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Article

A Replication-Defective Myxoma Virus Inducing Pro-Inflammatory Responses as Monotherapy and an Adjuvant to Chemo- and DC Immuno-Therapy for Ovarian Cancer

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Abstract: Myxoma virus (MYXV), a rabbit specific poxvirus and non-pathogenic to humans and mice, is an excellent candidate oncolytic virus for cancer therapy. MYXV also has immunotherapeutic benefits. In ovarian cancer (OC), immunosuppressive tumor associated macrophages (TAMs) are key to inhibit antitumor immunity while hindering therapeutic benefit by chemotherapy and dendritic cell (DC) vaccine. Because MYXV favors binding/entry of macrophages/monocytes, we examined the therapeutic potential of MYXV against TAMs. We found previously that a replication-defective MYXV with targeted deletion of an essential gene, *M062R*, designated $\Delta M062R$ MYXV, activated both host DNA sensing pathway and the SAMD9 pathway. Treatment with $\Delta M062R$ leads to therapeutic benefit as monotherapy comparable to wildtype replicating MYXV in preclinical models. Here we found that $\Delta M062R$ MYXV, when integrated with cisplatin and DC immunotherapy, further improved treatment benefit likely through promoting tumor antigen-specific T cell function. Moreover, we also tested $\Delta M062R$ MYXV in targeting human immunosuppressive TAMs from OC patient ascites in a co-culture system. We found that $\Delta M062R$ treatment subverted the immunosuppressive properties of TAMs and elevated the avidity of cytokine production in tumor antigen-specific CD4⁺ T cells. Overall, $\Delta M062R$ presents a promising immunotherapeutic platform and is as a beneficial adjuvant to chemotherapy and DC vaccine.

Keywords: oncolytic virus; myxoma virus; immunoregulatory; immunotherapy platform; host range factor; type 1 interferon; type 1 interferon stimulated genes; proinflammatory

1. Introduction

Cancer cells can cultivate and maintain the tumor environment to escape from host immune detection and elimination [1]. Once the tumor environment is established, cancer cells often evade targeted treatment regimes such as chemotherapy and even develop resistance and tolerance to repeated chemotherapy treatments, which eventually leads to irreversible progression in cancer cell growth and lethality in patients [2]. This is often the case for ovarian cancer (OC) patients [3]. Ovarian cancer is a leading cause of cancer death in women with over 200,000 death annually worldwide [4]. While platinum-based chemotherapy remains the standard care for OC patients and most patients initially responded, chemo-resistance arises frequently in patients [5,6] and tumor progression is especially shielded by an immunosuppressive tumor environment [3]. High grade serous OC (HGSOC) is the most common form of OC in women (over 70%) [3], and parameters indicating an immunosuppressive tumor environment suggest a poor prognosis in patients [7,8]. Targeting key elements of the OC immunosuppressive tumor environment, such as tumor associated macrophages (TAMs) [9] and Tregs [10], leads to development of novel therapeutics, for instance through the use

of dendritic cell (DC) vaccines [11,12]. In this study, we considered the promise of DC vaccines in treating immunosuppressive HGSOC and aim to develop combinatorial treatment strategy to further advance the therapeutic benefit.

Myxoma virus (MYXV) belongs to the poxvirus family and has a unique safety profile for therapeutic applications in humans and in preclinical mouse models [13]. These viruses also have a large genome capacity to allow extensive genetic engineering for therapeutic molecules, such as IL-12 [14], IL-15 [15], and TNF [16], to edit regional and systemic immune networks. Because WT MYXV has oncolytic properties by specifically infecting and killing cancer cells [17] and engineered mutant MYXVs can also activate host innate immune signaling [16,18,19], this virotherapy vector represents a novel approach to eliminate tumor cells while reprogramming the tumor environment for customized therapy. Besides utility as monotherapy, MYXV has its versatility as a complement for other therapeutics, e.g., chemotherapy (cisplatin [20] and gemcitabine [21]). In this study, we further explore the potential application of mutant MYXV for immunotherapy and as an adjuvant to chemotherapy and DC vaccination.

Although replicating oncolytic viruses can amplify killing of tumor cells, antiviral innate immunity often rapidly eliminates their presence in the tumor environment quickly [22]. However, despite their removal, their immunological impact of oncolytic viruses may continue to provide therapeutic benefit [23]. The debate on whether replication-competency determines the longevity and effectiveness of therapeutic immune responses continues. Our study provides another argument on the therapeutic benefit of replication-defective virus.

In this study, we tested MYXV virotherapy combined with cisplatin chemotherapy and a Th17 – inducing DC vaccine that has recently shown promise for treatment of advanced stage ovarian cancer patients [24]. We show that combinatorial treatments with MYXV improve survival in a mouse model of OC when compared with cisplatin or DC monotherapy. We further show that MYXV can alleviate immune suppression by patient-derived ovarian tumor-associated CD14⁺ macrophages (TAMs) in coculture with tumor antigen-specific CD4⁺ T cells.

2. Materials and Methods

Human Subjects

Deidentified ovarian cancer patient samples were provided by the Women's Oncology clinic, Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences (UAMS) and the Mayo Clinic Ovarian Cancer Research Program Biospecimens Bank under IRB-approved protocols. Ovarian tumor ascites samples were recovered at the time of surgery.

Cell Culture and Virus Stock

Patient ovarian cancer cells (KAJ343, OvCa-37, and OvCa-43)[20] were cultured in RPMI complete growth medium supplemented with glutamine, penicillin/streptomycin and 10% fetal bovine serum. KAJ343 and OvCa-43 were high-grade serous ovarian carcinomas. OvCa-37 underwent neoadjuvant chemotherapy, and diagnosis was based on paracentesis ascites rather than surgical pathology, which was not defined. BSC40 (ATCC CRL-2761), ID8 TP53^{-/-} (provided by Iain McNeish, Imperial College, London) [25], and ID8-SP17 cells (provided by Keith Knutson, Mayo Clinic, Jacksonville)[26] are cultured in DMEM complete medium. The complete growth medium (e.g., DMEM Lonza/BioWhittaker Catalog no 12-604Q) was supplemented with 10% FBS (Atlanta Biologicals, Minneapolis, MN), 2 mM glutamine (Corning Cellgro, Millipore Sigma, St. Louis, MO), and 100 µg per ml of Pen/Strep (Corning Cellgro, Millipore Sigma, St. Louis, MO); for RPMI1640 complete culture medium, in addition to FBS, glutamine, and Pen/Strep, 2-mercaptoethanol (MP biomedical, Solon, OH) was supplemented to a final concentration of 0.05 mM.

The viruses used were originally derived from Lausanne strain of myxoma virus (MYXV) (GenBank Accession NC_001132.2/AF170726.2). The MYXV M062R deletion mutant (Δ M062R) and

WT MYXV have been described previously [27]. Myxoma virus stocks were prepared on BSC-40 cells and purified with sucrose step gradient through ultracentrifugation as previously described [28].

RT Realtime (RT²) PCR

Human primary patient cancer cells KAJ434, OvCa-37 and OvCa-43 or murine ID8 derivatives ID8-TP53^{-/-} are seeded the day before. The next day cells are mock treated, or infected at an moi of 5 for either WT or ΔM062R MYXV [27]. At 24 hrs post-infection, cells were harvested with Direct-zol RNA Mini Prep kit (catalog # R2052, Zymo, Irvine, CA, USA) according to manufacturer standard protocol. RNA quality is examined by running on the RNA gel to check 28S and 18S integrity and photospectrometer to estimate concentration. Equal amounts of total RNA in a maximal volume of 6 μL are used for cDNA synthesis using NEB PhotoScript® First Strand cDNA Synthesis Kit (Catalog # E6300L, NEB Inc, Ipswich, MA) as instructed in the manufacturer standard protocol. using NEB ProtoScript First Strand cDNA Synthesis Kit (Catalog # E6300L, NEB Inc, Ipswich, MA) as instructed in the manufacturer standard protocol. Realtime PCR is conducted following manufacturer standard protocol (Luna Universal qPCR Master Mix, NEB Inc). Sybr green RT-PCR primers used in this study is listed in **Table 1**.

Table 1. RT-PCR primers.

Target Gene	Primer sequences
Human IFNβ	Fwd 5'- GCC ATC AGT CAC TTA AAC AGC -3'
	Rev 5'- GAA ACT GAA GAT CTC CTA GCC T - 3'
Human IL-12b	Fwd 5'- CAAAGGAGGCGAGGTTCTAA-3'
	Rev 5'- GCAGGTGAAACGTCCAGAATA-3'
Human Sp17	Fwd 5'- GGTTC CATAGGCAGTTCTTAC-3'
	Rev 5'- GGAAGGCAGCTTGGATTT-3'
Human RSAD2	Fwd 5'- AGT GCA ACT ACA AAT GCG GC -3'
	Rev 5'- CTT GCC CAG GTA TTC TCC CC -3'
Human CXCL-10	Fwd 5'- CTG TAC CTG CAT CAG CAT TAG TA -3'
	Rev 5'- GAC ATC TCT TCT CAC CCT TCT TT - 3'
Human IL15	Fwd 5'- AGCCAACTGGGTGAATGTAATA-3'
	Rev 5'- CATCTCCGGACTCAAGTGAAATA-3'
Human ISG54	Fwd 5'- AGCGAAGGTGTGCTTTGAGA -3'
	Rev 5'- GAGGGTCAATGGCGTTCTGA -3'
Human NFκB1A	Fwd 5'- CCCTACACCTTGCCTGTGAG -3'
	Rev 5'- TGACATCAGCACCCAAGGAC -3'
Human CCL3	Fwd 5'- CTCTCTGCAACCAAGTTCTC-3'
	Rev 5'- CTGCTCGTCTCAAAGTAGTC-3'
Murine Sp17	Fwd 5'- CTTTCTCCAACACCCACTAC-3'
	Rev 5'- CTTCATCTTCTTTACCTCTTCTCT-3'
Murine IL-10	Fwd 5'- AGGCGCTGTCATCGATTTCT -3'
	Rev 5'- ATGGCCTTGTAGACACCTTGG-3'

Murine CD40	Fwd 5'- GTAGGTCACCCCTGAGAACC-3'
	Rev 5'- ACAACCCGAACCATACACACAA-3'
Murine CX3CL1 [29]	Fwd 5'- GCTCCTAGCCCTGACCCATC-3'
	Rev 5'- AGCTGATAGCGGATGAGCAA-3'
Murine GM-CSF	Fwd 5'- CTGGCCCCATGTATAGCTGA-3'
	Rev 5'- ACAGTCCGTTTCCGGAGTTG-3'
Murine IL-6	Fwd 5'- TCAATATTAGAGTCTCAACCCCCA-3'
	Rev 5'- GAAGGCGCTTGTGGAGAAGG-3'
Murine iNOS [30]	Fwd 5'- ATCGACCCGTCACAGTATG-3'
	Rev 5'- GATGGACCCCAAGCAAGACT-3'
Murine TNF α	Fwd 5'-CCCTCACACTCACAAACCAC-3'
	Rev 5'- ACAAGGTACAACCCATCGGC-3'
Murine IFN β	Fwd 5'- AGATCTCTGCTCGGACCACC-3'
	Rev 5'- CGTGGGAGATGTCCTCAACT-3'
Murine CXCL10	Fwd 5'- ATGACGGGCCAGTGAGAATG -3'
	Rev 5'- TCGTGGCAATGATCTCAACAC-3'
Murine IRF3.2	Fwd 5'- CACTCCCCACGCTACACTC-3'
	Rev 5'- TCCCATCCCCAGTAGCATGAG-3'
Murine IRF7.2 [31]	Fwd 5'- TGCTGTTTGGAGACTGGCTAT-3'
	Rev 5'- TCCAAGCTCCCGGCTAAGT-3'
Murine IFN γ	Fwd 5'- CGGCACAGTCATTGAAAGCC-3'
	Rev 5'- TGTCACCATCCTTTTGCCAGT-3'
Murine CXCL1 [29]	Fwd 5'- GCTGGGATTACCTCAAGAA-3'
	Rev 5'- TCTCCGTTACTTGGGGACAC-3'
Murine CXCL3 [32]	Fwd 5'- CCACTCTCAAGGATGGTCAA -3'
	Rev 5'- GGATGGATCGCTTTTCTCTG -3'
Murine MCP-1 [30]	Fwd 5'- AGGTCCCTGTCATGCTTCTG-3'
	Rev 5'- GGATCATCTTGCTGGTGAAT-3'
Murine EGR1	Fwd 5'- CACCTGACCGCAGAGTCTTTT-3'
	Rev 5'- GCGGCCAGTATAGGTGATGG-3'

Tumor Model Establishment and Treatment

The animal study was approved by the UAMS Institutional Animal Care and Usage Committee (IACUC) at the University of Arkansas for Medical Sciences (UAMS). ID8-TP53^{-/-} and ID8-SP17 cells are used for mouse model studies. The tumor model establishment is similar to what we previously described for the intraperitoneal carcinomatosis model. Briefly, 4 studies were performed. (a) The “WT MYXV+ cisplatin+ DC vaccine” study. In this study, ID8-TP53^{-/-} tumor cells were injected at 3x10⁶ cells per mouse through intraperitoneal injection (IP), followed by WT MYXV treatment started on day 7 post-tumor establishment at 10⁸ pfu per injection with a total of 4 injections at 2 days apart. In the same study, for combination treatment, the cisplatin is at 3mg/Kg of dose with a regimen of every 3 days starting on day 16 post-tumor establishment for a total of 3 treatments. The DC vaccine started at 30 days post-tumor establishment for a total of 4 treatments at a frequency of every 7 days. (b) The

“late Cisplatin+ $\Delta M062R$ +DC vaccine” study with the ID8-TP53^{-/-} model. At 16 days post-tumor establishment at the same dose as in (a), cisplatin was given via IP route at 3 mg/Kg at the same frequency as in (a). On 28 days post-tumor establishment, $\Delta M062R$ treatment was provided at 10⁸ pfu per mouse via IP route for every 2 days for a total of 4 treatments. On 44 days post-tumor establishment, DC vaccine was given at the same frequency as in (a). The 3rd study (c) is called “early Cisplatin+ $\Delta M062R$ +DC vaccine” study. The same doses and frequencies of cisplatin, $\Delta M062R$, and DC vaccine as in (b) were used, but the treatment started at 7 days post-tumor establishment. For the ID8-SP17 HGSOC model (d), we tested targeted immunotherapy against tumor antigen Sp17. The tumor cells were injected at 10⁶ cells per mouse via IP route, and cisplatin was at 1 mg/Kg of dose with a regimen of one injection every 3 days; the $\Delta M062R$ treatment was first performed 6 days after the last cisplatin treatment and followed by DC vaccine 2 days afterwards, and the $\Delta M062R$ -DC vaccine mini-regimen was repeated for 4 times every 7 days.

Human Ovarian Cancer Ascites CD14⁺ Macrophages and CD4⁺ T Cell Co-Culture System

CD14⁺ TAMs were recovered from primary ovarian tumor ascites by CD14⁺ microbead and magnetic column purification (Miltenyi). Human CD4⁺ T cells specific for ovarian tumor peptide antigens were derived through stimulation with TADG14v peptide-loaded Th17-inducing dendritic cells, as previously described [33–35]. CD14⁺ ascites TAMs were either uninfected or infected with WT or $\Delta M062R$ MYXV (at a moi of 10, pre-incubated on ice for 1 hr, then washed one time with PBS). Ascites CD14⁺ TAMs (5 x 10⁵/well) were then co-cultured with ovarian tumor antigen--specific CD4⁺ T cells (5 x 10⁵/well) in 0.7 ml RPMI plus antibiotics, glutamine 5 x 10⁻⁵ M 2-mercatopethanol and 10% human AB serum (RPMI 10Hu). After 48 hr, tumor antigen peptide-loaded and irradiated (75 gray) autologous lymphoblastoid cells (2.5 x 10⁵ cells/well) were added in 0.2 ml RPMI/10Hu, and the cocultures incubated o/n in the presence of Brefeldin A. Where indicated, cocultures were treated with a STING inhibitor (1 μ M H-151) or a TBK-1 inhibitor (2 μ M BX-795). Following coculture, CD4⁺ T cells were stained with PE-anti-CD4 antibody, fixed and permeabilized with fixation/permeabilization buffer (Affymetrix eBioscience) and stained with APC anti-TNF antibody. Samples were analyzed with a FACSCalibur flow cytometer and CellQuest software.

Statistical Analyses

GraphaPrism 10.1 was used for statistical analyses. Survival study was performed using Kaplan-Meier analyses followed by Long-rank comparison. Statistical significance is defined by *p<0.05.

3. Results

3.1. Myxoma Virus Infection in Primary Cells of Human Ovarian Cancer Environment Stimulated Pro-Inflammatory Gene Expression and Up-Regulation of Sp17

Antigen Sp17 is a signature protein found in OC cells [36,37]. Expression of Sp17, however, is not found in normal female cells; thus Sp17 antigen may be an excellent target for OC immunotherapy [37–39]. We included Sp17 in our RT-PCR array as an indicator for cancer cells. Two MYXV viruses, a replicating WT and replication defective mutant virus ($\Delta M062R$), were used for the study. Primary OC patient ascites cells from 3 patients were mock treated or treated with either MYXV at an moi of 5-10 before examining the gene expression profile. MYXV treatment increased Sp17 expression (**Figure 1A-C**, red frame) in addition to up-regulation of IFN β and other proinflammatory molecules (e.g., IL12b and CXCL10) (**Figure 1**). We performed similar tests using the murine OC cell line ID8-TP53^{-/-} that was used for establishing a syngeneic OC model system as previously described [25]. We found that MYXV treatment increased expression of pro-inflammatory molecules (**Figure 2A**) similar to what we saw in human cells (**Figure 1**). Moreover, Sp17 expression is also up-regulated (**Figure 2A and 2B**, red frame) similarly as found in human cells from the cancer environment (**Figure 1**). Using an array panel to examine gene expression that shapes a pro-cancer

tumor environment, we found that $\Delta M062R$ treatment moderately altered gene expression to a lesser degree than wildtype (WT) MYXV (**Figure 2B**). This is consistent with our previous observation that $\Delta M062R$ treatment stimulates pro-inflammatory gene expression at both RNA and protein levels compared with WT MYXV infected cells and mock treated primary cells [18].

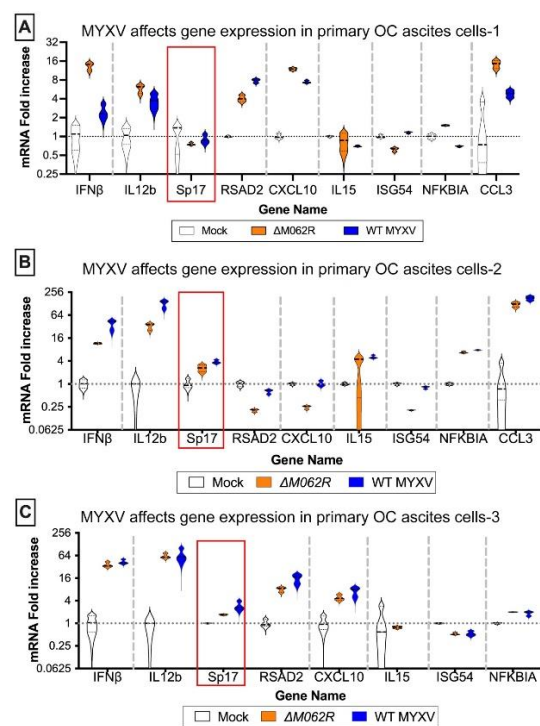


Figure 1. Myxoma virus treatments of primary human ovarian cancer ascites cells induce proinflammatory molecule expression and upregulation of Sp17. Ascites cells from OC patients, (A) OvCa37, (B) KAJ343, and (C) OvCa43, were mock treated or infected with MYXV, WT or $\Delta M062R$, at an MOI of 5 for 18 h before cells were harvested for RNA extraction and RT²-PCR to assay target gene expression levels for semi-quantitative comparison. Red frame marks the ovarian cancer cell specific antigen, Sp17, expression levels.

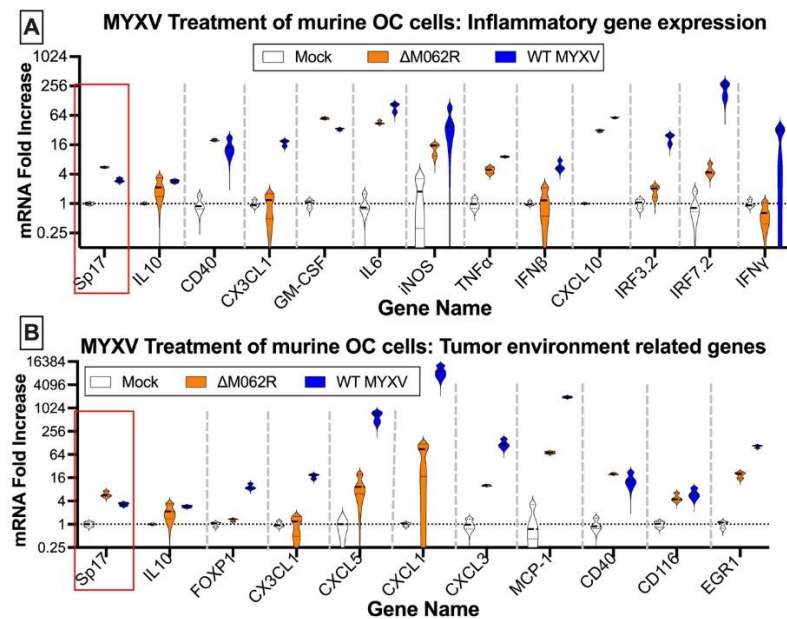


Figure 2. Myxoma virus treatments of murine ID8 TP53^{-/-} induce inflammatory gene expression and alter gene expression affecting tumor environment. Murine OC cells were mock treated or infected with MYXV at an MOI of 5 for 18 h before RNA extraction and RT²-PCR. Genes related to inflammatory responses (A) and contributing

to tumor environment maintenance (B) were examined for their expression affected by MYXV treatments. In either array, OC tumor marker, murine Sp17, was also examined.

3.2. Wildtype MYXV Treatment Proceeding Cisplatin Treatment Led to Moderate Therapeutic Benefit in a High Grade Serious Ovarian Cancer (HGSOC) Murine Syngeneic Model

The recent success of a DC vaccine trial in OC patients provided exciting and novel options targeting the unique ovarian tumor environment [24]. With both human and murine Sp17 expressed in OC cells, we proposed a proof-of-concept DC vaccination strategy of pulsing Sp17 peptide on DC cells for the in vivo study as previously described [26]. In our previous study, we found that WT MYXV treatment before cisplatin treatment provided better therapeutic benefit than either WT MYXV monotherapy or WT MYXV provided after cisplatin treatment [20]. The ability of replicating oncolytic WT MYXV to eliminate OC cells facilitated cisplatin control of tumor progression, hence the rationale of our combination treatment strategy. Thus, with a replicating oncolytic agent promoting the expression of tumor antigen, we tested whether DC vaccine may be a good option to prolong survival further. We indeed found that WT MYXV and DC provided a significant improvement of survival with and without cisplatin treatment (Figure 3A) with a median survival of 87 days for either regime. Mock treatment, cisplatin alone, WT MYXV alone, and WT MYXV plus cisplatin show a median survival of 50, 65, 72, and 75 days, respectively.

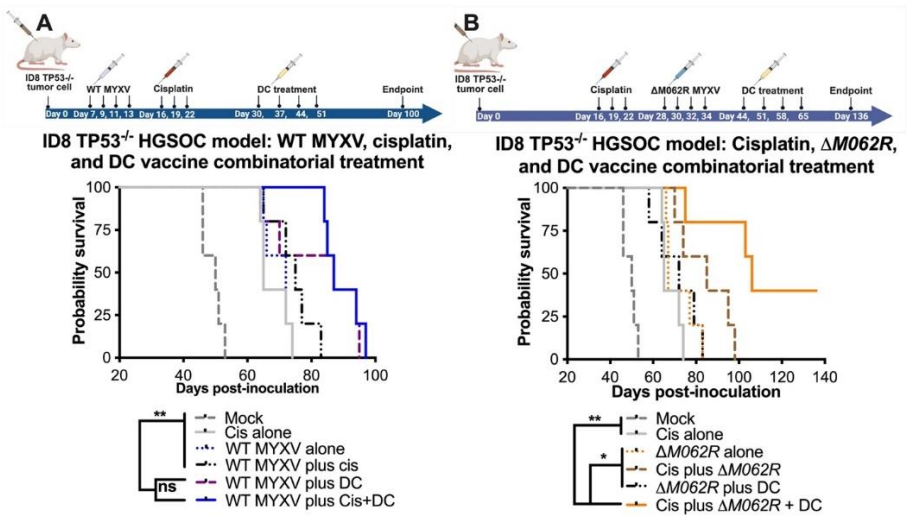


Figure 3. Myxoma virus treatments facilitate survival in the combinatorial treatment with cisplatin and DC vaccine. C57/BL6 mice were injected through intraperitoneal route (i.p.) with 3×10^6 ID8-TP53^{-/-} cells on day 0 and randomly grouped to 5 mice per treatment. Cisplatin was injected at 3 mg/kg i.p. on days 16, 19 and 22. WT-MYXV (10^8 pfu, i.p.) (A) was injected on days 7, 9, 11 and 13 (before cisplatin treatment), while ΔM062R MYXV (10^8 pfu i.p.) (B) was injected on days 28, 30, 32 and 34 (following cisplatin treatment). Sp17 peptide-loaded DC (10^6) were injected subcutaneously (s.c.) on days 30, 37, 44 and 51 for WT-MYXV recipients and days 44, 51, 58 and 65 for ΔM062R MYXV recipients. The log-rank (Mantel-Cox) test was conducted for Kaplan-Meier survival comparison. * $p < 0.05$, ** $p < 0.001$, n.s.: not significant.

3.3. M062R-Null (ΔM062R) MYXV Treatment as Monotherapy or in Combination with DC Vaccine Targeting Sp17 Antigen Showed Significant Treatment Benefit in the HGSOC Murine Syngeneic Models.

Due to the association of Sp17 antigen in OC with tumorigenicity, tumor progression, chemoresistance, and the immunosuppressiveness, Sp17 becomes a promising therapeutic target for vaccine development in treating OC [37,38,40,41]. In our study, the DC vaccine is indeed designed to target the antigenic epitopes of Sp17 [42].

In our previous study [20], we examined the therapeutic potential of a replication defective MYXV called *M062R*-null MYXV ($\Delta M062R$) as either monotherapy or in combination with chemotherapy. We found previously that $\Delta M062R$ treatment after cisplatin provided better survival [20]. Recently, we further examined the immunostimulatory effect of $\Delta M062R$ and found this virus superior in inducing proinflammatory responses in monocytes/macrophages by triggering DNA-sensing stimulated type 1 interferon responses (IFN-I) [18]. The effect induced by $\Delta M062R$ in myeloid cells is different from the conventional DNA-sensing stimulated IFN-I responses and promotes a potent pro-inflammatory outcome [18,43], indicating an additional mechanism governing the unique immunological state [43]. Thus, besides its being a potential adjuvant to cisplatin, we proposed to examine if $\Delta M062R$ has therapeutic potential for immunotherapy and may also be an adjuvant for DC vaccine. In the clinical trial of DC vaccine, all the patients were treated after platinum-based therapy and tumor debulking, thus we did not specifically examine DC vaccine as a monotherapy in our study. The ID8-TP53^{-/-} syngeneic model system portrays the conditions of HGSOc in humans [44], and it presents an aggressive form of OC (**Figure 3**). We found that combined treatment with cisplatin, $\Delta M062R$, and DC vaccine led to significant improvement in survival ($p < 0.0001$, Long-rank Mantel-Cox test, with a median survival of 106 days, **Figure 3B**) compared to mock treatment, cisplatin alone, and $\Delta M062R$ alone, with median survival of 50, 65, and 67 days, respectively. Moreover, while combination treatment of cisplatin- $\Delta M062R$ or $\Delta M062R$ -DC vaccine significantly improved survival compared to monotherapy with median survival of 85 and 72 days, respectively, cisplatin- $\Delta M062R$ -DC vaccine combinatorial regime is significantly better than the other two-regime treatments ($p < 0.0197$ and $p < 0.0127$, respectively, by Long-rank Mantel-Cox test). In this study, however, we did not evaluate the therapeutic effect of DC vaccine in facilitating cisplatin chemotherapy or $\Delta M062R$ virotherapy. We also did not examine whether $\Delta M062R$ therapeutic benefit is synergistic/additive based on the same mechanism of DC vaccine. We recognized that in this study the treatment of cisplatin or virotherapy was provided at a much later time, which were from days 16 or 28, respectively (see **Figure 3B** diagram on treatment schedule) than earlier studies of starting treatment at 7 days post-tumor establishment [20]. Although there is little evidence suggesting that a moderate delay in treatment would affect overall patient survival [45,46], we decided to investigate whether starting $\Delta M062R$ virotherapy or DC vaccine early in the combinatorial treatment offers any therapeutic benefit. We thus designed a follow-up study using the same ID8-TP53^{-/-} syngeneic model system that will be discussed next.

3.4. *M062R*-Null ($\Delta M062R$) MYXV Treatment in Combination with DC Vaccine Targeting Sp17 Antigen Significantly Improve the Treatment Outcome of Cisplatin.

In this second stage of study, we modified the treatment schedules of corresponding controls as shown in the diagram of **Figure 4**. We focused on the logistics of therapy design similar to a clinical setting and followed the following general principles, (1) to start the first treatment at 7 days post-tumor establishment, and (2) to start the DC vaccine at 8-9 days after the prior treatment for combination treatments. Although introducing $\Delta M062R$ treatment early as the first course of therapy (median survival of 59 days, $p = 0.0071$, Kaplan-Meier analysis by Long-rank Mandel-Cox test) significantly improved the survival compared to mock treated group (median survival of 49 days), it did not provide a dramatic edge to prolong survival over either cisplatin alone (median 75, $p = 0.0019$) or DC vaccine alone (median 75, $p = 0.0019$). This is consistent with our previous finding on the utility and timing of $\Delta M062R$ [20]. We also found that as a monotherapy, early initiation of DC vaccine (median survival of 75 days) is as effective as cisplatin (median survival of 75 days) at the same time frame in this model (no statistically significant difference). Although two-regime and three-regime (all $p < 0.0001$) combinatorial treatments significantly improved survival compared to mock, no significant difference among cisplatin plus $\Delta M062R$ (median survival 96 days), cisplatin plus DC (median survival 113 days), and cisplatin plus $\Delta M062R$ and DC vaccine (median survival 115 days) was observed, suggesting the importance of early cisplatin treatment in survival. Adding $\Delta M062R$ ($p = 0.0002$) or DC vaccine ($p < 0.0001$) to cisplatin significantly improved treatment outcome compared

to cisplatin alone. Clustered $\Delta M062R$ treatment (four consecutive doses at every two days) at an early time (7 days post tumor establishment) followed by clustered DC vaccine treatment (four consecutive doses every week) showed no difference in survival to DC vaccine alone starting at 7 days. This prompted us to investigate utilizing individual $\Delta M062R$ and DC vaccine sequentially in a combination treatment to exploit the immunostimulatory properties of $\Delta M062R$ in facilitating the effects of DC vaccine that is shown in the next section.

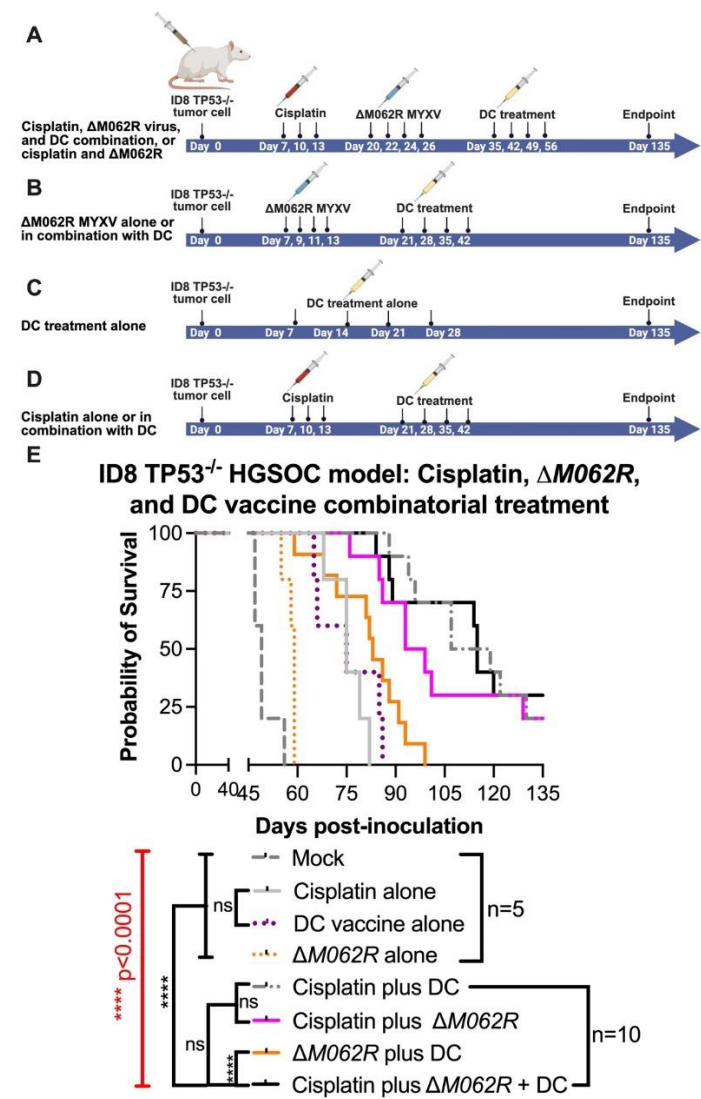


Figure 4. Treatment of $\Delta M062R$ further enhances therapeutic effect by DC vaccine and cisplatin in a combinatorial treatment. C57/BL6 mice were injected i.p. with 3×10^6 ID8-TP53^{-/-} cells on day 0. Five mice were randomly assigned per group for mock and the individual treatment controls, and 10 mice were assigned per group for double or triple combinatorial treatments. Regime design is shown in **A** for cisplatin plus $\Delta M062R$ and the triple combinatorial treatment. **B**. Treatment design for $\Delta M062R$ alone or combination of $\Delta M062R$ and DC vaccine. Treatment schedule of DC vaccine alone is shown in **C**. Treatment schedule of cisplatin alone or cisplatin plus DC vaccine schedule is shown in **D**. In general, treatment starts at 7 days after tumor establishment. Treatment with $\Delta M062R$ is at 10^8 pfu, i.p. every 2 days for a total of 4 injections. Cisplatin was injected i.p. at 3 mg/Kg every 3 days for a total of 3 treatments. DC vaccine is injected s.c. 8-9 days after the previous treatment (if it is a combination treatment) every 7 days for a total of 4 vaccinations. **E**. Kaplan-Meier survival curve is shown and $\Delta M062R$ plus DC provided superior therapeutic outcome with or without cisplatin. The log-rank (Mantel-Cox) test was conducted with statistical significance defined as the following, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, and n.s.: not significant.

Experimental Timeline:

- Day 0:** ID8-SP17 tumor cells (indicated by a syringe icon).
- Day 7, 10, 13:** Cisplatin treatment (indicated by a syringe icon).
- Day 19, 21:** $\Delta M062R$ MYXV DC Vaccine.
- Day 26, 28:** $\Delta M062R$ MYXV DC Vaccine.
- Day 33, 35:** $\Delta M062R$ MYXV DC Vaccine.
- Day 40, 42:** $\Delta M062R$ MYXV DC Vaccine.
- Day 152:** Endpoint.

Survival of ID8-SP17 HGSOC model

The Kaplan-Meier survival plot shows the Probability of Survival (Y-axis, 0 to 100) versus Days post-inoculation (X-axis, 0 to 152). The legend identifies the treatment groups:

- Mock (dotted line)
- Cisplatin alone (dashed line)
- DC alone (dotted line with open circles)
- $\Delta M062R$ alone (solid orange line)
- Cisplatin plus DC (dotted line with open squares)
- DC plus $\Delta M062R$ (solid blue line)
- Cisplatin plus $\Delta M062R$ (solid purple line)
- Cisplatin plus $\Delta M062R$ + DC (solid black line)

Statistical significance is indicated by asterisks (*, ***) and brackets. The survival curves show that the combination of Cisplatin plus $\Delta M062R$ + DC (solid black line) results in the highest survival probability, reaching approximately 60% at Day 152. The $\Delta M062R$ alone group (solid orange line) shows the lowest survival, dropping to 0% by Day 122.

Figure 5. Treatment with $\Delta M062R$ provides survival advantage in HGSOC model with antigen targeted immunotherapy. **A.** Treatment design. C57/BL6 mice were injected i.p. with 1×10^6 ID8-Sp17 cells at day 0. Animals were randomly assigned to each treatment group with 4 mice per group. Cisplatin, in single or combinatorial treatment, was injected i.p. at 1 mg/Kg starting at 7 days post tumor establishment for a total of 3 treatments every 3 days. In the cisplatin- $\Delta M062R$ -DC or $\Delta M062R$ -DC combination treatments, $\Delta M062R$ -DC was administered 2 days before each DC vaccine. The $\Delta M062R$ -DC treatment block is repeated 4 times every 7 days. In treatments where $\Delta M062R$ or DC is not coincident, the $\Delta M062R$ or DC treatment was given on the set days shown in the diagram. **B.** Kaplan-Meier analysis of outcomes showed the therapeutic benefit of $\Delta M062R$. The log-rank (Mantel-Cox) test was conducted with statistical significance defined as the following, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, and n.s.: not significant.

3.6. *M062R-Null MYXV Infection of Ovarian Cancer Patient Ascites CD14⁺ Cells Improved CD4⁺ T Cell Anti-Tumor Response in a Primary Cell Co-Culture System.*

It is known that MYXV favors infecting monocytes/macrophages [48], providing a mechanistic rationale of targeting myeloid cells for immunotherapy with these viral agents. Based on our prior finding on the potent immunostimulatory effect by $\Delta M062R$ in monocytes and macrophages [18,43], we further examined whether $\Delta M062R$ immunotherapeutic benefit is due to its effects on immunosuppressive tumor-associated monocyte/macrophages or TAM population in the tumor environment, designated immunosuppressive CD14⁺ tumor-associated macrophages (TAMs). We thus utilized a highly innovative co-culture system to test how OC patient CD4⁺ T cell functions are regulated in OC tumor environment. It is known that in OC CD14⁺ TAMs suppress the anti-tumor function of CD4⁺ T cells [33]. The key question is whether MYXV influences or even subverts immunosuppression by CD14⁺ TAMs to enable a more effective anti-tumor CD4⁺ T cell response. In this co-culture system, patient CD4⁺ T cells were stimulated with tumor antigen (TADG14v)[33] pulsed DC in the presence of TAMs with or without prior treatments. We then examined the status of CD4⁺ T cell activation reflected by TNF production via flow cytometry [33]. As shown in **Figure 6A**, in the absence of CD14⁺ TAMs, human CD4⁺ T cells responded to tumor antigen with TNF production (**Figure 6A** first column). In the presence of TAMs, however, upon the same stimulation of OC tumor antigen, CD4⁺ T cells presented significant reduction of TNF production (**Figure 6A** second column). Both WT and $\Delta M062R$ treatment of TAMs reversed the immunosuppressive effect by TAMs but with $\Delta M062R$ treatment leading to promotion of T cell activation. Shown in **Figure 6A** (top panel) is a representative result from an allogeneic co-culture system with CD4⁺ T cells and CD14⁺ TAMs from different donors. We also performed similar tests using autologous specimens for the co-culture system and achieved similar results (shown in **Figure 6A** bottom panel).

Our previous study showed that $\Delta M062R$ activated CD14⁺ monocytes/macrophages to promote proinflammatory responses regulated by both DNA-sensing via cGAS/STING/TBK1/IRF3 axis and SAMD9 pathway [18]. Using a STING antagonist, H151, and a TBK1 inhibitor, BX-795, we found that reversal of TAM immunosuppression by $\Delta M062R$ was not affected (**Figure 6B**). We thus conclude that therapeutic effect of $\Delta M062R$ against immunosuppressive TAMs is independent from cGAS/STING/TBK1/IRF3 axis and is likely determined by SAMD9 pathway. This is consistent with our observation that $\Delta M062R$ treatment induced a unique transcriptional landscape and cellular translation profile independent from DNA-sensing pathway [43].

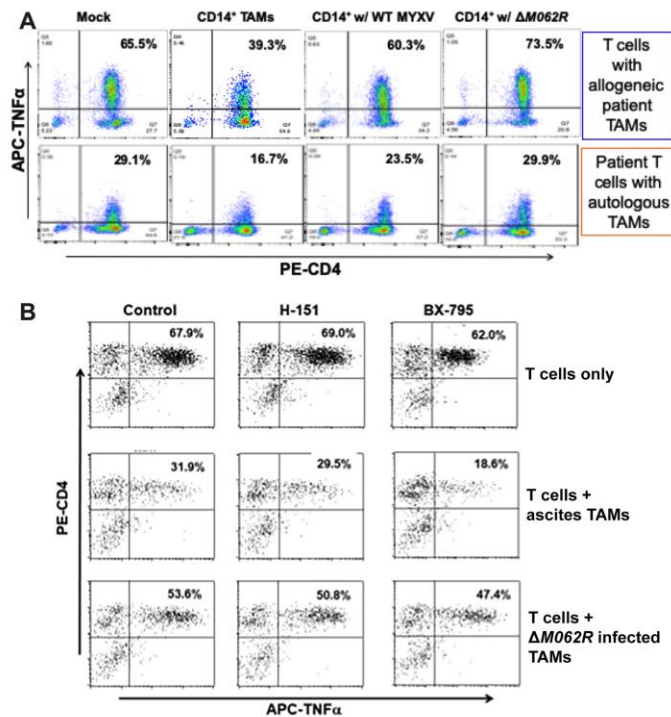


Figure 6. MYXV treatments to OC CD14⁺ TAMs promote CD4⁺ T cell activation against tumor antigen. A co-culture system with TADG14v peptide tumor antigen-specific CD4⁺ T cells and OC ascites CD14⁺ TAMs was used for this study. **A.** MYXV treatments of CD14⁺ TAMs reversed the immunosuppressive effect by TAMs. After incubating without any TAMs (mock), with untreated TAMs, with WT MYXV treated TAMs, or with $\Delta M062R$ treated TAMs, TNF α expression by allogeneic (top row) or autologous (bottom row) CD4⁺ T cells was determined following overnight stimulation with tumor peptide antigen-loaded autologous lymphoblastoid cells. A representative result from 3 individual patient TAM samples is shown. **B.** Reversal of TAM immunosuppression by $\Delta M062R$ is independent of the cGAS/STING/TBK1/IRF3 signaling axis. CD4⁺ T cell functionality was measured as described above in **A**. A STING antagonist, H-151 (1 μ M), and TBK1 kinase inhibitor, BX-795 (2 μ M), were used to inhibit different steps of the DNA sensing pathway in cultures of T cells alone (first row), T cells plus CD14⁺ TAMs (second row), or T cells plus $\Delta M062R$ MYXV treated TAMs (third row).

4. Discussion

MYXV was first formulated as oncolytic agent almost 2 decades ago [49] and have been examined for their therapeutic potential against multiple cancer types [50–53]. The WT MYXV and replication competent MYXVs armed with therapeutic molecules have been developed to purge metastatic [16,51] and hematological malignant cells [54–56] as well as being loaded on vehicle cells for a trojan-horse like delivery [19,57]. Furthermore, MYXV can be used in coordination with chemotherapeutic agents to achieve synergistic effect for improved treatment outcome in preclinical models [20,21,58,59]. Our previous study found that replication-competent WT MYXV provided better therapeutic benefit in an OC carcinomatosis model when it is used before cisplatin treatment [20]. Surprisingly, a replication-defective $\Delta M062R$ MYXV achieved superior therapeutic benefit as a monotherapy and as an adjuvant with cisplatin in combatting OC carcinomatosis [20]. In contrast with WT MYXV, the optimal schedule for $\Delta M062R$ MYXV is to be used after cisplatin treatment [20]. Infection by $\Delta M062R$ does not lead to progeny viral production [27,60], thus, treatment with $\Delta M062R$ does not cause amplifying oncolysis seen with WT MYXV. Our recent work demonstrated that

$\Delta M062R$ infection triggered proinflammatory cytokine/chemokine production in monocytes/macrophages [18]. Such proinflammatory response is triggered in part through DNA-sensing pathway leading to IFN-I production [18]. However, upon transcriptomic analyses on $\Delta M062R$ effect to monocytes/macrophages, a distinct feature from that of cGAS/STING/TBK1/IRF3 axis of DNA sensing pathway was observed in monocytes/macrophages by $\Delta M062R$ [43]. This unique response is likely due to the activation of another host immunoregulatory factor, SAMD9 that is the host target of viral M062 protein [27,61]. Activating the SAMD9 pathway by $\Delta M062R$ is associated with the immunotherapeutic potential of this replication-defective viral agent. Although the concept of immunotherapeutic potential of oncolytic viruses have been recognized [62], the therapeutic promise of a replication defective viral vector is not yet broadly recognized. In this study, we presented strong evidence that such a replication-defective $\Delta M062R$ MYXV can enhance immunotherapy for OC and alleviate ovarian TAM immunosuppression of DC-stimulated tumor antigen-specific human CD4⁺ T cell responses. The MYXV $\Delta M062R$ platform is an excellent option for immunotherapy in OC and can be utilized as an adjuvant to existing chemotherapy and DC vaccine.

The utility of $\Delta M062R$ in immunotherapy is due to its immunostimulatory property by activating DNA-sensing associated IFN-I induction and the SAMD9 pathway [18,43]. However, the timing and design of incorporating $\Delta M062R$ treatment is important to achieve therapeutic benefit. We found that providing $\Delta M062R$ early before the establishment of tumor environment (1 week after tumor cell inoculation) did not render significant benefit as monotherapy nor boosted the benefit of DC vaccine (**Figure 4B** and **4E**). On the other hand, when $\Delta M062R$ treatment is provided much later during disease progression (a month after tumor cell inoculation), as monotherapy it outperformed cisplatin monotherapy that started 2 weeks before $\Delta M062R$ treatment (**Figure 3B**). More importantly, adding $\Delta M062R$ treatment after chemotherapy in either clustered regime preceding DC vaccine (**Figure 3B** and **4E**) or individually administrated preceding DC vaccine (**Figure 5**) provided superior treatment advantage over chemotherapy alone, making $\Delta M062R$ regimens logical choices for therapeutic design in the clinical setting for OC patients, especially after tumor debulking and the initial chemotherapy. Among human leukocytes, MYXV presented preferred binding and entry in myeloid cells such as monocytes and macrophages [48], which establishes the rationale of using this viral vector against TAMs. To investigate possible mechanisms of $\Delta M062R$ -DC vaccine combinatorial treatment, we examined how $\Delta M062R$ influenced monocytes/macrophages to activate innate immune signaling events [18,43]. In OC TAMs, $\Delta M062R$ likely silences the expression of immunosuppressive cytokines/chemokines [20] or even promotes them to produce proinflammatory molecules. Alleviation of immunosuppression by CD14⁺ TAMs allows stronger CD4⁺ T cell responses to tumor antigen stimulation (**Figure 6A**). Besides being an informative predictor for prognosis [63], the immunosuppressive properties of the OC tumor environment also affect the efficacy of immunotherapy agents including DC vaccine [64] and CAR-T therapy [65,66]. The immunotherapeutic benefit of $\Delta M062R$ may not be restricted to monocytes/macrophages, as therapeutic benefit to influence T cell responses by MYXV has also been reported [67]. MYXV hijacks cells with short circuited IFN-I responses, often malignant cells or cells cultured in the tumor environment [68], but does not affect the functionality of normal cells such as hematopoietic stem cells [54,69], progenitor cells [54,69], and normal T cells [67,70], further rendering a safety profile of $\Delta M062R$ for clinical applications in the future.

5. Patents

A US patent resulting from the work reported in this manuscript, **US 12,128,099 B2**, was awarded to J.L. and M.J.C.

Author Contributions: Conceptualization, M.J.C and J.L.; methodology, M.J.C and J.L.; software, M.J.C. and J.L.; formal analysis, M.J.C and J.L.; investigation, M.J.C and J.L.; resources, J.L.; data curation, J.L.; writing—original draft preparation, J.L.; writing—review and editing, M.J.C and J.L.; visualization, J.L.; project administration,

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Abbreviations

The following abbreviations are used in this manuscript:

MYXV	Myxoma virus
OC	Ovarian cancer
DC	Dendritic cell
TAM	Tumor-associated macrophage
HGSOC	High grade serous ovarian cancer

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