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Prime-boost using Separate Oncolytic Viruses in Combination with Checkpoint Blockade Improves Anti-tumor Therapy

Elizabeth Ilett [1,2*], Timothy Kottke[1*], Jill Thompson[1], Karishma Rajani[1], Shane Zaidi[1,3], Laura Evgin[1], Matt Coffey[4], Christy Ralph[2], Rosa Diaz[5], Hardev Pandha[6], Kevin Harrington[3], Peter Selby[2], Richard Bram[7], Alan Melcher[2**], Richard Vile[1,2,7,**]

[1]Department of Molecular Medicine, Mayo Clinic, Rochester, MN 55905; [2]Leeds Institute of Cancer and Pathology, St. James' University Hospital, Leeds, UK; [3]The Institute of Cancer Research, 237 Fulham Road, London, SW3; [4]Oncolytics Biotech Incorporated, Calgary, Canada; [5]Vyriad, 221 1st Avenue SW, Suite 102, Rochester, MN; [6]University of Surrey, Guildford, UK; [7]Department of Immunology, Mayo Clinic, Rochester, MN 55905

[*] These authors contributed equally to this work; [**]Joint senior authors

Running Title: Prime-boost with different immunovirotherapies

Correspondence should be addressed to R.V. (vile.richard@mayo.edu) / Mayo Clinic, Guggenheim 18, 200 First Street SW, Rochester, MN 55905 / Phone: 507-284-3178 / FAX: 507-266-2122

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ABSTRACT

The anti-tumor effects associated with oncolytic virus therapy are mediated significantly through immune-mediated mechanisms which depends both on the type of virus and the route of delivery. Here, we show that intra-tumoral (i.t.) oncolysis by Reovirus induced the priming of a CD8+, Th1-type anti-tumor response. In contrast, systemically delivered VSV expressing a cDNA library of melanoma antigens (VSV-ASMEL) promoted a potent anti-tumor CD4+ Th17 response. Therefore, we hypothesised that combining the Reovirus-induced CD8+ T cell response, with the VSV-ASMEL CD4+ Th17 helper response, would produce enhanced anti-tumor activity. Consistent with this, priming with i.t. Reovirus, followed by an intra-venous VSV-ASMEL Th17 boost, significantly improved survival of mice bearing established subcutaneous (s.c.) B16 melanoma tumors. We also show that combination of either therapy alone with anti-PD-1 immune checkpoint blockade augmented both the Th1 response induced by systemically delivered Reovirus in combination with GM-CSF, and also the Th17 response induced by VSV-ASMEL. Significantly, anti-PD-1 also uncovered an anti-tumor Th1 response following VSV-ASMEL treatment that was not seen in the absence of checkpoint blockade. Finally, the combination of all three treatments (priming with systemically delivered Reovirus, followed by double boosting with systemic VSV-ASMEL and anti-PD-1) significantly enhanced survival, with long-term cures, compared to any individual, or double, combination therapies, associated with strong Th1 and Th17 responses to tumor antigens. Our data show that it is possible to generate fully systemic, highly effective anti-tumor immunovirotherapy by combining oncolytic viruses, along with immune checkpoint blockade, to induce complimentary mechanisms of anti-tumor immune responses.

INTRODUCTION

Oncolytic viruses (OV) are naturally occurring or genetically modified viruses that target tumor cells while largely sparing normal cells, dependent on a number of different mechanisms[1-3]. In this respect, it is now clear that the anti-tumor activity of these agents is, at least in part, dependent on immune responses raised to both the virus and tumor associated antigens released during the process of immunogenic tumor cell killing[4-6]. This concept is underscored by the recent FDA approval of talimogene laherparepvec (T-Vec, an HSV encoding GM-CSF), confirming the potential of OV as immunovirotherapeutic agents for cancer treatment.

The exact immune mechanisms through which OV induce anti-tumor responses depend upon multiple factors, including the type of virus used, the route of administration of the virus and the transgenes encoded. In this respect, we, and others, have shown that immune responses mediated by a range of OV encoding either tumor antigens (Ag), cytokines and/or co-stimulatory molecules, are effective in controlling tumor growth in pre-clinical models[7-10], with several of these agents being tested in clinical trials[11-13]. For example, Reovirus replication occurs in tumor cells with defective anti-viral PKR signalling resulting in oncolysis[14] but also generates potent anti-tumor immune responses, both innate and adaptive, which are highly important for tumor regression[15-18]. A number of Phase1/2 clinical trials of Reovirus serotype 3 Dearing (Oncolytics Biotech) have demonstrated it to be safe[19-21]. We have shown that, when delivered intra-tumorally (i.t.), Reovirus generates a Th1 anti-tumor response[22], which also correlates with our previous observations that Reovirus activates CTL[16, 17]. However, when delivered systemically in combination with GM-CSF, we showed that the anti-tumor immune response is also heavily dependent on innate mechanisms[23].

We have also developed an effective systemic immunovirotherapy against established tumors using Vesicular Stomatitis Virus (VSV) expressing either single, or multiple, tumor antigens. In particular, i.v. delivery of VSV expressing a cDNA library derived from either normal, or tumor, cells primed specific anti-tumor immune responses in models of melanoma, prostate cancer and brain tumors [10, 24, 25]. Interestingly, in all of these models, the anti-tumor immune

responses primed against tumor by expression of multiple tumor antigens encoded by the virally-expressed cDNA were dependent upon CD4+ Th17 cells[10, 24].

Normal immune responses to infection or injury are modulated at checkpoints to prevent them leading to uncontrolled immune cell proliferation and auto-immune disease. For example, Programmed cell death-1 (PD-1) is a receptor found on immune cells including T cells, B cells and monocytes[26] binding of which to one of its ligands, PD-L1 or PD-L2, inhibits immune cell activation. Expression of PD-L1 is found on many types of tumor[27] resulting in the ability of tumor cells to evade immune responses against them. Checkpoint inhibitors are antibodies which target these negative immune regulators or their ligands, including PD1/PD-L1, and have shown great promise as immune therapy for the treatment of at least a proportion of patients with melanoma and other cancers[28-30]. These data clearly suggest that these checkpoint inhibitors relieve repression of (weak) T cell responses against self tumor associated antigens, as well as against pathogens associated with infection and injury. Therefore, given that OV can prime anti-tumor T cell responses, several groups have proposed that the combination of OV therapy and checkpoint inhibition will be of immunotherapeutic value [22, 25, 31, 32].

In the current study, we hypothesised that a combination of two different forms of oncolytic viroimmunotherapy, which stimulate alternative CD8+ Th1 and CD4 helper Th17 mechanisms of anti-tumor immunity, could combine co-operatively or synergistically, along with immune checkpoint blockade, to enhance anti-tumor therapy. We show here a Th1/Th17 prime-boost treatment with two different viruses, both delivered systemically, was significantly more effective in controlling tumors than either single immunovirotherapy treatment alone. Further addition of immune checkpoint blockade with anti-PD-1, generated long term cures in mice treated with the triple combination therapy under experimental conditions where double therapies alone did not.

RESULTS

Reovirus primes a Th1 response, while VSV-cDNA primes a Th17 response against B16 melanoma.

Pooled cultures of splenocytes and lymph node (S/LN) cells from mice treated intra-tumorally (i.t.) with Reovirus, but not with PBS, secreted IFN-γ in response to B16 tumor cell lysates (**Fig.1A**). They also generated a Th1 recall response to a combination of the three VSV-expressed self antigens (VSV-NRAS, VSV-CYT-c, VSV-TYRP1), which we have previously described as rejection antigens for B16 tumors following treatment with a VSV-ASMEL cDNA library [24] (**Fig.1A**, VSV-combo). However, no IL-17 (< 50 pg/ml, data not shown) was detected as a result of i.t. Reovirus treatment indicating the absence of a Th17 immune response.

In this s.c. B16 model, we have shown that single agent Reovirus delivered i.t., but not intravenously (i.v.), was an effective anti-tumor therapy[33]. In contrast, established B16 tumors could be treated with a systemically delivered VSV-cDNA library (VSV-ASMEL – Altered Self Melanoma Eptiope Library)[10]. The anti-tumor response was dependent on CD4+ T cells and associated with a Th17 response against at least three dominant tumor Ag, NRAS, CYT-c and TYRP1[24]. Consistent with those data, splenocyte/LN cells from VSV-ASMEL-treated mice secreted IL-17 in response to either B16 lysate or to the VSV-combo (**Fig.1B**). In contrast, no IFN-γ was secreted on re-stimulation with B16 lysate or the VSV-combo (< 50 pg/ml, data not shown), indicating no significant detectable Th1-type response to this treatment. Therefore, i.t. Reovirus (Th1), and i.v. VSV-cDNA (Th17), prime different types of anti-tumor immune response.

Prime-boost using Reovirus and VSV-ASMEL improves anti-tumor therapy.

Therefore, we hypothesized that a combination of immunovirotherapies working through different immune mechanisms would enhance overall anti-tumor therapy in the context of a prime-boost strategy. Using sub-optimal individual treatments either alone, or in combination, to allow detection of improved efficacy, prime-boost with Reo/PBS, Reo/Reo, VSV-ASMEL/VSV-ASMEL, Reo/VSV-GFP and VSV-ASMEL/Reo all resulted in significantly improved survival compared to PBS/PBS treated controls (**Fig.2A**, $p<0.001$ for all). However,

prime-boost with Reo/VSV-ASMEL was a significantly better treatment than any of the other regimens (**Fig.2A**, p<0.001 Reo/VSV-ASMEL vs any other treatment). Increased survival following Reo/VSV-ASMEL prime boost was associated with a stronger Th1 recall response against B16 lysate, or the melanoma tumor antigen TYRP1, compared to that seen in mice treated with prime-boost Reo/PBS (**Fig.2B**, p = 0.0140, B16 lysate; p = 0.0023, TYRP1). There was a trend towards increased Th17 responses following prime-boost Reo/VSV-ASMEL treatment compared to PBS/VSV-ASMEL although this did not reach statistical significance (**Fig.2C**). IFN-γ or IL-17 recall responses to TC2 F/T lysate, a non-melanoma cell line, were minimal, indicating that the Th1 and Th17 responses were tumor-specific (**Figs.2B&C**).

Enhancement of systemic Reovirus therapy by checkpoint blockade is dependent on CD8 cells.

We have previously shown that systemically delivered Reovirus can be effective when used in combination with other agents such as GM-CSF, cyclophosphamide or VEGF[23, 33, 34] or in the context of ex vivo loaded cell carriage[18]. In this respect, pre-conditioning with GM-CSF prior to systemic Reovirus delivery, effectively treated B16 tumors dependent on innate immune responses[23]. As before[23], a suboptimal regimen of two cycles of GM-CSF/Reovirus significantly prolonged survival in C57Bl/6 mice bearing 5 day established B16 s.c. tumors (**Fig.3A**). Combination with anti-PD-1 checkpoint blockade resulted in significantly improved survival (**Fig.3A**, GM-CSF/Reovirus/anti-PD-1 vs GM-CSF/Reovirus alone, p = 0.0174). The low level Th1 response to tumor Ag following GM-CSF/Reovirus treatment was significantly improved by the addition of anti-PD-1 (**Fig.3B**, GM-CSF/Reovirus/anti-PD-1 vs GM-CSF/Reovirus, p = 0.0250). Previously we showed that GM-CSF/Reovirus therapy is largely mediated by innate effectors such as natural killer (NK) cells and monocytes[23]. Similarly, depletion of neither CD8, nor CD4, cells significantly affected survival after treatment with GM-CSF/Reovirus (**Fig.3C**). However, consistent with the improved Th1 response seen on addition of anti-PD1 (**Fig.3B**), depletion of CD8, but not CD4, cells significantly reduced

survival in mice treated with GM-CSF/Reovirus + anti-PD-1 (**Fig.3D**, p = 0.0135). No Th17 response was detected following GM-CSF/Reovirus treatment, with, or without, addition of anti-PD-1 (IL-17 < 20 pg/ml, data not shown). These data suggest that, although the effect of GM-CSF/Reovirus is mainly mediated via innate effectors, a low level Th1 response was also generated but did not contribute significantly to tumor control. However, in the presence of checkpoint blockade this weak Th1 response was significantly enhanced, which translated into improved overall survival.

Checkpoint inhibition improves VSV-ASMEL therapy and uncovers a Th1 anti-tumor response.

The addition of anti-PD-1 significantly prolonged survival of mice with established s.c. B16 tumors treated with VSV-ASMEL alone (**Fig.4A**, VSV-ASMEL + anti-PD-1 vs VSV-ASMEL + control IgG, p = 0.018). Improved survival following VSV-ASMEL + anti-PD-1 was associated with a significantly stronger Th17 recall response against B16 lysate compared to VSV-ASMEL alone (**Fig.4B**, p = 0.001). Furthermore, anti-PD-1 treatment uncovered a Th1 response to tumor as evidenced by production of IFN-γ from splenocyte/LN cells in response to B16 lysate (**Fig.4C**, p = 0.0014), which was not detectable in the absence of anti-PD-1.

Combined Th1/Th17 therapy, together with checkpoint inhibition, cures B16 melanoma.

Finally, we hypothesized that combining an innate-driven/Th1 Reovirus-induced anti-tumor response, with a Th17 VSV-ASMEL-induced response, both of which were enhanced with anti-PD-1 blockade, would generate more effective anti-tumor therapy than either alone. As before, GM-CSF/Reovirus was effective in treating s.c. B16 tumors (**Fig.5A**, p = 0.0004 vs PBS), while combination with anti-PD-1 further improved survival (**Figs.3A&5A**). As with i.t. Reovirus + VSV-ASMEL (**Fig.2A**), prime-boost with systemic GM-CSF/Reovirus followed by VSV-ASMEL, was superior to GM-CSF/Reovirus alone (**Fig.5A**). However, addition of anti-PD-1 to the GM-CSF/Reovirus/VSV-ASMEL prime-boost treatment was the only therapy able to generate long-term cures under these experimental conditions (**Fig.5A**, p < 0.01 vs GM-

CSF/reo, GM-CSF/reo/anti-PD-1, GM-CSF/VSV-ASMEL). Splenocyte/LN cultures from the long-term cured mice produced significantly higher levels of IFN-γ in response to B16 lysate than mice from any other treatment group which had been euthanised earlier due to tumor burden, (**Fig.5B**, p = 0.00006). This Th1 recall response included a specific component against the melanoma Ag TYRP1 (**Fig.5B**, p = 0.0216 vs control group). In addition, mice treated with GM-CSF/Reovirus/VSV-ASMEL + anti-PD-1had a significantly improved Th17 recall response compared to those treated with the prime-boost regimen without checkpoint blockade (**Fig.5C**, p = 0.0156). These data show that two separate oncolytic immunovirotherapies, working through different immune effector mechanisms, and combined with checkpoint blockade, can be effectively combined to eradicate established disease.

DISCUSSION

It is now clear that the efficacy of many oncolytic virus regimens depends upon an immune component. Thus, Reovirus is effective against B16OVA tumors which are not susceptible to direct oncolysis[17], and systemic VSV did not generate significant anti-tumor therapy in nude mice[35]. However, the immunological mechanisms of such effects will vary between virus types, routes of administration and transgenes encoded by the viruses. In this respect, we show here that, whereas i.t. injection of oncolytic Reovirus primed a Th1-type response to B16 s.c. tumors, systemic administration of the VSV-ASMEL cDNA library primed a Th17 response to tumor-specific Ag. Therefore, we hypothesized that combining complementary immunological effector pathways, induced by different oncolytic viruses, would generate improved immune-mediated anti-tumor therapy.

Repeated treatment with the same type of immunovirotherapy (Reo/Reo (Th1) or VSV-ASMEL/VSV-ASMEL (Th17)) resulted in prolonged survival compared to PBS-treated controls (**Fig.2A**). However, combination Reovirus/VSV-ASMEL (Th1/Th17) prime-boost treatment significantly improved survival compared to repeated single therapies (**Fig.2A**), associated with enhanced Th1, and, to a lesser extent, Th17 anti-tumor Ag responses, (**Figs.2B&C**). Interestingly, reversing the order of the prime-boost from Th1/Th17 to Th17/Th1 still

significantly improved survival compared to controls. However, this improvement was only comparable to single repeated immunovirotherapies and was significantly less effective than the Th1/Th17 prime-boost (**Fig.2A**). These data show that two different oncolytic viruses, each priming a different type of immune response, can be combined to produce significantly better therapy than either virus alone. Furthermore, the order in which the responses were induced was important (Th1 followed by Th17).

As part of our long term goal to develop delivery regimens for oncolytic immunovirotherapy which do not necessitate direct i.t. injection, we developed an effective systemic Reovirus therapy by pre-conditioning tumor-bearing mice with GM-CSF prior to i.v. Reovirus injection, which is mediated by NK cells and $CD11b^{+}$ monocytes[23]. We have also shown that Reovirus-mediated NK cell activation following i.t. Reovirus injection was augmented by anti-PD-1 leading to improved tumor therapy[22]. Therefore, we investigated whether anti-PD-1 could improve our systemic Reovirus treatment. **Fig.3A** shows that addition of anti-PD-1 treatment significantly enhanced survival of mice compared to GM-CSF/Reovirus alone. Significantly, this improvement in therapy was associated with an enhanced Th1 response to B16 tumor Ag, which was only minimally detected in the absence of anti-PD-1 (**Fig.3B**). The improved therapy was also dependent upon CD8+ T cells (**Figs.3B&D**), consistent with the mechanism of checkpoint blockade as acting predominantly via release of inhibition on T cells[36-38]. These data show that checkpoint blockade mechanistically enhanced systemic GM-CSF/Reovirus therapy by significantly augmenting an otherwise very weak CD8+ T cell dependent component which was associated with significantly better anti-tumor therapy.

Similarly, although therapy associated with systemic delivery of VSV-ASMEL was dependent upon CD4+ T cells and a Th17 response (**Fig.4B**), with no detectable Th1 response (**Fig.4C**), addition of anti-PD-1 uncovered a Th1 response to tumor Ag that was not detectable in the absence of checkpoint blockade (**Fig.4C**). As for the addition of anti-PD-1 to the GM-CSF/Reovirus regimen, uncovering of this anti-tumor Th1 response was associated with extended survival, and increased tumor cures, in vivo (**Fig.4A**). Anti-PD-1 also moderately enhanced the anti-tumor Th17 response against B16 tumor Ag (**Fig.4B**). We are currently

244 investigating the possibility that anti-PD-1 therapy acts so effectively to augment these
245 otherwise undetectable Th1 T cell responses (for both GM-CSF/Reovirus and VSV-ASMEL
246 treatments), through direct activity on suppressive cells such as MDSC or T_{reg} induced in
247 response to virotherapy.
248 Since the combination of GM-CSF/Reovirus and VSV-ASMEL therapy enhanced therapy
249 compared to either alone (**Fig.2**), and since both mono-immunovirotherapies were significantly
250 enhanced by anti-PD-1 checkpoint inhibition (**Figs.3&4**), we tested the combination of all three
251 therapies. As seen in **Fig.5**, the triple therapy (GM-CSF/Reovirus (innate immune mediated,
252 C8+T $Th1^{lo}$) + VSV-ASMEL boost (CD4+ Th17, $Th1^{lo}$) + anti-PD-1 (Th1 and Th17
253 enhancement) was significantly more effective than any of the double combinations, resulted
254 in tumor regression with 100% of the mice cured long term at day 70, and was associated with
255 very strong Th1 and Th17 responses to tumor antigens, including TYRP-1 (**Fig 5**).
256 Our data are consistent with a model in which primary treatment with GM-CSF/Reovirus leads
257 to initial tumor killing through virus delivery and innate immune activation[23]. This therapy
258 induced detectable, but very low level, Th1 responses against tumor antigens (**Fig.3B**). We
259 hypothesise that, critically, initial tumor killing releases a very broad range of tumor Ag, against
260 which only very weak anti-self T cell responses can be primed. Subsequent delivery of VSV-
261 ASMEL provides a similarly broad range of tumor Ag in the form of the cDNA library. These
262 stimulate CD4+ Th17 responses which can, therefore, provide additional help to the T cell
263 responses stimulated by the primary GM-CSF/Reovirus treatment (**Fig.2B&C**). Finally, late
264 boosting with anti-PD-1 further augments both the already enhanced Th1 and Th17 responses
265 against this broad range of tumor antigens leading to the potent and sustained therapy
266 observed in **Fig.5**.
267 Other studies have shown that heterologous prime-boost can generate efficient anti-tumor Ag-
268 specific therapy[39-41]. Our approach here moves beyond the use of different vectors encoding
269 specific antigens and uses the release of multiple antigens through oncolysis as the basis of
270 the priming step, which is then boosted by the use of the cDNA library. We believe that raising
271 T cell responses against multiple tumor antigens simultaneously reduces the ability of tumor

cells to escape immune pressure by developing antigen loss variants. Our approach here is also novel in that it specifically exploits the complementary immunological mechanisms by which two oncolytic viruses (Reovirus and VSV) stimulate anti-tumor immunity through different immune effectors.

In summary, we show here that it is possible to combine oncolytic viruses, which induce complimentary mechanisms of anti-tumor immune responses, along with immune checkpoint blockade, to generate fully systemic, highly effective anti-tumor immunovirotherapy.

MATERIALS AND METHODS

Cell lines. Murine B16 melanoma and TRAMP-C2 (TC2) prostate tumor cells were grown in DMEM (Life Technologies) supplemented with 10% (v/v) FCS (Life Technologies) and L-glutamine (Life Technologies). Cell lines were monitored routinely and found to be free of Mycoplasma infection.

Viruses. Wild type Reovirus type 3 (Dearing strain, REOLYSIN®) was obtained from Oncolytics Biotech (Calgary, Canada). Stock titers were measured by plaque assays on L929 cells. The ASMEL VSV-cDNA library was generated as previously reported[10, 24, 42]. Individual viral clones were isolated by limiting dilution as previously described[24, 42], expanded in BHK cells and purified by sucrose gradient centrifugation. VSV-GFP was manufactured as described[43].

In vivo experiments. 6-8 week old female C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All in vivo studies were approved by the Mayo IACUC. Mice were challenged subcutaneously with $2x10^5$ B16 melanoma cells in 100 µL PBS (HyClone). Tumors were measured 3 times per week, and mice were euthanized when tumors reached 1.0 cm diameter. Reovirus was administered i.v. at $5x10^7$ or i.t. at $1x10^8$ $TCID_{50}$ per injection; VSV-GFP and VSV-ASMEL were administered i.v. at $1x10^7$ pfu per injection. GM-CSF was administered i.p. at 300 ng/injection, as described previously[23], 1 cycle of GM-CSF/reo = GM-CSF i.p. on 3 consecutive days followed by Reovirus ($5x10^7TCID_{50}$) i.v. on the following 2 days. Anti-PD-1 (BioXcell, West Lebanon, NH) or control IgG (BioXcell) was given

i.v. at either 225 or 250 µg per injection as detailed in the figure legends. Anti-CD4 (GK1.5, BioXcell) or anti-CD8 antibodies (Lyt2.43, BioXcell) for cell depletions were administered i.p. at 100 µl per injection.

In vitro splenic re-stimulation of splenocytes/lymph nodes and enzyme-linked immunosorbent assay for IFN-γ/TNF-α. Spleen and lymph nodes (S/LN) were immediately excised from euthanized mice and dissociated in vitro to achieve single-cell suspensions. S/LN cells were pooled for each individual mouse. Red blood cells were lysed with ACK lysis buffer for 2 min. Cells were re-suspended in Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) + 5% FBS + 1% Pen-Strep + 40 µM 2-ME. Supernatants were harvested from 1 x 10^6 S/LN stimulated with one of the following: VSV-combination (VSV-NRAS, VSV-CYT-c, VSV-TYRP1) at MOI=1 per stimulation; 1 µg/ml synthetic H2-b-restricted peptides murine TRP-$2_{180–188}$ SVYDFFVWL ($H2K^b$), murine TRP-$1_{222–229}$ TAYRYHLL ($H2K^b$), human $gp100_{25–33}$ (Hgp100) KVPRNQDWL ($H2D^b$), murine $gp100_{25-33}$ (Mgp100) EGSRNQDWL ($H2D^b$) or with freeze-thaw lysates (equivalent to 1 x 10^6 tumor cells), from B16 (relevant) or TC2 (irrelevant) tumor cells every 24 h. Cell-free supernatants were collected at 48 or 72 h and tested by enzyme-linked immunosorbent assay for murine IFN-γ or murine IL-17 (BD Biosciences, San Jose, CA). The peptides were synthesized at Mayo Foundation Core Facility (Rochester, MN).

Statistics. Survival data from the animal studies were analyzed by the log-rank test using GraphPad Prism 6 Software. A Student's t-test analysis was applied for in vitro data. Statistical significance was determined at the level of $P < 0.05$.

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FIGURE LEGENDS

Figure 1: Reovirus primes a Th1 response, while VSV-cDNA primes a Th17 response against B16 melanoma. A&B. C57Bl/6 mice (4 per group) bearing 10 day established B16 tumors, received 6 i.t. injections of either PBS or Reovirus on days 10,12,14,17,19,21 (**A**), and C57Bl/6 mice (4 per group) bearing 5 day established B16 tumors, received 6 i.v. injections of either VSV-GFP or VSV-ASMEL on days 5,7,9,12,14,16. (**B**). At day 25, mice were euthanised, spleens and LN dissociated into single cell suspensions and re-stimulated with either: B16 F/T lysate; VSV-NRAS + VSV-CYT-c + VSV-TYRP1 (VSV-combo, total MOI=1 per re-stimulation) or peptide as indicated (1 μg/ml per re-stimulation), every 24 h. Supernatants were harvested after 48 h and tested for IFN-γ and IL-17 by ELISA. Graphs show values +SD (triplicate wells) for individual mice. *p<0.05, **p<0.01 two-tailed t-test.

Figure 2: Prime-boost using Reovirus and VSV-ASMEL improves anti-tumor therapy. A. C57Bl/6 mice (7 per group) bearing 10 day established B16 tumors, received 3 i.t. injections of either PBS, Reovirus or VSV-ASMEL on days 10,12,14 followed by 3 i.v. injections of either PBS/Reovirus/VSV-ASMEL on days 17,19,21 as indicated. Tumor measurements were taken 3x per week and mice euthanised when tumors reached 1.0 cm diameter. Graph shown is representative of n=2 individual experiments, ***p<0.001 Log-Rank test Reo/VSV-ASMEL compared to all other groups. **B&C.** At time of sacrifice due to tumor burden, S/LN were harvested from 3 mice per group. Single cell suspension cultures of S/LN were re-stimulated with either, B16 (relevant) or TC2 (irrelevant) F/T lysate, or TYRP1 peptide, every 24h. Supernatants were harvested after 72h and tested for IFN-γ and IL-17 by ELISA. Bars on graphs show values for individual mice. *p<0.05, **p<0.01 two-tailed t-test.

Figure 3: Enhancement of systemic Reovirus therapy by checkpoint blockade is dependent on CD8 cells. A&B. C57Bl/6 mice (7 per group) bearing 5 day established B16 tumors, were treated ± 2 cycles of GM-CSF/Reovirus beginning on days 5 and 12, then 3 injections of anti-PD-1 (250 μg) or control IgG on days 19,21,23. **A.** Tumors were measured

3x per week and mice euthanised when tumors reached 1.0 cm diameter. *p<0.05 Log-Rank test. **B.** S/LN were harvested at time of sacrifice (as indicated). Single cell suspension cultures of S/LN were re-stimulated with B16 F/T lysate every 24 h. Supernatants were harvested after 72 h and tested for IFN-γ by ELISA. Bars on graphs show values +SD (triplicate wells) for individual mice. *p<0.05 two-tailed t-test. **C&D.** C57Bl/6 mice (5 per group) bearing 5 day established B16 tumors, received 3 cycles of GM-CSF/Reovirus with co-injection of anti-CD4 or anti-CD8 depleting antibodies along with the GM-CSF, begining on days 5,12,19. Anti-PD-1 (250 µg) or control IgG was administered on days 19,21,23. Tumors were measured 3x per week and mice euthanised when tumors reached 1.0 cm diameter. **C.** Depletion of CD4 or CD8 cells on GM-CSF/Reovirus therapy; **D.** Depletion of CD4 or CD8 cells on GM-CSF/Reo/anti-PD-1 therapy. *p<0.05 Log-Rank test. **C&D** are results from the same experiment.

Figure 4: Checkpoint inhibition improves VSV-ASMEL therapy and uncovers a Th1 anti-tumor response. C57Bl/6 mice (7-8 per group) bearing 5 day established B16 tumors, received 6 injections of either VSV-GFP or VSV-ASMEL on days 5,7,9,12,14,16, followed by 6 injections of anti-PD-1 (250 µg) or control Ig on days 19,21,23,26,28,30. **A.** Tumor measurements were taken 3x per week and mice euthanised when tumors reached 1.0 cm diameter. Graph shown is representative of n=3 individual experiments, *p<0.05 Log-Rank test. **B&C.** S/LN were harvested from 4 mice/group at time of sacrifice. Single cell suspension cultures of S/LN were re-stimulated with B16 F/T lysate every 24 h. Supernatants were harvested after 72 h and tested for IL-17 (B) and IFN-γ (C) by ELISA. Bars on graphs show values +SD (triplicate wells) for individual mice. **p<0.01, ***p<0.001 two-tailed t-test.

Figure 5: Combined Th1/Th17 therapy, together with checkpoint inhibition, is effective in curing B16 melanoma. C57Bl/6 mice (7 per group) bearing 5 day established B16 tumors, received 2 'prime' cycles of either PBS or GM-CSF/Reovirus starting at days 5 and 12, then 3 'boost' injections of PBS or VSV-ASMEL on days 19,21,23. Anti-PD-1 (225 µg) or control IgG

was given on days 19,21,23,26,28,30. **A.** Tumor measurements were taken 3x per week and mice euthanised when tumors reached 1.0 cm diameter. Graph shown is representative of n=2 individual experiments, **p<0.01 Log-Rank test. **B&C.** S/LN were harvested from 3 mice/group at time of sacrifice (as indicated in C). Single cell suspension cultures of S/LN were re-stimulated with B16 F/T lysate or peptide as indicated, every 24 h. Supernatants were harvested after 72 h and tested for IFN-γ (B) and IL-17 (C) by ELISA. Bars on graphs show values +SD (triplicate wells) for individual mice. *p<0.05, ***p<0.001 two-tailed t-test.