

# A new MVA ancestor-derived oncolytic vaccinia virus induces immunogenic tumor cell death and robust antitumor immune responses

Juan J. Rojas,<sup>1,2,3</sup> Lien Van Hoecke,<sup>4,5</sup> Miquel Conesa,<sup>1,2</sup> Carmen Bueno-Merino,<sup>1,2</sup> Ana del Canizo,<sup>1,2</sup> Stephanie Riederer,<sup>3</sup> Maria Barcia,<sup>1,2</sup> Katrin Brosinski,<sup>3</sup> Michael H. Lehmann,<sup>3</sup> Asisa Volz,<sup>3,6</sup> Xavier Saelens,<sup>5,7,8</sup> and Gerd Sutter<sup>3,9</sup>

<sup>1</sup>Immunology Unit, Department of Pathology and Experimental Therapies, School of Medicine, University of Barcelona – UB, 08907 L'Hospitalet de Llobregat, Spain; <sup>2</sup>Immunity, Inflammation, and Cancer Group, Oncobell Program, Institut d'Investigació Biomèdica de Bellvitge - IDIBELL, 08908 L'Hospitalet de Llobregat, Spain; <sup>3</sup>Division of Virology, Institute for Infection Medicine and Zoonoses, Department of Veterinary Sciences, LMU Munich, 85764 Oberschleißheim, Germany; <sup>4</sup>VIB Center for Inflammation Research, VIB, 9052 Ghent, Belgium; <sup>5</sup>Department of Biomedical Molecular Biology, Ghent University, 9052 Ghent, Belgium; <sup>6</sup>Institute of Virology, University of Veterinary Medicine Hannover, 30559 Hannover, Germany; <sup>7</sup>VIB Center for Medical Biotechnology, VIB, 9052 Ghent, Belgium; <sup>8</sup>Department of Biochemistry and Microbiology, Ghent University, 9052 Ghent, Belgium; <sup>9</sup>German Center for Infection Research (DZIF), Partner Site Munich, 80539 Munich, Germany

Vaccinia viruses (VACVs) are versatile therapeutic agents and different features of various VACV strains allow for a broad range of therapeutic applications. Modified VACV Ankara (MVA) is a particularly altered VACV strain that is highly immunogenic, incapable of replicating in mammalian hosts, and broadly used as a safe vector for vaccination. Alternatively, Western Reserve (WR) or Copenhagen (Cop) are VACV strains that efficiently replicate in cancer cells and, therefore, are used to develop oncolytic viruses. However, the immune evasion capacity of WR or Cop hinders their ability to elicit antitumor immune responses, which is crucial for efficacy in the clinic. Here, we describe a new VACV strain named Immune-Oncolytic VACV Ankara (IOVA), which combines efficient replication in cancer cells with induction of immunogenic tumor cell death (ICD). IOVA was engineered from an MVA ancestor and shows superior cytotoxicity in tumor cells. In addition, the IOVA genome incorporates mutations that lead to massive fusogenesis of tumor cells, which contributes to improved antitumor effects. In syngeneic mouse tumor models, the induction of ICD results in robust antitumor immunity directed against tumor neo-epitopes and eradication of large established tumors. These data present IOVA as an improved immunotherapeutic oncolytic vector.

## INTRODUCTION

Virotherapy is a type of cancer immunotherapy that uses modified viruses to lyse cancer cells and elicit antitumor immune responses. Mechanistically, intratumoral virus replication acts as a potent danger signal for the immune system, decreasing tumor immune suppression and promoting infiltration of immune cells into the tumor.<sup>1</sup> Concurrently, virus-mediated lysis of tumor cells results in the release of tumor antigens that can be taken up by antigen-presenting cells to prime and activate tumor-specific cytotoxic T lymphocytes.<sup>2</sup> Thus,

the capacity of the virus to effectively replicate in, and kill cancer cells and to act as a danger signal within the tumor are key factors for achieving effective antitumor effects.

Vaccinia virus (VACV), the prototype human live virus vaccine used to eradicate smallpox,<sup>3</sup> is among the most promising vectors to develop oncolytic agents due to its fast and lytic replication cycle, an established safety record in humans, and its high capacity to incorporate transgenes.<sup>4</sup> Furthermore, VACV strains are highly immunogenic and this immunogenicity can be further improved by targeted deletion of specific viral genes to enhance tumor recognition by the immune system.<sup>5</sup>

Beside their use as oncolytic agents, VACV have also proved versatile and effective as vaccine platforms. Modified VACV Ankara (MVA) is a highly attenuated strain of VACV that exhibits defective replication in cells from mammalian hosts and has been broadly used to deliver antigens derived from heterologous pathogens.<sup>6</sup> MVA was obtained after 516 *in vitro* passages of the Chorioallantois VACV Ankara (CVA) strain in chicken embryo fibroblasts (CEFs). During these passages, CVA suffered a consecutive loss of genetic information, including many viral immune evasion genes that restricted its virulence and replicative capacity in mammalian cells.<sup>7</sup> Despite its inability to productively replicate in cancer cells, previous studies indicate that danger signals elicited after intratumoral administration of MVA are sufficient to induce systemic antitumor effects,<sup>8</sup> although further research is needed to elucidate the mechanisms of action.

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**Correspondence:** Juan J. Rojas, Department of Pathology and Experimental Therapies, University of Barcelona, Feixa Llarga, s/n, 08907 Hospitalet de Llobregat, Barcelona, Spain. **E-mail:** jrojas@ub.edu



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Over the last decade, the capacity of different anticancer therapeutics to induce immunogenic cell death (ICD) emerged as pivotal to achieve effective and long-term antitumor immunity.<sup>9</sup> ICD is a type of regulated cell death that can elicit adaptive immunity against dead cell-associated antigens.<sup>10</sup> Although different types of cell death are included under the term ICD (e.g., necroptosis and pyroptosis), all of them are characterized by the exposure or release of damage-associated molecular patterns (DAMPs), which are molecules able to activate and attract immune cells.<sup>11</sup> In the context of oncolytics, viruses such as Semliki Forest virus are described to induce potent ICD,<sup>12</sup> whereas strains of VACV used to date as oncolytics induce only limited ICD levels.<sup>12,13</sup> Together with its strong lytic capacity and ability to incorporate transgenes, an oncolytic VACV strain that also induces potent ICD may represent a highly promising immunotherapeutic virus.

Here, we analyzed the levels of ICD induced by different VACV strains and constructed a novel oncolytic VACV strain that combines effective replication in cancer cells with a unique capacity to induce ICD. This strain, named Immune-Oncolytic VACV Ankara (IOVA), showed greatly enhanced therapeutic activity mediated by antitumor immune responses. Importantly, IOVA also exhibited antitumor effects on untreated distal tumor sites and a decreased toxicity upon systemic delivery. In addition, since IOVA induces syncytia formation after infection of tumor cells, we studied the effects of tumor cell fusogenesis on the activation of antitumor immune responses and tumor growth.

## RESULTS

## VACV MVA induces ICD in tumor cells but poorly controls tumor growth in vivo

ICD-inducing therapies hold promise for complete eradication of and long-term protection against cancer.<sup>9</sup> To ascertain whether VACV induces ICD, we tested the capacity of different VACV strains to induce calreticulin (CALR) translocation after infecting tumor cells. MVA was able to significantly induce CALR translocation to the cell surface in HeLa cells, as analyzed by flow cytometry (Figure S1A). On the contrary, strains widely used to develop oncolytic VACV such as Western Reserve (WR) or Copenhagen (Cop) failed to induce CALR translocation. However, despite this effective induction of CALR exposure, compared with WR/TK<sup>-</sup>, intratumoral injection of MVA had a poorer antitumor effect in the B16 mouse melanoma model (Figure S1B). Since MVA fails to replicate in mammalian cells,<sup>14</sup> we hypothesized that the capacity to induce features of ICD combined with effective replication and lysis of tumor cells are key prerequisites for an oncolytic VACV that would be suitable as a curative anti-tumor agent.

#### IOVA: A novel oncolytic VACV based on an MVA ancestor virus

Since MVA sequentially acquired its many genomic mutations during more than 500 CEF passages,<sup>6</sup> we hypothesized that a virus from an intermediate CEF passage might still possess the capacity to replicate in cancer cells while having already acquired mutations that cause ICD. Thus, we tested virus preparations from different passages during MVA generation for their capacity to replicate in cancer cells and chose a clonal virus isolate from a passage earlier than 150, since replication in mammalian cells was compromised in later passages (data not shown). In the genome of the isolated virus, we truncated the gene encoding the viral thymidine kinase (TK, J2) to support selective replication in cancer cells, and inserted an mCherry expression cassette into this gene locus as a fluorescence marker of viral gene expression. We called the novel VACV strain IOVA (Immune-Oncolytic VACV Ankara) and fully sequenced its genome. IOVA-encoded proteins with mutations or deletions were compared with the parental CVA strain<sup>15</sup> (GenBank: AM501482.1), the VACV strain used as initial material for IOVA and MVA selection (Table S1). In addition, the identity of these proteins in the MVA strain (GenBank: AY603355.1) is also included.

## IOVA shows a fusogenic cytopathic effect, improved cytotoxicity, and efficient replication in cancer cells

After infection of cancer cells, IOVA showed a clear fusogenic phenotype (Figure 1A, Video S1). This phenotype was demonstrated in a wide panel of human and mouse cancer cells (Figure S2). By counting the number of nuclei within HeLa cells after infection, we observed that IOVA can induce syncytia enclosing more than 60 nuclei (Figure 1B). Genome analysis revealed that IOVA encodes a truncated A56 protein, which is described to confer a fusogenic phenotype to VACV.<sup>16</sup> To generate a non-fusogenic version of IOVA, we restored the original A56 sequence and generated the virus IOVA/A56<sup>+</sup>, which visually restored the non-fusogenic phenotype (Figure 1A), although syncytia with up to 10 nuclei could still be observed (Figure 1B).

To ascertain whether IOVA holds promise as an oncolytic candidate, we first tested its capacity to destroy tumor cells *in vitro* in a wide panel of tumor cells compared with a VACV of the WR strain carrying the same deletion as IOVA in the TK gene. This virus was chosen as a control because it represents the gold

#### Figure 1. VACV IOVA induces syncytia formation and increases destruction of tumor cells

(A) Fluorescent images of HeLa cells 48 h after infection with indicated viruses at a MOI of 5 or 0.05. mCherry (red) is encoded by the oncolytic VACV (scale bar, 200  $\mu$ m). (B) IOVA induces fusogenesis of tumor cells. HeLa cells were infected at a MOI of 0.2 and the number of nuclei per cell was counted 3 days after infection using Hoechst 33342 staining. The nuclei per cell of 50 representative cells are plotted  $\pm$  SD. (C) Comparative cytotoxicity in a panel of tumor cells. Human tumor cell lines (HeLa, 143B, and MCF7) or mouse tumor cell lines (CT26, LLC1, and Renca) were infected at a MOI of 1 or 10, respectively, and surviving cells were quantified 72 h after infection. The percent of dead cells  $\pm$  SD are depicted for six different replicates. (D) Viral production in a panel of tumor cells. The indicated tumor cell lines were infected at a MOI of 5, and 48 h after infection, viral yield was evaluated in quadruplicate by plaque assays. (E) Detection of viral factories. EdU staining was used to visualize viral factories within syncytia. HeLa cells were infected with the indicated viruses at a MOI of 0.05 and, 16 h after infection, cells were incubated with EdU for 4 h followed by EdU detection and Hoechst 33342 staining (scale bar, 5  $\mu$ m). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.



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standard strain for constructing oncolytic VACV.<sup>17</sup> When we assessed tumor cell destruction using a metabolic assay, IOVA showed an improved capacity to destroy tumor cells in all cell lines nuclei context destruction with WR/TK<sup>-</sup> (Figures 1C and S3A). Impor-

tested compared with WR/TK<sup>-</sup> (Figures 1C and S3A). Importantly, the non-fusogenic IOVA/A56<sup>+</sup> also showed stronger cytotoxicity than WR/TK<sup>-</sup> in most tumor cell lines, indicating that the fusogenic capacity of IOVA is not the main feature driving this enhanced killing capacity.

Next, we tested the replication capacity of IOVA in tumor cells. Not only was replication not diminished in cancer cells, but it was also significantly increased in cell lines such as HeLa or MCF-7 (Figures 1D and S3B). IOVA replication was only significantly lower in Renca cells compared with WR/TK<sup>-</sup>. Interestingly, compared with its non-fusogenic counterpart IOVA/A56<sup>+</sup>, IOVA demonstrated improved replication in cell lines such as HeLa, MCF-7, and LLC1. To demonstrate effective viral replication within syncytia, we added 5-ethynyl-2'-deoxyuridine (EdU) to the supernatant of HeLa cells infected with IOVA or controls. Subsequent conjugation of EdUlabeled DNA to fluor-azide allowed us to visualize viral factories within the cytoplasm of infected HeLa cells. Numerous viral factories were detected within large syncytia induced by IOVA infection, demonstrating that fusogenesis does not prevent VACV replication (Figure 1E).

The size of plaques formed in cell monolayers after infection can serve as an indicator of the viral capacity to destroy cells upon virus propagation. Therefore, we analyzed plaque diameters in our panel of cancer cells, excluding those not supporting the formation of distinct plaque lesions upon VACV infection (CT26, LLC1, and Renca). The plaques formed after infection with IOVA were significantly larger than those formed after infection with WR/TK<sup>-</sup> (Figures 2A and 2B). As we previously observed with the killing capacity and viral production, the plaques formed after the infection of IOVA or IOVA/ A56<sup>+</sup> were of similar size except in MCF-7 cells, demonstrating that the fusogenic phenotype is not triggering the large plaque phenotype. In addition, microscopic analysis revealed a distinct morphology of cells within plaques generated by infection with the non-fusogenic version of IOVA compared with WR/TK<sup>-</sup> (Figure 2C).

#### Truncations of A56 and A26 cooperate to induce fusogenesis

To further study the effect of fusogenesis on the *in vitro* oncolytic properties of VACV, we constructed a version of WR/TK<sup>-</sup> incorporating a deletion in the A56 sequence. Further analysis of IOVA's genome showed a mutation in the A26 protein, whose deletion has also been linked to fusogenesis.<sup>18</sup> To assess the importance of the A26 truncation, an A26-deleted and a double A56/A26-deleted version of WR/TK<sup>-</sup> were also generated. Microscopic analyses re-

vealed that viruses incorporating an A56 truncation but not a single A26 deletion had a fusogenic phenotype (Figure S4A). However, nuclei counting revealed that syncytia of up to 10 nuclei can be detected after infection of the single A26-deleted virus, correlating with numbers of IOVA/A56<sup>+</sup> (Figures 1B and S4B). Importantly, A26 truncation did have synergistic effects with A56 truncation, increasing the number of nuclei within syncytia to more than 40 (Figure S4B). Induction of massive fusogenesis by either single A56 or double A56/A26 deleted viruses was associated with an increased capacity of the viruses to destroy tumor cells (Figure S4D) or the diameter of plaques (Figures S4E and S4F).

#### IOVA induces hallmarks of ICD in cancer cells

Hallmarks of ICD include surface-exposed CALR as well as secretion of adenosine triphosphate (ATP), annexin A1, type I interferon, and high-mobility group box 1 (HMGB1).<sup>19</sup> First, we tested whether IOVA maintains the capacity of MVA to translocate CALR after infection. CALR on the surface of infected human tumor cells (HeLa, 143B, and MCF-7) was analyzed by flow cytometry and a fixable viability dye was used to discard cells with a disrupted plasma membrane. In all cell lines tested, infection with IOVA led to high level of CALR translocation, with CALR detected in more than 75% of cells (Figures 3A and 3B). After WR/TK<sup>-</sup> infection, an increased percentage of CALR<sup>+</sup> cells was only detected in 143B cells (<40%) and percentages of CALR<sup>+</sup> in HeLa and in MCF-7 were similar to mock-infected cells. Interestingly, CALR translocation was not associated with syncytia formation, since IOVA/ A56<sup>+</sup> also induced such an effect. In addition, infection with either IOVA or IOVA/A56<sup>+</sup> also led to increased secretion of HMGB1 and ATP by infected cells as determined by ELISA or luminescence assays (Figures 3C and 3D).

### IOVA replicates efficiently in mouse tumor models

Mice bearing Renca (mouse renal adenocarcinoma) or CT26 (mouse colon carcinoma) tumors were intratumorally injected with IOVA or controls to evaluate their capacity to replicate *in vivo*. Four days after intratumoral virus injection, vector-driven fluorescence from tumor tissues and viral growth were analyzed (Figure 4A). Fluorescence of tumors that were injected with WR/TK<sup>-</sup> or IOVA did not significantly differ (Figure 4B). When comparing IOVA with its non-fusogenic (A56<sup>+</sup>) version, increased virus loads were detected in the CT26 but not in the Renca model (Figure 4C). Similar replication kinetics were also observed when virus loads were analyzed at different time points after IOVA or WR/TK<sup>-</sup> administration (Figure S5).

Finally, immunohistochemical analysis revealed the presence of syncytia in those tumors administered with IOVA (Figure 4D), thus

Figure 2. VACV IOVA produces a large-plaque phenotype in cancer cells

Plaque assays were performed after infecting the indicated cells at a MOI of 0.05 with WR/TK<sup>-</sup>, IOVA, or IOVA/A56<sup>+</sup>. Seventy-two hours after infection, cells were stained with crystal violet. (A) Images of plaque phenotype. (B) Plaque diameter of 10 representative plaques  $\pm$  SD. (C) Microscopy images of plaque morphology (scale bar, 200  $\mu$ m). \*p < 0.05; \*\*\*p < 0.001.



Figure 3. VACV IOVA induces hallmarks of ICD in cancer cells

(A and B) Detection of CALR on the surface of infected cancer cells. Cancer cells were infected with the indicated viruses at a MOI of 5 and, 24 h after infection, CALR was detected by flow cytometry. A fixable viability dye eFluor520 was used to discard cells with a disrupted membrane. (A) Percentage of eFluor520<sup>-</sup>CALR<sup>+</sup> cells are plotted  $\pm$  SD for four different replicates. (B) Representative distribution of the CALR<sup>+</sup> population within HeLa cells after infection. (C and D) Release of HMGB1 (C) and ATP (D) to cell supernatants after infection. Cells were infected with the indicated viruses at a MOI of 5 and an ELISA assay was used to detect HMGB1 or luminescent detection kit to detect ATP. Data are plotted as fold change versus levels released after WR/TK<sup>-</sup> infection. \*p < 0.05; \*\*p < 0.01 \*\*\*p < 0.001.

demonstrating that fusogenesis mediated by IOVA infection also occurs *in vivo* in mouse tumors.

## IOVA improves antitumor efficacy after intratumoral administration and activates systemic antitumor immune responses

To test the antitumor efficacy of our IOVA strain, we evaluated the impact on tumor growth after intratumoral administration in the aggressive syngeneic B16 melanoma model (Figure 5A). IOVA administration not only induced a strong antitumor effect and significantly delayed tumor growth (Figures 5B and 5C), but also greatly increased the median survival time of mice compared with the WR/TK<sup>-</sup> control virus (Figure 5D). The non-fusogenic version IOVA/A56<sup>+</sup> also increased antitumor efficacy compared with WR/TK<sup>-</sup>, but to a lesser extent than the highly fusogenic IOVA.

We also evaluated whether intratumoral administration of IOVA into a primary tumor could result in systemic anti-tumor responses able to control the growth of distant, non-treated tumors. Thus, B16 cells were inoculated first into the left and, 3 days later, into the right flank of mice. When both tumors were established, IOVA or controls were intratumorally administered only into the left tumor, and the growth of both tumors was monitored (Figure 6A). The antitumor efficacy of the directly injected tumors was comparable with the results shown in Figure 5C, with IOVA and IOVA/A56<sup>+</sup> inducing a significant delay in tumor growth compared with WR/TK<sup>-</sup> (Figure 6D). In addition, administration of IOVA also delayed the growth of the untreated distal tumors (Figures 6B and 6C), whereas treatment with WR/TK<sup>-</sup> had no effect. Notably, treatment with IOVA again resulted in significantly better control of tumor growth and improved overall survival compared with its non-fuso-genic counterpart (Figures 6C–6E).

To test whether this improved control of tumor growth was associated with cellular antitumor immune responses, we evaluated the tumor epitope-specific T cell responses established after virus



## Figure 4. Replication of VACV IOVA and syncytia formation in tumor models *in vivo*

(A) Flow diagram of the experiment. Nine days before,  $5 \times 10^5$  Renca or CT26 cells were subcutaneously implanted in the flank of BALB/c mice. At day 0, a virus dose of  $1 \times 10^7$  PFU was intratumorally injected; mice were sacrificed and tumors were harvested 4 days later. (B) Quantification of virus-driven mCherry-specific fluorescence. Plotted are fluorescence of individual tumors and group means  $\pm$  SD. (C) Viral titers were determined by plaque assays after tumor homogenization. Titers obtained from each independent tumor are plotted with means  $\pm$  SD. (D) Detection of VACV by immunohistochemistry in paraffinembedded CT26 tumors. Larger infected cells compatible with syncytia (black arrow) were detected in IOVA-injected tumors (scale bar, 50 µm). ns, not significant; \*p < 0.05.



Figure 5. Antitumor activity of VACV IOVA after intratumoral administration

(A) Tumor implantation and treatment plan. C57BL/6 mice harboring subcutaneous B16-F10 tumors were randomized and injected twice (days 0 and 4) with an intratumoral dose of  $1 \times 10^7$  PFU of tested viruses. Injection of PBS was used as a control. (B) Tumor volume of individual animals, (C) mean tumor size after treatments, and (D) overall survival are plotted for 5–8 mice/group ± SEM. \*\*p < 0.001.

administration in the B16 tumor model using ELISpot assays. We evaluated the responses directed against the major histocompatibility complex class II-restricted neoepitope B16-M30 or its wild-type counterpart B16-WT30.<sup>20</sup> IOVA and IOVA/A56<sup>+</sup> increased T cell reactivity responses directed against the tumor neoepitope B16-M30, but not against the wild-type peptide (Figure 6F).

## Systemic administration of IOVA decreases both toxicities associated with VACV administration and tumor growth

Since systemic exposure is preferred for the treatment of metastatic cancer because it would allow the virus access to all tumor lesions, we tested whether intravenous injection of IOVA is feasible and whether it leads to improved antitumor efficacy. Remarkably, whereas



#### Figure 6. Intratumoral administration of IOVA also protects against distal tumors

(A) Tumor implantation and treatment plan. C57BL/6 mice harboring a primary and secondary (in the counterflank) B16-F10 tumor were injected twice (days 0 and 4) with an intratumoral dose of  $1 \times 10^7$  PFU of the tested viruses into the primary, left flank, tumor. (B) Tumor growth of untreated secondary tumor of individual animals, (C) mean secondary tumor size after treatments, (D) mean tumor size of the injected primary tumors, and (E) overall survival are plotted ± SEM for 5–8 mice per group. (F) IOVA induces antitumor T cell responses directed against tumor neoepitopes. Mice harboring B16-F10 tumors were treated twice (days 0 and 4) with an intratumoral dose of the indicated viruses or control PBS. At day 8 after virus administration, splenocytes were analyzed for their reactivity to the indicated peptides (neoepitope B16-M30 or wild-type B16-WT30) by IFN- $\gamma$  ELISPOT. Individual values of five mice/group are plotted with mean ± SD. \*\*p < 0.01; \*\*\*p < 0.001.

a single injection of  $1 \times 10^8$  PFU of WR/TK<sup>-</sup> leads to a profound body weight loss between days 1 and 5 after administration, both IOVA and IOVA/A56<sup>+</sup> only induced a slight decrease in body weight at day 1 followed by a rapid recovery (Figure 7A). In addition, a single systemic dose of IOVA or IOVA/A56<sup>+</sup> (Figures 7B and S6A) was able to significantly reduce tumor growth (Figures 7C, 7D, and S6B) and increase overall survival (Figures 7E and S6C) compared with WR/ TK<sup>-</sup>-treated mice harboring Renca or CT26 syngeneic tumors.

## Repeated intratumoral administrations of IOVA eradicate large established tumors and result in sustained protection

To test the antitumor potential of IOVA in a more clinically relevant setting, we treated mice harboring large established Renca tumors (volumes of  $\leq 200 \text{ mm}^3$ ) with three high-dose intratumoral injections of IOVA or virus control (WR/TK<sup>-</sup>) (Figure 8A). We chose this repeated administration regimen based on the clinical experience with talimogene laherparepvec,<sup>21</sup> the only oncolytic virus approved so far by the U.S. Food and Drug Administration and the European Medicines Agency). Importantly, IOVA treatment resulted in rejection of 61.5% of tumors (8/13), while WR/TK<sup>-</sup> only induced a tumor rejection of 15.4% (2/13) (Figures 8B and 8C). A significantly increased overall survival was also achieved with IOVA treatment (Figure 8D).

To demonstrate that IOVA administration not only activates an antitumor immunity, but also generates long-term memory responses, we investigated whether prior Renca tumor rejection could protect mice against subsequent Renca cell implantation. Mice that had previously rejected Renca tumors displayed complete protection against tumor rechallenge, whereas Renca tumors developed in the majority of naive mice (Figure 8E).

### DISCUSSION

Here we describe the construction of a novel oncolytic VACV, which we named IOVA (Immune-Oncolytic VACV Ankara). IOVA is engineered from an early virus isolate in the historical serial passage experiment that led to the generation of MVA, when the virus was still able to multiply in mammalian cells. Indeed, IOVA replicates efficiently in mammalian tumor cells *in vitro* and *in vivo*. In addition, a unique combination of genomic features allows IOVA to activate ICD and delay the growth of syngeneic mouse tumors not only by intratumoral treatment, but also in untreated distal tumors, a so-called abscopal effect. IOVA also elicits increased T cell reactivity directed against tumor neoepitopes and sustained protection against tumor rechallenge, and systemic administration showed a decrease in the toxicity usually associated with VACV therapy.

MVA was developed during the 1960s at the LMU University of Munich and the Bavarian Vaccine Institute in Munich after more than 516 passages in CEF cells in order to serve as a safer vaccine during the World Health Organization smallpox eradication campaign.<sup>22</sup> Nowadays, MVA is used worldwide for constructing safe recombinant vaccine candidates, some of which are currently being clinically tested for vaccination against betacoronaviruses, human immunodeficiency viruses, *Mycobacterium tuberculosis*, or *Plasmodium falciparum*.<sup>6</sup> In addition, MVA also serves as an effective platform for cancer vaccination.<sup>23</sup> Interestingly, despite its inability to replicate in cancer cells, MVA has also been used as an oncolytic vector and has been shown to induce systemic antitumor immunity and strong antitumor effects.<sup>8</sup> Mechanistically, it is reported that live or inactivated MVA infection leads to higher levels of interferon induction through the cGAS-STING cytosolic DNA-sensing pathway, and that Batf3dependent CD103<sup>+</sup>/CD8 $\alpha^+$  dendritic cells play an essential role in MVA-based therapy. Here, we have also shown that MVA is able to induce clear features of ICD as demonstrated by CALR translocation to the plasma membrane. Previously, it was described that MVA induces ICD of dendritic cells in the context of vaccination,<sup>24</sup> but this is the first time to our knowledge that ICD induction is demonstrated in cancer cells after MVA infection.

Despite this ICD induction, our results contradict previous reports in terms of antitumor efficacy: in our experiments, intratumoral administration of MVA resulted in discrete antitumor effects. We hypothesize that these differences are associated with different regimens of administration: in our experiments, mice receive two intratumoral administrations of MVA in 4 days, mimicking administration regimens typically used to deliver oncolytic VACV in preclinical settings.<sup>25</sup> In contrast, previous reports used a more frequent administration regimen with biweekly administration until the end of the experiment.<sup>8</sup> Such an intensive regimen, administering fresh virus every 3–4 days, may compensate for MVA's inability to replicate, thus facilitating antitumor effects. However, VACV replication plays an important role in clinical settings.

In this study, we used a VACV of the WR strain with a deleted TK gene (WR/TK<sup>-</sup>) as a control throughout the study. TK deletion is a well known safety factor<sup>26</sup> and such a virus is considered the gold standard strain for constructing oncolytic VACV,27-29 including candidate viruses already tested in the clinic.<sup>17,30</sup> Here, we demonstrate that such a virus is unable to induce ICD, confirming previous reports.<sup>12</sup> In addition, we also confirm that VACV Cop strain, which is also widely used to generate oncolytic viruses,<sup>31</sup> is similarly unable to induce ICD, in agreement with previous reports.<sup>13</sup> Consequently, strains of VACV used to date to construct oncolytic viruses replicate efficiently in cancer cells, but have a low immunogenicity and are unable to induce ICD. Conversely, MVA is unable to replicate in cancer cells but can efficiently induce ICD. Both viruses, in our experiments, display a limited antitumor activity, and we hypothesized that a VACV strain with the ability to combine effective replication in cancer cells and induction of ICD would lead to a more effective antitumor therapy.

From the MVA selection process of more than 500 passages in CEF cells, we still conserve viruses from different intermediate passages. As the MVA genome sequentially acquired deletions and mutations affecting its replicative capacity and increasing its immunogenicity,<sup>6,32</sup> we hypothesized that a virus from an earlier passage of this selection might still efficiently replicate in cancer cells but already



#### Figure 7. Toxicity and antitumor activity of VACV IOVA after systemic administration

(A) Body weight change after intravenous administration. Balb/c mice were injected intravenously with  $1 \times 10^8$  PEU of WR/TK<sup>-</sup>, IOVA, or IOVA/A56<sup>+</sup>. PBS was used as a control. Weight profiles of IOVA/A56<sup>+</sup> and IOVA-injected mice were similar to those injected with PBS, whereas WR/TK<sup>-</sup>-injected mice lost more than 10% in body weight at day 2 after virus injection. (B–E) IOVA strain improves anti-tumor activity after systemic administration. (B) Tumor implantation and treatment plan. Balb/c-bearing Renca tumors were treated with a single intravenous dose of the tested viruses (1 × 10<sup>8</sup> PEU/mouse). (C) Tumor size in individual animals, (D) mean tumor size after treatments, and (E) overall survival are plotted for 5–10 mice/group ± SEM. \*p < 0.01; \*\*p < 0.01;

harbor different genome alterations that allow for ICD induction. Indeed, IOVA, which was engineered from a clone isolated from an earlier passage to incorporate a TK gene deletion, replicated very efficiently in most of the human and mouse tumor cell lines tested, even with improved productivity in cell lines such as MCF-7 (breast cancer). Moreover, such replication occurred in fully established tumors *in vivo*. IOVA also demonstrated more efficient killing of cancer cells and generated larger plaques than the WR/TK<sup>-</sup> control virus.

Remarkably, a very distinct phenotype of dead cells within the virus plaques could clearly be observed when comparing WR/TK<sup>-</sup> with

IOVA infection. We hypothesized that this might be associated with a different mechanism of killing cancer cells, and thus investigated whether such mechanisms result in a more immunogenic outcome by evaluating the release or exposure of DAMPs defined as hallmarks of ICD<sup>11</sup>: translocation of CALR from the endoplasmic reticulum to the cell surface and release of HMGB1 and ATP. In all cancer cell lines tested, CALR translocation greatly increased when cells were infected with IOVA as opposed to WR/TK<sup>-</sup>. In HeLa, CALR was detected in only around 8% of cells after infection with WR/TK<sup>-</sup>, while IOVA infection increased this to more than 80% of cells, very similar to MVA-associated CALR translocation levels.



Figure 8. Antitumor activity and protective immunity induced by VACV IOVA after repeated high-dose intratumoral administrations

(A) Tumor implantation and treatment scheme. Balb/c mice harboring subcutaneous Renca tumors were randomized and injected thrice (on days 0, 3, and 6) with an intratumoral dose of  $5 \times 10^7$  PFU of the indicated viruses. Injection of PBS was used as a control. (B) Tumor size in individual animals, (C) mean tumor size after treatments, and (D) overall survival are plotted for 10–13 mice/group ± SEM. (E) Tumor rechallenge of recovered mice with Renca cells results in sustained protection. We implanted  $5 \times 10^5$  Renca cells in the contralateral flank of mice that had cleared tumor following IOVA treatment (n = 8). Age-matched naive mice (n = 10) were used as controls. CR, complete responses. \*\*p < 0.01; \*\*\*p < 0.001.

Furthermore, the release of HMGB1 to the supernatant increased in all cancer cell lines tested and elevated ATP levels were found in one out of three cell lines. IOVA's unique combination of effective viral replication plus ICD induction translated into outstanding antitumor efficacy in syngeneic tumor models after both local and systemic viral administration, leading to complete rejection of large established tumors when administered repeatedly. In addition, IOVA was able to elicit T cell responses directed against tumor neo-epitopes, protect against tumor rechallenge, and induce a pronounced delay in the growth of distal untreated tumors, demonstrating its potential to treat metastatic cancer.

Sequencing of the IOVA genome revealed its genomic characteristics and differences compared with its parental strain CVA and to MVA. Interestingly, although sharing different genomic features such as deletion of the A52-A55 region or mutations in N2 or K6, IOVA is not a direct progenitor of MVA since it contains unique mutations that do not exist in MVA. This category includes a frameshift mutation in A56 that leads to a shorter inactivated protein and mutations in N1 and A9 that translate into proteins that are 11 amino acids shorter and 5 amino acids longer, respectively. This is not surprising since selection processes such as the generation of MVA are not linear and intermediate passages contain a collection of mutant viruses with different genomic characteristics, not all of which are selected in subsequent passages.

IOVA incorporates mutations that change the amino acid sequence of CVA proteins in a total of 106 open reading frames (ORFs). Among these changes, we noted the inactivation of important VACV immune modulators. Several of these modulators that accumulated mutations are involved in NF- $\kappa$ B inhibition, such as C2, N1, A52, or B14,<sup>33–36</sup> while others target IRF3 and interferon-induced proteins, such as N2 or C9.<sup>37,38</sup> Other important mutated immune modulators include A53, a soluble TNF receptor,<sup>39</sup> M1, an inhibitor of apoptosis,<sup>40</sup> and A39, a secreted semaphorin homolog.<sup>41</sup> Finally, IOVA also includes several mutations in VACV proteins whose function is not to inhibit the immune system, such as K6, a putative monoglyceride lipase,<sup>42</sup> A51, a protein that promotes viral protein stability,<sup>43</sup> and B5, a structural membrane protein.<sup>44,45</sup>

Beside the mutations discussed, IOVA also includes mutations that truncate VACV proteins A56 and A26. A56 forms a fusion regulatory complex together with K2,46 and A26 is a fusion suppressor of mature virus.<sup>18</sup> As we demonstrate here, truncations of A56 and A26 add up to allow the formation of large syncytia after infecting tumor cells. Importantly, syncytia formation does not diminish VACV replication, as was observed with other oncolytic viruses,<sup>47</sup> and IOVA replicates to WR levels in most cancer cells and in fully established tumors in vivo. Our findings indicate that syncytia formation confers an advantage to oncolytic VACV in terms of cytotoxicity to cancer cells and antitumor efficacy in vivo. Recently, an oncolytic VACV with a truncated K2 protein (which forms the fusion regulatory complex with A56) was reported.48 Such a virus also induced fusogenesis of cancer cells, increased cytotoxicity, and an improvement in antitumor efficacy, comparable with our results. However, our results also indicate that the capacity of IOVA to induce ICD and elicit potent antitumor immune responses is independent of syncytia formation and relies on its unique combination of deletions and mutations within its genome.

Another important aspect observed with IOVA is a favorable safety profile. Although WR/TK<sup>–</sup> lacks TK to achieve selective replication in tumor cells, in this and previous works,<sup>49</sup> we demonstrated that a 10<sup>8</sup> PFU intravenous dose leads to up to 15% weight loss in mice. In contrast, intravenous administration of the same dose of IOVA results in only minor and transient weight loss. This could allow for treatments with increased doses and potentially better antitumor efficacies. Mechanistically, we hypothesize that this reduced toxicity profile is linked to increased immunogenicity of IOVA, which could help to clear the virus more rapidly from normal tissues. However, the immunosuppression of the tumor microenvironment<sup>50</sup> may allow for efficient viral replication of IOVA despite its higher immunogenicity profile.

In summary, IOVA represents a novel VACV with unique features that improve its performance as an oncolytic virus: it replicates with high efficiency in tumor cells, it has a safer toxicity profile, it elicits potent antitumor immune responses, and it profoundly controls tumor growth in various mouse tumor models. These results support the development of IOVA-based oncolytic VACV clinical candidates that incorporate different therapeutic transgenes.

## MATERIALS AND METHODS

## Cell lines

All cell lines used in this research (MA104, HeLa, MCF-7, Renca, LLC1, B16, and CT26 cells) were obtained from the American Type Culture Collection and maintained in recommended culture media containing 5%–10% fetal bovine serum and antibiotics at  $37^{\circ}$ C, 5% CO<sub>2</sub>. We used 11-day-old chicken embryos (specific pathogen-free (SPF) eggs, VALO BioMedia) to prepare primary CEF cells. Cell lines were regularly tested for mycoplasma contamination.

## Viruses

VACV strains MVA, Cop, and WR were previously described.<sup>6,14,51,52</sup>

Construction of WR/TK<sup>-</sup>, the VACV strain WR with a truncated viral TK gene and expressing mCherry, was described elsewhere.<sup>5</sup> WR/TK<sup>-</sup> served as the backbone for truncating VACV proteins A56 and A26. Single or double deleted viruses were generated by homologous recombination replacing the original gene sequences with a synthetic construct containing two 350-base pair DNA sequences upstream and downstream of the genomic site targeted for insertion. For homologous recombination and generation of the VACV deletion mutants, plasmid DNA containing the synthetic construct was transfected into MA104 cells that were previously infected with VACV WR/TK<sup>-</sup>. The deletion mutant viruses were clonally isolated by a positive-negative selection system based on GFP as a reporter and all genetic modifications were confirmed by PCR and sequencing. For the construction of the double A56 and A26 truncated virus, the homologous recombination process was repeated with the A26 shuttle plasmid once the A56 truncation was confirmed.

The novel oncolytic strain IOVA was engineered from a clonal virus isolated earlier than passage 150 of the more than 516 passages

performed in CEF cells during the selection process of MVA.<sup>7</sup> To enhance selective replication in cancer cells, IOVA was constructed by inactivating the viral TK gene in the isolated clone through insertion of an expression cassette for the mCherry reporter gene under transcriptional control of the VACV late promoter P11. IOVA/ A56<sup>+</sup> was constructed by homologous recombination between IOVA and a PCR product containing the wild-type sequence of the A56 protein and was isolated based on the loss of fusogenic phenotype and confirmed by PCR and DNA sequencing.

Viruses were purified by ultracentrifugation through sucrose cushions as previously described<sup>49,53</sup> and titrated by plaque assay in MA104 cells for replication-competent VACV or CEF cells for the MVA strain.

## VACV genomic DNA isolation, sequencing, and analysis

We used 10<sup>8</sup> plaque-forming units (PFU) from a purified virus preparation to isolate DNA with a commercially available kit (QIAmp DNA micro kit, Qiagen), and whole-genome sequences were determined and analyzed using high-throughput sequencing (Illumina & Oxford Nanopore Technology, Laboratory for Functional Genome Analysis, LMU). Benchling software was used to compare the sequence of IOVA ORFs with the published sequences of VACV strains CVA (GenBank: AM501482.1) and MVA (GenBank: AY603355.1). VACV Cop (GenBank: M35027.1) ORF nomenclature is used throughout this study since it is the most common in the literature.

## Virus growth assay and plaque diameter

We seeded  $2 \times 10^5$  cells in 24-well plates and infected at a multiplicity of infection (MOI, PFU/cell) of 5. One hour after infection, cells were washed with PBS and new pre-warmed medium was added. At different time points (0, 4, 12, 24, 48, and 72 h after infection), samples including cells and supernatant were harvested and frozen at  $-80^{\circ}$ C. Viral titer was determined by plaque assays after three freeze-thaw cycles. Viral yield was evaluated in quadruplicate in two independent experiments.

To assess the diameter of the plaques formed by the different viruses, cell cultures were infected at a MOI of 0.05, and 72 h after infection, the diameter of plaques was measured after staining with crystal violet. Ten representative plaques per group were measured.

### In vitro cytotoxicity assay

Cytotoxicity assays were performed by seeding  $5 \times 10^4$  cells in 96-well plates. Cells were infected with 1:5 serial dilutions starting at a MOI between 500 and 20, depending on the cell line used, and incubated at  $37^{\circ}$ C for 72 h. After three days, cells were checked for remaining metabolic activity using a non-radioactive cell proliferation assay (Promega) following the manufacturer's instructions. Three different replicates from two independent experiments were evaluated.

### Detection of viral factories and nuclei counting

HeLa cells were infected at a MOI of 0.05 with indicated viruses, and 16 h after infection, an EdU staining iFluor 488 Proliferation Kit (Ab-

cam) was used to detect replicating DNA in infected cells following manufacturer's recommendations. Hoechst 33342 staining was used to visualize nuclei and cells were analyzed using a Keyence BZ-X710 microscope (Keyence).

For counting nuclei within syncytia, HeLa cells were infected with indicated viruses at a MOI of 0.2, and 72 h after infection, Hoechst 33342 staining was used to visualize and count the number of nuclei included in one cell/syncytia under the microscope. The number of nuclei in 50 representative cells were counted.

## Analysis of HMGB1 and ATP release

We infected  $2 \times 10^5$  cells at a MOI of 5 with indicated viruses, and 24 h after infection, supernatants were harvested. A HMGB1 ELISA kit (Tecan) and a luminescent ATP detection assay kit (Abcam) were used (following the manufacturer's instructions) to determine HMGB1 and ATP, respectively, released after 24 h of infection. Two technical replicates of four independent samples were evaluated for each cell line.

#### Flow cytometry

Cells were infected with indicated viruses at a MOI of 5 and stained using an Alexa Fluor 405 Anti-CALR antibody (Ref ab210431, Abcam) 24 h after infection. A fixable viability dye eFluor 520 was used to discard cells with a disrupted plasma membrane (eBioscience) and data was acquired by a MACSQuant flow cytometer (Miltenyi Biotec) and analyzed using FlowJo software.

#### Mouse models

All animal experiments were approved either by the Government of Upper Bavaria or by the local ethics committee of the Universities of Ghent or Barcelona. Six- to 8-week-old female BALB/c (Renca and CT26 tumor models) or C57Bl/6 (B16 tumor model) mice were purchased from Charles River Laboratories and housed in an isolated cage unit with free access to food and water. Tumor cells for implantation were maintained in vitro at standard conditions. On the day of implantation, cells were trypsinized and  $5 \times 10^5$  cells were implanted in the left flank of mice. In a different experimental set-up, a second tumor was implanted on the right flank of mice 3 days after primary implantation. When tumors reached a volume of 50-200 mm<sup>3</sup>, cages harboring mice were randomized (block randomization) and viruses were administered. The number of animals necessary for each experiment was calculated using a G\*Power software Version 3.1.9.2 and the following settings: one tail, effect size d of 1, alpha error probability of 0.05, a power (1 - beta error)probability) of 0.8, and an allocation ratio N2/N1 of 1. Animals were excluded from the experiment only in the case of defective virus administration.

## Study of viral replication *in vivo* and immunohistochemistry of tumor sections

Tumors were established as described above. After randomization of the mice, they received at day 0 a single intratumoral virus dose of  $1 \times 10^7$  PFU. Mice were sacrificed at day 4 and tumors were

harvested, washed with PBS, and fluorescence signals from the tumors were acquired using a Geldoc imaging system (Bio-Rad) and quantified using ImageJ. To determine viral titer within tumors, mice were treated as described above and sacrificed at day 4 after viral administration. Tumors were harvested, weighed, and homogenized using metal beads and a tissue homogenizer (Qiagen). Virus titers were determined by plaque assays on MA104 cells. Four to five mice per group were used for this quantification.

In parallel, tumors were fixed in formalin and embedded in paraffin, and 4  $\mu$ m sections were cut using a microtome and stained with HE. Primary antibody for immunohistochemistry was a polyclonal rabbit anti-VACV antibody (1:1000, Ref BP1076, OriGene) and biotinylated secondary antibody was a goat anti-rabbit antibody (1:200, Ref BA-1000, Vector). Peroxidase-complexed avidin-biotin (ABC-HRP, PK-6100, Vector) and diaminobenzidine were used for visualization and hemalum as a counterstain.

#### In vivo antitumor activity

Tumors were established as described above. Mice were treated either with a single intravenous dose or with multiple intratumoral doses of indicated viruses. Mice were monitored and weighed daily, and tumors measured three times per week using a caliper; tumor size was calculated as the length  $\times$  width  $\times$  height in mm<sup>3</sup>. Mice were euthanized when tumors reached termination criteria. Fourteen mice per group were used to determine antitumor activity.

For Kaplan-Meier survival curves, the endpoint was established at 750 mm<sup>3</sup> or greater. Animals whose tumor size achieved the threshold were included as right-censored information.

#### IFN- $\gamma$ ELISPOT

Tumors were established as described above and mice treated twice with an intratumoral dose of indicated viruses. Five days after the second virus injection, mice were sacrificed and spleens harvested. We cultured  $2 \times 10^5$  cells for 48 h in anti-IFN-  $\gamma$  (MABTECH) pre-coated 96-well plates together with 2 µg/mL of peptides. The synthetic peptides used for restimulation were: B16-M30wt (PSKPSFQEFVD-WEKVSPELNSTD) and B16-M30mut (PSKPSFQEFVDWENVSPEL NSTD). An automated ELISPOT reader (A.EL.VIS Eli.Scan) was used for counting and analyzing. Five mice per group were used for ELISPOT assays.

## Tumor rechallenge experiments

Mice that had completely rejected subcutaneous Renca tumors were rechallenged with  $5 \times 10^5$  Renca cells at day 30 after tumor rejection and tumor growth was monitored for 30 days after rechallenge. Agematched naive mice were used as controls.

#### Statistical analysis

A one-way ANOVA and Tukey's multiple comparison test were used to analyze Figures 1, 2, 3, 4, 6F, S1, S3, and S4. In Figures 5C, 6C, 6D, 7A, 7D, 8C, S1B, S5B, and S6B, a two-way ANOVA and Bonferroni posttest were chosen to analyze tumor growth curves. A log rank test was used to analyze survival curves in Figures 5D, 6E, 7E, 8D, and S6C. A Fisher's exact test was used to analyze Figure 8E. In all cases, significance was achieved if the p value was les than 0.05.

## DATA AND CODE AVAILABILITY

The data presented in this study are available on request from the corresponding author.

#### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10. 1016/j.ymthe.2024.05.014.

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### AUTHOR CONTRIBUTIONS

Conceptualization: G.S. and J.J.R.; methodology: G.S., X.S., and J.J.R.; investigation: S.R., L.V.H., A.C., C.B.M., M.C., M.B., K.B., M.H.L., A.V., and J.J.R.; writing – original draft: J.J.R.; writing – review and editing: X.S., G.S., and J.J.R.; supervision: G.S., X.S., and J.J.R.; funding acquisition: G.S. and J.J.R.

## DECLARATION OF INTERESTS

J.J.R. and G.S. have filed the patent application WO2019106205A1 and are named as inventors on this patent application describing the use of immune oncolytic VACV.

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