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An approach to increase efficacy against triple negative breast cancer using an engineered multigenic vaccinia virus combined with CAR T-cell therapy

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Abstract. Triple negative breast cancer (TNBC) is an aggressive subtype with poor prognosis and limited treatment options. Conventional therapies often lead to systemic relapse, and cancer immunotherapy faces challenges such as insufficient immune activation and lack of tumor specificity. To overcome these limitations, we propose a novel strategy using an engineered oncolytic vaccinia virus (VV) combined with chimeric antigen receptor (CAR) T-cell therapy to treat TNBC. This oncolytic virus is designed to increase tumor specificity through the expression of anti-MUC-1 and ANTXR1 and to reprogram the tumor microenvironment (TME) by integrating PD-L1 inhibitor, CCL5, GM-CSF, and IL-2. This is expected to significantly increase recruitment and function of macrophages, NK cells, granulocytes, and T cells within the TNBC TME and to enhance tumor cell lysis. Additionally, the VV is expected to increase CAR T-cell proliferation and their specific targeting of TNBC cells. Our model provides a specific and immunologically potent treatment for TNBC. Future directions include addressing tumor heterogeneity and minimizing antiviral immune responses.

Keywords: Triple-negative breast cancer, oncolytic virus, vaccinia virus, CAR T-cell therapy.

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1. Introduction

1.1. Breast Cancer Overview

Breast cancer is the second most common cancer affecting women in the United States after skin cancer, responsible for approximately 30% of all new female cancers each year.[1] In addition, it is the second leading cause of cancer death for women after lung cancer.[1] Its etiology includes a combination of genetic, hormonal, environmental, and lifestyle factors. Inherited mutations in genes such as breast cancer gene (BRCA)1 and BRCA2, estrogen and progesterone use, and exposure to radiation can all promote breast cancer growth.[2] Breast cancer has several subtypes based on the presence or absence of hormone receptors (HR) and human epidermal growth factor receptor 2 (HER2) on the cancer cells. The four main subtypes include HR+/HER2+, HR+/HER2-, HR-/HER2+, and HR-/HER2-, which is also known as triple negative breast cancer (TNBC).[3]

1.2. TNBC Characteristics

Among these breast cancer subtypes, TNBC is a particularly aggressive form that accounts for 10-15% of all cases.4 It is characterized by the lack of three key receptors normally associated with breast cancer growth: estrogen receptor (ER), progesterone receptor (PR), and HER2.4 Due to the absence of these receptors, TNBC is resistant to hormonal therapies or drugs targeting HER2 receptors.4 TNBC tends to grow and develop more rapidly than other breast cancer types, with a higher risk of metastasizing to the brain, liver, and bones, and it has a higher recurrence rate.4 Compared to 91% 5-year relative survival rate for all breast cancer types, the overall 5-year survival for TNBC is only 77%, and this drops to 12% once the tumor has metastasized.4,5 TNBC is more common in women under 40, who are African American, and who has a mutation in the BRCA1 gene.4 Symptoms are similar to other breast cancers and can manifest as a lump/mass in the breast, abnormalities in breast shape, skin dimpling, breast pain, and nipple discharge.[6]

1.3. Current Therapeutic Challenges in TNBC

There are only a few current therapeutic options for TNBC, including surgery, adjuvant chemotherapy, and radiotherapy.[7] However, these conventional treatments often lead to systemic relapse.[8] Small molecule inhibitors, such as poly ADP ribose polymerase (PARP) and Bromodomain and Extra-Terminal motif (BET) inhibitors, tend to induce rapid resistance in many patients.[7] In recent years, cancer immunotherapy has emerged as a promising therapeutics, though with many challenges. New antibody-based treatments, such as immune checkpoint inhibitors, have targets that are only effective for a small number of TNBC patients.[9] For CAR T-cell therapy, challenges include poor trafficking to the tumor site and limited persistence of CAR T-cells. The TME also complicates CAR T-cell therapy due to the presence of immunosuppressive cells.[10] Another study, adoptive cell transfer therapy with primed peripheral lymphocytes and anti-PD-L1, has shown promise in inhibiting the growth and metastasis of TNBC, but not all patients responded to this treatment.[11] A recent study also reported the use of oncolytic adenoviruses expressing granulocyte macrophage colony-stimulating factor (Ad5/3-D24-GMCSF) with low doses of the chemotherapeutic cis-platin (CP) to enhance immunostimulatory function, but efficacy was limited, and many patients experienced side effects.[12]

1.4. Oncolytic Viruses: A Promising Tool Against TNBC

Oncolytic viruses (OVs) are a novel type of cancer immunotherapy that leverages the natural capability of viruses to infect and destroy tumor cells. These viruses are genetically engineered to selectively target tumor cells while leaving normal cells unharmed. OVs function through direct tumor cells lysis and stimulation of robust antitumor immunity.[13] This dual mechanism makes OVs a promising treatment, particularly for cancers that are resistant to conventional therapies, such as TNBC. Various types of viruses have been engineered for this purpose, including herpes simplex virus (HSV), adenoviruses, and vaccinia virus (VV).[13]

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2. Approach

2.1. Summary of Primary Research

In the following section, we will highlight the primary research articles that were chosen as foundational studies for our project. They provide us insights regarding innovative modifications that could be made to the oncolytic virus as well as their mechanism of action.

2.1.1. OV-Expressed PD-L1 Inhibitors Stimulates T Cell Functions.

This study provides a new approach to enhance antitumor immunity using oncolytic viruses.[14] The engineered OV was able to overcome the immunosuppressive TME by blocking the PD-L1 pathway and stimulating immune responses through GM-CSF. It turned out that treating mice containing colon adenocarcinoma (MC38) with this virus significantly enhanced T cell responses against tumor neoantigens. The OV was intratumorally injected into mice, and ten days later, their splenocytes showed increased production of interferon-gamma (IFN-γ) and T cell proliferation in response to a mix of neoantigen peptides. This indicates that a robust antitumor immune response has been elicited. The results suggest that the combined expression of PD-L1 inhibitors and GM-CSF through an OV can effectively boost antitumor immunity.

2.1.2. OV Expressing Cetuximab-CCL5 Fusion Protein Enhances Glioblastoma Treatment.

This study engineered an oncolytic herpes simplex virus (oHSV) which encodes for a bispecific fusion protein called OV-Cmab-CCL5.[15] One arm contains a single-chain variable fragment of cetuximab (an EGFR antibody), and the other arm contains C-C motif chemokine ligand 5 (CCL5), which is a chemokine crucial for leukocyte recruitment. It turns out that the engineered oHSV continuously produces CCL5 within the $EGFR^+$ glioblastoma TME. It greatly increases the infiltration and activation of various leukocytes, suppresses tumor EGFR signaling, and decreases tumor volume. These results suggest that OVs can be engineered to deliver chemokines to tumors in a highly specific, targeted manner, turning "cold" tumors, i.e. those not triggering a robust immune response, into "hot" ones. By combining chemokine activity with monoclonal antibody function, the engineered virus leads to enhanced immune cell-mediated antitumor response.

2.1.3. OV-Delivered CAR Antigens Increases Treatment Efficacy Against Solid Tumors.

This study combines oncolytic virotherapy with CAR T-cell therapy to improve the targeting of solid tumors.[16] Due to limited efficacy of CAR T, researchers engineered vaccinia viruses (VVs) by inserting transgenes into the viral thymidine kinase (TK) locus to selectively deliver CAR target mCD19 antigen to solid tumors, leading to the enhancement of mCD19 CAR T cell activity to target even tumor cells with low expression of cognate antigens. The TK-disrupted viruses can selectively spread, replicate, and transfect in malignant cells. The tendency of these viruses toward tumor cells comes from high TK activity of tumor cells, which is associated with fast nucleotide turnover and reduced CAR target expression on normal cells or infiltrating T cells. Infection with mCD19 VVs causes tumor cells to express mCD19, making them targets for recognition and destruction by mCD19 CAR T cells. This approach enhances the selective cytotoxicity of CAR T cells with matched antigens, demonstrating that oncolytic virus-driven antigen expression can improve the therapeutic effect of CAR T.

The studies discussed above have each demonstrated the ability of engineered OVs to deliver specific proteins and antigens for enhancing the immune response against various solid tumors. By expressing PD-L1 inhibitors, GM-CSF, CCL5, and CAR target antigens, these OVs have shown the ability to overcome the immunosuppressive TME and recruit various immune cells. Building on these results, we hypothesize that a unified OV model combining PD-L1 inhibition, chemokine-mediated immune cell recruitment, and targeted antigen delivery will more effectively treat TNBC.

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2.2. Method and Materials

2.2.1. General Approach

Oncolytic VV will be employed, which has a large genome size of 190kb that allows for multiple gene insertions.[12] Modifications will be made to the viral genome to encode for an inhibitor of PD-L1 (iPD-L1), CCL5, GM-CSF, interleukin (IL)-2, and the CAR antigen ANTXR1. Additionally, mutated viral glycoproteins (anti-MUC-1) will be selected to allow tight binding with the MUC-1 surface antigen on TNBC cells. After the primary OV treatment, CAR T cells specific to the tumor antigen ANTXR1 will be added to further enhance the antitumor response.

The engineered oncolytic VV is expected to increase the specificity of tumor targeting and oncolytic effect against TNBC. Anti-MUC-1 is a mutated envelope glycoprotein selected to increase the specificity of viral entry into TNBC cells via interaction with MUC-1. iPD-L1, CCL5, GM-CSF, and IL-2 will reprogram the immunosuppressive TME to recruit and activate T cells, NK cells, and macrophages. Finally, when used synergistically with CAR T cell therapy, the expression of the CAR antigen ANTXR1 will enhance CAR T cell function against TNBC.

Firstly, the VV envelope glycoprotein will be engineered to bind to MUC1 using yeast display approach. Secondly, it needs to be tested whether VVs that express anti-MUC-1 specifically infect TNBC with minimal effects on normal cells. Thirdly, TNBC cells protein expression and differences in the cytokine environment with and without OV treatment will be evaluated. Then, the effect of oncolytic VVs on ANTXR1 expression and CAR T cell proliferation will be measured. Lastly, it will be demonstrated that the combined treatment with OV and CAR T leads to better outcomes than either therapy alone.

2.2.2. Mice Model

Female wild-type BALB/c mice will be used in this study. The mice will be 6 weeks old and pathogen-free at the time of tumor transplantation and be given unrestricted access to standard laboratory diet and water. They will be monitored under controlled temperature and a 12-hour light and dark cycle.

2.2.3. Tumor Cell Line

The 4T1 murine mammary cancer cell line will be used for this study. 4T1 is originally derived from a spontaneously arising mammary tumor in BALB/c mice, and it shares substantial molecular features with human TNBC.[17] It is one of the most widely used breast cancer models and are used to specifically investigate TNBC.[17] 4T1 tumor cells can also spontaneously metastasize to distant organs.[17] This closely mimics human TNBC's aggressive and metastatic nature, and it could be advantageous for the purpose of analyzing the efficacy of our engineered OV.

2.2.4. Syngeneic Transplantation of 4T1

4T1 cells will be injected into the mammary fat pads of female BALB/c mice at 6 weeks old to establish primary tumors.

2.2.5. Choice of Oncolytic Virus

To better target TNBC, we decide to leverage the substantial coding capacity of VV. We will engineer VV to express multiple immunostimulatory transgenes. This approach allows us to introduce cDNAs coding for iPD-L1, CCL5, GM-CSF, IL-2, and ANTXR1, which is important for modifying the TME and enhancing antitumor immunity. Large DNA viruses, like VV, possess a substantial coding capacity, allowing for the encoding of multiple immunostimulatory transgenes, and this capacity enables the reshaping of the tumor immune microenvironment.[18] VV can accept 25 kb of foreign DNA.[19] The total length of gene insertions for iPD-L1 (290 amino acids; around 870 bases),[20] CCL5 (91 amino acids; around 273 bases),[21] GM-CSF (144 amino acids; around 432 bases),[22] IL-2 (149 amino acids; around 477 bases),[23] ANTXR1 (289 amino acids; 867 bases)[24] will be at least 2919 bases. This is well within the VV's capacity, making our OV design feasible.

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2.2.6. Method of OV and CAR T delivery

The recombinant VV will be delivered via both intratumoral and systemic injection to treat TNBC. For intratumoral delivery, the engineered VV will be directly injected into the tumor using a needle catheter. Systemic injection allows VV to circulate through the bloodstream around the whole body to recognize and attack the tumor. This method will be effective against metastasized TNBC. CAR T cells will be injected intravenously with an effector-to-target (E:T) ratio of 4:1.

2.2.7. Generation of TNBC-specific VV via Yeast Display

To develop a VV with enhanced specificity for targeting TNBC, we use a yeast display system to engineer the virus' envelope glycoprotein to bind to MUC-1, a mucin protein overexpressed on TNBC cells.[25] We first construct a comprehensive DNA library encoding various mutant forms of the VV envelope glycoprotein. The DNA library is then transformed into yeast cells, allowing the display of mutant glycoproteins on the yeast cell surface. The transformed yeast cells are cultured to create a large yeast display library and incubated with a fluorescently-labeled MUC-1 protein. Using fluorescence-activated cell sorting (FACS), we will select yeast cells having high fluorescence, which express glycoproteins with high binding affinity for MUC-1. DNA from these high-affinity yeast clones is then sequenced to determine which mutations are responsible for enhanced MUC-1 binding. Finally, the receptor-binding domain of the VV glycoprotein will be engineered to express the highest-affinity mutant glycoprotein (anti-MUC-1).

2.2.8. Bioluminescence Imaging

To evaluate targeting specificity, we propose to perform bioluminescence imaging on three groups of TNBC-bearing mice: VV-MUC-1, wt-VV, and VV with receptor-binding domain knockout. Mice TNBC will be injected with luciferin substrate prior to imaging. This allows production of bioluminescence signals by the luciferase-expressing VV upon infection of tumor cells. The intensity of the bioluminescence signals will be analyzed and compared between the groups.

2.2.9. Analysis of ANTXR1's effect on CAR T cell response

Tumor infiltrating cells will be analyzed by flow cytometry to examine how OV-induced expression of ANTXR1 can affect the response of CAR T cells. ANTXR1 (also known as TEM8) is a tumor associated antigen that is upregulated in TNBC. There are existing CAR T cells that specifically target $ANTXR1^+$ TNBC tumor cells.[26] For the experimental group, mice bearing TNBC will receive intratumoral injections of VV that encodes for ANTXR1, followed by CAR T cell infusion. The control group will be mice receiving wt-VV without the ANTXR1 gene, followed by CAR T treatment. To confirm that the difference in result is attributed to ANTXR1, we will also engineer a VV with mutated form of ANTXR1 that cannot bind to the chimeric antigen receptor. This will be injected into the mice, followed by CAR T cells. Cells will be stained with fluorophore-conjugated antibodies specific for CAR, T cell markers such as CD3, activation markers (e.g. CD69. CD25), and ANTXR1. Using flow cytometry, we will analyze the number, phenotype, and activation status of CAR T cells and endogenous T cells, from both the tumor and as control from the spleen.

2.2.10. Analysis of iPD-L1, CCL5, GM-CSF, and IL-2 Function Within the TME

The efficacy of OV-induced iPD-L1, CCL5, GM-CSF, and IL-2 on TNBC tumor cells will then be examined. The mice will be split into a total of 12 groups (2 control groups and 10 experimental groups). Table 1 and table 2 outline the groups of mice we plan to use for this experiment. Groups with one factor positive at a time (Group 3, 4, 5, 6) allows us to assess the individual effect of each factor on the immune response. The groups with 3 positive and 1 negative factor (Group 7, 8, 9, 10) allow us to see if there is an additive or amplified response when multiple immunostimulatory genes are present together. Using immunophenotyping by flowcytometry, we will analyze the recruitment of different types of immune cells and the expression level of inhibitory markers. This will provide insights into the immune landscape within the TME and the impact of different OV treatments. Specifically, we will evaluate T

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cells (CD3+), including helper (CD4+) and cytotoxic (CD8+) subsets, marked additionally by CD25 and CD69. NK cells will be assessed using NK1.1 and NKG2D. Macrophages will be marked by CD11b and F4/80. Dendritic cells will be marked by CD11c. Granulocytes will be marked by CD11b and Ly6G. Key effector cytokines will also be quantified, including IFN-γ, TNF-α, and granzyme B.

Table 1. Summary of control groups used in this study.

Group #	Treatment
1	Saline
2	Infection with wild-type VV

Table 2. Summary of experimental groups used in this study. OVs expressing different combinations of immunostimulatory genes will be tested to analyze the individual and synergistic effects of the therapeutic components. Plus sign (+) indicates the presence of a specific gene, and minus sign (-) indicates its absence.

Group #	Treatment					
	Anti-MUC-1	iPD-L1	CCL5	GM-CSF	IL-2	ANTXR1
3	+	+	-	-	-	+
4	+	-	+	-	-	+
5	+	-	-	+	-	+
6	+	-	-	-	+	+
7	+	-	+	+	+	+
8	+	+	-	+	+	+
9	+	+	+	-	+	+
10	+	+	+	+	-	+
11	+	-	-	-	-	+
12	+	+	+	+	+	+

2.2.11. Flow Cytometry and Intracellular Cytokine Staining

Using immunophenotyping by flow cytometry, we will analyze the recruitment of different types of immune cells and the expression level of inhibitory markers. By using fluorescently-labeled (anti-PD-L1, CCL5, GM-CSF and IL-2) antibodies, the expression levels of intracellular cytokines in cells can be detected, helping to assess the immune suppression status of tumor or immune cells.

The expression of effector cytoplasmic cytokines including IFN-γ and TNF-α will then be detected by intracellular cytokine staining (ICS) and flow cytometry. First, cell dissociation techniques will be used, followed by fixation. Second, cells are permeabilized using saponin or digitonin. Third, intracellular cytokines will be stained by fluorescent labeled antibodies, and their expression will be analyzed by flow cytometry.

2.2.12. Measurement of Tumor Size

Tumor size will be measured using bioluminescence imaging (BLI) at specific time points. BALB/c mice with luciferase-transfected tumor cells will be injected intraperitoneally with D-luciferin (150 mg/kg) before imaging. Anesthetized mice will be imaged using an IVIS Spectrum Imaging System, and the total photon flux within the tumor regions will be quantified to estimate tumor size over time.

2.2.13. Survival Rate Analysis

Mice will be monitored daily for signs of morbidity and survival data will be collected. Kaplan-Meier survival curves will be created to compare the various treatment groups with the control groups.

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3. Anticipated Results

3.1. Specific Entry of Engineered VV into TNBC Cells and Expression of Desired Proteins

The engineered VV (Figure 1) is expected to specifically target and infect TNBC cells via interaction between MUC-1 and anti-MUC-1. This will lead to the expression of the encoded proteins, including ANTXR1, iPD-L1, GM-CSF, CCL5, and IL-2. As depicted in Figure 2, this targeted delivery is expected to enhance the infiltration of NK cells, macrophages, and T cells into the TNBC TME, as well as blocking the immunosuppressive PD-1/PD-L1 interaction. Additionally, CAR T cell activation and proliferation will be stimulated via the interaction with ANTXR1.

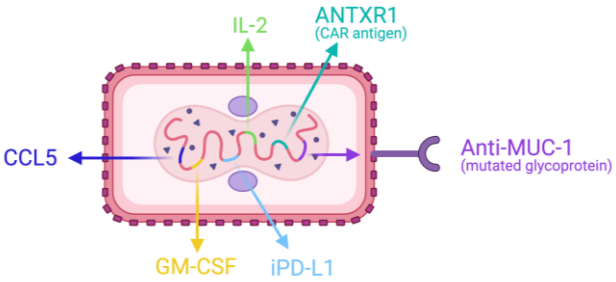

Figure 1. Schematic structure of the engineered oncolytic VV encoding for anti-MUC-1, ANTXR1, iPD-L1, GM-CSF, CCL5, and IL-2.

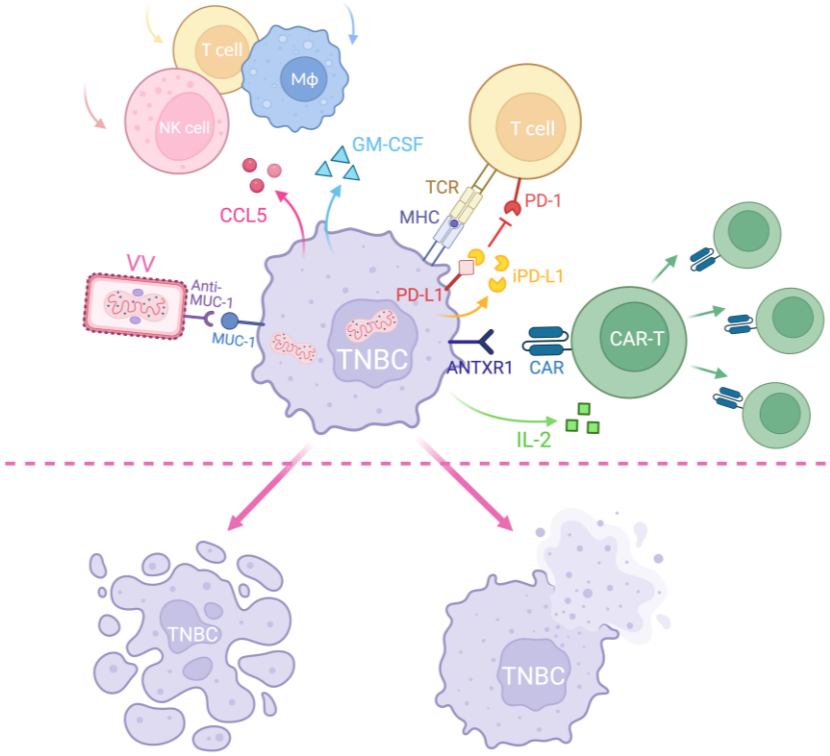

Figure 2. Schematic of the engineered oncolytic VV infecting TNBC cells via MUC-1 recognition, leading to production of iPD-L1, CCL5, GM-CSF, IL-2, and ANTXR1. This increases infiltration of NK cells, macrophages, and T cells into the TME. iPD-L1 blocks PD-1 and PD-L1 interaction, removing the "brake" on the immune system. Interaction between ANTXR1 and the CAR activates CAR-T cells and their proliferation. This eventually leads to tumor cell lysis or apoptosis.

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3.2. PD-L1 Inhibitor, CCL5, GM-CSF, and IL-2 Expression in the TME

For the different control and experimental groups of mice, group 12 (see Table 2) is treated with VV that expresses the whole combination of genes that we propose to insert. This is expected to have the highest infiltration of immune cells and antitumor effect. The number of macrophages, NK cells, $CD4^+$ and $CD8^+$ T cells will be highest for group 12 when compared to the control groups and knockout groups (Figure 3).

The grouping strategy is intended to reflect the varying levels of immune response and antitumor activity of the different OV gene combinations. For the outcome of the control groups, group 1 (saline) serves as the baseline for untreated conditions, characterized by the lowest survival rate, the largest tumor volume, and extensive metastasis. Group 2 (wt-VV) shows infection of both tumor and normal cells, with moderate activation of innate immune cells. However, there is no specific targeting of TNBC, resulting in only a slight decrease of tumor volume compared with the saline group.

In groups 3 to 6, the four genes under analysis (iPD-L1, CCL5, GM-CSF, and IL-2) are individually expressed. Group 3 (iPD-L1) demonstrates increased intertumoral T cell activation due to iPD-L1 expression. Group 4 (CCL5) shows increased infiltration of NK cells, macrophages, and T cells into the TME, leading to improved cell lysis, phagocytosis, and cytotoxic function, ultimately reducing tumor size and prolonging survival. Group 5 (GM-CSF) focuses on increased recruitment of granulocytes and macrophages with enhanced cytotoxic activity, including increased degranulation and reactive oxygen species production. Group 6 (IL-2) specifically targets TNBC tumor cells, leading to increased T cell proliferation and activation, resulting in enhanced antitumor activity and reduced tumor size.

Subsequently, in groups 7 to 10, the four genes under analysis are individually knocked out. Group 7 has lower expression of T cell activation markers compared to group 12, showing a moderate antitumor response due to the combined effect of CCL5, GM-CSF, and IL-2. However, it is less potent due to the absence of iPD-L1, and the high interaction between PD-1 and PD-L1 leads to an immunosuppressive TME. Group 8 exhibits less infiltration of leukocytes compared to group 12 due to the absence of CCL5, resulting in a less potent antitumor function and moderate tumor size reduction. Group 9 has reduced infiltration of macrophages and granulocytes due to the absence of GM-CSF. Group 10 experiences reduced activation and proliferation of T cells due to the absence of IL-2, leading to a less robust T cell cytotoxic function.

Group 11 and 12 are the special groups, with group 11 having all the targeted genes knocked out and group 12 having all the genes expressed. Group 11 involves specific viral infection of TNBC cells but shows a significantly less robust oncolytic effect. The recruitment of T cells, NK cells, and macrophages is relatively low, resulting in a relatively immunosuppressive TME. In contrast, group 12 has the highest number of NK cells, macrophages, and T cells among all groups, with enhanced NK cell lysis, macrophage phagocytosis, and T cell cytotoxicity. This group exhibits the highest survival rate and the most significant tumor size reduction due to the combined effect of all expressed genes.

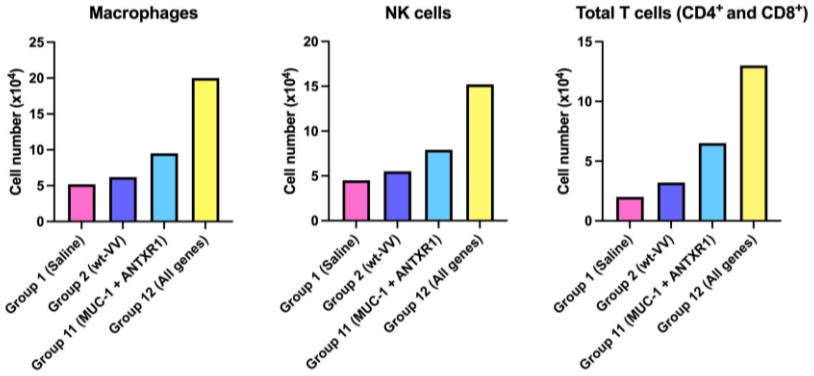

Figure 3. Predicted number of macrophages, NK cells, and total T cells ($CD4^+$ and $CD8^+$) for selected groups. Group 12 (all six genes present) is expected to have the highest number of these immune cells compared to control and knockout groups, due to the synergistic effect of the therapeutic components.

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3.3. Change in Tumor Growth in Response to Altered TME

Mice treated with oncolytic VV that expresses the whole combination of genes (Group 12) will theoretically lead to the most robust antitumor immune response and tumor size reduction (Figure 4 and 5).

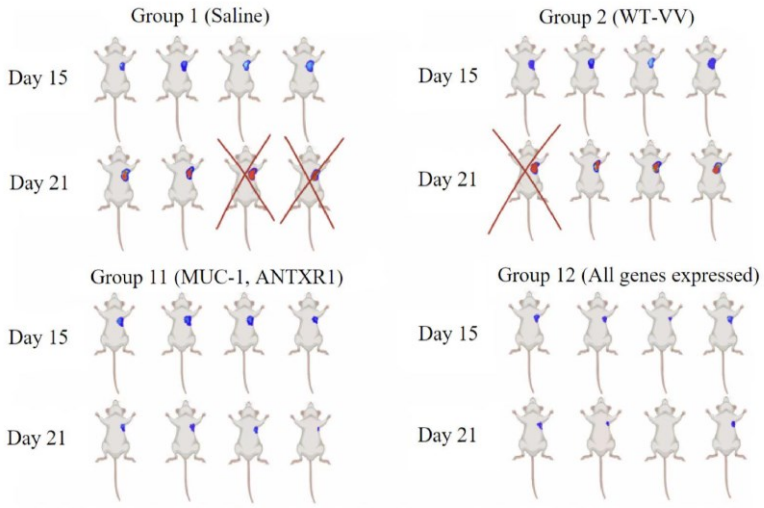

Figure 4. Predicted luciferase intensity data indicating TNBC tumor size. Group 12 is expected to have the most significant tumor size reduction due to the combined effect of all therapeutic genes. The control groups (saline and wt-VV) will lead to tumor growth and eventually death as the tumor progresses. Group 11, which expresses MUC-1 and ANTXR1 but lacks the other four immunostimulatory genes, is expected to reduce tumor size slightly compared to group 12.

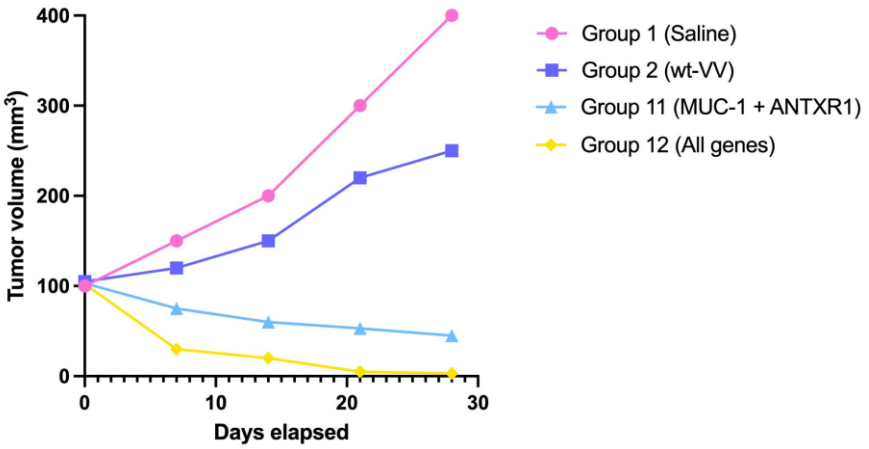

Figure 5. Anticipated tumor volume chart. Group 12, where all desired genes are expressed, will theoretically have the most significant tumor volume reduction. Group 11 (only MUC-1 and ANTXR1 expressed) will lead to slight tumor size reduction, whereas the control groups (groups 1 and 2) will show significant growth in tumor volume due to the lack of specific TNBC targeting and immunostimulatory molecules.

3.4. Survival Rate Analysis

Due to the combined effect of all six genes (anti-MUC-1, ANTXR1, iPD-L1, GM-CSF, CCL5, and IL-2), the overall survival rate is expected to be highest for Group 12 (Figure 6). This corresponds to the greatest reduction in tumor volume for this group.

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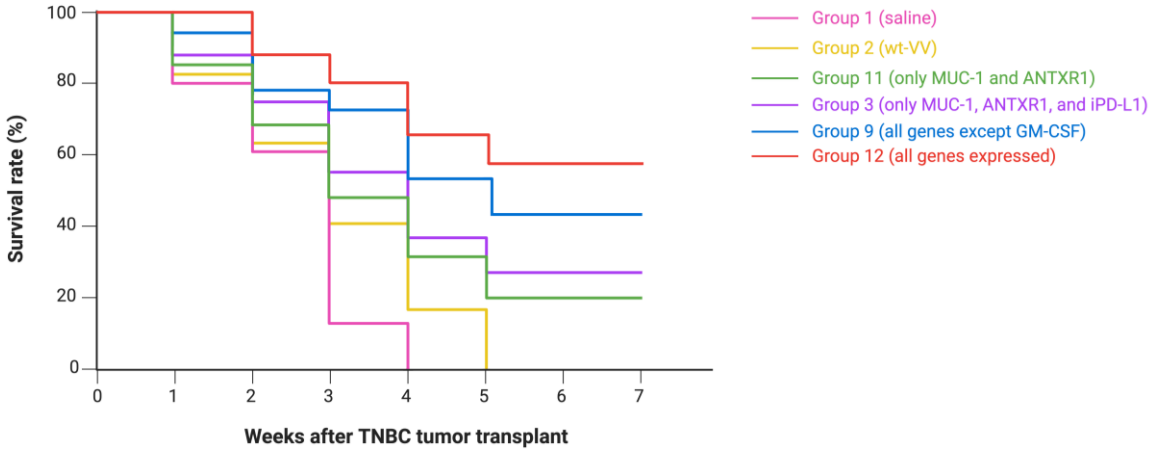

Figure 6. Predicted Kaplan-Meier survival curve for a select number of control and experimental groups. Group 12, where all genes we propose to insert are present, is expected to have the highest survival rate, with over 50% of treated mice still alive seven weeks after TNBC tumor transplantation. Group 9, where one therapeutic gene is being knocked out (GM-CSF), will lead to a slight reduction in survival rate, whereas groups with multiple gene knockouts (groups 3 and 11) will have more limited therapeutic effects against TNBC. Relatively early deaths are expected among mice in the control groups (groups 1 and 2) during the experiment.

4. Discussion

4.1. Significance of Combined Therapy

The engineered multigenic VV proposed in this study, in combination with CAR T-cell therapy, provides an innovative strategy for improving the treatment of TNBC, a subtype notoriously resistant to conventional therapies. The significance of this approach lies in its ability to combine many different therapeutic components into a single agent, thereby eliciting a robust and multifaceted antitumor response. One of the primary advantages is the ability of engineered VV to selectively infect and destroy TNBC cells, while simultaneously expressing therapeutic genes that modulate the TME and increase CAR T-cell targeting.

4.2. Increasing the Delivery Efficiency and Stability of the Oncolytic Virus

How does the engineering of the oncolytic viruses impact their delivery efficiency and stability? In our study, the engineering of VV includes multiple modifications aimed at enhancing its specificity and therapeutic efficacy against TNBC. The primary engineering modification involves altering the VV envelope glycoprotein to improve its binding to MUC1. As outline here, this can be achieved through techniques such as yeast display to alter the receptor-binding site. The impact on viral stability is significant: the engineered envelope glycoprotein must be correctly folded and integrated into the viral envelope. Improper folding or incorporation can lead to reduced viral stability and infectivity, compromising the virus's ability to maintain its structural integrity and effectively deliver its therapeutic payload into TNBC cells.

4.3. Potential Caveats

4.3.1. Off-target Toxicity of Engineered VV

Despite these promising aspects, there are some potential caveats that require further consideration. One is specificity and off-target effects. While MUC-1 is overexpressed in TNBC, it is also expressed on glandular epithelial cell surfaces, including those in respiratory and GI tract.[27] Therefore, if systemic

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injections are applied, the anti-MUC-1 expressing oncolytic VV may bind and deliver genes coding for ANTXR1 to these normal cells, causing the recognition and destruction of normal cells by CAR T cells, leading to certain side effects (Figure 7). To reduce this, we can engineer CAR T cells that co-express ANTXR1 CARs and synthetic Notch (synNotch) receptors that bind with NKG2DL, a highly expressed surface protein on TNBC cells.[28] This restricts the activation of T cells to the condition where the target cell expresses both MUC-1 and NKG2DL (Figure 8). However, the infection of normal cells is likely to remain harmless in our immunocompetent BALB/c mouse model. The reason why oncolytic VVs can persist in TNBC tumor is that tumor's immune evasion strategies can limit the effectiveness of antiviral immunity, but in normal cells, without the immunosuppressive TME, the immune system can help establish antiviral state in the cells, hence detect and clear viral infections in normal MUC-1 expressing epithelial cells.

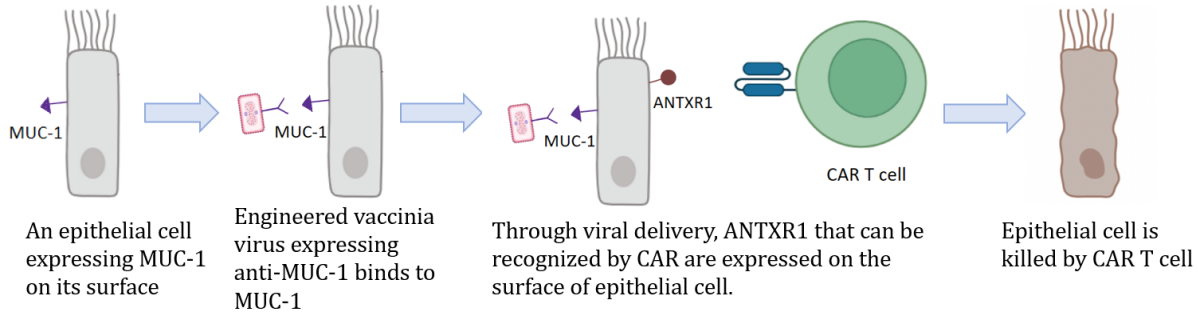

Figure 7. Off-target effects of the oncolytic VV due to other epithelial cells expressing MUC-1.

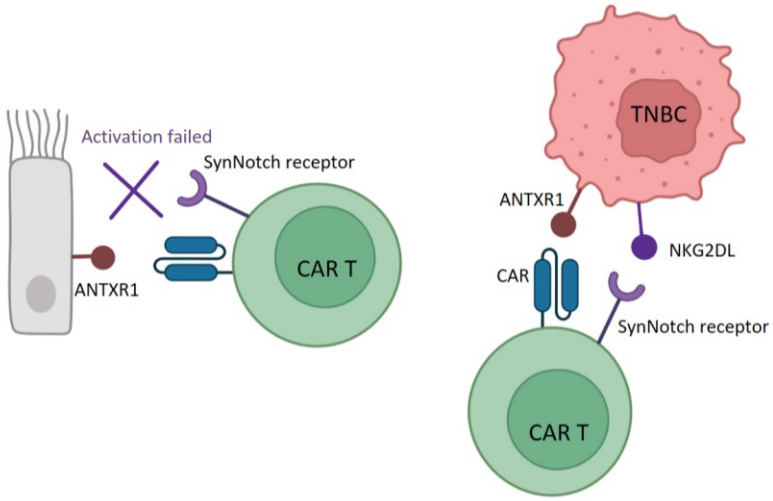

Figure 8. CAR T cells engineered to co-express CAR and synNotch receptor could potentially reduce off-target effects and enhance the therapeutic outcome against TNBC.

4.3.2. The Heterogeneity of TNBC Tumor Cells

Another caveat arises from the heterogeneity of TNBC tumor cells. Not all TNBC cells express high levels of MUC-1, but our proposed oncolytic VV model is highly selective and dependent upon the expression of MUC-1 (Figure 9). Since VV is responsible for delivering the CAR antigen (ANTXR1), CAR T cell activation is also dependent on VV entry into MUC-1-expressing tumor cells. Tumor heterogeneity means that TNBC cells with low levels of MUC-1 may be left unharmed, and these resistant tumor cells may proliferate without further treatment. This selective pressure could lead to a

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population of tumor cells that escape both the oncolytic and CAR T cell therapies. To address this, VV could be modified to express bispecific ligands that target two tumor-specific receptors, such as MUC-1 and NKG2DL. This dual targeting ensures that a broader range of TNBC cells are targeted, reducing the likelihood of resistant cell populations emerging. Alternatively, a combination of two OVs could be used, each engineered to target a specific TNBC receptor to allow a more comprehensive response.

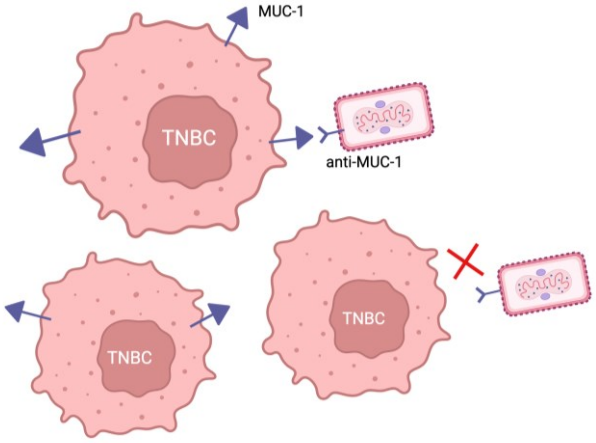

Figure 9. Heterogeneity of TNBC tumor cells. TNBC cells that express low levels of MUC-1 may be selected to proliferate *in vivo*.

4.3.3. Antiviral Immune Response

The immunogenicity of VV itself is a double-edged sword. While it can stimulate a robust antitumor immune response, its viral proteins can also provoke an antiviral immune response that attacks the virus before it exerts its full therapeutic effect. Neutralizing antibodies against VV, either pre-existing or developed during treatment, can inhibit VV replication. To balance the antitumor and antiviral response, we could engineer the VV to express immunomodulatory molecules, create a "stealth" VV variant that avoids recognition by pre-existing antibodies, or less optimally, use mild immunosuppressants. Repeated, low-dose injection of the VV may lead to a more sustained and manageable immune response compared to a single high-dose administration, reducing safety risks.

4.4. Other Alternative Approach

One possible alternative approach is to use other types of OVs, such as Herpes Simplex Virus (HSV). HSV has a large genome size of 152 kb, and this allows for multiple genetic modifications and insertion of foreign genes.[29] HSV is also highly immunogenic, and this can help trigger a strong antitumor immune response.[29] Additionally, HSV replicates in the nucleus of host cells and exploits the host's transcription and replication machinery. This means it likely has a strong ability to manipulate cellular processes for efficient viral replication within tumor cells.

5. Conclusion

Our strategy of combining an engineered multigenic VV with CAR T cell therapy is a novel approach for treating TNBC. By exploiting VV's large coding capacity to express multiple immunostimulatory genes, this OV overcomes the challenges of immune suppression and poor tumor specificity which have limited the efficacy of current TNBC therapies. It is designed to reprogram the TME, enhance immune cell infiltration, and improve CAR T cell targeting to destroy TNBC cells more effectively.

The experimental design includes generating a VV specific to MUC-1-expressing TNBC cells using the yeast display system, followed by *in vivo* testing in BALB/c mice transplanted with TNBC. The individual and combined effects of iPD-L1, CCL5, GM-CSF, and IL-2 will be assessed. The engineered VV is expected to significantly boost the recruitment and function of various leukocytes within the TME. Mice treated with the full combination of engineered VV and CAR T cells will show the highest survival

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rate and tumor size reduction. Future studies could focus on addressing TNBC tumor heterogeneity and off-target effects. Furthermore, the strategy of engineered VV in this study could be applied to other solid tumor types to enhance their treatments.

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