Retargeted and Multi-cytokine-Armed Herpes Virus Is a Potent Cancer Endovaccine for Local and Systemic Anti-tumor Treatment

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Oncolytic viruses (OVs) are novel anti-tumor agents with the ability to selectively infect and kill tumor cells while sparing normal tissue. Beyond tumor cytolysis, OVs are capable of priming an anti-tumor immune response via lysis and cross-presentation of locally expressed endogenous tumor antigens, acting as an “endovaccine.” The effectiveness of OVs, similar to other immunotherapies, can be hampered by an immunosuppressive tumor microenvironment. In this study, we modified a previously generated oncolytic herpes simplex virus (oHSV) retargeted to the human HER2 (hHER2) tumor molecule and encoding murine interleukin-12 (mIL-12), by insertion of a second immunomodulatory molecule, murine granulocyte-macrophage colony-stimulating factor (mGM-CSF), to maximize therapeutic efficacy. We assessed the efficacy of this double-armed virus (R-123) compared to singly expressing GM-CSF and IL-12 oHSVs in tumor-bearing mice. While monotherapies were poorly effective, combination with α-PD1 enhanced the anti-tumor response, with the highest efficacy of 100% response rate achieved by the combination of R-123 and α-PD1. Efficacy was T cell-dependent, and the induced immunity was long lasting and able to reject a second contralateral tumor. Importantly, systemic delivery of R-123 combined with α-PD1 was effective in inhibiting the development of tumor metastasis. As such, this approach could have a significant therapeutic impact paving the way for further development of this platform in cancer immunotherapy.

INTRODUCTION

Oncolytic virotherapy is emerging as a promising approach for the treatment of different cancer types. This approach takes advantage of the intrinsic ability of viruses to infect, replicate, and kill the target cells and at the same time to spread to other cells. The anti-tumor activity of oncolytic viruses (OVs) is not only a mere consequence of the direct cytopathic effect of the virus, but it is mainly linked to the induction of an immune response against the tumor. Because of the combined tumor killing via oncolysis and the ability to potently stimulate multiple arms of the immune system, OVs can be considered in situ tumor vaccines (“endovaccines”). The key desirable features of any OV are specificity, safety, and potency. Specificity is usually conferred by attenuating mutations, which cause preferential replication of the virus in tumor cells and not in healthy cells. This is, for example, the case of the talimogene laherparepvec (T-VEC), a type I herpes simplex virus (HSV), currently the only US Food and Drug Administration (FDA)-approved OV for the local treatment of metastatic melanoma. T-VEC is an attenuated HSV deleted of ICP34.5, a gene that plays a critical role in viral replication and in overcoming the host anti-viral responses. In normal cells, activation of the double-stranded RNA-dependent protein kinase PKR is one of the most effective cellular responses to block virus propagation. ICP34.5 protein overcomes the anti-viral responses by blocking PKR and thus allowing for viral replication. In many cancer cells, the PKR pathway is not active. Therefore, HSV lacking ICP34.5 can preferentially replicate in cancer cells and not in healthy cells. However, effective replication is not guaranteed in all tumor cells since some may still have an active PKR pathway. An alternative strategy to achieve cancer selectivity, independently of the intrinsic defects of tumor cells, consists in detargeting the viruses from their natural receptors and retargeting viral tropism to cancer-specific receptors via an encoded single-chain variable fragment (scFv) expressed on the surface of the virus. Such modifications preserve the full lytic capacity of wild-type (WT) HSV and overcome the limits of virus attenuation, hence enhancing potency in all cancer cells, in addition to gaining specificity. Most importantly, because retargeted oHSVs infect no other cells than the specifically targeted tumor cells, their safety profile is very good. Our previous preclinical work has demonstrated that systemic administration of HER2-retargeted HSV is safe in nude mice, by virtue of viral surface modifications causing tropism detargeting from the natural receptors, and retargeting to...
HER2. More recently, we confirmed the good safety profile also in immunocompetent mice. A key feature for a successful OV-based therapy is the capacity to induce an immunological response. OVs function as a “kick start” for an anti-tumor response. However, one of the major obstacles for effective T cell function is posed by the highly immunosuppressive tumor microenvironment (TME). OVs can counteract immunosuppression by encoding several immunostimulatory molecules, including checkpoint inhibitors, cytokines, and chemokines, to restore a more favorable inflammatory TME and potentiating the immunological response.11 A number of studies have indeed shown that OVs genetically armed with immunostimulatory molecules exert more robust therapeutic effects than do their non-engineered counterparts.12–14

The clinically approved HSV (T-VEC) also encodes for a stimulatory factor (granulocyte-macrophage colony-stimulating factor [GM-CSF]) to enhance the anti-tumor response by local recruitment of dendritic cells for antigen presentation.15 Despite the great therapeutic potential, OVs are still not powerful enough, especially in the situation of scarcely immunogenic tumors or in a highly suppressive microenvironment. Combinations of OVs and immune checkpoint blockade therapy have strong synergistic effects. In patients, combined treatment of T-VEC with ipilimumab or pembrolizumab resulted in higher efficacy than did either therapy alone.16,17 In this study, we propose the use of a next-generation targeted herpes virus (THV), which combines the following features: (1) WT backbone for efficient replication and killing of any tumor cells, (2) detargeting from natural receptors for improved safety, (3) retargeting to the tumor-specific antigen human HER2 (hHER2) to gain cancer specificity, and (4) an improved potency by encoding two immunomodulators (GM-CSF and IL-12) for induction of an effective adaptive immunological response. By using a stringent mouse model of tumors non-responsive to z-PD1 treatment and a stringent therapeutic setting of advanced large tumors, we showed that intratumoral THV treatment turned z-PD1 non-responsive tumors to responsive. The highest efficacy was achieved when using the “armed” virus. Intravenous delivery (i.v.) has the potential to reach both the primary tumor and metastatic lesions even when they are not clinically detectable. By exploiting the safety improvements gained with the retargeting to tumor cells of a not attenuated virus,9,10 we explored systemic treatment with THV. We demonstrated that a virus with such features is suitable for systemic delivery. The THV can target the metastatic tumor masses after i.v. injection, exerting a significant anti-tumor activity, also in a contest of pre-existing neutralizing immunity against HSV. This work establishes the proof of principle that immunologically potent THVs armed with multiple cytokines can cure large established tumors when combined with z-PD1 and become effective upon local and systemic delivery.

RESULTS

Generation of IL-12- and GM-CSF-Armed hHER2-Retargeted oHSV

By using a hHER2-retargeted oHSV, we have recently demonstrated higher anti-tumor efficacy when the virus is armed with murine interleukin-12 (mIL-12) relative to the unarmed virus.10 In an effort to further enhance tumor cytotoxicity and improve the immune effector response, we have engineered the hHER2-retargeted IL-12-armed oHSV R-11518 to express a further immunostimulatory cytokine, GM-CSF. IL-12 and GM-CSF were respectively inserted into the US1–US2 intergenic region and between the UL26 and UL27 intergenic region. The resulting double-armed virus, named R-123, was compared to the single-armed IL-12 virus (R-115), the single-armed GM-CSF virus (R-121), and to the unarmed counterpart (R-113) (Figure 1A). The R-121 virus was obtained by inserting the GM-CSF-encoding cassette into the UL26–UL27 intergenic region.

To confirm transgene expression and production of the two encoded cytokines, supernatants from virus-infected hHER2 cell cultures (SK-OV-3) were analyzed by enzyme-linked immunosorbent assay (ELISA) 24 and 48 h post-infection. The amounts of mIL-12 and GM-CSF produced by double-armed virus versus both single-armed viruses were very similar, indicating that the double insertion of the two transgenes did not affect the expression (Figure 1B). The four viruses R-113, R-115, R-121, and R-123 were finally compared in terms of viral growth and production by measuring the virus yield as plaque-forming units (PFU)/mL upon infection of the HER2-expressing SK-OV-3 cells. The average yield measured was found to be similar (Figure 1C).

Monotherapy with Cytokine-Armed THVs Is Not Sufficiently Effective to Eradicate Large Established Tumors

To evaluate the anti-tumor activity of the hHER2-retargeted OHSVs, we made use of a stably transduced Lewis lung carcinoma murine cell line expressing hHER2 (HER2-LLC1) for in vivo studies.10 The syngeneic C57BL/6 mice transgenic for hHER2 were used as the host mouse model, as no immune responses are generated against the transgenic product upon tumor implantation.10,17 By using the very same mouse model, we have demonstrated effective and superior efficacy of retargeted R-115 oHSV armed with IL-12 versus the unarmed counterpart in a therapeutic setting in which the treatment was performed 3 or 10 days after tumor implantation, considered early and late treatments, respectively.10 In order to be more stringent, we used for this study an advanced setting consisting of mice all bearing large tumors at the beginning of treatment. We have previously shown that the effectiveness of the immunotherapies can be better evaluated in a setting of advanced disease and that some therapies that are effective upon early treatments might fail in situations of advanced disease, given the highly immunosuppressive TME.20 Therefore, in the current study, mice were challenged with a subcutaneous (s.c.) injection of HER2-LLC1 cells. Tumors were allowed to grow until they reached an average volume of ≈110 mm3, in order to recapitulate a late therapeutic setting of large tumors. At this stage (day 0), treatment with R-113, R-115, R-121, or R-123 started. The THVs were injected intratumorally every 2–3 days for a total of five injections at a dose of 106 PFU each (Figure 2A). In this situation, the unarmed R-113 and the single-armed R-121 or R-115 virus had very limited efficacy when administered alone (10%–20%) (Figure 2B). A trend versus a better anti-tumor response was observed when tumor-bearing mice were treated with the double-armed R-123 virus, which elicited tumor regression in about 40% of the animals (Figure 2B).
Combination of α-PD1 and Double-Armed IL-12 and GM-CSF THV Induces Strong Anti-tumor Activity

To improve the therapeutic efficacy of the THV-based oncotherapy, the combination with α-PD1 was considered. Five viral injections were performed into the tumor mass, together with α-PD1 treatment according to the schedule depicted in Figure 3A. While monotherapies with either unarmed and armed THVs were moderately effective (Figure 2B), the combined treatment of R-113, R-115, R-121, or R-123 with α-PD1 led to a significant improvement in efficacy, and the percentage of tumor response was observed in 60%, 75%, 50%, and 100% of treated mice, respectively (Figure 3B). The most potent effect was observed in the R-123 and α-PD1 arm, with all mice.
responding to the combination therapy. These results suggest that (1) combination of OVs with checkpoint inhibitors is required to eradicate large tumors, and (2) the inclusion of the two encoded cytokines contributes to ameliorate the potency of the oHSV.

The Anti-tumor Activity of R-123/α-PD1 Is T Cell-Mediated
To investigate the mechanism of anti-tumor responses, mice undergoing complete tumor regression upon combined treatment were rechallenged with a second contralateral tumor inoculum.
Figure 3. Double-Armed IL-12 and GM-CSF R-123 Is Highly Effective in Combination with α-PD1
(A) Schematic of treatments. Mice were inoculated s.c. with HER2-LLC1 cells, randomized into six groups when tumors reached 110 mm³ as volume mean and treated with α-mPD1 antibody every 3–4 days until day 17, as stand-alone therapy or in combination with 10⁶ PFU/injection of R-113, R-115, R-121, or R-123. (B) Growth of vehicle, α-PD1, or combo-treated tumors. Each line represents an individual HER2-LLC1 tumor (n = 8-15) followed over time (dashed lines for non-responders and solid lines for responders). Percentages on the graphs indicate the rate of response as sum of complete and partial response (≥40% tumor shrinkage). Data are representative of three independent experiments. Statistical analysis was performed by a mid-p exact test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Protection from tumor rechallenge was observed in 100% of mice, indicative of a long-lasting adaptive T cell memory response (Figure 4A). To evaluate the role of the immune system on the effectiveness of R-123 and α-PD1, a series of experiments were performed in several conditions of immune cells depletion.
Selective depletion of CD8+ or CD4+ T cells affected in a significant manner the capability of the combined treatment of R-123 and α-PD1 to cure large s.c. tumors. In particular, CD4+ T cell depletion caused an almost complete abrogation of the anti-tumor effect, highlighting a major contribution of this lymphocyte population to the treatment efficacy. Depletion of CD8+ T cells also impacted the efficacy of the treatment, demonstrating that OV-mediated anti-tumor immunity depends on T cells (Figure 4B). We further depleted interferon (IFN)-γ, showing that the anti-tumor effect of R-123 and α-PD1 is IFN-γ-mediated (Figure S1). The contribution of the innate arm to the therapeutic effect was also explored by in vivo depletion of NK cells, showing in this case a minor impact on the response rate (Figure 4B). Interestingly, the combined treatment with R-123 and α-PD1 induced an overall increase in the number of T cells, including both CD4+ and CD8+ T cells present in the blood of treated mice versus untreated control mice (Figure 4C).

**Systemic Delivery of Armed THV in Combination with α-PD1 Effectively Controls Lung Metastatic Nodules**

OVs currently in various stages of clinical trials, or in clinical practice, e.g., T-VEC, are often administered to cancer patients by the intratumoral route, mainly because of safety reasons, consequent to the lack of specific targeting to cancer cells. There is intense interest in systemically deliverable OVs in order to treat patients with inaccessible tumors and particularly for those with metastatic diseases.

To explore the capability of hHER2-retargeted oHSVs to reach the target tumor and exert their anti-tumor activity after systemic administration, we considered a therapeutic setting of tumors diffuse to lungs, considered as a model of metastatic disease. LLCP cells are capable of developing cancerous pulmonary nodules when injected by tail vein. Therefore, HER2-LLC1 cells were administered through the mouse tail vein (day −3). Three days later, the viral and checkpoint inhibitor treatments started. The i.v. administration of R-123 was performed in combination with α-PD1. 2 weeks after the beginning of treatments, mouse lungs were harvested and analyzed for the development of tumor nodules (Figure 5A). Treatment with R-123 in combination with α-PD1 was highly effective in reducing the number of lung nodules, and it resulted in a significantly higher anti-tumor activity as compared to α-PD1 alone; the latter exhibited similar efficacy as the vehicle (Figure 5B). Beyond confirming the strong anti-tumor efficacy of the double cytokine-armed R-123, the model highlighted the possibility of exploring the systemic route of administration in the context of retargeted OVs. Pre-existing anti-herpes immunity is prevalent in the human population and may reduce the effectiveness of a systemic oncolytic treatment. Thus, the efficacy of the i.v. treatment in combination with α-PD1 was assessed in HSV-immunized mice. Animals were pre-immunized with a recombinant HSV-1 (F)-derived virus21 using the prime-boost schedule outlined in Figure 5C. As expected, immunization of mice with HSV led to development of neutralizing antibodies (NAbS) to HSV as determined by an in vitro viral neutralization assay (Figure 5D). To evaluate the effect of immunization on the activity of THV to prevent lung metastases formation, HSV-naive and HSV-immunized animals were inoculated i.v. with the HER2-LLC1 and then treated with R-123 in combination with α-PD1. Prior immunization with HSV did not impact the efficacy of treatment, as evidenced by the similar reduction of lung nodule formation observed in both HSV-naive and HSV immune-treated groups (Figure 5E).

**DISCUSSION**

OVs are considered highly promising tools to increase the efficacy of a checkpoint inhibitor. In this study, we have developed an approach based on the use of a fully replicative HER2-retargeted oHSV armed with IL-12 and GM-CSF to maximize therapeutic activity. By using a stringent setting of large murine tumors and a model refractory to α-PD1 monotherapy22,23 we demonstrated (1) highly synergistic effect of THV, unarmed and armed ones, with α-PD1, and (2) enhanced efficacy by potentiation of the viral payload to express multiple immunomodulatory agents. Our earlier in vivo studies demonstrated that the efficacy of retargeted oHSV as a stand-alone therapy, even when armed with IL-12 (R-115), is dramatically reduced when moving from an early therapeutic setting to late treatment, suggesting that the hostile microenvironment found in a setting of late treatment (e.g., in the presence of large established tumors) limits the anti-tumor activity of such viruses.10 In the present study, we confirmed the modest effect of unarmed and armed THV, either single or double armed, in mice bearing large tumors, suggesting that a combination with a checkpoint inhibitor might be beneficial to ameliorate the anti-tumor response. The combination of α-PD1 and double-armed IL-12 and GM-CSF (R-123) given intratumorally resulted in a 100% response, 80% of which included complete response with full eradication of advanced tumors. Importantly, note that these results were achieved by using a tumor cell line, HER2-LLC1, that is markedly less permissive to HSV infection than the human cancer cells and the syngeneic C57BL/6 mice, a strain among the most highly resistant to HSV infection.10,24

This model also showed resistance to α-PD1 treatment, as demonstrated by the absence of the therapeutic response upon α-PD1 administration. The resistance of these tumors to α-PD1 therapy was overcome with the addition of retargeted oHSVs, with the maximum therapeutic benefit achieved in the presence of the double-armed cytokine virus. This confirms and extends the notion that the use of OVs, particularly armed viruses, can be applied to optimize immunotherapy and overcome resistance to checkpoint blockade. One of the major limitations of checkpoint blockade includes situations in which the tumor is invisible to the immune system, the so-called “cold” and “excluded” tumors. It is likely that the addition of THV to the checkpoint blockade is able to generate an inflamed “hot” tumor environment, thereby reawakening anti-tumor immune responses, as also demonstrated by our studies,10 as well as by other studies.25 The therapeutic effect of R-123 and α-PD1 was T cell-mediated, likely attributable to their ability to produce IFN-γ, as shown by a significant reduction of anti-tumor efficacy by depleting CD4+ or CD8+ T cells or by blocking IFN-γ. Depletion of natural killer (NK) cells had no impact on the effectiveness of treatment. Indeed, induction of tumor-directed adaptive immune
responses has been widely recognized as a key mechanism to achieve long-term therapeutic success in the clinic.\textsuperscript{4,26}

More recently, a key role of infiltrating CD8\textsuperscript{+} T cells was documented in patients responding to a combination of T-VEC and pembrolizumab.\textsuperscript{17} Analysis of tumor biopsies demonstrated that T-VEC treatment increased the presence of CD8\textsuperscript{+} T cells in 75% of injected lesions and that the increase in CD8\textsuperscript{+} T cell density appeared to be associated with the response to combination therapy.\textsuperscript{17} The combined treatment of \(\alpha\)-PD1 and R-123 elicited a systemic anti-tumor effect and a long-lasting memory response, as proven by the full protection from the growth of contralateral, distant, untreated tumors. An increase in circulating CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell levels was observed upon treatment. A systemic increase in circulating CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells was also found in patients upon T-VEC administration, providing evidence for the generation of a systemic anti-tumor response.\textsuperscript{17}

**Figure 5. Systemic Delivery of R-123 Controls Lung Metastasis Development in Combination with \(\alpha\)-PD1**

(A) Schematic of lung metastatic setting. HER2-LLC1 cells were i.v. injected into the mouse tail vein. Treatments started after 3 days (d0): R-123 was i.v. delivered (five injections at 10\textsuperscript{8} PFU each), combined with \(\alpha\)-PD1. At day 14 lung nodules were counted. (B) Number of lung nodules in vehicle-treated (black symbols), \(\alpha\)-PD1-treated (gray symbols), or R-123/\(\alpha\)-PD1-treated (red symbols) mice. Bars show mean with SEM of two independent experiments; dots represent individual animals (n = 10–20). (C) Prime-boost immunization scheme with WT HSV. For the efficacy experiment, naive versus HSV immune mice were i.v. inoculated with HER2-LLC1 cells. Treatments started after 3 days (d0) and were performed as described in (A). (D) Anti-HSV antibody serum titers determined by plaque reduction neutralization test at week 12 after immunization. The y axis indicates the dilution factor at which the number of viral plaques was reduced by 50% compared to the control sample (virus alone). Bars show mean with SEM. (E) Numbers of lung nodules in untreated mice (black dots) versus treated mice, HSV naive mice (red dots), or HSV immune mice (blue dots) are shown. Bars show mean with SEM; dots represent individual animals (n = 8–12). Statistics were generated using an unpaired Mann-Whitney nonparametric test. *\(p < 0.05\), ****\(p < 0.001\).
We are currently investigating the induction of LLC1 neoantigen-specific T cell responses after combined treatment to better elucidate the mechanisms of immune-mediated anti-tumor responses. In line with our findings, the use of armed OVs has shown improved therapeutic benefit in clinical trials and preclinical models. Several viruses have been armed with IL-12 and tested in different tumor models, including oHSV, with the demonstration of enhanced efficacy. Multiple preclinical studies demonstrated that OVs armed with IL-12 yield better anti-tumor effects than does vectored GM-CSF. Consistent with previous studies assessing the effectiveness of cytokine-armed OHSV, our armed GM-CSF THV exerted similar efficacy than to that of the parental non-cytokine virus, while IL-12-armed THV showed a very strong anti-tumor activity. The contribution of GM-CSF was revealed when coupling GM-CSF and IL-12 in one single THV. The expression of multiple transgenes from a single oncolytic HSV has been recently reported with a potent antitumor effect and improved therapeutic effect in combination with PD1 blockade in murine models, supporting the use of the multiplearming strategy as a potent and versatile approach to develop new oncolytic immunotherapies for clinical use.

Prompted by the rate of response achieved upon local administration of R-123 combined with α-PDI, which is the highest achieved so far using a retargeted oHSV to treat established tumors, we next assessed whether the same approach might also be effective for i.v. delivery. The systemic administration of oHSV represents an appealing route of administration because it allows treatment of diffuse metastases as well as the primary lesions. Several types of OVs have been administered systemically for cancer therapy. However, to date, the most frequently used route in clinical studies is local administration, including the approved administration of T-VEC. The intratumoral route is preferred (1) to ensure safety by limiting the risk of replication in normal cells, and (2) to avoid pre-existing neutralizing antibodies that may dampen the efficacy of OVs. Recently, a first-in-human phase 1 study in young cancer patients demonstrated feasibility and tolerability of i.v. administration of an attenuated oHSV, although no evidence of intratumoral virus replication was found after systemic administration.

In the present study, for the first time, we showed that a retargeted and cytokine-armed oHSV is effective in controlling lung metastases formation when systemically injected, in combination with α-PDI. Systemic administration was safe, as no toxicity was observed in the i.v. treated mice, confirming our previous data. To further improve the safety of our THV, we have recently generated a new oHSV that combines the feature of retargeting with tumor replication conditioning as an option to be considered for i.v. delivery. Neutralizing antibodies are one of the main obstacles for systemically administered OVs given the high seroprevalence in the human population. We have demonstrated maintenance of therapeutic activity of systemically delivered R-123 in combination with α-PDI in mice immunized with HSV-1 virus showing anti-HSV neutralizing antibodies. By using a protocol of active immunization, we have likely induced also cellular immunity against HSV that may be beneficial for the anti-tumor effect, consistent with the observations by Ricca et al. While the current study suggests that i.v. delivery is feasible and can be effective to control tumor growth, it still represents a challenge because of several components impeding the optimal systemic delivery efficiency of OVs. Among them, the sequestration and subsequent clearance of the infused viruses by the host’s mononuclear phagocyte system and the inactivation by the host’s defense systems. In order to enable the virus to evade the host’s defense machinery and efficiently reach the tumor site, different strategies are being considered, such as virus capsid engineering, chemical modifications of virus capsid (e.g., PE-Glylation), use of carrier cells and potentiation of the virus by choice of optimal immunomodulators to strengthen the immune-mediated effect of oHSV. Our findings open the possibility to explore the clinical use of retargeted-armed oHSV for local and systemic treatment of cancer diseases and support combined immunotherapy of checkpoint blockade and endovaccines based on OVs.

MATERIALS AND METHODS

Mice

Tolerant hHER2-transgenic C57BL/6 mice (B6.Cg-Pds51Jcr/Tg[ERBB2]2299Wzw/J) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mouse colony management and all day-to-day care were performed by trained mouse house staff at Plaisant (Castel Romano, Italy). Female mice (6–8 weeks of age) were used for in vivo experiments.

Cell Cultures

The SK-OV-3 (human ovarian carcinoma) cell line was purchased from ATCC (Manassas, VA, USA). The HER2-LLC1 cell line was generated by Campadelli’s laboratory. The SK-OV-3 cells were cultured in RPMI 1640 medium-GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with heat-inactivated 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA). HER2-LLC1 cells were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% FBS, 1% (v/v) penicillin/streptomycin, 2 mM l-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), and 2 mM puromycin (Sigma-Aldrich/Merck, St. Louis, MO, USA) for selection. Both cell lines were maintained at 37° C in 5% CO2.

Viruses

R-113 corresponds to the previously described virus named R-LM113. R-115, described by Menotti et al., and R-121 are derivatives of R-113. They are single-armed THVs, expressing mIL-12 or murine GM-CSF (mGM-CSF), respectively. R-123 is a double-armed THV, derivative of R-115, expressing both cytokines. The viral sites of insertion are UL26–UL27 for mGM-CSF-expressing cassette and US1–US2 for mIL-12-expressing cassette. All viruses were cultivated and titrated by plaque assay in SK-OV-3 cells.

A WT HSV1, R-LM5, was used to induce the development of a pre-existing anti-HSV immunity in mice.
ELISA Assay
SK-OV-3 cells were seeded in 12-well plates at a density of 5 \times 10^5 cells/well 24 h before virus infection. Cells were infected with R-113, R-115, R-121, or R-123 at a multiplicity of infection (MOI) of 0.1 PFU/cell. 24 and 48 h after infection, supernatants were collected to check cytokine production. mIL-12 and mGM-CSF were quantified by means of an IL-12p70 mouse ELISA kit or GM-CSF mouse ELISA kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s instructions, respectively.

In Vivo Studies
For the metastatic tumor setting, 7.5 \times 10^5 HER2-LLC1 cells were injected i.v. into the mouse tail vein. After 3 days, viral and z-PD1 treatments started (day 0). THVs were administered five times at 10^7 PFU/injection every 2–3 days starting from day 0. α-murine (α-m)PD1, clone RMP1-14 (Bio X Cell, Lebanon, NH, USA), was administered intraperitoneally (i.p.) at a dosage of 200 µg twice a week from day 0 to day 10. On day 14, lungs were perfused with India ink (15%), harvested, and fixed in Fekete’s solution. Lung metastatic colonies were counted using a dissecting microscope.

In the condition of pre-existing immunity against HSV, 12 weeks before HER2-LLC1 implantation, mice were systemically (i.p.) injected with R-LM5 in a prime-boost regimen (week 0, week 3) at a dosage of 3 \times 10^6 PFU. Serum neutralization titers were measured over time by a plaque reduction neutralization test.

For the established tumor setting, 5 \times 10^5 HER2-LLC1 cells were s.c. injected into the mouse right flank. At day 0 treatments started in animals previously selected based on the tumor volume (tumor size average per group 110 mm^3), according to the doses and the regimen described above for the metastatic setting. α-PD1 antibody was administered in this setting until day 17. Tumor growth was measured using a digital caliper every 3–4 days. Tumor volume was calculated using the formula 0.5 \times \text{length} \times \text{width}^2, where the length was the longer dimension. Mice were sacrificed as soon as signs of distress or a tumor volume above 1,500 mm^3 occurred. 5 \times 10^5 HER2-LLC1 cells were also used for the contralateral tumor challenge, performed at day 40 in cured tumor-free mice.

To deplete immune cells subsets, α-mCD8, α-mCD4, or α-mNk (Bio X Cell, Lebanon, NH, USA) was used. In vivo depletion of IFN-γ was performed with α-mIFN-γ (Bio X Cell, Lebanon, NH, USA). Each antibody was administered five times by i.p. injection at a dosage of 200 µg, every 3–4 days from day 1 to day 15.

Ex Vivo Immune Analysis
Immune cell analysis was performed on blood 10 days post treatments start. Blood collected in heparin tubes was processed to remove erythrocytes; cells were then stained for viability with a Live/Dead fixable near-infrared (IR) dead cell stain kit (Thermo Fisher Scientific, Waltham, MA, USA). Surface staining was then performed to detect CD3^+ T cells and both CD4^+ and CD8^+ T cell subsets, with the following surface antibodies: allophycocyanin anti-mouse CD3e, phycoerythrin anti-mouse CD4, and peridinin chlorophyll protein (PerCP) anti-mouse CD8a (BD Biosciences, San Jose, CA, USA). Stained cells were acquired on a FACSCanto flow cytometer and analyzed using DIVA software (BD Biosciences). The same antibodies were used to check CD8^+ and CD4^+ T cell depletion. Fluorescein isothiocyanate anti-mouse CD335 (BioLegend, San Diego, CA, USA) was added for the NK cell depletion check.

Plaque Reduction Neutralization Test
SK-OV-3 cells were seeded in 12-well plates at a density of 5 \times 10^5 cells/well and cultured overnight at 37°C in 5% CO_2 until the cells became a monolayer. Heat-inactivated mouse sera were first diluted 20-fold in DMEM containing 1% heat-inactivated FBS and 1% (v/v) penicillin/streptomycin, followed by 3-fold serial dilutions from 1:60 to 1:131,220. An equal volume of HSV-1 viral dilution, containing 100 PFU of viruses, was mixed with the diluted samples and incubated at 37°C in 5% CO_2 for 30 min. Then, 0.35 mL of the total mixture was added to the SK-OV-3 cells and incubated for 1.5 h at 37°C on a shaker. The infection mixture was then removed and 1.5 mL of fresh culture medium (RPMI 1640 medium-Glutamax, supplemented with heat-inactivated 2.5% FBS and 1% [v/v] penicillin/streptomycin) was added to facilitate the viral entry. After 48h incubation, the cells were fixed with 96% EtOH (0.5 mL/well) for 10 min and then stained with 0.8 mL of Giemsa (Sigma-Aldrich/Merck, St. Louis, MO, USA) for 15 min. Plates were then washed and left to dry. The plaques were counted by use of a dissecting microscope. Virus control wells were infected with the same amount of virus as the testing wells mixed with serially diluted samples. Each test was carried out in triplicate.

The neutralizing titer was expressed as the dilution to which the number of plaques reduces by 50% compared to the control sample (virus alone).

Statistical Analysis
Statistical significance was calculated using the Mann-Whitney test, using Prism 6.0 software. The analysis of contingency data was performed using the mid-p exact test. The significant p values were assigned as *p <0.05, **p <0.01, ***p <0.001, and ****p <0.0001 and designated on the plots.

Study Approval
All experimental procedures were approved by the local animal Ethics Council and were performed in accordance with national and international laws and policies (EEC Council Directive 86/609; Italian Legislative Decree 26/14). The Ethics Committee of the Italian Ministry of Health approved this research.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
A.M.D, E.Scarselli, and A.N. conceived the experiments and supervised the study. A.M.D and E.Scarselli designed the experiments.
M.D.L., G.C., V.B., I.G., L.N., F.L., B.P., E.Sasso, S.P., G.F., C.G., and G.-C.F. conducted the research. M.D.L., A.M.D., and G.C. analyzed the data. G.C.F. and N.Z contributed to data interpretation. M.D.L. and A.M.D. wrote the manuscript.

CONFLICTS OF INTEREST
E. Scarselli and A.N. are founders of Nouscom S.R.L. G.C.-F. owns shares in Nouscom S.r.l. A.M.D, M.D.L., G.C., V.B., I.G., L.N., F.L., E. Sasso, and B.P. are employees of Nouscom S.r.l. The remaining authors declare no competing interests.

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