicting fold change in GFP⁺ cells $\pm$ SEM is representative of 3 biological replicates (F). Statistical significance was established by a repeated measure one-way analysis of variance (ANOVA) using Tukey's multiple-comparison test.

alone did not significantly alter CD69 expression at either the high (Fig. 5B and C) or low ratio (Fig. 5D and E). This demonstrates that any differences in cell death in 293Ts was not due to differential NK cell activation.

Identification of the VZV ORF4 granzyme B cleavage site. As ORF4 appeared to be able to protect 293Ts against NK cell-mediated cytotoxicity, we wanted to identify

FIG 4 VZV ORF4 can protect 293Ts against NK cell cytotoxicity at a low NK cell to target ratio. 293Ts were transfected to express GFP alone, HSV ICP27-GFP, HSV ICP4-YFP, VZV ORF4-GFP, VZV ORF62-GFP, or were untransfected. Two days posttransfection, 293Ts were incubated with IL-2-treated NK cells at an NK cell to target cell ratio of 1.5:1 or were cultured without NK cells. Cells were collected and analyzed via flow cytometry. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (A). Bar graph depicting fold change in live cells $\pm$ SEM is representative of 3 biological replicates (B). Representative histograms of CC3 MFI are displayed (C) and collated in a bar graph ($n = 3$, $\pm$ SEM) (D). GFP expression was measured in CTV⁺ live cells. Representative flow plots for both UT and NK cell-incubated 293Ts are displayed. GFP expression was measured in CTV⁺ live cells. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (E). Bar graph depicting fold change in GFP⁺ cells $\pm$ SEM is representative of 3 biological replicates (F). Statistical significance was established by a repeated measure one-way ANOVA using Tukey's multiple-comparison test. *, $P \leq 0.05$.

FIG 5 NK cell activation is not affected by transfected 293Ts at a high or low NK to target ratio. 293Ts were untransfected or were transfected to express GFP alone, VZV ORF4-GFP, VZV ORF62-GFP, ICP4-YFP, or HSV ICP27-GFP. Two days posttransfection, 293Ts were incubated with IL-2-treated NK cells isolated from PBMCs in an NK cell to target cell ratio of 5:1 (A to C) or 1.5:1 ratio (D and E) or were cultured without NK cells. Cells were collected and analyzed via flow cytometry. NK cells were identified as CD56⁺ CD3⁻, as seen in the representative flow plot (A). Isotype control staining for CD69 was performed on NK cells cultured alone. CD69 expression was evaluated by histogram (B and D) and MFI collated in a bar graph (C and E) ($n = 3$, $\pm$ SEM).

the granzyme B cleavage site and mutate it to determine whether the granzyme B cleavage site was responsible for this effect. Using the initial GraBCas predictions, as well as cleavage product size, we predicted the granzyme B cleavage site (Fig. 6A). Previous studies have shown that by converting the aspartic acid (D) residue in the cleavage site to a glutamic acid, the ability of granzyme B to cleave these sites is abolished (20, 21). The granzyme B cleavage site in Bid was identified in both reports via mutation of D75 to glutamic acid (20, 21). Site-directed mutagenesis was performed on pORF4 to mutate the aspartic acid residue via a single base pair substitution (Fig. 6A). Successful single base pair substitution was confirmed by sequencing, and the resulting plasmid was termed pORF4M. pORF4 and pORF4M were transfected into 293Ts, and lysates were collected for digest with granzyme B. pORF4M without granzyme B digest produced the same 75-kDa band as pORF4-GFP. When incubated with granzyme B, the 65-kDa cleavage product was absent from ORF4M-expressing cells, suggesting that the mutant abolished the ability of granzyme B to cleave this gene product (Fig. 6B). This was observed in 3 independent biological replicates, indicating that the granzyme B cleavage site was successfully identified in VZV ORF4.

The ability of VZV ORF4 to inhibit NK cell cytotoxicity is independent from the granzyme B cleavage site. To determine whether the granzyme B cleavage site in ORF4 was responsible for its ability to limit cell death mediated by NK cell-mediated cytotoxicity, the low-ratio cytotoxicity assay was performed with 293Ts expressing GFP, ORF4, or ORF4M and then compared to untransfected 293Ts. Similarly to results shown in Fig. 3 and 4, the fold frequency of live cells when cocultured with NK cells over cells alone was calculated (Fig. 7A and B). As demonstrated in Fig. 5B, following culture with NK cells, a higher frequency of live cells was noted in 293Ts expressing ORF4 compared

FIG 6 Identification of the ORF4 granzyme B cleavage site using site-directed mutagenesis. Potential sites of granzyme B cleavage were identified via GrasBCas (11). The aspartate residue in the cleavage site was changed to a glutamic acid via a single base pair substitution using PCR (A). 293Ts were transfected with 1 μ g of ORF4 or ORF4M plasmid. On day 2 posttransfection, cells were harvested and protein lysates generated for immunoblotting. Lysates were treated with 133 ng granzyme B (BioVision, USA). Blots were probed for GFP and GAPDH and are representative of 3 biological replicates (B). $\pm$ indicates untreated (–) and granzyme B treated (+). Solid arrows indicate protein size ladder.

to GFP-expressing or untransfected 293T control cells (Fig. 7A and B). Similarly to the previous assay, VZV ORF4-expressing cells showed on average 10% more live cells than did control cells and cells expressing other viral genes. Unexpectedly, ORF4M-expressing cells had a significantly higher fold change frequency of live cells compared to GFP-expressing cells (Fig. 7A and B). VZV ORF4M-expressing cells had on average 15% more live cells in the NK cell cytotoxicity assay than did control cells and cells expressing other viral genes. This trend is also observed in the CC3 MFI (Fig. 7C and D). When we examined live cells after NK cell coculture, significantly more GFP-expressing cells were present when 293Ts expressed ORF4M or ORF4 than in GFP-expressing 293Ts (Fig. 7E and F). Together, these data indicate that ORF4 has a previously unknown function that protects cells from NK cell-mediated cytotoxicity; however, this is not due to the presence of a granzyme B cleavage site.

VZV ORF4 does not protect 293Ts against staurosporine- or FasL-induced apoptosis. To determine whether the ability of VZV ORF4 to restrict cell death mediated by NK cell cytotoxicity was due to a broad antiapoptotic effect, ORF4-, ORF4M-, or GFP-expressing and untransfected 293Ts were treated with two independent apoptotic stimuli. As described previously, staurosporine induces the intrinsic apoptotic pathway, while FasL induces the extrinsic apoptotic pathway (18). Thus, treatment with these drugs would identify whether an antiapoptotic effect was specific

FIG 7 Both ORF4 and ORF4M can protect 293Ts from NK cell cytotoxicity at a low NK cell-to-target ratio. 293Ts were untransfected or were transfected to express GFP alone, VZV ORF4-GFP, or VZV ORF4M-GFP. Two days posttransfection, 293Ts were incubated with IL-2-treated NK cells isolated from PBMCs in an NK cell to target ratio of 1.5:1 or cultured without NK cells. Cells were collected and analyzed via flow cytometry. Representative flow plots are displayed (A). Bar graph depicting fold change in live cells $\pm$ SEM is representative of 3 biological replicates (B). Representative histograms of CC3 MFI are displayed (C) and collated in a bar graph ($n = 3$, $\pm$ SEM) (D). GFP expression was measured in CTV⁺ live cells. Representative flow plots for both untreated and NK cell-incubated 293Ts are displayed. GFP expression was measured in CTV⁺ live cells. Representative flow plots are displayed for untransfected, GFP, ORF4, and ORF4M 293Ts (E). Bar graph depicting fold change in GFP⁺ cells $\pm$ SEM is representative of 3 biological replicates (F). Statistical significance was established by a repeated measure one-way ANOVA using Tukey's multiple-comparison test. *, $P \leq 0.05$; ****, $P \leq 0.001$.

FIG 8 VZV ORF4 does not protect 293Ts against staurosporine- or FasL-induced apoptosis. 293Ts were untransfected or transfected with pGFP-C1, pORF4, or pORF4M. Two days posttransfection, 293Ts were treated with 1 μ M staurosporine (A and B) or 200 ng/ μ l FasL (C and D) or were left untreated for 5 h. Cells were stained with Zombie NIR, permeabilized, fixed, stained for CC3, and analyzed via flow cytometry. Flow plots displayed are representative of 3 biological replicates (A and C). Bar graph depicting fold change in live cells $\pm$ SEM is representative of 3 biological replicates (B and D). Statistical significance was established by a repeated measure one-way ANOVA using Tukey's multiple-comparison test.

to a particular pathway. However, there were no significant differences in the fold change in live cell frequencies between 293Ts expressing ORF4 or ORF4M compared to GFP-expressing or untransfected 293Ts for either staurosporine (Fig. 8A and B) or FasL treatment (Fig. 8C and D). This suggests that the ability of ORF4 to limit cell death through NK cell cytotoxicity is not mediated by inhibition of extrinsic or intrinsic apoptosis induction.

DISCUSSION

Granzyme B has been shown to be critical to the regulation of HSV-1 reactivation by the immune system; however, it is unknown why granzyme B does not induce apoptosis in this context. VZV is closely related to HSV-1, but much less is known regarding control of VZV reactivation and lytic infection by the immune system. Overall, this study aimed to identify HSV-1 and VZV proteins that could be cleaved by granzyme B and assess whether these proteins could inhibit NK cell-mediated cytotoxicity. This study demonstrates that there are multiple VZV and HSV-1 proteins that are susceptible to cleavage by granzyme B; however, this does not necessarily confer resistance to NK cell-mediated cytotoxicity. A novel function of VZV ORF4 was identified in protecting 293Ts against NK cell-mediated cytotoxicity at a low effector to target ratio. Surprisingly, mutation of the granzyme B cleavage site in ORF4 did not change the ability of ORF4 to inhibit NK cell-mediated cytotoxicity, suggesting that this function is independent of the cleavage site. The ability of VZV ORF4 to modulate NK cell cytotoxicity did not seem to be due to an ability to inhibit extrinsic or intrinsic apoptotic pathways. Altogether, this work highlights a novel immunomodulatory function of VZV ORF4 and a more extensive role for granzyme B in limiting alphaherpesvirus infection.

The role of granzyme B in viral infections has been previously addressed in the context of adenovirus. It has been identified that adenovirus L4-100K can inhibit granzyme B function and lymphokine-activated killer (LAK) cell-mediated cytotoxicity through the possession of its granzyme B cleavage site (6). In the cases of HSV-1 and VZV, it appears that in the context of NK cell-mediated cytotoxicity a granzyme B cleavage site is not sufficient to inhibit NK cell cytolytic function. This difference could be due to the NK cells in our system also being able to kill through the FasL pathway, while the 293Ts were Fas deficient in the adenovirus study (6). It is important to examine the role of Fas and other extrinsic apoptotic inducers in this context, as they could induce apoptosis in target cells when granzyme B is inhibited. Both VZV and HSV contain proteins that can inhibit both intrinsic and extrinsic apoptotic pathways (22, 23), and thus granzyme B cleavage of critical viral proteins may be a fail-safe mechanism to ensure inhibition of viral spread even when cell death pathways are inhibited.

NK cell/CTL granules contain other granzymes that could compensate for the inhibition of granzyme B. In the case of adenovirus, granzyme H was found to target adenovirus proteins that were crucial for viral DNA replication to inhibit viral replication (24). Therefore, it would be interesting to examine the role of other granzymes in the context of alphaherpesvirus infection. Interestingly, the viral proteins we identified as being cleaved by granzyme B (HSV ICP27, VZV ORF4, and VZV ORF62) all have critical roles in viral replication and/or host evasion (12, 14, 16, 25, 26). This suggests that in the context of VZV infection, granzyme B could limit viral replication and spread when cell death has been inhibited, and this warrants further research.

In our NK cell cytotoxicity assays, the other components of cytolytic granules could be masking the ability of viral proteins to inhibit granzyme B function. It would be of interest to determine if the expression of HSV and VZV viral proteins could inhibit granzyme B-mediated cytotoxicity in an isolated system. In the process of this study, several attempts were made to deliver recombinant granzyme B to target cells by the bioPORTER protein delivery system; however, this method of delivery was ineffective at inducing granzyme B-mediated apoptosis at the cell confluence necessary to perform these experiments (Fig. S2). Therefore, investigating different methods of protein delivery/generating recombinant granzyme B may be necessary to directly assess the ability of HSV/VZV proteins to inhibit granzyme B function *in vitro*.

A novel mechanism of VZV ORF4 to inhibit NK cell-mediated cytotoxicity was identified in this study; however, the mutation of the granzyme B cleavage site revealed this was not due to the cleavage site. We investigated the possibility that VZV ORF4 could inhibit apoptosis; however, it did not inhibit either FasL- or staurosporine-induced apoptosis. This finding suggests that ORF4 limits NK cell-mediated cell death through an alternative mechanism, such as regulating NK cell detection of the target.

VZV ORF4 is the homolog of HSV ICP27; however, in our NK cell assay, only ORF4 could protect against NK cell cytotoxicity. This suggests that the ORF4 mechanism of immunoevasion is distinct from potential overlapping functions with HSV ICP27. Our previous work has shown that VZV and HSV-1 differentially modulate the expression of ligands to the activating NKG2D receptor expressed on NK cells (27). Even though coculture with ORF4-expressing 293Ts did not affect CD69 expression on IL-2-treated NK cells, it could have affected expression of NK cell ligands such as NKG2D ligands. Future studies could measure expression of NK cell ligands such as NKG2D ligands on ORF4-expressing cells and relate this back to a broader assessment of NK cell function.

In HSV-1 infection in mice, it was found that CTLs could inhibit viral reactivation without cell death induction, and this was attributed to granzyme B cleavage of ICP4 (5). In VZV infection, both NK cells and CTLs have been identified in ganglia of patients who have experienced herpes zoster (9, 10). The CTLs in this context were granzyme B positive, and there was no indication of neuronal apoptosis (9). It would be interesting to investigate the role of VZV ORF62 and VZV ORF4 in protecting neurons from granzyme B-induced apoptosis. Additionally, using cell lines such as the SH-SY5Y cell line or neurons derived from induced pluripotent stem cells (iPSCs), it may be possible to determine whether granzyme B treatment of VZV infected neurons could inhibit virus spread and drive the virus into a latent-like state. This direction would likely be impeded by the difficulty in achieving effective delivery of granzyme B, as described above, but would be of interest to examine in highlighting a potential role for granzyme B in controlling VZV reactivation.

In summary, we have found novel HSV-1 and VZV proteins that can be cleaved by granzyme B, including HSV-1 ICP27, VZV ORF4, and VZV ORF62. In contrast to adenovirus infection, the possession of a granzyme B cleavage site in VZV and HSV-1 proteins was not sufficient to protect against NK cell-mediated cytotoxicity. For the first time, we report that VZV ORF4 has a novel function in limiting NK cell-mediated cytotoxicity through a granzyme B cleavage site-independent mechanism. VZV ORF4 alone was not able to protect cells against FasL- or staurosporine-induced apoptosis, indicating that its ability to inhibit NK cell apoptosis is not due to a broad inhibition of apoptotic pathways. Altogether, this study broadens our knowledge concerning both viral mechanisms of immune modulation and the effects of granzyme B on alphaherpesvirus pathogenesis.

MATERIALS AND METHODS

Cell lines. HEK293T cells (ATCC) and ARPE-19 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 IU/ml penicillin and streptomycin.

Plasmid construction and validation. PCR was performed to amplify HSV ICP27, VZV ORF62, and VZV ORF4 from HSV strain F or the VZV pOka genome, respectively, while incorporating restriction enzyme cleavage sites. PCR products were purified (Illustra GFX PCR DNA and gel band kit; GE Healthcare) and digested with SgrAI and NheI (VZV ORF62) or AgeI and NheI (HSV ICP27 and VZV ORF4). The backbone plasmid (pGFP-C1; Clontech) was also digested with AgeI and NheI and ligated with the VZV and HSV gene PCR products using T4 DNA ligase (NEB), and the resulting plasmids were termed pORF4, pORF62, and pICP27. In the resulting plasmids, GFP was tagged to the 3' end of the viral ORFs. Plasmids were transformed into *Escherichia coli* 5-alpha competent cells (NEB) and isolated using a plasmid DNA purification kit (NucleoBond Xtra Midi plasmid DNA purification kit; Macherey-Nagel). pICP4-YFP was generously gifted by Roger Everett from the MRC Centre, University of Glasgow (Glasgow, Scotland). Sequencing was performed to ensure that the gene products were inserted into the backbone correctly.

Plasmid DNA transfections. On the day prior to transfection, 6×10^5 293T cells were seeded into a well of a 6-well plate (Corning). On the day of transfection, 1 μ g of plasmid DNA was added to serum-free DMEM and FuGene HD (Promega) and was incubated for 15 min at room temperature (RT). The transfection mixtures were then added to previously seeded 293T cells. Two days posttransfection, GFP was imaged at $\times 10$ magnification via a fluorescence microscope (Zeiss Axio).

Generation of protein lysates and granzyme B digest. Plasmids were transfected into 293Ts as described above. Medium was removed from cells at day 2 posttransfection, and cells were washed with cold phosphate-buffered saline (PBS). Cells were resuspended in cell lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP-40, and 1% Triton X-100) for lysis before supernatant was collected and stored at -20°C .

Protein lysates were treated with various concentrations of recombinant granzyme B (initial cleavage assay, Millipore; remaining assays, Enzo Life Sciences) as indicated or were left untreated and were incubated at 37°C for 1 h. Samples were denatured by heating in reducing sample buffer (Bio-Rad) and resolved by SDS-PAGE on precast 4 to 20% polyacrylamide gels (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and probed with primary antibodies. This was followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam) and incubation with enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific). The blot was imaged using a ChemiDoc imaging system (Bio-Rad). The following primary antibodies were used: mouse anti-GFP (B-2; Santa Cruz Biotechnology) and rabbit anti-human GADPH (FL-335; Santa Cruz Biotechnology).

Site-directed mutagenesis. PCRs were designed to substitute an amino acid to convert an aspartic acid to a glutamic acid in the VZV ORF4 plasmid using the Q5 site-directed mutagenesis kit (NEB). The forward primer was TATTACCGAATCAAACCATC and the reverse primer was CAACAACCAAAGTTTCG. PCR products were ligated using the kinase, ligase, and DpnI (KLD) enzyme mix (NEB) and were transformed into chemically competent *Escherichia coli*. Colonies were selected, and the plasmid was isolated via miniprep kit (NucleoSpin; Macherey-Nagel) and sequenced to determine whether they contained the mutated plasmid. Plasmids with the correct sequence were transformed into *Escherichia coli* 5- α competent cells (NEB) and isolated using a plasmid DNA purification kit (NucleoBond Xtra Midi plasmid DNA purification kit; Macherey-Nagel). The resulting plasmid was termed pORF4M.

Peripheral blood mononuclear cell isolation and NK cell separation. Buffy coats collected from healthy human donors were sourced from the Australian Red Cross Blood Service, with ethics approval from the University of Sydney (Sydney, Australia). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). Cells were resuspended in PBS and cryopreserved. CD56⁺ NK cells were isolated from cryopreserved PBMCs via magnetic bead separation using CD56 microbeads (Miltenyi Biotec, Germany) as in (28). Cells were resuspended in RPMI supplemented with 10% FBS (Lonza) and 1% (vol/vol) penicillin/streptomycin (Life Technologies) and enumerated with a hemocytometer. NK cells were left untreated or were incubated overnight with 200 U/ml IL-2 Improved Sequence (IS) (Miltenyi Biotec) for use in NK cell cytotoxicity assays.

NK cell cytotoxicity assay. 293Ts were either transfected with GFP constructs or were left untransfected. Cells were trypsinized and washed twice with PBS. Cells were labeled with cell trace violet (CTV) (Thermo Fisher Scientific) for 1 h at 37°C so they were distinguishable from NK cells during subsequent flow cytometry analysis. Labeled cells were quenched with supplemented DMEM for 5 min at 37°C, washed once with supplemented DMEM, and seeded into a 12 well plate. Previously isolated NK cells were counted and added to 293Ts at a 5:1 or 1.5:1 ratio, as indicated, and incubated together for 4 h at 37°C on a rocking platform to prevent cells from becoming adherent. Cells were subsequently harvested for flow cytometry analysis.

Apoptosis induction assay. 293Ts were transfected with pGFP-C1, pORF4, and pORF4M, as described, and at day 2 posttransfection, cells were left untreated, treated with 1 μ M staurosporine (Sigma-Aldrich), or treated with 200 ng/ μ l FasL (Enzo Life Sciences) for 5 h and analyzed via flow cytometry as previously described (18).

Flow cytometry. Cells were collected and stained with Zombie NIR (BioLegend) per the manufacturer's instructions. For NK cell cytotoxicity assays, cells were surface stained with mouse anti-human CD56 BV605 (clone NCAM16.2; BD Biosciences), mouse anti-human CD3 PerCP-Cy5.5 (clone UCHT1; BD Biosciences), and mouse anti-human CD69 BV711 (clone FN50; BD Biosciences) at RT for 30 min. Cells were fixed and permeabilized with fix/perme buffer (BD) for 20 min at RT. Cells were stained with rabbit anti-human cleaved caspase 3 (CC3) phycoerythrin (PE) (clone C92-605; BD Biosciences) overnight at 4°C. Samples were acquired using an LSRFortessa flow cytometer. Data were analyzed with FlowJo software v10.0.6 (FlowJo, LLC, USA).

Immunofluorescence. Cells were grown on cover slips and transfected to express GFP alone or were left untransfected. At day 2 posttransfection, granzyme B (Enzo Life Sciences) was delivered to cells using the bioPORTER protein delivery system (Sigma-Aldrich) per the manufacturer's instructions. At 4 h posttreatment, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at RT. After washing with PBS, the cells were permeabilized with Triton X-100 (Sigma-Aldrich) for 10 min and blocked using 20% normal donkey serum (Sigma-Aldrich). The cells were incubated with rabbit anti-CC3 (D3E9, 1:150; Cell Signaling Technology) for 1 h at RT. Cells were washed with PBS, incubated with donkey anti-rabbit IgG 594 (1:250; Invitrogen) for 30 min, and washed with PBS, and coverslips were mounted on glass slides using Prolong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). Staining was visualized on a Nikon E widefield microscope, and images were taken using Nikon software. Images were pseudocolored using Fiji (Image J). For cell quantification, thresholds for individual channels were determined by using the Huang method to create a binary image. The watershed method was used to separate cell staining, and automatic counting was performed using Fiji (Image J).

Statistical analysis. *P* values were determined for flow cytometry analysis via a repeated measure one-way analysis of variance (ANOVA) using Tukey's multiple-comparison test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01140-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

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REFERENCES

- Valyi-Nagy T, Shukla D, Engelhard HH, Kavouras J, Scanlan P. 2007. Latency strategies of alphaherpesviruses: herpes simplex virus and varicella-zoster virus latency in neurons, p 1–36. *In* Minarovits J, Gonczol E, Valyi-Nagy T (ed), Latency strategies of herpesviruses. Springer, New York, NY.
- Kinchington PR, St Leger AJ, Guedon J-M, Hendricks RL. 2012. Herpes simplex virus and varicella zoster virus, the house guests who never leave. *Herpesviridae* 3:5. <https://doi.org/10.1186/2042-4280-3-5>.
- Zerboni L, Sen N, Oliver SL, Arvin AM. 2014. Molecular mechanisms of varicella zoster virus pathogenesis. *Nat Rev Microbiol* 12:197–210. <https://doi.org/10.1038/nrmicro3215>.
- Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. 2000. CD8⁺ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *J Exp Med* 191:1459–1466. <https://doi.org/10.1084/jem.191.9.1459>.
- Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. 2008. Noncytotoxic lytic granule-mediated CD8⁺ T cell inhibition of HSV-1 reactivation from neuronal latency. *Science* 322:268–271. <https://doi.org/10.1126/science.1164164>.
- Andrade F, Bull HG, Thornberry NA, Ketner GW, Casciola-Rosen LA, Rosen A. 2001. Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. *Immunity* 14: 751–761. [https://doi.org/10.1016/S1074-7613\(01\)00149-2](https://doi.org/10.1016/S1074-7613(01)00149-2).
- Pellet P, Roizman B. 2013. Herpesviridae, p 1802–1822. *In* Fields BN, Knipe DM, Howley PM (ed), Fields virology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
- Verjans GM, Hintzen RQ, van Dun JM, Poot A, Milikan JC, Laman JD, Langerak AW, Kinchington PR, Osterhaus AD. 2007. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. *Proc Natl Acad Sci U S A* 104:3496–3501. <https://doi.org/10.1073/pnas.0610847104>.
- Steain M, Sutherland JP, Rodriguez M, Cunningham AL, Slobedman B, Abendroth A. 2014. Analysis of T cell responses during active varicella-zoster virus reactivation in human ganglia. *J Virol* 88:2704–2716. <https://doi.org/10.1128/JVI.03445-13>.
- Gowrishankar K, Steain M, Cunningham AL, Rodriguez M, Blumbergs P, Slobedman B, Abendroth A. 2010. Characterization of the host immune response in human ganglia after herpes zoster. *J Virol* 84:8861–8870. <https://doi.org/10.1128/JVI.01020-10>.
- Backes C, Kuentzler J, Lenhof H-P, Comtesse N, Meese E. 2005. GraBCas: a bioinformatics tool for score-based prediction of caspase- and granzyme B-cleavage sites in protein sequences. *Nucleic Acids Res* 33: W208–W213. <https://doi.org/10.1093/nar/gki433>.
- Fontaine-Rodriguez EC, Knipe DM. 2008. Herpes simplex virus ICP27 increases translation of a subset of viral late mRNAs. *J Virol* 82: 3538–3545. <https://doi.org/10.1128/JVI.02395-07>.
- Sandri-Goldin RM. 2011. The many roles of the highly interactive HSV protein ICP27, a key regulator of infection. *Future Microbiol* 6:1261–1277. <https://doi.org/10.2217/fmb.11.119>.
- Cohen JI, Krogmann T, Ross JP, Pesnicak L, Prikhod'ko EA. 2005. Varicella-zoster virus ORF4 latency-associated protein is important for establishment of latency. *J Virol* 79:6969–6975. <https://doi.org/10.1128/JVI.79.11.6969-6975.2005>.
- Sen N, Sommer M, Che X, White K, Ruyechan WT, Arvin AM. 2010. Varicella-zoster virus immediate-early protein 62 blocks interferon regulatory factor 3 (IRF3) phosphorylation at key serine residues: a novel mechanism of IRF3 inhibition among herpesviruses. *J Virol* 84: 9240–9253. <https://doi.org/10.1128/JVI.01147-10>.
- Perera L, Mosca J, Sadeghi-Zadeh M, Ruyechan W, Hay J. 1992. The varicella-zoster virus immediate early protein, IE62, can positively regulate its cognate promoter. *Virology* 191:346–354. [https://doi.org/10.1016/0042-6822\(92\)90197-w](https://doi.org/10.1016/0042-6822(92)90197-w).
- Olofsson PE, Forslund E, Vanherberghen B, Chechet K, Mickelin O, Ahlin AR, Everhorn T, Önfelt B. 2014. Distinct migration and contact dynamics of resting and IL-2-activated human natural killer cells. *Front Immunol* 5:80. <https://doi.org/10.3389/fimmu.2014.00080>.
- Gerada C, Steain M, McSharry BP, Slobedman B, Abendroth A. 2018. Varicella-zoster virus ORF63 protects human neuronal and keratinocyte cell lines from apoptosis and changes its localization upon apoptosis induction. *J Virol* 92:e00338-18. <https://doi.org/10.1128/JVI.00338-18>.
- De Pelsmaeker S, Romero N, Vitale M, Favoreel HW. 2018. Herpesvirus evasion of natural killer cells. *J Virol* 92:e02105-17. <https://doi.org/10.1128/JVI.02105-17>.
- Li H, Zhu H, Xu C-j, Yuan J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 491–501. [https://doi.org/10.1016/S0092-8674\(00\)81590-1](https://doi.org/10.1016/S0092-8674(00)81590-1).
- Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K, Browne KA, Trapani J. 2000. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J Exp Med* 192:1403–1414. <https://doi.org/10.1084/jem.192.10.1403>.
- You Y, Cheng A-C, Wang M-S, Jia R-Y, Sun K-F, Yang Q, Wu Y, Zhu D, Chen S, Liu M-F, Zhao X-X, Chen X-Y. 2017. The suppression of apoptosis by α -herpesvirus. *Cell Death Dis* 8:e2749. <https://doi.org/10.1038/cddis.2017.139>.
- Afonina IS, Cullen SP, Martin SJ. 2010. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. *Immunol Rev* 235:105–116. <https://doi.org/10.1111/j.0105-2896.2010.00908.x>.
- Andrade F, Fellows E, Jenne DE, Rosen A, Young C. 2007. Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition. *EMBO J* 26:2148–2157. <https://doi.org/10.1038/sj.emboj.7601650>.
- Sandri-Goldin RM. 1998. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev* 12:868–879. <https://doi.org/10.1101/gad.12.6.868>.
- Peng H, He H, Hay J, Ruyechan WT. 2003. Interaction between the varicella zoster virus IE62 major transactivator and cellular transcription factor Sp1. *J Biol Chem* 278:38068–38075. <https://doi.org/10.1074/jbc.M302259200>.
- Campbell TM, McSharry BP, Steain M, Slobedman B, Abendroth A. 2015. Varicella-zoster virus and herpes simplex virus 1 differentially modulate NKG2D ligand expression during productive infection. *J Virol* 89: 7932–7943. <https://doi.org/10.1128/JVI.00292-15>.
- Campbell TM, McSharry BP, Steain M, Ashhurst TM, Slobedman B, Abendroth A. 2018. Varicella zoster virus productively infects human natural killer cells and manipulates phenotype. *PLoS Pathog* 14:e1006999. <https://doi.org/10.1371/journal.ppat.1006999>.