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Fusciello, Manlio

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Manlio Fusciello,[1,2,3,6] Erkko Ylösmäki,[1,2,3,6] Sara Feola,[1,2,3] Arttu Uoti,[1,2,3] Beatriz Martins,[1,2,3] Karri Aalto,[4] Firas Hamdan,[1,2,3] Jacopo Chiaro,[1,2,3] Salvatore Russo,[1,2,3] Tapani Viitala,[4] and Vincenzo Cerullo[1,2,3,5]

[1]Drug Research Program (DRP) ImmunoViroTherapy Lab (IVT), Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Viikinkaari 5E, 00790 Helsinki, Finland; [2]Helsinki Institute of Life Science (HiLIFE), University of Helsinki, Fabianinkatu 33, 00710 Helsinki, Finland; [3]Translational Immunology Program (TRIMM), Faculty of Medicine Helsinki University, University of Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland; [4]Pharmaceutical Biophysics Research Group, Drug Research Program, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [5]Department of Molecular Medicine and Medical Biotechnology, Naples University "Federico II", S. Pansini 5, Naples, Italy

Common vaccines for infectious diseases have been repurposed as cancer immunotherapies. The intratumoral administration of these repurposed vaccines can induce immune cell infiltration into the treated tumor. Here, we have used an approved trivalent live attenuated measles, mumps, and rubella (MMR) vaccine in our previously developed PeptiENV cancer vaccine platform. The intratumoral administration of this novel MMR-containing PeptiENV cancer vaccine significantly increased both intratumoral as well as systemic tumor-specific T cell responses. In addition, PeptiENV therapy, in combination with immune checkpoint inhibitor therapy, improved tumor growth control and survival as well as increased the number of mice responsive to immune checkpoint inhibitor therapy. Importantly, mice pre-vaccinated with the MMR vaccine responded equally well, if not better, to the PeptiENV therapy, indicating that pre-existing immunity against the MMR vaccine viruses does not compromise the use of this novel cancer vaccine platform.

INTRODUCTION

The measles, mumps, and rubella (MMR) vaccine is a trivalent vaccine that contains live attenuated strains of MMR viruses. MMR vaccines are indicated for the routine immunization of children for the prevention of measles, mumps, and rubella. Recently, common vaccines for infectious diseases, such as a seasonal influenza vaccine, rotavirus vaccines, and a vaccine against yellow fever, have been repurposed as intratumoral immunotherapies for the modulation of the tumor microenvironment (TME).[1–3] By intratumoral administration of these viral vaccines, they were able to elicit immunostimulatory effects, such as enhancement of infiltration of cytotoxic T cells (CTLs), natural killer cells and CD4^{+} Th1 T helper cells, reduction of T regulatory cells and in some cases, exert oncolytic properties.[1–5] Immune checkpoint inhibitors (ICIs), a novel class of therapeutic antibodies that target immune checkpoint molecules such as programmed death 1 (PD-1), programmed death

ligand 1, and cytotoxic T lymphocyte-associated antigen 4, can activate pre-existing anti-tumor immune responses.[6] ICIs have demonstrated induction of durable tumor regression in some cancer patients, with response rates of 10%–25% in the majority of cancers.[7] The shared feature of the patients responsive to ICI therapy seems to be that they have an existing anti-tumor immunity and immune cell infiltration in the tumor tissue before ICI therapy.[8,9] This pre-exiting anti-tumor immunity can then be enhanced and rendered functional by the ICI therapy. However, the remaining 75%–90% of the patients are not responding owing to various immune-suppressive properties of the TME, such as the lack of immune cell infiltration into the TME.[10] As a consequence, novel therapies that can modulate the TME and enhance the infiltration of tumor-specific CD8^{+} T cells into the TME to increase the number of patients benefiting from the ICI therapy are much needed. PeptiENV cancer vaccine platform consists of clinically relevant (oncolytic) enveloped viruses combined with tumor antigen peptides containing envelope-attaching or -anchoring moieties. By physically attaching tumor antigen peptides onto the envelope of enveloped viruses, PeptiENV platform can broaden the virus-induced immune response more toward the cancer. The TME-modulating effects of intratumorally administered therapeutic viruses combined with tumor antigen peptide-driven immune response can enhance anti-tumor immunity and enhance the infiltration of tumor-specific T cells into the TME.[11]
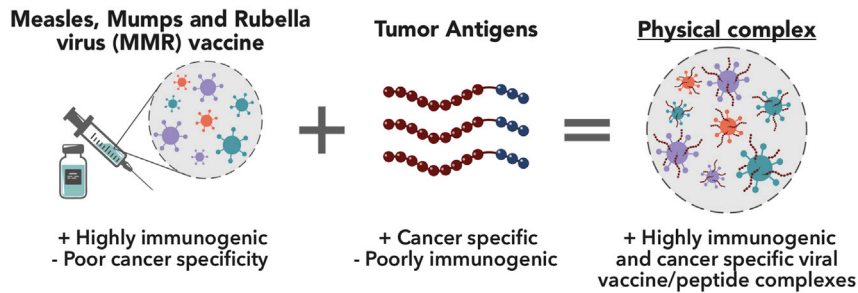
Figure 1. A schematic presentation of the PeptiENV cancer vaccine platform

Tumor antigens can be attached to the viral envelopes of MMR vaccine viruses by using a CPP sequence as an anchoring moiety. Anchor-modified peptides are complexed for 15 min with the MMR vaccine for allowing efficient attachment. Various different peptides, including MHC class I and II epitopes, can be delivered by the PeptiENV platform for potent activation of APCs and increased antigen-specific immunological responses.

Here we tested the use of an US Food and Drug Administration (FDA)/European Medicine Agency (EMA)-approved MMR vaccine (trade name Priorix) in the PeptiENV platform aiming to broaden the MMR vaccine-driven immune responses to include tumor antigens. The intratumoral administration of PeptiENV Priorix significantly increased the number of tumor-infiltrating tumor-specific T cells as well as systemic tumor-specific T cells. In addition, treatment with PeptiENV Priorix suppressed tumor growth in two murine models of melanoma. This platform was then tested in combination with ICI therapy. Although PeptiENV Priorix was efficient in controlling tumor growth as a monotherapy, combining PeptiENV Priorix with an anti-PD-1 ICI induced significant tumor growth control and increased the number of mice responding to ICI therapy. As a large number of people have been vaccinated with an MMR vaccine, we tested the efficacy of PeptiENV Priorix in mice pre-vaccinated with Priorix. Pre-existing immunity against the MMR vaccine did not decrease the efficacy of the PeptiENV Priorix platform; on the contrary, PeptiENV therapy in pre-vaccinated mice was very efficient and led to complete tumor rejection in one-half of the treated animals. The elegance of using the PeptiENV platform is that it allows the transformation of already approved vaccines, such as the MMR vaccine, or oncolytic enveloped viruses to be rapidly converted into efficient cancer vaccines.

RESULTS

MMR vaccine viruses can be coated with therapeutic peptides by using a cell-penetrating peptide sequence as an anchor

As all three attenuated virus strains of the MMR vaccine are enveloped viruses containing a lipid bilayer derived from the host cell membrane,[12] we hypothesized that therapeutic peptides could be attached onto the lipid bilayer of these enveloped viruses by using our recently developed peptide-based cancer vaccine platform PeptiENV[11] (see Figure 1 for a schematic presentation of the platform). For the attachment moiety, we used a cell-penetrating peptide (CPP) sequence derived from the HIV-1 Tat protein. This CPP sequence was tested by surface plasmon resonance (SPR) for its efficacy at anchoring therapeutic peptides onto the envelopes of the MMR viruses. The CPP moiety-containing peptide (CPP-ovalbumin [OVA]) had a robust affinity toward viral envelopes, and the affinity was specifically attributed to the CPP moiety, as peptide without the CPP moiety (OVA control peptide) had no affinity toward the viral envelopes (Figure 2A).

Antigen-presenting cells can readily present PeptiENV Priorix-delivered immunogenic antigens

Next, we tested whether PeptiENV platform can deliver therapeutic peptides into antigen-presenting cells (APCs) and if they can cross-present the MHC class I epitope from these peptides. Mouse dendritic cell (DC) line JAWSII was pulsed with PeptiENV-OVA Priorix, Priorix mixed with OVA control peptide, Priorix alone, or CPP-OVA peptide alone, and the efficiency of cross-presentation of the mature form of the epitope (SIINFEKL) was assessed by flow cytometry (Figure 2B). The efficacy of SIINFEKL cross-presentation delivered by PeptiENV-OVA Priorix (77% of the cells presenting) was similar to Priorix mixed with OVA control peptide (61%) and CPP-OVA peptide alone groups (84%), indicating that peptide coating does not affect the delivery or presentation of the peptides by the APCs. However, clear differences were seen in the ability to induce DC activation as measured by the increased expression of cluster of differentiation (CD) 86 and by the change in morphology toward more granular phenotype. Although the CPP-OVA peptide was efficiently presented by the DCs, no DC activation was seen. In contrast with the peptide administration, when Priorix was administered alone, robust DC activation, but not SIINFEKL, presentation was seen. When PeptiENV-OVA or Priorix mixed with OVA control peptide was administered, efficient presentation and DC activation was seen with both groups, indicating again that PeptiENV-OVA Priorix can act as a potent adjuvant for the delivered peptides.

Vaccination with PeptiENV Priorix induces a strong immune response against the therapeutic peptide coated onto the viral envelopes of the MMR viruses

To assess the significance of physically linking the therapeutic peptides onto the envelopes of the MMR viruses, we vaccinated naive C57BL/6JOlaHsd mice with Priorix coated with CPP-containing SIINFEKL peptide (PeptiENV-OVA Priorix) or Priorix mixed with OVA control peptide without the CPP moiety. Mice receiving only the Priorix vaccine or PBS (mock) were used as controls. After vaccination, mice were analyzed for the induction of systemic SIINFEKL-specific T cell responses by the interferon-gamma enzyme-linked immunospot (ELISPOT) assay (Figure 3). As expected, vaccination with Priorix or PBS did not induce any SIINFEKL-specific T cell responses. Vaccination with Priorix mixed with SIINFEKL peptide without the CPP moiety induced SIINFEKL-specific T cell responses, but the majority of the responses were moderate (average spot count 170 spots/10^6 cells). In contrast, PeptiENV Priorix-OVA induced
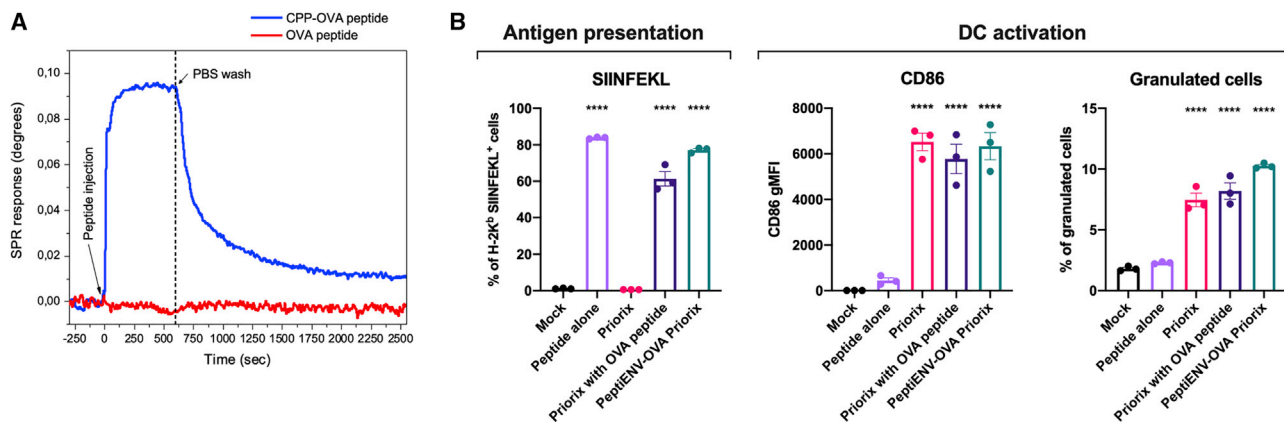
Figure 2. Characterization of the PeptiENV Priorix complexes

(A) SPR analysis of the interaction between the CPP-OVA and Priorix vaccine and OVA peptide without the attachment moiety and Priorix vaccine. (B) Mouse DC line JawsII was pulsed with PeptiENV-OVA Priorix, Priorix mixed with OVA peptide (a non-interacting control peptide), Priorix, CPP-containing SIINFEKL peptide alone or left unpulsed (Mock). Cross-presentation was determined by flow cytometry using APC-conjugated anti-H-2Kb bound to SIINFEKL. CD86 expression and changes in morphology of the cells (as measures of DC maturation and activation) were determined by flow cytometry. Each bar is the mean ± standard error of the mean of biological triplicates. Statistical analysis was performed with one-way ANOVA. ****$p < 0.0001$.

significantly enhanced SIINFEKL-specific T cell responses, highlighting the importance of physically linking the antigen and the adjuvant (average spot count 675 spots/10^6 cells).

Intratumoral PeptiENV-OVA Priorix treatment induces a robust systemic and intratumoral infiltration of tumor-specific T cells in a syngeneic mouse model of B16.OVA

To study the effects of the PeptiENV platform with Priorix on the anti-tumor immunity, we used a syngeneic mouse melanoma model B16 expressing chicken OVA as a model antigen.[13] Tumor-bearing mice were treated intratumorally with OVA-targeting PeptiENV (PeptiENV-OVA Priorix), Priorix, peptides alone or vehicle (mock). Treatment with PeptiENV-OVA Priorix showed a modest increase in tumor growth control as compared with the other treatment groups (Figure 4A). A tumor size threshold of 500 mm³ was set for defining the responders in each treatment group. CPP-containing SIINFEKL peptide alone, Priorix, and mock treatments did not have a significant effect on tumor growth, with two mice defined as responders to the therapy in each group. In contrast, PeptiENV-OVA Priorix treatment had a minor effect on tumor growth with five mice responding to the therapy. Next, we assessed the immunological effects induced by the different treatments. We did not see any differences in the number of tumor-infiltrating lymphocytes or in the number of cytotoxic CD8+ T cells infiltrated into the tumors between the different treatment groups. However, PeptiENV-OVA Priorix-treated mice had a significantly enhanced infiltration of tumor-specific CD8+ T cells into the TME as compared with the tumors of Priorix-, peptide alone-, or mock-treated mice (Figure 4B). In addition, we saw an increase of tumor-specific CD8+ T cells in tumor-draining lymph nodes of PeptiENV-OVA-treated animals as compared with the other groups (Figure 4C). In marked contrast with the Priorix-, peptide alone-, and mock-treated mice, a significant induction of

a systemic OVA-specific T cell response was also seen in the PeptiENV-OVA Priorix-treated mice (Figure 4D).

Intratumoral treatment with PeptiENV-Trp2 Priorix increases the number of responders to anti-PD-1 therapy, greatly improves tumor control, and induces systemic anti-tumor immune responses in a syngeneic mouse model of B16.F10.9/K1 melanoma

Next, we tested the PeptiENV Priorix platform in a syngeneic mouse model of B16.F10.9/K1 melanoma using a tumor-associated antigen derived from tyrosinase-related protein 2 (Trp2$_{180-188}$) in combination with anti-PD-1 ICI therapy. The B16.F10.9/K1 melanoma is a derivative of a B16.F10.9 melanoma that is engineered to be more responsive to cancer immunotherapies than the highly immunosuppressive parental strain.[14] Starting at 7 days after tumor engraftment, mice were treated with Priorix, PeptiENV-Trp2 Priorix, anti-PD-1 alone, Priorix in combination with anti-PD-1, or PeptiENV-Trp2 Priorix in combination with anti-PD-1 or were left untreated (mock). Priorix and PeptiENV-Trp2 Priorix treatments were given intratumorally and anti-PD-1 therapy was given intraperitoneally. Here, with the more immunogenic tumor model, a tumor size threshold of 250 mm³ was set for defining the responders in each treatment group. In contrast with the mock-treated animals, the Priorix- and anti-PD-1-treated groups showed modest tumor growth control with response rates of 10% and 18%, respectively. Priorix in combination with anti-PD-1-treated animals showed moderate tumor growth control with a 45% response rate. PeptiENV-Trp2 Priorix-treated animals showed efficient tumor growth control with a response rate of 60%. PeptiENV-Trp2 Priorix in combination with anti-PD-1-treated animals showed the most efficient tumor growth control, with a 91% response rate. By using this combination, the response rate for anti-PD-1 therapy was efficiently increased from 18% to 91%
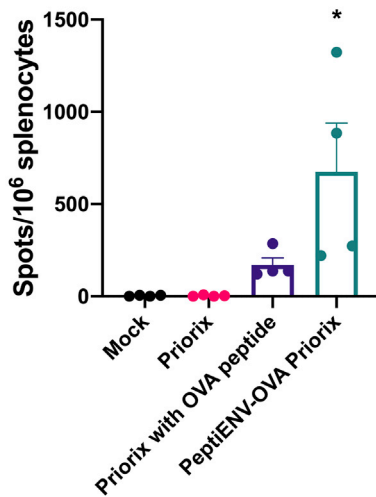
Systemic OVA-specific T cell response

Figure 3. PeptiENV enhances antigen-specific T cell responses by physically linking tumor antigen and the biological adjuvant

Naïve C57BL/6JOlaHsd mice (n = 4/group) were immunized with PeptiENV-OVA Priorix, Priorix mixed with OVA peptide (a non-interacting control peptide), Priorix, or PBS as a mock-treated group on days 0, 2, and 15. Five days after the last treatment, mice were sacrificed, and spleens were collected for the quantification of activated interferon-gamma (IFN-γ) secreting CD8$^+$ CTLs specific for the tumor epitope (SIINFEKL) by using a mouse interferon-gamma ELISPOT assay. Each bar is the mean ± standard error of the mean of biological quadruplicates. Statistical analysis was performed with one-way ANOVA. *$p < 0.05$.

(Figure 5A). All treatment groups increased the survival of the animals as compared with the mock group (Figure 5B); however, treatment with PeptiENV-Trp2 Priorix or PeptiENV-Trp2 Priorix in combination with anti-PD-1 increased most efficiently the survival of the animals, and by the time all mice in the mock group had died, 100% of the PeptiENV-Trp2 Priorix in combination with anti-PD-1-treated mice were still alive. To study systemic anti-tumor immunity elicited by the different treatments, surviving mice were rechallenged with a very high amount (1.2 × 10^6, twice the initial dose) of the same tumor cells into the contralateral flank. All naive mice (n = 5), used as engraftment controls, showed robust tumor growth and at day 7 after the rechallenge, had developed tumors with an average volume of 50 mm^3. In contrast, rechallenged mice in the Priorix in combination with anti-PD-1 (n = 5), PeptiENV-Trp2 Priorix (n = 5), and PeptiENV-Trp2 Priorix in combination with anti-PD-1 (n = 7) groups had significantly smaller tumors, with average volumes of 12.6 mm^3, 17.9 mm^3, and 10.9 mm^3, respectively. In addition, 20% of rechallenged mice in Priorix in combination with anti-PD-1 and in PeptiENV-Trp2 groups did not show secondary tumor growth as compared with 43% in the PeptiENV-Trp2 Priorix in combination with anti-PD-1 group, indicating an induction of a systemic anti-tumor immunity by these treatment modalities (Figure 5C).

Immunization of mice with MMR vaccine before intratumoral treatment with PeptiENV-Trp2 Priorix does not negatively affect treatment efficacy

Finally, we tested the effects of pre-existing MMR vaccine immunity on the anti-tumor efficacy of PeptiENV-Trp2 Priorix treatment. A cohort of mice were vaccinated with a dose equivalent to a dose given to humans (one vaccination dose) subcutaneously two times with 21 days between doses. A cohort of mice was left unvaccinated and used as naive controls. Fourteen days after the final vaccine dose, vaccinated mice and naive control mice were subcutaneously engrafted with B16.F10.9/K1 melanoma. Starting at 7 days after tumor engraftment, mice were treated with PeptiENV-Trp2 Priorix or left untreated (mock). Again, the tumor size threshold of 250 mm^3 was set for defining the responders in each treatment group. In contrast with vaccine-naive Mock-treated animals that did not show tumor growth control, the vaccine-naive PeptiENV-Trp2 Priorix-treated group showed efficient tumor growth control with a response rate of 63% (identical response rate as in the previous experiment). Similar to vaccine-naive Mock-treated animals, vaccinated Mock-treated animals did not show tumor growth control (only one mouse displayed tumor growth control, or poor tumor engraftment). In striking contrast with the Mock-treated animals, vaccinated PeptiENV-Trp2 Priorix-treated animals showed very robust tumor growth control (90% response rate), with 50% of mice completely rejecting the implanted tumor (50% complete response rate) (Figure 6). To assess the pre-vaccination-induced cellular immunity and PeptiENV-induced tumor-specific cellular responses, antigen-specific IFN-γ secretion was measured from splenocytes of the treated mice (Figure S1). Pre-vaccinated mice showed robust IFN-γ secretion upon stimulation with the Priorix vaccine, indicating efficient immunization by two doses of the Priorix vaccine. As expected, robust Trp2 peptide antigen-specific cellular responses were seen in the PeptiENV-Trp2 Priorix-treated mice.

DISCUSSION

In this study, we have exploited the immunostimulatory characteristics of a safe and widely used FDA- and EMA-approved vaccine against measles, mumps, and rubella, the MMR vaccine trade named Priorix. One vaccination dose of Priorix contains very low amounts of highly attenuated strains of MMR viruses. As all these vaccine viruses are enveloped, we set up to test the suitability of Priorix vaccine in our recently developed peptide-based cancer vaccine platform for enveloped viruses PeptiENV.[11] By using SPR analysis, we showed that the CPP sequence derived from HIV Tat protein can efficiently act as an attachment moiety when fused to the N-terminus of tumor antigens. Based on our earlier study, the addition of the CPP sequence to the therapeutic peptides does not affect the presentation of tumor epitopes from these CPP-containing peptides.[11] By using an immunodominant epitope from chicken OVA as a model antigen, we were able to study the characteristics of antigen presentation and concomitant DC activation by the PeptiENV platform. PeptiENV-OVA Priorix was able to efficiently deliver tumor antigens into DCs and induce their robust activation. Similar characteristics were also seen with Priorix mixed with OVA control peptide lacking the CPP attachment
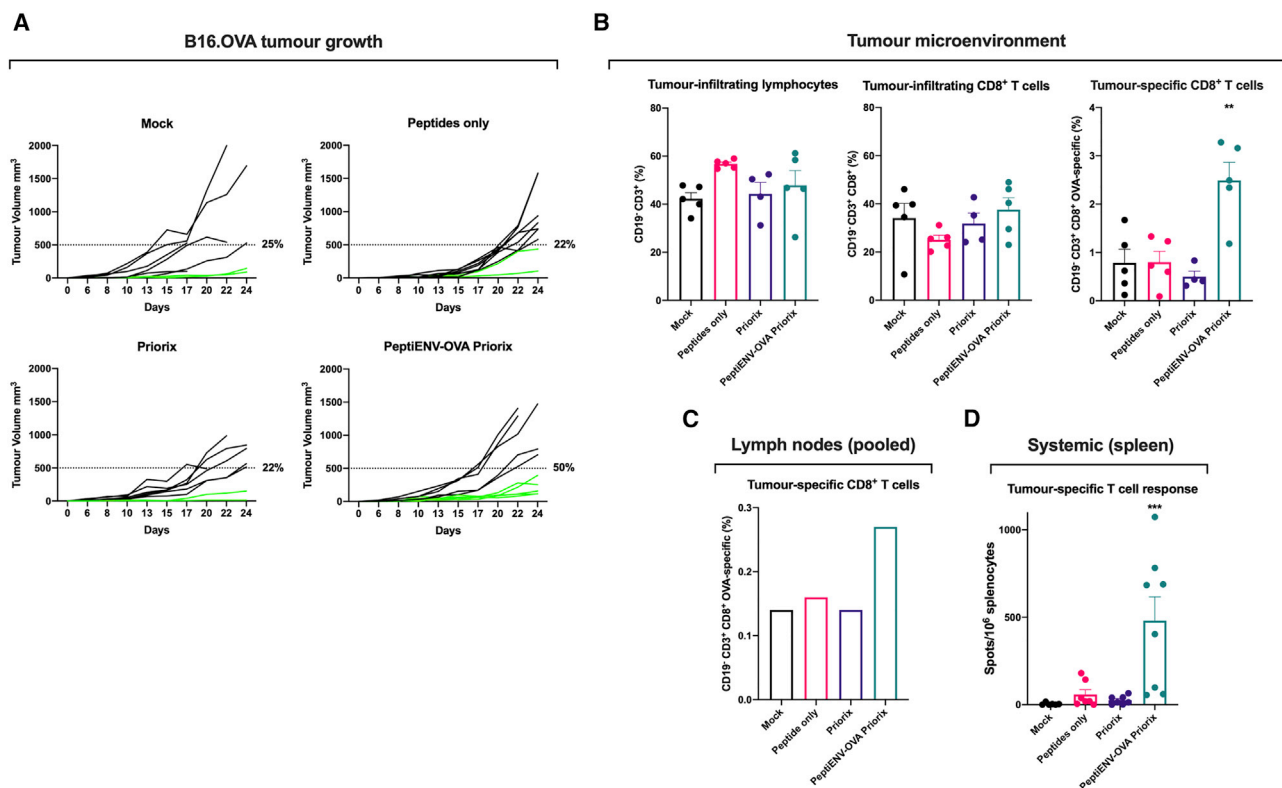
Figure 4. Intratumoral treatment with PeptiENV-OVA Priorix induces intratumoral and systemic tumor-specific T cell responses in a syngeneic mouse model of B16.OVA melanoma

(A–D) PeptiENV-OVA Priorix, Priorix, peptides alone or PBS as a mock was given intratumorally 6, 8, and 20 days after tumor implantation. Individual tumor growth curves for all treatment groups are shown. The number of mice in each group was 7–10. The percentage of responders is shown on the right side of the dotted line. Immunological analysis of (B) tumors (C) lymph nodes and (D) spleens of treated mice. Statistical analysis was performed with one-way ANOVA. **$p < 0.01$, ***$p < 0.001$.

moiety. These *in vitro* results are expected; pulsing DC monolayers in a cell culture dish does not take into account the differences in diffusion kinetics of peptides and the biological adjuvant (Priorix). Indeed, in *in vivo* vaccination setting, when the tumor antigen was physically linked to the Priorix viruses (PeptiENV-OVA Priorix), we saw significantly improved OVA antigen-specific T cell responses as compared with the situation where the peptide and Priorix did not have a physical linkage (Priorix with OVA peptide). The clear difference in OVA antigen-specific T cell responses between the non-linked (Priorix with OVA peptide) and the physically linked (PeptiENV-OVA Priorix) complexes is likely due to the limited amount of Priorix as the biological adjuvant, and in situations where the adjuvant is limited (such as when using low viral doses or intravenous administration) it might be highly beneficial to physically link the antigen to the adjuvant to increase the changes of them to reach the same APC for efficient induction of T cell responses.

After establishing the parameters of the physical complex and immunostimulatory potential of PeptiENV-OVA Priorix, we went on to test the effects of the PeptiENV Priorix platform on tumor growth and OVA antigen-specific T cell induction in a murine melanoma model of B16.OVA (expressing chicken OVA as a model antigen). Although

we saw only a modest tumor growth control with PeptiENV-OVA Priorix, we saw a significant increase in systemic and intratumoral OVA antigen-specific T cells as well as an increased number of OVA antigen-specific T cells in tumor-draining lymph nodes. These results prompted us to test the PeptiENV Priorix platform in combination with ICIs, as these therapies can benefit from the increased immune cell infiltration into the TME.[9] To test the effects of the PeptiENV Priorix platform in combination with anti-PD-1 ICI on tumor growth and tumor-specific T cell induction, we used a syngeneic mouse model of B16.F10.9/K1 melanoma together with a more relevant tumor epitope derived from tumor-associated antigen Trp2$_{(180–188)}$. Interestingly, both Priorix and anti-PD-1 monotherapies had a very minimal effect on tumor growth control; however, the combination therapy (Priorix + ICI) had an enhanced effect on tumor growth control. However, already as a monotherapy, PeptiENV-Trp2 Priorix had a robust effect on tumor growth control, and, when combined with anti-PD-1 therapy, the antitumoral effects were further enhanced.

Vaccine strains of measles and mumps have been extensively tested for oncolytic cancer virotherapy.[15,16] However, the number of viral particles given intratumorally have typically been at the level of 10^6 infectious viral particles per dose, which is 100–1000 times more
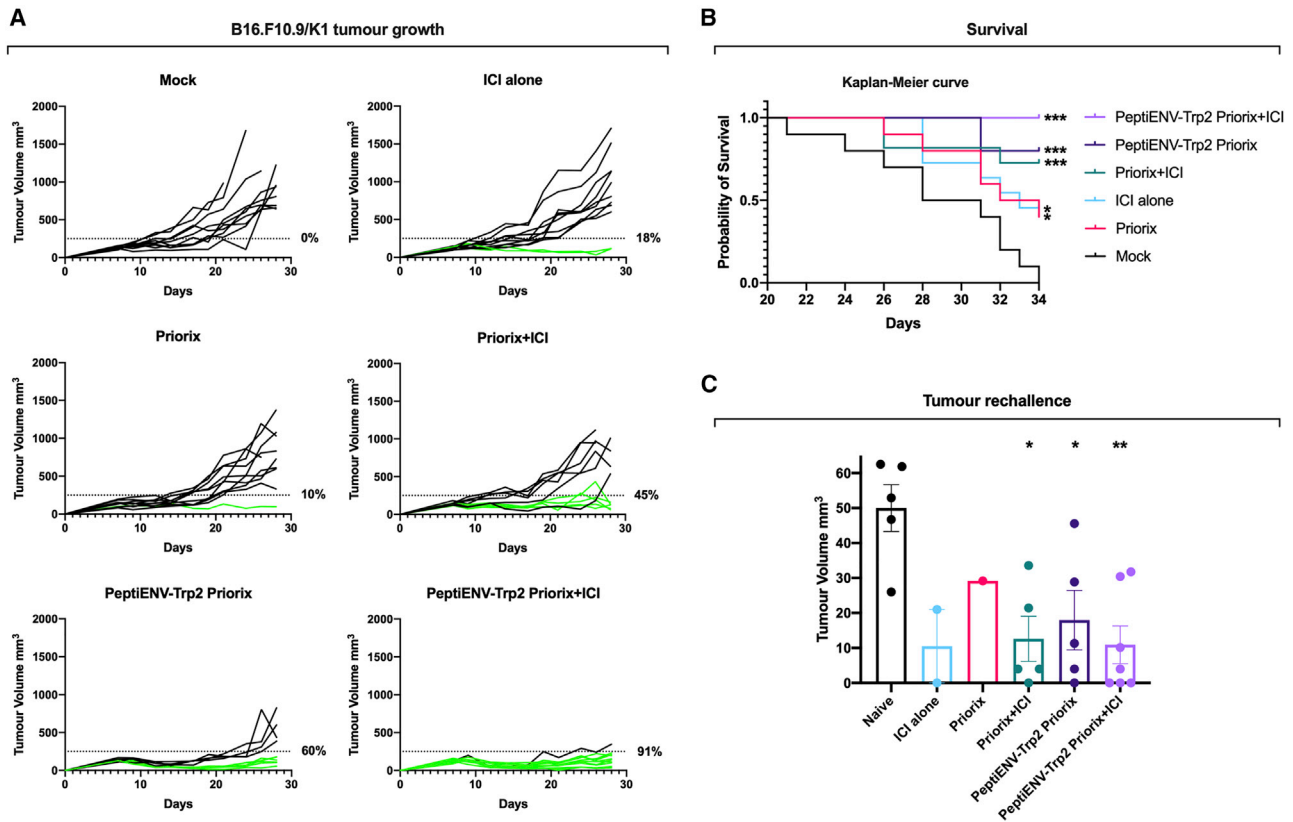
Figure 5. PeptiENV-Trp2 Priorix in combination with anti-PD1 improves tumor growth control and survival, and triggers a systemic anti-tumor memory response in a syngeneic mouse model of B16.F10.9/K1

(A) Anti-PD-1 alone, Priorix alone or in combination with anti-PD-1, and PeptiENV-Trp2 alone or in combination with anti-PD-1 was given Priorix or PeptiENV-Trp2 Priorix intratumorally 7, 9, and 21 days after tumor implantation and anti-PD-1 was given intraperitoneally three times a week, starting at day 14. Individual tumor growth curves for all treatment groups are shown. The percentage of responders is shown on the right side of the dotted line. The number of mice in each group was 10–11. (B) Kaplan-Meier survival curve for each treatment group. (C). Individual tumor volumes for rechallenged mice at day 7 after secondary tumor engraftment. Naïve mice (n = 5) were used as engraftment control. Statistical analysis was performed with one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

than what was used in this study (one vaccination dose of Priorix contains approximately $10^{3.0}$ CCID$_{50}$ and $10^{3.7}$ CCID$_{50}$ of measles and mumps, respectively). Indeed, we wanted to use the same FDA/EMA-approved dose that has safely been used in vaccination programs worldwide for vaccinating against measles, mumps, and rubella. With the dose used, we did not see any oncolysis-driven anti-tumoral effects by the Priorix vaccine; however, when combined with the PeptiENV platform, Priorix acted as an outstandingly potent biological adjuvant for the attached tumor antigen peptides and was able to induce anti-tumoral effects through the stimulation of tumor antigen-specific T cell immunity.

Recently, common live attenuated vaccines for various infectious diseases have been repurposed as intratumoral immunotherapy for the modulation of the TME.[1,3] As an example, Aznar et al.[1] successfully repurposed a live attenuated yellow fewer virus vaccine strain 17D as an intratumorally administered cancer immunotherapy. However, the dose used was much higher than the FDA/EMA-approved dose for

vaccination against the yellow fever virus (intratumoral dose of 4×10^6 plaque-forming units vs. vaccination dose of 10^3 infectious units). As the yellow fewer virus is an enveloped virus, it is intriguing to hypothesize that, by using the yellow fever vaccine in the PeptiENV platform, one could further enhance the number of intratumoral tumor-specific CD8$^+$ T cells and thus further increase the anti-tumor efficacy of the yellow fewer virus vaccine strain 17D and, possibly, allow for a decrease in the amount of vaccine virus used to minimize the risk of adverse events. We and others have previously shown that pre-existing immunity against the vaccine or viral vector used in intratumoral cancer treatment is not negatively affecting the therapeutic efficacy.[1,17] However, as hundreds of millions of people have been vaccinated against MMR, we tested the anti-cancer effects of PeptiENV Priorix in mice pre-immunized with Priorix vaccine. We did not see a decrease in the therapeutic efficacy of PeptiENV Priorix in mice pre-vaccinated with Priorix vaccine; in contrast, the therapeutic efficacy seemed to be slightly increased. These findings are in line with previous studies using the yellow fever vaccine strain 17D.[1]
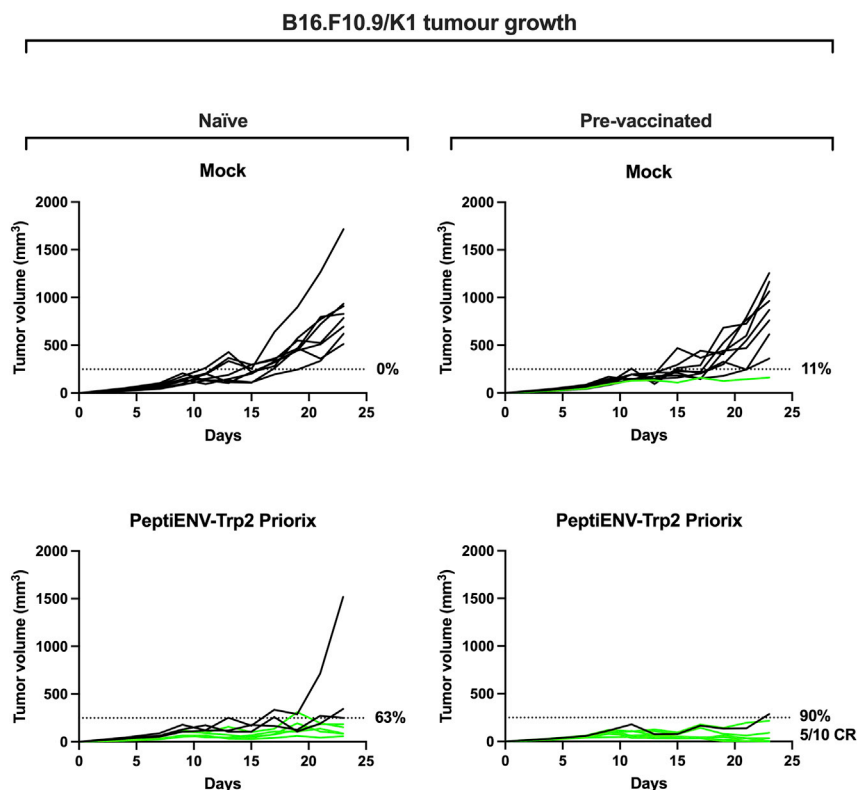
B16.F10.9/K1 tumour growth

Naïve

Pre-vaccinated

Mock

Mock

PeptiENV-Trp2 Priorix

PeptiENV-Trp2 Priorix

Figure 6. Pre-existing immunity against measles, mumps, and rubella does not negatively affect intratumoral PeptiENV-Trp2 Priorix immunotherapy

Mice pre-vaccinated with Priorix (2 doses, 21 days apart) or naive mice were engrafted with subcutaneous B16.F10.9/K1 melanoma, and intratumorally treated 7, 9, and 19 days after tumor implantation with PeptiENV-Trp2 Priorix or saline as the mock-treated group. Individual tumor growth curves for all treatment groups are shown. The percentage of responders is shown on the right side of the dotted line. The number of mice displaying complete response (CR) is also shown. The number of mice in each group was 8–10.

Recent studies have suggested that vaccination with the MMR vaccine may induce some cross-protective immunity against coronavirus disease 2019 (COVID-19),[18–21] and the efficacy of the MMR-induced cross-protection against COVID-19 is now being clinically tested (NCT04333732). However, the efficacy of the MMR vaccine to provide protection against COVID-19 could be significantly improved by the use of the PeptiENV platform with severe acute respiratory syndrome coronavirus 2-specific antigen peptides.

In summary, we demonstrated that an FDA/EMA-approved vaccine against MMR can be used as a potent biological adjuvant in the PeptiENV cancer vaccine platform. Treatment with PeptiENV Priorix induced robust tumor-specific T cell responses, and, when combined with ICI therapy, the number of mice responding to ICI therapy was markedly increased. As more safe, personalized cancer therapies are needed for the beneficial modulation of the TME for enhanced anti-tumor efficacy and for boosting of ICI therapies, there is a rationale to convert existing FDA/EMA-approved vaccines for infectious diseases to be used in these personalized and targeted approaches. The PeptiENV Priorix vaccine platform could be rapidly taken into clinical testing, particularly in combination with ICI therapies.

MATERIALS AND METHODS
Cell lines
The cell line B16.OVA, a mouse melanoma cell line expressing chicken OVA, was kindly provided by Prof. Richard Vile (Mayo Clinic). The B16.OVA cells were cultured in RPMI with 10% FBS (Life Technologies), 4 mM L-glutamine (Life Technologies), 1% penicillin/ streptomycin, and 5 mg/mL of geneticin (Life Technologies). B16F10.9/K1 cell line was kindly provided by Ludovic Martinet (INSERM, France) and was cultured in high glucose DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. The murine DC line JAWSII was purchased from ATCC and was cultured in alpha minimum essential medium with 20% FBS (Life Technologies), ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), and 5 ng/mL murine granulocyte macrophage colony stimulating factor (PeproTech).

MRR vaccine
The Priorix vaccine containing the live attenuated Schwarz measles, RIT 4385 mumps (derived from the Jeryl Lynn strain), and Wistar RA 27/3 rubella strains of viruses (GlaxoSmithKline Biologicals s.a.) was purchased from the University Pharmacy Ltd. A single vaccination dose of Priorix contains minimum of $10^{3.0}$ cell culture infectious dose 50% ($CCID_{50}$) of the Schwarz measles, $10^{3.7}$ $CCID_{50}$ of the RIT 4385 mumps, and $10^{3.0}$ $CCID_{50}$ of the Wistar R 27/3 rubella virus strains according to the vaccine provider.

Peptides
The peptides used in this study were: GRKKRRQRRRPQRWEKISIINFEKL, RWEKISIINFEKL, and SIINFEKL (containing an MHC class I-restricted epitope from chicken $OVA_{257-264}$). GRKKRRQRRRPQRWEKISVYDFFVWL (containing an MHC class I-restricted epitope from tyrosinase-related protein 2, $Trp2_{180–188}$). All peptides were purchased from Zhejiang Ontores Biotechnologies.

PeptiENV Priorix complex formation
One vaccination dose of Priorix reconstituted in sterile water for injection was complexed with 3 nM CPP-extended peptides (GRKKRR QRRRPQRWEKISIINFEKL or GRKKRRQRRRPQRWEKISVYDFF VWL) or 3 nM control peptide (RWEKISIINFEKL).

SPR

Measurements were performed using a multi-parametric SPR Navi 220A instrument (Bionavis Ltd). PBS (pH 7.4) was used as a running buffer during virus adsorption on the SPR sensor surface and during peptide-virus interaction measurements. A constant flow rate of 20 μL/min was used throughout the experiments, and temperature was set to +20°C. Laser light with a wavelength of 670 nm was used for surface plasmon excitation. A linear polyethyleneimine (LPEI, M_w = ~2,500 g/M)-coated Au sensor was used for the affinity measurements. LPEI renders the Au sensor slide highly positively charged, which allows for the efficient immobilization of MMR viruses onto the sensor. For testing the interaction between peptides and viral envelopes, 100 μM of the tested peptides (CPP-OVA or OVA control peptide without the CPP attachment moiety as a non-interacting control) were injected into a virus-coated channel and into an uncoated flow channel. The final SPR response for the peptide-virus interaction was then obtained by subtracting the SPR response in the reference channel from the measured response in the virus-coated channel.

JAWSII DC line cross-presentation experiments

JAWSII cells were seeded in 24-well plates (2.5×10^5 cells/well) and pulsed with PeptiENV-OVA Priorix, Priorix mixed with OVA control peptide without the CPP attachment moiety (RWEKISII NFEKL), or Priorix alone or were left unpulsed. After 24 h, cells were collected by scraping and stained with APC-conjugated anti-mouse H-2K^b bound to SIINFEKL antibody (141606, BioLegend) and PerCP-Cy5.5-conjugated anti-mouse CD86 antibody (105027, BioLegend) and analyzed by flow cytometry.

Animal experiments

The Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland (license number ESAVI/11895/2019) reviewed and approved all animal experiments described here. Tumor growth was measured every second day, starting on the day tumors were first treated. All injections and tumor measurements were performed under isoflurane anesthesia.

For the vaccination experiment using naive mice, 8- to 9-week-old immunocompetent female C57BL/6JOlaHsd mice were subcutaneously treated on days 0, 2, and 15 with one vaccination dose of Priorix alone, one vaccination dose of Priorix together with OVA control peptide, one vaccination dose of PeptiENV-OVA Priorix, or PBS as a mock-treated group. For performing immunological analysis, animals were sacrificed at day 20 and spleens were harvested. For the B16.OVA melanoma experiment, 8- to 9-week-old immunocompetent female C57BL/6JOlaHsd mice were injected in the right flank with 350,000 B16.OVA melanoma cells, and were treated 6, 8, and 20 days after tumor implantation with one vaccination dose of Priorix alone, one vaccination dose of PeptiENV-OVA Priorix, peptides alone, or PBS as a mock-treated group. For performing immunological analysis, animals were sacrificed at day 24 and tumors, spleens, and tumor-draining lymph nodes were harvested. For the B16F10.9/K1 melanoma experiment combined with anti-PD-1 therapy, 8- to 9-week-old im-

muno-competent female C57BL/6JOlaHsd mice were injected in the right flank with 600,000 B16F10.9/K1 cells together with a 3:2 ratio (v:v) of Matrigel Basement Membrane Matrix High Concentration (Corning), and were treated 7, 9, and 21 days after tumor implantation with one vaccination dose of Priorix alone, one vaccination dose of PeptiENV-Trp2 Priorix, or left untreated (mock). Anti-PD-1 (InVivoMab, clone RMP1-14) was injected intraperitoneally three times per week with 100 μg/dose starting at day 12 after tumor implantation. Tumor growth and survival was monitored until all mice in the control group had died or sacrificed (day 35 after tumor implantation). For the assessment of induction of systemic anti-tumor T cell responses, surviving mice were rechallenged with 1.2×10^6 B16F10.9/K1 cells in the left flank and followed for secondary tumor development and growth. For the pre-existing immunity experiment, 8- to 9-week-old immunocompetent female C57BL/6JOlaHsd mice were vaccinated with two vaccination doses of Priorix with 21 days between doses. Fourteen days after the final pre-immunization dose of Priorix, mice were injected in the right flank with 600m000 B16F10.9/K1 cells together with a 3:2 ratio (v:v) of Matrigel Basement Membrane Matrix High Concentration, and were treated 7, 9, and 19 days after tumor implantation with one vaccination dose of PeptiENV-Trp2 Priorix, or left untreated (mock). Naive mice were used as controls, with identical tumor implantation and treatment schedule.

ELISPOT assays

The number of SIINFEKL (OVA$_{257-264}$)-specific activated, IFN-γ secreting T cells were measured by ELISPOT assay (CTL) according to the manufacturer's instructions. Briefly, 2 μg SIINFEKL peptide was used to stimulate the APCs. After 3 days of stimulation, plates where stained and sent to CTL-Europe GmbH for counting of the spots.

IFN-γ ELISA

The levels of secreted IFN-γ from activated, Trp2 antigen-specific T cells were measured by mouse IFN-γ ELISA kit (eBioscience) according to the manufacturer's instructions. Briefly, 1×10^6 splenocytes were plated on 96-well plates and stimulated with 2 μg Trp2 peptide or Priorix vaccine. After 48 h of stimulation, the cell culture supernatant was collected and the levels of secreted IFN-γ was measured.

Flow cytometry

Antibodies used in the flow cytometric assays: TruStain FcX anti-mouse CD16/32 (101320, BioLegend), Peridinin-Chlorophyll-Protein anti-mouse CD19 (115531, BioLegend), Phycoerythrin) anti-mouse CD3e (550353, BD Pharmingen), and fluorescein isothiocyanate anti-mouse CD8 (A502-3B-E, ProImmune). APC-labelled H-2Kb/SIINFEKL pentamer (F093-84B-E, ProImmune) was used to measure the number of SIINFEKL epitope-specific T cells. Flow cytometry was performed using a BD Accuri 6C Plus (BD Biosciences) or a BD LSRFortessa (BD Biosciences) flow cytometer and FlowJo software v10 (BD Biosciences) was used for data analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software). For data analysis, one-way or two-way

ANOVA were used. All results are expressed as the mean ± standard error of the mean.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.04.002.

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AUTHORS CONTRIBUTIONS

E.Y., M.F., A.U., and V.C. conceived and planned the experiments. E.Y., M.F., A.U., S.F., F.H., J.C., K.A., and T.V. carried out the experiments. E.Y., M.F., A.U., B.M., S.F., F.H., J.C., K.A., T.V., and V.C. contributed to the interpretation of the results. E.Y. wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

DECLARATION OF INTERESTS

Vincenzo Cerullo is a co-founder and shareholder at VALO therapeutics.

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