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Reshaping the tumor microenvironment with oncolytic viruses, positive regulation of the immune synapse, and blockade of the immunosuppressive oncometabolic circuitry

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Abstract

Immune-related therapies have revolutionized the management of cancer. Oncolytic viruses are now considered part of the immunotherapy armamentarium and have shown promise in clinical trials of patients with glioblastoma. These studies have suggested that tumor microenvironment remodeling is required to achieve an effective response in solid tumors. Here, we showed that Delta-24-RGDOX (DNX-2440), an oncolytic adenovirus expressing the T cell activator OX40L, triggered antitumor immune responses. However, Delta-24-RGDOX also elicited paradoxical activation of the cytokine-driven immunosuppressive IDO-kynurenine-AhR circuitry. The IDO-kynurenine-AhR cascade had the dual effects of preventing optimum viral replication and decreasing the virus-initiated antitumor immune response. To enhance virotherapy, we combined Delta-24-RGDOX with clinically relevant IDO inhibitors. This combination therapy increased the frequency of activated CD8⁺ T cells and decreased the frequencies of immunosuppressive MDSC and Treg populations in animal models of gliomas and melanoma. Functional studies demonstrated that the IDO blockade-dependent activation of immune cells against tumor antigens could be reversed by the oncometabolite kynurenine. The concurrent targeting of effectors and suppressors in the tumor immune landscape significantly prolonged the survival of glioma- and melanoma-bearing mice. Our data identified the striking role of immunosuppressive pathways in the resistance of solid tumors to oncolytic virotherapy. Specifically, the activity of the tumor microenvironment IDO circuitry was responsible, at least partially, for the remodeling of local immunosuppression after tumor infection. Combining molecular and immune-related therapies may improve outcomes in human gliomas and other cancers treated with virotherapy.

Introduction

Natural human viral infections can induce remission in several types of cancers. During the pandemic, cancer patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have experienced complete remissions^{1,2}, illustrating the antitumor capacity of viruses. In one phase I study, the oncolytic virus Delta-24-RGD (DNX-2401) induced complete tumor regression in 20% of patients with recurrent glioblastoma³. Other findings in this clinical trial, including radiological signs of inflammation, pseudoprogression, and tumor infiltration by T-bet⁺ CD8⁺ T cells, strongly suggested that the antitumor effects of Delta-24-RGD were due in part to an antitumor immune response. These observations agreed with preclinical studies showing that Delta-24-RGD infection induces autophagy and immunogenic cell death in glioblastoma^{4,5}. To enhance the immune arm of this oncolytic virotherapy, we generated Delta-24-RGDOX (DNX-2440), a third-generation adenovirus that includes the T cell activator OX40-ligand (OX40L)^{6,7} in the backbone of Delta-24-RGD. Preclinical studies have shown that Delta-24-RGDOX induces a stronger T cell-mediated antitumor effect than parental Delta-24-RGD in glioblastoma and metastatic melanoma murine models^{6,7}. Based on these results, Delta-24-RGDOX is being tested in clinical trials for the treatment of malignant gliomas (NCT03714334) and liver metastases (NCT04714983).

Infection of tumors with viruses is an efficient method of recruiting cytotoxic CD8⁺ T cells^{3,6,7}; however, suppressive mechanisms within the tumor microenvironment restrain the effector immune response⁸. Therefore, improving the overall potency of virotherapy may require targeting inherent tumor immunosuppression while augmenting T cell activation. Notably, viral infections elicit immunosuppressive mechanisms that further hinder virus-induced antitumor responses⁹.

Indoleamine 2,3-dioxygenase (IDO) is expressed in the tumor microenvironment and induces immune privilege, an effect that is reversed by the administration of IDO inhibitors¹⁰. The catabolism of tryptophan (Trp) by IDO produces kynurenine (Kyn), a direct activator of aryl hydrocarbon receptor (AhR), promoting robust immunosuppression^{11,12}. The IDO-Kyn-AhR multicellular circuitry plays a fundamental role at the center of the immune synapse and acts as a bridge among the main immune cell populations in tumors¹³.

Activation of the IDO cascade is a major characteristic of several tumors¹⁴⁻¹⁶, including gliomas¹⁷, and correlates with poor prognoses^{17,18}. In addition to its tumor role, IDO is activated in various tissues and cell subsets following cytokine stimulation during infection¹⁹.

Combinations of therapeutic strategies are probably required to overcome treatment resistance and improve the efficacy of immunotherapies in solid tumors. The main objective of this work was to determine the therapeutic efficacy and immune effects of combined treatment with Delta-24-RGDOX and IDO inhibitors in solid tumors. We showed that simultaneous activation of the effector arm of the immune system, using oncolytic viruses and OX40-mediated T cell activation, and inhibition of the suppressor arm, with IDO inhibitors, produced robust remodeling of the tumor microenvironment, significantly enhancing viroimmunotherapy and leading to tumor eradication in immunocompetent animal models.

Results

Delta-24-RGDOX infection results in robust transcriptional modulation of the tumor microenvironment.

Solid tumors consist of both tumor-intrinsic and tumor-extrinsic phenotypic features, which shape the overall tumor microenvironment. RNA sequencing (RNA-seq) enables the unbiased cellular and molecular profiling of complex tissues to dissect intratumoral heterogeneity. Leveraging this approach, we performed bulk RNA-seq analyses of Delta-24-RGDOX-infected murine gliomas to comprehensively examine changes in the tumor transcriptome. These data showed drastic reshaping of the tumor microenvironment following intratumoral infection (Fig. 1A-B). Specifically, ingenuity pathway analysis (IPA) of Delta-24-RGDOX-infected tumors showed an enhancement of inflammation-related canonical pathways in these tumors compared with control tumors (Fig. 1C). We also performed IPA to identify altered upstream regulators in tumor treated with Delta-24-RGDOX infection. This analysis identified IFN γ , TNF, ILB, IFN α and STAT1 as candidate regulators of the transcriptional response to Delta-24-RGDOX infection (Fig. 1D). To explore whether these transcriptional programs were caused by oncolytic adenovirus infection or transgenic expression of OX40L, we performed similar RNA-seq analysis following infection with the parental oncolytic viral vector, Delta-24-RGD. We observed a comparable pattern in tumors treated with the parental Delta-24-RGD virus (Supplementary Fig. 1A-D). Crucially, these data confirmed our clinical and

preclinical results, which showed tumor-infiltrating population characteristics that we observed in surgical specimens from gliomas infected with Delta-24-RGD³. Similarly, our data were in agreement with changes in the immune populations observed in murine models of glioma and melanoma upon infection with oncolytic adenoviruses^{6,7}. Therefore, the RNA-seq analyses were consistent with previous data for human and murine tumors infected with an adenovirus obtained using other methodologies.

Based on the rationale that IDO is triggered by IFN γ ²⁰ and upregulated during viral infections⁹, we asked whether Delta-24-RGDOX treatment induces upregulation of the IDO network. We observed that a great number of IDO-related transcripts were upregulated after viroimmunotherapy, including those playing a pivotal role in maintaining glioma immunosuppression such as TGF β , CTLA-4, PD-1, and GATA-3 (Fig. 1E). With respect to estimated nontumor cell type abundances inferred from bulk tissue transcriptomes, the main impact was a remarkable increase in the CD8⁺ T cell proportion ($P = 0.0031$) and a trend toward upregulation of the natural killer (NK) cell proportion (Fig. 1F). Importantly, the use of Delta-24-RGD allowed us to dissect the role of the OX40L/OX40 costimulatory axis; whereas tumors infected with Delta-24-RGD showed an increase in CD8⁺ T cells, they did not show the same shift in the population from CD4⁺ T cells to CD8⁺ T cells that was seen in tumors infected with Delta-24-RGDOX. In addition, the increase in the NK cell population was much less prevalent in tumors infected with the parental virus (Supplementary Fig. 1E). Indirectly implying that the effects on these cell populations were specific, Delta-24-RGDOX infection resulted in a negligible effect on the transcriptional signatures of macrophages and dendritic cells.

Collectively, these analyses indicated that Delta-24-RGDOX induced a robust effect on the tumor microenvironment. The pleiotropic impact of Delta-24-RGDOX infection includes not only the expected activation of proinflammatory pathways but also negative impacts on regulators of the immune responses against viruses and tumors. Within the negative regulatory network, IDO is activated by Delta-24-RGDOX infection and appears to play a critical role in the re-emergence of the immunosuppressive response.

Delta-24-RGDOX elicits IDO expression and activation in gliomas.

We next aimed to confirm the effect of Delta-24-RGDOX on the activity of the IDO pathway by assessing the production of IDO-related metabolites, such as Kyn, and the

expression of key downstream targets of IDO, including the transcription factor AhR²¹⁻²³. Several proteins that are directly or indirectly activated by adenovirus infection via activation of cytokines can modulate IDO expression. Of note, IDO was the first gene discovered to be inducible by IFN γ ²⁰, a potent mediator of antiviral immune responses. In addition, IDO is regulated by other molecular mechanisms activated during infection, such as the STAT/JAK²⁴ and IFN type I transduction signaling pathways²⁵⁻²⁷.

In *in vitro* experiments, we observed a remarkable increase in IDO expression in human and murine glioma cells following treatment with IFN γ (Supplementary Fig. 2). We also determined that infection of GL261-5 and GSC-005 murine glioma cells with Delta-24-RGDOX triggered a six- to seven-fold increase in IDO levels, as assessed by qRT-PCR (Fig. 2A). Additionally, infection of intracranial GL261-5-derived gliomas with Delta-24-RGDOX led to a significant increase in IDO levels *in vivo*, and we observed a similar pattern in orthotopically implanted breast tumors (Fig. 2B-C).

Kyn is a product of the metabolic activity of IDO²⁸, and therefore, we next sought to ascertain whether infection of murine tumors with Delta-24-RGDOX increased the production of this oncogenic metabolite *in vivo*. In agreement with the increased IDO mRNA expression observed in murine glioma cell lines upon viral infection, both GL261-5- and GSC-005-derived tumors infected with Delta-24-RGDOX showed a significantly higher Kyn/Trp ratio than control mouse brain tumors (Fig. 2D), as determined by liquid chromatography–mass spectrometry, indicating the presence of an active IDO cascade in these tumors.

The IDO-mediated catabolism of Trp and subsequent production of Kyn suggested the potential activation of AhR, a key downstream target of IDO directly activated by Kyn^{11,29}. Importantly, AhR has dual functions as a transcriptional target for immune escape by viruses^{30,31} and a promoter of immunosuppression in solid tumors, including gliomas³²⁻³⁵. Using AhR reporter cells expressing a luciferase reporter functionally linked to an AhR-responsive promoter, we assessed the transcriptional activity of AhR in a panel of glioma cell lines during active adenoviral infection. We found that Delta-24-RGDOX treatment significantly increased AhR transcriptional activity (Fig. 2E). Interestingly, viral infection enhanced AhR transcriptional function with more efficiency in glioma cells than in control HeLa cells. Because the binding of Kyn to AhR promotes AhR translocation to the nucleus³², we analyzed the expression and subcellular localization of AhR in cancer cells infected with Delta-24-RGDOX using

immunofluorescence. Compared with uninfected cells, infected cells showed significant increases in the whole-cell and nuclear intensity of AhR as well as a remarkable increase in the percentage of cells displaying nuclear AhR (Fig. 2F-H). Importantly, the Delta-24-RGDOX-mediated changes were similar to the subcellular trafficking of AhR observed when uninfected cultures were treated with Kyn (Fig. 2F-H).

These results suggested that following Delta-24-RGDOX infection, there was substantial activation of the immunosuppressive IDO-Kyn-AhR cascade. The increased expression of the oncometabolite Kyn and upregulation of the transcriptional activity of AhR strongly suggested that infection of a tumor is followed by activation of the IDO circuitry with subsequent maintenance of an immunosuppressive environment.

The Delta-24-RGDOX replication capability and cytotoxic effect are preserved under IDO inhibition.

To ascertain the effect of IDO activity specifically on virus oncolytic activity, we assessed the viral infectivity, viral replication, and virus-induced cytopathic effect of Delta-24-RGDOX in a panel of human and murine glioma cell lines (Fig. 3). We observed that treating glioma cultures with a direct IDO enzyme inhibitor did not modify the activity of Delta-24-RGDOX in any of the human or murine cancer cell lines tested. Thus, the quantitative levels of progeny virion production and expression of viral proteins were unchanged during IDO inhibition (Fig. 3A, B, F, G). Corroborating these results, a timepoint analysis of cell viability showed that the oncolytic effect was similar in cells treated with Delta-24-RGDOX alone or in combination with IDO inhibitors (Fig. 3C, D, E, H, I). These results suggested that the potency of Delta-24-RGDOX remained unchanged under conditions of IDO inhibition.

The combination of Delta-24-RGDOX and IDO inhibitors enhances immune activation in murine models of glioma.

To determine the phenotypic changes within the tumor microenvironment following the combined administration of Delta-24-RGDOX and an IDO inhibitor, we performed RNA-seq analysis of GL261-5 tumors from mice treated with PBS, an IDO inhibitor, Delta-24-RGDOX, or a combination of the IDO inhibitor and Delta-24-RGDOX. Hierarchical clustering of genes and samples showed that the transcriptome signatures of PBS- and IDO inhibitor-treated mice

were not different. In contrast, Delta-24-RGDOX infection resulted in significant alterations in gene pathways, making Delta-24-RGDOX-treated mice distinct from PBS- and IDO inhibitor-treated mice (Fig. 4A and C). There was also a clear difference in the global transcriptome signature between Delta-24-RGDOX- and combination therapy-treated mice (Fig. 4A and C). Furthermore, network analyses of differentially expressed genes demonstrated that the addition of IDO inhibition to Delta-24-RGDOX infection reverted the effects of the adenovirus on several key immune-related transcripts that were induced during adenovirus infection, such as immune checkpoint regulators, inflammation-related cytokines, Toll-like receptors 7 and 9 and components of the STING pathway (Fig. 4C).

The predicted immune cell composition and transcriptome signature showed minimal differences between PBS- and IDO inhibitor-treated brain tumors (Fig. 4B). Interestingly, upon treatment with Delta-24-RGDOX, the estimated percentage of CD8⁺ T cells almost doubled compared to that in the group treated with IDO inhibition alone (Delta-24-RGDOX: 23.7%; IDO inhibitor: 12.0%); the combination of Delta-24-RGDOX and the IDO inhibitor (combination: 26.2%; PBS: 16.3%) did not show significant changes in the CD8⁺ T cell transcriptome frequency compared to Delta-24-RGDOX alone (Fig. 4B). The combination therapy-treated group displayed the lowest predicted frequencies of NK cells (combination: 1.9%; IDO inhibitor: 3.7%; RGDOX: 6.7%), which have been shown to have antiviral activity³⁶, and dendritic cells (combination: 3.4%; PBS: 7.4%; IDO inhibitor: 11.4%; RGDOX: 17.3%), which have been implicated in tumor tolerance³⁷ (Fig. 4B).

The altered immune responses of combination therapy-treated mice versus Delta-24-RGDOX-treated mice confirmed and complemented the results that showed increased activation of the IDO network in Delta-24-RGDOX-treated mice and decreased IDO activation in combination therapy-treated mice, compared to mice in the control group (Fig. 4C-D). These data also demonstrated the ability of the IDO inhibitor to decrease the number of IDO-related transcripts induced in tumors infected with the adenovirus (Fig. 4D). Remarkably, the combination treatment decreased the expression of more than 90% of the upstream and downstream IDO-related transcripts induced upon Delta-24-RGDOX treatment.

Together, these results indicate that the combination of IDO inhibition with Delta-24-RGDOX infection modulates the tumor microenvironment by two means, promoting an adaptive immune response while decreasing immunosuppression caused by virus-induced IDO activation.

These collective findings strengthen our rationale for combining Delta-24-RGDOX infection with IDO inhibition for the treatment of gliomas.

Combination of Delta-24-RGDOX infection with IDO inhibition prolongs the survival of glioma-bearing mice.

The results from several clinical studies using oncolytic viruses^{3,38-41}, chimeric antigen receptor T cells⁴²⁻⁴⁴, or anti-PD-1 and anti-PD-L1 antibodies^{45,46} have shown that single cancer immunotherapies are unlikely to circumvent the immune evasion mechanisms of gliomas. Therefore, we aimed to test a three-pronged therapy in immunocompetent animal models. First, to ascertain whether IDO inhibition impairs oncolytic ability *in vivo*, we treated GL261-5 tumor-bearing IDO-knockout (KO) mice with Delta-24-RGDOX. In this context of IDO inhibition in the tumor microenvironment, Delta-24-RGDOX prolonged median survival and produced 20% long-term survivors (Supplementary Fig. 3). We then compared the survival of glioma-bearing IDO-KO mice and wild-type mice treated with Delta-24-RGDOX or PBS (Fig. 5A-B). As expected, all the PBS-treated mice succumbed to their tumors, with a median survival time of 44 days, regardless of the mouse genotype. Importantly, Delta-24-RGDOX treatment resulted in a longer median survival duration and a higher percentage of long-term survivors in the IDO-KO mice than in the wild-type mice ($P = 0.0142$). To further determine the therapeutic efficacy of Delta-24-RGDOX combined with IDO inhibition, we tested this treatment in the intracranial GL261-5 tumor-bearing mouse model (Fig. 5C-D). The median survival times of the control-treated mice and IDO inhibitor-treated mice were 38.5 and 37.5 days, respectively. As expected, the mice treated with Delta-24-RGDOX had significantly prolonged survival, with a median survival time of 46.5 days. In addition, 20% of the mice treated with Delta-24-RGDOX survived for 120 days (the last timepoint tested). Notably, mice treated with a combination of Delta-24-RGDOX and an IDO inhibitor had the greatest therapeutic benefit, with a median survival time of 108.5 days and a remarkable long-term survival (120 days) rate of 50% ($P = 0.0295$; vs. Delta-24-RGDOX).

Next, we asked whether the antiglioma effect of the combined Delta-24-RGDOX and IDO inhibitor therapy is mediated by the immune system. To address this question, we performed rechallenge experiments with long-term survivors in the combination therapy group using intracranial injection of GL261-5 cells into the contralateral hemisphere (Fig. 5C and E).

As expected, treatment-naïve mice injected with GL261-5 cells died within 50 days, but the mice treated with Delta-24-RGDOX and the IDO inhibitor survived 100 days with no symptoms of disease. These data unequivocally indicated the immune nature of the treatment response, as the combination of Delta-24-RGDOX and IDO inhibitors generated immune memory against glioma antigens.

To confirm these results, we tested the combination therapy in another murine glioma model. To this end, we intracranially implanted GSC-005 cells in C57BL/6 mice and treated the mice with either PBS or Delta-24-RGDOX with or without the IDO inhibitor (Fig. 5C and F). The PBS- and IDO inhibitor-treated mice had median survival times of 38.5 or 42 days, respectively, and 100% of the mice died due to tumor growth. As expected, treatment of these brain tumor-bearing mice with Delta-24-RGDOX prolonged survival compared to control treatment, with a median survival time of 53 days ($P = 0.02$) but no long-term survivors. Similar to the GL261-5 model, the GSC-005 tumor-bearing mice treated with the combination of Delta-24-RGDOX and the IDO inhibitor exhibited the longest median survival (63 days) and 44% long-term survivors, indicating a trend of higher long-term survival rate than that achieved with Delta-24-RGDOX alone.

To test whether the combination therapy was also effective in other solid tumors, we treated intracranial B16 melanoma-bearing mice with PBS or with Delta-24-RGDOX with or without an IDO inhibitor (Fig. 5G). The mice treated with Delta-24-RGDOX alone had a median survival time that was twice that of the PBS-treated mice, none of which survived to 10 days after tumor implantation. The mice treated with the combination therapy had significantly prolonged survival and a long-term survival rate of 14%. These results indicate that the combination of Delta-24-RGDOX and an IDO inhibitor is therapeutically superior to virus treatment alone in animal models of glioblastoma and intracranial melanoma.

In agreement with the survival data, analysis of tissue sections of brains harvested from mice injected with either GL261-5 glioma cells or GSC-005 glioma cells in the different treatment groups at 15 and 24 days showed progressive tumor regression in the mice treated with the combination therapy (Fig. 5H-I; Supplementary Figs. 4-5). Additionally, neither the GL261-5 nor GSC-005 brain tumor-bearing animals treated with the combination therapy exhibited significant changes in weight over the course of treatment, indicating the tolerability of this treatment (Supplementary Fig. 6).

In summary, antitumor treatment with the combination of Delta-24-RGDOX and an IDO inhibitor is superior to treatment with either agent alone and significantly prolongs survival, generates long-term survivors and induces antiglioma immune memory in immunocompetent animal models of cancer.

The anticancer effect of combination treatment with Delta-24-RGDOX infection plus IDO inhibition is dependent upon CD4⁺ T cell activity.

To further understand the immune-mediated mechanisms underlying the anticancer effect of the combination of Delta-24-RGDOX infection plus IDO inhibition, we assessed the phenotypic changes in the lymphocyte population following treatment. Mice were treated as described in Fig. 6A, and flow cytometric analyses revealed a significant influx of CD45⁺CD3⁺ T cells in both GL261-5 and GSC-005 tumor-bearing mice treated with Delta-24-RGDOX compared to control-treated mice (Fig. 6B). These results were confirmed with immunohistochemical assessments of CD3⁺ cells in the two murine brain tumor models (Fig. 6C; Supplementary Fig. 7). Compared with mice receiving monotherapy or control treatment, those receiving Delta-24-RGDOX and an IDO inhibitor had significantly higher absolute counts of CD3⁺, CD4⁺, and CD8⁺ T cell populations (Fig. 6B, D, E).

Based on our RNA-seq findings suggesting a link between IDO and an immunosuppressive tumor microenvironment following Delta-24-RGDOX infection and on previous reports of IDO activation resulting in increasing frequencies of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)⁴⁷, we next assessed whether these two cell populations underwent changes in treated tumors. Gene set enrichment analyses of RNA-seq data showed positive enrichment of gene sets associated with Tregs (normalized enrichment score of 1.375, $P = 0.0$, FDR q-value = 0.0439) and MDSCs (normalized enrichment score of 1.352, $P = 0.0$, FDR q-value = 0.0642) in tumors treated with Delta-24-RGDOX compared with control-treated tumors (Fig. 6F, left panels). In contrast, the combination of Delta-24-RGDOX infection with IDO inhibition led to a reversal of these findings, shifting the tumor microenvironment away from an immunosuppressive phenotype (Fig. 6F, right). These results were confirmed by flow cytometric analyses of the tumor microenvironment of virus- and combination-treated mice (Fig. 6G). Importantly, whereas Delta-24-RGDOX alone increased

Treg and MDSC populations, Delta-24-RGDOX plus the IDO inhibitor significantly decreased these crucial immunosuppressive populations.

Since CD4⁺ T cells constitute the target of the OX40 ligand/receptor synapse⁴⁸, we aimed to understand the extent to which these cells contributed to the therapeutic efficacy of the combination of Delta-24-RGDOX infection and IDO inhibition in murine glioblastoma. To this end, we performed a survival experiment in which CD4⁺ T cells were depleted using anti-CD4 antibodies (Fig. 7A-C). Our results showed that in mice with a decreased population of CD4⁺ T cells, the antiglioma effect of the combination treatment was abolished, and the median survival of the combination-treated CD4⁺ T cell-depleted mice was similar to that of control-treated mice (Fig. 7D). This loss of the anticancer effect indicates that the combined therapy depends on the CD4⁺ T helper cell population to induce maximal anticancer effects and produce long-term survivors. We also examined the effect of depletion of Tregs, a CD4⁺ cell population, on the therapeutic activity of the combination treatment. We observed that depleting 85% of the CD25⁺ population (Fig. 7B-C) had no significant deleterious effect on the survival of mice receiving the combination treatment (Fig. 7D). These results were consistent with the observations that the combination treatment induced a significant reduction in Tregs (Fig. 6G) and suggested that blocking IDO already caused functionally relevant inactivation of Tregs. Additionally, these analyses also suggested that the effect observed with the depletion of CD4⁺ cells was most likely due to the reduction in the CD4⁺ T helper cell population.

In summary, our data indicated that the combination of Delta-24-RGDOX infection with IDO inhibition resulted in intratumoral infiltration of several T cell populations, such as CD45⁺CD3⁺, CD45⁺CD3⁺CD4⁺ and CD45⁺CD3⁺CD8⁺ cells. Of interest, we detected paradoxical activation of immunosuppressive populations, including MDSCs and Tregs, by Delta-24-RGDOX, which was partially reversed by the addition of IDO inhibitors. Furthermore, the depletion of CD4⁺ cells, the main target of the OX40 ligand/receptor synapse⁴⁸, abrogated the antitumor efficacy of this combination therapy. These findings underscored the critical role of the CD4⁺ T cell population, but not the Treg population, in the eradication of tumors treated with Delta-24-RGDOX and an IDO inhibitor.

Treatment with Delta-24-RGDOX infection and IDO inhibition led to functional activation of antitumor immune cells.

To study the function of T cells in response to treatment, we performed coculture-based functional immune assays. Thus, splenocytes from GL261-5 tumor-bearing mice treated with Delta-24-RGDOX with or without IDO inhibitors were cocultured with uninfected or Delta-24-RGDOX-infected glioma cells to assess antitumor immune responses (Fig. 8A-D). We showed that splenocytes isolated from mice treated with the combination therapy displayed robust immune activation against Delta-24-RGDOX-infected glioma cells, as determined by measuring the secreted levels of the Th1 cytokines IFN γ and IL-2 (Fig. 8C). Importantly, we detected the same trend of immune activation against uninfected glioma cells (Fig. 8B), suggesting that tumor infection resulted in antigen spreading that led to recognition of tumor antigens. We also evaluated this T-cell activation at an earlier timepoint, which further confirmed the antitumor activation of splenocytes against uninfected cells (Supplementary Fig. 8).

We then sought to dissect the negative role of Kyn in T cell activation using similar functional assays (Fig. 8D). To this end, we challenged the effect of IDO inhibition by enriching the culture medium with Kyn and then examined the activity of splenocytes cocultured with glioma cells⁴⁹. As expected, the secretion of IL-2 was increased in cocultures of splenocytes isolated from virus-treated mice compared to control-treated mice when either uninfected cells or virus-infected cells were included, validating the virus-induced activation of T cells against both tumor antigens and viral antigens (Fig. 8D). Interestingly, the addition of Kyn partially decreased T cell functionality by reducing IL-2 secretion, evidencing that Kyn had a negative effect on virus-activated splenocytes (Fig. 8D)⁴⁹.

These functional assays showed the robust effect of IDO inhibition on the activation of immune cells against cancer cells and the significant reversion of this immune activation by downstream activators of the IDO signaling circuitry. In addition, these data indirectly suggested that the IDO-Kyn circuitry has a role in counteracting the antitumoral virus-mediated activation of T cells.

Discussion

A recent plethora of clinical trials in adults and children with brain tumors have shown that oncolytic viruses prolong the survival of a small percentage of patients (<20%)^{3,38-41}. Importantly, these studies have also shown that viroimmunotherapy induces T cell infiltration into brain tumors^{3,38-41}. Such findings support the paradigm-shifting concept that complete tumor debulking by virotherapy requires the elicitation of antitumor immune responses following the initial oncolytic effect. Therefore, further enhancement of the immune arm of this treatment approach may be required to increase the percentage of positive responders⁵⁰. Here, we showed that the infection of a tumor with the armed oncolytic adenovirus Delta-24-RGDOX was followed by a striking activation of the immune response. Intriguingly, RNA-seq analyses further revealed that Delta-24-RGDOX infection promoted the paradoxical activation of immunosuppressive pathways, including the IDO cascade, and immunosuppressive populations, such Tregs and MDSCs. This double-edged activation of positive and negative immune regulators is occasionally observed during viral infections⁵¹⁻⁵³. As exemplified by the transcriptional signature of the treated gliomas in this study, viral infection activates immune checkpoint regulators, including CTLA-4, PD-1, and IDO⁵⁴. This counterintuitive effect of tumor infection was confirmed by *in vitro* and *in vivo* studies showing that Delta-24-RGDOX infection is followed by the upregulation and activation of the IDO cascade, a notable finding that is in agreement with previous reports showing that IDO is upregulated in human glioma cells upon exposure to the major antiviral cytokine IFN γ , which is also a transcriptional regulator of IDO^{20,55}. Thus, our results are consistent with the cell and tissue upregulation of IDO following cytokine stimulation in the context of infection, autoimmunity, and cancer¹⁹.

Downstream of IDO, AhR is activated by viral infection⁵⁶ and induces immunosuppression^{11,12,57-59}, which in turn can favor cancer progression. In human patients with glioblastoma, the expression of AhR is associated with a poor prognosis⁶⁰. Thus, the initial effect of Delta-24-RGDOX may be counteracted by the expression of the IDO-AhR cascade and the subsequent prevention of an antitumor immune response. Therefore, we hypothesized that targeting the IDO pathway should have a double-positive effect: on the one hand, improving virotherapy and, on the other hand, decreasing intrinsic tumor immunosuppression (Fig. 9).

Of further clinical relevance, IDO and AhR are potential druggable targets in malignant gliomas^{17,60-62}. In this work, we demonstrated that Delta-24-RGDOX combined with IDO

inhibitors induced prolonged survival and generated a higher rate of long-term survival than control treatments in murine models of glioma and intracranial melanoma. The addition of IDO inhibitors is required to induce anticancer effects in several models of cancer. For example, HPV16-E6-E7/HRAS-driven lung epithelial cancer was suppressed by only the combination of HPV16 E7 vaccines, OX40 agonists, and IDO inhibitors⁶³. Furthermore, Berrong *et al*⁶³ showed that while the addition of anti-OX40 to an antigen-specific cancer vaccine moderately enhanced therapeutic efficacy, it was the addition of an IDO inhibitor to this treatment that eventually led to complete regression of established tumors in 60% of treated mice. Additionally, in agreement with our data, Sagiv-Barfi and colleagues combined intratumoral delivery of an adenovirus-related TLR9 ligand with OX40 activation to increase anticancer T cell responses⁶⁴.

The IDO-Kyn-AhR pathway potentiates systemic toxicity during viral infection^{30,31}. Moreover, activation of the IDO-Kyn-AhR cascade worsens influenza virus infection⁶⁵⁻⁶⁸. In addition, constitutive AhR activation reduces the type I IFN antiviral response²³. Therefore, the inhibition of the IDO circuitry should likely limit the potential systemic toxicity of Delta-24-RGDOX in clinical trials. In this study, we did not observe any sign of toxicity in mice treated with the combination therapy.

The OX40 pathway is predominantly active in CD4⁺ T cells⁶⁹. In our models, the anticancer effect of Delta-24-RGDOX infection combined with IDO inhibition was due to the elicitation of a robust antitumor immune response. Thus, we observed increased absolute counts of tumor infiltrating CD4⁺ and CD8⁺ T lymphocytes in combination treated tumors. These observations aligned with our functional studies showing that Kyn negatively regulated T cell responses to viral or tumor antigens. Furthermore, antibody depletion of CD4⁺ T cells abolished the antitumor effect of the combination therapy, but the anticancer effect was unaffected when Tregs were depleted. Together, the T cell depletion studies indicate that the T helper cell response is required to elicit antitumor immune responses and that IDO inhibition mimics Treg inhibition.

Our data also indicated that the virus-mediated upregulation of the IDO cascade was associated with intriguing increases in the MDSC and Treg populations, which were partially counteracted by IDO inhibition. In this regard, IDO orchestrates immunosuppressive effects through Treg-dependent recruitment and activation of MDSCs⁴⁷. Furthermore, IDO expression in solid tumors is strongly associated with Treg and MDSC infiltration, which can be reversed by

IDO inhibition⁴⁷. Moreover, IDO activity inhibits the proliferation of antigen-specific T lymphocytes, inducing tumor tolerance^{59,70-73}, and further inhibits T cell-based adaptive immunity by promoting the differentiation of Tregs in tumors^{15,74-76}, which can be induced by Kyn¹¹ and result in the suppression of antigen-specific T cell responses^{49,77}. It is important to note that in our cell system, the addition of Kyn to cocultures of splenocytes from treated mice and cancer cells efficiently suppressed the activation of the immune cells elicited by IDO inhibition.

In conclusion, our findings reveal that immunosuppressive pathways play a prominent role in the resistance of solid tumors to oncolytic virotherapy. Furthermore, the activity of the tumor microenvironment IDO circuitry is responsible, at least partially, for the remodeling of local immunosuppression after tumor infection. Combining molecular and immune-related therapies may improve outcomes in human gliomas and other cancers treated with virotherapy. These highly translatable studies should propel the development of a clinical trial to test the safety and efficacy of the combination of oncolytic adenoviruses armed with T cell activators and IDO inhibitors in patients with glioblastoma and other solid tumors.

Data availability

All data are available in the main text or the supplementary materials.

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Author contributions

Conceptualization and design: T.T.N., H.J., F.F.L., M.M.A., D.A.W., C.G.-M., J.F. Development of methodology and acquisition of data: T.T.N., D.H.S., S.S., Y.R.-M., X.F., J.G., L.Z., E.L., K.L.L. Data analysis: T.T.N., S.K.S., H.J., S.S., C.A.-B., F.G.V. Supervision: C.G.-M., J.F. Writing, review, and/or revision of the manuscript: T.T.N., C.G.-M., J.F.

Competing interests

M.M.A., H.J., F.F.L., C.G.-M., and J.F. report license agreements with DNATRIX, Inc. C.G.-M. and J.F. are founders of DNATRIX, Inc. MMA reports DNATRIX-sponsored research not related to this work.

Additional information

Supplementary information is submitted with this manuscript.

Figure 1: Delta-24-RGDOX treatment remodels the tumor microenvironment.

Fig. 1. Delta-24-RGDOX treatment remodels the tumor microenvironment. Differentially expressed genes in tumors treated with PBS versus tumors treated with Delta-24-RGDOX were utilized for clustering, gene ontology (GO) biological process enrichment analysis, and pathway and network analyses. (A) Heatmap comparing the transcriptional signatures of intracranial GL261-5–derived tumors treated with PBS or Delta-24-RGDOX. The log₂-normalized expression levels of genes with significant adjusted *P*-values (<0.05) across samples are shown. The color scale is shown above the heatmap. (B) GO biological process enrichment results for tumors treated with PBS or Delta-24-RGDOX. The five most significant GO biological processes are shown. GO biological processes significantly associated with PBS-treated tumors are marked as ‘+’, whereas those significantly associated with Delta-24-RGDOX–treated tumors are marked as ‘–’. (C) The five most significantly altered canonical pathways in tumors treated with PBS versus Delta-24-RGDOX. Activation z-scores are plotted in the graph. [†]Hypercytokinemia/hyperchemokinemias in the pathogenesis of influenza; ^{††}neuroinflammation signaling pathway. (D) The five most significantly altered upstream regulators in tumors treated with PBS versus Delta-24-RGDOX. Activation z-scores are plotted in the graph. The negative z-scores represent activation in Delta-24-RGDOX. (E) IDO1 network genes with significantly altered expression levels in tumors treated with PBS or Delta-24-RGDOX. The color intensity indicates the log₂ fold-change (green = activation) levels for each gene in PBS-treated tumors versus Delta-24-RGDOX–treated tumors. (F) The prediction of the immune cell composition in GL261-5–derived brain tumors treated with PBS or Delta-24-RGDOX. The percentages of various immune cell populations in each sample are presented in the graph. The color code for the various immune cell types is shown to the right of the graph.

Figure 2: Oncolytic adenoviruses induce the expression and activation of the IDO-Kyn-AhR cascade *in vivo* and *in vitro*.

Fig. 2. Oncolytic adenoviruses induce the expression and activation of the IDO-Kyn-AhR cascade *in vivo* and *in vitro*. (A) IDO expression in GL261-5 and GSC-005 murine glioma cells in response to Delta-24-RGDOX infection. Cells were infected with Delta-24-RGDOX (100-150 multiplicities of infection [MOIs]) over 48 h. RNA was extracted, and the relative levels of IDO were measured using qRT-PCR; GAPDH or β -Actin was used as a housekeeping gene control. The column graph shows $2^{-(Ct^{GAPDH \text{ or } \beta\text{-Actin}} - Ct^{IDO})}$ results normalized to the mock-infected control. (B and C) Relative IDO expression in GL261-5 or 4T1.2 tumors in response to oncolytic adenovirus treatment *in vivo*. In (B and/or D), the mice were implanted intracranially with GL261-5 or GSC-005 cells and intratumorally treated with PBS, Delta-24-RGD, or Delta-24-RGDOX; brain tumors were collected and flash-frozen on day 12. In (C), the mice were implanted with 4T1.2 cells in the right mammary fat pad and treated with PBS, D24-RGDOX, or indoximod (IDOi); tumors were collected and flash-frozen on day 36. RNA was extracted and analyzed as described in (A). (D) The Kyn and Trp metabolite levels in murine gliomas following the indicated treatments (described in [B]) were quantified using liquid chromatography–mass spectrometry. Data are presented as the ratios $\pm$ SDs of Kyn concentrations to Trp concentrations. (E) AhR activity in human GSC lines and HeLa cells in response to Delta-24-RGDOX infection. Cells were mock-infected or infected with Delta-24-RGDOX (50 MOI) over 48 h, and the transcriptional activity of AhR in the cells was quantified by evaluating the supernatants. Controls included medium (negative), medium containing the AhR agonist MeBio (0.32 nM), and medium containing Kyn (25 μ M). HeLa cells were used as a positive control. (F) AhR expression and nuclear translocation upon Delta-24-RGDOX treatment. HeLa cells were infected with Delta-24-RGDOX (25 MOI) over 48 h or treated with Kyn (positive control) and then immunostained for AhR detection. Representative images of AhR-FITC staining, DAPI (nuclear) staining, and merged FITC/DAPI staining are shown. Scale bars, 50 μ m. (G) Quantification of the mean fluorescence intensity of AhR in whole cells and nuclear compartments. (H) Frequency of nuclear AhR-positive cells after the indicated treatments, as analyzed using ImageJ software. Data are shown as the means $\pm$ SDs. P-values were generated by using a two-tailed Student's t-test.

Figure 3: The viral activity of Delta-24-RGDOX is preserved in the context of IDO inhibition.

Fig. 3. The viral activity of Delta-24-RGDOX is preserved in the context of IDO inhibition.

(A) Viral replication of Delta-24-RGDOX in human GSCs with or without an IDO inhibitor (IDOi). The viral concentration (pfu/ml) at 48 h after cell infection with Delta-24-RGDOX (25 MOI) alone or in combination with an IDOi (BGB-7204; 150 nM) was determined using a hexon titration assay performed with HEK293 cells. Initial viral input: $\log_{10}(6.57)$ pfu/ml. (B) Expression of the viral protein E1A in whole-cell lysates of human GSCs at 48 h after treatment with Delta-24-RGDOX (25 MOI) with or without an IDOi (BGB-7204; 150 nM), as assessed by Western blotting. Tubulin was used as a loading control. UVi, UV-inactivated Delta-24-RGDOX. (C-E) Viability of human GSCs infected with Delta-24-RGDOX (25 MOI) in the presence or absence of an IDOi (BGB-7204; 150 nM). (F) Viral replication of Delta-24-RGDOX at 48 h after infection of murine glioma cells (100-150 MOIs) with or without treatment with an IDOi (BGB-7204; 50 nM), as explained in (A). Initial viral input: $\log_{10}(7.18)$ to $\log_{10}(7.35)$ pfu/ml. (G) Expression of the viral protein E1A in murine glioma cells at 48 h after treatment with Delta-24-RGDOX (100-150 MOI) alone or in combination with an IDOi (BGB-7204; 50 nM), as explained in (B). (H-I) Viability of murine glioma cells infected with Delta-24-RGDOX (100-150 MOIs) and treated with or without an IDOi (BGB-7204; 50 nM). Data are shown as the means $\pm$ SDs. P-values were derived by using a two-tailed Student's t-test.

Figure 4: IDO inhibition modulates the tumor microenvironment of Delta-24-RGDOX treated murine brain tumors.

Fig. 4. IDO inhibition modulates the tumor microenvironment of Delta-24-RGDOX-treated murine brain tumors. Differentially expressed genes in tumors treated with PBS, the IDO inhibitor (IDOi) indoximod, Delta-24-RGDOX (RGDOX), or the IDOi and Delta-24-RGDOX (combo) utilized for clustering, immune population prediction, and pathway and network analyses. **(A)** Heatmap comparing the transcriptional signatures of intracranial GL261-5–derived tumors treated with PBS, the IDOi, Delta-24-RGDOX, or the combination therapy. Tumors were established in these mice for 21 days, and the mice underwent treatment for 14 days. The log₂-normalized averaged expression levels of genes with significant adjusted *P*-values (<0.05) across sample groups are shown. The color scale is shown above the heatmap. **(B)** The prediction of the immune cell composition in GL261-5–derived brain tumors in the indicated treatment group. The percentages of various immune cell populations in each sample are presented in the graph. The color code for the various immune cell types is shown at the bottom of the graph. **(C)** Heatmap or **(D)** pathway representation of IDO1 network genes with significantly altered expression levels in tumors; treatment group comparisons are indicated. The color scale is shown above the heatmap. The color intensity of the pathway representation graphics indicates the log₂ fold-change levels for each gene in the specified treatment group comparison; gray represents unchanged, red represents activation, and green represents inhibition.

830 **Figure 5: Combined treatment with Delta-24-RGDOX and IDO inhibition results in an enhanced therapeutic effect and tumor regression.**

Fig. 5. Combined treatment with Delta-24-RGDOX infection and IDO inhibition results in an enhanced therapeutic effect and tumor regression. (A) Treatment schedule of wild-type (WT) and IDO-KO C57BL/6 mice bearing intracranial (ic) GSC-005 tumors. GSC-005 cells were implanted intracranially (ic) on day 0, and the mice were randomly assigned to receive intratumoral (it) injection of either Delta-24-RGDOX or vehicle. Survival was monitored for up to 200 days. (B) Kaplan–Meier survival curves of the mice included in the experiment depicted in (A). (C) Treatment schedule of C57BL/6 mice bearing intracranial tumors that were treated with Delta-24-RGDOX alone or in combination with an IDO inhibitor (IDOi). OG, oral gavage. (D) Kaplan–Meier survival curves of intracranial GL261-5 tumor-bearing C57BL/6 mice treated with vehicle or with Delta-24-RGDOX alone or in combination with the IDOi 1-methyl-DL-tryptophan (1MT; n=10/group). (E) Long-term survivors previously treated with the combination therapy described in (D) were rechallenged with an intracranial injection of GL216-5 cells into the contralateral hemisphere, and their survival was compared with that of control treatment-naïve mice (n=5/group). (F) Kaplan–Meier survival curves of intracranial GSC-005 tumor-bearing C57BL/6 mice treated with vehicle or with Delta-24-RGDOX alone or in combination with the IDOi BGB-7204 (n=9-10/group). (G) Kaplan–Meier survival curves of intracranial B16 melanoma-bearing C57BL/6 mice treated with PBS or with Delta-24-RGDOX alone or in combination with the IDOi indoximod (n=6 or 7 per group). (H and I) The brains of intracranial GL261-5 or GSC-005 tumor-bearing C57BL/6 mice were subjected to histopathological analyses on day 15 (GL261-5) or 24 (GSC-005) after treatment with PBS, Delta-24-RGDOX, indoximod, or the combination therapy (n=5-8/group). Representative images of H&E staining (H) and the average tumor surface areas (I) were acquired using Aperio ImageScope software. Scale bar, 2 mm. P-values were derived with the log-rank test (B, D-G), a two-tailed Student's t-test (I), or an ordinary one-way ANOVA (I).

Figure 6: Combined Delta-24-RGDOX and IDO inhibitor treatment increases intratumoral T cells and decreases immunosuppressive cell populations.

Fig. 6. Combined Delta-24-RGDOX and IDO inhibitor treatment increases intratumoral T cells and decreases immunosuppressive cell populations. (A) Treatment timeline for the analysis of immune cell populations. C57BL/6 mice were intracranially (ic) implanted with GL261-5 or GSC-005 cells and randomly assigned to receive PBS (control), an IDO inhibitor (IDOi; GL261-5: indoximod. GSC-005: BGB-7204), Delta-24-RGDOX, or Delta-24-RGDOX plus IDOi. On day 24, brains were collected, stained as indicated, and analyzed using flow cytometry. Parallel experiments were performed for immunohistochemical analyses of the brains. it, intratumorally; OG, oral gavage. (B) Column graphs show the absolute numbers of CD45⁺CD3⁺ cells per tumor-containing brain hemisphere in the indicated murine glioma model. (C) Representative CD3 immunohistochemistry images for the indicated treatment groups. Images were acquired using Aperio ImageScope pathology slide viewing software. Scale bar, 100 μ m. (D and E) Column graphs show the absolute numbers of CD4⁺ (D) and CD8⁺ (E) T cells per hemisphere in the indicated murine glioma models. (F) Enrichment plots for the Treg (top) and MDSC (bottom) gene sets in PBS-treated versus Delta-24-RGDOX–treated or Delta-24-RGDOX- versus Combo–treated GL261-5 brain tumor RNA. (G) Column graphs show the absolute numbers of CD4⁺CD25⁺FOXP3⁺ Tregs per hemisphere (top) and CD45⁺GR1⁺CD11b⁺ MDSCs per hemisphere (bottom). Data are shown as the means $\pm$ SDs (n = 3). P-values were derived with an ordinary one-way ANOVA (B, D-E) or a two-tailed Student's t-test (G).

Figure 7: Therapeutic efficacy of combined Delta-24-RGDOX infection and IDO inhibition requires CD4+ T-cell activity but not regulatory T-cell activity.

Fig. 7. The therapeutic efficacy of combined Delta-24-RGDOX infection and IDO inhibition requires CD4⁺ T cell activity but not Treg activity. (A) Treatment schedule for Delta-24-RGDOX given alone or in combination with an IDO inhibitor (IDOi) in the presence of anti-CD4 or anti-CD25 depleting antibodies. Mice were intracranially (ic) implanted with GL261-5 cells and randomly assigned to receive intratumoral (it) injections of PBS or the combination of Delta-24-RGDOX and the IDOi indoximod (n=10 or 11 per group, respectively). An IgG control antibody and the anti-CD4 and anti-CD25 depleting antibodies were administered intraperitoneally. OG, oral gavage. (B) Flow cytometry plots showing CD4⁺, CD8⁺, and CD4⁺CD25⁺ T cell populations in the spleen of treatment-naïve mice and mice treated with IgG2b, anti-CD4, or anti-CD25 at 38 days after tumor implantation. (C) Column graphs show the frequencies of CD4⁺ or CD25⁺ T cells in the indicated treatment groups. (D) Kaplan–Meier survival curves of GL261-5 tumor-bearing C57BL/6 mice in the different treatment groups. Data are shown as the means ± SDs. P-values were derived with a two-tailed Student's t-test (C) or the log-rank test (D).

Figure 8: Combined oncolytic virus and IDO inhibitor treatment enhances anti-viral and anti-tumor T-cell activation.

Fig. 8. Combined oncolytic virus and IDO inhibitor treatment enhances antiviral and antitumor T cell activation. (A) Treatment timeline for the functional splenocyte assays. C57BL/6 mice were intracranially (ic) implanted with GL261-5 cells and randomly assigned to receive PBS, the IDO inhibitor (IDOi) indoximod, Delta-24-RGDOX, or Delta-24-RGDOX plus indoximod. On day 24, splenocytes from the tumor-bearing mice were cocultured with the indicated prefixed target cells for 48 h, and the concentrations of secreted IFN γ or IL-2 were assessed by ELISA. it, intratumoral; OG, oral gavage. (B-D) Column graphs show the levels of secreted IL-2 (top) or IFN γ (bottom) in cocultures containing splenocytes from mice in the indicated treatment groups and mock-infected GL261-5 cells (B), GL261-5 cells infected with Delta-24-RGDOX (C), or uninfected or Delta-24-RGDOX-infected GL261-5 cells with or without kynurenine (Kyn, 25 μ M) supplementation in the medium (D). Data are shown as the means $\pm$ SDs (n=3). Indicated P-values were evaluated using a two-tailed Student's t-test.

Figure 9: Schematic illustration of the reshaping of the tumor microenvironment by the inhibition of virotherapy and IDO inhibition.

Fig. 9. Schematic illustration of the reshaping of the tumor microenvironment by the inhibitory effects of virotherapy and IDO inhibition. The glioma microenvironment is characterized by a paucity of T cells, with the few cells present skewed toward the Th2 phenotype. This Gata-3-driven Th2 phenotype promotes IL-4-based functions and the recruitment of MDSCs, Tregs, and anergic PD-1⁺ or CTLA-4⁺ T cells. The infection of gliomas with oncolytic viruses, such as Delta-24-RGDOX, induces a Th1 phenotype with increased production of IL-12 and/or IFN γ . However, viral infection activates both the IFN γ -driven IDO-Kyn-AhR cascade and the maintenance of immunosuppression via the activation of MDSCs and Tregs. The addition of IDO inhibitors to virotherapy results in an imbalance in signaling with a predominance of Th1 and CD8⁺ cytotoxic T cells, leading to tumor eradication. (Figure created with BioRender.com.)

Supplementary Files

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