


# Leveraging immune memory against measles virus as an antitumor strategy in a preclinical model of aggressive squamous cell carcinoma

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## ABSTRACT

Viral antigens are among the strongest elicitors of immune responses. A significant proportion of the human population already carries pre-existing immunity against several childhood viruses, which could potentially be leveraged to fight cancer. We sought to provide proof of concept in mouse models that a pre-existing measles virus (MeV) immunity can be redirected to inhibit tumor growth by directly forcing expression of cognate antigens in the tumor. To this end, we designed DNA vaccines against known MeV cytotoxic and helper T epitopes, and administered these intradermally to mice that were subsequently challenged with syngeneic squamous cancer cells engineered to either express the cognate antigens or not. Alternatively, established wild-type tumors in vaccinated animals were treated intratumorally with in vitro transcribed mRNA encoding the cognate epitopes. Vaccination generated MeV cytotoxic T lymphocyte (CTL) immunity in mice as demonstrated by enhanced interferon gamma production, antigen-specific T cell proliferation, and CTL-mediated specific killing of antigen-pulsed target cells. When challenged with syngeneic tumor cells engineered to express the cognate antigens, 77% of MeV-vaccinated mice rejected the tumor versus 21% in control cohorts. Antitumor responses were largely dependent on the presence of CD8<sup>+</sup> cells. Significant protection was observed even when only 25% of the tumor bulk expressed cognate antigens. We therefore tested the strategy therapeutically, allowing tumors to develop in vaccinated mice before intratumoral injection with Viromer nanoparticles complexed with mRNA encoding the cognate antigens. Treatment significantly enhanced overall survival compared with controls, including complete tumor regression in 25% of mice. Our results indicate that redirecting pre-existing viral immunity to fight cancer is a viable alternative that could meaningfully complement current cancer immune therapies such as personalized cancer vaccines and checkpoint inhibitor blockade.

## BACKGROUND

Prophylactic vaccination, arguably one of the most notable achievements of modern

medicine,<sup>1</sup> works because our immune systems can be educated to recognize and remember an antigen. The pre-existing immunity generated by vaccination is recalled on subsequent re-exposure to the antigen, resulting in a more rapid and vigorous defense response. Based on this simple yet ingenious tenet, the implementation of mass vaccination programs has accounted for the near-elimination of common childhood virus-mediated diseases that once claimed millions of lives globally. Consequently, a significant proportion of the human population, especially in developed countries, carries a pre-existing immunity against these viruses.

In the context of cancer, active immunization has shown efficacy in preventing malignancies associated with tumorigenic viruses, such as human papilloma virus and hepatitis B virus, principally by protecting the host from infection. For tumors without viral etiology, cancer vaccines can only be applied therapeutically, when the tumor is already present. In this setting, several factors critically impact the success of cancer vaccination. One such factor is the immunosuppressive state tumors induce in patients by actively engaging inhibitory checkpoints and tolerance mechanisms designed to protect the host from self-damage.<sup>1,2</sup> Cancer vaccines based on tumor-associated antigens, which are largely derived from ‘self’ proteins that are overexpressed in cancer cells,<sup>2,3</sup> exhibit limited efficacy because they are unable to effectively circumvent this tolerogenic state. As such, the choice of antigen represents the single most important component of cancer vaccine design.<sup>2</sup> Ideal antigens include foreign antigens, for example, those derived

from viruses, and neo-antigens arising from mutated proteins, as neither are subject to central tolerance. While certain mutations are associated with specific tumors, every tumor has a unique mutational landscape, with only a fraction of mutations shared between patients.<sup>4</sup> This has spurred strategies that combine next-generation sequencing approaches (to map the entire tumor mutanome) with computational methods (to rationally select vaccine targets), in order to generate personalized vaccines. Such strategies have shown impressive success and great promise in patients with melanoma.<sup>5,6</sup> Yet, they are unlikely to be accessible to the majority of patients with cancer.

Instead, we rationalized that pre-existing infectious immunity against common childhood pathogens, such as measles virus (MeV), can be leveraged to effectively fight established tumors. The immune memory generated prior to the establishment of a tumor-mediated suppressive microenvironment could be recalled by directly forcing expression of cognate antigens in the tumor. Here, we provide proof of concept of the feasibility of this approach in a preclinical model of aggressive squamous cell carcinoma (SCC).

## METHODS

All methods, including ethics approvals, are described in detail in the online supplemental information.

### DNA vaccines

DNA plasmid vaccines used in this study are depicted in figure 1, and the description of their generation and the amino acid sequences encoded by each shown in online supplemental figure S1. The vaccines included coding sequences for the murine interleukin 2 (IL-2) signal sequence, and cytotoxic T lymphocyte (CTL) (H-2K<sup>k</sup>-restricted, N52-60 and N81-88) and T helper (Th) (F288) epitopes derived from MeV nucleocapsid (N) and fusion (F) proteins previously shown to be immunogenic,<sup>7</sup> subcloned into the pCI vector (Promega, Madison, Wisconsin, USA).

### MeV immunization

All experiments were performed in 4–6-week-old female C3H/He mice (haplotype H-2K<sup>k</sup>). Mice were immunized intradermally three times, 14 days apart, via gene gun-mediated delivery of DNA vaccines. At day 7 after the final boost, we evaluated the quality of the immune responses generated by standard interferon gamma (IFN $\gamma$ )-ELISPOT, T cell proliferation, and in vivo CTL assays (figure 1B).

### Tumor challenge

For tumor challenge experiments, mice were intradermally injected with syngeneic tumor cells (SCCVII)<sup>8</sup> that had been engineered to express the MeV cognate antigens or not. Where indicated, mice were injected intraperitoneally with a CD8-depleting antibody (clone YTS 169.4,

BioXCell) 1 day prior to tumor injection (day –1), and at days 3 and 7 post-tumor injection. Mice were monitored every 2 days for tumor development and sacrificed when tumor volume was  $\geq 500 \text{ mm}^3$ . Time to development of measurable tumors was noted as tumor-free skin survival.

For treatment studies, the indicated therapeutic molecules were injected directly into the tumor mass. Briefly, complexing of RNA (either in vitro transcribed mRNA or a control non-encoding RNA oligonucleotide as indicated) to Viomer IN VIVO nanoparticles (Lipocalyx GmbH) was performed according to manufacturer's protocols and used within 2 hours. Each tumor was treated with 8  $\mu\text{g}$  RNA in a 25  $\mu\text{L}$  volume.

## Statistics

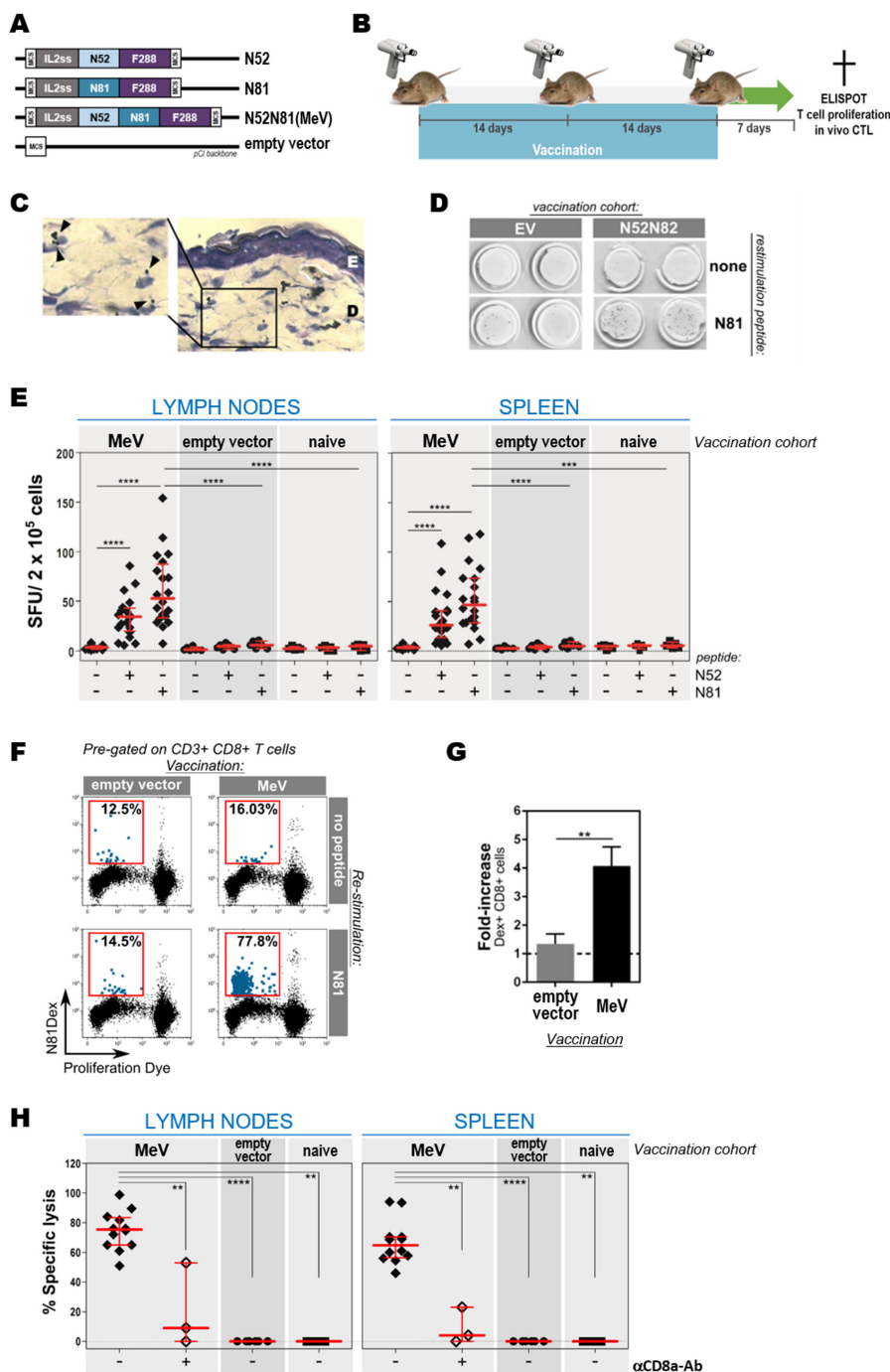
All statistical analyses were performed using GraphPad Prism software. Normal distribution of the data was evaluated prior to applying parametric or non-parametric tests. Statistical tests performed are indicated in figure legends. Significance levels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

## RESULTS

### Establishment of a murine model for MeV cell-mediated immunity

We first established MeV immunity in C3H/He mice (online supplemental information) by delivering vaccine vectors encoding both CTL and T helper MeV-derived epitopes intradermally to the dendritic cell-rich region of the skin by gene bombardment (figure 1A–C), in line with accumulating evidence of the efficacy of this immunization route in the context of cancer vaccination, as well as in the generation of memory tissue-resident T cells.<sup>9,10</sup>

We confirmed the quality of the immune responses generated by IFN $\gamma$ -ELISPOT, T cell proliferation, and in vivo CTL assays (figure 1D–H). Briefly, vaccination of mice with a tandem CTL construct N52N81 (henceforth also referred to as MeV vaccine) resulted in an increase in IFN $\gamma$ -producing cells in both lymph nodes and spleens (figure 1D,E, online supplemental figure S2). IFN $\gamma$  production was most robust when restimulation was performed with the N81 peptide (online supplemental figure S2), which was identified in these experiments to be the immunodominant CTL epitope. This allowed us to generate a fluorescein isothiocyanate-labeled H-2K<sup>k</sup>-N81 dextramer (N81Dex), which we used to identify antigen-specific CD8<sup>+</sup> T cells from MeV-vaccinated mice by flow cytometry (online supplemental figure S3A). In T cell proliferation assays, CD8<sup>+</sup> N81Dex<sup>+</sup> cells demonstrated enhanced proliferation upon restimulation with the cognate peptide in vitro (figure 1F,G, online supplemental figure S3B–D). We additionally confirmed that humoral responses against N81 were not generated by this immunization protocol (online supplemental figure S3E). Finally, in in vivo functional CTL-killing assays, adoptive transfer of N81-pulsed and unpulsed splenocytes into MeV-vaccinated mice resulted in specific depletion



**Figure 1** Vaccination induced robust MeV CTL immunity in mice. (A) MeV DNA plasmid vaccine was designed to encode the IL-2 signal sequence (IL2ss), H-2K<sup>k</sup>-restricted CTL epitopes (N52 and N81) and a promiscuous helper T cell (Th1) epitope (F288). (B) Mice were immunized with the indicated vaccines a total of three times, with 14 days between vaccinations, and the quality of the immunity generated was evaluated 7 days after the last vaccination via IFN $\gamma$ -ELISPOT, T cell proliferation, and in vivo CTL assays. (C) Gene gun immunization delivers vaccine-coated bullets (black triangles) to DC-rich dermis. E=epidermis, D=dermis. The lymph node and spleen cells isolated from vaccinated or naïve mice (MeV: n=20; empty vector: n=7; naïve: n=5) were cultured in IFN $\gamma$ -specific ELISPOT plates in the absence or presence of either N52 or N81 cognate antigenic peptides. Captured IFN $\gamma$ -Ab complex spots (D) were counted and plotted for each organ separately (E). A custom synthesized dextramer (N81Dex) was used to identify N81-specific CD8<sup>+</sup> T cells by flow cytometry. (F) Representative plots from T cell proliferation assays showing the percentage of the N81Dex<sup>+</sup> CD8<sup>+</sup> T cells with decreased proliferation dye intensity compared with day 0. (G) Graphical summary showing fold-increase in the percentage of dextramer (Dex)<sup>+</sup> CD8<sup>+</sup> T cells at the end of T cell proliferation assays following stimulation with N81-pulsed cells (empty vector: n=4; MeV: n=7). (H) In vivo CTL assays demonstrated up to 80% specific lysis of N81-pulsed target cells in MeV-vaccinated mice (n=11) compared with controls (empty vector: n=4; naïve: n=4), which was markedly reduced when MeV-immunized mice were pretreated with an anti-CD8 $\alpha$  blocking antibody (n=3). Statistical analyses to compare groups, Mann-Whitney (GraphPad Prism). \*P $\leq$ 0.05; \*\*p $\leq$ 0.01; \*\*\*p $\leq$ 0.001; \*\*\*\*p $\leq$ 0.0001. CTL, cytotoxic T lymphocyte; DC, dendritic cell; IFN $\gamma$ , interferon gamma; IL-2, interleukin 2; MeV, measles virus.



of only the antigen-pulsed target cells (figure 1H, online supplemental figure S4A,B). Pretreatment of mice with an anti-CD8 blocking antibody (online supplemental figure S4C) abrogated the effect, indicating that specific target-cell killing was mediated by the action of CD8<sup>+</sup> cells (figure 1H). Together, these data demonstrate successful establishment of a robust MeV CTL immunity in the mice.

### MeV-vaccinated mice reject tumors engineered to express cognate antigen

We next challenged MeV-vaccinated mice intradermally with syngeneic tumor cells that were engineered to express the cognate epitopes (SCCVII\_N81F288) (online supplemental figure S5A–C) and monitored tumor development (figure 2A). From our previous experience, intradermal injection of unmodified (parental) SCCVII cells into naïve C3H/He recipients led to engraftment rates of 84.6% (11/13 mice), with a median time to measurable tumor development of 9 days (data not shown). We observed similar engraftment rates of 80%–86% when mock-transduced cells (SCCVII\_mock) were intradermally injected into mice, regardless of whether the recipients had been immunized against MeV or not (table 1, cohorts A3 and A4). When SCCVII\_N81F288 cells were injected into mice that did not harbor MeV immunity (table 1, cohorts A1 and A2), tumor engraftment rates dropped to 58.9%–60%, which is attributable to the expression of immunogenic epitopes by the cells capable of stimulating de novo immune responses in the mice. Nevertheless, comparing these various control settings without match in MeV immunity status and MeV antigen expression on the engrafted tumor cells, no significant differences in tumor development were observed (table 1 and figure 2B). Altogether, 36 out of 53 mice (79%) in the various control cohorts (A1–A4) developed tumors within a median period of 13 days (table 1 and figure 2B,C).

In contrast, when MeV-vaccinated mice (n=22) were subsequently challenged with SCCVII\_N81F288 cells, only 5 (23%) developed tumors, while 77% remained tumor-free, representing a significant protection from tumor engraftment (table 1, cohort A5 and figure 2C,  $p=0.0002$ , Log-rank Mantel-Cox). CD8<sup>+</sup> cell depletion (online supplemental figure S6) prior to tumor cell transplantation completely abrogated the tumor protection afforded by vaccination, resulting in tumor development within a median period of 8 days (cohort A6 in table 1 and figure 2C,  $p<0.0001$ , Log-rank Mantel-Cox), indicating the importance of CD8-mediated interactions in the anti-tumor response. Of note, other immune cell subsets that might support the antitumor response, such as natural killer T cells and some dendritic cells, also express CD8a. Thus, the more rapid onset of tumors in this cohort potentially reflects the loss of all these different CD8<sup>+</sup> cell subsets.

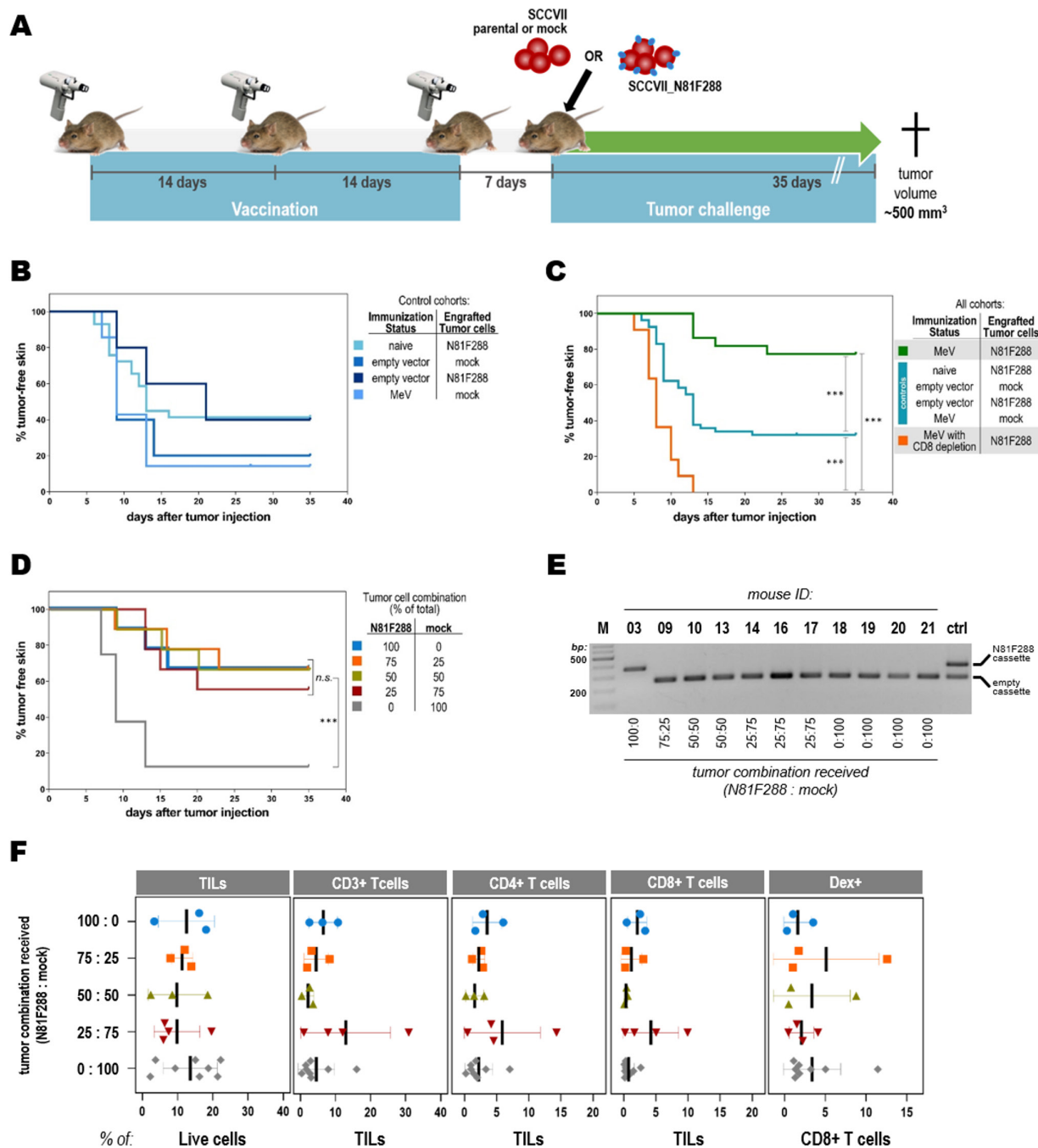
As the application of our approach in a therapeutic setting would rely on efficient delivery of the cognate epitopes into tumor cells, we investigated the level of epitope expression within the tumor bulk that would be

necessary to achieve significant protection. SCCVII\_mock and SCCVII\_N81F288 tumor cells were mixed at defined ratios and injected into MeV-vaccinated mice, which were then monitored for tumor development (table 1, cohorts B1–B5 and figure 2D). Mice injected with 100% SCCVII\_mock cells developed tumors with a prevalence of 87.5% in a median period of 9 days. In contrast, in all other cohorts, that is, when a fraction of the tumor cells comprised SCCVII\_N81F288 cells, the prevalence of tumor development ranged from 33.4%–44.4% (mean 36.1%,  $p<0.0001$ ). Intriguingly, we observed no significant differences in the level of protection whether the SCCVII\_N81F288 cells represented 25% or 100% of the bulk population (table 1, cohorts B1–B5 and figure 2D). PCR analyses of genomic DNA extracted from the tumors that developed revealed the loss of the N81F288 cassette, indicating specific antitumor responses and clearance of SCCVII\_N81F288 cells (figure 2E). In a proportion of the mice, this antigen-specific immunity also resulted in elimination of SCCVII\_mock cells. These results also suggest that an in vivo tumor transfection efficiency of 25%–50% would be sufficient to induce significant anti-tumor responses, a level achievable with current nanoparticle technologies.

We also investigated the presence of N81-specific CD8<sup>+</sup> T cells in tumors that developed in these MeV-vaccinated mice. Flow cytometric analyses of single cell suspensions following tumor dissociation demonstrated that CD8<sup>+</sup> N81Dex<sup>+</sup> T cells were present in all tumors that developed, regardless of whether or not the original inoculum contained N81-expressing cells (figure 2F, online supplemental figure S7A). At the end-point of this experiment, the relative distribution of N81Dex<sup>+</sup> cells within the CD8<sup>+</sup> T cell population did not appear to correlate with the level of N81 expression in the original tumor inoculum or the time to tumor development (data not shown). The observation that in MeV-vaccinated mice, CD8<sup>+</sup> N81Dex<sup>+</sup> T cells could also be found in tumors that developed entirely from SCCVII\_mock cells is in line with recently published data demonstrating that virus-specific memory T cells generated from previous exposure extend their surveillance to tumors that subsequently develop in the host.<sup>11</sup> Incubation of tumor slices, harvested from either MeV-vaccinated or naïve mice, with N81 peptide for 9 hours in vitro resulted in a more robust induction of IFN $\gamma$  in tumors from vaccinated mice as compared with naïve mice (online supplemental figure S7B,C), suggesting that memory CD8<sup>+</sup> T cells are already in place and ready to be reactivated and that they do not need to be recruited first into the tumor from the blood.

### In vivo tumor therapy controls tumor outgrowth

Viromer nanoparticles (Lipocalyx GmBH) represent a recently developed class of polymers designed for efficient nucleic acid delivery. Successful in vivo application and delivery of RNA molecules has been reported with these reagents in the context of vaccination,<sup>12</sup> immune cell reprogramming<sup>13</sup> and treatment of inflammatory



**Figure 2** Antitumor responses in MeV vaccinated mice. (A) SCCVII cells that either expressed the cognate MeV antigens (N81F288) or not (mock) were injected intradermally into MeV-vaccinated, empty vector-vaccinated, or unvaccinated (naïve) C3H mice. The development of tumors was monitored over 35 days. (B) Tumor-free survival of the different control cohorts (cohorts A1–A4 in table 1) without match between MeV immunization status and expression of cognate antigen on the engrafted tumor cells. No significant differences in time to tumor development were observed. Please refer to table 1 for sample numbers in each cohort. (C) Combined tumor-free skin survival of control cohorts (table 1 cohorts A1–A4) compared with experimental cohorts in which SCCVII\_N81F288 tumor cells were engrafted into MeV-vaccinated mice that had previously received anti-CD8 blocking antibody (cohort A6, table 1) or not (A5, table 1). Sample numbers in each cohort are listed in table 1. (D) SCCVII\_N81F288 and SCCVII\_mock cells were mixed at defined ratios, injected intradermally into MeV-vaccinated mice and tumor growth was monitored over 35 days. Time to tumor development is depicted. Sample numbers in each cohort are listed in table 1. (E) Genomic DNA was extracted from tumors that developed and PCR analyses demonstrated that cells carrying the N81F288 cassette were specifically deleted from the tumor bulk in MeV-vaccinated mice (ctrl=control, ie, PCR reaction with genomic DNA isolated from input cell mixture of 50:50, N81F288:mock). (F) Single cell suspensions were prepared from the tumors that developed (n=22) and analyzed by flow cytometry for the presence of N81Dex+CD8+ T cells. The relative distribution of lymphocytes, total CD3+ T cells, CD4+ and CD8+ T cells, and N81Dex+CD8+ T cells are depicted graphically. Statistical analyses, Log-rank (Mantel-Cox) test (GraphPad Prism). \* $P < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . MeV, measles virus; TILs, tumor-infiltrating lymphocytes.

**Table 1** Tumor engraftment experiments

## A. Engraftment of antigen-expressing (N81F288) or antigen-non expressing (mock) tumor cells

Cohort	n	Immunization status	Engrafted tumor cells	Median time to tumor development (days)	Tumor-free at 35 days
A1	29	Naïve	N81F288	13	12/29 (41.4%)
A2	5	Empty vector	N81F288	21	2/5 (40%)
A3	5	Empty vector	Mock	9	1/5 (20%)
A4	14	MeV	Mock	9	2/14 (14%)
A5	22	MeV	N81F288	Undefined	17/22 (77.3%)
A6	11	MeV with CD8+ depletion	N81F288	8	0/11 (0%)

## B. Tumor challenge with different N81F288:mock cell ratios

Cohort	n	Immunization status	Engrafted tumor cells (N81F288 : mock)	Median time to tumor development (days)	Tumor-free at 35 days
B1	9	MeV	100:0	Undefined	6/9 (66.7%)
B2	9	MeV	75:25	Undefined	6/9 (66.7%)
B3	9	MeV	50:50	Undefined	6/9 (66.7%)
B4	9	MeV	25:75	Undefined	5/9 (55.6%)
B5	9	MeV	0:100	9	1/8 (12.5%)

disorders.<sup>14</sup> First, we confirmed the suitability of this in vivo delivery reagent by complexing in vitro-transcribed (IVT) mRNA encoding enhanced green fluorescence protein (GFP) with Viomer IN VIVO reagent and direct injection of the complexes into a fully established intradermal tumor. A second tumor on the contralateral side of the mouse was left untreated as a control. Tumors were harvested 24 hours later and stained for GFP expression. Additionally, we stained for CD45 and CD11c in order to delineate immune cells and in particular dendritic cells within the tumor. Immunofluorescence microscopy showed that, compared with the untreated control, we could observe robust GFP expression throughout the tumor tissue with the Viomer IN VIVO transfection reagent (figure 3B), indicating that dendritic cells and other immune subsets can also be efficiently transfected with this reagent as previously observed.<sup>13 14</sup> Notably, presentation of cognate antigens by dendritic cells potentially results in a more efficient reactivation of memory CTLs.<sup>15</sup>

To simulate a cancer therapeutic setting, we engrafted MeV-vaccinated mice with SCCVII\_mock cells, which do not express the cognate epitopes, and waited for tumors to develop. Palpable tumors ( $\leq 40\text{mm}^3$ ) were treated with direct intratumoral injections (up to two injections, 4 days apart) of Viomer IN VIVO complexed with IVT-mRNA encoding the N81F288 cassette (Viomers/N81F288) (figure 3A). Treatment with either PBS or Viomer complexed with a non-coding RNA (Viomers/CTRL) served as controls. We monitored tumor development in mice over a 6-week period following treatment start. Mice in the PBS and Viomer/CTRL treatment cohorts showed rapid tumor growth, with all reaching the

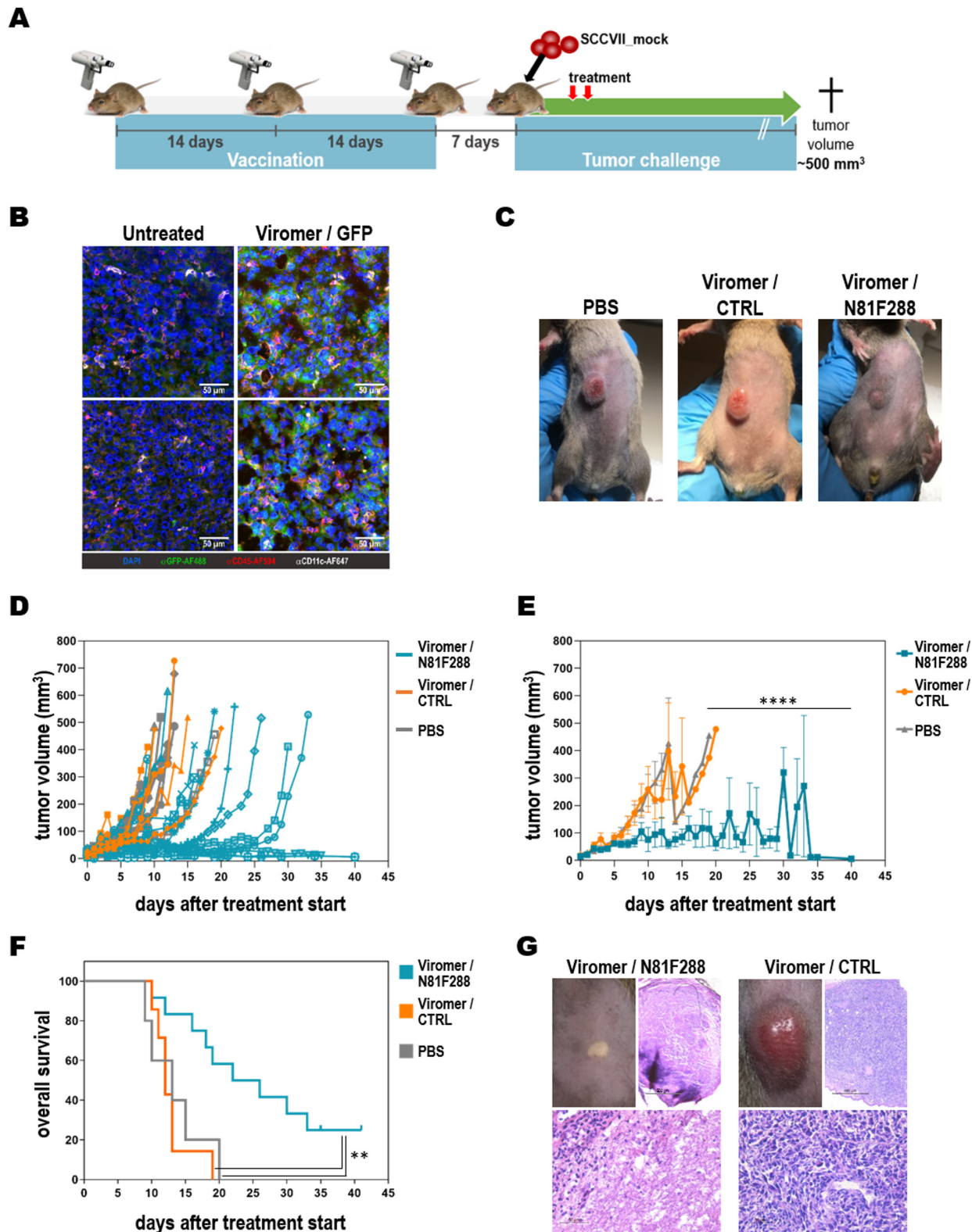
experimental endpoint (tumor volume  $\geq 500\text{mm}^3$ ) within 20 days of treatment start (figure 3C–E). In contrast, growth of tumors treated with Viomer/N81F288 was significantly reduced, resulting in a significant increase in overall survival of the mice compared with controls (Log-rank (Mantel-Cox) test, Viomer/N81F288 versus Viomer/CTRL,  $p=0.0079$ ; Viomer/N81F288 versus PBS,  $p=0.0029$ ) (figure 3F). In 3 of the 12 mice in this cohort (25%), tumor outgrowth was completely inhibited and at the end of the study, H&E staining revealed that the remaining mass consisted of epidermal (keratin) cysts (figure 3G).

## DISCUSSION/CONCLUSION

C3H/He mice engrafted with SCCVII cells are a well-established model of an aggressively growing tumor<sup>8</sup> with low immunogenicity.<sup>16</sup> Yet our data demonstrate that a re-called immune memory against a few MeV CTL and T-helper epitopes could be harnessed to effectively control tumor outgrowth and achieved a significant survival benefit following delivery of mRNA encoding the cognate epitopes directly into the tumor mass. With the continuing improvements in epitope-based vaccine design,<sup>4 17</sup> more robust responses can be expected with multiple epitopes, multiple treatments, improved application mode, and combination with other immune therapy strategies.

Beyond the requirement for CD8-mediated responses, there is currently incomplete understanding of the ongoing immune responses in this therapeutic setting, which represents a limitation of the study. Immunofluorescence analysis for CD4, CD8, CD11b, and Gr1





**Figure 3** In vivo tumor therapy. (A) Schematic of tumor therapy setting. SCCVII\_mock tumors ( $\leq 40 \text{ mm}^3$ ) developing in MeV-vaccinated mice were treated by intratumoral injections with PBS ( $n=7$ ), Viromer/CTRL ( $n=5$ ), or Viromer/N81F288 ( $n=12$ ) formulations and tumor development monitored for up to 6 weeks. (B) Immunofluorescence staining of GFP expression in tumor 24 hours after injection with Viromer/GFP complexes. (C) Representative pictures of tumor development in mice at day 10 after start of treatment. Mean tumor growth ( $\pm \text{SEM}$ ) observed in individual mice (D) and each treatment cohort (E) (Viromer/N81F288,  $n=12$ ; Viromer/CTRL,  $n=5$ ; PBS,  $n=7$ ) over time.  $P < 0.0001$ , one-way ANOVA repeated measures mixed effects model (REML, GraphPad Prism). (F) Overall survival of mice in each treatment cohort. Statistical analyses Log-rank (Mantel-Cox) test. Viromer/N81F288 vs Viromer/CTRL,  $p=0.0079$ ; Viromer/N81F288 vs PBS,  $p=0.0029$ . (G) Histological analyses of keratin cyst in a mouse treated with Viromer/N81F288 vs tumor treated with Viromer/CTRL complexes. CTRL, control; PBS, phosphate-buffered saline.

surface markers, as well as pan-cytokeratin, in outgrowing tumors harvested at the end point of the study (ie, when tumor volume reached approximately 500 mm<sup>3</sup>) could not detect observable differences in the immune infiltrate between treatments (online supplemental figure S8). However, this can be expected, since at this stage when tumor volumes are doubling within 24–48 hours, robust antitumor immune responses are likely no longer detectable. High-resolution profiling by single cell RNA-seq should be able to provide insight into the different immune subsets that contribute to ongoing antitumor effects observed during therapy and should be the focus of future studies. Such analyses will be important for the further development of this strategy by, for example, enabling the rationale design of combination therapies that either boost antitumor effector subsets or curb tumor-supportive cell populations.

While the success of recent trials based on personalized tumor neoantigen epitope vaccines ensures their place in the armamentarium of immune therapies against cancer, we believe the strategy we outline here offers some advantages. Viral antigens are among the strongest elicitors of immune responses and, therefore, have the potential to break tolerance and reprogram the immunosuppressive tumor microenvironment, a crucial aspect also leveraged by oncolytic viral therapy.<sup>18</sup> Recent evidence in mouse models shows the infiltration of tumors with virus-specific T cells following treatment with the FDA-approved oncolytic virus Talimogene laherparepvec (T-VEC).<sup>19</sup> T-VEC is a modified herpes simplex virus (HSV) that is administered intratumorally and has been engineered for enhanced and selective replication in tumor cells. It additionally carries two copies of the human GM-CSF gene to promote the development of systemic antitumor immunity. The additional influx of HSV-specific T cells into tumors potentially supports these antitumor mechanisms by changing the immunosuppressive tumor microenvironment into an immunologically reactive one. In this regard, intratumoral immunotherapy as a means of converting immunologically ‘cold’ tumors into ‘hot’ ones is increasingly reported in the literature. This includes intratumoral electroporation of an IL12-encoding plasmid (a.k.a. tavokinogene telseplasmid, Immuno-Pulse IT-tavo-EP) in advanced melanomas,<sup>20</sup> Merkel cell carcinomas,<sup>21</sup> and breast tumors.<sup>22</sup> IL-12 promotes adaptive type I cell-mediated immunity, which is the type of immune response triggered by viral infection. Control of tumor growth has recently been reported in a mouse model of melanoma following repeated intratumoral injections with the seasonal influenza vaccine.<sup>23</sup> Likewise, similar to the strategy we propose here, complete regression of several basaloid SCCs in an elderly patient was also recently reported by first generating systemic viral immunity by vaccination with Gardasil-9, followed by repeated injection of the vaccine intratumorally into the largest tumors over the course of 1 year.<sup>24</sup> As Gardasil-9 is a protein-based vaccine that contains no live oncolytic virus, the antitumor effects observed in this case were

likely initiated by the local recalled immune response to the viral proteins injected into the tumors, although this needs to be verified with immunophenotyping studies. A current limitation of intratumoral immunotherapy approaches is the need for repeated injections. Nevertheless, these observations suggest the potency of a strategy based on antiviral immunity, especially given the observation that virus-specific memory T cells from previous exposure are already present within developing tumors and can be repurposed for cancer immunotherapy.<sup>11</sup>

It has recently been reported that induction of regulatory B cell activity within the tumor, which results in production of immunosuppressive IL-10, can effectively counter antitumor immune responses.<sup>23</sup> Our strategy here favors the use of multiepitope vaccines based on CTL and Th epitopes, which is expected to induce more directed (ie, T cell-mediated) and potent immune responses, with a potential additional gain in safety.<sup>25</sup> While the immunogenic CTL epitopes of well-studied viruses such as MeV have been identified and validated based on decades of empirical evidence, the selection of immunogenic (ie, CTL) tumor neoepitopes currently relies on classical predictive algorithms that have been mostly validated on viral epitopes and may not be well suited to mutated self-peptides,<sup>26</sup> thereby requiring additional validation steps. Tumor heterogeneity also imposes a risk of selecting neoepitopes present only within certain tumor subclones. Additionally, tumor evolution, in part driven by constant immune pressure, might select for clones that have lost such highly immunogenic tumor epitopes, for example, by mutation, or alternatively by downmodulating expression of major histocompatibility complex (MHC) Class I or  $\beta$ 2 microglobulin, rendering the tumor ‘invisible’ to the immune system. While our strategy of forced robust expression of select cognate viral antigens in the tumor potentially circumvents the former, it would not be able to overcome the loss of tumor immunogenicity due to reduced antigen presentation. Of note, this limitation is shared by several immune therapeutic strategies that rely on CD8+ CTL responses for antitumor activity and has been shown to result in immune evasion and therapy failure.<sup>5 27–29</sup> However, molecules that can boost MHC Class I antigen presentation and such as IFN $\gamma$ ,<sup>30</sup> as well as other factors that might aid antitumor responses, including IL-12 as already described above,<sup>20–22</sup> IL-2,<sup>31</sup> or alarmins,<sup>32</sup> can additionally be encoded into the vector and delivered directly where they are needed to augment therapeutic efficacy.

Since the target antigens to be introduced into the tumor are known, this strategy also offers the opportunity to pre-engineer and bank patient-autologous antigen-specific CAR T cells ready for use. This may be particularly attractive for patient populations at high risk for cancer development, such as patients with the rare genetic skin fragility disease recessive dystrophic epidermolysis bullosa (RDEB). Continuous cycles of wounding and associated inflammation in RDEB drive the early onset of life-threatening cutaneous SCCs (median age at



diagnosis, 32 years) with an incidence of >90% by age 55 years,<sup>33</sup> warranting the development of prophylactic anti-tumor strategies for this patient group. However, RDEB tumors are not associated with any known viral etiology and mutational analysis has highlighted a mutation spectrum as complex as other tumor entities,<sup>34</sup> hindering the prediction of which mutations and mutagenic neoepitopes will likely arise in patients. From a clinical standpoint, the therapeutic strategy outlined here, which is based on intratumoral application, is also feasible for RDEB-SCC, which grows cutaneously and remains preferentially lymphogenic long before exhibiting distant metastases, as well as other inoperable skin tumors.

Leveraging pre-existing viral immunity to fight cancer would make efficient use of already existing personal defense arsenals within the population, adding an important perspective to ongoing discussions in the general population over mass vaccinations, particularly in the face of the current pandemic. Together with continuing advances in nanoparticle-based delivery technologies, the mRNA-based intratumoral strategy for repurposing of a pre-existing viral immunity as outlined here represents a viable alternative that can meaningfully complement current cancer immune-therapies, including personalized cancer vaccine and checkpoint inhibitor blockade.

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