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ORIGINAL ARTICLE

Expressing human interleukin-15 from oncolytic vesicular stomatitis virus improves survival in a murine metastatic colon adenocarcinoma model through the enhancement of anti-tumor immunity

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In this study, we sought to enhance the potency of an oncolytic virus, vesicular stomatitis virus (VSV), by inserting a transgene encoding a highly secreted version of human interleukin-15 (IL-15). IL-15 has shown promise as an immunotherapeutic cytokine, as it is able to enhance both natural killer (NK) and T-cell responses, but it has not yet been tested as a therapeutic transgene in the context of viral oncolysis. The transgene was modified to ensure enhanced secretion of IL-15 from infected cells, leading to strong localized expression from infected CT-26 tumors *in vivo*. This localized expression in the tumor microenvironment led to a clear enhancement to anti-tumoral T-cell responses and enhanced survival, while additional IL-15 administration systemically failed to further enhance the therapy. Overall, the transient localized expression of IL-15 in the tumour by an oncolytic virus was able to induce stronger anti-tumoral immunity in a murine model of colon carcinoma.
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Keywords: oncolytic virus; VSV; IL-15; colon cancer

Introduction

Oncolytic viruses (OVs) have been shown to be a promising new therapeutic in the treatment of cancer and have been found to not only be safe, but have shown good efficacy in both preclinical models and clinical trials.[1–3] Although there are very promising results for several different OVs, there is still room for improvement of these therapeutics, including through the modulation of the anti-tumor immune responses induced during OV therapy.[4]

Vesicular stomatitis virus (VSV) is one such OV that has shown promise in pre-clinical models.[3,5,6] VSV is an enveloped negative-sense, single-stranded RNA virus that is exquisitely sensitive to type I interferon (IFN).[7] VSV is specifically targeted to replicate in the tumor utilizing its sensitivity to IFN along with the observation that tumors are generally hypo-responsive to type I IFN.[5] Our oncolytic VSV vector harbors a deletion of methionine 51 in the matrix protein, allowing for type I IFN to be produced from infected cells.[6,8] Therefore, normal cells will be protected from infection, while the hypo-responsive tumor cells will still remain susceptible. VSV, when delivered systemically, has been shown to improve survival of mice in various tumor models;[9–11] however, it has been difficult to achieve high proportions of long-term survivors with VSV alone. The improvements observed in some cases have been associated with a requirement of adaptive immune responses following oncolytic therapy. Therefore, it may be possible to further improve the efficacy of VSV therapeutics by engineering these viruses to improve on the anti-tumor immune responses induced following OV therapy.

Interleukin-15 (IL-15) is a pleiotropic cytokine that plays a role in numerous innate and adaptive responses to both pathogens and in cancer. IL-15 is a member of the 4-α helix family of cytokines and signals through a receptor complex that includes both the IL-15-specific receptor α chain (IL-15/Rα) and the β γ common chain.[12] IL-15 has been shown to play important roles in numerous immunological processes, including natural killer (NK) cell proliferation and activation,[13,14] CD8 T-cell activation, in particular, memory cell development[15] and is able to rescue tolerized T cells *ex vivo*.[16] As well, the treatment of established tumors with IL-15 improves

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survival[17] and pre-association of IL-15 to its receptor α chain further enhances NK and CD8 T-cell activation, proliferation[18,19] and anti-tumour activity. Finally, human (h)IL-15 has also been added to immunotherapeutic[20] and oncolytic vectors[21] in hopes of improving their therapeutic efficacy.

In this study, the expression of a highly secreted form of optimized human IL-15 (opt.hIL-15) from our oncolytic VSV vector was tested in two models of CT26 colon cancer. hIL-15 has previously been used in the treatment of murine tumor models[17,19] and has also been used in the construction of IL-15 transgenic mice where the over-expression of hIL-15 led to increases in NK cell and CD8 T-cell frequencies.[22] We have shown that the addition of opt.hIL-15 to VSV improved survival of treated mice compared to controls. This increased survival was found to be significantly correlated to the levels of anti-tumor CD8 T-cell immune responses. The addition of exogenous IL-15 cytokine increased the systemic levels of NK cells, but not CD8 T cells before viral treatment. However, this did not further improve on the efficacy of VSV viral treatment alone. These data indicate that inclusion of an IL-15 transgene in an OV can enhance anti-tumoral immunity and improve treatment efficacy.

Materials and methods

Cloning of opt.hIL-15

Opt.hIL-15 was polymerase chain reaction amplified from plasmid containing opt.hIL-15 transcript variant 3 cDNA to add on *Xho*I (5′) and *Nhe*I (3′) cloning sites to facilitate construction of ΔM51 VSV/opt.hIL-15.[23] The recombinant VSV-opt.hIL-15 was rescued as described previously.[24] Briefly, A549 were transfected using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) with 3 μg of the genome plasmid containing hIL-15, along with the helper plasmids VSV-P, VSV-N and VSV-L (0.75, 1.5, 0.75 μg, respectively), and subsequently infected with vaccinia virus expressing T7 polymerase. The rescued virus was passaged on Vero cells, then plaque purified twice, amplified and titered on Vero cells.

Cells and viruses

Vero cells and CT26 colon carcinoma cells were cultured in α-modified Eagle's medium with 10% fetal bovine serum (Gibco, Burlington, ON, Canada), 2 mM L-glutamine, 100 U ml penicillin and 100 μg ml streptomycin. Cells were cultured at 37 °C with 5% CO_2.

The production of virus for use in animal experiments was performed in HEK293 T cells. Infections were carried out at a multiplicity of infection of 0.01 for 24 h. Supernatants were collected and cell debris was cleared by centrifugation and filtration through a 0.2 μM filter. The virus was then pelleted from the cleared supernatant, resuspended and banded on a continuous 5–40% sucrose gradient made in phosphate-buffered saline. The visible band of virus was then collected from the gradient and dialyzed against phosphate-buffered saline, divided into small aliquots and stored at −80 °C.

Enzyme-linked immunosorbent assay

The presence and concentration of hIL-15 was determined by enzyme-linked immunosorbent assay (ELISA) using a human IL-15 ELISA kit as per the manufacturer's recommendations (R&D Systems, Minneapolis, MN). For assessment of the ability of ΔM51 VSV/opt.hIL-15 to direct the secretion of hIL-15, Vero cells were infected at a multiplicity of infection of 5 for 7 h, a time-point where all cells were still viable. Supernatants were collected, centrifuged and analyzed by ELISA. Human IL-15 expression was also assessed *in vivo*. Balb/c mice bearing subcutaneous CT26 tumors were treated intravenously with 5×10^8 pore-forming unit of ΔM51 VSV/GFP, ΔM51 VSV/opt.hIL-15 or ultraviolet-inactivated VSV. Tumors were removed 72 h after infection, homogenized and hIL-15 was analyzed in the tissue homogenate by ELISA.

Tumor studies

Female 4–6-week-old Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA). All animal experiments were conducted with approval from the McMaster University Animal Research Ethics Board. Subcutaneous tumors were established by anesthetizing mice with 5% isofluorane and injecting 5×10^5 CT26 cells subcutaneously in 100 μl saline on the flank of the mouse at a viability of no <95%. Tumor-bearing mice were then treated with three doses of 5×10^8 pore-forming unit of the indicated virus every other day once tumors reached a mean of 10–20 mm^3. Mice were then monitored and tumor volumes were measured and calculated using the following formula: $4/3 \times \pi \times L/2 \times (W/2)^2$, where '*L*' and '*W*' refer to tumor length and width, respectively. For CT26 lung metastases studies, 3×10^5 CT26 were injected intravenously in 200 μl of saline. At 12 days after tumor injection, mice were treated with three doses of 5×10^8 pore-forming unit of the indicated virus every other day. In some groups, mice were pretreated before virus delivery with hIL-15 receptor α chain (hIL-15/Rα). In all, 0.5 μg of hIL-15 (Peprotech, Rocky Hill, NJ) and 1 μg of hIL-15/Rα (Peprotech) were incubated for 30 min at 37 °C to facilitate binding before intraperitoneal injection in 400 μl of saline. Mice were monitored for signs of illness, including hunched posture, ruffled fur, lethargy and difficulty breathing and were euthanized when mice presented with obvious respiratory distress as per our Animal Use Protocol.

Analysis of immune responses

At the indicated time-points, blood was collected from the mice and processed by ACK red blood cell lysis (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 M Na_2EDTA, pH 7.2–7.4). To analyze the effect of delivering exogenous hIL-15/Rα complex, peripheral blood mononuclear cells were subjected to a surface stain with anti-CD3, -CD8 and -DX5 (pan NK). To analyze the tumor- and viral-specific immune responses, peripheral blood mononuclear cells were re-stimulated with peptides from VSV-N (N-MPYLIDFGL-C) or CT-26 AH1 (N-SPSYVYHQF-C) (Peprotech) for 6 h at 37 °C. At 2 h into the stimulation,

brefeldin A was added to the cultures. The peripheral blood mononuclear cells were then stained with anti-CD3, -CD8, -CD4 and -IFN-γ. All samples were analyzed on a FACSCanto (BD Biosciences, Mississauga, ON, Canada). FlowJo (Tree Star, Ashland, OR) software was used to analyze all FACS data. All antibodies and brefeldinA were purchased from BD Biosciences.

Statistical analyses

All data were analyzed using the GraphPad Prism 4.0 and mean ± s.e.m. is shown. In experiments containing fewer than three groups, a Student's *t*-test was performed. When more than three groups were compared, a one-way analysis of variance was performed with a Bonferonni's *post-hoc* test comparing all groups to the control group. For survival curves, a log-rank test was performed to test for significant differences and a linear regression was performed to assess for correlation. In all experiments, a Grubbs' test was performed to identify outliers and these data points were excluded from the analyses.

Results

Recombinant VSV generated to express hIL-15 optimized for enhanced secretion

As hIL-15 is poorly secreted in its natural form, we replaced its natural leader sequence with Igκ immunoglobulin leader sequence, as described previously,[25] to facilitate optimal secretion of hIL-15 from ΔM51 VSV/opt.hIL-15-infected cells. This opt.hIL-15 was polymerase chain reaction amplified and cloned into pVSV-XNΔM51 (Figure 1a). Recombinant VSV/opt.hIL-15 was then rescued using standard techniques. The addition of the opt.hIL-15 transgene did not impact viral replication as the replication kinetics of ΔM51 VSV/opt.hIL-15 were similar to ΔM51 VSV/GFP in a one-step growth curve using Vero cells (Figure 1b).

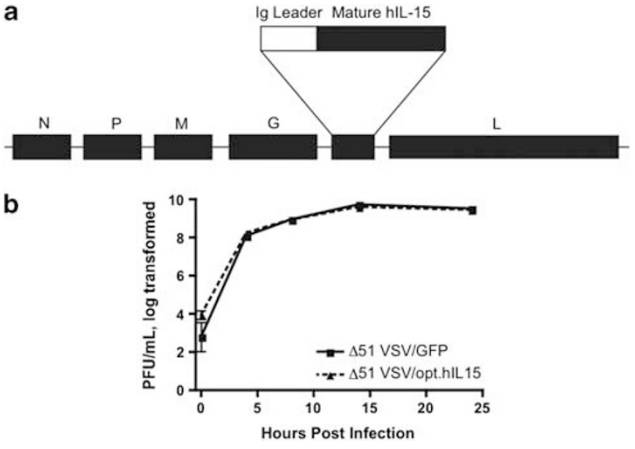

Figure 1 Construction of ΔM51 vesicular stomatitis virus (VSV)/optimized human interleukin-15 (opt.hIL-15). (**a**) Opt.hIL-15 optimized for high-level secretion was cloned into the ΔM51 VSV genome between the viral glycoprotein and polymerase. (**b**) To assess viral growth kinetics, Vero monolayers were infected in duplicate with ΔM51 VSV/GFP and ΔM51 VSV/opt.hIL-15 at a multiplicity of infection of 5. Samples were collected at 0, 4, 8, 12 and 24 h after infection and titered in duplicate on Vero cells using standard plaque assay technique. The mean ± s.e.m. of log-transformed titers are shown.

To determine whether hIL-15 was secreted from ΔM51 VSV/opt.hIL-15-infected cells, Vero cells were infected with ΔM51 VSV/opt.hIL-15 or ΔM51 VSV/GFP at a multiplicity of infection of 5. At 7 h after infection, a time-point when infected cells were still alive, hIL-15 was only present in the supernatants of ΔM51 VSV/opt.hIL-15-infected cells ($P<0.001$; Figure 2a). To ensure that hIL-15 was also expressed *in vivo*, mice bearing subcutaneous CT26 tumors were infected with ΔM51 VSV/GFP and ΔM51 VSV/opt.hIL-15, and subsequently tested for hIL-15 levels. Balb/c mice bearing 10-day-old CT26 subcutaneous tumors were treated intravenously with ΔM51 VSV/GFP, ΔM51 VSV/opt.hIL-15 or ultraviolet-inactivated ΔM51 VSV/GFP. At 48 h after infection, tumors were removed and hIL-15 levels were measured in tumor homogenates by ELISA. Only tumors treated with ΔM51 VSV/opt.hIL-15 were found to have high levels of hIL-15 present (Figure 2b).

The addition of hIL-15 to oncolytic VSV did not improve VSV efficacy in a subcutaneous CT26 model

To determine whether the addition of hIL-15 to our oncolytic VSV vector improved on treatment outcomes, we started by testing ΔM51 VSV/opt.hIL-15 in a subcutaneous CT26 colon carcinoma model. Mice were engrafted with CT26 on the flank, and when the mean tumor volumes reached 50 mm^3, they were treated intravenously with ΔM51 VSV/GFP or ΔM51 VSV/opt.hIL-15 given every other day for a total of three treatments. Unfortunately, in this model the parental GFP-expressing virus cured 80% of the mice not allowing us to detect an improvement in outcome when using the ΔM51 VSV/opt.hIL-15 vector, which also cured 80% of the mice (Figure 3).

ΔM51 VSV/opt.hIL-15 performs better than ΔM51 VSV/GFP in CT26 lung mets model

Both ΔM51 VSV/GFP and ΔM51 VSV/opt.hIL-15 performed very well in the subcutaneous CT26 model, hence we moved into the more challenging model of CT26 lung metastases. To this end, Balb/c mice bearing 12-day-old CT26 lung metastases were treated with three doses of ΔM51 VSV/GFP or ΔM51 VSV/opt.hIL-15 intravenously every other day (Figure 4a). In this version of the CT26 tumor model, we have been unable to cure mice using the parental virus and once again all of the ΔM51 VSV/GFP-treated animals performed better than those treated with phosphate-buffered saline ($P<0.0001$, log-rank test), but ultimately succumbed to their tumors. We were however able to cure 50% (5 of 10) of the ΔM51 VSV/opt.hIL-15-treated animals (Figure 4b), a significant improvement over the GFP-expressing parental virus ($P=0.0022$, log-rank test). To ensure that the differences observed were not due to variability in tumor engraftment, tumor burden as assessed 12 days post-engraftment, which is the time of treatment. In Supplementary

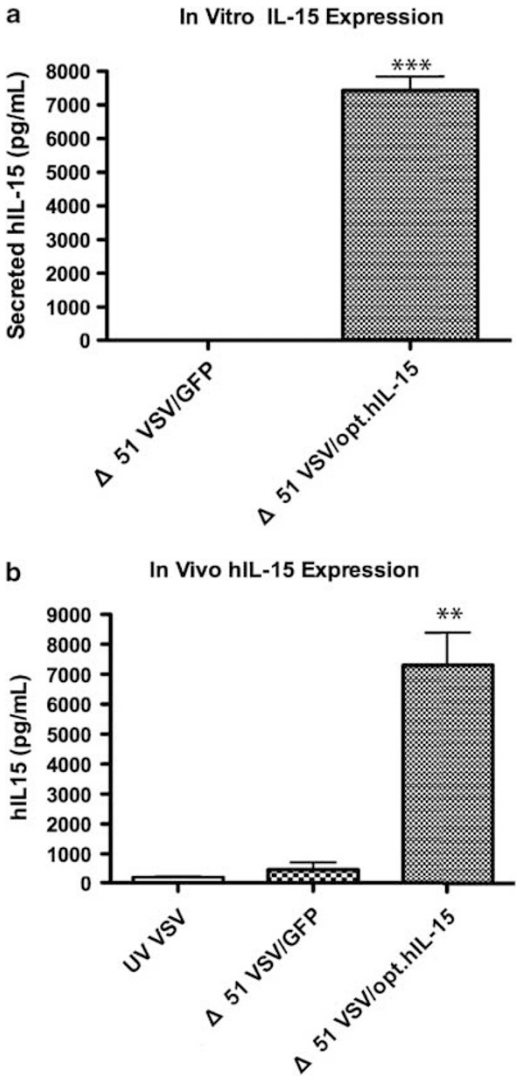

Figure 2 Expression of human interleukin-15 (hIL-15) from ΔM51 vesicular stomatitis virus (VSV)/optimized hIL-15 (opt.hIL-15) *in vitro* and *in vivo*. (**a**) Vero cells were infected in duplicate with ΔM51 VSV/green fluorescent protein (GFP) or ΔM51 VSV/opt.hIL-15 at a multiplicity of infection of 5 in the minimal media for 45 min, followed by another 6.25 h for a total of 7 h, a time-point where all cells are still viable. Supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) and hIL-15 was only detected in ΔM51 VSV/opt.hIL-15-infected cells. Data are mean ± s.e.m.; $n=2$; ***$P=0.003$. (**b**) hIL-15 expression was assessed *in vivo* in a subcutaneous CT26 tumor model. Tumor-bearing Balb/c mice were treated with 5×10^8 pore-forming unit (PFU) of ultraviolet (UV) VSV ($n=2$), ΔM51 VSV/GFP ($n=3$) or ΔM51 VSV/opt.hIL-15 ($n=3$). At 72 h after treatment, tumors were removed and hIL-15 was only detected by ELISA in homogenates from ΔM51 VSV/opt.hIL-15-treated tumors. Data are mean ± s.e.m.; **$P<0.01$.

Figure 1A, it can be seen that tumor burden was equal in all mice analyzed. We then investigated CD8+ T-cell responses vs the tumor (AH1 endogenous MuLV gp70 epitope expressed in CT26 N-SPSYVYHQF-C)[26] and the OV (nucleocapsid epitope N-MPYLIDFGL-C)[27] 9 days post-infection. At this time-point, we saw that inclusion of the opt.hIL-15 transgene greatly enhanced anti-tumoral

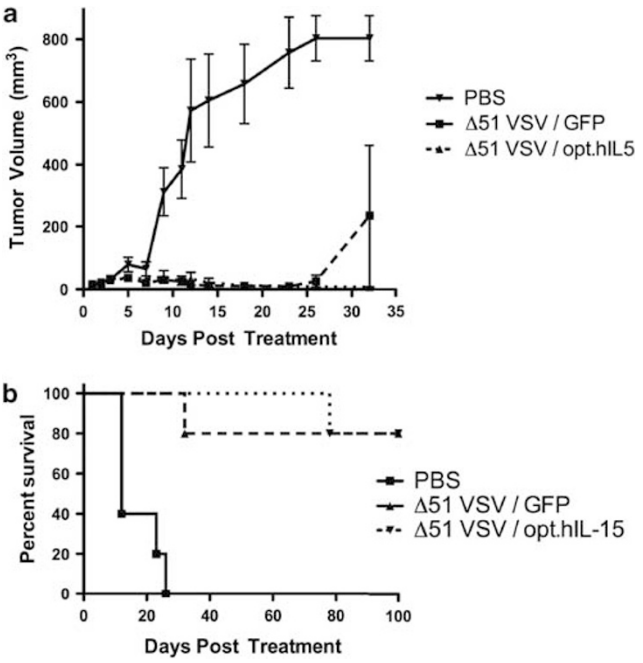

Figure 3 Treatment of subcutaneous CT26 tumors with ΔM51 vesicular stomatitis virus (VSV)/optimized human interleukin-15 (opt.hIL-15). Mice bearing established tumors were treated with phosphate-buffered saline (PBS) ($n=5$) ΔM51 VSV/green fluorescent protein (GFP) ($n=5$) or ΔM51 VSV/opt.hIL-15 ($n=5$). Once tumors reached a mean of ~10–20 mm^3, mice were treated with 5×10^8 pore-forming unit (PFU) of the indicated virus intravenously every other day for a total of three treatments. (**a**) Tumor volume (calculated as an ellipse, where volume $=4/3\times\pi\times L/2\times(W/2)^2$) is shown as mean ± s.e.m. and (**b**) survival were monitored and are depicted above.

immunity (Figure 4c; $P<0.05$; Student's *t*-test), while not modifying the immune response vs the vector (Figure 4d). Thus, transient expression of a secreted form of IL-15 from within the infected tumor enabled the generation of anti-tumoral immunity, leading to eradication of CT26 lung mets and long-term survivors.

Pretreatment with IL-15/Rα increased peripheral NK counts

We reasoned that as hIL-15 expression by ΔM51 VSV/opt.hIL-15 was very transient only being produced during viral replication, and some effects of IL-15 take as much as a week to manifest,[18,28] we decided to pretreat tumor-bearing animals with recombinant hIL-15 + hIL-15/Rα (Figure 5a). It has been previously demonstrated that the soluble cytokine is more bioavailable and effective *in vivo* when complexed with the soluble receptor α chain,[18,29] and that this hIL-15/Rα complex expands NK and CD8 memory T-cell numbers.[18] Thus, we pretreated mice with hIL-15/Rα before ΔM51 VSV/opt.hIL-15 or ΔM51 VSV/GFP treatments in an attempt to improve the immune response generated by oncolytic VSV treatment. Mice treated with hIL-15/Rα were assessed before viral treatment to determine the effects of hIL-15/Rα on peripheral NK and CD8 T cells. Mice that received hIL-15/Rα were found to have a significantly higher

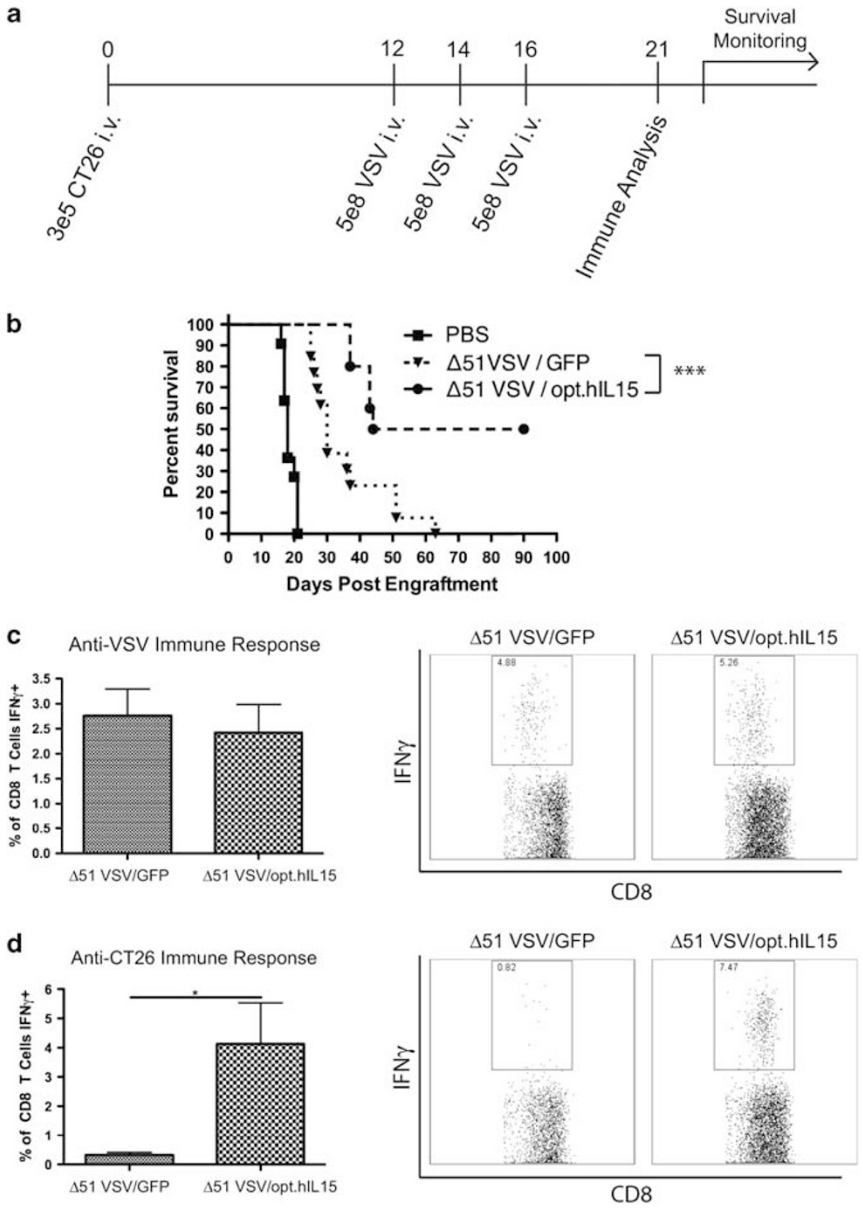

Figure 4 Expression of human interleukin-15 (hIL-15) from vesicular stomatitis virus (VSV) significantly improves oncolytic virus (OV) therapy. (**a**) Outline of Experimental design. (**b**) Survival curve of treated mice; phosphate-buffered saline (PBS), $n=7$; ΔM51 VSV/green fluorescent protein (GFP), $n=10$; ΔM51 VSV/optimized human interleukin-15 (opt.hIL-15), $n=10$; ***$P<0.0001$. On day 21, 9 days after starting OV treatment, blood was collected to assess the anti-tumor and anti-viral immune responses. Peripheral blood mononuclear cells (PBMCs) were stimulated *ex vivo* with the appropriate peptides and the analyzed for activation by flow cytometry using interferon-γ (IFNγ). Anti-tumor and anti-viral immune responses are represented in (**c**) and (**d**), respectively. Data are mean ± s.e.m. combined from 2 to 3 independent experiments; ΔM51 VSV/GFP, $n=13$; ΔM51 VSV/opt.hIL-15, $n=10$; *$P<0.05$.

percentage of peripheral DX5 + NK cells ($P<0.0001$; Figure 5b). However, the hIL-15/Rα did not affect their activation level as assessed by surface expression of the activation marker CD69 (Figure 5c). We did not find any difference in the peripheral CD8 T-cell frequencies in mice treated with hIL-15/Rα (Figure 5d).

Pretreatment with IL-15/Rα failed to enhance anti-tumoral immunity or survival

The addition of hIL-15/Rα cytokine complex to either VSV therapy did not improve on the anti-tumor immune response elicited by either virus alone (Figure 6a). Although the anti-tumor response in the ΔM51 VSV/GFP + hIL-15/Rα-treated mice was increased compared with ΔM51 VSV/GFP alone, this result did not reach significance as assessed by a one-way analysis of variance. Surprisingly, the addition of hIL-15/Rα pretreatment before oncolytic viral delivery led to significantly reduced anti-viral CD8 T-cell responses ($P=0.0029$, Student's *t*-test) (Figure 6bii). The addition of this soluble cytokine pretreatment failed to modify the survival outcomes generated by either virus (Figure 6c), indicating that

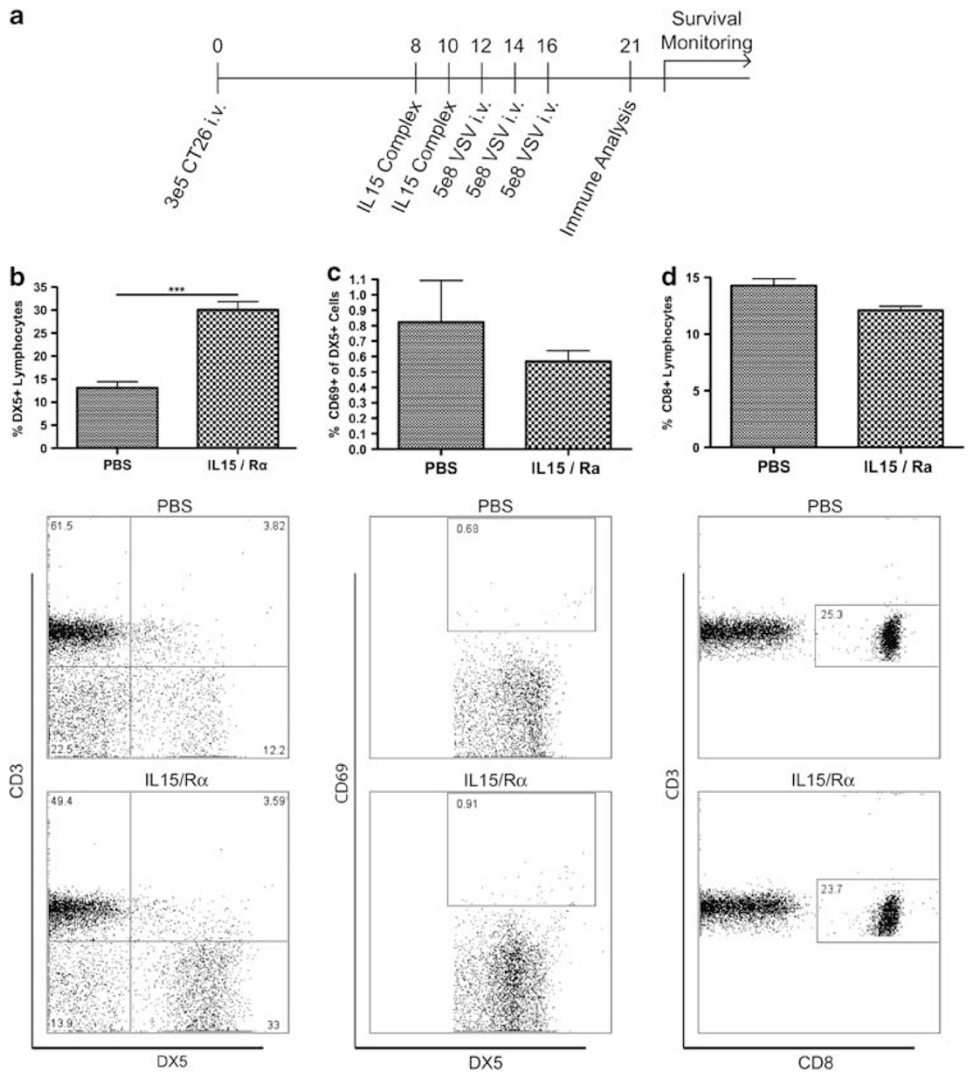

Figure 5 Pretreatment of CT26 lung met tumors with human interleukin-15 receptor α chain (hIL-15/Rα) increases peripheral natural killer (NK) cell frequencies. (**a**) Experimental outline for the treatment of mice with hIL-15/Rα before oncolytic virus (OV) delivery. NK cells (**b**), activated NK cells (**c**) and CD8 T cells (**d**) were analyzed in peripheral blood mononuclear cells (PBMCs) before viral delivery to assess the impact of hIL-15/Rα on the tumor-bearing mice. Blood was collected 8 h before viral delivery and analyzed by the flow cytometry. Data are mean ± s.e.m. combined from two independent experiments; phosphate-buffered saline (PBS), $n=8$; hIL-15/Rα, $n=33$; ***$P<0.0001$.

while the expression of IL-15 from the OV is quite transient, local production is more useful in this setting than is systemic pretreatment in enhancing the therapy. Ultimately, there was a significant correlation between the magnitude of anti-tumoral CD8 T-cell response and survival (Figure 7), indicating that the enhancement of this response by virally encoded IL-15 contributed top therapeutic enhancement.

Discussion

Oncolytic viral therapy is an emerging strategy for the treatment of cancer. Although the virus infects and kills tumor cells, it is unlikely that the virus alone will be able to destroy entire tumors through viral oncolysis on its own as there are many barriers to viral spread through solid tumors *in vivo*.[30] Therefore, we feel that it would be beneficial to harness the host immune response in an effort to augment the therapeutic effect of OVs. Transgenes have been added to oncolytic VSV vectors in an attempt to improve on efficacy through modulation of the immune response, with varying impacts on therapeutic outcome.[9,31–34] A number of groups have added cytokines, including IL-4, IL-12 and IL-23, into VSV in an effort to polarize the adaptive immune response to improve on anti-tumor immune responses. Although there was some improvement over parental control viruses, the immune responses were either not measured

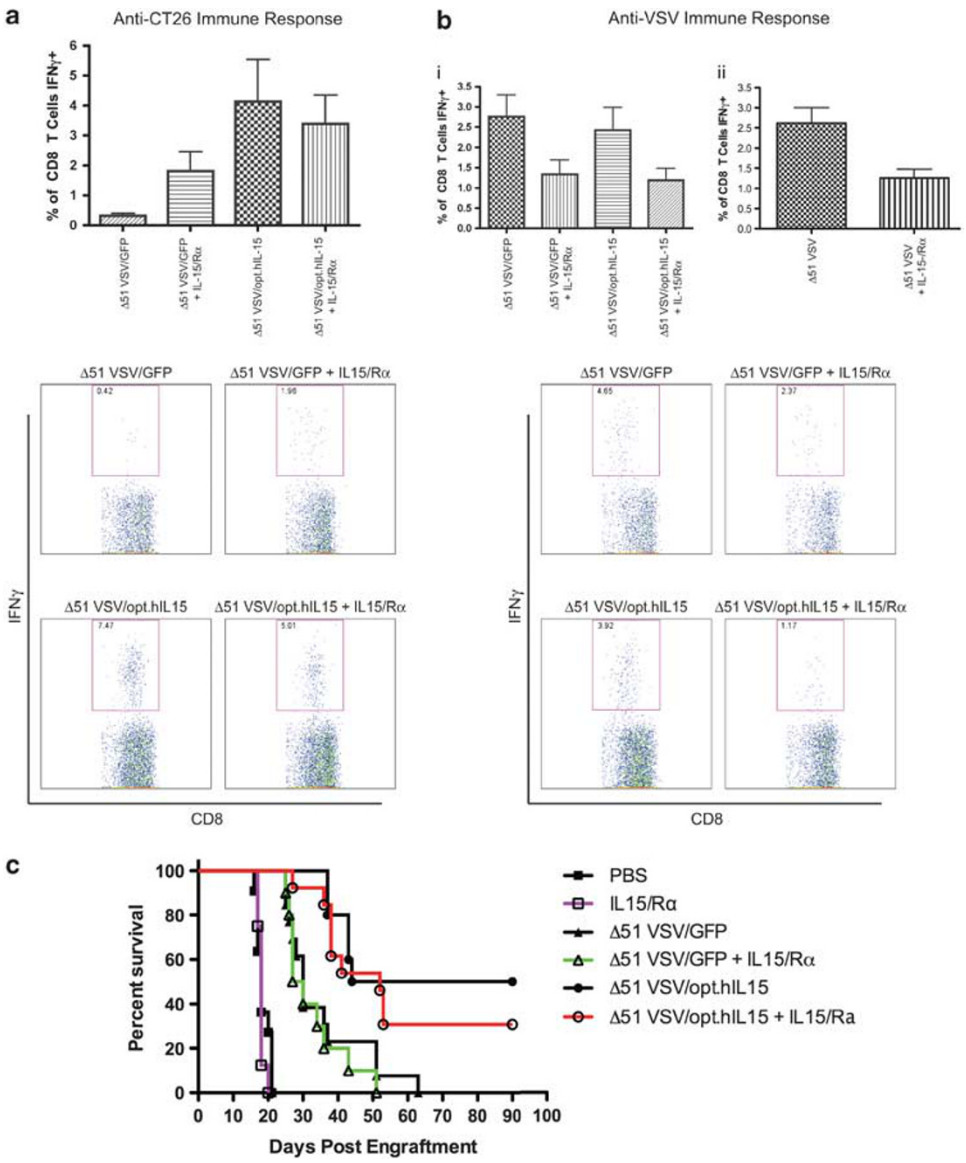

Figure 6 Pretreatment with human interleukin-15 receptor α chain (hIL-15/Rα) does not improve on treatment with either virus alone. (**a**) Blood was collected 9 days after the start of oncolytic virus (OV) treatment to assess the anti-tumor and anti-viral immune responses. Peripheral blood mononuclear cells (PBMCs) were stimulated *ex vivo* with the appropriate peptides and analyzed for activation by flow cytometry using interferon-γ (IFNγ). Anti-tumor and anti-viral immune responses are represented in (**a**) and (**b**), respectively. Data are mean ± s.e.m. combined from 2 to 3 independent experiments; ΔM51 vesicular stomatitis virus (VSV)/green fluorescent protein (GFP), $n=13$; ΔM51 VSV/GFP + hIL-15/Rα, $n=11$; ΔM51 VSV/opt.hIL-15, $n=10$; ΔM51 VSV/opt.hIL-15 + hIL-15/Rα, $n=13$; $^{*}P<0.05$; $^{**}P<0.01$. (**c**) Kaplan–Meier survival curve of the indicated treatments. There are three pairs of survival curves (phosphate-buffered saline (PBS), ΔM51 VSV/GFP and ΔM51 VSV/opt.hIL-15; +/−hIL-15/Rα). Each of the ΔM51 VSV/GFP and ΔM51 VSV/opt.hIL-15 pairs are significantly different from PBS and each other. There was no significant difference between groups within a pairing (log-rank test; ΔM51 VSV/opt.hIL-15 vs PBS, $P<0.0001$, ΔM51 VSV/opt.hIL-15 vs hIL-15/Rα, $P<0.0001$, ΔM51 VSV/opt.hIL-15 vs ΔM51 VSV/GFP, $P=0.0017$, ΔM51 VSV/opt.hIL-15 vs ΔM51 VSV/GFP + hIL-15/Rα, $P=0.0006$, ΔM51 VSV/opt.hIL-15 vs ΔM51 VSV/opt.hIL-15 + hIL-15/Rα, $P=$ NS).

in these studies or showed no, or only very minor, increases to immune responses, leaving room for further improvement.[31–34] Alternatively, co-stimulatory molecules have been expressed from the OV VSV vector again with no improvement over control viruses in survival or induction of immune responses.[9] To improve on the oncolytic efficacy of VSV, we have added a highly secreted form of human IL-15 to our oncolytic VSV vector for its immuno-stimulatory effects on innate and acquired immunity.[12,15,16,35]

The addition of the hIL-15 transgene did not impair viral replication *in vitro* and led to high levels of secretion of hIL-15 from virally infected cells both *in vitro* and *in vivo*. We predicted that expression of IL-15 from our virus would enhance therapy, as it has been shown previously that IL-15 expressed from transfected tumors

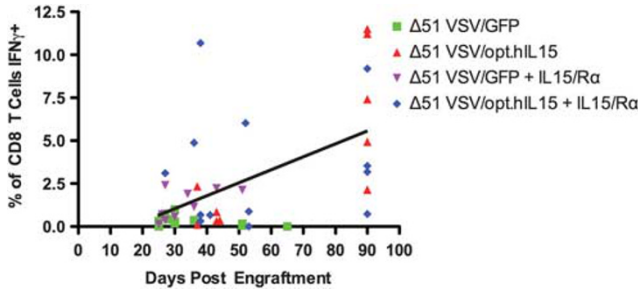

Figure 7 Survival correlates with anti-tumor immune response. The magnitude of the anti-tumor immune response 9 days after the start of oncolytic virus (OV) treatment was plotted against time to end point and is presented above. Data points represent the individual mice that survived to immune analysis. There is a significant correlation between survival and anti-tumor immune response, $P<0.0001$, $r^2=0.317$.

in vivo impacts on tumor growth and subsequent immune responses.[17,25,36]

We failed to see an enhancement to therapy when we first tested our viruses in the subcutaneous version of the CT26 model as the parental GFP-expressing virus was already very efficacious in this setting. We suspect that this stems from guaranteed delivery of virus to the one and only tumor target, followed by the significant impact on vascular function and massive hypoxic death induced in this setting.[37,38] Therefore, we moved into the more challenging model of CT26 lung metastases where we are unable to cure mice of their tumors with ΔM51 VSV/GFP, possibly due to a failure to infect every tumor nodule by the intravenous route of delivery accompanied by a reduced induction of hypoxia of these smaller tumor nodules in the lung.

As expected, mice bearing CT26 lung metastases could not be cured of their disease when treated with ΔM51 VSV/GFP. However, high levels of expression of hIL-15 during ΔM51 VSV/opt.hIL-15 treatment was able to improve therapy, leading to a significant improvement in survival and ultimately leading to 50% of ΔM51 VSV/opt.hIL-15-treated mice being cured. We suspect that the improvement seen in the ΔM51 VSV/opt.hIL-15-treated mice is due to the enhanced anti-tumor CD8 T-cell responses induced as there was a correlation between the magnitude of this response and survival across all groups.

Treatment of the tumor-bearing mice with recombinant IL-15/Ra complex led to a systemic increase in NK cell numbers as expected. This failed to directly impact tumor growth as a monotherapy and also failed to enhance oncolytic viral therapy. However, delivery of IL-15/Ra cytokine complex before oncolytic viral therapy led to a reduction in the anti-viral CD8 T-cell response. We speculate that this is indicative of a reduced load of viral antigen in these mice due to an attenuation of viral replication mediated by an enhanced NK cell response. Thus, the cytokine pretreatment may have provided a minor enhancement to anti-tumoral responses, while also enhancing innate anti-viral responses largely canceling any impact on therapy. Concurrent, or even subsequent, cytokine administration may provide anti-tumoral benefits without attenuating viral oncolysis.

In summary, the addition of a highly secreted form of IL-15 to an oncolytic VSV vector led to enhanced therapeutic outcomes under circumstances where viral oncolysis alone was unable to mediate durable cures. This enhanced efficacy was correlated with increased anti-tumoral immune responses following intravenous viral administration.

Conflict of interest

The authors declare no conflict of interest.

References

1 Cattaneo R, Miest T, Shashkova EV, Barry MA. Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. *Nat Rev Microbiol* 2008; **6**: 529–540.
2 Breitbach CJ, Reid T, Burke J, Bell JC, Kirn DH. Navigating the clinical development landscape for oncolytic viruses and other cancer therapeutics: no shortcuts on the road to approval. *Cytokine Growth Factor Rev* 2010; **21**: 85–89.
3 Parato KA, Senger D, Forsyth PAJ, Bell JC. Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* 2005; **5**: 965–976.
4 Prestwich RJ, Harrington KJ, Pandha HS, Vile RG, Melcher AA, Errington F. Oncolytic viruses: a novel form of immunotherapy. *Expert Rev Anticancer Ther* 2008; **8**: 1581–1588.
5 Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N *et al*. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* 2000; **6**: 821–825.
6 Stojdl D, Lichty B, tenOever B, Paterson J, Power A, Knowles S *et al*. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 2003; **4**: 263–275.
7 Lichty B, Power A, Stojdl D, Bell J. Vesicular stomatitis virus: re-inventing the bullet. *Trends Mol Med* 2004; **10**: 210–216.
8 von Kobbe C, van Deursen J, Rodrigues J, Sitterlin D, Bachi A, Wu X *et al*. Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Mol Cell* 2000; **6**: 1243–1252.
9 Galivo F, Diaz RM, Thanarajasingam U, Jevremovic D, Wongthida P, Thompson J *et al*. Interference of CD40L-mediated tumor immunotherapy by oncolytic vesicular stomatitis virus. *Hum Gene Ther* 2010; **21**: 439–450.
10 Lun X, Senger DL, Alain T, Oprea A, Parato K, Stojdl D *et al*. Effects of intravenously administered recombinant vesicular stomatitis virus (VSV(deltaM51)) on multifocal and invasive gliomas. *J Natl Cancer Inst* 2006; **98**: 1546–1557.
11 Ebert O, Harbaran S, Shinozaki K, Woo S. Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. *Cancer Gene Ther* 2005; **12**: 350–358.

12 Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol* 1999; **17**: 19–49.
13 Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, Paxton R *et al*. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med* 1994; **180**: 1395–1403.
14 Ohteki T. Critical role for IL-15 in innate immunity. *Curr Mol Med* 2002; **2**: 371–380.
15 Kokaji AI, Hockley DL, Kane KP. IL-15 transpresentation augments CD8+ T cell activation and is required for optimal recall responses by central memory CD8+ T cells. *J Immunol* 2008; **180**: 4391–4401.
16 Teague RM, Sather BD, Sacks JA, Huang MZ, Dossett ML, Morimoto J *et al*. Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. *Nat Med* 2006; **12**: 335–341.
17 Ugen KE, Kutzler MA, Marrero B, Westover J, Coppola D, Weiner DB *et al*. Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-15-expressing plasmid followed by *in vivo* electroporation. *Cancer Gene Ther* 2006; **13**: 969–974.
18 Dubois S, Patel HJ, Zhang M, Waldmann TA, Müller JR. Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. *J Immunol* 2008; **180**: 2099–2106.
19 Wu Z, Xu Y. IL-15R alpha-IgG1-Fc enhances IL-2 and IL-15 anti-tumor action through NK and CD8+ T cells proliferation and activation. *J Mol Cell Biol* 2010; **2**: 217–222.
20 Yiang G-T, Harn H-J, Yu Y-L, Hu S-C, Hung Y-T, Hsieh C-J *et al*. Immunotherapy: rAAV2 expressing interleukin-15 inhibits HeLa cell tumor growth in mice. *J Biomed Sci* 2009; **16**: 47.
21 Liu J, Wennier S, Reinhard M, Roy E, MacNeill A, McFadden G. Myxoma virus expressing interleukin-15 fails to cause lethal myxomatosis in European rabbits. *J Virol* 2009; **83**: 5933–5938.
22 Marks-Konczalik J, Dubois S, Losi JM, Sabzevari H, Yamada N, Feigenbaum L *et al*. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc Natl Acad Sci USA* 2000; **97**: 11445–11450.
23 Davies E, Reid S, Medina MF, Lichty B, Ashkar AA. IL-15 has innate anti-tumor activity independent of NK and CD8 T cells. *J Leukoc Biol* 2010; **88**: 529–536.
24 Lawson N, Stillman E, Whitt M, Rose J. Recombinant vesicular stomatitis viruses from DNA. *Proc Natl Acad Sci USA* 1995; **92**: 4477–4481.
25 Araki A, Hazama S, Yoshimura K, Yoshino S, Iizuka N, Oka M. Tumor secreting high levels of IL-15 induces specific immunity to low immunogenic colon adenocarcinoma via CD8+ T cells. *Int J Mol Med* 2004; **14**: 571–576.
26 Casares N, Lasarte JJ, de Cerio AL, Sarobe P, Ruiz M, Melero I *et al*. Immunization with a tumor-associated CTL epitope plus a tumor-related or unrelated Th1 helper peptide elicits protective CTL immunity. *Eur J Immunol* 2001; **31**: 1780–1789.
27 Ramsburg EA, Publicover JM, Coppock D, Rose JK. Requirement for CD4 T cell help in maintenance of memory CD8 T cell responses is epitope dependent. *J Immunol* 2007; **178**: 6350–6358.
28 Bessard A, Solé V, Bouchaud G, Quéméner A, Jacques Y. High antitumor activity of RLI, an interleukin-15 (IL-15)-IL-15 receptor alpha fusion protein, in metastatic melanoma and colorectal cancer. *Mol Cancer Therap* 2009; **8**: 2736–2745.
29 Stoklasek TA, Schluns KS, Lefrançois L. Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity *in vivo*. *J Immunol* 2006; **177**: 6072–6080.
30 Smith E, Breznik J, Lichty B. Strategies to enhance viral penetration of solid tumors. *Hum Gene Ther* 2011; **22**: 1053–1060.
31 Miller JM, Bidula SM, Jensen TM, Reiss CS. Vesicular stomatitis virus modified with single chain IL-23 exhibits oncolytic activity against tumor cells *in vitro* and *in vivo*. *Int J Infereron Cytokine Mediat Res* 2010; **2010**: 63–72.
32 Shin E, Wanna G, Choi B, Aguila D, Ebert O, Genden E *et al*. Interleukin-12 expression enhances vesicular stomatitis virus oncolytic therapy in murine squamous cell carcinoma. *Laryngoscope* 2007; **117**: 210–214.
33 Fernandez M, Porosnicu M, Markovic D, Barber GN. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. *J Virol* 2002; **76**: 895–904.
34 Willmon CL, Saloura V, Fridlender ZG, Wongthida P, Diaz RM, Thompson J *et al*. Expression of IFN-beta enhances both efficacy and safety of oncolytic vesicular stomatitis virus for therapy of mesothelioma. *Cancer Res* 2009; **69**: 7713–7720.
35 Sato N, Patel HJ, Waldmann TA, Tagaya Y. The IL-15/IL-15Ralpha on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells. *Proc Natl Acad Sci USA* 2007; **104**: 588–593.
36 Tasaki K, Yoshida Y, Miyauchi M, Maeda T, Takenaga K, Kouzu T *et al*. Transduction of murine colon carcinoma cells with interleukin-15 gene induces antitumor effects in immunocompetent and immunocompromised hosts. *Cancer Gene Ther* 2000; **7**: 255–261.
37 Breitbach CJ, De Silva NS, Falls TJ, Aladl U, Evgin L, Paterson J *et al*. Targeting tumor vasculature with an oncolytic virus. *Mol Ther* 2011; **19**: 886–894.
38 Breitbach C, Paterson J, Lemay C, Falls T, McGuire A, Parato K *et al*. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Mol Ther* 2007; **15**: 1686–1693.

Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)