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Comparison of the oncolytic activity of a replication-competent and a replication-deficient herpes simplex virus 1

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Abstract

In 2015, the oncolytic herpes simplex virus 1 (HSV-1) T-VEC (talimogene laherparepvec) was approved for intratumoral injection in non-resectable malignant melanoma. To determine whether viral replication is required for oncolytic activity, we compared replication-deficient HSV-1 *d*106S with replication-competent T-VEC. High infectious doses of HSV-1 *d*106S killed melanoma ($n = 10$), head-and-neck squamous cell carcinoma ($n = 11$), and chondrosarcoma cell lines ($n = 2$) significantly faster than T-VEC as measured by MTT metabolic activity, while low doses of T-VEC were more effective over time. HSV-1 *d*106S and, to a lesser extent T-VEC, triggered caspase-dependent early apoptosis as shown by pan-caspase inhibition and specific induction of caspases 3/7, 8, and 9. HSV-1 *d*106S induced a higher ratio of apoptosis-inducing infected cell protein (ICP) 0 to apoptosis-blocking ICP6 than T-VEC. T-VEC was oncolytic for an extended period

of time as viral replication continued, which could be partially blocked by the antiviral drug aciclovir. High doses of T-VEC, but not HSV-1 *d*106S, increased interferon-β mRNA as part of the intrinsic immune response. When markers of immunogenic cell death were assessed, ATP was released more efficiently in the context of T-VEC than HSV-1 *d*106S infection, whereas HMGB1 was induced comparatively well. Overall, the early oncolytic effect on three different tumour entities was stronger with the non-replicative strain, while the replication-competent virus elicited a stronger innate immune response and more pronounced immunogenic cell death.

KEYWORDS
apoptosis, cancer, human, tumour immunology, viral

INTRODUCTION

Oncolytic viruses, in particular oncolytic herpes simplex viruses (HSVs), have left the experimental stage and moved from cell culture and animal experimentation towards patient application. This translational development was initiated and driven by the encouraging results of the OPTiM trial, in which T-VEC (talimogene laherparepvec) was the first oncolytic herpes virus to show a significant improvement compared to subcutaneous application of GM-CSF in durable response rate and overall survival in patients suffering from unresectable stage IIIB to IV melanoma [1, 2].

Since then, oncolytic viruses have been further tailored to express transgenes for immune modulation [3]. While T-VEC codes for GM-CSF to promote recruitment of dendritic cells [4], 2nd and 3rd generation oncolytic herpes viruses carry transgenes like IL-12 [5–9], IL-15 [10, 11], or IL-18 [12] to create a more pro-inflammatory tumour microenvironment. Newer strains carry transgenes for the expression of CD40L [13] and checkpoint inhibitor anti-PD-1 [14]. One of the most recent strains is equipped with five transgenes, namely IL-12, the extracellular domain of FLT3 ligand, CCL4, and antagonists of PD-1 and CTLA-4 [15].

A growing number of trials are being conducted using various oncolytic herpes viruses for the treatment of different tumour entities [16]. A remarkable success was recently reported in a phase I study of paediatric high-grade gliomas, a difficult-to-treat tumour, in which the oncolytic HSV-1 G207 strain was applied stereotactically [17]. Like T-VEC, this virus has a deletion of the neurovirulence gene γ34.5, but additionally a deletion of infected cell protein (ICP) 6, which attenuates virus replication [17].

A non-replicative virus may offer advantages in terms of clinical safety, for example, back mutation or recombination with virulent HSV is less likely. The latest viral strains with receptor retargeting [18, 19] may be particularly well suited for systemic use to find and target distant metastases. For this application, a replication-deficient virus could be used with fewer concerns. Therefore, the aim of our study was to compare the oncolytic effects of the replication-deficient HSV-1 *d*106S with the state-of-the art replication-competent T-VEC strain. Are both viruses equally efficient in triggering cell death? Is the type of cell death different? Is HSV-1 *d*106S as efficient as T-VEC in inducing immunogenic cell death [20]?

MATERIALS AND METHODS

Cell lines

Melanoma cell lines IGR-1, IGR-37, IGR-39, SK-MEL-3, SK-MEL-30 (obtained from the German Collection of Microorganisms and Cell Cultures DSMZ, Braunschweig, Germany), and AXBI, LIWE-7, ARST-1, UMBY, ICNI-5li (established at the Department of Dermatology, University Hospital Erlangen, Germany) [21], as well as human head-and-neck squamous carcinoma cell lines (HNSCC) PCI-1, PCI-4, PCI-4a, PCI-6, PCI-8, PCI-9, PCI-13, PCI-15, PCI-37a, PCI-52, PCI-68 (kindly provided by Prof. Dr. Theresa L. Whiteside, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA) were propagated in DMEM (ThermoFisher Scientific, Schwerte, Germany) supplemented with 10% heat-inactivated FCS, 90 U/mL streptomycin, 0.3 mg/mL glutamine, and 200 U/mL penicillin (all PAN Biotech, Aidenbach, Germany). HTB94 (SW 1353) and Hs 819.T chondrosarcoma cell lines (both from the American Type Culture Collection ATCC, Manassas, VA, USA) were cultured in supplemented DMEM-F12 (Merck, Darmstadt, Germany) and DMEM (ATCC), respectively. Before infection, cells were seeded and cultured overnight to allow adherent growth. All cell lines were checked quarterly for mycoplasma contamination.

Generation of viral stocks

T-VEC (Imlygic®) was obtained from Amgen (Munich, Germany). HSV-1 *d*106S, derived from the HSV-1 *d*106 virus [22], is replication-deficient due to deletions of essential viral genes and promoter regions (ICP4, ICP22, ICP27, ICP47), and expresses GFP under the control of the CMV promoter [23]. T-VEC was propagated and titrated in Vero cells, and HSV-1 *d*106S in Vero cells providing the missing ICP4 and ICP27/47 *in trans* (E11 cells), as described previously [24, 25]. For the preparation of T-VEC stocks, cultures were harvested after Vero cells were lysed. To account for the reduced replication capacity of HSV-1 *d*106S, E11 cells were harvested in 10% of the culture supernatant, when the cells showed a pronounced cytopathic effect, but before cell lysis. Both preparations were subjected to three freeze–thaw cycles. Cellular debris was removed by centrifugation and subsequent filtration through 0.45 µm pore filters (Roth, Karlsruhe, Germany). Aliquots were stored at −80°C.

To analyse the infectivity of viral stocks, the 50% tissue culture infectious dose ($TCID_{50}$) was determined according to the method of Reed and Muench [26]. For this purpose, stocks were serially diluted fourfold and each dilution was used to infect eight cell culture wells. After incubation for 3–4 days, infected cell cultures were identified by cytopathic effects using light microscopy. This approach allows to calculate the volume of the viral stock infecting a cell culture with a probability of 50% and corresponds to 0.5 plaque forming units (PFU). Infectivity of viral stocks was determined as mean of at least three experiments. Titration of an HSV-1 *d*106S stock by cytopathic effects and expression of GFP yielded comparable viral titres (Supplementary Table 1). The ratio of virus particles to infectious virus, determined by quantitative PCR and limiting dilution, was six times higher in HSV-1 *d*106S compared to T-VEC stocks (Supplementary Table 2).

The expression of GM-CSF from T-VEC-infected tumour cell lines was measured in cell culture supernatants using the Luminex-based ProcartaPlexHuman GM-CSF Simplex kit and the respective human basic kit (ThermoFisher Scientific).

Cell viability assay

The cell viability of tumour cell lines was analysed using 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT). In brief, 10 000 cells/well were infected in 96-well plates. After 2 days (unless indicated otherwise), 10 µL MTT (Biomol, Hamburg, Germany) dissolved at 5 mg/mL in DPBS was added and incubated for 1–2 h, followed by addition of 100 µL detergent (10% SDS, 10 mM HCl). Absorbance was measured at 595 nm after incubation overnight. Mock-infected cells served as controls. MTT data of melanoma cell lines infected with T-VEC at MOI 1 (multiplicity of infection; one infectious particle per cell) [25] and of chondrosarcoma cell line HTB-94 infected with T-VEC at MOI 10 [27] were recently reported and are included here for comparison.

Reverse transcriptase-quantitative PCR analyses

A total of 300 000 cells/well were infected in 12-well plates. After 18 h, RNA was extracted from cell pellets using the RNeasy Mini kit (Qiagen, Hilden, Germany) and transcription of IFN-β, ICP0, ICP6 and housekeeping β-glucuronidase (GUS) was analysed by RT-qPCR. Primers and probes are listed in Supplementary Table 3.

Apoptosis and necroptosis assays

The effect of various inhibitors of the oncolytic virus-induced cell death was assessed using the lactate dehydrogenase (LDH)-based Cytotoxicity Detection Kit (Merck). For this purpose, 10 000 melanoma cells/well were seeded in a 96-well plate and the medium was replaced the next day by fresh medium containing only 5% FCS. Cells were exposed to pan-caspase inhibitor Q-VD-OPh (Hycultec, Beutelsbach, Germany; QVD; 50 µM), which rescued apoptosis induced by recombinant TRAIL (Apronex, Jesenice u Prahy, Czech Republic; data not shown), and necroptosis inhibitors necrosulfonamid (Bio-Techne, Wiesbaden-Nordenstadt; NSA; 18.75 µM), GSK'873 (Merck; GSK; 1.25 µM) and necrostatin-1 (Biozol, Eching, Germany; NEC; 0.625 µM) for 30 min prior to infection with HSV-1 *d*106S and T-VEC at MOI 1. LDH activity was measured 48 h post infection (p.i.) according to the manufacturer's recommendations. Wells lysed with 1% Triton X-100 (Sigma-Aldrich, Darmstadt, Germany) served as positive controls (100% cytotoxicity).

To analyse caspase activation, 10 000 melanoma cells/well were infected in 96-well plates and activity of caspases 1, 3/7, 8 and 9 was determined 24 h p.i. using Caspase-Glo Assay Systems (Promega, Walldorf, Germany) according to the manufacturer's recommendations. Caspases 1, 8, and 9 were measured in the presence of the inhibitor MG-132 and caspase 1 was additionally measured in the presence of the inhibitor Ac-YVAD-CHO. Staurosporine (Selleck Chemicals, Planegg, Germany) was used as positive control at a final concentration of 1 µM. Control stocks prepared from lysates of

uninfected Vero and E11 cells were tested for induction of caspase 3/7 activation to rule out effects by residual cellular components within the viral stocks.

Quantification of adenosine triphosphate and high motility group box 1 (HMGB1)

After plating and infecting 300 000 cells/well (MOI 1 and 10), supernatants of mock-, HSV-1 *d*106S- and T-VEC-infected cell cultures were harvested at 42 h p.i., passed through 0.45 µm pore size syringe filters and stored at −80°C. Intracellular ATP concentrations were measured after subjecting uninfected cells to two freeze–thaw cycles, which served as controls. ATP levels were determined using an ATP bioluminescent assay kit (Sigma-Aldrich). The manufacturer's protocol was changed to measure standards and samples in a 1:10 dilution with sterile water. HMGB1 levels were quantified with the HMGB1 Human ProcartaPlex Simplex Kit (ThermoFisher Scientific) according to the manufacturer's instructions using the Luminex 100 system (Austin, TX, USA) and LiquiChip Analyser Software (Qiagen).

Statistics

Multiple data sets were analysed using repeated measures or ordinary one-way ANOVA tests with Tukey's, Dunnett's or Sidak's correction for multiple comparisons, as appropriate. Two-sided *p*-values <0.05 were considered significant.

RESULTS

High doses of HSV-1 *d*106S kill three tumour entities more efficiently than T-VEC

The oncolytic activity of replication-competent T-VEC has been described in vitro and in vivo [1, 2, 4]. To find out whether a replication-deficient viral strain can exert a similar effect, three different tumour entities (melanoma, HNSCC, chondrosarcoma) were infected with replication-deficient HSV-1 *d*106S in comparison to replication-competent T-VEC using three different MOI (0.1, 1 and 10).

Two days p.i. with HSV-1 *d*106S, a clear dose-dependent oncolytic effect was observed in 10 melanoma cell lines (Figure 1a). With the exception of one melanoma cell line (AXBI), the viability of cell lines infected with MOI 10 decreased below 20%. In contrast, the dose-dependent oncolytic effect was less pronounced in the replication-competent strain T-VEC (Figure 1b). Here, the viability of all cell lines infected at MOI 10 was above 20%, and in three cell lines even above 40%.

When the data of all melanoma cell lines were combined, HSV-1 *d*106S showed significantly increased oncolytic activity with increasing MOI ($p < 0.001$) (Figure 1c). In contrast, the oncolytic activity of T-VEC did not increase further between MOI 1 and 10 (Figure 1d). A similar discrepancy between the two viruses was observed in 11 HNSCC (Figure 2) and 2 chondrosarcoma cell lines (Figure 3), with the limitation that individual HNSCCs were less responsive to HSV-1 *d*106S in particular. Overall, we observed a dose-dependent oncolytic effect for the replication-deficient HSV-1 *d*106S strain, which was limited at high doses of T-VEC.

Since the HSV-1 *d*106S preparations contained six times more genome equivalents than T-VEC (Supplementary Table 2), the experiment was repeated using respectively reduced amounts of HSV-1 *d*106S. Compared to T-VEC infection (MOI 1), HSV-1 *d*106S adjusted for the number of virus particles (MOI 0.17) induced less cell death in all melanoma cells lines ($p < 0.05$) (Supplementary Figure 1). At high infectious doses, however, the differences were less pronounced with T-VEC (MOI 10) and HSV-1 *d*106S (MOI 1.7) being significantly more active in SK-MEL-3 and IGR-39 cells, respectively.

Low doses of T-VEC are more active than HSV-1 *d*106S over time

In a next step, we monitored the oncolytic activity of both viruses in three melanoma cell lines (IGR-1, IGR-39, SK-MEL-3) for 6 days. While the oncolytic activity of HSV-1 *d*106S peaked at day 2 p.i., cytotoxicity of T-VEC further increased (Figure 1e and Supplementary Figure 2). This phenotype was particularly evident at low infectious doses, consistent with the replicative capacity of T-VEC.

GM-CSF production does not affect HSV-1-induced short-term oncolytic activity

In contrast to HSV-1 *d*106S, T-VEC did not show increased oncolytic activity at a high MOI (Figure 1d). One explanation may be the induction of GM-CSF production by this virus. GM-CSF is reportedly secreted in HNSCC, skin carcinoma, glioma and lung cancer tissue

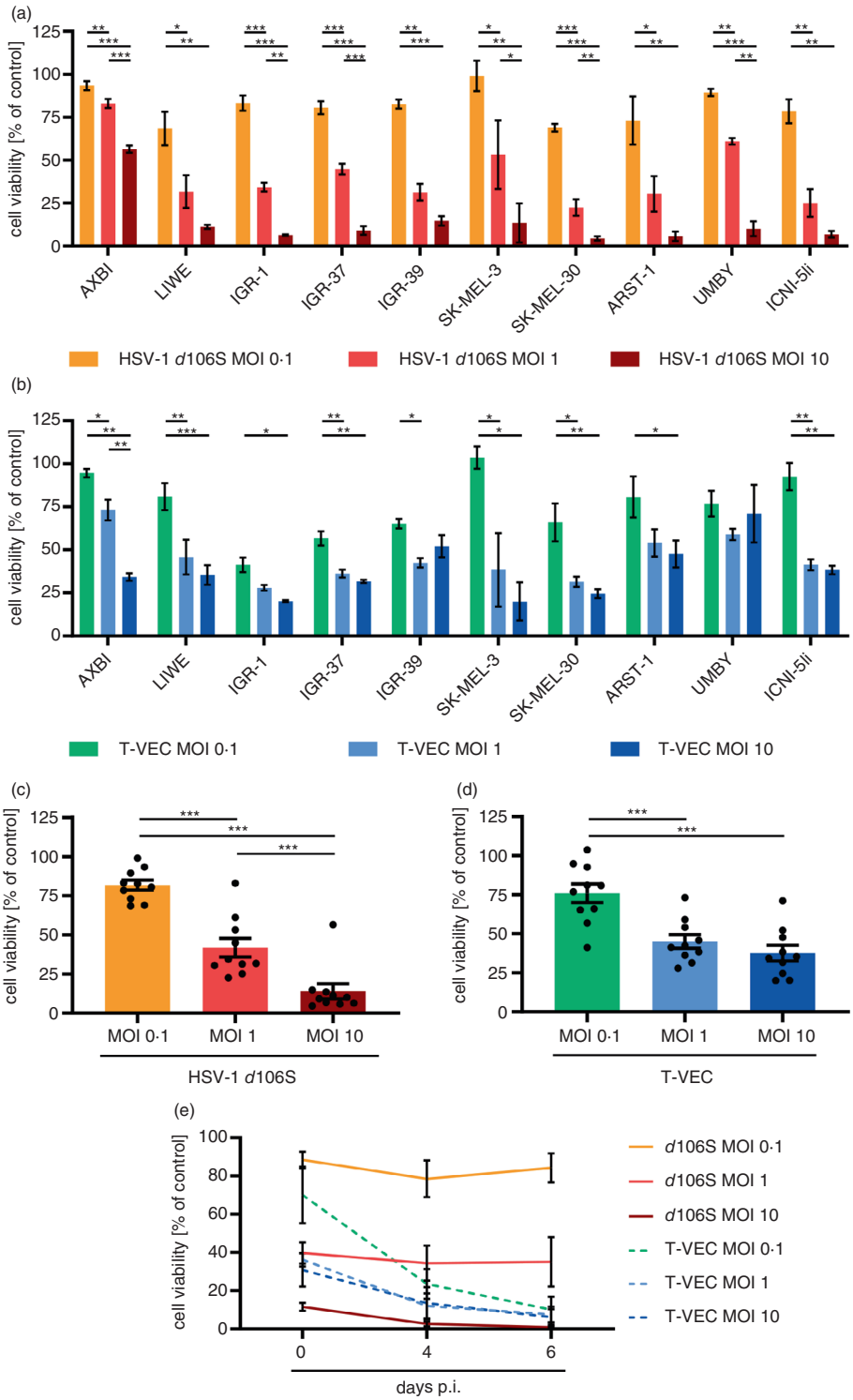

FIGURE 1 Direct oncolytic activity of replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC in malignant melanoma. The viability of 10 melanoma cell lines was analysed 2 days post infection (p.i.) with (a, c) HSV-1 *d*106S and (b, d) T-VEC at increasing multiplicities of infection (MOI) of 0.1, 1 and 10. Viability of infected cells was calculated as percentage of the MTT-based metabolic activity of uninfected control cells. Data show mean and standard error of (a, b) each cell line determined in three independent experiments, and (c, d) all 10 cell lines, depending on the MOI used for infection. To compare the oncolytic activity induced by different MOI of the respective virus in melanoma cell lines, repeated measures one-way ANOVA with Tukey's correction was performed; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (e) Oncolytic activity of HSV-1 *d*106S and T-VEC at increasing MOI over a period of 2, 4 and 6 days p.i.. Data show mean and standard error of the viability of IGR-1, IGR-39 and SK-MEL-3 cells depending on the MOI used for infection. Viability was determined in three to five independent experiments for each cell line. The results for individual cell lines are shown in Supplementary Figure 2.

cultures, directly stimulating tumour growth in an autocrine or paracrine manner [28], which appears to contribute to poor clinical outcome in HNSCC [29].

In a first step, we studied GM-CSF production in melanoma (IGR-1, IGR-39, SK-MEL-30), HNSCC (PCI-52), and chondrosarcoma (HTB-94) cell lines. After 24 h of infection with T-VEC (MOI 1 and 10), GM-CSF concentrations in cell culture supernatants increased to 6.9–12.6 ng/mL and 13.8–17.8 ng/mL, respectively (Figure 4a). When melanoma cell lines IGR-39 and SK-MEL-30 were infected with HSV-1 *d*106S or T-VEC (MOI 0.1, 1, 10) in the presence of 20 ng/mL GM-CSF, oncolytic activity was not affected (Figure 4b). Thus, induction of GM-CSF production by T-VEC did not explain the observed differences of both viruses in tumour cell killing.

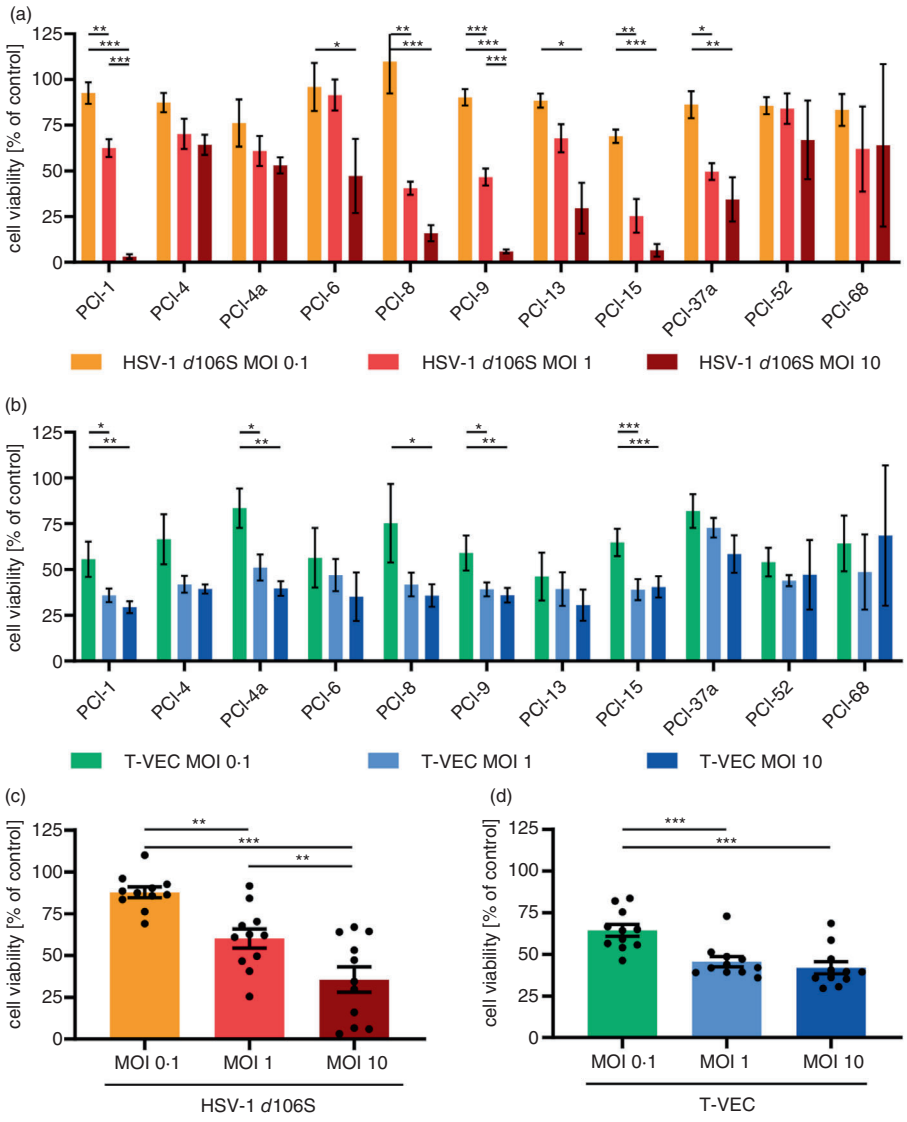

FIGURE 2 Oncolytic activity of replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC in head-and-neck squamous carcinoma. The viability of 11 head-and-neck squamous carcinoma cell lines was analysed 2 days p.i. with (a, c) HSV-1 *d*106S and (b, d) T-VEC at increasing multiplicities of infection (MOI). Viability of infected cells was calculated as percentage of the MTT-based metabolic activity of uninfected control cells. Data show mean and standard error of (a, b) each cell line determined in 3–5 independent experiments, and (c, d) all 11 cell lines, depending on the MOI used for infection. To compare the oncolytic activity induced by different MOI of the respective virus in head-and-neck cancer cell lines, repeated measures one-way ANOVA with Tukey's correction was performed; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

HSV-1 *d*106S and T-VEC induce early caspase-dependent apoptosis

We next aimed to investigate the nature of cell death following oncolytic HSV-1 infection. Apoptotic cell death was blocked by pan-caspase inhibitor Q-VD-OPh (QVD), necroptosis by receptor-interacting protein 1 (RIP-1) inhibitor necrostatin-1 (NEC), mixed lineage kinase domain-like protein (MLKL) inhibitor necrosulfonamid (NSA), and receptor interacting protein kinase 3 (RIPK3) inhibitor GSK'873 (GSK), using published concentrations [30, 31].

Virus-induced cytotoxicity was determined using LDH release assay. QVD significantly rescued viability of IGR-37, IGR-39 and SK-MEL-3 cells upon HSV-1 *d*106S and T-VEC infection, and of IGR-1 and SK-MEL-30 cells upon HSV-1 *d*106S infection, while viability in mock-infected IGR-1 and IGR-37 cells was only slightly affected ($p < 0.05$) (Figure 5). In contrast, NSA and NEC had no effect on cytotoxicity. GSK was mildly toxic in uninfected IGR-39 cells and moderately toxic in T-VEC-infected SK-MEL-30 cells. Aggregation of results revealed that both oncolytic viruses triggered caspase-dependent cell death 2 days p.i., whereas necroptotic cell death did not appear to be involved.

To determine which caspases contribute to cell death, activity of caspases 1, 3/7, 8 and 9 was measured 24 h p.i. (Figure 6). Caspases 8 and 9 were activated in most cell lines after infection with HSV-1 *d*106S (MOI 10), while caspases 3/7 responded to MOI 1 and 10 of this virus. T-VEC (MOI 1 and 10) activated caspases 3/7 in most cell lines, albeit at lower levels than HSV-1 *d*106S. Significant activation of caspases 8 and 9 by T-VEC could not be observed at either MOI in most cell lines. To exclude effects mediated by residual cellular components in the virus stocks, control stocks were prepared from lysates of uninfected Vero and E11 cells in a similar manner as from infected cells; these preparations did not induce

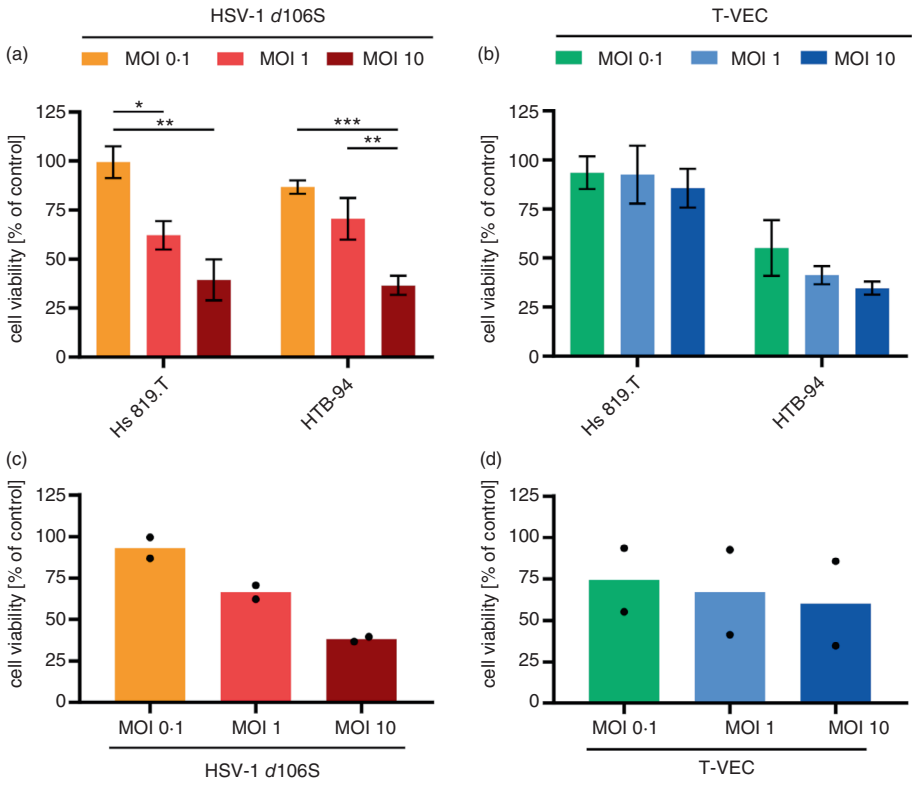

FIGURE 3 Oncolytic activity of replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC in chondrosarcoma. The viability of two chondrosarcoma cell lines (Hs 819.T, HTB-94) was analysed 2 days p.i. with (a, c) HSV-1 *d*106S and (b, d) T-VEC at increasing multiplicities of infection (MOI). Viability of infected cells was calculated as percentage of the MTT-based metabolic activity of uninfected control cells. (a, b) Mean and standard error of each cell line determined in 4–5 independent experiments. To compare the oncolytic activity induced by different MOI of the respective virus in chondrosarcoma cell lines, repeated measures one-way ANOVA with Tukey's correction was performed; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (c, d) Mean of the two cell lines for the respective MOI used for infection.

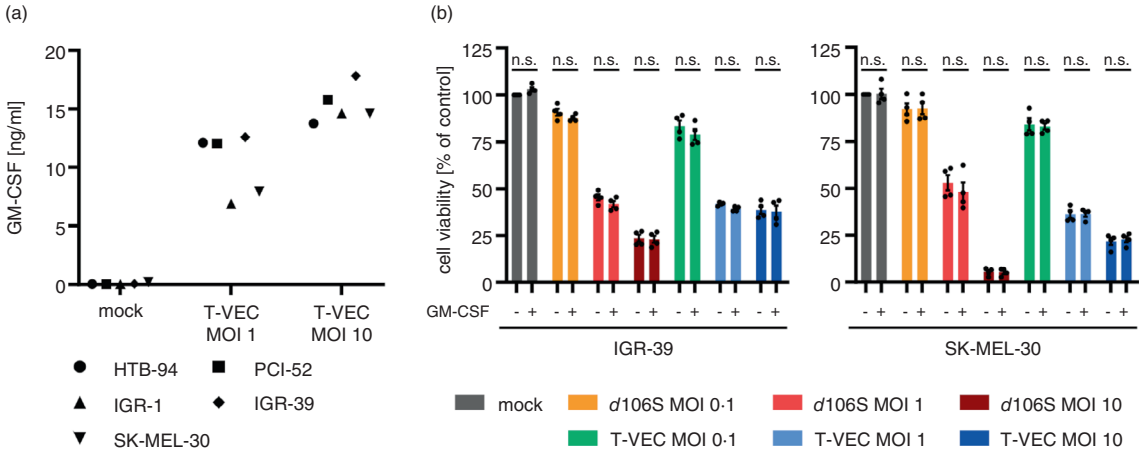

FIGURE 4 Effect of GM-CSF on the oncolytic activity of replication-deficient HSV-1 *d*106S and replication-competent strain T-VEC. (a) Induction of GM-CSF expression in one chondrosarcoma (HTB-94), one head-and-neck squamous carcinoma (PCI-52) and three melanoma cell lines (IGR-1, IGR-39, SK-MEL-30) 24 h p.i. with T-VEC at multiplicities of infection (MOI) 1 and 10. (b) Oncolytic activity of HSV-1 *d*106S and T-VEC in melanoma cell lines IGR-39 and SK-MEL-30 at MOI 0.1, 1 and 10, analysed without and with addition of GM-CSF (20 ng/mL) using the MTT assay. For each condition repeated measures one-way ANOVA with Sidak's correction was used to compare cell viability of cultures in the absence and presence of exogenous GM-CSF, which revealed no significant differences. Mean and standard error of four independent experiments are shown.

caspase 3/7 activation (Supplementary Figure 3). Caspase 1, known to be upregulated in pyroptosis [32], was induced only to a very low extent by both viruses (Supplementary Figure 4). Overall, these results confirm the data obtained with the pan-caspase inhibitor and suggest that apoptosis mediated by caspases 3/7, 8 and 9 plays a major role in HSV-1 *d*106S-induced cell death.

Ratio of ICP0/ICP6 mRNA expression is higher in HSV-1 *d*106S compared to T-VEC

To find out whether the pronounced early cell death by HSV-1 *d*106S is caused by the induction of different ICP profiles, we analysed ICP0 and ICP6 mRNA expression in five melanoma cell lines 18 h p.i.. HSV-1 *d*106S

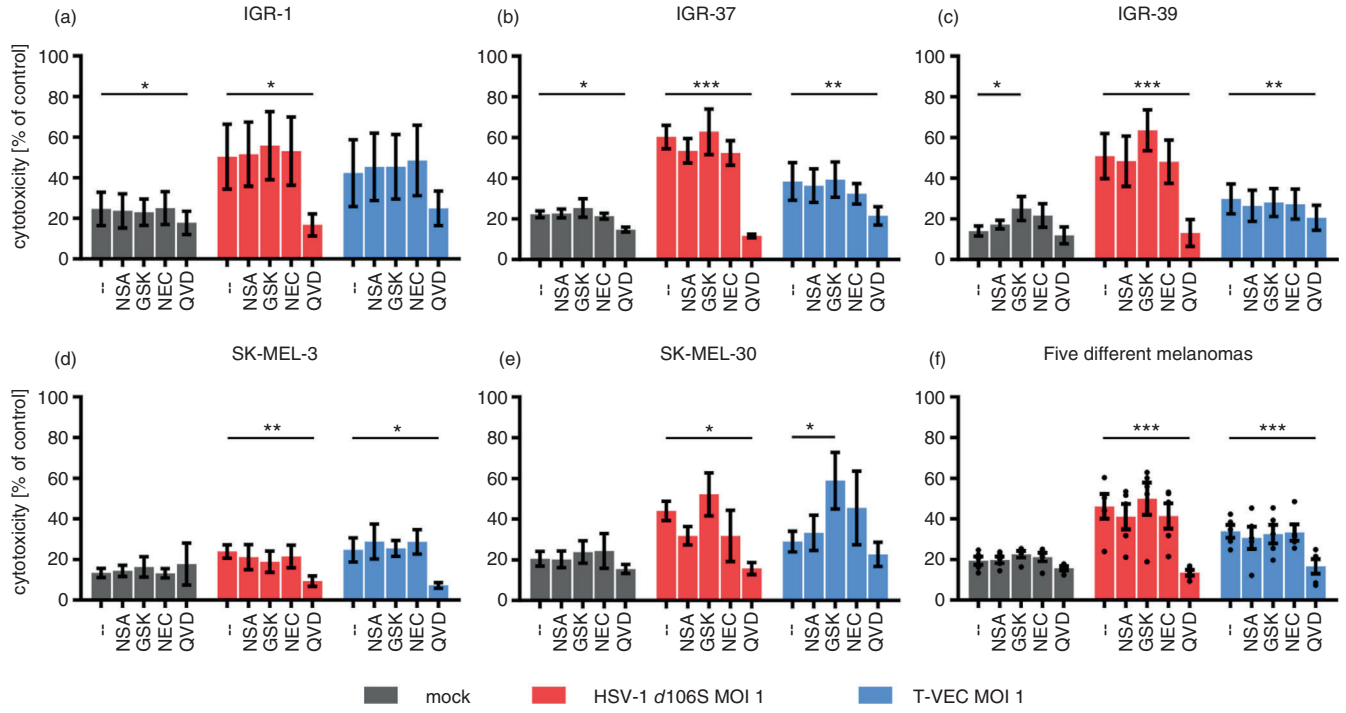

FIGURE 5 Inhibition of the oncolytic activity of replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC by a pan-caspase inhibitor and three necroptosis inhibitors. (a–e) Five melanoma cell lines were exposed to the pan-caspase inhibitor Q-VD-OPh (QVD; 50 μM) and necroptosis inhibitors necrosulfonamide (NSA; 18.75 μM), GSK'873 (GSK; 1.25 μM) and necrostatin-1 (NEC; 0.625 μM) prior to infection with HSV-1 *d*106S and T-VEC at a multiplicity of infection (MOI) of 1. Two days p.i., the oncolytic activity was determined by LDH release. Wells lysed with 1% Triton X-100 served as controls and were considered 100% cytotoxic. Data represent mean and standard error of 3–4 independent experiments. (f) Mean and standard error of all five melanoma cell lines. Within each condition (mock, *d*106S and T-VEC), repeated measures one-way ANOVA with Dunnett's correction was used to compare all treatments to uninhibited cultures; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

expresses only ICP0 and ICP6 due to deletions of immediate early genes in the viral genome [22], whereas T-VEC expresses all ICPs except ICP34.5 and ICP47, allowing a productive replication cycle [33]. ICP0 induces microtubule disassembly in the early phase of HSV-1 replication [34] and causes early apoptosis [35], while ICP6 blocks apoptosis [36] and necroptosis by inhibiting receptor interacting protein 3 (RIP3) and Caspase-8 [37].

In SK-MEL-3 cells, HSV-1 *d*106S infection induced a significantly higher ICP0/ICP6 mRNA ratio than T-VEC (Figure 7). The other melanoma cell lines showed a similar ICP profile, although the difference was not significant. In summary, higher levels of apoptosis-inducing ICP0 compared to apoptosis- and necroptosis-blocking ICP6 may support early cell death by HSV-1 *d*106S, while increased ICP6 levels in T-VEC-infected cells may delay cell death and thus support further viral replication.

T-VEC replication induces late cell death

To directly investigate the role of viral replication in HSV-1-induced oncolytic cell death, we examined the effect of the DNA polymerase inhibitor aciclovir. T-VEC, but not HSV-1 *d*106S, elicited a cytopathic effect characteristic of lytic HSV-1 replication with cells ballooning and detaching from the bottom, which was reduced in the presence of aciclovir (Figure 8a). Aciclovir induced a small but significant rescue from cell death in T-VEC-infected IGR-1 and SK-MEL-30 cells at day 2 p.i., which became more prominent at day 4 p.i. and was also detectable in IGR-39 cells (although not significant). In contrast, cell killing induced by replication-deficient HSV-1 *d*106S was not affected by aciclovir (Figure 8b). These results argue that viral replication contributes to late-stage cell killing by T-VEC.

Early host innate immune response is elicited by high doses of T-VEC, not HSV-1 *d*106S

Intracellular pattern recognition receptors such as cyclic GMP-AMP-synthase activate a variety of IFN-regulating transcription factors and genes that induce type I IFN production [38]. Type I IFNs activate first-line antiviral

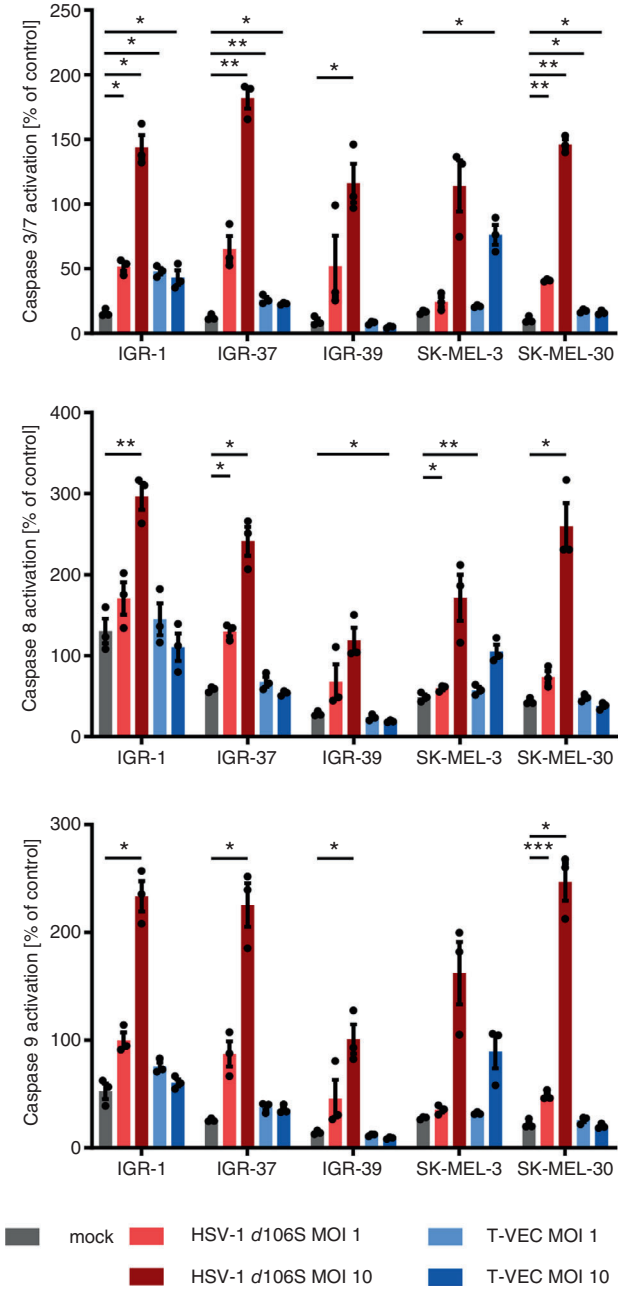

FIGURE 6 Activation of caspases 3/7, 8 and 9 in five melanoma cell lines infected with replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC (MOI 1 and 10). Caspase activity was assayed 24 h p.i. using caspase-Glo assay systems. Concentrations were normalized to the respective induction by 1 μM staurosporine. Data represent mean and standard error of three independent experiments. Induction of caspase 1 activity was minimal (see Supplementary Figure 4). Repeated measures one-way ANOVA with Dunnett's correction for the comparison of all treatments to mock-infected cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

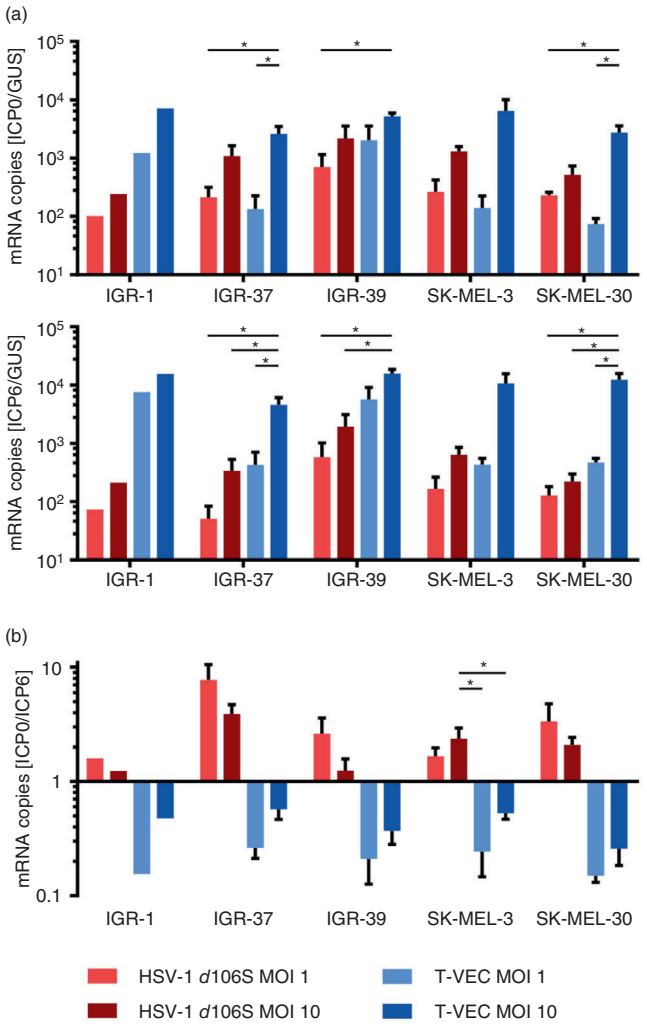

FIGURE 7 Induction of infected cell protein (ICP) 0 and 6 expression upon infection of melanoma cell lines with replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC. (a) Induction of ICP0 and ICP6 mRNA in five melanoma cell lines upon infection with HSV-1 *d*106S and T-VEC (MOI 1 and 10), quantified in the cell pellets at 18 h p.i. and normalized for housekeeping β-glucuronidase (GUS) using RT-qPCR. (b) Ratio of the normalized expression of ICP0 and ICP6 within the infected cell lines. Data represent mean and standard error of three independent experiments for all cell lines except for IGR-1 (mean of two independent experiments). Within each cell line, repeated measures one-way ANOVA with Tukey's correction was performed; *$p < 0.05$.

cellular defence mechanisms that inhibit multiple steps of the viral replication cycle [39]. To characterize the early host immune response, we examined the expression of IFN-β in four melanoma cell lines (IGR-1, IGR-37, IGR-39, SK-MEL-30) 18 h p.i. using RT-qPCR.

T-VEC, but not HSV-1 *d*106S, induced significantly increased levels of IFN-β in cell lines IGR-39 (MOI 1) and IGR-1, IGR-37 and SK-MEL-30 (MOI 10) compared to mock-infected cells ($p < 0.05$) (Figure 9). These data indicated that T-VEC, in contrast to HSV-1 *d*106S, stimulated the innate immune response more efficiently.

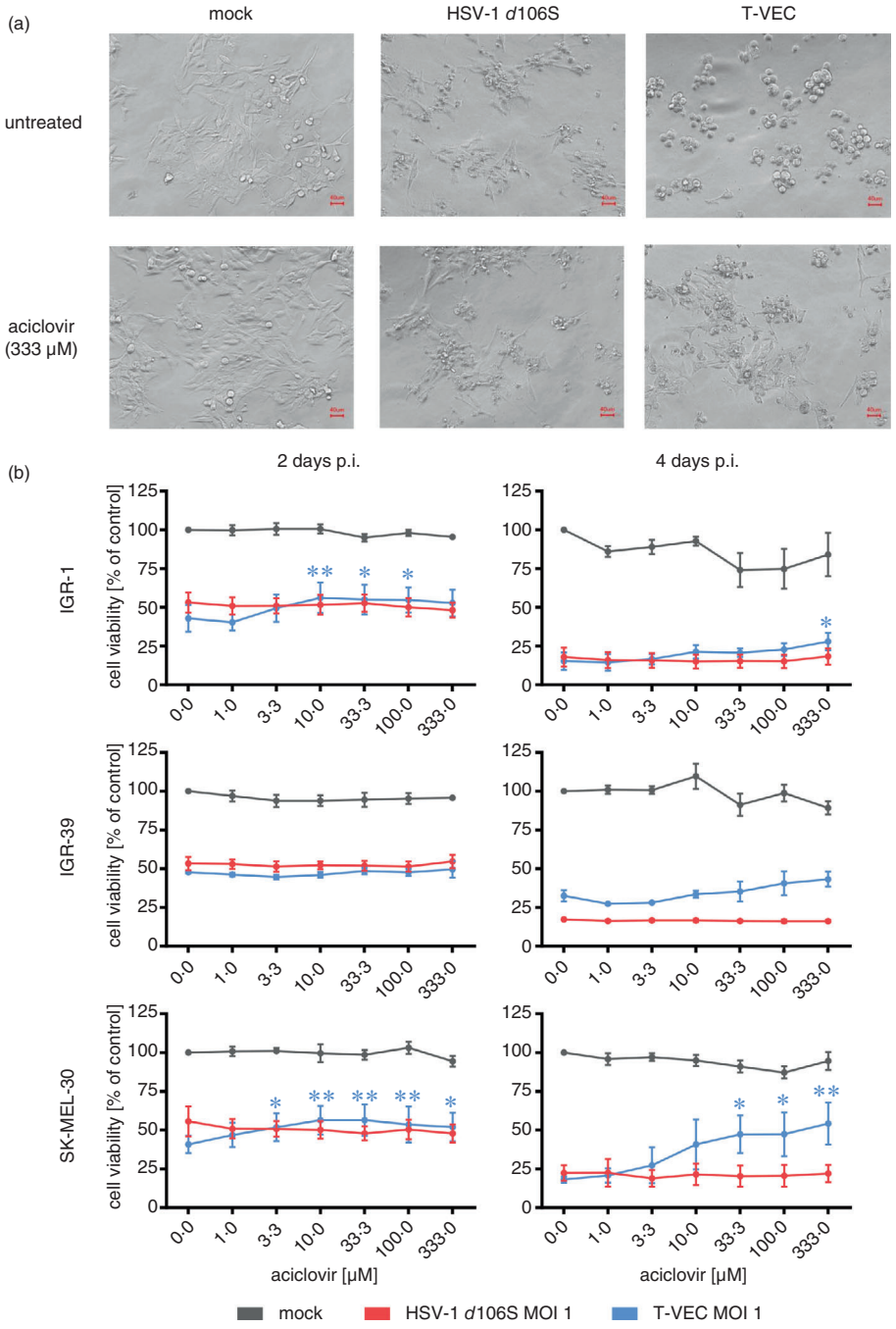

FIGURE 8 Effect of aciclovir on the oncolytic activity of replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC. Three melanoma cell lines were infected with HSV-1 *d*106S and T-VEC at a multiplicity of infection (MOI) of 1 and cultivated in the presence of increasing concentrations of the DNA polymerase inhibitor aciclovir. (a) Cell morphology of SK-MEL-30 cells was recorded by light microscopy 2 days p.i.. (b) Viability of IGR-1, IGR-39 and SK-MEL-30 cells was monitored 2 and 4 days p.i. by determining MTT metabolic activity and normalized to uninfected cells without aciclovir. Data show mean and standard error of 4 and 3 independent experiments for each cell line 2 and 4 days p.i., respectively. Repeated measures one-way ANOVA with Dunnett's correction was performed to compare infected cell viability in the presence of aciclovir to cultures infected in the absence of aciclovir.
$^{*}p < 0.05$, $^{**}p < 0.01$.

T-VEC induced more pronounced immunogenic cell death compared to HSV-1 *d*106S

The release of ATP and HMGB1 from cells infected with oncolytic herpes viruses is a key feature of immunogenic cell death, contributing to the recruitment of HSV- and tumour antigen-specific CD8+ T cells and inducing a proinflammatory tumour milieu [40]. To explore the potential of T-VEC and HSV-1 *d*106S in this regard, we quantified ATP release in cell culture supernatants 42 h p.i..

Despite a high variability in these experiments, likely due to rapid ATP turnover in cell culture supernatants by ectonucleotidases [41], we observed ATP release in four out of five melanoma cell lines infected with T-VEC (MOI 1 and 10) but not with HSV-1 *d*106S (Figure 10a–f). For T-VEC MOI 10, we observed a significantly increased release of ATP in IGR-1 and a tendency towards higher extracellular ATP concentrations in SK-MEL-3 and SK-MEL-30 cells. Notably, infected IGR-39 cells did not release ATP.

In addition, we measured the concentration of HMGB1 in these supernatants (Figure 10g–l). T-VEC

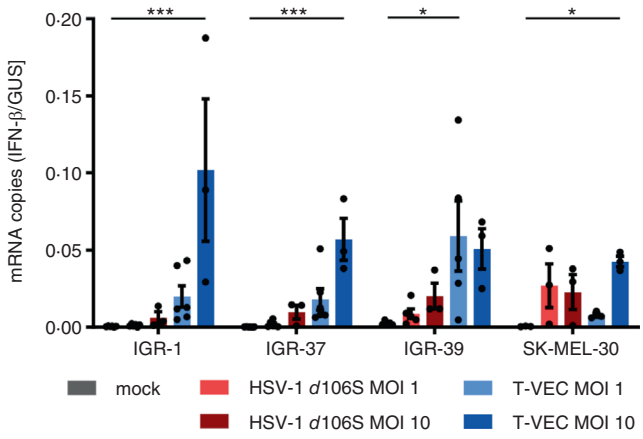

FIGURE 9 Induction of interferon (IFN)-β expression in melanoma cell lines upon infection with replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC. Induction of IFN-β production in four melanoma cell lines, analysed 18 h after infection with HSV-1 *d*106S and T-VEC (MOI 1 and 10). Intracellular IFN-β mRNA levels were normalized to housekeeping β-glucuronidase (GUS). Mean and standard error of 3–6 independent experiments are shown. Within each cell line, ordinary one-way ANOVA with Dunnett's correction was used for multiple comparisons with mock-infected cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

induced HMGB1 in IGR-1 and IGR-37 cells (both MOI 1 and 10), while HSV-1 *d*106S stimulated HMGB1 release in IGR-1 (MOI 10), IGR-37 (MOI 1 and 10), IGR-39 (MOI 1), and SK-MEL-30 (MOI 10) cells ($p < 0.05$). When ATP and HMGB1 data were combined, T-VEC appeared to stimulate immunogenic cell death slightly better than HSV-1 *d*106S.

DISCUSSION

The use of viruses for the treatment of tumours, especially melanoma, has recently received increasing attention. The classical understanding of an oncolytic virus is the induction of cell death by lytic replication in the tumour cell. Most recently, however, oncolytic activity has been observed in vitro and in vivo in replication-attenuated viruses [17, 42], which raises the question of whether replication is necessarily associated with anti-tumour activity. In this context, we described an anti-tumour activity of the infectious, but replication-deficient HSV-1 *d*106S, which affected the viability of melanoma cells, but not human foreskin fibroblasts [21].

Our present study intended to investigate the role of viral replication in the oncolytic activity of herpes viruses in three different tumour entities with a head-to-head comparison of HSV-1 *d*106S to the state-of-the-art T-VEC strain. The tumour cells reflected a broad range of clinical isolates and established cell lines and showed large variability in terms of oncolytic effect. Replication-deficient HSV-1 *d*106S proved to be more effective and faster oncolytic at high MOI, while replication-competent T-VEC was more efficient over time at low MOI. Besides viral replication capacity, other factors may contribute to these differences, for example, the different backbones of the two viral strains. Importantly, we were able to rule out an impact of GM-CSF produced by T-VEC (Figure 4b), reported to stimulate tumour growth in an autocrine or paracrine manner [28]. In addition, viral stocks were purified in a slightly different manner; however, we could exclude an impact of residual cellular debris. In conclusion, HSV-1 *d*106S appears to be advantageous when a tumour mass needs to be killed in short time, provided that sufficient amounts of virus can be delivered to and distributed within the lesion. T-VEC is advantageous when not all tumour cells are infected during inoculation, because the virus continues to replicate. However, 'more is more' may not apply to T-VEC, as higher infectious doses do not appear to enhance oncolysis.

Cell death induced by HSV-1 *d*106S was mainly caused by apoptosis, as shown by pan-caspase inhibitor QVD and pronounced induction of caspases 3/7/8/9. A comparable effect of QVD was observed in T-VEC-infected cells, which was surprising as its cytopathic effect with ballooning cells differed from the shrinkage and nuclear condensation upon HSV-1 *d*106S infection (Supplementary Figure 5). Necroptosis, a form of controlled pyrogenic cell death that occurs in different situations including infection [30], and pyroptosis [32] played only a minor role. In this respect, necroptosis was suppressed in human cells through ICP6, which inhibits the interaction of RIPK1 and 3 [37]. RIPK3 [43] and MLKL [44] are key molecules for necroptosis and are often suppressed in HSV-infected human cells or necroptosis-deficient cancer cells. In contrast, apoptosis played an important role in the early cell death induced by both viruses.

Our results show that the ICP0/ICP6 mRNA ratio shifted between the two viruses. T-VEC expressed ICP0 to a lesser extent than ICP6, the large subunit of the ribonucleotide reductase that contributes to viral replication [45]. Besides ICP6, T-VEC expresses other viral proteins such as ICP4, ICP27, Us3, Us5 and gD, which block apoptosis [46–50]. A replicative virus may benefit from this profile because only living cells produce viral progeny. In contrast, HSV-1 *d*106S was originally developed for vaccination purposes [51] to sustain antigen expression independent of viral replication. Since HSV-1 *d*106S expressed less ICP6 relative to ICP0, the latter may predominate in cells infected with this viral strain and induce apoptosis [35]. Notably, the

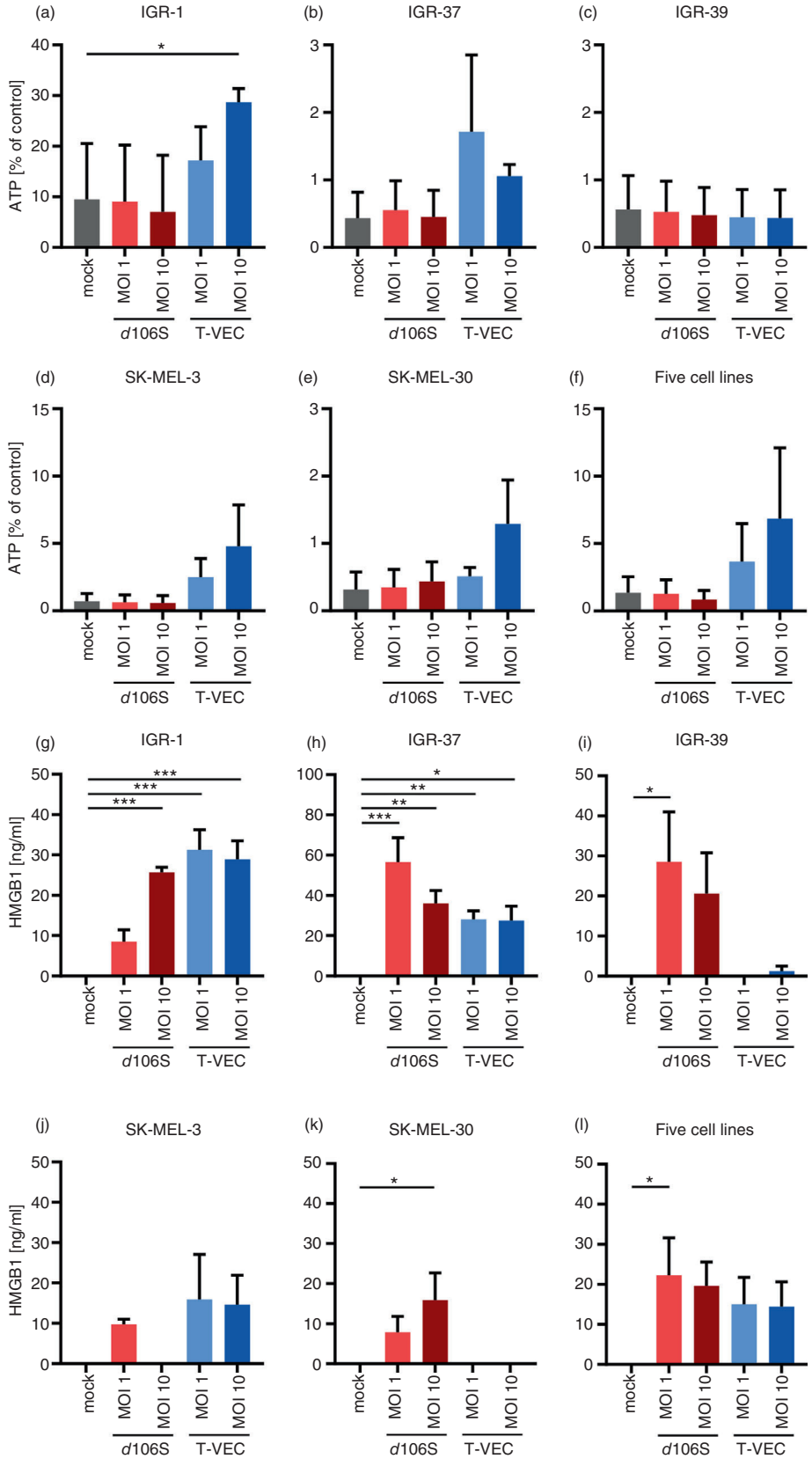

FIGURE 10 Induction of immunogenic cell death in melanoma cell lines after infection with replication-deficient herpes simplex virus 1 (HSV-1) *d*106S and replication-competent strain T-VEC. (a–e) Adenosine 5′-triphosphate (ATP) concentrations were measured in the supernatants of five melanoma cell lines 42 h p.i.. Cell lysates prepared by subjecting mock-infected cells to two freeze–thaw cycles served as positive controls. The ATP contents in lysates of IGR-37 (800.3 nM), IGR-39 (1696.0 nM), SK-MEL-3 (992.6 nM), and SK-MEL-30 (1126.4 nM) cell lines were comparable, while lysates derived from IGR-1 contained only 29.6 nM ATP. Data represent mean and standard error of four independent experiments. (f) Mean and standard error of the five melanoma cell lines analysed for ATP release. (g–k) High mobility group box 1 protein (HMGB1) concentrations measured in the supernatants of five melanoma cell lines 42 h p.i.. Data represent mean and standard error of four (IGR-1, IGR-37, IGR-39) and three independent experiments (SK-MEL-3, SK-MEL-30). HMGB1 was undetectable in all mock-treated cell cultures. (l) Mean and standard error of the five melanoma cell lines analysed for HMGB1 release. Repeated measures one-way ANOVA with Dunnett's correction was used for the comparison of all treatments to mock-infected cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

ICP6-depleted strain G207 showed attenuated replication, yet achieved striking antitumor activity in paediatric gliomas [17].

Viral replication was directly blocked by DNA polymerase inhibitor aciclovir. HSV-1 *d*106S has increased sensitivity to aciclovir in a complementary cell line

providing the deleted ICP4 and ICP27 genes in trans [23]. Theoretically, aciclovir could have suppressed its oncolytic activity as well; however, the infected cells did not show improved viability at early or late stage. In contrast, aciclovir rescued cell survival after infection with T-VEC. This effect was already detectable with two melanoma cell lines at day 2, but became particularly evident at day 4 p.i., demonstrating that viral replication is an essential component of late cell death induced by T-VEC. Possibly, expression of late genes after genome replication is involved here.

T-VEC in particular induced IFN-β production. This production can be directly induced in the infected cell or in bystander cells via transfer of cGAMP through gap junctions or anion channels [52, 53]. Type I IFNs are antiproliferative and thus contribute to tumour regression, but also induce an antiviral state, which in turn can inhibit T-VEC replication. Besides innate immune responses, type I IFNs also trigger adaptive immunity. STING signalling and the release of IFN-β are critical for the immunogenicity of dying cells engulfed by antigen-presenting cells [54]. Hyperphosphorylation of translation initiation factor eIF2α by IFN-β-induced protein kinase R triggers reduced viral protein translation [39] and exposes ecto-calreticulin at the outer plasma membrane, serving as a potent immunogenic 'eat-me' signal for tumour-associated DCs [55]. Other potent chemoattractants are ATP and HMGB1 released from virus-infected cells [40]. In addition, T-VEC contributes to maturation of BDCA-1+ myeloid dendritic cells [20]. Immunogenic cell death and induction of memory T cells are required for long-lasting antitumor immunity that protects against tumour recurrence [56]. In our experiments, high doses of T-VEC were able to trigger IFN-β induction and release of ATP and HMGB1 in several cell lines, while replication-deficient HSV-1 *d*106S was less effective in this respect. On the contrary, this lower induction of inflammation by HSV-1 *d*106S may be advantageous for an application in the central nervous system, as has been shown for the replication-attenuated G207 strain in paediatric gliomas [17].

In applications requiring enhanced immune activation, immunogenicity of non-replicative HSV-1 may be enhanced by insertion of different transgenes, for which HSV-1 *d*106S provides specific options. In this respect, immunomodulatory transgenes such as IL-12 [5–7] and IL-15 [10] could increase the influx of dendritic cells and improve oncolytic activity. As has recently been shown in an animal model, HSV-1 *d*106S delays tumour development, but a durable effect with tumour equilibrium was observed only with a modified version expressing IL-12 [9]. Transgenes for the expression of antibodies against PD-1 [14] or the CD47 'don't eat me' signal [57], and the insertion of CD40L [13] may further favourably modulate the tumour microenvironment and increase cytotoxic T-cell responses. The use of potent adjuvants, such as Quillaja saponaria extract, included in the recently approved glycoprotein vaccine against zoster, may contribute to increased IFN-γ and CD4+ T-cell responses [58]. In addition, HSV-1 *d*106S may be used with specific chemotherapeutic agents to further enhance the oncolytic activity [59–61], thereby combining the oncolytic potential of replication-deficient herpes viruses with enhanced immunogenicity.

In conclusion, our study showed that short-term oncolytic activity is not necessarily related to herpes virus replication in tumour cells. However, replication is required for the spread in tumour tissue, especially when not all tumour cells are infected in the first round. Virus-induced cell death was mainly due to apoptosis in the early phase and virus replication in the late phase. Replication was associated with a more pronounced innate immune response and immunogenic cell death. Overall, viral replication apparently modifies the properties of oncolytic herpes viruses, which should be exploited for the specific needs of each tumour entity.

AUTHOR CONTRIBUTIONS

Conceptualization, G.L., B.Sm. and P.S.; methodology, G.L., B.Sm. and P.S.; formal analysis, G.L., A.W., C.L.M., K.R., B.Sm. and P.S.; investigation, G.L., A.W., C.L.M., B.H., V.K., S.Hi., L.G., S.L., M.T. and A.R.; resources, G.K. and M.E. (provided reagents and expertise on the mechanisms of cell death), D.M.K. and N.D. (provided HSV-1 *d*106S and E11 cell line), B.S.-T. and S.G. (provided melanoma cell lines), R.J.B. (provided head-and-neck squamous carcinoma cell lines), and S.S. (provided chondrosarcoma cell lines), B.Sw. and S. Ha. (contributed T-VEC and clinical expertise on T-VEC application); data curation, G.L., B.Sm. and P.S.; writing—original draft preparation, B.Sm. and P.S.; writing—review and editing, G.L., D.M.K., S.H., B. Sm. and P.S.; visualization, G.L., A.W. and C.L.M; supervision, B.Sm. and P.S.; funding acquisition, S.H., D.M.K. and B.Sm. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

D.M.K. is a co-inventor on patents held by Harvard University that includes claims on the use of replication-defective mutant viruses such as *d*106S vaccine vector and immunomodulatory agent. S.H. has received consulting fees, speaker honoraria and travel support from Amgen. Amgen had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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