Neoadjuvant oncolytic virotherapy before surgery sensitizes triple-negative breast cancer to immune checkpoint therapy

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Triple-negative breast cancer (TNBC) is an aggressive disease for which treatment options are limited and associated with severe toxicities. Immunotherapeutic approaches like immune checkpoint inhibitors (ICIs) are a potential strategy, but clinical trials have demonstrated limited success in this patient cohort. Clinical studies using ICIs have revealed that patients with preexisting antitumor immunity are the most responsive. Given that oncolytic viruses (OVs) induce antitumor immunity, we investigated their use as an ICI-sensitizing approach. Using a therapeutic model that mimics the course of treatment for women with newly diagnosed TNBC, we demonstrate that early OV treatment coupled with surgical resection provides long-term benefits. OV therapy sensitizes otherwise refractory TNBC to immune checkpoint blockade, preventing relapse in most of the treated animals. We suggest that OV therapy in combination with immune checkpoint blockade warrants testing as a neoadjuvant treatment option in the window of opportunity between TNBC diagnosis and surgical resection.

INTRODUCTION

The treatment paradigm for newly diagnosed breast cancer includes surgical resection of the primary mass and is often followed by radio- and chemotherapy, which have well-documented quality of life-compromising toxicities (1, 2). An increasing number of patients now receive chemotherapeutic treatment before surgery. Less toxic and more effective therapeutic strategies to complement surgery are still needed, especially for triple-negative breast cancer (TNBC), which is the deadliest form of the disease, with the highest death rate in the first 2 years and the worst overall survival of all breast cancer types (3, 4). Immune checkpoint inhibitors (ICIs), which are particularly effective for highly immunogenic cancers (5), are considered a promising strategy for TNBC patients; however, results from the first-in-human clinical trial investigating the use of ICIs for TNBC treatment demonstrated benefits for only a minority of patients (6). Two additional studies are currently testing the efficacy of immune checkpoint blockade for TNBC treatment, but the results are still unknown [(4) and NCT02838823]. It is reasonable to suggest that for intractable diseases like TNBC, a combination of complementary therapeutic strategies has a better chance of success. Furthermore, given the rapid progression of TNBC, candidate therapies are more likely to be effective early in the disease, when tumor burden is minimal. It is now well established that oncolytic viruses (OVs) induce antitumor immunity (7), and therefore, we tested the use of OV therapy in combination with ICI treatments in a “window of opportunity” surgery model. Our OV of choice was the Maraba rhabdovirus, which is effective in a variety of mouse tumor models (8) and is now being tested in phase 1/2 studies (NCT02285816 and NCT02879760). We found that Maraba treatment before surgery efficiently triggers antitumor immunity that is effectively enabled by ICI treatment after surgery. Our findings support the idea of administering Maraba in the window of opportunity that separates the initial diagnosis of TNBC and tumor resection to minimize TNBC recurrence and open the possibility of treating relapsing patients with ICIs. Our treatment strategy could greatly improve the outcome of TNBC patients and merits consideration for clinical testing.

RESULTS

Maraba OV therapy affects both primary and metastatic lesions

We assessed the potential of Maraba-based OV for breast cancer therapy in previously described patient-derived xenografts (PDXs) of TNBC (HCI001; Fig. 1, A and B) and luminal B breast cancer (HCI003; fig. S1A) (9). Tumors were established and subsequently treated by a single direct intratumoral injection of 10⁶ plaque-forming units (PFU) of Maraba virus. Histological analysis of the cytokeratin-18–positive tumor tissue revealed robust infection in both models 36 hours after treatment. The hematoxylin and eosin (H&E) staining of both PDX models do not allow the assessment of the contribution of the immune system to the efficacy of an OV product, and so, we tested Maraba in three different syngeneic TNBC models (10–12). When implanted orthotopically, 4T1, EMT6, and E0771 tumors naturally form metastases to the lungs, liver, brain, and bones, recapitulating the disease course of advanced human TNBC. As seen with the PDX tumors, direct injection of Maraba virus into 4T1 tumors initiated virus infection and tumor killing (Fig. 1D and fig. S1, B and C). Despite this initial virally mediated oncolysis, overall tumor control and long-term survival of

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4T1-bearing animals were minimal (Fig. S2A). Similar results were found using Maraba OV therapeutically in the E0771 and EMT6 tumor models (Fig. S2, B and C). These results are consistent with previous findings showing that murine cancer cells have a range of susceptibilities to Maraba (8) and that the 4T1 and EMT6 tumor models are particularly refractory to virus treatment in vivo (12). Because most TNBC patients undergo resection of their untreated, or chemotherapy-treated, primary lesions, we treated 4T1 tumors with Maraba, surgically removed the primary lesion 3 days later, and then sacrificed the treated animals 25 days later to allow the enumeration of the sites of spontaneous metastatic growth. Fewer and smaller internal (Fig. 1E and Fig. S3A) and surface (Fig. 1F and Fig. S3B) lung tumors were observed with intratumoral Maraba treatment, with 50% of the animals being metastasis-free, whereas all of the control animals displayed lung tumors (Fig. 1F and Fig. S3B). Similar findings were obtained using the EMT6 (G, n = 10) and E0771 (H, n = 10) tumor models.

Neoadjuvant OV therapy generates a long-lasting personalized antitumor immune response

The reduction of lung tumor nodules after direct injection of Maraba into tumors in the fat pad suggested that even limited virally mediated oncolysis could reduce metastatic progression, perhaps through the initiation of an antitumor immune response. To determine whether viral oncolysis–mediated antitumor immunity could prevent relapse, we developed a rechallenge model to force the reemergence of the disease. In this setting, primary tumors are treated intratumorally with Maraba and surgically resected, and secondary tumors are implanted in the fat pad and left untreated (Fig. 2A). In all three TNBC models tested, the initial Maraba treatment provided protection against the secondary tumors without the need for additional virus treatments, and at least 20% of the animals showed a complete response (Fig. 2, B to D). The effect was dependent on virus replication because ultraviolet (UV)–inactivated Maraba provided no protection in this rechallenge model (Fig. S4A). Intravenous infusion of Maraba virus was even more efficient than local administration in this rechallenge setting, with 40% of the mice disease-free for the duration of the experiment (Fig. 2E). We repeated our tumor rechallenge experiment using T cell–deficient mice and found the therapeutic benefits to be completely lost (Fig. 2F), supporting the idea that a functional immune system is required for Maraba to confer protection in the window of opportunity setting. To test the specificity of this immune response, we applied our tumor rechallenge model to a group of animals for which different primary and secondary tumors were implanted (Fig. 2G and Fig. S4B). We observed that when the challenge tumors were genotypically distinct from the initial malignancy, no survival benefits were conferred by neoadjuvant OV therapy.

In long-term survival studies using Maraba virus in the 4T1 model, we classified treatment outcomes into four groups. A small number...
of animals in both untreated and treated groups died relatively early after secondary tumor implantation [found dead by the Animal Care and Veterinary Service (ACVS) staff], and although they had detectable tumor burden, it was not possible to ascribe a cause of death. A second cohort reached the endpoint tumor size of 1500 mm$^3$ in the injected fat pad as determined by the ACVS guidelines at our institute and were euthanized (tumor burden). A third group of animals had extensive lung disease and were determined to be at endpoint by the ACVS staff because of respiratory distress. Finally, a fourth group showed no evidence of the disease 100 days after secondary tumor implantation and was classified as “complete responders.” In the control group, animals deteriorated more rapidly and had extensive lung disease (Fig. 3A). In contrast, the treated animals survived longer, fewer had evidence of metastatic lung disease, and almost 25% of animals had no evidence of tumor growth despite both a primary and secondary tumor challenge. Similar results were obtained using the EMT6 and E0771 tumor rechallenge models (fig. S5). When looking at the spontaneous lung metastasis in T cell–deficient mice,

**Fig. 2. Maraba treatment results in complete responses in the window of opportunity setting.** (A) Schematic representation of the treatment schedule used for the tumor rechallenge model. (B to D) Tumor growth and Kaplan-Meier survival curves obtained using 4T1 (B), EMT6 (C), or E0771 (D) cells in the tumor rechallenge model ($n = 10$ mice per group per experiment). Maraba treatments were administered intratumorally. NT, no treatment. (E and F) The same experiment as in (B) was repeated using intravenous delivery of Maraba virus (E) or immunocompromised CD-1 nude mice (F). (G) Primary EMT6 or 4T1 tumors were treated with Maraba or left untreated and were resected, and all animals were rechallenged with 4T1 tumors. The dotted lines indicate the time of Maraba treatment. Statistical analysis for tumor measurements: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (unpaired multiple two-tailed t test). Statistical analysis for survival curves: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Mantel-Cox test).
we found that early Maraba treatment had no effect (fig. S6). The 4T1 complete responders were challenged for a third time via tumor implantation with 4T1 and EMT6 cells on opposite fat pads. We found that 100% of the EMT6 tumors were able to grow in these complete responders; however, only 1 of 10 mice had a detectable 4T1 tumor at the endpoint tumor size of 1500 mm³ for the EMT6 complete responders; however, only 1 of 10 mice had a detectable 4T1 tumor at the endpoint tumor size of 1500 mm³ for the EMT6 tumors on the opposite flank (Fig. 3B). These results are consistent with neoadjuvant Maraba therapy initiating antitumor immunity that is both prophylactic and therapeutic.

**Maraba virus infection alters the local tumor microenvironment**

Our observation that active Maraba infection was required to induce antitumor immunity suggested that a component of the activity may be related to changes in the tumor-induced local immunosuppressive microenvironment (13). To test this idea, we carried out transcriptome analysis of 4T1 and EMT6 tumor cells treated with either active or UV-inactivated Maraba virus. We found that during infection with Maraba virus, numerous genes were up-regulated compared to uninfected tumors or tumors treated with UV-inactivated Maraba virus (Fig. 4, A and B). Gene ontology analysis revealed that most of the top 10 enriched pathways were linked to immune responses (Fig. 4, C and D). The increase in proinflammatory cytokine mRNAs was mirrored at the protein level after Maraba infection (fig. S7, A and B). To extend these findings to the human disease, we assessed the induction of a subset of chemokines in vitro using the MDA-MB-231, BT-549, and HS578T human TNBC cell lines (fig. S7C) (14, 15) and our luminal B and TNBC PDX models (fig. S7D). Our results show the presence of viral genomes and the induction of CCL5 and CXCL11 after Maraba infection. Upon Maraba treatment, signal transducer and activator of transcription 1 (STAT1), the nuclear factor κB (NFκB) subunit p65, and interferon regulatory factor 3 (IRF3) are activated (Fig. 4E and fig. S8A). In an attempt to determine the mechanism by which Maraba triggers the expression of proinflammatory factors, we knocked down retinoic acid-inducible gene 1 (RIG-1) and myeloid differentiation primary response 88 (MYD88) in our cell lines using small interfering RNAs (siRNAs) and found that both STAT1 and IRF3 phosphorylation were impaired in the absence of RIG-1 (Fig. 4E). Consistent with this, the induction of Ccl5, Cxcl10, and Il-6 by Maraba was impaired by RIG-1 knockdown (Fig. 4F). MYD88 knockdown also impaired the induction of Il-6, Cxcl10, and Cc5 in some cell lines (Fig. 4F), which highlights the importance of both pathways for the inflammatory response induced in response to virus infection. We also investigated the importance of inflammasome activation upon Maraba infection by measuring the concentration of interleukin-1β (IL-1β) in the culture supernatant 24 hours after infection. We found the IL-1β concentration to be below the limit of detection in all cell lines (fig. S8B), indicating the absence of inflammasome activation in these conditions.

To determine whether the chemokines produced by Maraba-infected cells could recruit immune cells more efficiently compared to uninfected cells, we used a Boyden chamber migration assay. Our results show the improved chemotactic activity of the medium conditioned by Maraba-infected cells (Fig. 5A). When using antibodies to block various chemokines, we found that CCL2, CCL5, CXCL9, CXCL10, and CXCL11 all had minor but significant (P = 0.0105 to <0.0001) effects on the chemotactic activity of the virus-conditioned medium (Fig. 5B and fig. S9). In vivo, we assessed the immune cell composition of 4T1 tumors after Maraba treatment (see gating strategy in fig. S10). Our results show that the tumors contained significantly (P = 0.0008) more immune cells after the administration of Maraba (Fig. 5C) and that blockade of CXCR3 (the receptor of CXCL9, CXCL10, and CXCL11) prevented this immune recruitment (Fig. 5D). Furthermore, histological analysis of the T cell content of 4T1 tumors revealed a greater quantity of T cells and deeper infiltration into the tumors after Maraba treatment (Fig. 5E and fig. S11). We then assessed the generation of antitumor immunity by interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) analysis. Our data show a strong response against 4T1 cells, but not EMT6 cells, after Maraba treatment of 4T1 tumor-bearing animals (Fig. 5F). We evaluated the role of IFN-α/β, IFN-γ, and CXCR3 in the Maraba-induced IFN-γ ELISPOT assay and found that blockade of IFN-α receptor 1 (IFN-αR1), but not CXCR3 or IFN-γ, impaired the induction of antitumor immunity (Fig. 5G). These results are in line with the absence of therapeutic benefits observed when using different primary and secondary tumor types (Fig. 2G) and confirm the specificity of the Maraba-induced antitumor immunity.

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**Fig. 3. Local Maraba treatment of TNBC tumors provides long-term systemic protection.** (A) Time of death of the 4T1 tumor-bearing animals in the rechallenge model. The cause of death is color-coded. (B) Maraba-treated mice (n = 10) from 4T1 tumor rechallenge experiments that were complete responders 100 days or more after tumor seeding were rechallenged with 4T1 and EMT6 cells. The right graph shows the percentage of tumors that could be detected before the animals reached end point. Statistical analysis according to the multiple unpaired t test: **P < 0.01, ***P < 0.001.
Fig. 4. Maraba infection triggers inflammation. (A and B) Microarray analysis of 4T1 (A) or EMT6 (B) cells infected with Maraba or ultraviolet (UV)–inactivated Maraba. The heat map includes the top genes that were enriched more than fourfold as compared to uninfected cells. R1 and R2 represent distinct experimental replicates. (C and D) Top 10 gene ontology enrichments of the genes induced by Maraba in the 4T1 (C) or EMT6 (D) model. FDR, false discovery rate. (E) Western blot analysis of 4T1 and EMT6 cell lysates. The cells were transfected with different small interfering RNAs (siRNAs) for 24 hours and infected with Maraba at a multiplicity of infection of 1 for an additional 12 hours before sample collection. STAT1, signal transducer and activator of transcription 1; pSTAT1, phospho-STAT1; IRF3, interferon regulatory factor 3; pIRF3, phospho-IRF3; VSV, vesicular stomatitis virus. (F) The same samples were also analyzed by quantitative polymerase chain reaction (n = 4) for the expression of different genes. Statistical analysis by unpaired t test with Welch’s correction: *P < 0.05, **P < 0.01, ***P < 0.001.
Maraba treatment sensitizes 4T1 tumors to immune checkpoint blockade

Proinflammatory signals are required to activate immune cells and generate antitumor immunity but also often trigger the expression of the immune checkpoint molecule programmed death ligand 1 (PD-L1) (16). Our transcriptome and flow cytometry analysis revealed the up-regulation of PD-L1 at both the mRNA level and the protein level by Maraba in both 4T1 and EMT6 cells (Fig. 4, A and B) and at the protein level in 4T1, EMT6, and E0771 cells (Fig. 6A). In addition, when using an antibody to block the receptor for both IFN-α and IFN-β, PD-L1 up-regulation was completely blocked for the 4T1 cells but not significantly blocked for the EMT6 or E0771 cells, suggesting that other factors are responsible for PD-L1 induction in these cell lines.

The increased expression of PD-L1 after virus treatment suggests that although antitumor T cells are generated by Maraba therapy, their therapeutic activity may be mitigated by the tumor-specific expression of PD-L1. Furthermore, we observed a tumor-specific increase in regulatory T cells after OV therapy (Fig. 6B). These observations suggest that Maraba therapy could be augmented by anti–programmed cell death protein 1 (PD-1) therapy. In primary tumors, Maraba treatment in combination with immune checkpoint blockade significantly \(P < 0.0001\) slows tumor growth in the 4T1 model compared to untreated or animals receiving either ICIs or Maraba monotherapy (Fig. 6C and fig. S12), although even the combination treatment is unable to completely eradicate the tumors. In contrast, when Maraba neoadjuvant therapy is coupled with surgical resection of the primary tumor, animals

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Fig. 5. Maraba induces antitumor T cell immunity. (A) Migration of splenocytes induced by conditioned medium from uninfected or infected 4T1 or EMT6 cells \((n = 4\) experimental replicates). (B) The same experiment was performed in the 4T1 cell line in the presence of control or blocking antibodies (Ab) \((n = 4\) experimental replicates; unpaired two-tailed \(t\) test with Welch’s correction, \(^{*}P < 0.05\), \(^{**}P < 0.01\), and \(^{***}P < 0.001\)). (C and D) Percentage of cells within 4T1 tumors that stained positive for CD45 as quantified by flow cytometry 10 days (C) or 2 days (D) after Maraba treatment with and without CXCR3-blocking antibodies \((n = 5\) mice; unpaired two-tailed \(t\) test with Welch’s correction, \(^{**}P < 0.01\) and \(^{***}P < 0.001\)). (E) Immunohistological assessment of CD3+ cells in 4T1 tumors at the time of resection in the tumor rechallenge model. Scale bars, 100 \(\mu\)m. (F) Interferon-γ (IFN-γ) ELISPOT (enzyme-linked immunospot) analysis of splenocytes harvested 10 days after treatment from mice treated with Maraba for five consecutive days \((n = 3\) to 5 mice per group). Restim, ex vivo restimulation with cells. (G) The same experiment was repeated with or without blocking antibodies administered intraperitoneally \((n = 5\) mice per group; unpaired two-tailed \(t\) test, \(^{**}P < 0.01\) and \(^{***}P < 0.001\)). The cells were restimulated ex vivo with EMT6 or 4T1 cells. IFN-αR1, IFN-α receptor 1.
respond well to immune checkpoint therapy (see treatment regimen in Fig. 6D) with 60 to 90% of animals experiencing a complete response after tumor rechallenge (Fig. 6, E to G). This is in stark contrast to animals undergoing primary tumor resection without prior virus therapy, wherein immune checkpoint therapy is completely ineffective in all three TNBC models tested.

**DISCUSSION**

It has now been established that certain OVs can initiate clinically relevant antitumor immune responses after locoregional therapy of skin lesions (17–19), particularly in the case of melanoma. This in situ vaccine effect is thought to be a consequence of the release of tumor-associated antigens in the presence of viral pathogen-associated molecular patterns and other danger signals (7). In addition, some OVs appear to induce immunogenic cell death rather than autophagy (20, 21). Oncolytic rhabdoviruses like Maraba and vesicular stomatitis virus (VSV) have been shown to enhance natural killer cell–mediated killing of tumor cells (22), dendritic cell maturation, up-regulation of antigen presentation by tumor cells (23), and the production of proinflammatory cytokines and chemokines (23–26). Here, we demonstrate that the production of several chemokines and cytokines by

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**Fig. 6. Maraba treatment sensitizes 4T1 tumors to immune checkpoint blockade.** (A) Cell surface programmed death ligand 1 (PD-L1) expression on 4T1, EMT6, and E0771 cells after an incubation with Maraba-infected 4T1 cell–conditioned medium with or without IFN-αR1–blocking antibody. MFV, mean fluorescence value; MRB, Maraba. (B) Mice with 4T1 tumors were treated intratumorally with Maraba (days 7 to 11) and sacrificed on day 20. The graph shows the percentage of T cells that were FOXP3+. Two-tailed unpaired t test, **P < 0.01. (C) Mice bearing 4T1 tumors were treated intratumorally with Maraba (days 7 to 11) followed by anti-cytotoxic T lymphocyte–associated protein 4 (CTLA4) and anti–PD-1 (programmed cell death protein 1) antibodies injected intraperitoneally on days 21, 23, 25, 27, and 29. The tumors were measured on day 31 (three separate experiments). The graph shows the volume of each tumor relative to the average volume of the tumors from the control group. Unpaired two-tailed t test, *P < 0.05, **P < 0.01, ***P < 0.001. ICI, immune checkpoint inhibitor. (D) Treatment schedule used in (E), (F), and (G). (E to G) Tumor growth and Kaplan-Meier survival analysis of 4T1 (E), EMT6 (F), or E0771 (G) tumor-bearing mice using the tumor rechallenge model. The dotted lines indicate the days of Maraba treatment. Unpaired multiple two-tailed t test, *P < 0.05, **P < 0.01, ***P < 0.001. The symbol “#” denotes the difference between NT and Maraba + ICI. The symbol “x” denotes the difference between ICI and Maraba + ICI. For survival curves, **P < 0.01, ***P < 0.001 (Mantel-Cox test).
tumor cells relies on both RIG-I and MYD88 signaling, depending on the tumor model. Considering cancer heterogeneity, this finding highlights the importance of using a therapeutic agent such as Maraba virus that has the capacity to simultaneously stimulate several pathways. Furthermore, we show that immune cell recruitment to the tumor microenvironment is dependent on one or several chemokines binding to CXCR3; however, this chemotraction does not seem critical for the induction of antitumor immunity. Rather, we demonstrate that immune stimulation by type I IFNs produced in response to OV treatment is required for the optimal induction of antitumor immunity. It is reasonable to suggest that antitumor immune responses initiated by OV infection could be enhanced by combination with immune checkpoint therapy, and in earlier mouse studies, Zamarin et al. (27) showed that the combination of an oncolytic Newcastle disease virus and anti-CTLA4 therapy produces curative systemic immune responses. In their study, the combination therapy was only effective when very small tumors (3 days after implantation) were treated, suggesting that in mouse models, where the time from implantation to end point is very short (2 to 4 weeks), rapid tumor growth can outstrip the generation and enablement of effective antitumor immune responses. Here, the combination of ICIs with Maraba OV therapy could slow the growth of aggressive TNBC but did not lead to complete response. However, if infected tumors were allowed to progress and then surgically resected before the end point, then mice could withstand rechallenge of even higher doses of the originally seeded tumor. This suggests a role for OV therapeutics as neoadjuvants before surgery. We feel that this may be a particularly relevant application for women newly diagnosed with TNBC. Our finding that the intravenous infusion of OV is more effective than intratumoral therapy suggests that the oncolytic therapeutic may be effective in generating antitumor immunity and in attacking the sites of minimal residual disease. We suggest that surgical reduction of tumor burden allows time for an effective OV-induced antitumor immune response to be initiated, which then plays a role in ongoing immune surveillance to prevent relapse. Our findings are in line with the ones obtained by Samson et al. (28), where they show that the intravenous infusion of oncolytic reovirus to glioblastoma patients promotes T cell recruitment to the tumors and leads to the up-regulation of PD-L1 by tumor cells. Consistent with what we observe using Maraba virus in our TNBC models, they also show that reovirus treatment administered before PD-1 blockade improved outcome in a murine orthotropic brain tumor model. Excitingly, results from a phase 1b trial investigating the use of the OV talimogene laherparepvec before PD-1 blockade for the treatment of advanced melanoma were recently published and support the combination (29). The best outcomes were observed in patients that showed increased PD-L1 expression upon treatment. Here, we demonstrate that Maraba virus also induces PD-L1 expression, therefore supporting the combination with immune checkpoint blockade. Currently, surgery followed by adjuvant chemotherapy of patients with TNBC is insufficient, because survival is 2 years or less for most patients (4). We suggest that although adjuvant chemotherapy may have some impact on minimal residual disease, its negative impact on the patient’s immune system could be contributing to patient relapse (30).

The oncolytic version of Maraba virus used in this study is currently undergoing clinical investigation (NCT02285816 and NCT02879760); thus, safety data and a therapeutic dosing regimen still need to be established. Because window of opportunity studies are an already established paradigm for TNBC patients (31), we believe that a version of the treatment strategy tested in this study could be rapidly translated to the clinic with the potential to substantially improve the outcome and quality of life for TNBC patients.

Of course, logistical challenges to the clinical testing of this approach still remain. To date, OV trials have largely been carried out in advanced cancer patients, whereas we propose that, at least for TNBC patients, the data presented here provide a rationale for treating very early in disease progression. In general, the infusion of replicating virus therapeutics has been very well tolerated in a number of clinical studies; however, there are often acute flu-like symptoms associated with therapy (32–34). Given the bleak outlook for most TNBC patients, we suggest that early treatment of an otherwise apparently healthy patient with a replicating virus therapeutic is warranted. Similarly, ICIs have been largely tested in TNBC patients with advanced disease; however, we argue that there is a rationale and an urgent need to test these therapeutics in patients early in their diagnosis when they have more robust immune systems and minimal disease burden.

MATERIALS AND METHODS

Study design

The objective of this study was to investigate the long-term immune-mediated effects of early oncolytic virotherapy in treatment-resistant TNBC models. We developed a model that recapitulates the treatment course of TNBC patients. Using this model, we forced relapse by challenging the animals with high doses of cancer cells upon surgical removal of their treated tumors. We used a minimum of 10 mice per group per experiment and obtained consistent results. The animals for which the primary tumors relapsed at the primary site of implantation were excluded from our study (about 1 of 100 mice).

Cell lines and culture

Vero kidney epithelial; 4T1, EMT6, and E0771 murine mammary carcinoma; and MDA-MB-231, BT-549, and Hs578T human TNBC cell lines were purchased from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Corning Cellgro) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and cultured at 37°C with 5% CO2.

Virus production and quantification

The virus used in this study is the clinical candidate Maraba MG1. Maraba is a single-stranded negative-sense RNA virus that replicates in the cytoplasm of infected cells. The expansion and purification of Maraba was previously described (8). Briefly, Vero cells were infected at a multiplicity of infection (MOI) of 0.01 for 24 hours before harvesting, filtration [0.22-μm bottle top filter (Millipore)], and centrifugation (90 min at 30,100g) of the culture supernatant. The pellet was resuspended in Dulbecco’s phosphate-buffered saline (DPBS) (Corning Cellgro) and stored at −80°C.

Viral titers were determined by plaque assay. Briefly, serially diluted samples were transferred to monolayers of Vero cells, incubated for 1 hour, and then overlaid with 0.5% agarose/DMEM supplemented with 10% FBS. Plaques were counted 24 hours later.

Irradiated virus

Maraba was UV-inactivated by exposure to 120 mJ/cm² for 2 min using a Spectrolinker XL-1000 UV cross-linker as described previously (22). Irradiation results in cross-linking of the viral RNA genome and therefore prevents viral replication.
Small interfering RNA

The siRNAs were all purchased from Dharmaco (M-053455-01-0005, M-065328-01-0005, and M-063057-00-0005) and transfected using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol 24 hours before virus infection.

Western blot analysis

Cells transfected with siRNAs for 24 hours were infected with Maraba at an MOI of 1 for 12 hours. Lysates were generated using NP-40 lysis buffer supplemented with 1 M NaF, 200 mM Na3VO4, and protease inhibitor cocktail (all from Roche). Cleared lysates were migrated on NuPAGE 4 to 12% bis-tris protein gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes (GE Healthcare). The following antibodies were used in this study: anti-mouse p65, anti–phospho p65, anti–IRF3, anti–phospho-IRF3, anti–STAT1, anti–phospho-STAT1, anti–IκBα (inhibitor of NFκB), anti–RIG-I, and anti–actin (Cell Signaling Technology), anti–SVS (house-made) (35), and anti-mouse MYD88 (Abcam).

Flow cytometry

Splenocytes were processed as previously described (35). Briefly, spleens were harvested and mashed through a 70-μm strainer (Thermo Fisher Scientific) before lysis of red blood cells using ammonium chloride potassium lysis buffer and resuspension in fluorescence-activated cell sorting buffer (PBS and 3% FBS). For tumor cell extraction, we used the mouse tumor cocktail (Miltenyi) according to the manufacturer’s protocol with gentleMACS tubes and a gentleMACS Dissociator (Miltenyi). Cells were stained using various combinations of CD45, CD3, CD4, FOXP3, and PD-L1 antibodies (all from BD Biosciences). The stained cells were then fixed using IC fixation buffer (eBioscience). For intranuclear staining, the FOXP3 staining buffer set was used (eBioscience). Flow cytometry analysis was performed on a CyAn ADP 9 (Beckman Coulter). Anti-mouse IFN-αR1 was purchased from BioXCell (clone MAR1-5A3) and used at the final concentration of 15 μg/ml.

Enzyme-linked immunospot

Splenocytes were collected 10 days after the last virus treatment, and a mouse IFN-γ ELISPOT was performed according to the manufacturer’s protocol (Mabtech). The incubation was performed in serum-free DMEM at a splenocyte-to-tumor cell ratio of 5:1.

Cytometric bead array and ELISA

Cytometric bead array quantification of mouse IFN-γ, IL-6, tumor necrosis factor–α, and CCL2 (BD Biosciences) and enzyme-linked immunosorbent assay (ELISA) quantification of mouse IFN-β (R&D Systems), and mouse IL-1β (Antibodies Online) were performed according to the manufacturer’s protocols.

Migration assay

Conditioned medium was generated by infecting EMT6 or 4T1 cells with Maraba for 24 hours at an MOI of 3 in serum-free DMEM. The virus was removed by filtration using Amicon centrifugal filters with a cutoff of 50 kDa (Millipore). To assess the chemotactic activity, 50,000 freshly harvested splenocytes were allowed to migrate toward the virus-cleared conditioned medium for 24 hours in a Boyden chamber 96-well plate with 3-μm membrane pores (BioVision), and the cells that migrated to the bottom chamber were quantified by fluorometric assay using a Fluoroskan Ascent FL (Thermo LabSystems) according to the manufacturer’s protocol (BioVision). The antibodies were used at the final concentrations listed below. The isotype control (BE0083; 10 μg/ml) was purchased from BioXCell, and anti-mouse CCL2 (AF-479; 25 μg/ml), CCL5 (AF418; 0.3 μg/ml), CXCL9 (AF492; 20 μg/ml), CXCL10 (AF-466; 25 μg/ml), and CXCL11 (MAB572; 2 μg/ml) were all from R&D Systems.

In vivo experiments and tumor models

All experiments were performed in accordance with the University of Ottawa ACVS guidelines. 4T1 and EMT6 tumors were implanted subcutaneously into the left flank of BALB/c or nude mice, whereas E0771 tumors were implanted into C57BL/6 mice (Charles River Laboratories). For the orthotopic models, 105 cells were injected into the second right mammary fat pad. For treatments, the virus (108 PFU) in a total volume of 100 μl of PBS was injected intratumorally or intravenously on days 7 to 11 (unless specified otherwise) using insulin syringes (The Stevens Co.). The ICIs [anti-CD8 (clone RMPI-14, BioXCell) and anti-CTLA4 (clone 9D9, BioXCell)] were injected intraperitoneally at a dose of 100 μg each every second day (on days 21, 23, 25, 27, and 29) for a total of five injections, as depicted in Fig. 6D.

For the experiments using blocking antibodies (Fig. 5, D and G), 200 μg of the corresponding antibody was administered intraperitoneally to the animals on day 10, and 108 PFU of Maraba were administered intratumorally on days 10 (simultaneously with antibody), 11, and 12. The following antibodies were all from BioXCell: isotype control, anti-CXCR3 (CXCR3-173), anti–IFN-γ (XMG1.2), and anti–IFN-αR1 (MAR1-5A3). Animals were sacrificed on day 12 (Fig. 5D) or day 20 (Fig. 5G).

For the tumor rechallenge model, 106 cells were injected subcutaneously into the left flank of the animals. The tumors were treated at the indicated time points and resected 7 days after the first treatment. The skin surrounding the tumors was also removed to minimize the risk of regrowth at the primary site of tumor implantation. Four days after surgery, a higher dose of tumor cells (3 × 105 cells) was seeded into the second right fat pad. The mice were rechallenged a second time more than 100 days after tumor seeding with 3 × 105 EMT6 and 4T1 cells intra-fat pad bilaterally.

Histological analysis

Tumors were fixed in 10% buffered formalin phosphate (Thermo Fisher Scientific) for 48 hours and embedded in paraffin. The sections were stained using H&E or the specified antibodies after rehydration (pH 6) was used for heat-mediated antigen retrieval. The following antibodies were used for staining of the different cells: CD3 (Abcam), Maraba (house-made) (12), and cytokeratin-18 (Abcam). The signal was quantified using the Aperio ScanScope software.

Lung metastasis analysis

4T1, EMT6, or E0771 cells were orthotopically implanted as described above and treated with PBS or 108 PFU of Maraba on days 7 to 11. The tumors were resected on day 14, all animals were sacrificed on day 45, and the lungs were either fixed in formalin, sectioned, and stained with H&E before measurement using the Aperio ScanScope software or perfused with a solution of 50% India ink (Super Black) in PBS and fixed using Fekete’s solution (70% ethanol, 10% formaldehyde, and 5% acetic acid) before counting of surface tumors.

PDX models

Previously described PDXs were grown in NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice as described
previously (9, 36). HCl001 was initially isolated from a stage 4 basal-like TNBC primary tumor, and HCl003 was initially isolated from a stage 3A luminal B primary lesion that tested estrogen receptor–positive, progesterone receptor–positive, and human epidermal growth factor receptor 2–negative. More details can be found in the original study describing the models (9). The tumors were treated when they reached 1000 mm$^3$ (day 40 for HCl001 and day 63 for HCl003) and collected 36 hours later for quantitative polymerase chain reaction and histological analysis.

**Quantitative polymerase chain reaction**

RNA was extracted using the RNeasy RNA extraction kit (Qiagen) for quantitative polymerase chain reaction and histological analysis.

**Microarray**

Monolayers of 4T1 or EMT6 cells were treated at an MOI of 3 for 24 hours with either Maraba or UV-inactivated Maraba. Culture supernatants were collected for cytometric bead array and ELISA analysis, and the RNA was extracted from the cells as described above for microarray analysis. Experimental duplicate total RNA samples were processed by the Center for Applied Genomics at the Hospital for Sick Children for microarray analysis on a MouseGene2.0-st Affymetrix chip. Raw files were analyzed using the Transcriptome Analysis Console v3.0 (Affymetrix) software. Normalized transcript expression values were further processed with R. Heat maps were produced using the R package “pheatmap” v1.0.8. Gene Ontology term enrichment analysis was performed using the online EnrichR tool (PMID 27141961). Genes selected for enrichment analysis are the subset of genes up-regulated by Maraba infection relative to noninfected cells.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 6.0 as described in the figure legends. Data are means ± SD.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/10/422/eaao1641/DC1

**REFERENCES AND NOTES**


Immunotherapy has emerged as a promising approach for cancer treatment, particularly in combination with checkpoint blockade. Oncolytic virotherapy promotes intratumoral T cell infiltration and improves anti-PD-1 checkpoint blockade.


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Neoadjuvant oncolytic virotherapy before surgery sensitizes triple-negative breast cancer to immune checkpoint therapy

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Viruses team up with cancer immunotherapy

Immune checkpoint inhibitors have shown great promise for cancer therapy, but they do not treat all cancers, and neither breast nor brain tumors are usually treatable with these drugs. However, Bourgeois-Daigneault et al. discovered a way to address this for breast cancer, and Samson et al. discovered a way to address this for brain tumors. In both cases, the authors found that oncolytic virus treatment given early, before surgical resection, alters the antitumor immune response and potentiates the effects of subsequent treatment with immune checkpoint inhibitors. Although these studies differ in the details of their methods and the immune effects induced by the oncolytic viruses, they indicate the potential of such viruses for enhancing the potential of checkpoint therapy and expanding it to new types of cancer.