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Oncolytic herpes simplex virus infects myeloma cells *in vitro* and *in vivo*

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Because most patients with multiple myeloma (MM) develop resistance to current regimens, novel approaches are needed. Genetically modified, replication-competent oncolytic viruses exhibit high tropism for tumor cells regardless of cancer stage and prior treatment. Receptors of oncolytic herpes simplex virus 1 (oHSV-1), NECTIN-1, and HVEM are expressed on MM cells, prompting us to investigate the use of oHSV-1 against MM. Using oHSV-1-expressing GFP, we found a dose-dependent increase in the GFP$^+$ signal in MM cell lines and primary MM cells. Whereas NECTIN-1 expression is variable among MM cells, we discovered that HVEM is ubiquitously and highly expressed on all samples tested. Expression of HVEM was consistently higher on CD138$^+$/CD38$^+$ plasma cells than in non-plasma cells. HVEM blocking demonstrated the requirement of this receptor for infection. However, we observed that, although oHSV-1 could efficiently infect and kill all MM cell lines tested, no viral replication occurred. Instead, we identified that oHSV-1 induced MM cell apoptosis via caspase-3 cleavage. We further noted that oHSV-1 yielded a significant decrease in tumor volume in two mouse xenograft models. Therefore, oHSV-1 warrants exploration as a novel potentially effective treatment option in MM, and HVEM should be investigated as a possible therapeutic target.

INTRODUCTION

Multiple myeloma (MM) is a plasma cell cancer currently treated with combinations of cereblon-binding drugs (e.g., the immunomodulatory drug thalidomide and its derivatives), proteasome inhibitors (PIs, bortezomib and carfilzomib as examples), CD38-targeted monoclonal antibodies (daratumumab and isatuximab), and steroids. A subset of patients with low-risk disease may live 10–20 years with current therapy, but aggressive myeloma develops resistance quickly, conferring survival of only 1–5 years. Although most patients respond to initial treatment, a pattern of multiple relapses and disease resistance to multi-drug regimens results in an aggressive cancer to which patients succumb.

Oncolytic virus (OV) therapy is a novel treatment strategy defined by tumor cell killing mediated by viruses with high tropism for tumor cells regardless of cancer stage and prior treatment. Interestingly, MM has several features that make it an ideal target for OV, including overexpression of several cell surface proteins that are generally used as viral entry receptors, and mutations in signaling pathways that sensitize MM cells to viral infection.[1–3] Genetically engineered oncolytic herpes simplex virus 1 (oHSV-1) is an enveloped, double-stranded DNA virus that specifically targets and kills a myriad of solid tumors, including glioma, melanoma, and breast, prostate, colon, ovarian, and pancreatic cancer. Increased therapeutic efficacy of OV therapies was observed when oHSV-1-modified viruses were used in combination with traditional therapies such as radiotherapy and chemotherapy, providing an attractive strategy to pursue in the clinic.[4,5] Moreover, oHSV-1 vectors expressing "suicide" genes (thymidine kinase, cytosine deaminase, rat cytochrome P450) or immune stimulatory genes (e.g., interleukin [IL-12], granulocyte-macrophage colony-stimulating factor [GM-CSF]) have been constructed to maximize tumor destruction through multimodal therapeutic mechanisms,[6] and the virus has been evaluated for safety and efficacy in multiple clinical trials.[7] The recent US Food and Drug Administration (FDA) approval of intratumoral injection of talimogene laherparepvec (T-VEC), an oHSV-1 for advanced melanoma, emphasizes its anti-cancer potential.[8] The safety of

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administering Seprehvir, another modified oHSV-1 (HSV1716), systemically was recently reported in the first intravenous phase 1 trial in cancer patients with extra-cranial solid tumors.[9]

However, until very recently, there had not been reports of intravenous oHSV-1 viruses in hematologic malignancies or information on associated potential entry receptors on blood cancer cells. While this manuscript was in communication, two recent reports highlighted that the third-generation oncolytic HSV-1, T-01, could infect and kill human cell lines and primary cells derived from various lineages of hematological malignancies[10] and that the oncolytic effect of T-01 could be augmented by the immunomodulatory drug lenalidomide in the treatment of plasma cell neoplasms.[11] However, we had first reported on the possibility of using HSV-1 in OV therapy of MM.[12] Since herpes virus entry mediator (HVEM, CD270, tumor necrosis factor receptor superfamily member 14 [TNFRSF14]) is overexpressed not only on myeloid and lymphoid cells, but also on primary MM cells and plasma cell leukemias,[13] in this work, we decided to investigate whether this receptor could be used by oHSV-1 to infect and kill MM cells. We show that oHSV-1 can infect and kill several MM cell lines and primary MM cells through viral-mediated induction of programmed cell death via the canonical apoptotic pathway *in vitro* and *in vivo*. Aligned with this finding, we show that both MM cell lines and primary MM cells have high expression of HVEM and variable levels of another HSV-1 receptor, NECTIN-1.[14] Furthermore, among several other tumor types, HVEM expression is the highest in MM and is positively correlated with patient survival, supporting the idea that HVEM can be further studied for its potential role as a future candidate for cell therapy and as a prognostic biomarker for MM.

RESULTS

oHSV-1 infects myeloma cell lines and primary myeloma cells

To explore whether oHSV-1 could infect MM cells, we used genetically engineered HSV-1 (HSVQ) that is deleted for both copies of viral ICP34.5 and possesses a gene-disrupting insertion of green fluorescent protein (GFP) within the viral UL39 locus encoding for the ICP6 gene (Figure S1A).[15] In this study, we have referred to this construct HSVQ as oHSV-1. We treated myeloma cells with increasing concentrations of GFP-expressing oHSV-1 and observed a dose-dependent increase in GFP^+ signal in MM cell lines (MM.1S, LP-1, and NCI-H929) (Figures S1B–S1D) at 24 h post-infection, and a further increase in MM.1S at 48 h (Figure 1A). Efficient infection was observed independently of genetic modifications in the backbone of the oHSV-1 vector (Figure 1B) when MM cell lines were treated with RAMBO,[16] which expresses human anti-angiogenic Vasculostatin-120 (Vstat120) under the viral IE4/5 promoter, and rQNestin34.5,[17] which expresses viral ICP34.5 under the nestin promoter in the same attenuated HSVQ (oHSV-1) construct (Figure S1A). All three viruses, namely, HSVQ (oHSV-1), RAMBO, and rQnestin34.5, express GFP under the same viral ICP6 promoter at the UL39 locus.[15–17] Furthermore, all MM cell lines tested (U266, RPMI 8226, NCI-H929, and LP-1) displayed a significant GFP^+ signal even at a low multiplicity of infection (MOI of 0.1) (Figure 1C) at 24 h after treatment. A highly efficient infection was also observed in primary $CD138^+$ MM cells obtained from bone marrow (BM) aspirates but not in the cellular fraction depleted of MM cells ($CD138^-$ BM fraction) (Figure 1D). Heat inactivation of the virus denatures its surface proteins and inhibits its interaction with virus receptors on target cells;[18] this process prevented viral entry into MM.1S cells even at a saturating MOI of 2.0 (Figure S1E), suggesting that oHSV-1 infection of myeloma cells is dependent at least partially on surface receptor expression. The cellular receptors NECTIN-1 and HVEM can both mediate oHSV-1 entry in a complex multistep process through binding of the major viral receptor-binding protein glycoprotein D (gD).[19] In our study, we observed reduction in surface NECTIN-1 levels in MM cell lines (MM.1S, RPMI 8226, KMS11, and L363) upon viral treatment for 24 h at an MOI of 0.1 (Figure 1E), indicating NECTIN-1 to be involved in the process of viral uptake.[10,20]

The HSV-1 receptor HVEM is highly expressed in myeloma cells

To correlate surface expression of receptors with infection efficiency, we next measured NECTIN-1 and HVEM expression in myeloma cells. We observed that, out of the five highly susceptible MM cell lines, NECTIN-1 was highly expressed only in MM.1S and RPMI 8226 cells, whereas it had a considerably lower surface expression in L363, U266, and H929 cells (Figure 2A; Figure S2A). These data are consistent with the fact that *PVRL1*, the mRNA that encodes NECTIN-1, was variably expressed in primary myeloma samples, as evident using data from the CoMMpass trial (ClinicalTrials.gov: NCT01454297) comprising 768 newly diagnosed MM patients (Figure 2B). On the contrary, we found that HVEM is ubiquitously and highly expressed not only in MM cell lines (Figure 2C; Figure S2A) but also in primary $CD138^+$ MM cells in all MM samples (3.3–62.7 fragments per kilobase per million reads [FPKM]) (Figure 2D), as evident using data from the CoMMpass trial. As a positive control, expression of B cell maturation antigen (BCMA) (Figure 2E), a known marker of MM that predicts outcome for MM patients and is a targetable antigen for novel therapies,[21] was also similarly measured under such conditions. Aligned with these data, high HVEM surface expression (mean fluorescence index [MFI] 1,664 ± 1,045) was also observed by flow cytometry in $CD138^+/CD38^+$ plasma cells, from BM fractions of MM patients ($n = 10$), but it was practically undetectable (MFI 264.8 ± 103.9, $p = 0.002$) in the BM $CD138^-$ fraction (non-MM cells) (Figures 2F and 2G). Interestingly, in two (RPMI 8226 and H929) out of four MM cell lines we tested, an HVEM-blocking antibody was able to impair oHSV-1 infection of MM cells, an effect that was not observed (RPMI 8226) or only partially observed (NCI-H929) when a NECTIN-1-blocking antibody was used (Figures S2B and S2C), indicating that besides NECTIN-1,[10] HVEM is also involved in the process of viral entry into MM cells.[22] Additionally, gene expression data downloaded from the Genomic Data Commons (GDC; https://portal.gdc.cancer.gov/) for 15 different cancer types (Table S1) also revealed MM to express HVEM to the greatest extent among all cell types tested (Figure 2H). In contrast, NECTIN-1 is not expressed as highly in MM (Figure S2D).

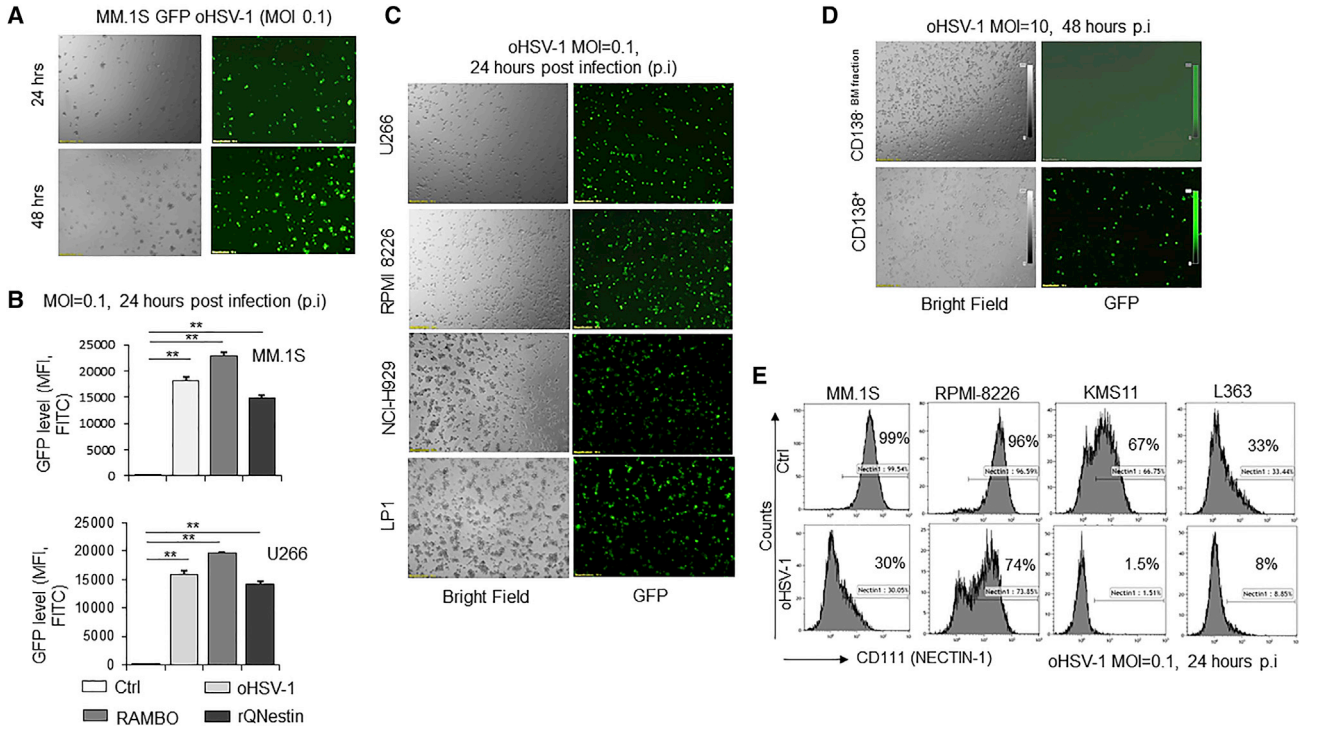

Figure 1. Oncolytic herpes simplex virus 1 (oHSV-1) infects MM cell lines and primary myeloma cells

(A) MM cell line MM.1S was infected with GFP-expressing oHSV-1 at a multiplicity of infection (MOI) of 0.1. Twenty-four hours and 48 h post-infection (p.i.), cells were observed under a fluorescence microscope, which showed greater infection and hence GFP expression over time. (B) MM.1S and U266 cell lines were infected with differently modified oHSV-1 (HSVQ referred to here as oHSV-1, RAMBO, and rQnestin34.5) at an MOI of 0.1. Twenty-four hours after treatment, GFP^+ cells were analyzed by flow cytometry, and high infection efficiency was observed independently of the genetic modifications of the backbone oHSV-1 vector for both cell lines. (C) Several MM cell lines (U266, RPMI 8226, NCI-H929, and LP1) were infected with oHSV-1 at an MOI of 0.1 for 24 h. Cells observed under a fluorescence microscope showed significant GFP^+ signals in all MM cell lines tested even at low an MOI of 0.1. (D) Primary $CD138^+$ MM cells and $CD138^-$ fractions isolated from bone marrow (BM) aspirates of MM patients were infected with oHSV-1 at an MOI of 10. Forty-eight hours after infection, cells were observed under a fluorescence microscope for the presence of GFP. (E) Flow cytometry analysis showing reduction in surface NECTIN-1 expression in MM cell lines (MM.1S, RPMI 8226, KMS11, and L363) upon oHSV-1 treatment (MOI of 0.1) for 24 h. See also Figure S1. Data are reported as mean ± SD of three to four experiments. *$p < 0.05$, **$p < 0.001$.

Moreover, Kaplan-Meier analysis of MM patients from the CoMMpass trial showed that patients with comparatively lower HVEM (TNFRSF14) expression experienced worse overall survival (OS) and progression-free survival (PFS) (Figure S3A). To further understand the reason, we annotated HVEM expression by myeloma subtype defined by gene expression.[21] This test revealed that the Maf (MF), proliferation (PR), and Mmset (MS) gene expression subtypes, which generally have poor outcomes, also expressed lower levels of HVEM, whereas the hyperdiploid (HY) subtype, which is associated with better prognosis,[21] expressed the highest levels of HVEM among the subtypes tested (Figure S3B). In contrast, NECTIN-1 (PVRL1) is not expressed as highly and does not correlate with response to therapy or survival (Figures S3C and S3D).

oHSV-1 kills MM cells independent of viral replication

To test whether oHSV-1 could kill MM cells, we infected MM.1S, U266, and RPMI 8226 cell lines with oHSV-1 at increasing MOI and time. A cell proliferation assay indicated that, compared to control, oHSV-1 could reduce proliferation of all three MM cell lines tested (Figure 3A). Although normal human hematopoietic cells are resistant to oHSV-1 infection,[23] to examine whether direct lytic replication[24] was responsible for oHSV-1-induced eradication of malignant MM cells, we carried out a replication assay as previously described.[15] Surprisingly, it was observed that, although oHSV-1 could efficiently infect and kill all of these cell lines, no viral replication occurred during the process (Figure 3B). To examine whether a structural protein of the oHSV-1 virion is responsible for inducing cell death, we heat-inactivated (HI) oHSV-1 according to a previous protocol.[18] It was observed that HI oHSV-1 failed to induce cell death in MM cells at indicated MOIs and time points (Figure 3C). To determine whether viral particles that are shed into the culture medium trigger cell death, we harvested conditioned medium (CM) in which MM.1S cells were infected with oHSV-1 (MOI of 0.1, 1, and 5) for 10 h, neutralized free virus with 0.4% human immunoglobulin G (IgG), and used this medium to treat freshly seeded MM.1S cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays showed that the CM failed to induce apoptosis in the uninfected, CM-treated MM.1S cells at the indicated time points (Figure 3C).

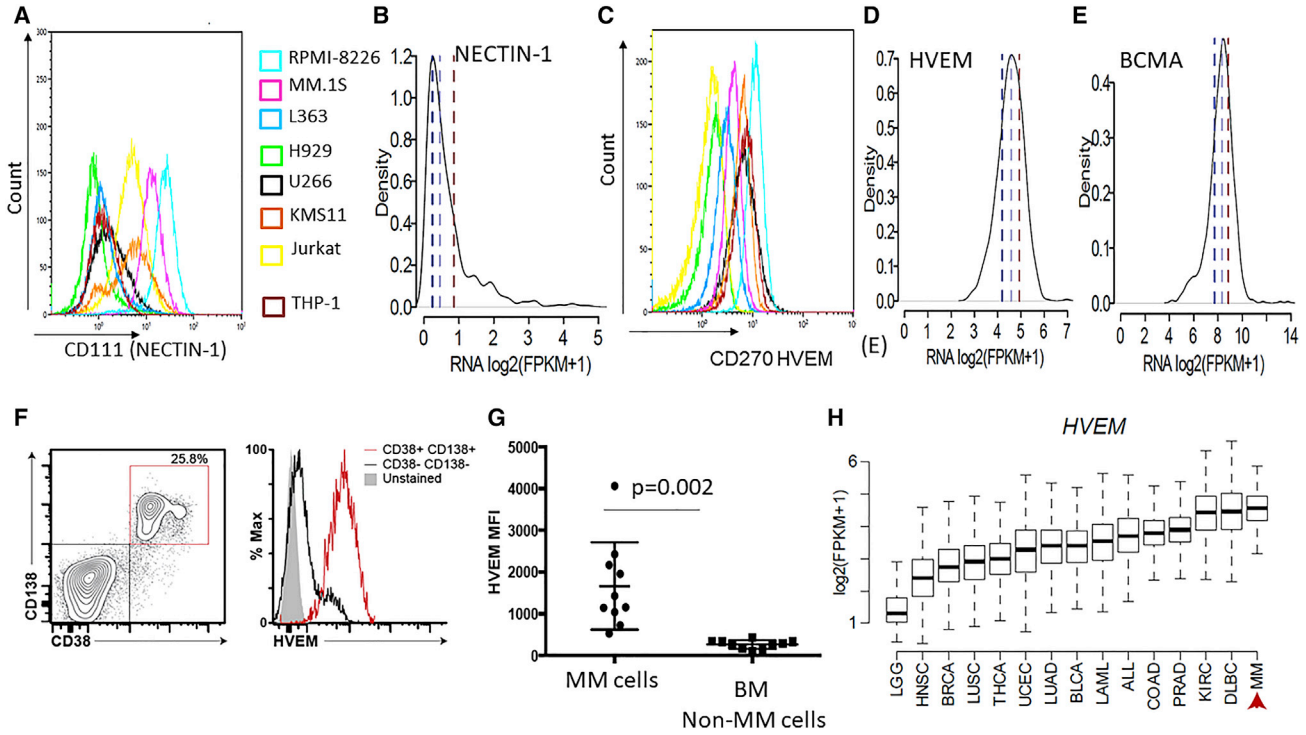

Figure 2. HVEM is highly expressed on MM cell lines and primary myeloma cells

(A and C) Indicated cancer cell lines were stained with antibodies for (A) NECTIN-1 (CD111) and (C) HVEM (CD270). Overlay images show relative surface NECTIN-1 levels and HVEM levels on different cell lines with respect to their individual isotype control. (B, D, and E) Distribution of (B) NECTIN-1, (D) HVEM, and (E) BCMA expression in 768 newly diagnosed patients with MM from the CoMMpass trial. Density plots of expression are expressed in $\log_2$(FPKM+1). The first quartile (blue), median (gray), and third quartile (red) of expression are denoted by dashed lines. (F) Representative flow cytometry dot plot demonstrating gating strategy for plasma cells ($CD138^+$, $CD38^+$) and HVEM staining in plasma cells versus non-plasma cells versus unstained cells. (G) Mean florescence intensity of HVEM in plasma cells versus non-plasma cells from 10 myeloma BM aspirates. (H) Cancer types are sorted by median expression for HVEM and include data from the Genomic Data Commons. Boxplots show the median and first and third quartiles of expression, with the whiskers extending to the most extreme data point within 1.5 times the interquartile range. LAML, acute myeloid leukemia; DLBC, lymphoid neoplasm diffuse large B cell lymphoma; ALL, acute lymphoblastic leukemia; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; HNSC, head and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; LGG, brain lower grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PRAD, prostate adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma. See also Figures S2 and S3 and Table S1. Data are reported as mean ± SD; n=10, p=0.0002.

These observations demonstrated that infection by live viruses is needed to induce cytotoxicity in MM despite the lack of viral replication. Furthermore, to examine whether oHSV-1 infection created sufficient endoplasmic reticulum (ER) stress to lead to apoptosis, we treated MM.1S, U266, and RPMI 8266 cell lines with oHSV-1 at an MOI of 0.1 for 6 h. Immunoblotting showed no significant change in the activation levels of known ER stress response markers PERK and Bip/GRP78[25] as well as subsequent unfolded protein response (UPR) markers Hsp40 and Hsp90[26] in infected cells compared to control (Figure 3D), indicating a lack of involvement of ER stress response pathways in the process.

oHSV-1 kills MM cells through viral-mediated induction of programmed cell death

Our data suggested that oHSV-1 kills human MM cells via a mechanism that is not dependent on lytic viral replication. There is evidence of replication-independent, abrupt myxoma-induced MM cell death via apoptosis, and oHSV-1 induced apoptosis even in the case of productive viral infection in human gastric cancer cells[27] and HEp2 cells.[28] To test apoptosis, we infected MM.1S and U266 cells with oHSV-1 at an MOI of 0.1. The virus-induced cytotoxicity as assayed 24 h post-infection revealed significant induction of both early ($annexin^+$/7-aminoactinomycin D [7-AAD]$^-$) and late ($annexin^+$/$7\text{-}AAD^+$) apoptosis by oHSV-1 (Figure 4A). To measure induction of the classical apoptotic pathway, we next assessed caspase-3 cleavage and PARP cleavage in MM.1S, U266, and RPMI 8266 cells under similar conditions. An immunoblot assay displayed cleavage of the apoptotic effector caspase-3, as well as cleavage of PARP, a marker for late-stage apoptosis in all three MM cell lines (Figure 4B). Furthermore, we pre-incubated MM.1S cells with a pan-caspase inhibitor (Z-VAD-FMK) and subsequently infected them with oHSV-1 (MOI of 0.1 for 24 h). Annexin/7-AAD staining revealed that, although 46% of cells were killed by oHSV-1, the percentages of early ($annexin^+$/$7\text{-}AAD^-$) and late ($annexin^+$/$7\text{-}AAD^+$) apoptotic cells taken together

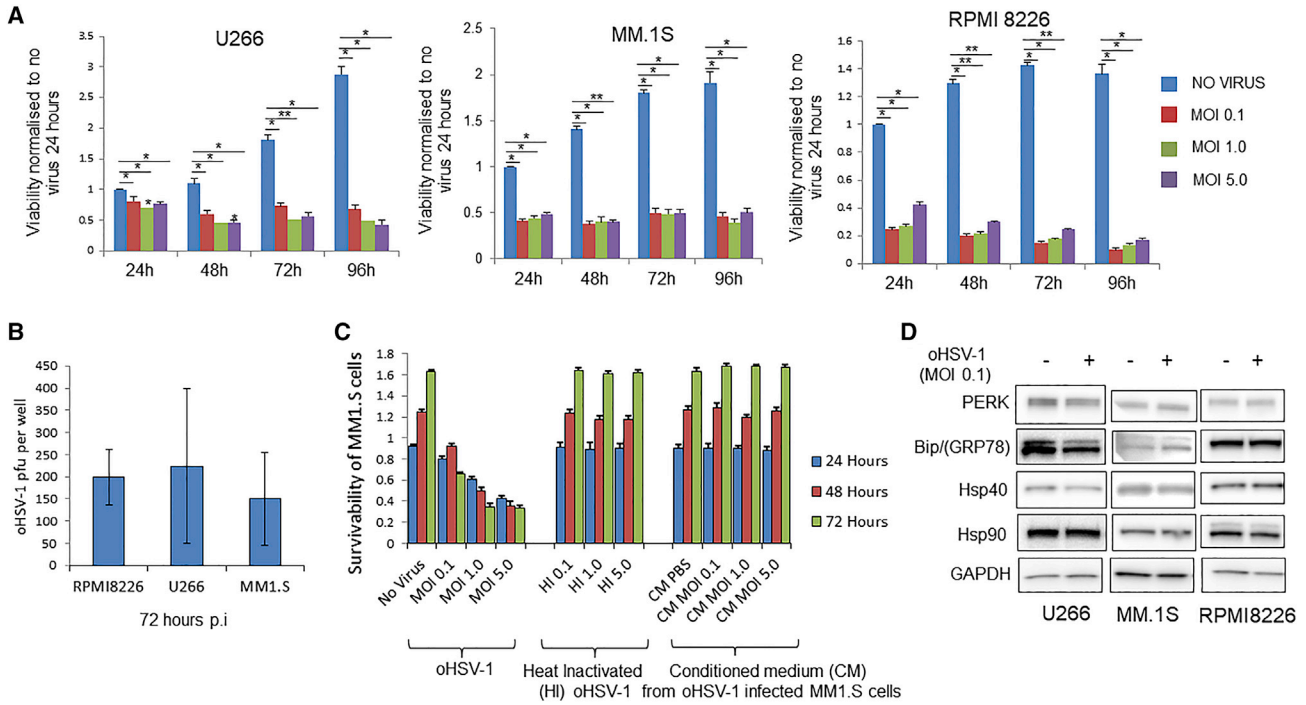

Figure 3. oHSV-1 kills MM cells independent of viral replication

(A) U266, MM.1S, and RPMI 8226 cells were infected with oHSV-1 at MOIs of 0.1, 1, and 5. Control and infected cells were harvested 24, 48, 72, and 96 h following infection, and cell proliferation was measured by an MTT assay. (B) U266, MM.1S, and RPMI 8226 cells were infected with oHSV-1 at an MOI of 0.01. Seventy-two hours following infection, virus from infected cells and medium was used to infect Vero cells for a replication assay. The number of plaques obtained were counted and compared with the initial viral titer. (C) MM.1S cells were treated with live oHSV-1, heat-inactivated (HI) oHSV-1, and conditioned medium (CM) obtained from oHSV-1-treated cells at MOIs of 0.1, 1, and 5. An MTT assay was performed to determine cell viability under such conditions. (D) U266, MM.1S, and RPMI 8226 cells were infected with oHSV-1 at an MOI of 0.1. Six hours after infection, cells were harvested and immunoblot analysis was performed to determine levels of ER stress response markers, namely PERK, Bip (GRP78), and unfolded protein response markers Hsp40 and Hsp90. The protein levels were normalized with respective levels of GAPDH. Data are reported as mean ± SD of three to four experiments. *$p < 0.05$, **$p < 0.001$.

were gradually reduced to 39%, 32%, and 25% in the presence of increasing concentrations of the pan-caspase inhibitor (10, 20, and 50 μM Z-VAD-FMK, respectively) (Figure 4C). On the contrary, use of venetoclax (5 μM), an inhibitor of anti-apoptotic BCL2 (B cell leukemia/lymphoma 2) that induces caspase-3 cleavage,[29] further potentiated oHSV-1-mediated apoptosis when given to MM.1S and NCI-H929 cells pre-treated with oHSV-1 (MOI of 0.1) (Figures S4A–S4D).

oHSV-1 infects and induces apoptosis in primary myeloma (MM) cells

To assess whether oHSV-1 induces apoptosis in primary MM cells, we infected BM aspirates and peripheral blood (PB) isolated from the same MM patients with oHSV-1 at an MOI of 5. While annexin/7-AAD staining performed 48 h post-infection revealed greater induction of apoptosis (41%) compared to uninfected control (12%) in BM aspirates, a similarly increased induction of apoptosis was not observed in oHSV-1-treated PB samples (17%) compared to control cells (10%) (Figure 5A). A cytotoxicity assay analogously revealed induction of apoptosis in BM aspirates of MM patients at both 24 and 48 h post-infection at MOIs of 5 and 10, in contrast to similarly treated PB isolated from MM patients (Figure 5B). To ascertain whether oHSV-1 induced apoptosis preferentially in the $CD138^+$ malignant plasma cell fraction, we isolated the $CD138^+$ cell population from BM aspirates of MM patients and infected it with oHSV-1 at MOIs of 5 and 10. Cytotoxicity assays revealed that oHSV-1 induced higher cell death in the $CD138^+$ population compared to the $CD138^-$ population isolated from BM aspirates (Figure 5C). Next, to determine whether this difference in killing was due to differences in the susceptibility of these subpopulations to infection, we treated BM aspirates, PB, and $CD138^+$ and $CD138^-$ cell populations derived from BM aspirates of MM patients with oHSV-1 (MOI of 10) for 48 h. Fluorescence microscopy revealed that oHSV-1 could efficiently infect BM aspirates in comparison to PB (Figure 5D) and the $CD138^+$ fraction derived from BM aspirates in comparison to the $CD138^-$ fraction obtained from the same MM patient (Figure 5E), as is also demonstrated in Figure 1D.

oHSV-1 has potent *in vivo* anti-myeloma efficacy

We next investigated the anti-myeloma effects of oHSV-1 *in vivo* in two different MM xenograft mice models. Six- to 8-week-old non-obese diabetic (NOD) severe combined immunodeficiency (SCID)

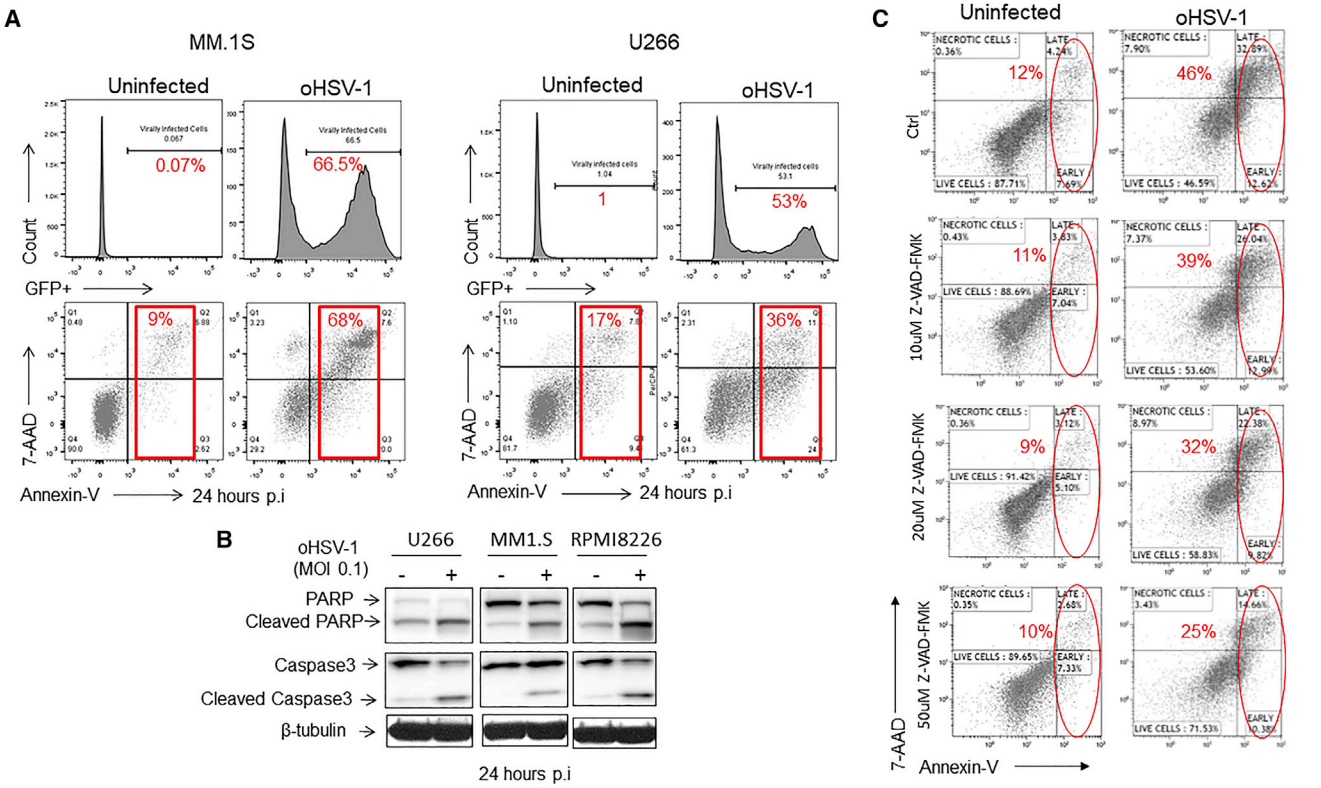

Figure 4. oHSV-1 kills MM cells through viral-mediated induction of programmed cell death

(A) MM.1S and U266 cells were infected with oHSV-1 at an MOI of 0.1. Twenty-four hours after infection, uninfected and oHSV-1-treated cells were stained with annexin V/7-AAD to determine the percentage of late apoptotic cells by flow cytometry. (B) U266, MM.1S, and RPMI 8226 cells were infected with oHSV-1 at an MOI of 0.1 for 24 h. Immunoblot analysis was performed with lysates from harvested cells to determine levels of caspase-3, cleaved caspase-3, PARP, and cleaved PARP. The protein levels were normalized with respect to β-tubulin levels. (C) MM.1S cells were pre-treated with increasing concentrations (0, 10, 20, and 50 μM) of a pan-caspase inhibitor (Z-VAD-FMK) for 2 h. Uninfected cells and cells infected with oHSV-1 (MOI of 0.1) were stained with annexin V/7-AAD after 24 h to determine the percentage of late apoptotic cells by flow cytometry.

gamma (NSG) mice were subcutaneously injected with 12.5×10^6 MM.1S or NCI-H929 cells in their right flank. On formation of palpable tumors, they were treated with 10^6 plaque-forming units (PFU) of oHSV-1 or with saline twice a week for 2 weeks. Figure 6A and 6C show that, while saline-treated tumors grew rapidly, tumor growth in both MM.1S (n = 7, p = 0.00338) and NIH-H929 (n = 7, p = 0.00214) xenograft models was significantly reduced upon treatment with oHSV-1. Figures 6B and 6D show representative images of mice bearing tumors and the tumors extracted from them in both models. These results clearly demonstrate efficient anti-myeloma effects of oHSV-1 *in vivo*.

DISCUSSION

This work shows that oHSV-1 can infect MM cell lines with high efficiency. HSV-1 receptor density on host cells is directly correlated with virus entry efficacy.[30] The key interaction governing HSV-1 entry into host cells occurs through virus surface gD binding to HVEM, NECTIN-1, or 3-*O*-sulfated heparan sulfate (3-OS HS), and in some cases NECTIN-2.[25,31] NECTIN-1 is reported to be the most efficient receptor, and low NECTIN-1 expression levels have been theorized to limit responses to HSV-1-mediated OV therapy in several malignancies.[32,33] Based on expression patterns, HVEM is thought to act as the principal receptor for HSV on lymphoid cells, with NECTIN-1 on epithelial and neuronal cells.[34,35] However, cells often express both NECTIN-1 and HVEM receptors, making their individual contributions to viral entry difficult to assess.[30] Although NECTIN-1 appeared to have a dominant effect on determining T-01 viral entry and oncolysis in a broad range of hematological malignancies, including myeloid-, B cell-, and T cell-derived tumors,[10] a closer inspection in this work, specifically of MM cell lines and primary MM samples comprising 768 newly diagnosed MM patients (CoMMpass trial; ClinicalTrials.gov: NCT01454297), revealed that the oHSV-1 surface receptor HVEM is highly and ubiquitously expressed on the surface of MM cell lines and in primary MM cells both at the mRNA and protein levels. Furthermore, we show that MM expresses HVEM at some of the highest levels of any tumor type, and analysis of MM patient data from the CoMMpass trial revealed that HVEM expression levels are positively correlated with OS and PFS of MM patients, indicating that the virus receptor may be prognostic. On the contrary, in regard to MM, surface NECTIN-1 expression could not account for

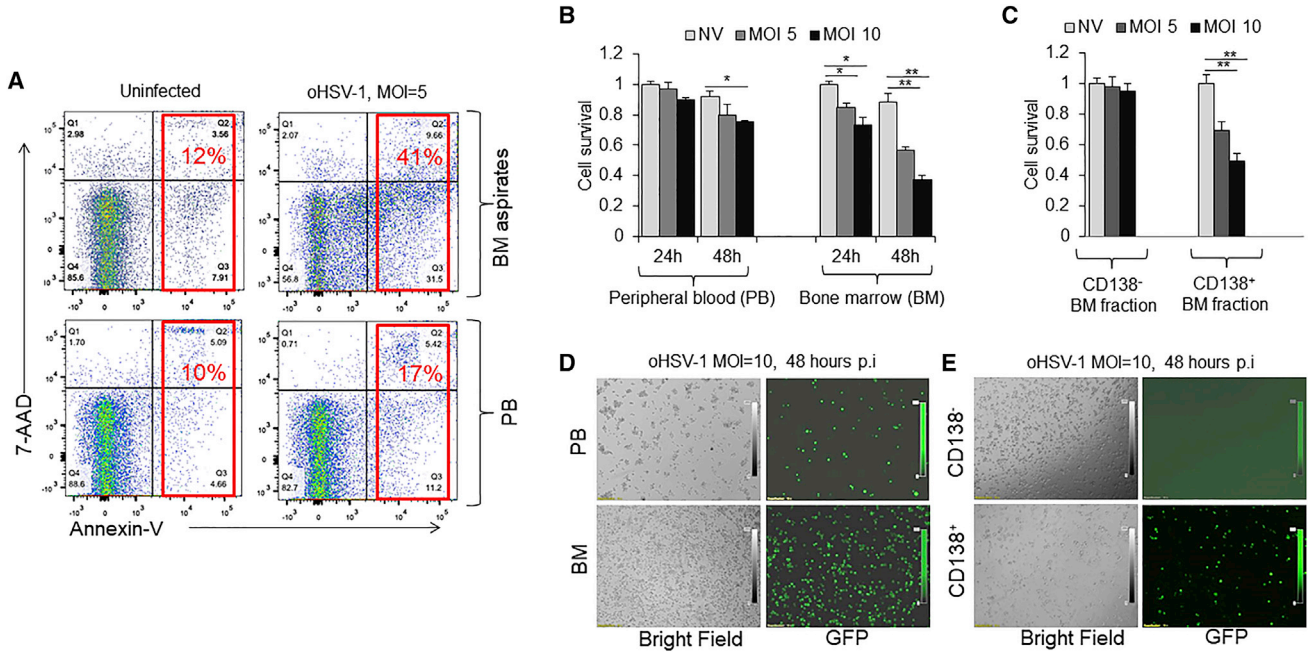

Figure 5. oHSV-1 infects and induces apoptosis in primary myeloma (MM) cells

(A) Primary MM cells derived from BM aspirates and peripheral blood (PB) of the same MM patients were separately infected with oHSV-1 at an MOI of 5. Forty-eight hours following infection, cells were stained with annexin V/7-AAD to determine the percentage of late apoptotic cells by flow cytometric analysis. (B) Primary MM cells derived from BM aspirates and PB of the same MM patients were separately infected with oHSV-1 at MOIs of 5 and 10. Percentage of viable cells was measured at indicated time points. (C) The $CD138^+$ cell population was isolated from the entire cellular extract derived from BM aspirates of MM patients, and $CD138^+$ and $CD138^-$ fractions were similarly treated with oHSV-1 (MOIs of 5 and 10) for 24 and 48 h, and the percentage of viable cells was determined. (D) Primary MM cells derived from BM aspirates and PB of the same MM patients, and also (E) $CD138^+$ and $CD138^-$ fractions isolated from BM aspirates, as previously shown in Figure 1D, were separately infected with oHSV-1 at an MOI of 10. Forty-eight hours after infection, cells were observed under a fluorescence microscope for the presence of GFP. Data are reported as mean ± SD of three to four experiments. $^*p < 0.05$, $^{**}p < 0.001$.

the efficiency of oHSV-1 infection across all of the MM cell lines tested. Since an HVEM blocking antibody prevented oHSV-1 infection of MM cells, we think that the high levels of HVEM make MM cells highly susceptible to HSV infection. This result is also consistent with the finding that none of the $HVEM^-$ MM cell lines was susceptible to infection by T-01.[10] Thus, this work highlights the potential for oHSV-1 and its receptor HVEM as novel anti-MM treatment approaches. OVs lead to anti-neoplastic effects mainly by (1) induction of immune responses to viral- and/or tumor-specific antigens, (2) direct lysis of cancer cells, and (3) vascular breakdown within the tumor microenvironment. Vaccinia virus and HSV can infect endothelial cells of tumor vasculature, disrupting it and causing indirect death of cells that may not be infected by virus.[36,37]

Our data demonstrate that, while oHSV-1 treatment had a profound effect on MM growth *in vitro*, there was no evidence of viral replication and lysis in these cells. We found evidence of OV-induced cell death by activation of ER stress response pathways.[38] ER stress response also plays a critical role in the survival of MM cells, and induction of ER stress response pathways occurs in both reovirus-induced MM cell death[39] and in the oHSV-1-infected cervical carcinoma cell line HeLa.[38] However, MM cell lines treated with oHSV-1 did not show activation of an ER stress response or unfolded protein response (UPR), ruling out this mechanism of cell death in MM. Because our data suggested that oHSV-1 kills MM cells via a mechanism independent of lytic viral replication, we next turned our attention to apoptosis. Although viral infection often leads to an apoptotic response by a cell in order to protect other cells from a similar fate,[40] there is evidence of oHSV-1-induced apoptosis even with productive viral infection in human gastric cancer cells[27] and HEp2 cells.[28] Myxoma induces rapid cellular apoptosis in MM at a rate such that it aborts the virus replication cycle prior to generation of progeny virus.[41,42] In this work, MM cell lines infected with oHSV-1 showed similar induction of apoptosis by cleavage of apoptotic effector caspase-3 and PARP, a marker for late-stage apoptosis. Furthermore, the partial rescue from oHSV-1-mediated apoptosis in the presence of a pan-caspase inhibitor (Z-VAD-FMK) in MM cells and further potentiation in the presence of venetoclax, which induces caspase-3 cleavage,[29] assert the involvement of caspase-3 in the process. These results suggest that oHSV-1 kills MM cells through a viral-mediated induction of programmed cell death. However, since caspase inhibition alone failed to completely rescue MM cells from oHSV-1-induced cell death, the finding suggests involvement of other mechanisms in the process. Survival of myeloma cells may be influenced by

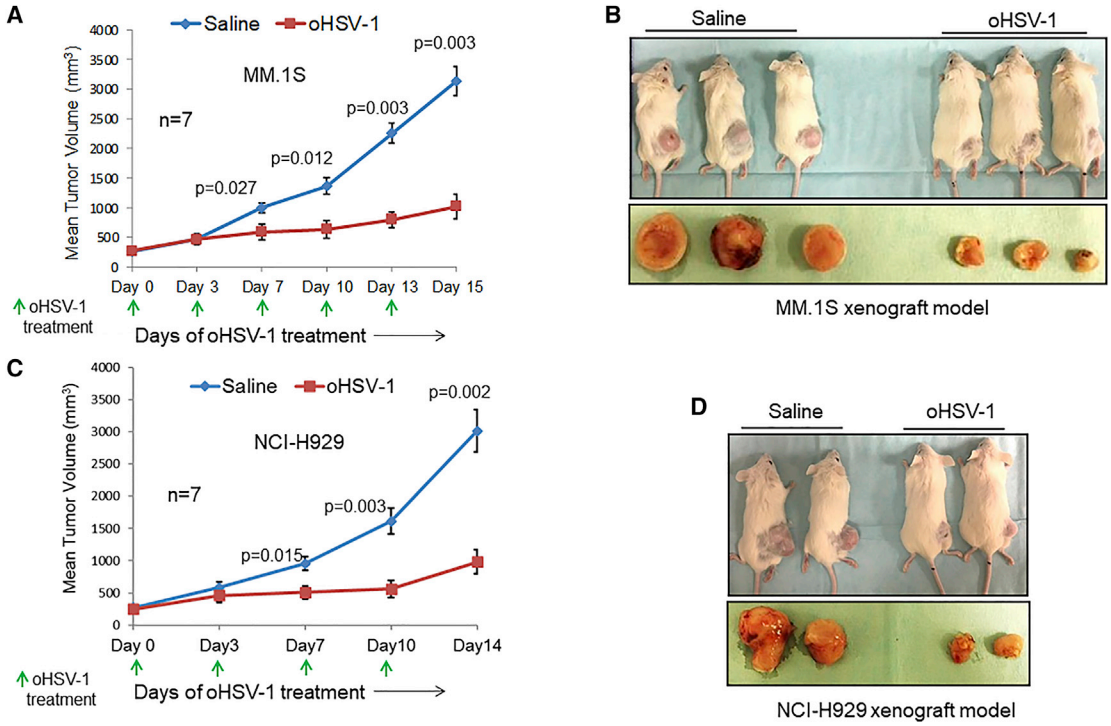

Figure 6. oHSV-1 shows potent *in vivo* anti-myeloma efficacy

Six to 8-week-old NSG mice were subcutaneously injected with 12.5 × 10^6 MM.1S or NCI-H929 cells in their right flank. On the formation of palpable tumors, they were treated with 10^6 PFU of oHSV-1 or with saline twice a week for 2 weeks. (A) Time course of tumor growth of MM.1S cell line. Mice were sacrificed on day 15 after the first treatment with oHSV-1, and tumor volumes were measured. (B) Representative image of mice bearing subcutaneous MM.1S xenografts/tumors treated either with saline or oHSV-1 and the corresponding extracted tumors. (C) Time course of tumor growth of the NCI-H929 cell line. (D) Representative image of mice bearing subcutaneous NCI-H929 xenografts/tumors treated either with saline or oHSV-1 and the corresponding extracted tumors. A significant decrease in tumor volume was observed with oHSV-1 in both the MM.1S xenograft model (n = 7, p = 0.00338) and the NCI-H929 xenograft model (n = 7, p = 0.00214) compared to those in saline-treated mice.

autophagy,[43] and reovirus-mediated oncolysis in MM cells is caused by both induction of apoptosis and autophagy.[44] Autophagic responses of infected tumor cells can either prevent autophagy activation[45] or can induce autophagic cell death in squamous cell carcinoma.[46] Interestingly, the oHSV-1 viral γ34.5 gene contains a Beclin1-binding domain that inhibits the progression of autophagosomes.[47] Hence, similar to other γ34.5 gene-deficient oHSV-1, the oHSV-1 construct used in this study lacking both copies of the viral γ34.5 gene might therefore activate both autophagic and apoptotic cell death in MM cells. Furthermore, it has been recently reported that oncolytic HSV-1, T-01, induces direct oncolysis and immune activation governed by plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells in plasma cell neoplasms.[11]

Additionally, oHSV-1 also induced apoptosis in primary MM cells. It was observed to be higher in the total cellular fraction isolated from BM aspirates of patients with MM, compared to that isolated from PB, and also in the BM $CD138^+$ cellular fraction compared to the BM $CD138^-$ cellular fraction. Such differences may be associated with the specific high level of expression of HVEM in MM plasma cells compared to other BM cellular fractions. Thus, on the basis of our observation, it is reasonable to explore the possibility of using surface HVEM as a potential biomarker in MM. Our standpoint is supported by two different xenograft mouse models of MM that indicate potent anti-MM efficacy of oHSV-1 even *in vivo*. We also speculate that plasma cells may be susceptible to naturally occurring HSV-1 infection, which may result in the observed aberrant interferon signaling,[48] a hypothesis that requires further testing.

Can recombinant HSV-1 move forward clinically? HSV-1 is the first approved OV, T-VEC, for the treatment of melanoma via intratumoral injection.[8,49] HSV spreads locally within the injected tumor and kills tumor cells by *in situ* necroptosis in the regional lymph nodes.[50] However, detailed analysis of individual lesion response rates showed complete responses in 46% of injected lesions, 30% of uninjected non-visceral lesions, and only 9% of uninjected visceral lesions;[51] evidently, direct infection is important for patients with metastatic disease. MM is a systemic hematologic malignancy with heterogeneous marrow infiltration, which makes intratumoral injection unattractive. Intravenous OV administration is a challenge, as the bloodstream dilutes the virus, circulating antiviral antibodies can remove the agent, and local macrophages sequester viruses before reaching the tumor. Thus, it is imperative to develop strategies to overcome these host immune viral responses. To this end,

cyclophosphamide has been shown to be a suitable immunosuppressant in animal models and in early clinical trials with measles virus, herpes virus, and reovirus.[52–54] It is noteworthy that cyclophosphamide, which is an approved therapeutic for MM,[55–57] when given in a metronomic regimen[54] sufficient to prolong viral dissemination in MM patients may facilitate anti-tumor efficacy[54] in combination with systemic oHSV-1. Moreover, our group was the first to successfully deliver Reolysin (pelareorep) as a systemically administered viral oncolytic in patients with relapsed MM.[58] Thus, promising approaches to deliver viruses intravenously include the use of immunosuppressants, the future use of viruses with low seroprevalence in humans,[41] and using monocytes to transport OVs to tumors.[59] Taken together, the results obtained in this work provide a platform for further exploring the possibility of using oHSV-1 in the treatment of MM. The combined use of approaches ensuring improved viral dissemination, thereby maximizing the efficacy of HSV-1 oncolytic viral therapy, might represent an available approach for MM when conventional treatments fail. Additionally, the highly expressed virus receptor HVEM can be further studied for its potential role as a future candidate for cell therapy and also as a prognostic biomarker for MM.

MATERIALS AND METHODS

Cell culture

MM cell lines (MM.1S, NCI-H929, RPMI 8226, LP1, U266, L363, and KMS11) were purchased from ATCC. The cell lines were cultured in RPMI 1640 supplemented with 10% or 2% (prior to infection) fetal bovine serum (FBS) (catalog no. 019K8420, Sigma), 100 IU/mL penicillin, and 100 μg/mL streptomycin. African green monkey kidney epithelial Vero cells were cultured in DMEM supplemented with 10% or 2% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

HVEM and NECTIN-1 expression from CoMMpass

RNA sequencing (RNA-seq) data from the CoMMpass trial (ClinicalTrials.gov: NCT01454297) were downloaded from dbGaP (phs000748.v7) and processed as previously described.[60] Briefly, RNA-seq expression was determined by FPKM normalization calculated using the summarizeOverlaps function of the GenomicRanges package (v1.38.0) in R (v3.6.2) using the Ensembl transcript database (GRCh37.74) without the use of IMGT-defined Ig genes. Clinical outcome data used were from interim analysis.[15]

HVEM and NECTIN-1 expression across common cancer types from GDC

Gene expression data were downloaded from GDC (https://portal.gdc.cancer.gov/) using the gdc-client (v1.5.0). FPKM normalized read counts were the result of harmonized GDC data processing mapping to the GRCh38 reference genome with the STAR aligner and GENCODE (v22) transcripts. Data from primary tumors generated by TCGA, TARGET, Beat AML, CTSP-DLBCL1, NCICCR-DLBCL, and MMRF CoMMpass projects were combined in R (v3.6.3), and universally unique identifiers (UUIDs) were annotated with the GDC (v1.10.0) package. Cancer types with more than 400 specimens were included in the final analysis (Table S1).

oHSV-1

Recombinant HSV-1 (rHSVQ1), used and denoted in this work as oHSV-1 (Figure S1A), is a first-generation OV deleted with both copies of γ34.5 encoding the neurovirulence factor ICP34.5 and ICP6 inactivated by insertion of GFP in the UL39 locus encoding the large subunit of the viral ribonucleotide reductase as previously described.[15,16] Upon infection in normal cells, γ34.5 gene product ICP34.5 targets host shutoff of protein synthesis, enabling the wild-type virus to replicate in host cells. With deletion of the γ34.5 gene and insertional inactivation of viral ICP6 in recombinant, attenuated oHSV-1, the virus can only replicate in cancer cells that can complement these mutations, thereby conferring several important safety advantages.[61–66] Additionally, genetically modified HSV-1 virus constructs, namely RAMBO,[16] which expresses human Vstat120 under viral IE4/5 promoter, and rQnestin34.5, which expresses viral ICP34.5 under the regulation of a glioma-specific nestin promoter[17] within the same oHSV-1 backbone (Figure S1A), were used to determine efficacy of HSV-1 infection of myeloma cells independent of genetic modification.

Primary samples and CD138$^+$ plasma cell isolation

Primary samples of blood and BM aspirates from MM patients at The Ohio State University were obtained from an Institutional Review Board (IRB)-approved protocol (ClinicalTrials.gov: NCT01408225); patient samples from Emory University were also collected from an IRB-approved protocol (Winship 2226/IRB00057236). BM mononuclear cells were isolated using Ficoll gradient centrifugation. CD138$^+$ plasma cells were purified from all cellular fractions of BM aspirates by human whole blood CD138 MicroBeads (catalog no. 140-000-673, Miltenyi Biotec) following the manufacturer's instructions.

Virus propagation and plaque formation assays

Recombinant HSV1 (oHSV-1) was propagated in Vero cells. Three days after infection, secreted virus and virus-infected Vero cells were harvested, subjected to repeated freeze-thaw cycles, and sonicated to release the viruses completely, after which cell debris was cleared by centrifugation (4,000 × *g*, 20 min). Virus containing supernatant was filtered to remove cell debris and further pelleted by centrifugation at 13,000 × *g* for 1 h. The virus pellet was dissolved in 10 mL of sterile normal saline and purified by 30% sucrose gradient ultracentrifugation at 13,000 × *g* for 90 min using a Sorvall RC-5C Plus ultracentrifuge. The titer (PFU/mL) of the resulting virus was determined by PFU assay in Vero cells.[15] For viral infections, myeloma cells were treated with oHSV-1 in RPMI 1640 medium with 2% FBS at the indicated MOI at 37°C and harvested at described time points.

Analysis of oHSV-1 infection in cultured myeloma cells

To measure initiation of early viral gene expression or infection, human myeloma cell lines and primary cells were treated with oHSV-1 at the indicated MOIs and then analyzed at the indicated time points for the expression of GFP using fluorescence microscopy or flow cytometry.

Virus replication assay

To measure completion of the viral replication cycle and production of new infectious progeny virus, replication assays were performed. The indicated MM cell lines were infected with oHSV-1 at an MOI of 0.01, corresponding to 500 PFU/mL. Human serum IgG was added between 15 and 16 h of infection. Seventy-two hours after infection, secreted virus and virus-infected MM cells were harvested, subjected to repeated freeze-thaw cycles, sonicated, and centrifuged to obtain supernatant with live viruses. The supernatant was then serially diluted and titrated on Vero cells as described earlier.[15] The number of plaques formed on Vero cells 72 h after infection with the supernatant was compared with that from the initial virus titer used to infect MM cell lines, thereby determining the replication efficacy of the virus within MM cells.

Reagents

Human serum IgG (catalog no. 14506) was purchased from Sigma. Pan-caspase 3 inhibitor Z-VAD-FMK (catalog no. G723A, Promega) was used at concentrations 10, 20, and 50 μM. Venetoclax (catalog no. 4762, Tocris Bioscience) was used at concentration of 5 μM. HVEM antibody (D-5) (catalog no. sc-365971, Santa Cruz) and NECTIN-1 antibody (F-10) (catalog no. sc-271063, Santa Cruz) were used for receptor blocking.

Cell proliferation assay

Myeloma cells (5×10^4) were seeded in 100 μL of RPMI 1640 medium with 2% FBS per well in 96-well plates in quadruplicate. The cells were infected with live oHSV-1 or with HI (100°C for 1 h) oHSV-1 or with CM of oHSV-1-treated myeloma cells at indicated MOIs and kept incubated for the indicated time points. Cell proliferation was assessed using an aqueous non-radioactive cell proliferation assay kit (Promega).

Flow cytometry

Analysis of *in vitro* infection and killing of myeloma cell lines and primary cells by GFP-expressing oHSV-1 was done at the indicated time points by staining control and infected cells with V450 annexin V (catalog no. 560506, Becton Dickinson) and 7-AAD (catalog no. 559925, BD Pharmingen) following the manufacturer's protocol and quantitating GFP^+ and early ($annexin^+/7\text{-}AAD^-$) and late apoptotic cells ($annexin\ V^+/7\text{-}AAD^+$) by flow cytometry. Data were analyzed using FlowJo 2.0 (Tree Star, Ashland, OR, USA). Alexa Fluor 647 mouse anti-human HVEM (CD270, catalog no. 56441, BD Pharmingen) and mouse anti-human NECTIN-1 (CD111)-phycoerythrin (PE) (catalog no. 130-103-833, Miltenyi Biotec) were used to determine HVEM and NECTIN-1 expression, respectively, and flow data were analyzed using Kaluza software (Beckman Coulter). To measure HVEM (CD270) expression on plasma cells, cells isolated from BM extracts were stained with anti-CD138 fluorescein isothiocyanate (FITC) (Becton Dickinson), CD38 V450 (Becton Dickinson), and CD270 allophycocyanin (APC) (Becton Dickinson) to distinguish MM cells ($CD138^+$, $CD38^+$) from all other BM non-MM cells and then analyzed by flow cytometry.

In vitro blocking of HVEM and NECTIN-1 receptors during oHSV-1 infection

1×10^6 MM cells (MM.1S, NCI-H929, RPMI 8266, and L363) were incubated in growth medium with 10 μg/mL HVEM antibody (D-5) (catalog no. sc-365971, Santa Cruz) and/or NECTIN-1 antibody (F-10) (catalog no. sc-271063, Santa Cruz) for 30 min, then infected with an MOI of 0.1 oHSV-1. After 24 h the percentage levels of infected GFP^+ cells, as well as HVEM and NECTIN-1 expression, were assessed by flow cytometry.

Western blotting

Cell lysates were prepared in 1× radioimmunoprecipitation assay (RIPA) lysis buffer, quantitated by the bicinchoninic acid (BCA) method, run on 4%–20% gradient SDS-PAGE gels, and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for at least 60 min with 5% nonfat dry milk in TBS-T (25 mM Tris, 150 mM NaCl, 2 mM KCl, 0.1% Tween 20 [pH 7.4]) and incubated overnight at 4°C with the indicated primary antibody diluted (1:1,000) in 2% nonfat dry milk in TBS-T. Appropriate secondary antibody incubations were done at room temperature for 2 h, and blots were treated with chemiluminescent substrate (Pierce Biotechnology) before exposure. For western blots, the primary antibodies used were caspase-3 rabbit Ab (catalog no. 9662S, Cell Signaling Technology), cleaved caspase-3 (catalog no. 9664S, Cell Signaling Technology), PARP rabbit Ab (catalog no. 9542S, Cell Signaling Technology), PERK (catalog no. 5683S, Cell Signaling Technology), Hsp90 rabbit Ab (catalog no. 4877S, Cell Signaling Technology), Hsp40 rabbit Ab (catalog no. 4871P, Cell Signaling Technology), Bip/GRP78 rabbit Ab (catalog no. 3177S, Cell Signaling Technology), GAPDH mouse Ab (catalog no. 97166S, Cell Signaling Technology), and β-tubulin rabbit Ab (catalog no. 2146S, Cell Signaling Technology). The secondary antibodies used were anti-rabbit IgG horseradish peroxidase (HRP)-linked whole Ab (catalog no. NA934, Sigma) and anti-rabbit IgG HRP-linked whole Ab (catalog no. NXA931, Sigma).

Mouse experiments

All animal studies were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee (IACUC). Mice were housed under a 12-h light/12-h dark cycle with food and water *ad libitum*. A cohort of 6- to 8-week-old male and female NSG mice was injected subcutaneously with 12.5×10^6 MM.1S or NCI-H929 cells in their right flank. At the time of presence of palpable tumors, the mice were randomly distributed into two experimental groups each and intratumorally (subcutaneously) treated twice a week for 2 weeks with (1) sterile normal saline, 100 μL (control group, n = 7); or (2) oHSV-1, 10^6 PFU in 100 μL of sterile normal saline (oHSV-1-treated group, n = 7). Tumor measurements (by caliper) were taken three times a week before control mice tumors reached the endpoint volume (30 days after tumor implantation), and mice were humanely euthanized. Because of the characteristic irregular tumor shape using the MM.1S or NCI-H929 cell line, tumor volume (in mm^3) was estimated by measuring the long side (mm) times the short side (mm) times the height (mm) of the tumor. The volumes of the

tumors (in mm^3) between control and oHSV-1-treated groups were compared to determine the *in vivo* anti-myeloma efficacy of the virus.

Statistical analysis

For *in vitro* experiments, data are reported as mean ± SD of three to four experiments. For experiments involving two groups, we have performed a two-tailed unpaired Mann-Whitney test. For experiments involving multiple groups, statistical analysis was conducted by one-way ANOVA followed by a post hoc Tukey's honestly significant difference (HSD) test (*$p < 0.05$, **$p < 0.001$) for pairwise comparisons between groups to determine statistical significance between various pairs of mean. For animal experiments involving two groups, a non-parametric Mann-Whitney test was done (*$p < 0.05$, **$p < 0.001$) and the data are shown as mean ± SEM. All statistical analyses were performed using GraphPad software (GraphPad, La Jolla, CA, USA).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.02.009.

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AUTHOR CONTRIBUTIONS

J.G. performed most of the experiments, A.D. and E.G.G. performed cell line experiments focused on Nectin and HVEM staining and blocking antibodies. M.M. performed work on HSV virus amplification and experiments associated with the effect of HSV + venetoclax in different myeloma cell lines. E.C., J.Y.Y., and L.R. helped perform animal experiments. L.R. and A.C.J.-R. performed flow cytometry on MM cell lines to determine infection. B.G.B. and V.A.G. analyzed the CoMMpass data and performed flow cytometry on myeloma BM samples for HVEM surface expression. J.G., C.C.H., A.D., and F.P. wrote the manuscript. J.F.S. scientifically corrected the manuscript. D.W.S., S.T.R., A.K., L.H.B., and B.K. revised the manuscript. All of the authors have contributed substantially to the analysis and interpretation of data, revising the manuscript for important content, and final approval.

DECLARATION OF INTERESTS

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