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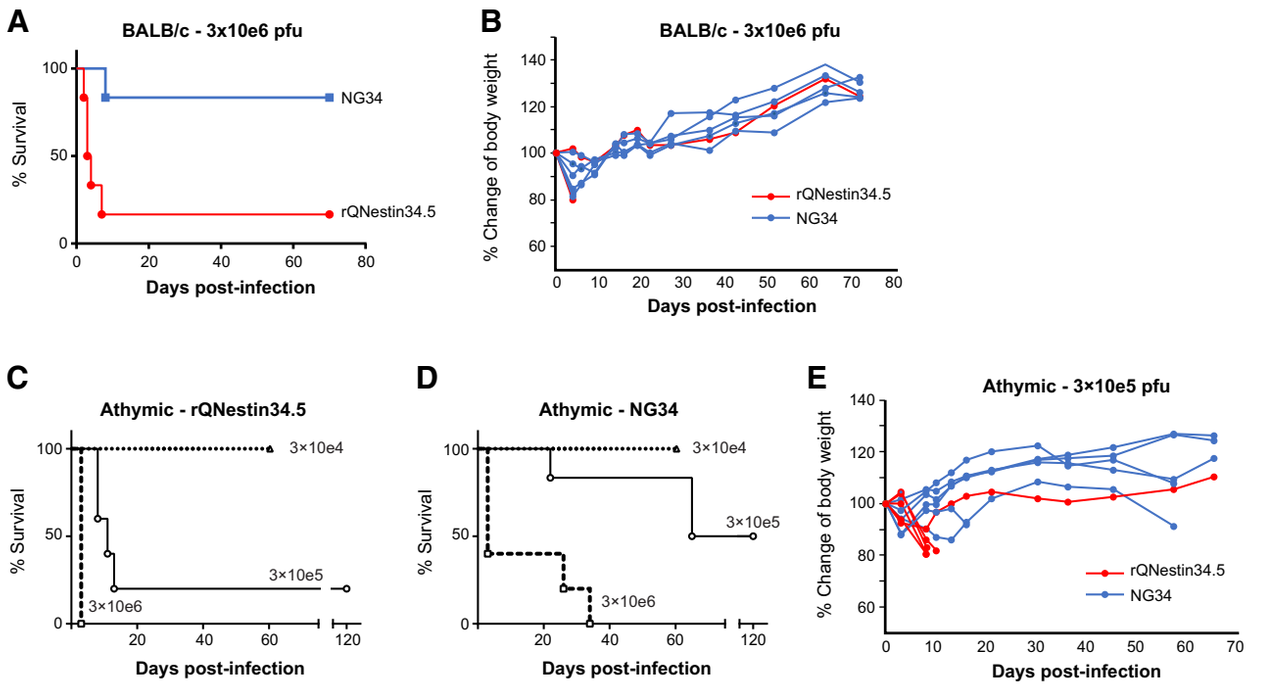

Figure 5.

Decreased mortality after intracerebral injection of NG34 versus rQNestin34.5. **A–D,** Survival of BALB/c (**A**) and athymic nude mice (**C** and **D**) inoculated intracerebrally with 3×10^6 pfu (**A** and **B**) or three different doses of either rQNestin34.5 (**C**) or NG34 (**D**). Kaplan–Meier survival curves were analyzed with Gehan–Breslow–Wilcoxon test, where $P < 0.001$ (**A**) and $P < 0.05$ (3×10^5 pfu of rQNestin344.5 in **C** and NG34 in **D**). Triangle with dot line; 3×10^4 pfu, circle; 3×10^5 pfu, and square with dot line; 3×10^6 pfu. Body weights of individual animals were also plotted in **B** (BALB/c; with doses of 3×10^6 pfu) and **E** (athymic; with doses of 3×10^5 pfu).

to be more toxic in athymic nude mice compared with immunocompetent BALB/c mice despite the lower intracerebral dose, we compared the transcription profiles of type I IFNs, TNFα, IL1β, IL27, and IL6 between these two mouse strains, four days after brain inoculation with NG34 at a dose of 3×10^5 pfus. Supplementary Figure S6 shows that the most significant change was an elevation in IL6 and IL27 in BALB/c versus athymic mice treated with NG34, while elevations of the other tested cytokines was fairly similar between athymic and BALB/c mice. Histopathologic analysis of the brains of athymic mice that exhibited signs of neurotoxicity at day 3 (3×10^6 pfu) or day 5 (3×10^5 pfu) after infection showed broad areas of HSV-1 antigenicity (Fig. 6A and C), along the needle injection tract (Fig. 6A). There was colocalization of positive HSV antigenicity with neuron ($NeuN^+$) and glia ($GFAP^+$) antigenicity in cerebral cortex (Supplementary Fig. S7). We observed different level of recruitment of innate immune cells in analyzed brains (Fig. 6E–L). Parenchymal infiltrates of $CD45^+$ immune cells and $Iba1^+$ microglia–positive areas were more prominent at the lower dose of NG34 (Fig. 6F and J), whereas $CD45^+$ cell infiltrates were less apparent for either oHSV at 3×10^6 pfu (Fig. 6E and G). Accumulation of $Iba1^+$ microglia within the anti-$HSV\text{-}1^+$ brain region and hemisphere was not apparent with rQNestin34.5 (Fig. 6K). Taken together, these studies showed that NG34 appeared to have an improved neurotoxicity profile when compared with rQNestin34.5. It also suggested that the degree of innate immune cell infiltration in $HSV\text{-}1^+$ regions may vary based on dose.

Discussion

Oncolytic virus (OV) therapy has now become a clinical reality with regulatory approval of the first product for melanoma (i.e., Imlygic, T-VEC, also known as OncoVEX-GM-CSF; ref. 34) and several other OVs being in advanced phases of clinical trials (35). For other cancers, such as GBM, OVs should also provide promising results. All clinical trials of oHSVs up-to-date have utilized constructs where the viral *ICP34.5* gene is deleted or defective in some form to minimize neurovirulence to normal brain. However, the lack of ICP34.5 also significantly attenuates the capacity of the oHSV to sustain robust replication in infected GBM cells. To overcome this obstacle, we have engineered and preclinically tested rQNestin34.5 (8), an oHSV where one copy of the viral *ICP34.5* gene is reinserted under control of the cellular nestin promoter, as nestin is highly expressed in GBM in adult human brain (36–39). A phase I clinical trial of this agent against recurrent GBM is currently actively accruing patients and is supported by an FDA-approved IND. However, spurious expression of ICP34.5 still carries a theoretical risk of neurotoxicity. We thus reasoned that the human *GADD34* gene, a mammalian ortholog of HSV ICP34.5, could be a substitute that might enable the same level of viral replication in infected GBM cells as wild-type ICP34.5-positive oHSV, yet still display the reduced neurotoxicity of ICP34.5-negative oHSV. Here we show that (i) newly engineered oHSV NG34 replicates in GBM cells *in vitro* with similar kinetics as those exhibited by rQNestin34.5; (ii) the dose response

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Table 1. 50% Effective dose of oHSV in GBM and non-GBM cell lines

	MOI (x10^{-3}), 95% Confidence intervals			
	rHSVQ		NG34	
	ED50	R-Seq	ED50	R-Seq
U251	22.67 – 47.63	0.8947	2.620 – 5.436	0.8728
U2OS	15.11 – 41.57	0.7516	7.172 – 15.90	0.8516
G9Rluc	34.63 – 70.44	0.9173	4.827 – 7.922	0.9587
G30	9.439 – 16.87	0.9392	1.962 – 3.150	0.9588
G83	25.08 – 54.51	0.9028	4.424 – 9.737	0.8810
G326	17.87 – 43.33	0.8850	3.564 – 7.058	0.9237
G528	86.39 – 270.7	0.6973	26.66 – 64.31	0.8706

NOTE: Enhanced glioma cytotoxicity effect with GADD34-encoding $\gamma_1$34.5-null NG34 versus original $\gamma_1$34.5-null rHSVQ virus. Intracellular ATP was measured 3 days after oHSV infection with either rHSVQ or NG34 or rQNestin34.5 in GBM (U251, G9Rluc and G30) and non-GBM (U2OS) cells at 20,000 cells per a well of 96-well plates for cell viability assay. Data with three replicates were normalized with maximum and minimum values before calculating ranges of 50% effective doses (ED50) and values of R^2 at 95% confidence intervals. These plots with nonlinear dose–response curves are also provided in Supplementary Fig. S1.

of NG34 toxicity shown in GBM cells is equivalent to, or in some cases even better when compared with rQNestin34.5; (iii) the *in vivo* antitumor efficacy of NG34 in two human orthotopic GBM models in athymic mice is similar to that of rQNestin34.5; (iv) NG34 also shows significant antitumor efficacy in a syngeneic mouse GBM model; and (v) intracerebral injection of NG34 in brains of immunocompetent and athymic mice shows significantly better tolerability when compared with rQNestin34.5. Taken together, these results demonstrate that, NG34 and rQNestin34.5 possess similar antitumor efficacy against GBM models, but NG34 appears to be less toxic when injected into mice brains without tumor.

As previously reported by others (18–20), we confirmed that GADD34 expression prevents phosphorylation of eIF2α at the serine-51 residue after infection with a $\gamma_1$34.5-null HSV (Fig. 2B). The translation initiation factor eIF2α is one subunit of the ternary EIF2 complex, whose formation is modulated by the phosphorylation of eIF2α (40). The eIF2 complex is primarily responsible for the binding of the initiator methionyl-tRNA to the 40S ribosomal subunit and catalyzes the initiation of protein synthesis (18). In response to HSV-1 infection, cells (including GBM cells) immediately activate PKR-mediated phosphorylation of eIF2α and suppress viral protein synthesis. HSV-1 ICP34.5 counteracts this process by dephosphorylating eIF2α through its binding to and transport of the PP1 phosphatase to eIF2 within the HSV-1–infected cell (12, 17). The carboxyl-terminal PP1 binding domain of mammalian GADD34 and viral ICP34.5 are both conserved as PP1-interacting proteins that lead to the dephosphorylation of eIF2α via the activity of PP1 (13). It has been reported that upregulation of cellular GADD34 can enhance the activity of oHSV-1 in glioma in the context of stress responses, such as treatment with temozolomide or culture under hypoxic conditions (41, 42). In addition to the NG34 approach we describe here, others have also engineered oHSV to modify or duplicate ICP34.5 function to enhance oHSV replication in tumors while minimizing ICP34.5 neurotoxicity. A study by Rabkin and colleagues demonstrated that Δ68H(-6) virus, an oHSV where the Beclin1-binding domain of the *$\gamma_1$34.5* gene was deleted, was highly neuroattenuated compared with HSV-1 that expresses wild-type ICP34.5 in A/J mice (10). On the basis of the finding that the HSV1 Us11 also suppresses phosphorylation of eIF2α (11, 17), Todo and colleagues engineered a $\gamma_1$34.5-null G47Δ oHSV encoding a *Us11* gene under transcriptional control of the immediate-early *Us12* promoter (43) and this oHSV (G47Δ) is being tested in clinical trials for GBM patients in Japan (44). In another approach, the TRS1 and IRS1 gene products (C130 and C134, respectively) of human cytomegalovirus have been engineered into an ICP34.5-null oHSV, as they have been shown to substitute for ICP34.5 function (45).

Wild-type HSV-1 neurotoxicity during the viral lytic cycle has been extensively studied (46, 47). Intracerebral inoculation of GADD34-encoding NG34 reduced mouse lethality when compared with injection of the ICP34.5-encoding rQNestin34.5, but did not eliminate neurotoxicity completely. It is interesting to speculate on why a human protein such as GADD34 would still show some extent of neurotoxicity when expressed from an oHSV. To provide possible explanations for this finding, we should consider two general topics: the first relates to the spurious expression of GADD34 or ICP34.5 in normal neural cells, while

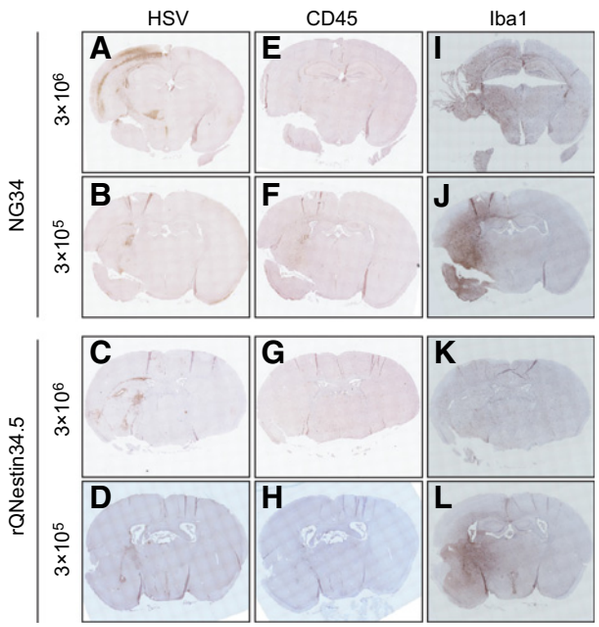

Figure 6.
IHC of the brains of athymic mice after the HSV-1 inoculation. Brains with 3×10^6 pfu were obtained from the euthanized mice at a terminal point (day 3) during the toxicity study in Fig. 5C and **D**. Brains with the 3×10^5 pfu were independently prepared for this study and obtained from the mice at day 5 after viral injection. The sections from the paraffin-embedded tissues were stained with anti-HSV1/2 (**A–D**), anti-CD45 (**E–H**), or anti-Iba1 (**I–L**) antibodies.

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the second relates to the direct involvement of GADD34 in neurotoxicity. In regard to the first topic, the Nestin promoter/enhancer transcriptional element drives expression of GADD34 in NG34 and ICP34.5 in rQNestin34.5. The Nestin enhancer should be transcriptionally active only in GBM cells and inactive in normal neural cells. A couple of explanations could be entertained (i) there is low-level expression of nestin in normal brain cells, that produces sufficient amount of GADD34 or ICP34.5 for progeny production leading to neurotoxicity, and/or (ii) there is transcriptional leakage of *GADD34* or *ICP34.5* gene controlled under the hybrid Nestin/Hsp68 promoter and gene regulatory elements in NG34 or rQNestin34.5 that leads to their protein production. We believe that the first explanation is more likely based on the data we have in hand. For the rQNestin34.5 IND application, we performed extensive studies related to nestin expression in mouse brains as well as in adult human brains. We have found that the brains of young adult mice do express enough nestin that can be detected by IHC, particularly in tanycytes around the ependymal layers of the ventricle (data not shown). However, brains of human adults do not exhibit expression of nestin detectable by IHC, either in brain tissues surrounding a GBM, or brains after radiation or chemotherapy, and brain areas around ventricles (data not shown). There have also been several reports to show that nestin is not expressed in adult human brains or, if it is, it is discreetly located in sparse areas of deep brain nuclei (36–39). These human studies would bring concern that data obtained from mice may overestimate the neurotoxicity of oHSVs where nestin transcriptional elements are driving expression of viral genes associated with neurovirulence. The second explanation is less likely, that the *hsp68* gene promoter without enhancer elements does possess some transcriptional leakage (data not shown). However, we did not observe progeny virions in primary tissue culture cells such as astrocytes and smooth muscle cells (data not shown). Compared to GADD34-null and ICP34.5-null rHSVQ virus, cytotoxicity of NG34 was not significant in non-nestin–expressing U2OS cells. Thus, we believe that the transcriptional leakage explanation is possible but not likely to contribute to *in vivo* neurotoxicity.

The ICP34.5$^+$ rQNestin34.5 oHSV exhibited higher neurotoxicity than the GADD34$^+$ NG34. Orvedahi and colleagues showed that inhibiting neuronal autophagy by ICP34.5 leads to fatal HSV-1 encephalitis in mice (48). Autophagy is especially important for nondividing neuronal tissue to maintain cellular homeostasis and protein's quality control, as well as to prevent neurodegeneration. Inhibition of the autophagy flux has been shown to be detrimental to neuronal protection after traumatic brain injury, which would promote neurodegenerative disorders. Interestingly, GADD34 expression during periods of cellular stress may promote autophagy (21–23). In addition to the high binding affinity of ICP34.5 to Beclin-1 (GADD34 does not bind to Beclin-1), ICP34.5 also regulates the IFN-I pathway via an interaction between the cellular TANK binding kinase I (TBK1) and the amino-terminus of ICP34.5 (49, 50). IFN-I signal the cascade of antiviral innate immune responses that modulate viral replication. Hence ICP34.5 may also facilitate neurovirulence through the regulation of IFN-I response in mice, a function that GADD34 is not known to possess (51). This could thus provide an additional explanation of why ICP34.5 may be more neurotoxic than GADD34. Finally, ICP34.5 also provides structural functions as part of the tegument compartment of viral particles (52). The ICP34.5 protein in rQNestin34.5 thus enters into cells, such as neuron and astrocytes, which may be nonpermissive for replication but still infection-susceptible: this by itself, can be neurotoxic even in the absence of active viral gene expression. Instead, GADD34 is not a structural component of the HSV-1 virion, and thus would not be transmitted in the absence of active gene expression. This may help to limit anti-HSV T-cell immunity mediated through autophagy in cells with primary infection with an ICP34.5$^+$ virus (53). The quick turn-over of GADD34 protein also would limit its toxicity (54). It should be also noted that the neurotoxicity of GADD34 may also depend on HSV strains and the context of experimental settings. In an experimental mouse stroke model, McCabe and colleagues reported that GADD34 restores virulence of the $\gamma_1$34.5-null HSV1716 virus, constructed from HSV17$^+$ strain, which is highly neurovirulent compared with the F strain used as backbone for our oHSVs (55, 56). The intracerebral inoculation experiment also demonstrated that immunocompetent BALB/c mice tolerated NG34 more than athymic mice. Except for a difference in increased IL6 and IL27 elevation, both mice responded to NG34 with similar elevation of other tested cytokines. Mice with intact immune systems are more likely to resist NG34 infection better than immunodeficient mice. The role of the differential IL6 and IL27 elevation can also be an interesting topic for discussion. Published studies report that IL6, as an acute phase reactant, promotes humoral immunity and lineage commitment in the Th17 subset of helper T cells, which athymic mice lack (57, 58). Beyond adaptive immunity, IL6 can also contribute to restrict HSV-1 neurotoxicity. Microglia produce IL6 upon HSV-1 infection to prevent neuronal loss during acute infection with HSV-1 (59). Our data seems to show that acute infection with high doses of rQNestin34.5 did not have as much Iba-positive microglia as observed at low-dose infection, suggesting that microglia are an important player in the survival from acute infection and protection from neuronal loss. It is also reported that IL6 is regulated via the GADD34–PP1 pathway but it is not clear whether NG34-expressing GADD34 contribute to this IL6 pathway (60). We also found a surge of IL27 expression upon NG34 infection. IL27 is a member of the IL6 cytokine family and may regulate antiviral T-cell immunity at the acute phase and contribute to protection in BALB/c mice (61). Since IL27 is produced by microglia and macrophages in the CNS upon viral infection (62) and we observed enrichment of microglia and CD45$^+$ cells within HSV-1–positive brain area, IL27 may mark the immune response of innate immune cells upon HSV-1 infection. The upregulation of IL1β, IFNβ, and TNFα instead may derive from innate immune cells present in both athymic and immunocompetent mice and contribute to the transition from innate to adaptive immunity (63). In addition, GADD34 expressed by NG34 can promote PP1-mediated dephosphorylation of TSC1, I-kB kinase (IKK), and TGFβ receptor 1 (TGFβR1; refs. 19, 21, 22, 31, 64, 65). The persistent PP1 interaction of GADD34 may also disturb the functionality of other PP1-interacting protein complexes, as PP1 is a major phosphoprotein phosphatase of protein Ser/Thr phosphatases, and forms as many as 650 distinct complexes (31).

Despite the reduced neurotoxicity of NG34 compared with rQNestin34.5, there was still evidence of positive HSV antigenicity in normal brain cells upon inoculation. IHC appeared to show that this antigenicity occurred in cells that were neurons or astrocytes. Interestingly, we know that the trauma from needle injection seems to upregulate nestin-positive reactive glia in mice and that there are a considerable number of nestin-positive

neurons in the brain of mice, including the subpendymal zone and along the walls of the third ventricle (data not shown). This nestin positivity in mice brains will thus allow for probable replication of the engineered oHSVs used in our study in mice.

In summary, we show that a novel oncolytic HSV-1 encoding GADD34, NG34, can provide an alternative to expression of ICP34.5 to enhance viral replication and minimize neurotoxicity. Although there have not been neurotoxicities to date with oHSVs in clinical trials, all current oHSVs lack ICP34.5 function. rQNestin34.5 is the first ICP34.5-positive oHSV to be injected in humans with cancer under a current IND. Although it is not known whether a neurotoxic MTD will be encountered with this particular oHSV, finding one would not be unexpected. In this context, NG34 may represent a possible solution for such an eventuality. Additional preclinical testing in animal models may thus be warranted to justify its use in clinical practices via an IND.

Disclosure of Potential Conflicts of Interest

H. Nakashima and E.A. Chiocca are listed as co-inventors on a provisional patent application on the actual virus construct: NG34, that is owned by Partners/Brigham and Women's Hospital. W.F. Goins is a consultant/advisory board member for Oncorus. D.A. Reardon reports receiving speakers bureau honoraria from Bristol-Myers Squibb, EMD Serono, Genentech, Merck, and Regeneron. A.C. Anderson reports receiving commercial research grants from and is a consultant/advisory board member for Potenza Therapeutics and Tizona Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Writing, review, and/or revision of the manuscript: H. Nakashima, T. Nguyen, C. Passaro, H. Ito, W.F. Goins, D.A. Reardon, E.A. Chiocca

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Nakashima, K. Kasai, W.F. Goins, I. Shaikh, I. Nakano, V. Kuchroo, E.A. Chiocca

Study supervision: H. Nakashima, E.A. Chiocca

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