AMERICAN SOCIETY of
GENE & CELL
THERAPY

APOBEC3 Mediates Resistance
to Oncolytic Viral Therapy

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Tumor cells frequently evade applied therapies through the accumulation of genomic mutations and rapid evolution. In the case of oncolytic virotherapy, understanding the mechanisms by which cancer cells develop resistance to infection and lysis is critical to the development of more effective viral-based platforms. Here, we identify APOBEC3 as an important factor that restricts the potency of oncolytic vesicular stomatitis virus (VSV). We show that VSV infection of B16 murine melanoma cells upregulated APOBEC3 in an IFN-β-dependent manner, which was responsible for the evolution of virus-resistant cell populations and suggested that APOBEC3 expression promoted the acquisition of a virus-resistant phenotype. Knockdown of APOBEC3 in B16 cells diminished their capacity to develop resistance to VSV infection *in vitro* and enhanced the therapeutic effect of VSV *in vivo*. Similarly, overexpression of human APOBEC3B promoted the acquisition of resistance to oncolytic VSV both *in vitro* and *in vivo*. Finally, we demonstrate that APOBEC3B expression had a direct effect on the fitness of VSV, an RNA virus that has not previously been identified as restricted by APOBEC3B. This research identifies APOBEC3 enzymes as key players to target in order to improve the efficacy of viral or broader nucleic acid-based therapeutic platforms.

INTRODUCTION

Oncolytic virotherapy has been developed for the treatment of cancer as it combines tumor-tropic cytotoxicity with a highly inflammatory anti-viral response that can activate cellular anti-tumor responses. Strategies such as tropism targeting and arming of the virus with immune stimulatory cytokines to promote the recruitment of immune cells to the tumor have significantly improved the inherent anti-cancer properties of viral therapy.[1,2] U.S. Food and Drug Administration (FDA) approval of the herpes simplex virus (HSV)-based viral therapy talimogene laherparepvec (Tvec), sold as Imlygic, in 2015 demonstrates the clinical significance of these viral-based platforms.[3,4] Despite our ability to enhance viral-mediated tumor cell killing and

immune activation, clinical responses are observed in only a subset of patients.[5,6] To continue to improve upon these responses, identifying restriction factors that limit viral infection or replication and that promote resistance to virus replication and spread will be critical. To this end, we have identified a novel mechanism by which tumor cells develop resistance to oncolytic vesicular stomatitis virus (VSV).

Tumor cells mutate rapidly, giving them a genetic plasticity that allows them to evolve and survive cytotoxic pressure from aggressive therapies.[7] Oncolytic viral therapy exploits genetic and epigenetic defects in the type I interferon (IFN) signaling pathway that frequently occur in tumor cells and render them more susceptible to viral infection and oncolysis.[8] Nonetheless, the heterogeneity of mutations both between tumor types, and even within a single tumor, results in tumors that are able to respond to type I IFN to varying degrees.[9,10] Although IFN-mediated resistance to immunotherapy modalities, including viral therapy, have been described,[9–12] the specific mechanisms by which tumor cells develop innate anti-viral resistance still need to be identified. Among the IFN-inducible anti-viral factors, the APOBEC family of cytosine deaminases has been shown to be involved in both viral resistance and cancer progression.[13,14]

The APOBEC3 family of enzymes catalyzes the deamination of cytosine to uracil on single-stranded DNA (ssDNA). The resulting mutation generates a cytosine-to-thymine (C-to-T) transition and less frequently a cytosine-to-guanine (C-to-G) transversion.[15] APOBEC3-mediated viral restriction has largely been characterized as a response to retroviral infection, although the APOBEC family
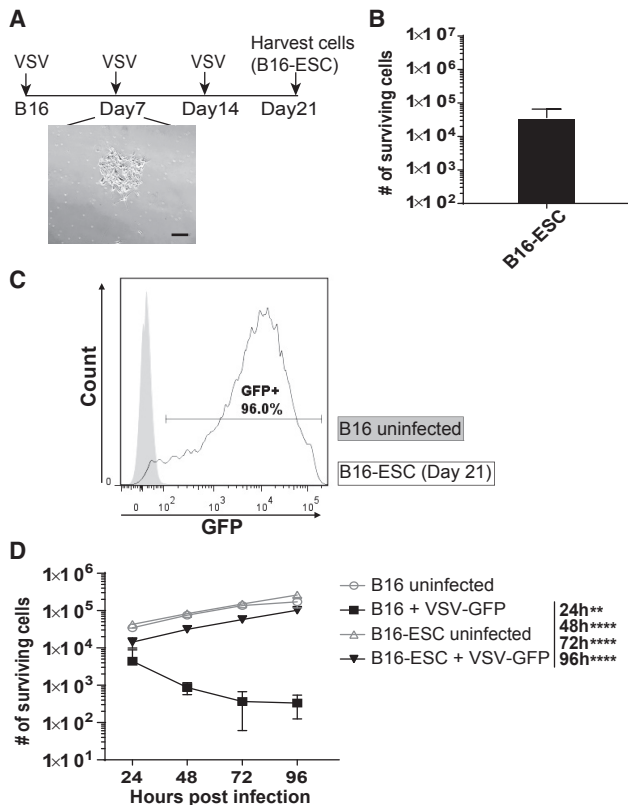
Figure 1. Generation of VSV-Resistant Tumor Cell Populations

(A) Schematic of the generation of VSV-resistant tumor cell populations. 2×10^4 B16 cells were infected at an MOI of 0.01 with VSV-GFP. Wells were washed every 2 days to remove dead cells. 7 days later, surviving cells were visible in the wells (inset). The scale bar represents 0.25 mm. On day 7, wells were trypsinized and cells were washed 3× in PBS before being replated and reinfected at an MOI of 0.01 (VSV-GFP). (B) On day 21 after the first infection, surviving cells (B16-ESCs) were counted. (C) Flow cytometry of B16-ESCs 21 days following initial VSV infection (open histogram) and uninfected parental B16 cells (gray histogram) for GFP expression. Events shown are gated from live cell populations. (D) 2×10^4 B16 cells or B16-ESCs were mock infected or infected at an MOI of 0.01 with VSV-GFP. The number of surviving cells was counted 24, 48, 72, and 96 hr after infection. Means ± SD of triplicate wells are shown. ns, not significant; $p > 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$.

of proteins also mutates cellular genomic DNA, viral DNA, and viral RNA genomes.[16,17] In addition to acting as viral restriction factors, overexpression of the APOBEC3 family of proteins occurs in many cancer types, and a strong correlation exists between APOBEC3 upregulation, genomic mutation burden, therapeutic resistance, and poor prognosis.[18,19] The human genome encodes seven distinct APOBEC3 proteins, while only one APOBEC3 is encoded in the murine genome.[20,21] Specifically, upregulation of human APOBEC3B (hAPOBEC3B), which localizes within the nucleus, has been directly implicated with increased mutational burden among many human cancers and is associated with chemotherapeutic resistance.[22,23] We were therefore interested in determining whether both murine APOBEC3 (mAPOBEC3) and hAPOBEC3B

can contribute to the generation of tumor cell populations that are resistant to oncolytic virotherapy.

We demonstrate here that VSV, a negative-sense RNA virus, mediated the upregulation of APOBEC3 in tumor cells. Elevated APOBEC3 expression was associated with the development of virus-resistant tumor cell clones, and knockdown of APOBEC3 significantly improved VSV therapy *in vivo*. Furthermore, we demonstrate that virus passaged through APOBEC3B-overexpressing cells was significantly less fit than parental virus. Altogether, our data demonstrate that APOBEC3 is a key factor that restricts the efficacy of oncolytic viral therapy.

RESULTS

Resistant Tumor Cell Populations Emerge after Oncolytic Virus Treatment

We have observed that intratumoral treatment of mice bearing established subcutaneous B16 melanoma tumors with VSV resulted in initial responsiveness to therapy followed by recurrence of the tumor after treatment.[24] These observations suggest that either a pre-existing population of virus-resistant tumor cell clones is present within the tumor or that virus infection/replication drives the evolution of such populations. To investigate how tumor cells acquire resistance to viral therapy, murine B16 melanoma cells were infected with repetitive cycles of VSV-GFP at a low MOI (0.01) *in vitro* (Figure 1A). Seven days after initial infection, surviving cells could be recovered, often as small, distinct colonies (Figure 1A, inset). After two further rounds of infection at an MOI of 0.01, viable populations of VSV-resistant cells were recoverable by day 21 after the first infection (B16- escape [ESC]) (Figure 1B). We found that more than 95% of the live B16-ESC cells were positive for GFP expression, indicating that they had been infected by the virus (Figure 1C). Most B16-ESCs at day 21 were both infected and alive, suggesting that the ESC population was significantly more resistant to VSV-mediated oncolysis than unselected parental B16 cells. To confirm that these colonies contained cells that were genuinely resistant to the oncolytic VSV, we infected either B16 parental cells or B16-ESCs with VSV-GFP at an MOI of 0.01 and counted the number of surviving cells after infection. As expected, B16-ESCs infected with VSV-GFP were able to resist infection and grew at a rate similar to uninfected controls (Figure 1D).

Oncolytic VSV Resistance Is Associated with an IFN-Dependent Upregulation of APOBEC3

The selectivity of oncolytic VSV for tumor cells depends upon their defective IFN signaling pathways. However, a range of IFN responsiveness exists for various tumor types.[9,10] Consistent with this, VSV-GFP infection of B16 cells induced moderate levels of type I IFN expression 48 hours after infection (Figure 2A). Therefore, we hypothesized that the acquisition of resistance to VSV oncolysis (Figure 1) may be associated with the expression of IFN-inducible genes in infected cells. In this regard, the APOBEC3 family of cytosine deaminases is well characterized as IFN-inducible genes that can increase the mutational burden within tumor cells, allowing them to
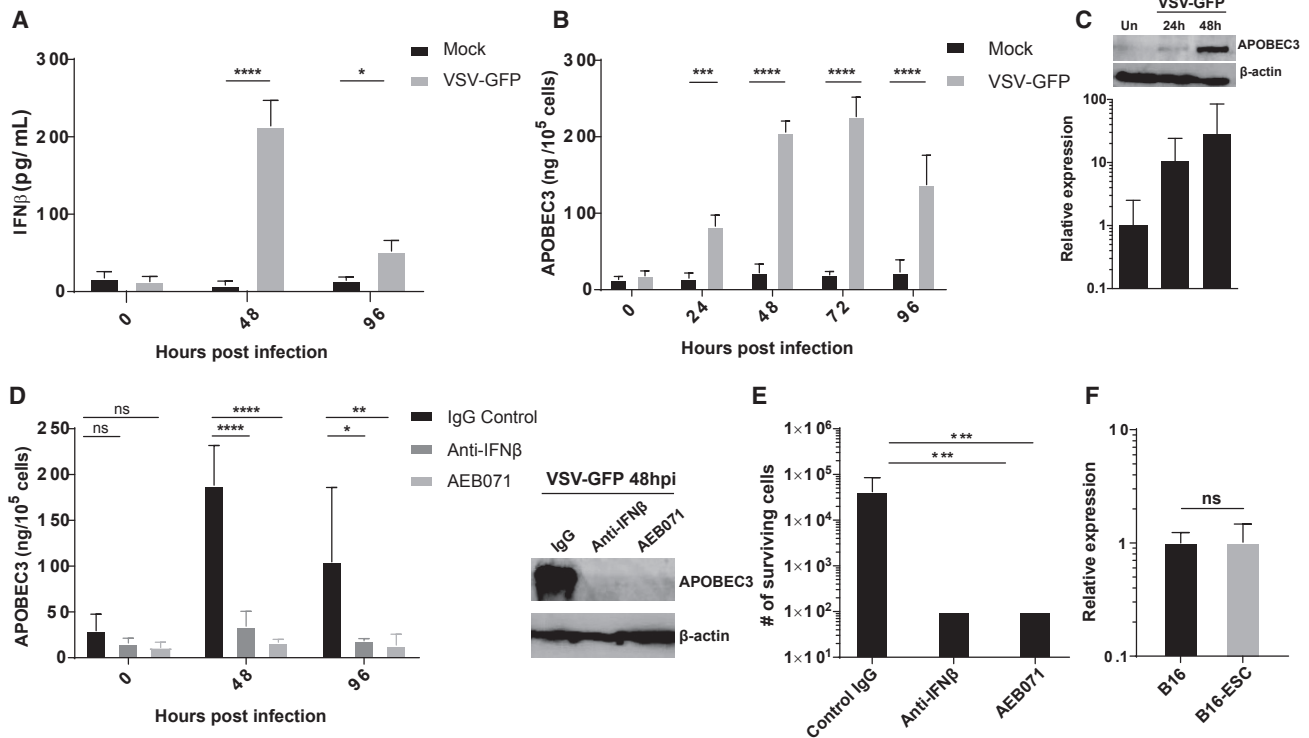
Figure 2. VSV Resistance Is Associated with IFN-β-Dependent APOBEC3 Induction

(A) Levels of IFN-β were measured from supernatants of wells in which 10^5 B16 cells were either mock infected or infected with VSV-GFP at an MOI of 0.01 at 0 (pre-infection), 48, or 96 hr after treatment. (B) Levels of murine APOBEC3 were measured from lysates of wells in which 10^5 B16 cells were either mock infected or were infected with VSV-GFP at an MOI of 0.01 at 0 (pre-infection), 24, 48, 72, or 96 hr after treatment. (C) Expression of murine APOBEC3 was measured by western blot (upper panel) and qRT-PCR (lower panel) from 10^5 B16 cells infected with VSV-GFP at an MOI of 0.01 at 24 and 48 hr after infection. Levels were normalized to uninfected control. (D) Levels of murine APOBEC3 were measured by ELISA (left panel) and western blot (right panel) from 10^5 B16 cells infected with VSV-GFP at an MOI of 0.01 at 0 (pre-infection), 48, or 96 hr after treatment with a control IgG, a polyclonal anti-IFN-β antibody, or AEB071 (10 μM). (E) 2×10^4 B16 cells were infected at an MOI of 0.01 with VSV-GFP with a control IgG, a polyclonal anti-IFN-β antibody, or AEB071 (10 μM) (triplicate wells per treatment). Wells were washed every 2 days to remove dead cells. On day 7, wells were trypsinized and cells were washed 3× in PBS before being replated and reinfected at an MOI of 0.01 (VSV-GFP). This was repeated for one more cycle of 7-day infection. On day 21 after the first infection, surviving cells were counted. (F) Expression of APOBEC3 RNA was measured by qRT-PCR from 10^5 B16 cells or B16-ESCs. Means ± SD of triplicate wells are shown. ns, not significant; p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

evolve and evade an applied therapy.[14,23] Therefore, we investigated whether APOBEC3 may play a role in the development of resistance to oncolytic VSV therapy. APOBEC3 was coordinately expressed with IFN-β following VSV infection, as shown by ELISA and validated by western blot and qRT-PCR (Figures 2B and 2C). In addition, antibody-mediated blockade of IFN-β during VSV infection almost abolished APOBEC3 induction (Figure 2D), as did the inhibitor of protein kinase C nuclear factor κB (PKC-NF-κB) signaling AEB071, previously reported as an inhibitor of APOBEC3 induction through the PKC pathway.[25,26] The emergence of VSV-resistant cell populations (B16-ESCs) was also significantly inhibited by either antibody blockade of IFN-β or PKC-NF-κB signaling inhibition by AEB071 (Figure 2E). Levels of mAPOBEC3 began to decline by 96 hours after infection (Figure 2B) and were not significantly different in B16-ESCs compared to B16 parental cells at a basal level (Figure 2F). These data indicate that VSV induces a transient expres-

sion of APOBEC3 similar to that seen with classical IFN-stimulated gene induction.

Altogether, these data led us to hypothesize that VSV-mediated induction of both type I IFN signaling and APOBEC3 expression is associated with enhanced tumor cell escape from viral oncolysis.

Increased IFN-β Expression Enhances Tumor Cell Resistance

Consistent with a role for type I IFN-induced genes being a key component in the *de novo* generation of oncolysis-resistant tumor cells, low MOI infection of B16 cells for 7 days with VSV-IFN-β[27] generated more VSV-resistant colonies than did infection with VSV-GFP (Figure 3A). Inhibition of PKC-NF-κB signaling by AEB071 during VSV-GFP or VSV-IFN-β infection significantly inhibited the outgrowth of resistant colonies (Figure 3A). Moreover, significantly more VSV-resistant cells were recovered from
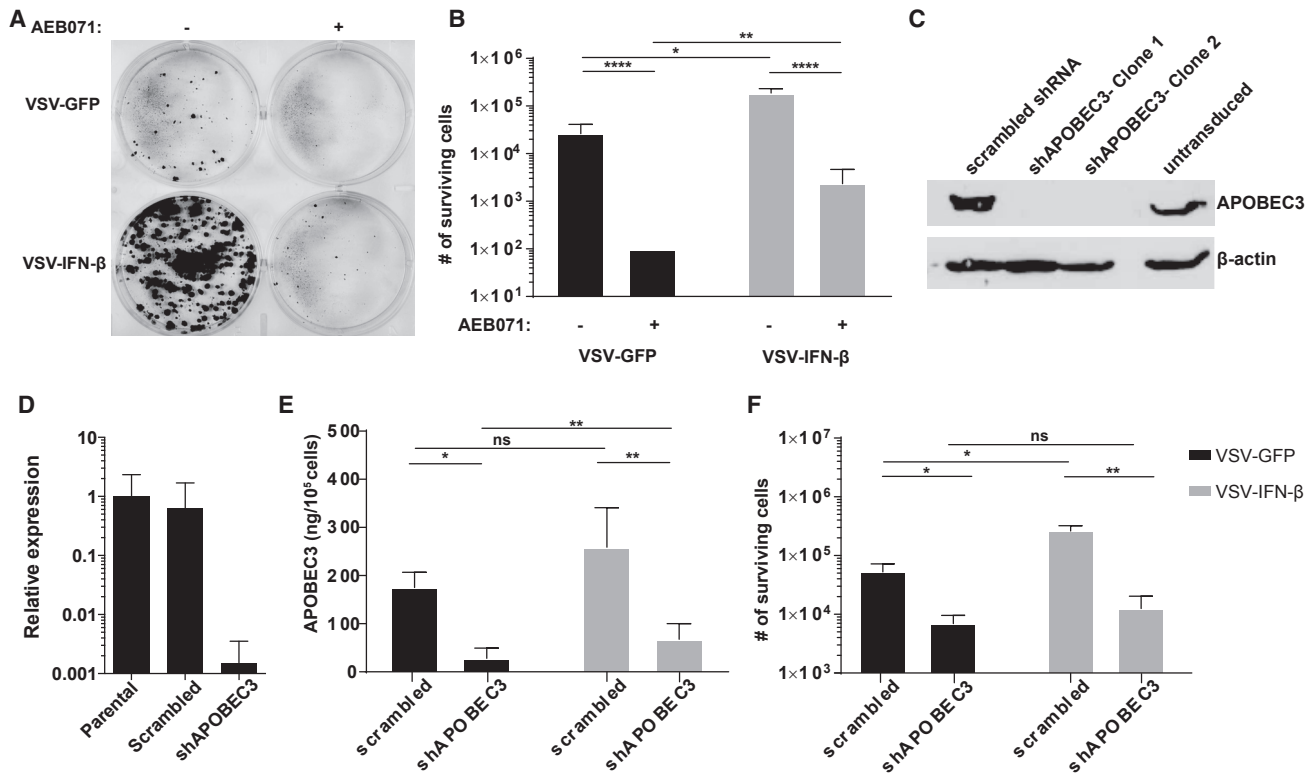
Figure 3. Knockdown of APOBEC3 Reduces the Generation of Virus-Resistant Tumor Cell Populations

(A) 2×10^4 B16 cells were infected at an MOI of 0.01 with VSV-GFP or VSV-IFN-β in the presence or absence of AEB071 (10 μM). Wells were washed every 2 days to remove dead cells. 7 days later, surviving cells were visualized by crystal violet staining. (B) 2×10^4 B16 cells were infected at an MOI of 0.01 with VSV-GFP or VSV-IFN-β in the presence or absence of AEB071 (10 μM). Wells were washed every 2 days to remove dead cells. On day 7, wells were trypsinized and cells were washed $3\times$ in PBS before being replated and reinfected at an MOI of 0.01 (VSV-GFP or VSV-IFN-β). This was repeated for one more cycle of 7-day infection. On day 21 after the first infection, surviving cells were counted. (C) Western blot detection of murine APOBEC3 levels in B16 cells transduced with scrambled shRNA, shAPOBEC3 clone 1, or shAPOBEC3 clone 2 or left untransduced. (D) qRT-PCR of APOBEC3 transcripts from parental B16 or B16 transduced with scrambled shRNA or shAPOBEC3 RNA. (E) Levels of murine APOBEC3 were measured from 10^5 B16(shAPOBEC3) or B16(scrambled shRNA) cells infected with VSV-GFP or VSV-IFN-β at an MOI of 0.01 at 48 hr after treatment. (F) 2×10^4 B16(shAPOBEC3) or B16(scrambled) cells were infected at an MOI of 0.01 with VSV-GFP or VSV-IFN-β. Wells were washed every 2 days to remove dead cells. On day 7, wells were trypsinized and cells were washed $3\times$ in PBS before being replated and reinfected at an MOI of 0.01 (VSV-GFP or VSV-IFN-β). This was repeated for one more cycle of 7-day infection. On day 21 after the first infection, surviving cells were counted. Means ± SD of triplicate wells are shown. ns, not significant; $p > 0.05$; $*p \leq 0.05$; $**p \leq 0.01$; $****p \leq 0.0001$.

repeated virus infection, as in Figure 1A, if cells were infected with VSV-IFN-β compared to VSV-GFP (Figure 3B). As seen previously, inhibition of PKC-NF-κB signaling significantly inhibited escape from VSV-IFN-β and, more effectively, from VSV-GFP (Figure 3B). These data show that IFN-β enhances the generation of B16-ESC populations through a signaling pathway that also induces APOBEC3 expression in infected tumor cells.

To demonstrate that APOBEC3 contributed to the acquisition of resistance to VSV infection, B16 cells in which expression of APOBEC3 had been knocked down with short hairpin RNA (shRNA), as shown by ELISA and validated by western blot and qRT-PCR (Figures 3C–3E), were infected with VSV-GFP or VSV-IFN-β. Overexpression of IFN-β from VSV led to increased, although not significant, levels of APOBEC3 (Figure 3E) from those induced by VSV-GFP. The infection of B16(shAPOBEC3) cells with either VSV-

GFP or VSV-IFN-β virus led to both significantly lower levels of APOBEC3 expression (Figure 3E), as well as significantly fewer VSV-resistant B16-ESC populations (Figure 3F), than did infection of B16 cells transduced with a scrambled, control shRNA (Figures 3C and 3F). These data indicate that although APOBEC3 is unlikely to be the only IFN-inducible response factor that contributes to viral resistance, it significantly affects the generation of cells that are able to evade VSV therapy.

APOBEC3 Inhibition Reduces Escape from VSV Therapy

To investigate whether APOBEC3 played an important role in the *in vivo* generation of resistance to viral oncolytic therapy, mice bearing 3-day established B16 parental, B16 (scrambled RNA), or B16(shAPOBEC3) tumors were injected with nine doses of VSV-GFP at the site of tumor cell injection. Over two separate experiments, fewer than 10% of mice injected with B16(shAPOBEC3) tumors
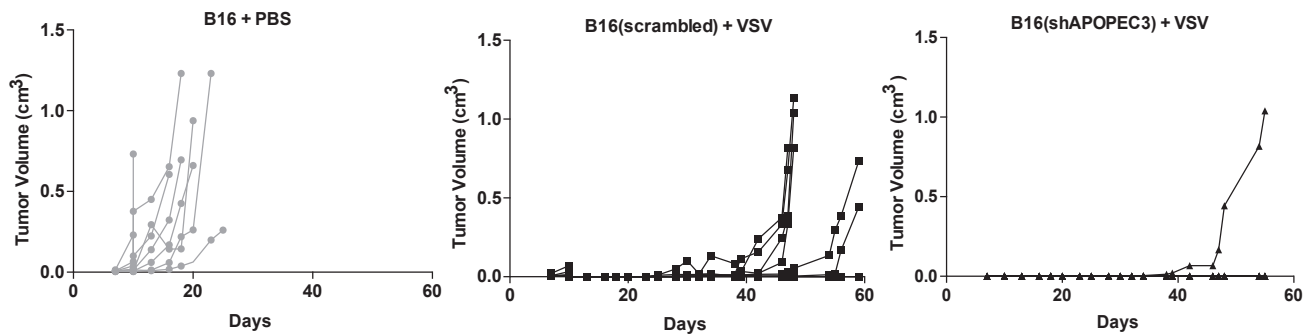
Figure 4. Knockdown of APOBEC3 Improves VSV Therapy *In Vivo*

C57BL/6 mice were injected subcutaneously with 2×10^5 B16(shAPOBEC3) or B16(scrambled) cells. On days 3, 5, 7, 10, 12, 14, 17, 19, and 21, the site of tumor cell injection was injected with PBS or VSV-GFP (1×10^8 PFU). The number of mice with detectable tumors (>0.2 cm diameter) at day 55 is shown. Cumulative results from two separate experiments; n = 8 mice per group.

escaped VSV therapy to form recurrent tumors (Figure 4). In contrast, 65% of mice with B16(scrambled) tumors escaped VSV treatment to form recurrent tumors (Figure 4). All PBS-injected mice developed tumors and required euthanasia by day 25. These data support the hypothesis that the expression of APOBEC3 contributes to the outgrowth of VSV-resistant tumors.

hAPOBEC3B Plays a Role in Resistance to Oncolytic VSV Therapy

hAPOBEC3B has been reported to be involved in the generation of acquired chemotherapeutic resistance.[23] We were therefore interested in whether hAPOBEC3B may recapitulate the function of mAPOBEC3 to drive the development of VSV-resistant populations in a human Mel888 xenograft tumor model. Thus, VSV vectors expressing four shRNAs against hAPOBEC3B individually or in combination (shRNAs 1–4) were generated, and knockdown of hAPOBEC3B in Mel888 cells infected with each vector was confirmed by western blot (Figure 5A). VSV expressing shRNAs 1–3, as well as the combination of all 4 shRNAs, dramatically reduced the expression of hAPOBEC3B. A non-specific band (**) was observed in all VSV-infected cells, suggesting cross reactivity of the antibody against a VSV antigen. This was confirmed by infection with VSV-GFP, which did not result in a reduction in hAPOBEC3B expression but did show the non-specific band (Figure 5A). Similar to induction of mAPOBEC3 in B16 cells (Figure 2), VSV-mediated induction of APOBEC3B in Mel888 cells was confirmed using a shorter exposure than that used in Figure 5A, left panel, which was overexposed to detect any APOBEC3B protein expressed after shRNA knockdown (Figure 5A, right panel). Treatment of mice bearing subcutaneous Mel888 tumors with nine doses of VSV expressing the combination of shRNAs (VSV-shAPOBEC3B) significantly improved survival compared to treatment with VSV-GFP or PBS (Figure 5B). VSV-shAPOBEC3B-treated mice had controlled tumor growth out to 50 days in contrast to VSV-GFP mice, which all succumbed to tumor before day 40 (Figure 5C). These data support a role for hAPOBEC3B in promoting resistance to oncolytic VSV therapy.

Tumors Overexpressing hAPOBEC3B Readily Escape VSV Therapy

To test this hypothesis, a mutated, enzymatically non-functional (Figure 6A, lower panel), or a fully functional hAPOBEC3B protein (Figure 6A, upper panel) was overexpressed in tumor cells by transduction with a retroviral vector, and expression was confirmed by western blot and qRT-PCR (Figures 6B and 6C). Robust hAPOBEC3B expression was observed 72 hr after transduction and decreased to a lower, yet still elevated, level compared to parental controls by 2 weeks. This could be due to toxicity associated with overexpression of hAPOBEC3B, which may act as a mutagen, selecting for cells that express a lower level of hAPOBEC3B. B16 cells overexpressing the mutated, non-functional APOBEC3B developed resistance to VSV following a low MOI infection with VSV-GFP over 21 days (Figure 6E) at a rate similar to that of parental B16 cells. The growth rate of the B16-APOBEC3B cell line was not greater than that of the B16-APOBEC3B MUT or parental B16 cell lines after 5 repeated passages for 96 hr (out to day 20), indicating that the increased frequency of VSV escape in the B16-APOBEC3B cell line was not due to altered growth rates associated with overexpression of APOBEC3B (Figure 6D). However, B16 cells overexpressing functional APOBEC3B were better able to resist VSV infection compared to either wild-type B16 or B16-APOBEC3B MUT (Figure 6E). These effects were not specific to the B16 cell line, because increased resistance to VSV oncolysis was observed in both the murine glioma GL261 and the human melanoma Mel888 cell lines, when engineered to overexpress APOBEC3B, after the 21-day treatment period (Figures 6F and 6G). Transient overexpression of the APOBEC3B protein, but not the APOBEC3B MUT protein, was associated with a significant decrease in viability of infected B16 cells (Figure 6H). These data show that the deaminase-competent APOBEC3B protein exerts significant toxicity—possibly through its geno-toxic, mutator activity—which is absent from the deaminase-incompetent APOBEC3B MUT protein. However, despite the toxicity of APOBEC3B expression, its expression allows for greater levels of cell survival upon VSV infection (Figures 6E and 6F), suggesting that it allows for selection of a virus-resistant phenotype.
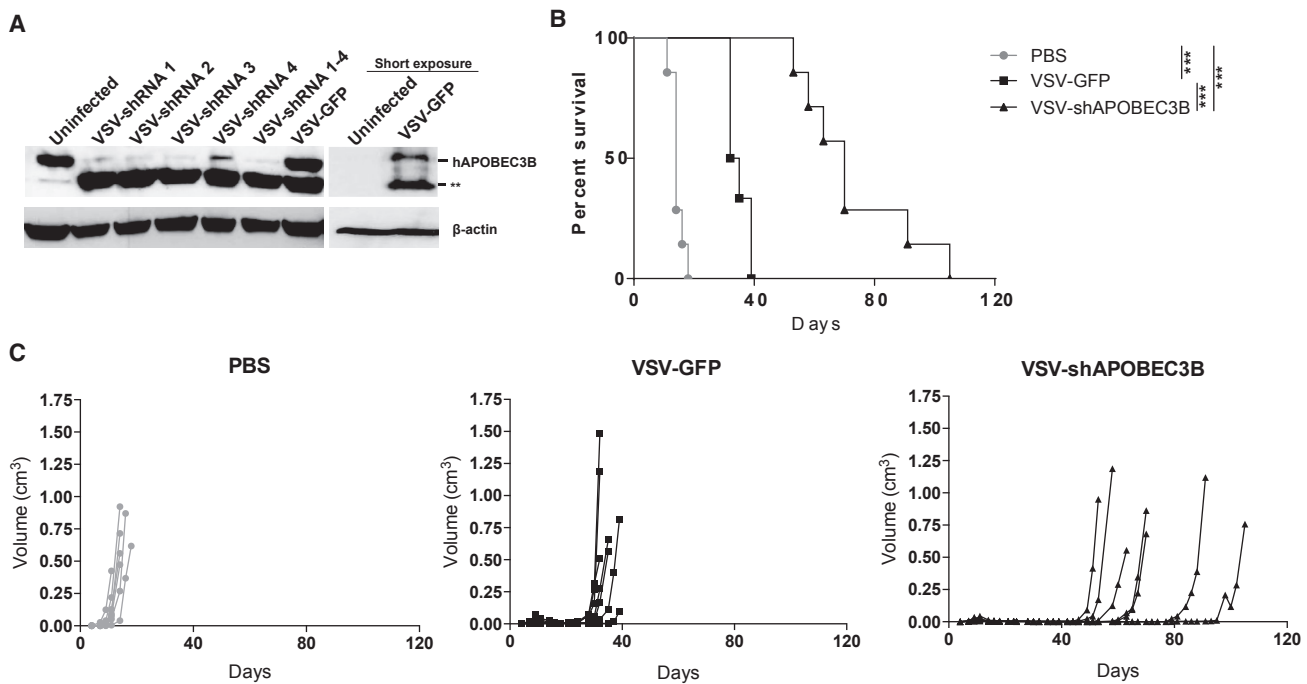
Figure 5. Knockdown of Human APOBEC3B Improves VSV Therapy in Human Melanomas

(A) Left panel: western blot of hAPOBEC3B expressed in Mel888 cells infected with VSV encoding shRNAs against hAPOBEC3B at an MOI of 0.01. Right panel: western blot of hAPOBEC3B expressed in uninfected Mel888 cells or in Mel888 infected with VSV-GFP at an MOI of 0.01 at a shorter exposure than in the left panel. (B and C) Kaplan-Meier survival (B) and tumor volumes (C) of nude mice that were injected subcutaneously with 10^6 Mel888 cells. On days 3, 5, 7, 10, 12, 14, 17, 19, and 21, tumors were injected with PBS, 10^7 PFU VSV-GFP, or a combined dose of 10^7 PFU of VSV-shAPOBEC3 (shRNAs 1–4); n = 7 mice per group. **p $\leq$ 0.01; ***p $\leq$ 0.001.

Consistent with the *in vitro* data (Figure 6E), B16 tumors overexpressing hAPOBEC3B treated with nine intratumoral injections of VSV-GFP escaped VSV therapy significantly more quickly than control tumors expressing the mutated APOBEC3B (Figures 7A and 7B). B16-APOBEC3B MUT-expressing cells were used as the negative control in these experiments, because previous data indicated target cell killing is equivalent between B16 parental and B16-APOBEC3B MUT cell lines (Figure 6E). In addition, low but detectable levels of VSV-GFP were recovered from most B16-APOBEC3B MUT tumors at sacrifice (Figure 7C). In contrast, no virus was recovered from B16-APOBEC3B treated tumors (Figure 7C). These data confirm that overexpression of APOBEC3B in tumors increased the rate at which tumors escaped from oncolytic virotherapy and suggested that it may reduce the fitness of the oncolytic virus.

hAPOBEC3B Overexpression Reduces VSV Fitness

Our data have shown that APOBEC3 contributed to generation of a virus-resistant phenotype of infected tumor cells (Figure 4). In addition, given the lack of recoverable virus from APOBEC3B-overexpressing tumors (Figure 7C), and consistent with its role as a restriction factor against viral infection, we hypothesized that upregulation or overexpression of APOBEC3 or APOBEC3B, respectively, may also directly affect VSV fitness. Approximately 10-fold less virus was recovered after repeated passage through B16-APOBEC3B cells compared to passage through B16-APOBEC3B MUT cells (Fig-

ure 8A). Moreover, when used to reinfect parental B16 cells at an MOI of 0.01, VSV recovered from 21-day passage through B16-APOBEC3B cells was significantly less cytolytic than both stock virus and virus recovered from B16-APOBEC3B MUT cells (Figure 8B). To further characterize the loss of fitness of the virus recovered from APOBEC3B-overexpressing cells, the burden of defective interfering particles (DIPs) in the viral population following multiple passages through either B16-APOBEC3B MUT or B16-APOBEC3B cells was quantified. DIP content in each viral passage was quantified by measuring the ability of a viral preparation collected after passage to interfere with infection with a stock virus.[28] After a single passage through either B16-APOBEC3B or B16-APOBEC3B MUT cell lines, virus recovered after passage interfered equally with infection of target cells by the stock virus, indicating similar DIP contents (Figure 8C). However, following five *in vitro* passages, virus recovered from B16-APOBEC3B cells contained significantly more interfering DIPs than did virus recovered from B16-APOBEC3B MUT cells (Figure 8C). These data showed that there are more non-functional particles in the supernatant recovered from APOBEC3B-overexpressing cells, suggesting that APOBEC3B may directly mutate the virus genome. We are characterizing the APOBEC3B-induced mutations within the viral genome that lead to this increased DIP content and reduced viral fitness. Altogether, these data suggest that APOBEC3B results in decreased oncolytic activity by both facilitating the emergence of tumor cells resistant to oncolytic virus activity and restricting
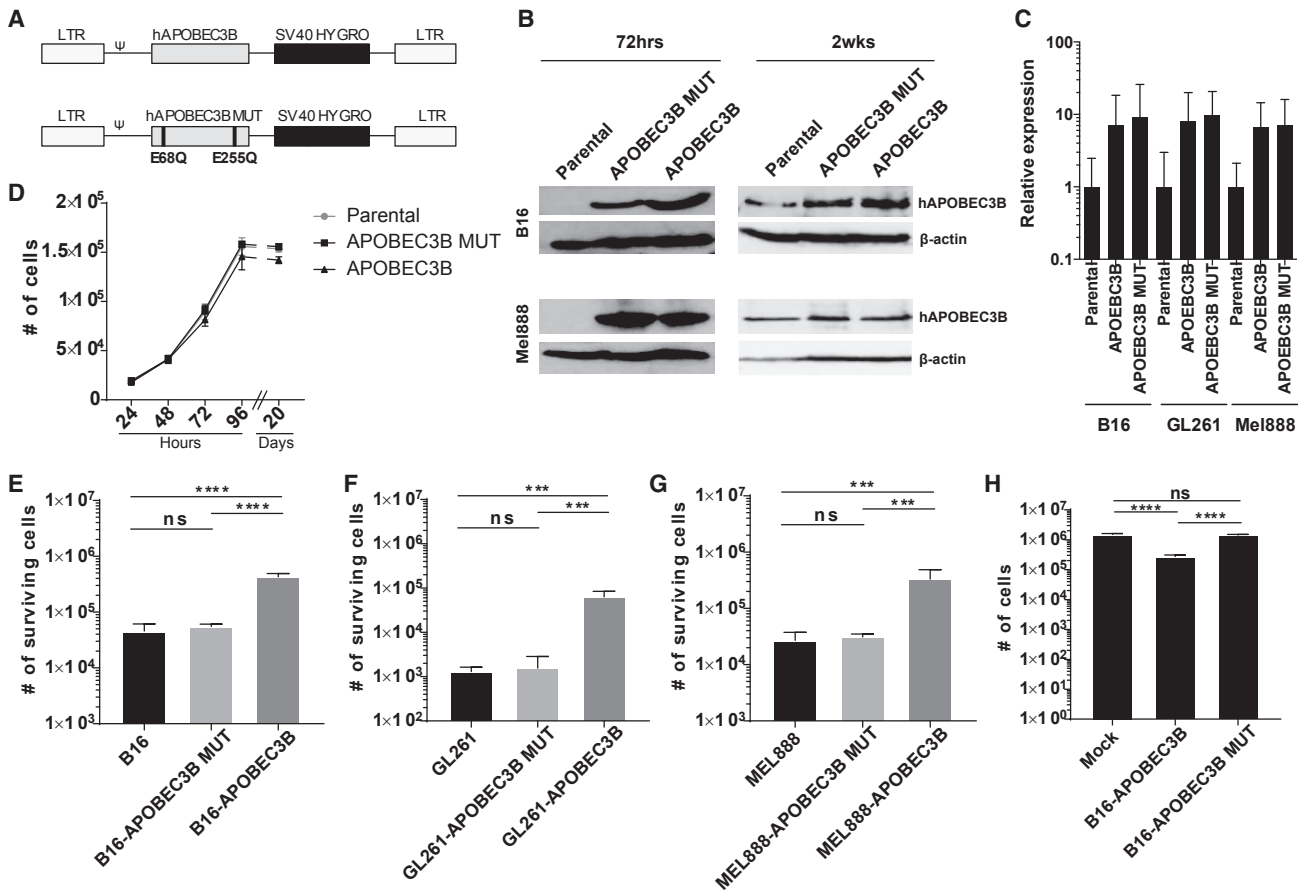
Figure 6. Overexpression of Human APOBEC3B Enhances Tumor Cell Escape *In Vitro*

(A) Tumor cell lines were engineered to overexpress human APOBEC3B following infection with either pBABE-Hygro APOBEC3B or pBABE-Hygro APOBEC3B MUT. 48 hr after infection, bulk populations of cells were selected in hygromycin 2 weeks and used for experiments. (B and C) 10^5 murine B16 or human Mel888 cells were mock infected (lane 1) or were infected at an MOI of 10 with APOBEC3B MUT-expressing viruses (lane 2) or APOBEC3B-expressing viruses (lane 3). Expression of hAPOBEC3B was assayed by western blot (B) at 72 hr or 14 days after infection or by qRT-PCR at 72 hr after infection (C). (D) 10^4 B16 parental, B16 APOBEC3B MUT-overexpressing, and B16-APOBEC3B-overexpressing cells were plated in triplicate wells per cell line, and growth was measured over 96 hr by counting viable cells. Cells were also grown for 96 hr and then trypsinized, viable cells were counted, and 10^4 viable cells were replated and grown for a further 96 hr. This regimen was repeated for a total of 20 days in culture. The number of viable cells at the end of the 20-day culture was measured as shown. (The 21-day point represents the number of cells of each line grown in the last 96-hr period from an initial plating of 10^4 viable cells.) (E) 2×10^4 B16-APOBEC3B or B16-APOBEC3B MUT cells were infected at an MOI of 0.01 with VSV-GFP (triplicate wells per treatment). Wells were washed every 2 days to remove dead cells. On day 7, wells were trypsinized and cells were washed $3\times$ in PBS before being replated and reinfected at an MOI of 0.01 (VSV-GFP). This was repeated for one more cycle of 7-day infection. On day 21 after the first infection, surviving cells were counted. (F and G) 2×10^4 GL261-APOBEC3B or GL261-APOBEC3B MUT cells (F) or MEL888-APOBEC3B or MEL888-APOBEC3B MUT cells (G) were infected at an MOI of 0.01 with VSV-GFP (triplicate wells per treatment). Wells were washed every 2 days to remove dead cells. On day 7, wells were trypsinized and cells were washed $3\times$ in PBS before being replated and reinfected at an MOI of 0.01 (VSV-GFP). This was repeated for one more cycle of 7-day infection. On day 21 after the first infection, surviving cells were counted. Means ± SD of triplicate wells are shown. ns, not significant; $p > 0.05$; ***$p \leq 0.001$; ****$p \leq 0.0001$. (H) Cell survival 120 hr following infection of 10^5 B16 parental cells with no virus or with pBABE-Hygro APOBEC3B or pBABE-Hygro APOBEC3B MUT viruses (MOI of ~10) in the presence of polybrene (triplicate wells per infection).

the fitness of the virus directly by altering the viability of progeny particles.

DISCUSSION

The tumor tropism of oncolytic viruses is associated with an impaired innate anti-viral signaling response frequently characterized in malignancies. However, the ability of tumor cells to produce and/or respond to type I IFN can differ greatly. Such heterogeneity is likely to be a significant factor affecting the variability in responses observed in clinical trials with oncolytic viral therapies. To understand the mechanisms underlying escape from oncolytic virus therapy, we studied tumor cells that became resistant to oncolytic VSV over several rounds of infection. The development of virus-resistant cells was associated with the upregulation of the mAPOBEC3 cytosine
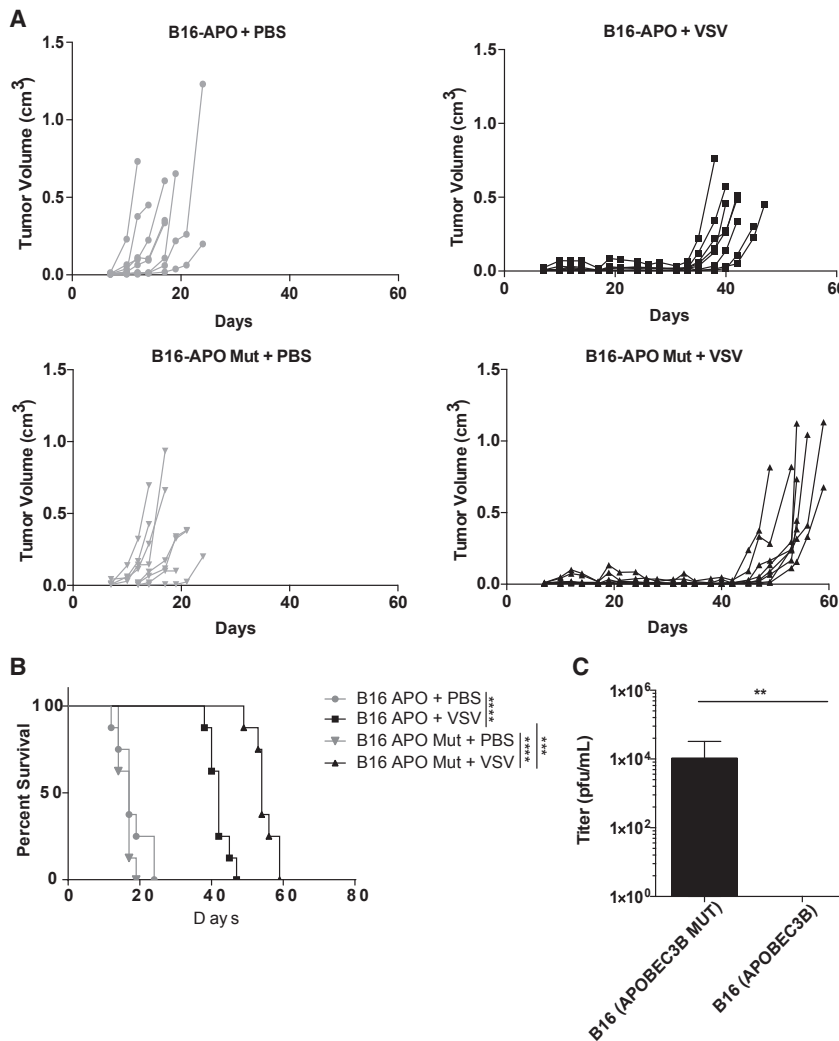
Figure 7. Overexpression of APOBEC3B Decreases the Efficacy of VSV Therapy *In Vivo*

(A and B) C57BL/6 mice were injected subcutaneously with 2×10^5 B16-APOBEC3B or B16-APOBEC3B MUT cells. On days 10, 12, 14, 17, 19, 21, 24, 26, and 28, the site of tumor cell injection was injected with PBS or VSV-GFP (1×10^8 PFU). Tumor growth (A) and overall survival (B) are shown. The number of mice with detectable tumors (>0.2 cm diameter) at day 55 is shown. Cumulative results from two separate experiments; n = 8 mice per group. (C) Tumors were collected at time of sacrifice, and viral titer was determined by plaque assay. Means ± SD of triplicate wells are shown. ns, not significant; p > 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

We observed a significant upregulation of APOBEC3 48–72 hours post-infection coincident with the upregulation of IFN-β (Figures 2A–2C). Blockade of IFN-β, or PKC signaling with the small molecule inhibitor AEB071, blocked APOBEC3 expression following infection and correspondingly led to a reduction in the number of surviving virus-resistant cells (Figures 2D and 2E). Moreover, infection of B16 cells with VSV expressing murine IFN-β significantly increased the number of treatment-resistant cells even after a single round of infection (Figure 3A), an effect that was abrogated by AEB071 (Figures 3A and 3B). Many classical type I IFN-stimulated genes have been identified as mediators of resistance to VSV viral therapy, and the use of broadly acting JAK/STAT inhibitors has been suggested to counteract these IFN responses to improve oncolytic therapy.[11,35] However, our data here are the first to identify APOBEC3 as a key type I IFN-stimulated gene that contributes to resistance to oncolytic viral therapy.

deaminase in an IFN-β-dependent manner. Our results here identify a role for APOBEC3 as an important factor that restricts the efficacy of oncolytic virotherapy.

The APOBEC3 family of proteins has been shown to be important in the restriction of retroviruses, herpesviruses, and hepatitis viruses.[13,19,29,30] One mechanism of APOBEC3-mediated retrovirus mutagenesis involves the deamination of cytosine to uracil on ssDNA during the reverse transcription of the viral genome.[31] APOBEC3 proteins have also been shown to act on cellular RNA substrates[32,33] and were implicated to mediate resistance to cornavirus infection, a positive-sense RNA virus.[34] In addition, APOBEC3 has mutagenic activity directly on cellular DNA and has been identified as a key driver for tumorigenesis and disease progression.[14,16,18] The dual properties of APOBEC3 as both an anti-viral restriction factor and a cancer mutation driver led us to hypothesize that APOBEC3 may play a role in restricting oncolytic viral infection and/or replication in tumor cells (Figure 1).

Consistent with the role of APOBEC3 activity in mediating the development of resistance to VSV oncolysis, fewer VSV-resistant surviving cells were recovered following *in vitro* infection with VSV-GFP or VSV-IFN-β if APOBEC3 expression was inhibited using shRNA (Figure 3F). In addition, the *in vivo* efficacy of VSV oncolytic therapy was significantly improved by APOBEC3 knockdown. Less than 10% of mice implanted with tumors expressing APOBEC3 shRNA developed tumors after an aggressive treatment of nine doses of intratumoral VSV-GFP, whereas tumor outgrowth was observed in 100% of mice implanted with B16 parental cells and 65% of mice implanted with B16 cells expressing a scrambled shRNA (Figure 4). Furthermore, specific targeting of hAPOBEC3B by shRNA expressed from VSV significantly improved the control of tumor growth and survival of nude mice bearing human Mel888 melanomas (Figure 5B), thereby implicating the human APOBEC3 family in the acquisition of
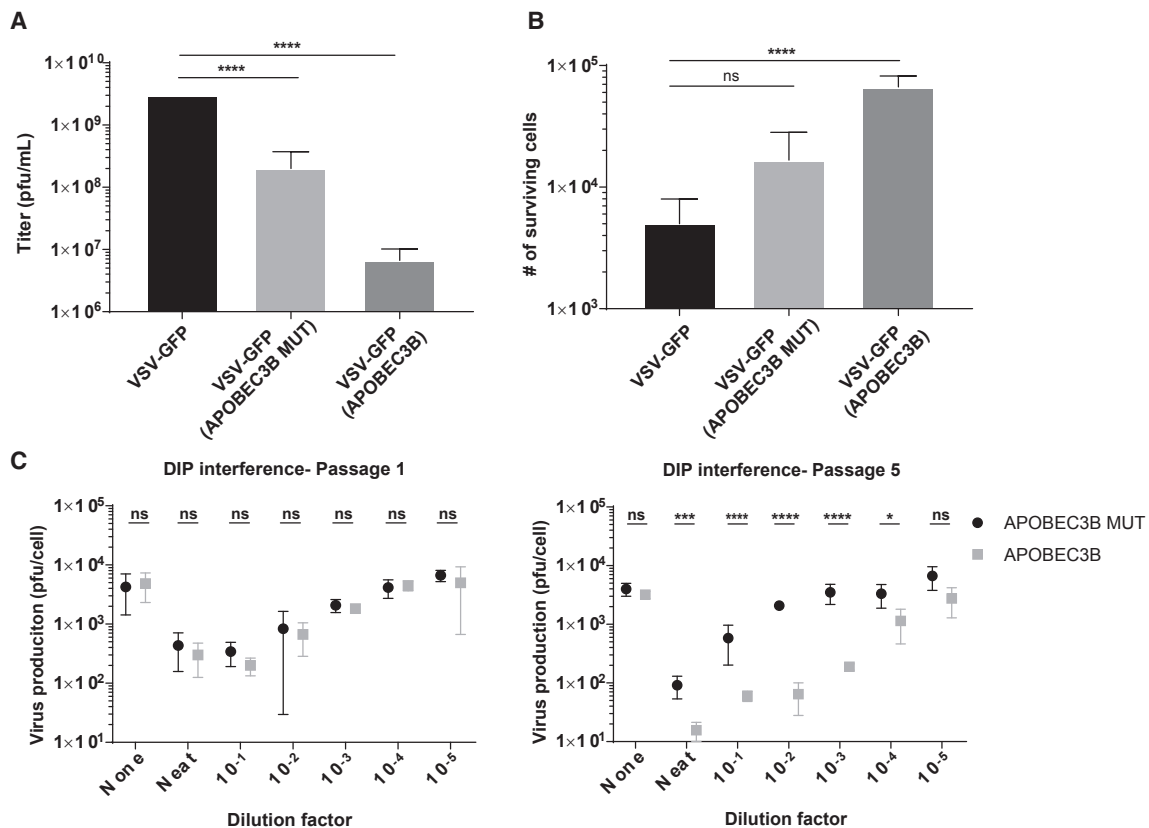
Figure 8. APOBEC3B Directly Affects the Fitness of VSV

(A) 2×10^4 B16-APOBEC3B or B16-APOBEC3B MUT cells were infected at an MOI of 0.01 with VSV-GFP from a parental stock of titer 5×10^9 PFU/mL (triplicate wells per treatment). Wells were washed every 2 days to remove dead cells. On day 7, supernatant was removed, the wells were trypsinized, and cells were washed $3\times$ in PBS before being replated. These replated cells were then reinfected with the 7-day virus supernatants for a further 7 days. This was repeated for one more cycle of 7-day infection. On day 21 after the first infection, virus was recovered (VSV-ESC) and titered. (B) Parental stock VSV-GFP or VSV-ESC viruses were used to infect 10^5 parental B16 cells at an MOI of 0.01, as determined from the titers in (A). 5 days after infection, surviving cells were counted. (C) 1×10^6 B16-APOBEC3B MUT or B16-APOBEC3B cells were infected at an MOI of 0.01 for 24 hr. Virus from the supernatant was collected, and 1 mL of supernatant was used to reinfect a fresh monolayer of 1×10^6 B16-APOBEC3B MUT or B16-APOBEC3B cells. Viral supernatants collected from serial passage were diluted from 10^{-1}–10^{-6} and used to infect 2×10^4 BHK cells. The virus was allowed to adsorb for 1 hr at 37°C and then were washed from the well, followed by infection with VSV-GFP stock virus at an MOI of 20. Supernatant was collected 24 hr after infection and analyzed by plaque assay to determine the titer of virus produced. Means ± SD of triplicate wells are shown. ns, not significant; p > 0.05; *p ≤ 0.05; ***p ≤ 0.001; ****p ≤ 0.0001.

resistance to oncolytic viral therapy. The human genome encodes six additional APOBEC3 proteins, with distinct patterns of expression, subcellular localizations, and biochemical and anti-viral activities.[18,36] In particular, human APOBEC3A, APOBEC3F, and APOBEC3G are also known to be IFN inducible.[37,38] Therefore, it will be of great interest to expand the studies described here to investigate the role of these additional human APOBEC3 enzymes in restricting the efficacy of VSV or other oncolytic viruses.

Our data indicate that inhibition of APOBEC3 enzymes may be a viable strategy to improve VSV oncolytic viral therapy *in vivo*. The implications for viral restriction by APOBEC3 extend beyond oncolytic therapy and include gene therapy protocols using retroviral or lentiviral vectors whose natural life cycle has been largely associated with APOBEC3 restriction. Although there are no clinically approved

inhibitors of APOBEC3B enzymes, the use of inhibitors such as AEB071 may indirectly block APOBEC3 upregulation, and targeted knockdown of APOBEC3 by shRNA delivered from a viral vector may be a viable strategy to circumvent APOBEC3-mediated viral resistance.

When tumor cells overexpressing hAPOBEC3B were treated with VSV for 21 days *in vitro* (Figures 6E–6G), we observed a significant increase in the number of virus-resistant, surviving cells compared to APOBEC3B MUT-overexpressing cells. APOBEC3B-associated anti-viral resistance did not depend on cell or tumor type, as overexpression of functional APOBEC3B in murine glioblastoma GL261 and human melanoma MEL888 cells also resulted in increased numbers of virus-resistant surviving cells after treatment (Figures 6F and 6G). Similarly, VSV treatment was better able to control

B16-APOBEC3B MUT tumors compared to B16-APOBEC3B tumors (Figures 7A and 7B). Virus was not recovered from B16-APOBEC3B tumors at the time of sacrifice, while titers up to 10^4 plaque-forming units (PFU)/mL were recovered from B16-APOBEC3B MUT tumors, suggesting that APOBEC3B had a direct effect on viral replication and persistence *in vivo* (Figure 7C).

We observed that overexpression of APOBEC3B is toxic in that elevated levels of APOBEC3B were seen within 72 hr after transfection or infection and then returned to lower levels within 2 weeks (Figure 6B). To explain this observation, in which APOBEC3B expression returns to background levels in the long term, we hypothesize that mutagenesis by APOBEC3B is tolerable up to a certain threshold within any given cell, over and above which critical mutations are induced, leading to cell lethality. In those cells in which overexpression of APOBEC3B does not reach this threshold, and in which APOBEC3B overexpression is selected against, cell survival is possible. This is consistent with our observation that transient overexpression of APOBEC3B, but not APOBEC3B MUT, was associated with a significant decrease in viability of infected B16 cells (Figure 6H) and that the long-term growth rate of B16 APOBEC3B cells was lower than that of B16 APOBEC3B MUT or B16 parental cells (Figure 6D). However, we observed a similar trend in the reduction of APOBEC3B MUT expression over 14 days (Figure 6B). In this respect, we cannot exclude that additional deaminase-independent activities of APOBEC3B may be preserved in the APOBEC3B MUT protein and that these additional deaminase-independent activities of the APOBEC3B and APOBEC3B MUT proteins may help to select only those cells in which expression of the transgene is selectively downregulated.

To understand whether APOBEC3 had a direct effect on the viral platform, VSV-GFP was passaged through B16-APOBEC3B MUT or B16-APOBEC3B cells. Approximately 10-fold less VSV-GFP was recovered from serial passage through APOBEC3B-overexpressing cells compared to virus recovered from APOBEC3B MUT-overexpressing cells (Figure 8A). In addition, virus recovered from several passages through APOBEC3B-overexpressing cells was significantly less oncolytic in parental B16 cells than either virus recovered from APOBEC3B MUT-overexpressing cells or parental stock virus (Figure 8B). Analysis of the virus generated after a single passage, although each cell line revealed a similar induction of the cytopathic effect and no significant difference in the content of DIPs (Figure 8C). However, after five passages, virus recovered from B16-APOBEC3B cells contained significantly more DIPs, as measured by its ability to interfere with infection of cells by a standard stock of virus, hence confirming that hAPOBEC3B activity directly affected the fitness of the viral progeny generated during infection (Figure 8C). To our knowledge, this is the first report of APOBEC3-mediated anti-viral resistance to VSV infection. We are testing two hypotheses to explain these observations. First, direct mutagenic activity of APOBEC3B on the VSV RNA genome would generate mutations that decrease the fitness of the viral quasispecies.

Alternatively, APOBEC3B activity on the host cell population may generate cellular environments in which viral replication is significantly inhibited. To distinguish between these two possibilities, we are sequencing the viruses that emerge from passage through B16 parental, B16-APOBEC3B MUT, or B16-APOBEC3B cells, as well as characterizing changes in the anti-viral phenotype of the APOBEC3B-modified cells.

Altogether, our data show that oncolytic viral infection with VSV induces a type I IFN-dependent upregulation of APOBEC3 that promotes the generation of virus-resistant tumor cell populations and reduces the oncolytic fitness of the virus. Our results identify APOBEC3 as a rational target to improve the efficacy of oncolytic platforms and other viral-based therapies. We are investigating the particular targets of APOBEC3-mediated mutation that are responsible for tumor cell escape from VSV oncolysis, as well as the viral genomic targets that reduce viral fitness. Our preliminary studies have confirmed that APOBEC3 overexpression generates significant levels of cellular genomic mutations, and we are testing the hypothesis that at least some of these act to decrease viral replication. Finally, we are pursuing strategies that target APOBEC3 during virus infection to improve the therapeutic outcome of oncolytic viral therapy.

MATERIALS AND METHODS

Cell Lines

Murine melanoma B16 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). B16TK cells were derived from a B16.F1 clone transfected with a plasmid expressing the herpes simplex virus 1 thymidine kinase (HSV-1 TK) gene. Following stable selection in 1.25 μg/mL puromycin, these cells were shown to be sensitive to ganciclovir (Cymevene) at 5 μg/mL.[39–41] B16-APOBEC3B and B16-APOBEC3B MUT cells were maintained in DMEM supplemented with 10% FBS at 37°C in 10% CO_2 and selected in hygromycin (200 μg/mL). Baby hamster kidney (BHK) cells were cultures in DMEM supplemented with 10% FBS. All cell lines were maintained at 37°C in 10% CO_2 and regularly shown to be free of *Mycoplasma* infection.

Mice

6- to 8-week-old female C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine).

Viruses

VSV was generated from pXN2 cDNA plasmid using the established reverse genetics system in BHK cells.[42] All transgenes were inserted between viral G and L protein using the *Xho*I and *Nhe*I restriction sites. Virus titers were determined by plaque assay on BHK cells.

Generation of Virus-Resistant Cell Lines

B16 cells were infected at an MOI of 0.01 (VSV) for 1 hr, washed with PBS, and then incubated for 7 days. Dead cells were removed every 2 days by washing with PBS. After 7 days, the cells were collected

by detachment with trypsin and replated. These cells were subjected to two repeated rounds of infection as previously described. After 21 days, or three total rounds of infection, the remaining virus-escaped cells were collected. This protocol was performed in the presence or absence of anti-IFN-β antibody (rabbit polyclonal anti-mouse IFN beta; PBL InterferonSource, Piscataway, New Jersey), PKC signaling inhibitor (AEB071, 10 μM; MedChemExpress, Monmouth Junction, New Jersey), or control immunoglobulin G (IgG) (Chrome Pure anti-rabbit IgG, catalog no. 011-000-003; Jackson Laboratory).

Protein Quantification
mAPOBEC3 was measured by western blot using a rabbit monoclonal anti-APOBEC3 (184990; Abcam, San Francisco, California); hAPOBEC3B was measured by western blot using a rabbit polyclonal anti-APOBEC3B PA5-11430 (Thermo Fisher Scientific). mAPOBEC3 was measured by rabbit monoclonal anti-hAPOBEC3B ELISA (Abcam, San Francisco, California), which reacts with both hAPOBEC3B and mAPOBEC3, according to the manufacturer's instructions. Murine IFN-β was measured by direct ELISA (R&D Systems) according to the manufacturer's instructions.

qRT-PCR
RNA was prepared with the RNeasy Mini Kit (QIAGEN, Valencia, California). 1 μg total RNA was reverse transcribed in a 20 μL volume using oligo-(dT) primers using the First Strand cDNA Synthesis Kit (Roche, Indianapolis, Indiana). A cDNA equivalent of 1 ng RNA was amplified by PCR with gene-specific primers using GAPDH as loading control (mgapdh sense, 5′-TCATGACCACAGTCCATGCC-3′; mgapdh antisense, 5′-TCAGCTCTGGGATGACCTTG-3′; APOBEC3 sense, 5′-ATGGGACCATTCTGTCTGGGA-3′; and APOBEC3 antisense, 5′-TCAAGACACGGGGGTCCAAG-3′). qRT-PCR was carried out using a LightCycler480 SYBR Green I Master kit and a LightCycler480 instrument (Roche) according to the manufacturer's instructions. The $\Delta\Delta C_T$ method was used to calculate the fold change in expression level of APOBEC3 relative to GAPDH and normalized to an untreated calibrator sample.

APOBEC Knockdown and Overexpression
Four separate mouse unique 29-mer shRNA retroviral constructs and a single scrambled shRNA-encoding retroviral construct (OriGene, Rockville, Maryland) were transfected into the GP+E86 ecotropic packaging cell line, and supernatant was used to infect B16TK cells to generate the B16TK (shRNA APOBEC3) and B16TK (scrambled shRNA) populations, respectively. In addition, a single scrambled negative control non-effective shRNA cassette was similarly packaged and used to infect B16TK cells to generate B16TK (scrambled shRNA) cells.

B16TK tumor cell lines were engineered to overexpress hAPOBEC3B or a catalytically inactive form of the protein APOBEC3B MUT, following infection with either pBABE-Hygro APOBEC3B or pBABE-Hygro APOBEC3B MUT, provided by Dr. Reuben Harris[43] (Figure 6A). Forty-eight hours after infection, bulk populations of cells were selected in hygromycin for 2 weeks and used for experiments.

DIP Assay
B16-APOBEC3B MUT-overexpressing or B16-APOBEC3B-overexpressing cells were infected with VSV-GFP at an MOI of 0.01 and incubated for 24 hr. Supernatant was collected, and 1 mL of supernatant was used to infect a fresh monolayer of cells. This was repeated out to five passages. The DIP assay was done by serially diluting passage 1 and passage 5 of VSV-GFP from B16-APOBEC3B MUT or B16-APOBEC3B cells (1:10 to 1:100,000). Fresh BHK cells were seeded the day before in triplicate wells, and diluted viral supernatants were allowed to adsorb for 1 hr. Stock VSV-GFP virus was then added at an MOI of 20 (8×10^5 PFU/well) and incubated for 1 hr. Cells were then washed 3× in PBS, and fresh supernatant was added. Supernatant was collected 24 hr after infection and titered by plaque assay by limiting dilution on BHK cells.

In Vivo Experiments
All *in vivo* studies were approved by the Institutional Animal Care and Use Committee at Mayo Clinic. Mice were challenged subcutaneously with 2×10^5 B16TK melanoma cells in 100 μL PBS (HyClone, Logan, Utah). All virus injections were delivered intratumorally in a 50 μL volume. Tumors were measured 3× per week, and mice were euthanized when tumors reached 1.0 cm in diameter. Mice were sacrificed upon emergence of neurological symptoms or weight loss.

Statistics
Survival curves were analyzed by the log-rank test. Student's t tests, one-way ANOVA, and two-way ANOVA were applied for *in vitro* assays as appropriate. Statistical significance was set at $p < 0.05$ for all experiments.

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