Genetically stable poliovirus vectors activate dendritic cells and prime antitumor CD8 T cell immunity

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Viruses naturally engage innate immunity, induce antigen presentation, and mediate CD8 T cell priming against foreign antigens. Polioviruses can provide a context optimal for generating antigen-specific CD8 T cells, as they have natural tropism for dendritic cells, preeminent inducers of CD8 T cell immunity; elicit Th1-promoting inflammation; and lack interference with innate or adaptive immunity. However, notorious genetic instability and underlying neuropathogenicity has hampered poliovirus-based vector applications. Here we devised a strategy based on the polio:rhinovirus chimera PVSRIPO, devoid of viral neuropathogenicity after intracerebral inoculation in human subjects, for stable expression of exogenous antigens. PVSRIPO vectors infect, activate, and induce epitope presentation in DCs in vitro; they recruit and activate DCs with Th1-dominant cytokine profiles at the injection site in vivo. They efficiently prime tumor antigen-specific CD8 T cells in vivo, induce CD8 T cell migration to the tumor site, delay tumor growth and enhance survival in murine tumor models.
Bolstering the potency or frequency of antitumor cytotoxic T lymphocyte (CTL) responses has achieved durable responses in cancer patients\(^1\)-\(^5\). Generating such responses in situ requires an appropriate costimulatory context coinciding with presentation of target antigen by MHC class I. Viral vectors offer a compelling approach to engage CTLs by providing intracellular expression of both, pathogen-associated molecular patterns and the desired antigen. This recapitulates the cues CTLs have evolved to respond to upon intracellular pathogen infection; in essence portraying the target antigen as a viral signature.

A clinically feasible poliovirus vector platform has remained elusive, despite obvious advantages: tropism for antigen-presenting cells\(^6\), a rapid lifecycle capable of propagating vector-encoded antigen, no interference with immune activation and antigen presentation, and robust engagement of inflammatory responses. We report the engineering of genetically stable vectors based on the highly attenuated PVSRIPO [type 1 poliovirus (Sabin) live-attenuated vaccine containing a rhinovirus type 2 internal ribosomal entry site (IRES)]\(^8\). PVSRIPO has inherent tropism for macrophages and DCs in primates\(^6\), and exhibits an unusual phenotype in such cells, characterized by lingering, sublethal infection and profound proinflammatory stimulation\(^9\). PVSRIPO is devoid of neuropathogenicity, even after high-dose intracerebral inoculation in non-human primates\(^10\) and in human subjects with recurrent WHO grade IV malignant glioma (glioblastoma; GBM)\(^11\). Intratumoral delivery of PVSRIPO has achieved clinical and radiographic responses with durable long-term survival (>36 months) in 21% of patients with recurrent GBM, an indication notorious for lacking immune engagement\(^11\).

Unlike the extremely heterogeneous adult malignant gliomas, the predominantly pediatric Diffuse Midline Gliomas (DMG) frequently are defined by a loss in histone 3 (H3K27) trimethylation\(^12\),\(^13\). Approximately 80% of DMGs of the pons [Diffuse Intrinsic Pontine Glioma (DIPG)] express H3.3\(^{K27M}\), which also occurs in spinal and thalamic DMGs. In addition, H3.3\(^{G34R/V}\) is associated with hemispheric high grade gliomas in pediatric patients\(^12\). Loss of H3K27 trimethylation has been implicated in aberrant gene expression control and tumorigenesis\(^14\).

H3.3\(^{K27M}\) is a high affinity HLA-A2-restricted tumor neoantigen and T cells bearing the H3.3\(^{K27M}\)-specific TCR lysed H3.3\(^{K27M}\)-positive glioma cells in vitro and in vivo\(^15\). Devising effective immunotherapies for DMG/DIPG is an urgent mandate, as there is no standard treatment for this invariably fatal disease with dismal prognosis. We describe a genetically stable PVSRIPO-based vector platform to generate antitumor responses against the H3.3\(^{K27M}\) epitope. We show that PVSRIPO-based vectors target DCs to: elicit sublethal viral translation and propagation resulting in expression of foreign epitopes in a highly adjuvanted context; induce DC maturation markers; provoke type I/III interferon (IFN) release; present the H3.3\(^{K27M}\) epitope to T cells; generate locoregional proinflammatory activation, immune cell infiltration and DC activation in vivo; and trigger DC migration to immunization site-draining lymph nodes in vivo, where they express the H3.3\(^{K27M}\) epitope. Vector immunization induces CD8\(^+\) T cell infiltration into tumors, and exhibits significant anti-tumor efficacy in immunocompetent rodent tumor models.

### Results

**Genetically stable PVSRIPO vectors.** Among diverse proposals for poliovirus-derived vectors, “polyprotein fusion” approaches are most advanced\(^16\)-\(^20\). Enterovirus + strand RNA genomes encode a single polyprotein, which is processed by two viral proteases (2A\(^{PRO}\), 3C\(^{PRO}\)). For polyprotein fusion, foreign polypeptides are N-terminally or internally fused to the polyprotein and released via an engineered viral protease cleavage site (Fig. 1a, b)\(^16\). Poliovirus polyprotein fusion vectors delivering simian immunodeficiency virus (SIV)-derived polypeptides or the ovalbumin (OVA) model antigen were tested in non-human primates\(^16\),\(^18\) and in human CD155-transgenic (hCD155-4g) mice\(^20\). Effective humoral, CD4\(^+\) and CD8\(^+\) T cell responses\(^16\)-\(^20\) protected macaques against lethal SIV challenge\(^15\). Yet, this vector design, where burdensome foreign sequences were simply added to the viral genome, triggered rapid deletion events\(^22\). We reasoned that to achieve genetic stability, foreign inserts must be functionally integrated into the genome, in order to contribute to viral fitness. All enterovirus genomes contain a cryptic AUG at the base of stem loop domain (SLD) VI of the IRES (Fig. 1a)\(^22\). Placing the cryptic AUG into KOZAK context and deleting SLD VI yields viable virus with growth deficits\(^23\),\(^24\). We used this scenario to incentivize insert retention, by putting the cryptic AUG into KOZAK context and replacing SLD VI with a coding region for foreign polypeptides (Fig. 1b, g).

We constructed a vector for the OVA class I epitope (SIINFEKL) among these guidelines (mOVA1; Fig. 1b), using a mouse-adapted PVSRIPO variant (mRIPO)\(^9\) as backbone (to enable proof-of-principle immunization tests in mice). We assessed genetic stability through 20 serial passages in HeLa cells. Corroborating earlier findings\(^23\), the OVA insert was retained over 20 passages. A single nt adaption emerged in passage 10: mixed C609/A that became A609 by passage 20 (Fig. 1d), suggesting a fitness advantage conveyed by A609. C609A is a missense mutation (L/M; Fig. 1d) that does not affect SIINFEKL epitope processing and presentation. Cloned mOVA2 carrying the A609 substitution remained genetically intact through 20 serial passages in HeLa cells (Fig. 1e); mOVA2 formed larger plaques with enhanced growth compared to mOVA1 (Fig. 1f).

A vector delivering the DMG/DIPG H3.3\(^{K27M}\) epitope [RIPO (H3.3); Fig. 1g] remained unchanged after 20 serial passages in HeLa cells (Supplementary Fig. 1). RIPO(H3.3)’s plaque size and growth kinetics were enhanced compared to PVSRIPO with SLD VI deleted [RIPO\(^{SI\text{O}}\); Fig. 1h], suggesting that the foreign insert conveys a fitness advantage. RIPO(H3.3) retained the neuron-specific translation deficit of its PVSRIPO parent, which defines neuroattenuation in vivo\(^25\),\(^26\). This was evident as deficient RIPO(H3.3) growth in HEK293 cells (adrenal precursor cells of neuroectodermal origin\(^27\) (Supplementary Fig. 2). HEK293 cells accurately reflect the neuroattenuation phenotypes of PVSRIPO and the poliovirus (Sabin) vaccines\(^28\),\(^29\). The PVSRIPO platform can be flexibly adapted to accommodate diverse inserts; we derived genetically stable vectors expressing peptides encoding the isocitrate dehydrogenase 2 (IDH2) R172G mutation, or SIV p55 Gag-derived peptides (Supplementary Fig. 3).

**mOVA2 initiates translation from the intended AUG.** The C609A substitution in mOVA2 resulted in tandem, in-frame AUGs separated by 18nt; both of these were in ideal KOZAK context (A/G . . . AUG A) (Fig. 1g). mOVA2 acquiring a new in-frame AUG in poor KOZAK context (C . . . AUG A) (Fig. 1g) remained unchanged after 20 serial passages in HeLa cells (Supplementary Fig. 1). RIPO(H3.3)’s plaque size and growth kinetics were enhanced compared to PVSRIPO with SLD VI deleted [RIPO\(^{SI\text{O}}\); Fig. 1h], suggesting that the foreign insert conveys a fitness advantage. RIPO(H3.3) retained the neuron-specific translation deficit of its PVSRIPO parent, which defines neuroattenuation in vivo\(^25\),\(^26\). This was evident as deficient RIPO(H3.3) growth in HEK293 cells (adrenal precursor cells of neuroectodermal origin\(^27\) (Supplementary Fig. 2). HEK293 cells accurately reflect the neuroattenuation phenotypes of PVSRIPO and the poliovirus (Sabin) vaccines\(^28\),\(^29\). The PVSRIPO platform can be flexibly adapted to accommodate diverse inserts; we derived genetically stable vectors expressing peptides encoding the isocitrate dehydrogenase 2 (IDH2) R172G mutation, or SIV p55 Gag-derived peptides (Supplementary Fig. 3).
initiation AUG at nt 588 in optimal KOZAK context is preferred (Supplementary Fig. 4).

**mOVA2 and RIPO(H3.3) express and process foreign epitopes.**

With our vector design, the H3.3K27M/OVA epitopes are fused to the viral P1 or VP0 precursors and processed by 2Apro cleavage (Fig. 2a). An H3.3K27M-specific antibody probe detected such H3.3K27M-fusion polypeptides in RIPO(H3.3)-infected HeLa cells (Fig. 2b). These appeared early (H3.3K27M-P1 peaked at 3hpi) and declined thereafter, presumably due to viral proteolytic processing. Processing of the H3.3K27M-VP0 fusion product was delayed compared to H3.3K27M-P1, in line with the poliovirus processing sequence (Fig. 2b)30. See Supplementary Fig. 5 for viral fusion-polypeptide expression analyses in mOVA2-infected HeLa cells. Expression of viral H3.3K27M/OVA-fusion proteins with our vector design corroborates our studies of initiation codon usage (Supplementary Fig. 4).

**Sublethal infection of hCD155-tg mouse BMDCs with mOVA2.**

Monocytes, macrophages, and DCs express the poliovirus receptor
CD155 and are natural, high priority poliovirus targets in pri-
mates. Accordingly, poliovirus infects monocytes, macrophages, and DCs in vitro,31,32. In line with productive infection in pri-
mate CD11c+ macrophages/DCs in vivo, poliovirus infection yields cytopathogenic virus replication and host protein synthesis shut-off in human DCs derived from PBMCs. In contrast, PVSRIPO exhibits a sublethal phenotype in human DCs, with protracted, low-level viral translation and propagation, accompanied by sus-

tained, potent type I dominated IFN activation.

We tested mOVA2-infected DCs for: viral translation and cytokotoxicity; proinflammatory cytokine production; expression and presentation of OVA class I epitope; and antigen-specific activation of CD8+ cytotoxic T cells. To this end, we established bone marrow-derived DC (BMDC) cultures from hCD155-tg mice either differentiated with GMCSF/LI4 (GMCSF-BMDCs; Fig. 3a) or with Fms-related tyrosine kinase 3 ligand (FLT3L-BMDCs; Fig. 3b). The latter consist of a mixture of CD8α+ (Batf3+ DCs) and CD8α− DCs. CD8α− DCs have a prominent role in eliciting CTL responses,35,36. GMCSF− (Fig. 3a) or FLT3L− (Fig. 3b) BMDCs were either mock-infected, or infected with mOVA2 at a multiplicity of infection (MOI) of 10. As a positive control for proin-
flamatory stimulation, we used lipopolysaccharide (LPS) (Fig. 3a, b).

mOVA2 infection of GMCSF- and FLT3L-BMDCs revealed the absence of eIF4G cleavage, the hallmark of poliovirus cytopathogenicity.37 We did not detect productive viral translation, i.e. viral 2C/2BC, in GMCSF-BMDCs (Fig. 3a). In contrast, viral protein synthesis was abundant in FLT3L-BMDCs (Fig. 3b). Viral translation peaked early (12hpi) and gradually declined without evidence for cytopathogenicity (i.e. eIF4G cleavage), indicating an ability of the FLT3L-BMDCs to prevent cytoxic damage stemming from viral translation (Fig. 3b).
mOVA2-induced type I IFN-dominant activation of mouse BMDCs. Both, GMCSF- and FLT3L-BMDCs responded to mOVA2 infection with type I IFN activation signatures, e.g. induction of p-STAT1(Y701), STAT1, TAP1 and ISG15 (Fig. 3a, b). This response was delayed in FLT3L- relative to GMCSF- BMDCs, especially at 12hpi (compare mOVA2-infected to LPS-
treated cultures; Fig. 3a, b). Results of repeat assays are shown in Supplementary Fig. 6a, b. mOVA2 infection led to the upregu-
ation of DC maturation markers (CD40/80/86) and PD-L1 in a

pattern that was qualitatively similar, but quantitatively diverging in FLT3L- vs. GMCSF-BMDCs (Fig. 3a, b). Levels of MHC I and -II remained unchanged in mOVA2-infected GMCSF- and FLT3L-BMDCs throughout the assay (Supplementary Fig. 6a, b). Delayed type I IFN responses in FLT3L-BMDCs were also evident.
in cytokine release assays in mOVA2-infected GMCSF- (Fig. 3c) and FLT3L-BMDCs (Fig. 3d). In both, GMCSF- and FLT3L-BMDCs, substantial responses were recorded for type 1 IFNs (IFNα/β), CCL5 (RANTES) and CXCL10 (Fig. 3c, d) (other tested cytokines had low or absent responses; Supplementary Fig. 7a, b).

While proinflammatory cytokine responses in FLT3L-BMDCs were delayed, their amplitude was higher compared to GMCSF-BMDCs (Fig. 3c, d). Collectively, these findings suggest that mOVA2 infection of BMDCs stimulated their antigen-presenting and T cell co-stimulation capacity.
mOVA2-infected BMDCs present SIINFEKL epitope. To test if mOVA2-infected and activated DCs present the SIINFEKL epitope and stimulate T cell function, we infected GMCSF-BMDCs with mOVA2 (MOI 10) and co-cultured them with either OT-I (SIINFEKL-specific) or naïve CD8+ T cells. We compared IFN-γ and granzyme B production by mOVA2-infected- to unstimulated-; SIINFEKL peptide-, LPS-, LPS + SIINFEKL-, mRIPO- or mRIPO + SIINFEKL-stimulated GMCSF-BMDCs (Fig. 3e). mOVA2 infection alone mirrored the response to LPS + SIINFEKL-stimulation (Fig. 3e). mRIPO was devoid of activity in this assay, but mRIPO + SIINFEKL-stimulation instigated responses that exceeded those to SIINFEKL-stimulation alone (Fig. 3e). These observations indicate expression, processing, and presentation of foreign polypeptides by PVSRIPO-based vectors in DCs, and illustrate the costimulatory potential of PVSRIPO-based vector infection.

Adjuvant effects of mOVA2 in vivo. A salient argument for viral vector-delivery of antigens is local adjuvancy from the stimuli provided by virus challenge. To test this, we immunized hCD155-tg mice with either mOVA2 or DMEM (Mock) by the intramuscular (i.m.) route and isolated skeletal muscle tissue from the inoculation site to assess the presence of mOVA2 by plaque assay; isolated skeletal muscle tissue from the inoculation site (24hpi) for tests of cytokine responses (Fig. 4a); compare to in vitro BMDC infection in Fig. 3c, d; Supplementary Fig. 7); assessed local immune cell infiltrates by flow cytometry (Fig. 4b); and tested DC maturation status by evaluating CD40 and CD86 expression on local infiltrating DCs (Fig. 4c).

A breadth of cytokines was induced by mOVA2 infection in vivo (Fig. 4a). The four principal responses to mOVA2 infection of DCs in vitro, IFNα/β, CCL5, CXCL10 (Fig. 3c, d), and IFNγ evident in the OT-I assay (Fig. 3e), also occurred in vivo (Fig. 4a). As suggested by the pattern of the local cytokine/chemokine signature, there was profuse recruitment of immune cells, e.g. DCs, macrophages and T cells, to the site of immunization (Fig. 4b). Confirming data with in vitro infection of BMDCs with mOVA2 (Fig. 3a, b), local DCs acquired maturation markers (CD40 and CD86), with CD40 being the most responsive (Fig. 4c).

Locoregional inflammation elicited by i.m. mRIPO-vector administration, immune cell infiltration, and costimulatory DC activation indicated that local DC interaction with vector, and (infected) DC migration to draining lymph nodes may occur in vivo. Therefore, we examined antigen expression in (popliteal) lymph nodes draining the vector inoculation site in vivo. To this end, we used the mouse-adapted form of RIPO (H3.3) [mRIPO(H3.3)] for i.m. immunization of hCD155-tg mice, because a suitable antibody for immunohistochemistry (IHC) of the H3.3K27M epitope is available. H3.3K27M IHC revealed that mRIPO(H3.3) had drained to popliteal lymph nodes in H3.3K27M–expressing cells, possibly migrated mRIPO (H3.3)-infected DCs (Fig. 4d).

As reported previously for wt poliovirus after i.m. inoculation in hCD155-tg mice38, the extent of mRIPO(H3.3) propagation in skeletal muscle at the inoculation site was very modest (Supplementary Fig. 12). Sequencing of mRIPO(H3.3) virus isolated from skeletal muscle tissue at the immunization site (at day 4 after i.m. immunization) revealed that the foreign insert remained genetically intact (Supplementary Fig. 13). This corroborated our data on genetic stability during in vitro passaging (Supplementary Fig. 1).
Therefore, to assess if mOVA2-instigated CD8 T cell responses exert anti-tumor efficacy, we challenged the B16F10.9-OVA tumor model in hCD155-tg mice. In mOVA2-immunized mice, tumor progress was significantly slowed with a significant survival benefit compared to mRIPOδ6-immunized mice (Fig. 6a, b). To mechanistically correlate these findings with mOVA2’s capacity to induce tumor antigen-specific CD8 T cell responses (Fig. 5a–d), we studied B16F10.9-OVA tumor immune infiltrates (Fig. 6c). This is not feasible with the approach described for Fig. 6a, b, because the mOVA2-induced treatment effect reduced tumor size at the testing interval (day 11), preventing comparison of mRIPOδ6- vs. mOVA2-immunized animals in similarly sized tumors (Fig. 6a). Therefore, B16F10.9-OVA tumors were implanted (day 0) followed by i.m. immunization with mRIPOδ6 or mOVA2 (day 1) and tumors were isolated at day 11 for analyses of CD8 and CD4 T cell infiltration by flow cytometry (Fig. 6c;...
Supplementary Fig. 17). mOVA2-immunized mice had significantly higher T cell (CD3+) infiltration in the tumor, when compared to mRIPOδ6-immunized mice (Fig. 6c). Also, the CD8:CD4 T cell ratio was significantly skewed towards CD8 T cells in mOVA2-immunized mice (Fig. 6c). This suggests that SIINFEKL-specific CD8 T cells generated by mOVA2 immunization migrate to and infiltrate the tumor and mediate the decrease in tumor burden.

Next, we assessed if the therapy effect of mOVA2 immunization correlates with the enhanced frequency of SIINFEKL-specific CD8 T cells [compared to poly(I:C) + SIINFEKL; Fig. 5a–c]; and depends on the presence of the OVA antigen in the tumor target. To this end, hCD155-tg mice were immunized with mRIPOδ6 or mOVA2 or with poly(I:C) or poly(I:C) + SIINFEKL peptide by i.m. inoculation (as described for Fig. 5a–c). Immunized mice received bilateral tumor implants: ipsilateral B16F10.9-OVA and contralateral B16F10.9 (Fig. 6d). None of the immunization regimens altered the progression of B16F10.9 tumors, lacking OVA (Fig. 6d). Only mOVA2 immunization prevented growth of the ipsilateral B16F10.9-OVA tumors (Fig. 6d, e). Thus, the
Fig. 5 mOVA2 and mRIPO(H3.3) induced epitope-specific CD8 T cell responses in vivo. a, b IFN-γ ELISPOT showing generation of SIINFEKL-specific CD8 T cells upon i.m. immunization of hCD155-tg mice with mOVA2. Cells were either left unstimulated or stimulated with peptide (SIINFEKL or MOG negative control); Concanaavalin A stimulation (positive control) is shown in Supplementary Fig. 14. c, d H2Kb-SIINFEKL pentamer staining of peripheral blood, gated on CD11b+CD45+ cells, showing % CD8 T cells that are SIINFEKL-specific. e, f HLA-A2-RMSAPSTGGV tetramer staining of peripheral blood, gated as in (c, d), showing % CD8 T cells that are RMSAPSTGGV-specific upon i.m. immunization of AAD_hCD155-tg mice with Complete Freund’s Adjuvant (CFA) and RMSAPSTGGV peptide emulsion, or with mRIPO(H3.3). e, f The gating strategy employed is shown in Supplementary Fig. 15. a, d, e Asterisks denote statistical significance between groups determined by RM-ANOVA protected Tukey’s test. a, c, e Error bars denote SEM. a, c, e All experiments were performed twice and representative results are shown. Error bars denote SEM. a, c, e All experiments were performed twice and representative results are shown.

Fig. 6 mOVA2 immunization delays tumor growth and induces tumor CD8 T cell infiltration. a Mice were immunized with either mOVA2 or mRIPO66 on day 0, boosted on day 14, B16F10.9-OVA cells were implanted subcutaneously 3 weeks later, and tumor volumes were monitored (mice were euthanized when tumors reached 1000 mm3). mOVA2-immunized mice survived significantly longer than mRIPO66 treated animals (P = 0.0017, Log-rank (Mantel-Cox) test, n = 10). A repeat assay including a mock-immunized group is shown in Supplementary Fig. 18. b, c Tumor progression in individual mice from experiment in (a). B16F10.9-OVA tumors from mOVA2-immunized mice had elevated CD8 T cell infiltration compared to mRIPO66-immunized mice. The gating strategy is shown in Supplementary Fig. 17. Two-tailed t-test, n = 4. *P < 0.05, **P < 0.01. d The anti-tumor effect of mOVA2 immunization correlates with SIINFEKL CD8 T cell frequency and is specific to tumors expressing OVA. Mice were immunized with mRIPO66, mOVA2, poly(I:C), or poly(I:C) + SIINFEKL as in Fig. 5a, followed by bilateral B16F10.9 and B16F10.9-OVA tumor implantation as shown (left panel). Tumor progression was monitored until reaching >1000 mm3 in the first test animal (asterisks; this occurred in the contralateral B16F10.9 tumors in all treatment groups). mRIPO66 n = 9, mOVA2 n = 8, poly(I:C) + SIINFEKL n = 8, poly(I:C) n = 10. All error bars represent SEM; n = number of animals.
antitumor effect of mOVA2 immunization is due to its capacity to instigate CD8 T cell responses against a tumor-specific antigen, as evident in ELISPOT and pentamer analyses (Fig. 5a–c).

mRIPO(H3.3) enhances survival in a mouse glioma model. We evaluated mRIPO(H3.3) immunization in an immunocompetent malignant glioma model transduced with HLA-A2 (AAD) and the H3.3K27M variant (the gene products of mouse and human H3F3A are 100% conserved) (Fig. 7a, b). Of the available syngeneic mouse glioma models, we favor the 20-methylcholanthrene-induced CT2A cell line because it recapitulates the notorious aggressive growth and immunotherapy resistance of the human disease.41 CT2A_AADH3.3K27M tumors were orthotopically implanted in AAD_hCD155 transgenic mice express hCD155 in brain; Hela cells were used as a positive control (the differences in hCD155 electrophoretic mobility are due to differential glycosylation). a AAD_hCD155 transgenic mice were immunized by i.m. inoculation (day 1), implanted with CT2A_AADH3.3K27M cells for orthotopic tumor initiation (day 7), boosted with the same regimen (day 14), and followed for assessment of weight and neurological status. Mice were euthanized after losing 15% of their max. weight. mRIPO(H3.3)-immunized mice survived significantly longer than their mRIPO66-immunized littermates [P = 0.0121, Log-rank (Mantel-Cox) test; mRIPO66 n = 9 animals, mRIPO(H3.3) n = 10]. A replicate is shown in Supplementary Fig. 19. (f) CD8 depletion abrogates the anti-tumor effect of mRIPO(H3.3) immunization. The assay was performed as in (e), with CD8 depletion antibody administered every 4 days starting at 7 days prior to tumor implantation [P = 0.3493, Log-rank (Mantel-Cox) test; mRIPO66 + αCD8 n = 10 animals; mRIPO(H3.3) + αCD8 n = 11].

RIPO(H3.3)-induces type I IFN activation of human DCs. We evaluated RIPO(H3.3) in primary human DCs to validate the vector phenotype, and to test presentation of the H3.3K27M antigen to T cells. Infection of human monocyte-derived DCs (from HLA-A2 donors) with RIPO(H3.3) (Fig. 8a; repeat analyses are shown in Supplementary Fig. 20a) did not elicit a cytotoxic response, consistent with the mouse BMDC analyses of mOVA2 (Fig. 3a, b). RIPO(H3.3) translation in human DCs was more efficient than mOVA2 in murine GMCSF-BMDCs, likely reflecting host-specificity (Fig. 8a; compare Fig. 3a). RIPO(H3.3) translation in human DCs closely tracked viral propagation, established in one-step growth curve assays (Fig. 8b). Viral propagation was productive, if limited, with a ~10-fold surge in virus progeny upon initial infection to reach a maximum of ~0.05 pfu per cell by 24hpi (Fig. 8b). Restricted RIPO(H3.3) propagation in human DCs is consistent with lacking cytotoxicity.9 Increases in viral progeny were detected up to ~36hpi (Fig. 8d).

RIPO(H3.3)-infected human DCs exhibited IFN-dominant proinflammatory activation and maturation (Fig. 8a, c, d) with sustained type I IFN signatures, e.g. p-STAT1(Y701) and STAT1/ISG15 induction (Fig. 8a). RIPO(H3.3) infection had similar effects on DC maturation (CD40, −80/86, CCR7) and PD-L1 in human DCs as mOVA2 in mouse BMDCs; however, the effect was more pronounced in PVSRIPO’s natural human host (Fig. 8c; compare Fig. 3a, b). RIPO(H3.3) did not affect MHC I/II surface expression in human DCs, as in murine BMDCs (Supplementary Figs. 6a, b; 20b).

Consistent with immunoblot analyses in human DCs (Fig. 8a), and the cytokine response in mOVA2-infected BMDCs (Fig. 3c, d), RIPO(H3.3) infection of human DCs induced a potent type I (IFN-α/β) and III (IFN-λ1) IFN-dominant response (Fig. 8d;
Fig. 8 RIPO(H3.3) activates human DCs and mediates H3.3K27M presentation to CD8 T cells. **a** Immunoblot of primary human DCs treated with poly(I:C) or cGAMP or infected with RIPO(H3.3). **b** One-step growth curve showing virus replication in primary human DCs (n = 2); the experiment was repeated twice and a representative series is shown. **c** Surface co-stimulatory molecule expression on DCs after infection. The gating strategy is shown in Supplementary Fig. 20c. Asterisks depicts significant difference from Mock group as determined by Two-way RM-ANOVA protected Dunnett’s post hoc test; 0–36 hpi n = 4, 48 hpi n = 3. **d** Cytokine induction profile in RIPO(H3.3) infected DCs. Asterisks denote significant difference from Mock 0 group as determined by one-way ANOVA protected Dunnett’s post hoc test; n = 3. **e** Illustration of activation of DCs and antigen presentation to CD8 T cells. **f** Primary DC and Jurkat-TCR co-culture showing RIPO(H3.3)-infected DCs can present the H3.3K27M antigen to CD8 T cells. Asterisks denotes significant difference between groups as determined by one-way ANOVA protected Tukey’s post hoc test, n = 3 independent repeats. Experiment was repeated twice. All error bars denote SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
IFN-λ1 was not part of the mouse panel). Compared to mouse BMDCs, RIPO(H3.3)-infected human DCs exhibited a broader proinflammatory response, including IL-6, IL-12, TNF-α, and IFNγ (Fig. 8d). See Supplementary Fig. 21 for complete analyses.

**RIPO(H3.3)-infected DCs present H3.3K27M to CD8 T cells.** Events stemming from RIPO(H3.3)-infection of human DCs indicate broad type I/III IFN-dominant engagement, DC maturation/antigen presentation enhancement, and T cell recruitment and co-stimulation (Fig. 8e). To confirm RIPO (H3.3)-mediated H3.3K27M epitope expression in human DCs and its presentation to CD8 T cells, we used a Jurkat T cell clone expressing CD8 and a H3.3K27M-specific TCR (Fig. 8e). Human DCs were left unstimulated, treated with the H3.3K27M peptide, infected with RIPO66, or infected with RIPO(H3.3) and co-cultured with the H3.3K27M-TCR+, CD8+ Jurkat cells (J76CD8 + TCR+) (Fig. 8f). Robust IL-2 release by co-cultured J76CD8 + TCR+ only occurred with RIPO(H3.3) infection (Fig. 8f). IL-2 production after DC stimulation with the H3.3K27M peptide was greatly stimulated by adding phorbol ester (TPA) to the culture; in contrast, RIPO(H3.3) infection yielded similar levels of J76CD8 + TCR+ activation without TPA (Fig. 8f). Our data summarized in Fig. 8e show that RIPO(H3.3) infects human DCs and provides the 3 required signals for generating antigenic CD8 T cells, i.e., MHCI-epitope complexes; maturation marker induction; and proinflammatory cytokines.

**Discussion**

Failure of early cancer vaccines—usually short peptides—were attributed to lacking DC engagement43. Above all, peptide vaccination without proper DC costimulation and proinflammatory cytokines can induce tolerance or T cell anergy44-46. We show that pharmacokinetic problems of peptide vaccination, e.g, poor uptake/presentation by DCs43, are resolved with PVSRIPO vectors. Due to poliovirus’ tropism for CD11c+ antigen-presenting cells6, PVSRIPO vectors naturally target them for infection. RIPO(H3.3)-vectored epitope expression eliminates the need for specialized antigen cross-presenting cells such as CD141+ (BDCA3+) DCs, required with peptide approaches47. Rather, in RIPO(H3.3)-infected DCs, the epitope is loaded onto MHCI class I through the classical pathway.

To stimulate DCs in cancer vaccine regimens, many synthetic adjuvants have been enlisted. Yet, these agents incite broad, indiscriminate stimulation, potentially out-of-sync with the kinetics and distribution of antigen presentation. With RIPO (H3.3), type I/III-dominant IFN release and costimulatory activation occur in the very cells expressing the H3.3K27M signature, in step with lingering viral RNA replication. The importance of coincident epitope expression and costimulatory activation in DCs is evident in our studies: CD8 T cell responses to ‘empty’ mRIPO66 (providing the same adjuvancy as mOVA2) combined with SIINFEKL peptide were far inferior to mOVA2.

Type I-dominant IFN responses, elicited by RIPO(H3.3) infection of DCs, provide a fitting costimulatory context for the induction of CD8 T cell responses48-52. Protracted, sublethal viral propagation in DCs produced robust, sustained proinflammatory cytokine release, in step with co-stimulatory molecule upregulation and epitope stimulation. These events are crucial for CD8 T cell priming53.

PVSRIPO’s DC-stimulating phenotype starkly contrasts with the immune evasion and suppression programs of many human pathogenic viruses, which coalesce on DCs and target MHCI functions54. Picornaviruses, small RNA viruses with extreme genetic austerity, lack the capacity to interfere with host adaptive immunity. Thus, unlike most human viral pathogens, PVSRIPO does not interfere with MHC I expression, antigen loading or presentation.

Tumor antigens often resemble self-antigens and are prone to tolerance. The host innate response to picornavirus/PVSRIPO infection is coordinated by the Melanoma-Differentiation Associated Protein 5 (MDA5) pattern recognition receptor55. Activation of MDA5 (e.g. by endogenous RNA) has been linked to breaches of self-tolerance and autoimmunity56. Thus, MDA5-mediated inflammation may be particularly apt to instigate CD8 T cell responses against weak tumor antigens.

PVSRIPO vectors elicit tumor antigen-specific CTLs capable of infiltrating distant tumors, reduce tumor burden and significantly increase survival in immunocompetent mouse tumor models. The vector ‘IRES cassette’ accommodates any insert, provided that the salient design principles of our approach are considered. Thus, we outline a clinically feasible enterovirus vector approach based on PVSRIPO that has a clinical track record of sale administration, has empirical evidence for genetic stability, and is capable of unique proinflammatory engagement of DCs for priming of CD8 T cells.

**Methods**

**Vector cloning, serial passage, one-step growth curves.** See Supplementary Table 1 for primers. To generate mOVA1-2 and RIPO(H3.3), the foreign insert was PCR synthesized with primers mOVA1-2/5′- and mOVA3′- or RIPO(H3.3)-3′/5′, respectively. A corresponding IRES fragment, spanning from the PmlI site in the cloverleaf of mRIPO (mRIPO66, mOVA1-5′) or PVSRIPO [RIPO(H3.3)] to the conserved cryptic AUG was generated with primers IRES-5′/3′. The mOVA2-flag insert was generated as described above, with the mOVA2-flag-5′ primer instead of mOVA2-5′. mOVA2s carrying an upstream AUG in poor Kozak context, or a frameshift in the foreign insert, were generated as described above with primers mOVA2-PC-5′ or mOVA2-PS-5′ substituting for mOVA2-PC-2′, respectively, and priming mOVA2-PC-2′ replaced for the former. Fusion of the foreign insert + IRES fragment as templates using flanking primers IRES-5′ mOVA1-3′ (mOVA1-2’d and derivatives) or IRES-5′/RIPO(H3.3)-3′/RIPO(H3.3)] yielded segments encompassing the IRES/foreign inserts for mOVA1-2 and RIPO (H3.3). mRIPO66 was generated using IRES-5′ and IRES66-3′ and the mRIPO IRES as template. These were inserted into the full-length cDNA clone of PVSRIPO digested with PmlI (mRIPO66, mOVA1-2 and derivatives) or EcoRI-Sacl (RIPOH3.3). In vitro synthesis of infectious RNA was performed using MEGAscript T7 (Thermo, AM1334) for deriving vectors54,55. For serial passage, 3 × 106 HeLa cells in 60 mm dishes were infected with vector (MOI 1; 24 h), freeze-thawed, the resulting lysate centrifuged to pellet debris, and 100 μl of the supernatant used to infect another HeLa cell culture (MOI of ~1). This procedure was repeated 20 times. Total RNA from infected cells at passages 3, 10, and 20 was isolated using TRIzol (ThermoFisher), reverse transcription was performed using Superscript IV (ThermoFisher) with primer Seq-3’, and the region encompassing the entire IRES, sequences coding for the foreign epitope and the adjoining viral polyprotein were PCR-amplified from cDNA with primers IRES-5′ and Seq-3’ for sequencing. One-step growth curves were performed according to established standards53,54.

**Rodent tumor models, immunization, T cell depletion.** All procedures involving vertebrate animals were performed under a Duke IACUC-approved protocol. Homozygous hCD115-tg C57Bl6 mice are maintained as a breeding colony; they were originally obtained from S. Koike57. 5 × 105 B16F10.9-OVA murine melanoma cells were implanted orthotopically in hCD115-tg mice56. AAD-tg C57Bl6 mice (Jackson Labs, strain #004191) express an interspecies hybrid class I MHC gene, AAD, which contains the a-1 and -2 domains of the human HLA-A2.1 gene and the a3 transmembrane and cytoplasmic domains of the mouse H-2Db gene, under the direction of the human HLA-A2.1 promoter. Heterozygous hCD115- and AAD-tg mice were obtained by crossing hCD115-tg mice with AAD-tg mice. Murine CT2A AAD-H3.K27M malignant gliomas were implanted intracerebrally in hCD115/AAD-tg mice as follows. Mice were anesthetized (isoflurane), scalp sterilized (betadine and 70% ethanol) and an incision was made along the sagittal suture to expose the bregma. A hole was drilled in the skull near the right coronal suture (2 mm right/0.5 mm anterior to the bregma) and a 30 gauge needle was used to inoculate 106 CT2A AAD-H3.K27M cells in 5 μl of methylcellulose suspension (at 3.33 μl/min) at a depth of 3.6 mm. Mice were monitored for clinical symptoms and neurological deficits, and weighed daily after tumor implant; mice were euthanized when they had lost 15% of their max. weight or when they became symptomatic as defined in our IACUC-approved protocol. For i.m. vector immunizations, mice 6–12 weeks of age were inoculated in the gastrocnemius muscle (108–109 pfu). A boost dose was administered on day 14 in
the contralateral leg (10^5–10^7 pfu). For SiINFEKl immunization, 50 μg of poly(1:10) (high molecular weight Vaccograde; Invivogen) was mixed with 50 μg of SiINFEKl peptide (Invivogen) for immunization/boost as described above. For H3.3K27M peptide immunization, 50 μg of RSMAAPGGV (Peptide 2.0) was emulsified in Complete Freund’s Adjuvant (Sigma) and administered intraperitoneally. A boost dose was administered on day 14 with 50 μg of RSMAAPGGV emulsified in Incomplete Freund’s Adjuvant (Sigma). For CD8 T cell depletion, anti-CD8 antibody (BioXCell) was administered on day 7 before tumor implantation, and continuing every 4 days thereafter.

**Immunoblot/inmunoprecipitation, flow cytometry, and IHC.** Immunoblots and immunoprecipitation were performed using standardized protocols with the antibodies listed in Supplementary Table 2. For flow cytometry, cells were washed and resuspended in 100 μL FACS buffer (phosphate-buffered saline, 2% fetal bovine serum) and 10 μg/mL of antibody (Invitrogen) were added (FC-blocked; BioLegend), stained with the appropriate antibodies (Supplementary Table 2), washed and resuspended in 250 μL FACS buffer to be analyzed on a BD LSRFortessaX20. Compensation and data were analyzed using Flowjo. H2Kb-SiINFEKl pentamer (Promimmune) staining was performed with blood seven days post boost-immunization according to manufacturer. The HLA-A2+RSMAAPGGV tetramer was synthesized by the NIH Tetramer Core Facility. hCD155_AAD-tg mice were immunized with vector or peptide and bled one week after boost. Blood was lysed with ACK Lysing buffer (Gibco, A1049201), washed (FACs buffer), stained with tetramer (30 min, 20 °C), washed, FC-blocked (see above), stained with CD8, CD19 and CD11b antibodies and analyzed as described above. For IHC, lymph nodes were fixed in 100% formalin, embedded in paraffin, cut (3 μm), mounted on slides, deparaffinized, rehydrated, stained with anti-CD8, anti-CD19 and anti-CD11b antibodies and analyzed as described above. For IHC, lymph nodes were harvested from euthanized mice, fixed in 4% paraformaldehyde, dehydrated in 70% ethanol, paraffin-embedded and sectioned (7 μM). Mounted sections were stained using the Ventana Discovery Ultra platform (Research Immunohistochemistry Lab, Duke Dept. of Pathology).

**ELISA, cytokine bead array, and ELISPOT.** We used Ready-Set-Go ELISA kits (ThermoFisher) for Granzyme B, IFNγ (and human) IL2 ELISAs. Cytokine Bead Arrays were performed using Bioplex mouse (#740061) and human (#740390) antiviruses LEGENDplex kits. Mouse skeletal muscle tissues were minced in 500 μL of DMEM medium, centrifuged, and the resulting supernatants were used for LEGENDplex analyses. For ELISPOTs, plates (Millipore, MSISP4W10) were pre-activated with 35% ethanol, washed 6 times with PBS, and coated with 10 μg/mL of antibody (Invitrogen; AN18, 12.4, 4C). Plates were blocked with R10 medium [RPMI, 10% FBS, non-essential amino acids, 1 x 2-mercaptoethanol (Gibco 21985023) (2 h, 37 °C) prior to splenocyte addition. Splenocytes were processed into single cell suspension, red blood cells lysed using ACK Lysing buffer, and splenocytes washed with R10 medium. Cells were plated at a density of 5 x 10^5 well in triplicate, and remained unstimulated or stimulated with 10 μg/mL of peptide [OVA257–264 (Invitrogen); myeloid-oligodendrocyte glycoprotein (MOG55–75) (Sigma)]; 4 μg/mL ConcanaValin A (Sigma) and incubated (24 h, 37 °C). After incubation, the plates were washed with PBS/0.05% Tween, incubated with biotin-labeled anti-mouse IFNγ (R4-6A2 (BioTech); 1 μg/mL (2 h, 37 °C), washed, treated with Avidin Peroxidase Complex (VectaStain, 1 h) and developed with AEC ELIspot substrate. All ELISPOT assays were evaluated externally by ZellNet Consulting.

**Cell lines, primary cultures, co-culture assays.** Cell lines: We used HuLa R19-11 and HEK293 cells for virus propagation and one-step growth curve assays. B16F10.9 murine melanoma cells were obtained from ATCC; derivation of HEK293 cells for virus propagation and one-step growth curve assays. Primary cultures: Primary cultures were established from wild type and H3.3(K27M)-mutated brain tumours of H3.3K27M-, H3.3(K27M)-mutated DIPG patient

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Additional information

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