Title: Deciphering the immunomodulatory capacity of oncolytic vaccinia virus to enhance the immune response to breast cancer

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Abstract:
Vaccinia virus (VACV) is a double stranded DNA virus that devotes a large portion of its 200 Kbp genome to suppressing and manipulating the immune response of its host. Here, we investigated how targeted removal of immunomodulatory genes from the VACV genome impacted immune cells in the tumor microenvironment with the intention of improving the therapeutic efficacy of VACV in breast cancer. We performed a head-to-head comparison of six mutant oncolytic VACVs, each harbouring deletions in genes that modulate different cellular pathways, such as nucleotide metabolism, apoptosis, inflammation, and chemokine and interferon signalling. We found that even minor changes to the VACV genome can impact the immune cell compartment in the tumor microenvironment. Viral genome modifications had the capacity to alter lymphocytic and myeloid cell compositions in tumors and spleens, PD-1 expression, the amount of virus, and the percentage of tumor-targeted CD8+ T cells. We observed that while some gene deletions improved responses in the non-immunogenic 4T1 tumor model, very little therapeutic improvement was seen in the immunogenic HER2/neu TuBo model with the various genome modifications. We observed that the most promising candidate genes for deletion were those which interfere with interferon signalling. Collectively, this research helped focus attention on the pathways that modulate the immune response in the context of VACV oncolytic virotherapy. They also suggest that the greatest benefits to be obtained with these treatments may not always be seen in “hot tumors”.

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Introduction:
Oncolytic viruses preferentially infect and kill cancer cells while promoting antitumor immunity, classifying them as a form of immunotherapy(1). The FDA’s approval of talimogene laherparepvec (T-VEC) in 2016 increased confidence in this growing field of immunotherapy, however oncolytic viruses have not yielded the same type of clinical success as other immunotherapeutic approaches(1). Thus, efforts are underway to improve the efficacy of oncolytic virus therapy.

Vaccinia virus (VACV) is a large (200 kbp) double-stranded DNA virus and a promising oncolytic agent(2). VACV has many attributes which make it a suitable cancer therapeutic, including a large coding capacity for transgenes, easy manipulation, a good safety profile, and the inability to integrate into the host genome(2). VACVs are in clinical trials(2), and there is continuing research aimed at improving the long lasting antitumor immunity, by encoding cytokines like GM-CSF or IL-2 into the virus genome(3,4). VACV encodes many gene products that can suppress host immune responses, which undermines the purpose(s) served by the aforementioned transgenes(2,5). An alternative approach to increase the immunogenicity of oncolytic virotherapy involves deleting virus-encoded immunomodulatory genes, a strategy which has improved immune responses to recombinant vaccine vectors(6–10). Although reports show that this strategy offers promise as a way of improving oncolytic therapies(11), the “one-off” nature of each report (i.e. often investigating only one candidate gene per study and sometimes simultaneously encoding additional transgenes), makes it difficult to identify which gene targets and immune pathways offer the most promise.

Here, we examined the effects of deleting individual VACV immunomodulatory genes on the virus-infected tumor microenvironment (TME). Specifically, we determined whether any one of the several antiviral pathways targeted by VACV affects the efficacy of virotherapy in breast tumor models. We performed a head-to-head comparison of six isogenic mutant oncolytic VACVs, each harbouring gene deletions that modulate different cellular pathways [nucleotide metabolism, apoptosis, inflammation, and chemokine and interferon signalling (Table 1)]. All of these viruses were also mutated in the thymidine kinase locus (J2R), and were compared to a ΔJ2R mutant, as this is a common attenuating mutation(2).

We determined how these deletions altered the immune cell compartment in the TME of two orthotopic and syngeneic mouse breast tumor models. Curiously, we observed that some gene deletions improved responses in the non-immunogenic 4T1 tumor model, but very little therapeutic improvement was seen in the immunogenic HER2/neu TuBo model. The most promising candidate genes for deletion were those previously identified as interfering with the IFN response, either directly [B8R(12)/B18R(13)], or indirectly by inhibiting components of the signalling pathways [C6L(14,15), N1L(16)]. Collectively, this research helped determine which genetically-encoded viral components were suppressing immune responses and should perhaps be removed to improve the oncolytic activity of VACV. It also suggested
that although these virus mutations could modulate the TME of immunogenic tumours, the greatest benefits could be attained in the context of non-immunogenic tumour models.

Materials and Methods:

Cell lines and culture conditions
TuBo mouse mammary carcinoma cells, provided by Dr. Landuzzi in 2016 (Istituto Ortopedico Rizzoli)(17), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 2mM L-glutamine, 100U/mL anti-mycotic/antibiotic, 1X non-essential amino acids and 1mM sodium pyruvate supplemented with 20% fetal bovine serum (FBS). Cells were passed through a syringe prior to plating to separate cell clumps. TuBo cells were authenticated upon receipt, showing them to be of mouse origin and free from adventitious agents (Charles River Mouse Essential Clear Panel and Contamination Panel). 4T1 cells (ATCC RRID:CVCL_0125) received in 2008 from ATCC, were cultured in DMEM supplemented as above, containing 10% FBS. BSC40 cells (ATCC RRID: CVCV_3657), received in 2005 from ATCC, were grown in minimal essential medium supplemented as above using 5% FetalGro bovine growth serum (RMBIO). Cells were passaged fewer than 20 weeks, and routinely tested for mycoplasma. 4T1 and BSC40 cells were not reauthenticated in the last five years.

Viruses
All viruses were derived from a clonal isolate of VACV strain Western Reserve (WR) using traditional homologous recombination techniques (Fig. 1A)(18–20). The parental ∆J2R virus was constructed using a modified version of the pSC66 plasmid to insert a yellow fluorescent protein (YFP)/guanine phosphoribosyltransferase (GPT) cassette flanked by loxP sites into the J2R gene locus. The selectable marker was then removed by growing the virus on BSC40 cells in the presence of cre-recombinase as described in by Rintoul et al., 2011(18), yielding a virus with the J2R gene disrupted by a loxP site. To create the remaining viruses, we used the pDGloxFK0DE3 plasmid bearing a copy of a GPT marker fused to either mCherry fluorescent protein (∆F4L, previously described (20), ∆F1L, ∆C6L, ∆A41L, ∆B8R) or YFP (∆K7R, ∆N1L, ∆B18R), flanked by regions of homology to the target gene to allow site-specific recombination. BSC40 cells were infected with ∆J2R VACV at an MOI of 2 and transfected two hours later with 2 μg of linearized plasmids using Lipofectamine 2000 (Invitrogen). The progeny viruses were harvested 24 hours later and purified using two rounds of drug selection under mycophenolic acid (MPA) and three rounds of plaque picking under agar. PCR (primers found in Supplemental Table S1) and full genome sequencing were used to confirm virus identity.

To produce virus stocks, BSC40 cells were infected for 72 hours, harvested and lysed by Dounce homogenization using a tight borosilicate glass tissue grinder with 0.05 mm clearance (Wheaton cat#06-
435A). Virus particles were purified on a 36% sucrose cushion and viral titers were determined by plaque assay on BSC40 cells. PCR was used to confirm the purity of the virus stock using the same primers found in Supplemental Table S1.

In vitro experiments
Multi-step growth curves and cytotoxicity assays were performed as previously described(20). For multistep growth curves, cells at 70% confluence were infected with VACV at a MOI of 0.03 for one hour. The virus inoculum was then removed and replaced with fresh media. At various time points post infection, cells and media were harvested by collection with a cell scraper and freeze-thawed three times at -80°C to lyse cells. The samples were diluted, plated in triplicate on BSC-40 cells and incubated for 48 hours in media containing 1% carboxymethylcellulose. Cells were fixed and stained with crystal violet and plaques counted to determine viral titers.

For cytotoxicity assays, cells were seeded in 96-well plates and infected with indicated viruses. After three days, the media was replaced with fresh cell culture media containing 44 μM resazurin (Sigma-Aldrich). Plates were incubated for four hours at 37°C, and fluorescence was measured using a microplate reader with 560-nm excitation and 590-nm emission filters.

In vivo animal models
All animal studies were carried out at the University of Alberta, Edmonton Alberta, and conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta.

Female BALB/c mice between 6-8 weeks of age were ordered from Charles River (Saint Constant, Canada). Animals were housed in HEPA-filtered ventilated cages in a biosafety level 2 containment suite at the University of Alberta Health Sciences Laboratory Animal Services facility in groups of four and five and given at least 7 days to acclimate to the housing facility. Environmental conditions were a temperature of 21°C ±2°C, humidity of 55% ±10%, lighting of 350 lux and a 12:12 light:dark cycle with lights on at 0700 and off at 1900. Animals were housed in 595x380x200 mm cages (Ehret, Germany) and given access to mouse maintenance food (LabDiet, St.Louis, MO) and water ad libitum. Environmental enrichment included bedding (Warrensburg, NY), one red tinted mouse tunnel (Bio-Serv, Flemington NJ), one 50mm x 50mm Nestlet (Ancare Bellmore, NY) and one 4-8g portion-controlled nesting material (Anderson Lab Bedding Maumee, Ohio).
To establish orthotopic tumors, tumor cells were trypsinized, washed twice with cold PBS, and concentrated in PBS to yield the final cell count desired for injection in 25 uL of PBS. Immediately prior to injection, 25 uL of Matrigel was mixed with 25 uL of cells containing 1x10^6 TuBo cells, or 1x10^4 4T1 cells per mouse. The cells were injected into the inguinal mammary fat pad of mice below the fourth nipple. Palpable tumors were detected after about eight days in both the TuBo and 4T1 tumor models.

Mice were randomized into treatment groups using Google’s random number generator (https://www.google.com/search?q=random+number). Viruses were sonicated and diluted to 2x10^8 plaque forming units (PFU)/mL with PBS. Mice were anaesthetized with isofluorane and injected intratumorally with 50 ul of virus yielding a dose of 1x10^7 PFU/tumor. Animals received 2 additional doses administered at 48-hour intervals. Tumor growth was measured twice per week with calipers, and tumor volume was calculated using the equation: \( V = \frac{1}{24} \pi L(W+H)^2 \). Mice were euthanized by CO\(_2\) inhalation at tumor burden endpoint (1500 mm\(^3\)), or at first indication of illness in the 4T1 tumor model (hunched posture, ruffled fur, or weight loss exceeding 10% of body weight).

**Tissue processing**

Spleens were mashed through a 70 \( \mu \)m cell strainer into isolation buffer (PBS + 2% heat-inactivated FBS (HI-FBS) + 0.5 mM EDTA) using the rubber end of a 3 mL syringe, centrifuged at 300 \( x \) g for 5 minutes and resuspended in 3 mL of 1X red blood cell lysis buffer (eBioscience) for 5 minutes. RBC lysis was stopped by the addition of 10 mL isolation buffer followed by centrifugation at 300 \( x \) g for 5 minutes and then washed twice with isolation buffer.

Tumors were collected into HBSS and cut into pieces. Tumor pieces were added to a GentleMACS C-tube containing 5 mL Roswell-Park Memorial Institute (RPMI) medium containing 0.5 mg/mL collagenase type 1A (Sigma-Aldrich), 10 \( \mu \)g/mL of DNAse I (Roche) and 10% HI-FBS. Tumors were dissociated using the m_impTumor01.01 protocol on a GentleMACs dissociator (Miltenyi Biotec). The digested tumor samples in enzymatic cocktail were incubated while shaking for 30 minutes at 37\( ^\circ \)C, filtered through a 70 \( \mu \)m cell strainer into PBS containing 2% HI-FBS and centrifuged at 500 \( x \) g for 5 minutes. Cells were resuspended in 40% Percoll (GE Healthcare) in HBSS and overlaid onto 80% Percoll. After centrifugation at 325 \( x \) g for 30 minutes, leukocytes at the interface between the 40% and 80% fraction were collected and washed twice in PBS, while tumor cells at the top of the 40% fraction were collected for virus titering.

Lung processing for quantification of metastases was previously described(21). Briefly, lungs were collected and rinsed twice in Hank’s buffered salt solution (HBSS). The lungs were minced for three minutes using a scalpel blade and incubated in HBSS containing 2mg/mL collagenase at 4\( ^\circ \)C for 75
minutes. The cells were then passed through a 70 μm cell strainer and washed twice with HBSS. After dilution, cells were plated in fully supplemented 10% FBS DMEM containing 60 μM of 6-thioguananine and cultured, undisturbed, at 37°C. After two weeks the colonies were fixed in 100% methanol, stained with methylene blue, and counted for quantification of metastases.

Flow cytometry staining

Two million splenocytes were aliquoted into individual wells of 96 well plates, with remaining splenocytes pooled for fluorescence minus one (FMO) gating controls, where all but one antibody is added to the pooled samples to use for setting appropriate gates during analysis. For tumor samples, the entirety of the cells isolated from tumors were split between 2 wells (for TuBO experiments) or 1 well (4T1 experiment) with 5% of each sample volume removed for use as a pooled sample for FMOs and controls. Cells were washed in PBS and stained with fixable viability dye eflour506 (Invitrogen, Cat. 65-086614). This and all subsequent staining steps were performed for 30 minutes at 4°C away from light. Fc receptors were blocked using the CD16/CD32 antibody (BioLegend). Cells were stained with antibodies described in Supplemental Table S2 and fixed prior to intracellular staining. Samples were run on the BD Fortessa X20 Flow Cytometer and analyzed with FlowJo. A representative example of the cell-gating strategy can be found in Supplemental Fig. S1.

Cytokine analysis

Blood was collected from mice at endpoint by cardiac puncture by inserting a needle into the chest cavity of mice under anaesthesia and drawing blood from the heart. The animals were then immediately euthanized, and blood was allowed to clot for 30 minutes at room temperature. Blood was centrifuged at 1000 x g for 10 minutes at 4°C and serum was collected and frozen at -20°C. Serum cytokines were then sent for analysis by Eve Technologies (Calgary, AB Canada) using the Mouse Cytokine 31-plex discovery assay to measure the following analytes: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX (not validated), MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF.

Statistical analysis

Data were analyzed using GraphPad Prism 7. If data were determined to be normally distributed by the Shapiro-Wilk normality and the Kolmogorov-Smirnov test, parametric one-way ANOVA testing was performed with Sidak’s multiple comparisons testing. If data was not parametric, Kruskal-Wallis testing was performed with Dunn’s multiple comparisons testing. Significance was determined if p ≤ 0.05. Tumor growth curves were analyzed using a two-way ANOVA to compare tumor volumes at multiple times.
points. Survival data were analyzed by both the log-rank (Mantel-Cox) and Gehan Brexlow Wilcoxon testing. Significance threshold was adjusted for multiple comparisons, where significance threshold set as \( p=0.05/K \) where \( K \) is the number of comparisons being made.

**Results**

**Mutation of VACV immunomodulatory genes**

Previous studies have identified that deletion of VACV F1L, K7R, N1L, C6L, A41L, and B8R plus B18R genes enhance immune responses to recombinant vaccine vectors *in vivo* (6–9,12,13,16,22–25) (Table 1). Except for B18R alone, most have not been previously investigated in the context of cancer virotherapy(11,26). For comparison we also deleted F4L, the small subunit of ribonucleotide reductase, as this mutation renders recombinant viruses highly tumor selective while leaving the virus genes regulating immune signalling pathways intact(20).

**Growth properties of mutant VACV *in vitro*.**

When we examined the relationship between virus amplification *in vitro* and cell killing over 72 hr, most of the mutations had little effect on either phenotype when compared to the \( \Delta J2R \) virus (Fig. 1B,C). In particular, the viruses were equally cytotoxic in both TuBo and 4T1 cells when plated at equal MOIs (Fig. 1C). The \( \Delta F4L\Delta J2R \) virus was an exception to this rule, where two different phenotypes were observed depending upon the cell type. In TuBo cells the \( \Delta F4L\Delta J2R \) virus grew more slowly than the \( \Delta J2R \) virus and was 30% less cytotoxic than other viruses at comparable MOIs (Fig. 1B,C). However, this effect was cell-line dependent as the \( \Delta F4L\Delta J2R \) virus grew quite poorly in 4T1 cells and yet exhibited comparable levels of cell killing as seen with the other viruses. Collectively, these data show that deleting the aforementioned immunomodulatory genes has little effect on VACV growth and does not affect its cytotoxicity *in vitro*, whereas deleting the F4L gene can sometimes inhibit replication and reduce cytotoxicity, at least in certain (TuBo) cells.

**All of the VACV mutant strains were safe *in vivo*, and viral replication was restricted to tumors.**

The safety and biodistribution of the mutant VACVs were tested *in vivo* in two different orthotopic and syngeneic models of breast cancer. After the tumors became palpable (~30 mm³), three doses of each virus at \( 1\times10^7 \) PFU or PBS as an injection control were given by intratumoral injection at 48 hr intervals (Fig. 2A).

No significant weight loss or adverse events associated with virus treatment were observed in either model, indicating that all of the recombinant viruses were safe (Fig. 2B, C). Live viruses were isolated from most of the TuBo tumors three weeks after virus treatment, although VACV \( \Delta F4L\Delta J2R \)
was not detected in any sample (Fig. 2D). In 4T1 tumors, which grow more rapidly than TuBo tumors, virus growth and biodistribution were assessed two weeks after virus treatment. In this model, virus was recovered from tumors in all the groups (Fig.2E), but not in spleens, ovaries, kidneys or liver, suggesting that these are tumor selective viruses. Collectively, these data suggest that all the recombinant viruses are safe, and that virus replication is restricted to tumors without dissemination to distant organs.

**Antitumor activity of VACVs in TuBo and 4T1 breast cancer tumor models**

Next, we monitored tumor regression, mouse survival, and assessed the immune response after treatment with the recombinant VACVs in both TuBo (Fig 3A-O) and 4T1 (Fig. 4A-O) breast tumor models. TuBo tumors are derived from a mammary carcinoma of HER2/neu transgenic mice, and therefore over-express rat HER2/neu(17). It is considered an immunogenic HER2+ mammary tumor model and is also characterized by relatively slow growth with no ability to form metastasis(27). The 4T1 tumor model is derived from a spontaneously-arising BALB/c mammary tumor, is poorly immunogenic, has rapid growth, and is highly metastatic(28). We can find no reports of 4T1-specific neo-antigens that can be used to detect tumor-specific T-cell responses in this model. Due to the rapid growth and metastasis of 4T1 tumors to the lungs, it was an animal welfare issue, rather than tumor volume, that was used to determine the endpoint. Mice were euthanized at the first behavioural indicators of distress, mainly the cessation of grooming, weight loss, and squinted eyes or hunched posture.

Tumors were treated as described above (Fig. 2A) and tumor growth was compared to both untreated controls (PBS) and to VACV ΔJ2R-treated controls (Fig. 3A, 4A). The VACV ΔJ2R mutant significantly decreased the rate of tumor growth in the TuBo model compared to the PBS-treated controls (Fig. 3A). However, none of the recombinant VACV produced any further improvement in the antitumor response compared to ΔJ2R. In fact, ΔF4LAJ2R and ΔF1LΔJ2R showed significantly higher tumor volumes at day 60 when compared to ΔJ2R-treated tumors (Fig. 3A). The tumor volumes after treatment with ΔK7RΔJ2R, ΔN1LΔJ2R, ΔC6LΔJ2R, ΔA41LΔJ2R, or ΔB8RΔB18RΔJ2R viruses (at 60 days) were not statistically different compared to ΔJ2R alone (Fig. 3A). We examined the long-term survival, following the animals out to 300 days post-treatment. Virus treatment initially appeared to significantly increase survival relative to PBS treatment (Fig. 3B). Gehan-Breslow Wilcoxon significance testing of survival curves yielded p=0.05 when comparing survival of PBS treated mice to ΔC6LΔJ2R treated mice, and p=0.03 when comparing ΔJ2R treated mice to ΔF4LΔJ2R. However, these values were deemed not significant, as the Bonferroni-corrected multiple comparisons threshold for significance was determined to be p<0.003. These results showed that no virus treatment significantly impacted mouse survival relative to untreated mice, and nor did any gene deletion significantly change survival compared to the parental ΔJ2R virus in this model (Supplemental Fig. S2A).
Although no significant changes in overall survival were observed, the ΔB8RΔB18RΔJ2R virus treatment produced the highest median survival time of 192.5 days, followed by ΔC6LΔJ2R and ΔN1LΔJ2R viruses at 163 and 159.5 days, respectively, while the median survival of PBS controls was just 87.5 days (Fig. 3B). The ΔB8RΔB18RΔJ2R and ΔN1LΔJ2R treatments also yielded the highest number of complete responses (CR) with 4/8 CR (50%) per group, determined as no detectable tumor mass at the end of the experiment (Fig. 3B, Supplemental Fig. S2A). Treatment with VACV ΔC6LΔJ2R produced 3/8 CR (37.5%) and one partial response (PR). The ΔJ2R, ΔF1LΔJ2R, and ΔK7RΔJ2R mutants each also showed 3/8 (37.5%) CRs, followed by ΔA41LΔJ2R with 2/8 CR (25%) and ΔF4LΔJ2R with 1/8 CR (12.5%) (Fig. 3B, Supplemental Fig. S2A). No CRs were seen with PBS-treated tumors although one PR was observed (Fig. 3B, Supplemental Fig. S2A). To determine if treated mice had acquired antitumor immunity, mice with stable or undetectable tumors at day 150 were re-challenged with fresh TuBo cells in the opposite mammary fat pad. While tumors appeared within 10 days in all of the naïve age-matched control mice, all but one of the 24 mice that had cleared a TuBo tumor rejected the implanted cells. The one exception was a mouse treated with VACV ΔF1LΔJ2R. It developed a palpable tumor that still eventually cleared (Fig. 3C).

In the more aggressive, non-immunogenic 4T1 tumor model, all of the virus treatments delayed tumor growth at day 18 relative to the PBS-treated tumors (Fig. 4A). It was also quite striking that in this model, most of the new viruses, except the ΔF4LΔJ2R and ΔF1LΔJ2R strains, improved survival relative to ΔJ2R treated mice. This correlated with TuBo data where we saw that the ΔF4LΔJ2R and ΔF1LΔJ2R VACV were less effective than the ΔJ2R control virus at slowing growth. Although the gained survival benefit was statistically significant, the actual extension of lifespan was minor (Fig. 4B, Supplemental Fig. S2B). The median survival of VACV ΔC6LΔJ2R-treated mice was 26 days compared with 22 days for PBS treated (Fig. 4B, Supplemental Fig. S2B). This difference was probably explained by the fact that the VACV ΔC6LΔJ2R-treated mice had an average of 1000 metastatic cells in the lungs when they reached endpoint, whereas 10- and 100-fold more cells were detected in the ΔJ2R- and PBS-treated animals, respectively (Fig. 4C). Collectively, these data showed that additional immunogenic deletions did not significantly improve survival in a TuBo immunogenic tumor model and may only have minor impacts on tumor regression, median survival length and incidences of complete and partial responses compared to ΔJ2R. However, in non-immunogenic 4T1 tumors, immunogenic gene-deleted VACVs imparted a survival benefit relative to the parental ΔJ2R virus. Moreover, where benefits could be detected they were associated with mutations in the B18R/B8R, C6L, N1L, and K7R genes, all of which modulated the interferon response (12–16,22). The F1L and F4L mutations, which promote apoptosis (24,29) and tumor specificity (20), respectively, did not offer advantages in these breast cancer models,
VACV altered the composition of the lymphocyte and myeloid cells in tumor-bearing mice

We next investigated the effects these recombinant VACVs might have had on the immune response in treated mice by characterizing the immune cell compartment in tumors and spleens. To determine if virus treatments caused lasting alterations to the TME we waited three weeks after the last virus injection in the TuBo model, and two weeks in the 4T1 model to assay immune-cell subtypes in the tumors and spleens.

Overall, the immune cell composition within tumors varied greatly between the two tumor models, but the recombinant VACVs did not cause drastic alterations compared to PBS treated tumors (Fig. 3D, 4D, Supplemental Fig S3). VACV ΔF4LΔJ2R treated tumors appeared to have reduced numbers of purported polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) infiltrating tumors in the TuBo model (Fig. 3D), and VACV ΔF1LΔJ2R-treated tumors appeared to have less immune cells overall in the 4T1 tumor model (Fig. 4D). All of the virus treatments increased the percentage of tumor infiltrating CD8⁺ T-cells and decreased the numbers of suppressive regulatory T cells (Tregs) (Fig. 3E,G, 4E,G). The magnitude of these changes was larger in the TuBo model compared to the 4T1 model, increasing the percentage of CD8⁺ T cells by approximately 30% and 15%, respectively, compared to PBS-treated tumors. However, none of the additional VACV gene deletions significantly altered these percentages compared to tumors infected with the VACV ΔJ2R parent strain. This suggests that no single deletion of one of these genes can substantially alter the levels of tumor infiltrating lymphocytes (TILs). The one exception was ΔB18RΔB8RΔJ2R, where we detected a reduction in the percentage of CD4⁺ T cells in TuBo tumors compared to tumors treated with ΔJ2R (Fig. 3F). PMN-MDSCs, which are potent suppressors of cytotoxic T cell activity(32), increased in tumors after all virus treatments except for those treated with ΔJ2RΔF4L (Fig.3I).

More subtle changes were detected in the immune compartment of the spleen and these differed from what was seen in the tumors. Virus treatment, independent of mutations, altered the overall composition of the spleens compared to PBS-treated mice in TuBo tumors, but not in 4T1 (Fig. 3,4 J-O). In either model, no obvious differences in overall splenocyte composition were found between virus mutants (Fig. 3J, 4J). Rather, differences were apparent between virus treatments when assessing more minute cellular characteristics (Fig. 3,4 K-O). We observed a decrease in the overall myeloid/MDSC populations in the spleens of mice treated with VACV in the TuBo tumor model compared to the 4T1 tumor model (Fig. 3,4 J). This was not surprising, given that accumulation of these suppressor cells in the
spleen is usually directly associated with tumor burden(32). We detected decreased percentages of CD8⁺ T cells in the spleens of mice treated with the ΔF4LΔJ2R, ΔK7RΔJ2R, and ΔN1LΔJ2R viruses compared to tumors treated with VACV ΔJ2R (Fig. 3 K,L). This might have explained why ΔK7RΔJ2R and ΔN1LΔJ2R-treated tumors had increased percentages of CD4⁺ T cells. Similar effects were seen in the spleens of 4T1 tumor-bearing mice but with less magnitude.

Expression of programmed cell death protein (PD-1) and its ligand, PD-L1, were measured in tumors and spleens (Fig. 5A-G, Supplemental Fig. S4 and S5). VACV ΔJ2R decreased PD-1 expression on Tregs in TuBo spleens and tumors relative to the PBS-treated controls (Fig. 5 B,C), an effect not observed in the non-immunogenic 4T1 tumor model (Fig. 5D,E). Alternatively, VACV ΔK7RΔJ2R did not cause this PD-1 down regulation on Tregs, which appeared more similar to Tregs isolated from untreated tumors. It was difficult to generalize effects on PD-1 expression, as each virus affected PD-1 expression differently. Compared to tumors treated with VACV ΔJ2R, ΔA41LΔJ2R infections were associated with up-regulated PD-1 expression on both CD4⁺ and CD8⁺ T cells in 4T1 mouse spleens, but not in tumors, while a VACV ΔB8RB18RΔJ2R infection was associated with an increase in PD-1 on CD8⁺ cells in the spleen. When analyzing PD-L1 expression on CD11b-CD3- cells isolated from TuBo tumors, we noted that VACV ΔF4LΔJ2R caused a marked upregulation of PD-L1 (Fig. 5 F,G), which may have partially explained the poor antitumor response relative to VACV ΔJ2R treatment. These observations show that different virus mutations have different effects on PD-1 and PD-L1 expression.

Intratumoral virus treatments caused systemic changes in circulating serum cytokines (Supplemental Fig. S6A-E). Mice treated with VACV ΔK7RΔJ2R exhibited reduced neutrophil-recruiting cytokine CXCL5 in serum, compared to mice treated with ΔJ2R (Supplemental Fig. S6A). The presence of VACV, independent of any specific genetic modification, caused a significant reduction in circulating granulocyte colony stimulating factor (G-CSF) (Supplemental Fig. S6B). This was associated with a marked reduction of granulocytes in the spleens of VACV-treated mice, and an overall increase in monocytes (Supplemental Fig. S6C-E).

Collectively, these data showed that the effects on the TME at the site of infection were dominated by virus-dependent recruitment of tumor-infiltrating CD8⁺ T-cells and were, with one exception, not especially sensitive to alterations in the genetic composition of these viruses. However, the deletion of immunomodulatory or metabolic genes from the VACV genome could differentially affect the overall composition of the lymphocytic and myeloid cell population in a tumor-bearing host, even weeks after virus treatment. In some instances, changes to the TME were still seen even after the virus had been cleared from the tumor, as was seen in the TuBo tumors recovered from VACV ΔF4LΔJ2R. In this case, no replicating virus was isolated from the tumors three weeks after virus treatment (Fig. 2D). These virus
infections also had idiosyncratic effects on checkpoint protein expression and alter circulating serum cytokines with effects on the splenocyte composition.

**Effects of VACV mutations on antigen specific CD8+ T cell responses to tumor and virus**

A primary goal of virotherapy is to increase antitumor immunity while limiting anti-viral immunity. We used H2-Kd-restricted tetramers, specific for CD8+ T cells recognizing immunodominant epitopes of TuBo tumors and vaccinia virus, to determine if these VACV mutations altered the percentages of antitumor versus anti-viral splenic CD8+ T cells (Fig. 6A). Specifically these experiments were designed to detect CD8+ T-cells recognizing the p66 rat HER2/neu or VACV A52R peptides(33,34). The PBS-treated mice exhibited a small increase in the percentage of p66 HER2/neu specific CD8+ T cells compared to mice that had not encountered tumor; 0.83% compared to 0.33% respectively (Fig. 6B). This basal frequency of tumor-targeting T cells was not unexpected and may have explained why some tumor regression was seen even in PBS treated controls. All of the virus treatments further increased these percentages of tumor-targeting T cells, with averages ranging from 1.1% of CD8+ T cells after ΔA41LΔJ2R treatment to 3.3% after ΔB8RB18RΔJ2R virus treatment. When compared to the parental ΔJ2R virus, the ΔK7RΔJ2R and ΔB8R18RAΔJ2R mutants most improved the percentage of p66 HER2/neu targeting T cells with the latter treatment invoking a statistically significant 3-fold increase relative to mice treated with VACV ΔJ2R (Fig. 6B).

All of these virus treatments enhanced CD8+ T cell responses to the VACV A52R tetramer with no background (<0.2%) detected in naïve and PBS-treated animals (Fig. 6C). Treatment with the ΔB8RΔB18RΔJ2R mutant significantly increased these percentages relative to VACV ΔJ2R treatments. This suggests that this virus was perhaps the most immunogenic of all the mutants tested, as it increased the percentage of T cells targeting both the tumor and viral antigens. A significant increase in antiviral CD8+ T cell responses was also seen in animals treated with VACV ΔF4LΔJ2R relative to VACV ΔJ2R treatment (Fig. 6C) although without inducing a parallel increase in p66 HER2/neu specific T cell percentages (Fig. 6B, Supplemental Fig.S7). The same trend was also seen in animals treated with VACV ΔA41LΔJ2R. The greatest (ΔB8RΔB18RΔJ2R) and least (ΔF4LΔJ2R; ΔA41LΔJ2R) numbers of long-term survivors were seen when comparing these viruses, pointing again to the critical value of balancing antitumor and anti-viral cellular immune response.

**Discussion:**

 Numerous oncolytic VACVs have been developed that harbour deletions of various viral genes(2), and many target genes have been suggested for removal from VACV to increase antitumor immunity(2). To
our knowledge, however, there have been no systematic attempts at deciphering which gene deletions have an impact on a tumor bearing host, and what the relative contribution of gene removal has in the overall immune response against the virus in this context. Here we sought to decipher how removal of genes from the VACV genome could impact immune cells in the TME post-oncolytic virus treatment. We removed eight different genes from VACV and compared the effects of these deletions in head-to-head comparisons in immune-competent breast tumor models.

Removing individual genes from the VACV genome did indeed have the capacity to induce sustained changes to the TME that persist up to three weeks after the final administration of virus. The presence of virus alone was capable of altering the overall immune cell composition in spleens and tumors. However, there did not appear to be drastic alterations between viruses where immune-modulating genes had been removed (with the exception of ΔF4LΔJ2R which showed decreased immune cells in the tumor overall). Rather, removing immune-modulating genes had more subtle effects on different cellular parameters, for instance, by improving the percentages of tumor antigen specific CD8\(^+\) T cells, or upregulating PD-1 expression on CD8\(^+\) T cells. In considering this information, rather than using immune-modulating gene deletions to cause drastic changes in the response to virotherapy, these deletions might be better suited for refining and fine-tuning immune responses to achieve a particular outcome. For instance, ΔB8RΔB18RΔJ2R increased tumor-specific CD8\(^+\) T cell percentages and increased PD-1 expression, thus a combination of this particular gene-deleted virus with PD-1 checkpoint blockade may be particularly beneficial. In agreement with this notion, a B18R-deleted virus on its own demonstrates significant survival benefits when combined with anti-CTLA4(35), but perhaps anti-PD-1 may be more therapeutically beneficial in this context.

We also observed that gene deleted viruses imparted a more significant survival benefit in our non-immunogenic 4T1 tumor model than they did in our immunogenic TuBo tumor model, despite these viruses causing more drastic changes to the TME in the TuBo model. This suggests that minor tweaks to the virus genome used to manipulate the TME even slightly may be more beneficial in non-immunogenic or more aggressive tumor models, where slight changes in the immune response may impart more therapeutic benefits.

Throughout these studies we determined a few candidate gene deletions that warrant further analysis. In the TuBo tumor model, ΔB8RΔB18RΔJ2R and ΔN1LΔJ2R had more complete responses than ΔJ2R. These two viruses, along with ΔC6LΔJ2R improved median survival. In the 4T1 tumor model these viruses all statistically improved survival relative to ΔJ2R treatment. These are all viruses with gene deletions that either directly inhibit interferon responses [ΔB8R(12), ΔB18R(13)], or indirectly interfere with interferon signalling through NF-κB or IRF3 inhibition [ΔC6L(14,15), ΔN1L(16),
This suggests that removal of VACV genes that inhibit interferon signalling may be a promising strategy for improving oncolytic VACV therapy. This is in agreement with previous studies that show targeting IFN pathways by deletion of B18R enhances therapeutic efficacy of oncolytic VACV in cancer models, however this may be improved with the additional removal of B8R(11,35). This is not surprising given the mounting body of evidence suggesting that IFN signalling is critical for the success of cancer immunotherapies(36–38).

A few VACV deletions in the models tested did not cause any survival improvement or therapeutic enhancement. VACV ΔF1LΔJ2R did not cause substantial changes to the immune response relative to ΔJ2R and was less effective than ΔJ2R alone in controlling tumor growth in the TuBo tumor model. It also did not impart a survival benefit in the 4T1 tumor model compared to ΔJ2R, whereas the rest of our immunogenic knockout viruses did improve this response. It can be noted, however, that a ΔF1LΔJ2R virus is more effective than ΔJ2R alone in treating subcutaneous colon tumors in mice(39). Similarly, ΔF4LΔJ2R VACV, which demonstrated superior safety and equal treatment efficacy in bladder tumor models compared to a ΔJ2R VACV(20), was not therapeutically superior in 4T1 or TuBo breast tumor models. This suggests that certain oncolytic viruses and the immunological changes they induce may be beneficial in one circumstance or tumor type, while being potentially unhelpful in other tumor types.

Future studies will be needed to assess if a combination of multiple of these proposed viral gene-targets further enhances oncolytic efficacy. Based on the current study we would propose a virus with multiple deletions of ΔB8R, ΔB18R, ΔC6L and ΔN1L. There are a few caveats with this strategy. One study investigating VACV as a vaccine vector found that while ΔK7R, ΔN1L and ΔC6L gene deletions on their own increased immune responses and vaccine efficacy, a triple-deleted mutant was significantly less effective, presumably due to increased virus attenuation and clearance(9). It will be interesting to determine if the same effect would be true in a cancer model, however, it is likely in any scenario that an appropriate balance must be found between immunogenic gene removal and virus attenuation in order to maximize therapeutic efficacy. The second challenge with combining multiple deletions stems from a manufacturing perspective. Creation of a virus with multiple gene targets categorically deleted would also be challenging using the traditional targeted methods utilized in this study, as we are limited based on the number of combinations of selectable markers used to isolate our desired viruses, and by the number of loxP sites a virus can contain without major genomic rearrangements. Although this strategy is challenging, it should be noted here that a previous study reported on the use of an oncolytic VACV with deletions in A48R, B18R, C11R and J2R demonstrating that such a virus is possible to selectively manufacture(40). With the advent of new synthetic biology approaches, the tools exist to create ‘designer’
viruses with any variety of genetic changes desired(41). Moving forward, it will be easier to rapidly create viruses with multiple gene deletions to maximize immune responses catered to the specific requirements of the tumor type in addition to therapeutic gene insertion and the expression of antigenic targets.

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<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Protein Product Function</th>
<th>Pathway</th>
<th>In vivo immune response in single gene-deleted virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4L</td>
<td>VACWR-043</td>
<td>Small subunit of ribonucleotide reductase.</td>
<td>Nucleotide biogenesis</td>
<td>Induces antitumor immune responses in bladder cancer models, with superior safety compared to ΔJ2R-only virus(20).</td>
</tr>
<tr>
<td>F1L</td>
<td>VACWR-040</td>
<td>Inhibitor of apoptosis and inflammasome.</td>
<td>Apoptosis and inflammasome signaling</td>
<td>Inhibits apoptosis and inflammasome activation, decreases caspase-1 and IL1b secretion to increase inflammation in lungs of infected mice(24,29).</td>
</tr>
<tr>
<td>K7R</td>
<td>VACWR-039</td>
<td>Binds TLR proteins, inhibits NF-κB, IRF3. Inhibits heterochromatin methylation.</td>
<td>NF-κB &amp; IRF3 signaling(42), heterochromatin regulation(43)</td>
<td>Increases natural killer (NK) cell infiltration and CD8⁺ T cells, and enhances MHCII presentation and cytolysis by NK cells and VACV specific CD8⁺ T cells(44).</td>
</tr>
<tr>
<td>N1L</td>
<td>VACWR-028</td>
<td>Virulence factor. NF-κB inhibitor, possible apoptosis inhibitor.</td>
<td>NF-κB and IRF3 signaling(16), apoptosis signaling(45) (disputed)(46)</td>
<td>Enhances CD8⁺ T cell effector and memory responses, and increases T cell cytotoxicity(47).</td>
</tr>
<tr>
<td>C6L</td>
<td>VACWR-022</td>
<td>IRF3 inhibitor, IFN inhibitor through degradation of histone deacetylase 4(15).</td>
<td>IRF3 and IFN signaling(14)</td>
<td>Enhances VACV specific T cells with increased cytotoxicity, and induces protection against challenge from a lethal dose of WR in a vaccination model (48).</td>
</tr>
<tr>
<td>A41L</td>
<td>VACWR-166</td>
<td>Secreted protein. Proposed to disrupt chemokine concentration gradients(30,31).</td>
<td>Chemokine binding</td>
<td>Increases VACV CD8⁺ T cell responses, and enhances protection against challenge(8). Mutant is cleared more readily due to increased infiltration of leukocytes(49).</td>
</tr>
<tr>
<td>B18R</td>
<td>VACWR-199</td>
<td>Binds type I IFN (Soluble and surface), more effective against IFNα than β(13).</td>
<td>IFN signaling</td>
<td>When combined, enhances adaptive immune responses to HIV antigens(50). Increases HIV and VACV-specific T-cell responses compared to Toll-like receptor (TLR) targeting VACVs(10).</td>
</tr>
<tr>
<td>B8R</td>
<td>VACWR-190</td>
<td>Soluble, binds secreted interferon gamma(12).</td>
<td>IFN signaling</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Target genes deleted to generate immunostimulatory oncolytic VACVs
Figure Legends

Figure 1. Mutant VACVs and *in vitro* growth properties  A) Genomic maps of mutant VACV constructs. *neo*, neomycin gene; *gusA*, β-glucuronidase gene; *lacZ*, β-galactosidase gene; *ITR*, inverted terminal repeat; *GPT*, guanine phosphoribosyltransferase; *Yfp*, yellow fluorescent protein; loxP, target loxP DNA sequence; *mCh*, mCherry fluorescent protein; nm, no marker. B) Viral growth kinetics of mutant VACVs on TuBo and 4T1 cells. Significance calculated at the 72 hour time point relative to ΔJ2R VACV using one-way ANOVA shown in the figure legends. C) Alamar blue cytotoxicity assay of cell viability 72 hours post infection in TuBo and 4T1 cells. Two-way ANOVA comparing cell viability after each virus treatment relative to parental ΔJ2R was used with Dunnett’s multiple comparisons correction and is shown on the graph at applicable points. Error bars denote +/- SEM from three experimental replicates. *p<0.05, **p<0.01 ***p<0.001 ****p<0.0001.

Figure 2. All immunomodulatory VACVs were safe *in vivo*, and virus replication was restricted to tumors. A. Experimental design to assess safety, biodistribution, and immune-microenvironment changes after treatment with mutant oncolytic viruses. 8-13 week old female BALB/C mice were injected with tumor cells into the mammary fat pad. Once palpable, three intratumoral 1x10⁷ PFU doses of VACVs were administered. Mice were euthanized after 14 days (4T1), or 21 days (TuBo). Tumors and organs were collected for immune-analysis and viral titering. B&C. Weight change from baseline of mice during treatment with oncolytic VACVs in the TuBo(B) and 4T1(C) tumor model. D&E. Virus titers isolated from tumors where dotted line shows the limit of detection. ND- not detected.

Figure 3. Mutant VACVs altered immune responses and therapeutic outcomes in TuBo tumors. A. Average tumor volume until first death observed in each group. Error bars show ± 1 SD from mean. Two-way ANOVA was used to evaluate differences in tumor growth over time compared to ΔJ2R treatment until first non-censored death at day 60; *p<0.05, **p<0.001, ****p<0.0001. N=8-9 mice per group. B. Kaplan-Meir survival plot of data shown in A. CR=complete responses, MS= Median survival. C. Individual tumor volumes of mice re-challenged with TuBo tumors in the opposite mammary fat pad after stable disease to determine establishment of antitumor immunity. D. Flow cytometric quantification of numbers (D) and percentages (E-I) of immune cells in tumors. J. Overall average splenocyte composition as a percent of total cells isolated from spleens. K-O. Immune-cell percentages from spleens of mice after treatment. Organs were harvested three weeks after virus treatment. Statistical analysis was performed using one-way ANOVA where data was shown to be parametric, or Kruskal-Wallis testing when data was
non-parametric; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data points represent individual mice in E-I and K-O with error bars denoting 95% CIs, and average values for groups in D & J.

**Figure 4. Mutant VACVs altered immune responses and therapeutic outcomes in 4T1 tumors.**

A. Average tumor volume per group until first death observed in each group. Error bars show ± 1SD from mean. Two-way ANOVA used to evaluate differences in tumor growth over time compared to ΔJ2R treatment until first death observed at day 18; *p<0.05, **P<0.001, ****P<0.0001. N=8-9 mice per group. B. Kaplan-Meir survival plot of data shown in A. C. Lung metastasis analysis as determined by colony formation assays of digested lung tissue plated under 6-thioguanine. D. Flow cytometric quantification of numbers (D) and percentages (E-I) of immune cells in tumors. J. Overall average splenocyte composition as a percent of total cells isolated from spleens. K-O. Immune-cell percentages from spleens of mice after treatment. Organs were harvested three weeks after virus treatment. Statistical analysis was performed using one-way ANOVA where data was shown to be parametric, or Kruskal-Wallis testing when data was non-parametric; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data points represent individual mice in E-I and K-O with error bars denoting 95% CIs, and average values for groups in D & J.

**Figure 5: Modifications to the VACV genome altered immune checkpoint expression on immune cells in tumors and spleens.**

A. Representative histograms of PD-1 expression on CD8+ T cells, CD4+ T cells, and T_{regs} from spleens. B-E) Median fluorescence intensity (MFI) on lymphocytes from spleens (B, D) or tumors (C, E) of mice with TuBo (B&C) and 4T1 (D&E) tumors. F. Representative histogram of PD-L1 expression from CD3- CD11b- cells in TuBo tumors. G. MFI of PD-L1 on CD11b- CD3- cells from TuBo tumors. Statistical analysis was performed using one-way ANOVA where data was shown to be parametric, or Kruskal-Wallis testing when data was non-parametric; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data points represent individual mice in B-E & G with error bars denoting 95% CIs.

**Figure 6: Modifications to the VACV genome altered antigen-specific T cell responses against tumor and virus**

A. Representative flow plots of splenocytes stained with H2K$^d$ tetramers specific for p66 HER2/neu of TuBo tumors (y-axis) and A52R of VACV (x-axis). Representative flow plots selected for sample with median value of p66 staining. B, C. Frequency of tetramer+ CD8+ T cells as described in A where each symbol represents one mouse. Error bars denote 95% CIs from mean values. Statistical significance determined using one-way ANOVA with Dunnett’s multiple comparisons testing. FMO-fluorescence minus one gating control.
Figure 1

A

\[ \Delta J2R^{mm} \]

\[ \Delta F4L \Delta J2R^{mCh} \]

\[ \Delta F1L^{mCh} \Delta J2R^{mm} \]

\[ \Delta K7R^{Yfp} \Delta J2R^{mm} \]

\[ \Delta N1L^{Yfp} \Delta J2R^{mm} \]

\[ \Delta C6L^{mCh} \Delta J2R^{mm} \]

\[ \Delta A41L^{mCh} \Delta J2R^{mm} \]

\[ \Delta B8R^{mCh} \Delta B18R^{mm} \Delta J2R^{mm} \]

B

Virus Amplification

TuBo

4T1

C

Cytotoxicity

TuBo

4T1
**Figure 2**

A. BALB/C

| N=5 |

- Virus injection x3
- 10^7 PFU Intratumoral
- Euthanize mice and collect organs

MFP injection

B. TuBo Model Animal Weights

![Graph showing weight change over time for TuBo model animals.]

- PBS
- ΔJ2R
- ΔF4ΔJ2R
- ΔF1ΔJ2R
- ΔK7RΔJ2R
- ΔN1LΔJ2R
- ΔC6LΔJ2R
- ΔA41LΔJ2R
- ΔB18RΔB8RΔJ2R

Average weight change from baseline (g)

Days post virus injection

C. 4T1 Model Animal Weights

![Graph showing weight change over time for 4T1 model animals.]

- PBS
- ΔJ2R
- ΔF4ΔJ2R
- ΔF1ΔJ2R
- ΔK7RΔJ2R
- ΔN1LΔJ2R
- ΔC6LΔJ2R
- ΔA41LΔJ2R
- ΔB18RΔB8RΔJ2R

Average weight change from baseline (g)

Days post virus injection

D. TuBo tumor titers

![Graph showing tumor titers for TuBo model animals.]

E. 4T1 tumor titers

![Graph showing tumor titers for 4T1 model animals.]

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Figure 5

A

CD8+ T cells

CD4+ T cells

T-regulatory cells

B

TuBo Spleen

MFI PD-1 on CD8+ T cells

MFI PD-1 on CD4+ T cells

MFI PD-1 on T_reg

C

TuBo Tumor

MFI PD-1 on CD8+ T cells

MFI PD-1 on CD4+ T cells

MFI PD-1 on T_reg

D

4T1 Spleen

MFI PD-1 on CD8+ T cells

MFI PD-1 on CD4+ T cells

MFI PD-1 on T_reg

E

4T1 Tumor

MFI PD-1 on CD8+ T cells

MFI PD-1 on CD4+ T cells

MFI PD-1 on T_reg

F

TuBo Tumor

PD-L1 MFI on CD11b- CD3- cells

G

TuBo Tumor

PD-L1 MFI on CD11b- CD3- cells

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Figure 6

A

FMO VACV tetramer  FMO TuBo tetramer  Naive Mouse  PBS

J2R  F4L2R  F1L2R  K7R2R

Q1  0.09  0.67  1.40  2.76
Q2  0.14  0.071  0.086  0.12
Q3  5.13  2.91  2.14  0.13
Q4  96.3  93.4  93.4  84.8

J2R  F4L2R  F1L2R  K7R2R

Q1  0.99  0.70  1.36  0.89
Q2  0.14  0.071  0.086  0.12
Q3  5.13  2.91  2.14  0.13
Q4  96.3  93.4  93.4  84.8

J2R  F4L2R  F1L2R  K7R2R

Q1  0.99  0.70  1.36  0.89
Q2  0.14  0.071  0.086  0.12
Q3  5.13  2.91  2.14  0.13
Q4  96.3  93.4  93.4  84.8

N1L2R  C6L2R  A41L2R  B8R18R12R

Q1  2.40  2.60  2.62  2.46
Q2  0.95  0.14  0.097  0.16
Q3  5.90  2.96  3.12  8.02
Q4  94.8  94.0  94.0  89.6

N1L2R  C6L2R  A41L2R  B8R18R12R

Q1  2.40  2.60  2.62  2.46
Q2  0.95  0.14  0.097  0.16
Q3  5.90  2.96  3.12  8.02
Q4  94.8  94.0  94.0  89.6

VACV A52R tetramer-BV421

B

%TuBo HER2/neu P66 tetramer+

*p=0.046

% TuBo tetramer+ of CD8+

Naive  PBS  ΔA4ΔL2R  ΔA4L2R  ΔA4ΔL2R  ΔA4L2R  ΔA4ΔL2R  ΔA4ΔL2R  ΔA4ΔL2R  ΔA4ΔL2R

C

% VACV A52R tetramer+

% TuBo tetramer+ of CD8+

Naive  PBS  ΔA2R2R  ΔA2R2R  ΔA2R2R  ΔA2R2R  ΔA2R2R  ΔA2R2R  ΔA2R2R  ΔA2R2R

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Cancer Immunology Research

Deciphering the immunomodulatory capacity of oncolytic vaccinia virus to enhance the immune response to breast cancer

Brittany A Umer, Ryan S Noyce, Brian C Franczak, et al.


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