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

Background Oncolytic viruses are considered part of immunotherapy and have shown promise in preclinical experiments and clinical trials. Results from these studies have suggested that tumor microenvironment remodeling is required to achieve an effective response in solid tumors. Here, we assess the extent to which targeting specific mechanisms underlying the immunosuppressive tumor microenvironment optimizes viroimmunotherapy.

Methods We used RNA-seq analyses to analyze the transcriptome, and validated the results using Q-PCR, flow cytometry, and immunofluorescence. Viral activity was analyzed by replication assays and viral titration. Kyn and Trp metabolite levels were quantified using liquid chromatography–mass spectrometry. Aryl hydrocarbon receptor (AhR) activation was analyzed by examination of promoter activity. Therapeutic efficacy was assessed by tumor histopathology and survival in syngeneic murine models of gliomas, including Indoleamine 2,3-dioxygenase (IDO)-/ mice. Flow cytometry was used for immunophenotyping and quantification of cell populations. Immune activation was examined in co-cultures of immune and cancer cells. T-cell depletion was used to identify the role played by specific cell populations. Rechallenge experiments were performed to identify the development of anti-tumor memory.

Results Bulk RNA-seq analyses showed the activation of the immunosuppressive IDO-kyurenine-AhR circuitry in response to Delta-24-RGDOX infection of tumors. To overcome the effect of this pivotal pathway, we combined Delta-24-RGDOX with clinically relevant IDO inhibitors. The combination therapy increased the frequency of CD8+ T cells and decreased the rate of myeloid-derived suppressor cell and immunosuppressive Treg tumor populations in animal models of solid tumors. Functional studies demonstrated that IDO-blockade-dependent activation of immune cells against tumor antigens could be reversed by the oncometabolite kynurenine. The concurrent targeting of the effectors and suppressors of the tumor immune landscape significantly prolonged the survival in animal models of orthotopic gliomas.

Conclusions Our data identified for the first time the in vivo role of IDO-dependent immunosuppressive pathways in the resistance of solid tumors to oncolytic adenoviruses. Specifically, the IDO-Kyn-AhR activity was responsible for the resurfacing of local immunosuppression and resistance to therapy, which was ablated through IDO inhibition. Our data indicate that combined molecular and immune

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Oncolytic viruses are promising antiglioma agents. We have reported that 20% of patients with glioma treated with a single intratumoral injection of an oncolytic adenovirus underwent complete or partial responses; however, in addition to driving an anti-tumor immune response, the infection of a tumor elicits the resurfacing of an immunosuppressive environment that opposes and counteracts the tumor eradication of the tumor.

WHAT THIS STUDY ADDS

⇒ In this work, we combined an oncolytic virus, which carries a positive activator of T-cells, with the inhibition of the Indoleamine 2,3-dioxygenase (IDO) cascade, a key player in the tumor immunosuppressive environment. This therapeutic strategy reshaped the tumor microenvironment in favor of an antitumor immune response and prolonged the survival of glioma-bearing mice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, OR POLICY

⇒ According to our data, the design of future clinical trials with oncolytic adenoviruses should consider the addition of IDO inhibitors to enhance the immune-mediated aspect of the antitumor effect.
therapy may improve outcomes in human gliomas and other cancers treated with virotherapy.

INTRODUCTION

Natural types of human viral infections can induce remission in several types of cancers. During the pandemic, patients with cancer infected with SARS-CoV-2 have experienced complete remissions, 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 glioblastoma. 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. 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) 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. Based on these preclinical results, Delta-24-RGDOX is now 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, however, suppressive mechanisms within the tumor microenvironment restrain the effector immune response. 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 responses. 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 inhibitors. The catabolism of tryptophan (Trp) by IDO produces kynurenine (Kyn), a direct activator of aryl hydrocarbon receptor (AhR), promoting robust immunosuppression. The IDO-AhR multicycuitry plays a fundamental role at the center of the immune synapse and acts as a bridge among the main immune cell populations in tumors.

Activation of the IDO cascade is a major characteristic of several tumors, including gliomas, and correlates with poor prognoses. In addition to its role in tumors, IDO is activated in various tissues and cell subsets following cytokine stimulation during infection. Several clinical trials are examining IDO inhibitors as anticancer drugs. Most of these inhibitors (epacadostat, BMS-986205, navoximod, KHK2455, and BGB-7294) are direct IDO enzymatic inhibitors, whereas indoximod is a Trp mimetic that imitates an artificial Trp-sufficiency signal on immune cells in an attempt to reverse IDO pathway-mediated suppression.

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.

METHODS

Methods are included in online supplemental materials.

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 (figure 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 (figure 1C). We also performed IPA to identify altered upstream regulators in tumor treated with Delta-24-RGDOX infection. This analysis identified IFNγ, TNF, IL1B, IFNα, and STAT1 as candidate regulators of the transcriptional response to Delta-24-RGDOX infection (figure 1D). 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) (figure 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. These data agreed with changes in the immune populations observed in murine models of glioma and melanoma on infection with oncolytic adenoviruses, as well as those observed in surgical specimens from gliomas infected with Delta-24-RGD.

Based on the rationale that IDO is triggered by IFNγ and upregulated during viral infections, we asked...
Figure 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 used for clustering, gene ontology (GO) biological process enrichment analysis, and pathway and network analyses. Analyses of tumors were performed on day 12, 5 days after the first dose of Delta-24-RGDOX. (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 vs Delta-24-RGDOX. Activation z-scores are plotted in the graph. †Hypercytokinemia/hyperchemokinemia 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) 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. (F) 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 vs Delta-24-RGDOX-treated tumors. IDO, indoleamine 2,3-dioxygenase.
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 TGFβ, CTLA-4, PD-1, and GATA-3 (figure 1F).

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-related pathway 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 AhR.22–24 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 activation of cytokines can modulate IDO expression. Of note, IDO was the first gene discovered to be inducible by cytokines, such as IFN-γ

Collectively, these analyses indicated that Delta-24-RGDOX increased Kyn/Tryptophan (T) ratio than control mouse brain tumors (figure 2C) and increased IDO expression in human and murine glioma cells following treatment with IFN-γ, a potent mediator of antiviral immune responses. In addition, IDO is regulated by other molecular mechanisms activated during infection, such as the JAK/STAT26 and IFN type I transduction signaling pathways.27–29

In in vitro experiments, we observed a remarkable increase in IDO expression in human and murine glioma cells following treatment with IFNγ (online supplemental figure S1). We also determined that infection of GL261-5 and GSC-005 murine glioma cells with Delta-24-RGDOX triggered a sixfold to sevenfold increase in IDO levels, as assessed by qRT-PCR (figure 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 (figure 2B,C).

Kyn is a product of the metabolic activity of IDO, 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 on viral infection, both GL261-5- and GSC-005-derived tumors infected with Delta-24-RGDOX showed a significantly higher Kyn/Tryptophan ratio than control mouse brain tumors (figure 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.31 Importantly, AhR has dual functions as a transcriptional target for immune escape by viruses and a promoter of immunosuppression in solid tumors, including gliomas.34–37 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 human glioma cell lines using supernatant of infected cells. We found that Delta-24-RGDOX treatment significantly increased AhR transcriptional activity (figure 2E), suggesting the treatment with the oncolytic adenovirus will have a effect on the IDO-Ahr pathway in infected cells and, multiplying the initial effect, in neighboring cells. Because the binding of Kyn to AhR promotes AhR translocation to the nucleus,34 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 (figure 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 (figure 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 (online supplemental figure S2). 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 (online supplemental figure 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 (online supplemental figure 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...
Figure 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 hours. 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^(-ΔΔCt) results normalized to the mock-infected control.
(B, C) Relative IDO expression in GL261-5 or 4T1.2 tumors in response to oncolytic adenovirus treatment in vivo. In (B), the mice were implanted intracranially with GL261-5 or GSC-005 cells and intratumorally treated with PBS 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) Relative UV fingerprints of 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±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 hours, 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 μM). HeLa cells were used as a positive control.
(F) AhR expression and nuclear translocation on Delta-24-RGDOX treatment. HeLa cells were infected with Delta-24-RGDOX (25 MOI) over 48 hours or treated with Kyn (positive control) and then immunostained for AhR expression. Representative images of AhR–FITC staining, DAPI (nuclear) staining, and merged FITC/DAPI staining are shown. Scale bars, 50 μm.
(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.

AhR, aryl hydrocarbon receptor.
performed RNA-seq analysis of GL261-5 tumors from mice treated with PBS, an IDO inhibitor or Delta-24-RGDOX alone or in combination. Hierarchical clustering of genes and samples showed that the transcriptome signatures of PBS-treated 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 (figure 3A,C). There was also a clear difference in the global transcriptome signature between Delta-24-RGDOX- and combination therapy-treated mice (figure 3A,C). Furthermore, network analyses of differentially expressed genes demonstrated that the addition of IDO network 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 stimulator of interferon genes (STING) pathway (figure 3C).

The predicted immune cell composition and transcriptome signature showed minimal differences between PBS-treated and IDO inhibitor-treated brain tumors (figure 3B). Interestingly, upon treatment with Delta-24-RGDOX, the estimated percentage of CD8 T cells almost doubled compared with that in the group treated with IDO inhibition alone (Delta-24-RGDOX: 23.7%; IDO inhibitor: 12.0%; PBS: 16.3%); the combination of Delta-24-RGDOX and the IDO inhibitor did not show significant changes in the CD8+ T cell transcriptome frequency compared with Delta-24-RGDOX alone (combination: 26.2%) (figure 3B).

The altered immune responses of combination therapy vs Delta-24-RGDOX-treated mice confirmed and complemented the results that showed increased activation of the IDO network in Delta-24-RGDOX-treated mice. In this experiment, performed 14 days after the first viral administration, we did not observe the upregulation of the IDO transcript detected 5 days after viral treatment (figure 2), but the IDO network was still activated. Additionally, we observed a decrease in IDO pathway activation in tumors from combination therapy-treated mice compared to mice in the control group (figure 3C,D). These data also illustrated the ability of the IDO inhibitor to decrease the number of IDO-related transcripts induced in tumors infected with the adenovirus (figure 3D). Remarkably, the combination treatment decreased the expression of more than 90% of the upstream and downstream IDO-related transcripts induced on 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 pathway 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, 38-41 chimeric antigen receptor T cells, 39-44 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 a genetically mediated IDO inhibition in the tumor microenvironment, Delta-24-RGDOX prolonged median survival and produced 20% long-term survivors (online supplemental figure S3). We then compared the survival of glioma-bearing IDO-KO mice and wild-type mice treated with Delta-24-RGDOX or PBS (figure 4A,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 (figure 4C,D). The median survival times of the control-treated mice and the IDO inhibitor 1MT-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 the 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=0.004; vs Delta-24-RGDOX).

Next, we asked whether the antiglioma effect of the combined Delta-24-RGDOX and IDO inhibitor therapy is immune mediated. 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 (figure 4C,E). As expected, treatment-naive 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 (figure 4C,F). In this case, we used BGB-7204, a clinical-grade CNS-penetrating...
IDO1 enzyme inhibitor. The PBS-treated 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 with control treatment, with a median survival time of 53 days (p=0.02). Similar to the GL261-5 model, the GSC-005 tumor-bearing mice treated with the

**Figure 3** 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. Examination of tumors were performed on day 21, 14 days after the first dose of Delta-24-RGDOX. (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, in the experiment delineated in (A). 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, green represents activation, and red represents inhibition. IDO, indoleamine 2,3-dioxygenase.
Figure 4  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 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-naive 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, H) 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 (G) and the average tumor surface areas (H) were acquired using Aperio ImageScope software. Scale bar, 2 mm. P values were derived with the log-rank test (B, D–F) and a two-tailed Student’s t-test (G–I). The difference between the arms Delta-24-RGDOX and Delta-24-RGDOX+IDOi was calculated using the restricted mean survival Qme (RMST) to account for the long-term survivors. IDO, indoleamine 2,3-dioxygenase.
combination of Delta-24-RGDOX and the IDO inhibitor exhibited the longest median survival (63 days) and resulted in 44% long-term survivors, showing a significant long-term survival rate in comparison to Delta-24-RGDOX as a single treatment (p=0.04).

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 showed progressive tumor regression in the mice treated with the combination therapy (figure 4G,H; online supplemental figures S4, S5). Of note, 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 (online supplemental figure S6).

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 antitumor effect of combination treatment with Delta-24-RGDOX infection plus IDO inhibition is dependent on CD4+ T cell activity

To further understand the immune-mediated mechanisms underlying the antitumor effect of the combination of Delta-24-RGDOX infection plus IDO inhibition, we assessed the phenotypic changes in the lymphocyte population following treatment (figure 5A). 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 with control-treated mice (figure 5B). These results were confirmed with immunohistochemical assessments of CD3+ T cells in the two murine brain tumor models (figure 5C; online supplemental figure S7). Analysis of absolute counts of intratumoral CD3+, CD4+, and CD8+ T cell populations showed a significant difference among the treatment groups (figure 5B, D and 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), 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 (figure 5F, 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 (figure 5F, right panels). These results were confirmed by flow cytometric analyses of the tumor microenvironment of virus-treated and combination-treated mice (figure 5G).

Importantly, whereas Delta-24-RGDOX alone increased Treg and MDSC populations, Delta-24-RGDOX plus the IDO inhibitor significantly decreased these crucial immunosuppressive populations. Similar results were observed from the analysis of CD4+CD25+FoxP3+, which have been considered Tregs precursors (48 49) (online supplemental figure S8).

Since CD4+ T cells constitute the target of the OX40 ligand/receptor synapse,46 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 glioblastoma. In this case, we used Indoximod as IDO inhibitor, which is being tested in clinical trials. Our results showed that combination of Delta-24-RGDOX with Indoximod was more potent than the combination using 1MT (figure 4D). To analyze the role of CD4+ T cells, we performed these cells using anti-CD4 antibodies (figure 6A–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 (figure 6D). This loss of the antitumor effect indicates that the combined therapy depends on the CD4+ T helper cell population to induce maximal antitumor effects and produce long-term survivors.

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 synapse,46 abrogated the antitumor efficacy of this combination therapy. These findings underscored the critical role of the CD4+ T cell population in the eradication of tumors treated with Delta-24-RGDOX and an IDO inhibitor.

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

To study the immune response to treatment, we performed coculture-based functional immune assays. 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 (figure 7A–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.
Figure 5  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 µm. (D, E) Column graphs show the absolute numbers of CD4+ (D) and CD8+ (E) T cells per hemisphere in the indicated murine glioma model. (F) Enrichment plots for the Treg (top) and MDSC (bottom) gene sets in PBS-treated versus Delta-24-RGDOX-treated or Delta-24-RGDOX-treated 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±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). ANOVA, analysis of variance; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid-derived suppressor cell.
IFNγ and IL-2 (figure 7C). Importantly, we detected the same trend of immune activation against uninfected glioma cells (figure 7B), suggesting that tumor infection resulted in antigen spreading that led to recognition of tumor antigens. We also evaluated immune activation at an earlier timepoint, which further confirmed the anti-tumor activation of splenocytes against uninfected cells (online supplemental figure S9).
We then sought to dissect the negative role of Kyn in immune activation using similar functional assays (figure 7D). 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 cells. As expected, the secretion of IL-2 was increased in cocultures of splenocytes isolated from virus-treated mice compared with control-treated mice when either uninfected cells or virus-infected cells were included, validating the virus-induced immune activation against tumor antigens (figure 7D). Interestingly, the addition of Kyn partially decreased immune cell activation by reducing IL-2 secretion, providing evidence that Kyn had a negative effect on virus-activated splenocytes (figure 7D).

These functional assays showed the robust effect of IDO inhibition on the activation of immune cells against cancer cells and the significant reversal 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 anti-tumoral virus-mediated immune activation.

**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%). Importantly, these studies have also shown that viroimmunotherapy induces T cell infiltration into brain tumors. 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 responders, and we previously reported that the infection of a tumor with the armed oncolytic adenovirus Delta-24-RGDOX was followed by a striking activation, and significantly more robust than that triggered by the previous generation Delta-24-RGD, of the immune response in murine syngeneic models of both glioma and melanoma.

Here, we show that RNA-seq analyses revealed that Delta-24-RGDOX infection also 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 infections.

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 IDO. 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 IFNγ, which is also a transcriptional regulator of IDO. Thus, our results are consistent with the cell and tissue upregulation of IDO following cytokine stimulation in the context of infection, autoimmunity, and cancer.

Downstream of IDO, AhR is activated by viral infection and induces immunosuppression, which in turn can favor cancer progression. In human patients with glioblastoma, the expression of AhR is associated with a poor prognosis. 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.

Of further clinical relevance, IDO and AhR are potential druggable targets in malignant gliomas. 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 gliomas. 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 inhibitors. Furthermore, Berrong et al 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 et al combined intratumoral delivery of an adenvirus-related TLR9 ligand with OX40 activation to increase anticancer T cell responses.

The IDO-Kyn-AhR pathway potentiates systemic toxicity during viral infection. Moreover, activation of the IDO-Kyn-AhR cascade worsens influenza virus infection. In addition, constitutive AhR activation reduces the type I IFN antiviral response. Based on these data, although in this study we did not observe any sign of toxicity, the inhibition of the IDO circuitry should likely limit the potential systemic toxicity of Delta-24-RGDOX in clinical trials.

The OX40 pathway is predominantly active in CD4+ T cells. 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, indicating that the T helper cell response is required to elicit antitumor immune responses.

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 in solid tumors immunosuppressive effects through Treg-dependent recruitment and activation of MDSCs, which can be reversed by IDO inhibition. Moreover, IDO activity inhibits the proliferation of antigen-specific T lymphocytes, inducing tumor tolerance, and further inhibits T cell–based adaptive immunity by promoting the differentiation of Tregs in tumors, which can be induced by Kyn and result in the suppression of antigen-specific T cell responses. 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 Delta-24-RGDOX.

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.

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Supplemental material
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Supplementary Methods

Cell lines. The murine glioma cell lines GL261 (Tumor Bank Repository, National Cancer Institute, Frederick, MD), GL261-5 (a clone with slower in vivo growth kinetics), and human HeLa and HEK293 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium with nutrient mixture F12 (DMEM/F12) (Corning). Murine GSC-005 glioma cells (kindly provided by I.M. Verma, The Salk Institute for Biological Studies, CA) were maintained in DMEM/F12 supplemented with N2 (1x; Invitrogen), fibroblast growth factor-2 (20 ng/ml; PeproTech), epidermal growth factor (20 ng/ml; Promega), and heparin (50 μg/ml; Sigma). The murine breast cancer cell line 4T1.2 expressing the luciferase gene (kindly provided by C. Bartholomeusz, MD Anderson Cancer Center, TX) was maintained in RPMI 1640 medium (Corning). The human glioma-like stem cell lines GSC11, GSC20, GSC7-2, GSC13, and GSC8-11 were cultured in DMEM/F12 with B27 (1x; Invitrogen), antibiotic-antimitotic (1x; ThermoFisher Scientific), fibroblast growth factor-2 (20 ng/ml), and epidermal growth factor (20 ng/ml). Cultures, except for cultures of GSCs, were supplemented with 10% fetal bovine serum (HyClone Laboratories) and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin; Corning). All cells were kept at 37°C in a humidified atmosphere containing 5% CO2.

Oncolytic adenoviruses. A previously constructed oncolytic adenovirus, Delta-24-RGDOX, was propagated in human lung carcinoma A549 cells. Virions were collected and purified using the Adenopure kit (Puresyn, Inc.) following the manufacturer’s instructions. Viral titers and replication were determined by measuring the plaque-forming units per ml (pfu/ml) using conventional methods. Briefly, HEK293 cells (2.5 × 10^5) were incubated in 24-well plates with serial dilutions of the viral stock. Forty-eight hours later, cultures were fixed with 100% ice-cold methanol for 10 min at −20°C. Cells were stained for hexon expression using an anti-adenovirus polyclonal antibody (Millipore Sigma, AB1056; 1 h, 37°C) followed by secondary staining with a biotinylated anti-goat IgG antibody (H+L, Vector Biolabs, BA-5000; 1 h, 37°C). The Vector Vectastain ABC kit (Vector Biolabs, PK-4000) and ImmPACT DAB peroxidase substrate kit (Vector Biolabs, SK-4105) were utilized for the visualization of positive cells. Hexon-stained areas were counted under a light microscope (20× objective) in 10 individual fields per well. In wells with viral dilutions showing 5-50 positive cells/field, the viral titer was calculated using the following formula: pfu/ml = [(mean number of positive cells/field) × (fields/well)] / [volume virus (ml) × dilution factor]. The antibodies used are shown in Supplementary Table 1.

Viral replication assay. To determine the replication ability of Delta-24-RGDOX in the adherent cell lines used in this study, we seeded 1.5 × 10^5 cells/well in a 12-well plate and counted the cells after letting them attach overnight to calculate the appropriate amount of Delta-24-RGDOX needed to achieve specified multiplicities of infection (MOIs). Delta-24-RGDOX was diluted in PBS to achieve the specified MOIs and added dropwise to the attached cells, which were cultured in 200 μl of serum-free media for 20 min at 37°C and shaken every 5 min to disperse the virus. The serum-free media was then removed, and the cells were cultured in 1 ml of complete medium. Non-adherent cells were cultured with diluted Delta-24-RGDOX in 200 μl of serum-free media for 20 min at 37°C and shaken every 5 min to disperse the virus, and then 800 μl of complete medium was added. After a 48-h incubation period, adherent and non-adherent cells and supernatant were collected, flash-frozen and thawed 3 times, and then centrifuged at 500 × g for 5 min. The supernatants were used for viral titration in HEK293 cells, as explained in the “Oncolytic adenoviruses” section above.
Viral-induced cytopathic ability of virus assay. To determine the virus-induced cytopathic ability of Delta-24-RGDOX, we seeded $1 \times 10^4$ cells/well in a 96-well plate (75 µl media/well). An additional 75 µl of media containing the appropriate treatment was then added to the cells. The virus-induced cytopathic ability was measured every 24 h for up to 168 h by quantifying ATP levels using the ViralToxGlo Assay (Promega) according to the manufacturer’s protocol.

IDO inhibitors. The IDO inhibitors indoximod and 1-methyl-DL-tryptophan (1MT; Sigma-Aldrich) were suspended in PBS containing 3-mm glass balls (Thomas Scientific, #3000), which was rotated overnight to help re-suspend the drug. The clinical grade direct IDO enzyme inhibitor BGB-7204 (BeiGene; kindly provided by D. Wainwright, Northwestern University, IL), whose pharmacokinetic/pharmacodynamics properties have been reported previously, was suspended in Ora-Plus oral suspending vehicle (Perrigo). For in vitro use of these inhibitors, DMSO (Sigma) was used as the diluent.

In vivo studies. For glioma implantations, $5 \times 10^4$ GL261-5 or GSC-005 cells/mouse or $1 \times 10^3$ B16-F10 cells/mouse were implanted into the caudate nucleus of 7- to 10-week-old male or female C57BL/6 mice using a guide-screw system as described previously. The mice were then randomly assigned to experimental groups. Treatment began 7 days after tumor cell implantation. Delta-24-RGDOX adenoviruses ($5 \times 10^7$ – $1 \times 10^8$ pfu/dose) were injected intratumorally on days 7, 9, and 11. For 4T1.2 breast tumor implantations, $1 \times 10^4$ 4T1.2 cells/mouse were injected into the right mammary pads of 7- to 10-week-old female BALB/c mice; intratumoral Delta-24-RGDOX ($1 \times 10^8$ pfu/dose) injections were administered on days 10, 14, 16, 18, and 21. Indoximod (275 mg/kg), 1MT (400 mg/kg), or BGB-7204 (100mg/kg) was administered twice daily, by oral gavage, 5 days/week, from days 7-35 after tumor implantation. Mice surviving 120 days were re-challenged with a new intracranial tumor injection using GL261-5 cells ($5 \times 10^4$ cells/mouse) on the contralateral side of the initial tumor injection. For the depletion studies, anti-CD4 depletion antibodies or rat IgG2b isotype control antibodies were administered to mice intraperitoneally starting on day 4 after tumor implantation and every 4 days thereafter until day 36. C57BL/6 IDO-knockout (KO) mice (B6.129-Ido1tm1Alm/J; stock no. 005867) were purchased from The Jackson Laboratory and bred in MD Anderson Cancer Center’s Research Animal Support Facility. The depletion antibodies used are shown in Supplementary Table 1. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of MD Anderson Cancer Center and National Institutes of Health and United States Department of Agriculture guidelines.

Preparation of single-cell suspensions from murine brains and spleens. Spleens and tumor-bearing brain hemispheres were collected from the mice. Initial suspensions were obtained by cutting the tissue or grinding the organs, filtering them through 100-µm cell strainers (Fisher Scientific), and then placing the cell suspension in RPMI 1640 medium (10 ml/sample). All tissues were pelleted by centrifugation (500 x g for 7 min at room temperature [RT]). The spleen-derived pellet was re-suspended in Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich) to lyse the red blood cells according to the manufacturer’s instructions. Then, the cell suspension was brought up to 20 ml/sample with RPMI 1640 medium to stop the lysis reaction and washed once in 1x PBS. The brain-derived pellet was washed once with HBSS (Corning), and then the cells were incubated in 8 pg/ml Liberase TM (Millipore Sigma) for 15 min at 37°C, washed again with HBSS, and then resuspended in 40% Percoll (1.130 g/ml; GE Healthcare), which was overlaid on top of 80% Percoll at a 1:1 ratio. The cells were centrifuged for 20 min at 500 x g at RT with an acceleration of 1 and a deceleration of 0, and the lymphocye gradient
interphase was collected and washed once with PBS. The cells from the brains and spleens were pelleted by centrifugation at 500 × g for 7 min at RT and finally re-suspended in FACS buffer (PBS containing 10 mM HEPES, 2 mM EDTA, and 1% fetal bovine serum).

**RNA sequencing and data analysis.** Total RNA was extracted from flash-frozen tumor using the RNeasy Plus Mini Kit (Qiagen). Sequencing was performed by Novogene. RNA quality control was performed to measure quantification using Nanodrop, test RNA degradation or potential contamination using agarose gel electrophoresis, and check for RNA integrity using Agilent 2100 Bioanalyzer System. Library construction was developed from the mRNA of eukaryotic organisms, which was enriched using oligo(dT) beads. This eukaryotic mRNA was then fragmented randomly in fragmentation buffer, and then cDNA was synthesized using random hexamers and reverse transcriptase. After first-strand synthesis, a custom second-strand synthesis buffer (Illumina) was added with dNTPs, RNase H, and *Escherichia coli* polymerase I to generate the second strand by nick translation. The final cDNA library was ready after a round of purification, terminal repair, A-tailing, sequencing adapter ligation, size selection, and PCR enrichment. Sequencing was performed using HiSeq machines (Illumina). Key steps of the RNA-seq data analysis included the evaluation of the quality of the reads by fastqc (fastqc/0.11.8) followed by the removal of the sequencing adapters and unpaired reads by trimmomatic (trimmomatic/0.33) 8. These trimmed FASTQ files were used to map reads to the mouse genome /ENSEMBL.mus_musculus.release-75 using the STAR aligner (star/2.6.0b) 9. Feature counts were extracted from the resulting .bam files by subread (subread/1.6.3) 10. Estimates of unwanted variations in the raw read counts across samples were determined using the remove unwanted variation (RUV) method on the Galaxy platform, which estimates the factors of unwanted variation using replicate samples 11. The RUV method uses the empirical Bayes approach to estimate a moderated t-statistic. These estimates were used as batch factors in DESeq2 analyses to determine differentially expressed genes in the group comparisons 12. For the preparation of heatmaps, the log2 normalized values for genes with significant adjusted P-values (<0.05) from the DESeq2 analyses were utilized; both rows and columns were hierarchically clustered using 1 − r, where r is the Pearson correlation, with average linkage. For gene ontology (GO) enrichment analysis, the log2 fold change (FC) values for genes with significant adjusted P-values (<0.05) from the DESeq2 PBS vs. RGDOX were used; enrichment analyses were performed on http://www.pantherdb.org/, with false discovery rate correction. Briefly, the genes with significant fold change are compared against each ontology category in panther database. The values for the genes in each ontology category are compared statistically to the overall distribution of values to look for coordinated shifts across that category. The cutoffs for the ingenuity pathway analyses (IPAs) were P-values ≤0.05 and log2 FCs of ±1; activation z-scores are plotted on graphs. An activation z-score of ±2 was considered significant. The expression levels of genes with significant FCs and P-values in the sample group comparisons analysis were overlaid onto the IDO1 network curated from the IPA knowledge base. For the prediction of immune cell compositions as inferred from RNA-seq data, we input transcripts per million for each sample to the seq-ImmucC platform 13, and the predicted percentages of various immune cell populations in each sample were plotted in Excel. The gene set enrichment analyses were performed with GSEA_4.0.3 software, and the whole-genome expression profiles of PBS- and Delta-24-RGDOX–treated tumors were used as inputs. The Treg gene set (Supplementary Data File 1) was curated from the IPA knowledge base, and the MSDC gene set (Supplementary Data File 2) was based on a previous publication 14.
Aryl-hydrocarbon-receptor activity assay. For the assessment of the activity of aryl-hydrocarbon-receptor (AhR) induced by Delta-24-RGDOX, cells were mock-infected or infected with Delta-24-RGDOX (50 MOIs) and incubated for 48 h. The transcriptional activity of AhR in cell supernatants was quantified using an AhR assay kit (Indigo Biosciences), which utilizes AhR reporter cells expressing a luciferase reporter gene functionally linked to an AhR-responsive promoter. The protocol was performed according to the manufacturer’s instructions.

AhR immunofluorescence. HeLa cells were seeded in a 96-well black-sided, clear-bottom tissue culture plate (Corning) at a density of 1 × 10^4 cells per well. Cells were mock-treated, treated with kynurenine (150 µM; Santa Cruz Biotechnology), or treated with Delta-24-RGDOX (25 MOI) in complete media for 48 h. Then, antibody immunofluorescence staining was performed by following the procedures provided by the antibody manufacturer. Briefly, cells were washed with PBS and fixed with 4% formaldehyde in PBS for 10 min at RT. After the wells were washed 3 times with wash buffer (2 mg/ml BSA in PBS), the cells were permeabilized with 0.2% Triton X-100 for 30 min at RT in a moist chamber. After permeabilization, the cells were washed 3 times and overlaid with the primary antibody against AhR (Santa Cruz Biotechnology) containing 2 mg/ml BSA. After overnight incubation, the cells were washed 3 times and incubated with the FITC-conjugated secondary antibody (Invitrogen, Alexa Fluor 488 goat anti-mouse) for 1 h at RT. The cells were washed 4 times and then stained with DAPI (Invitrogen; 1 µg/ml). The cells were then overlaid with mounting medium and imaged using an Axiovert 200 inverted microscope (Zeiss). The cellular and nuclear AhR intensities were quantified using ImageJ software (NIH). The antibodies and their working dilutions are shown in Supplementary Table 1.

Quantitative real-time polymerase chain reaction. For the detection of mRNA levels from cultured cells, Trizol reagent was used according to the established routine protocol. For the detection of mRNA levels from freshly dissected tissue samples, the RNeasy Plus Mini Kit (Qiagen) was used. One microgram of total RNA was reverse-transcribed into mRNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Primers were purchased from Sigma Aldrich (human IDO, R: TGGAGGAACGTAGACGCACT, F: TTCAGTGCTTTTGACGTCTCG; mouse IDO, R: TTGCGGGGCGACACCTTTTGC, F: CCCACACTGAGCACGGACGG). The RT-PCRs were conducted in a 96-well plate with a holding stage of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C, followed by an infinite hold at 4°C. Relative gene expression was calculated using the 2^−ΔΔCt method by normalizing the threshold cycle (Ct) values of the gene of interest to the Ct values of the internal housekeeping gene.

Western blotting. For the detection of protein expression, cell lysates were prepared using RIPA lysis buffer (20 mM HEPES pH 7.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 5 mM sodium pyrophosphate, 80 mM β-glycerophosphate, 50 mM NaF, and 0.1% SDS) plus freshly added protease inhibitor cocktail (1x; Sigma-Aldrich), the proteasome inhibitor MG-132 (1 µM; Calbiochem), and phosphatase inhibitor cocktail 3 (2.5 mg/ml; Sigma-Aldrich). Cell lysates were flash-frozen in liquid nitrogen and thawed 3 times and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected, and the concentration of protein was measured using Bradford reagent (Bio-Rad). DTT (50 mM; ThermoFisher) and NuPAGE LDS sample buffer (ThermoFisher) were added to 10-15 µg of total protein. The samples were heated at 95°C for 5 min, run on 4-20% Novex Tris-Glycine gels.
(Invitrogen), and then transferred to a PVDF membrane (ThermoFisher) and probed with the primary antibodies overnight at 4°C and then subjected to secondary antibody staining for 1 h at RT the next day. Protein bands were visualized using Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer). The antibodies and working dilutions used are shown in Supplementary Table 1.

**Liquid chromatography–mass spectrometry.** For the detection of IDO activity, the kynurenine and tryptophan levels of flash-frozen brain tumors from mice in the different treatment groups were measured by the Mass Spectrometry Facility at the University of Texas Medical Branch, Galveston, TX. Liquid extraction was used to extract polar metabolites from the frozen brain tissue. Briefly, 400 µl of 80% methanol was added to the frozen tissue. Zirconia/silica beads (1-mm diameter) were added to the tube. The tissue was broken down using a bead beater by pulsing the tissue for 45 s. The resulting solution was kept on ice for 30 min to precipitate the proteins. Next, 400 µl of chloroform was added, and the tube was pulsed in the bead beater for an additional 45 s and kept on ice for 15 min. Then, 200 µl of water was added to induce phase separation. The tube was centrifuged, and the upper aqueous phase containing the amino acids/amines was carefully transferred to another tube. Of the 500-µl aqueous phase, 250 µl was dried under vacuum. The dried metabolites were resuspended in 40 µl of buffer used for the derivatization of the amino acids/amines. Of this, 10 µl was taken for further derivatization and analysis by liquid chromatography–mass spectrometry (LC-MS). Standard calibration curves of known concentrations of amino acids, including tryptophan and kynurenine, were made. Before analysis by LC-MS, 5 µM of stable isotope-labeled amino acids, including tryptophan and kynurenine, in 10 µl were added to each sample. The concentrations of kynurenine or tryptophan in the analysis sample were then calculated using linear regression on the standard concentrations by plotting the area ratio (the ratio of the peak area of analyte to the peak area of the internal standard) and the known concentrations. Concentrations of kynurenine or tryptophan were normalized to tumor mass.

**Flow cytometry analysis.** For the analysis of cell surface protein expression, single cell suspensions from murine brains and spleens in a 96-well round-bottom plate were first blocked in anti-mouse CD16/CD32 Fc Block diluted with FACS buffer and then washed once with 250 µl of cold PBS. The cells were then incubated in 100 µl of Fixable Viability Stain 780 (1:1000; BD Biosciences) at 4°C in the dark for 30 min and then washed once with 250 µl of cold PBS. Then, the cells were incubated in 100 µl of primary antibody solution diluted in FACS buffer. After incubation at 4°C in the dark for 30 min, the cells were washed once with 250 µl of cold PBS. For the analysis of intracellular proteins, cells were stained with the eBioscience FOXP3/Transcription Factor Staining Buffer Set (Invitrogen) following the manufacturer’s instructions. The cells were finally re-suspended in 0.3 ml of FACS buffer containing 123count eBeads Counting Beads (Invitrogen) to acquire an accurate output for absolute cell count. The stained cells were analyzed using the FACSCelesta flow cytometer (BD Biosciences). FlowJo software, version 10 (FlowJo, LLC), was used for the analysis. To control for the technique and to arrange population gates accurately, we generated fluorescence-minus-one samples for each antibody using pooled spleen cells from brain tumor–bearing mice in different treatment groups. The antibodies and working dilutions used are shown in Supplementary Table 1.

**Analysis of splenocyte stimulation in co-cultures with target cells.** Target cells were seeded and treated with the specified agents. Four hours later, mouse interferon gamma (IFNγ; 100 units/ml; ProSpec) was added to the cultures. Forty-eight hours after viral infection, the cells were detached with 2 mM EDTA in PBS, fixed with 1% paraformaldehyde, and cleaned with lysine (0.1 M) wash solution.
A total of $2 \times 10^4$ fixed cells were seeded in 96-well round-bottom dishes. For immune cell activation, pre-fixed target cells were co-cultured with splenocytes ($5 \times 10^5$/well) in RPMI 1640 medium containing 100 μg/ml penicillin (Corning), 100 μg/ml streptomycin (Corning), and 55 μM beta-mercaptoethanol (Gibco) for 48 h. Then, the concentration of IFN-γ or interleukin-2 (IL-2) in the supernatant was assessed with standard ELISA (IFN-γ or IL-2 DuoSet ELISA, R&D Systems) according to the manufacturer’s instructions.

**Histopathological staining.** Tumor-bearing mouse brains were fixed in 10% buffered formalin for 24 h, transferred to 70% ethanol, and then embedded in paraffin for slide sectioning. Paraffin-embedded sections of the mouse brain tumors were deparaffinized at 60°C for 1 h and rehydrated with xylene and ethanol following conventional procedures. For hematoxylin and eosin staining, brain tumor sections were stained with Harris hematoxylin (Fisher Scientific) and Eosin-Y solution (Fisher Scientific) and mounted with Cytoseal 60 (Thermo Scientific). For CD3 immunohistochemistry, antigens were retrieved from the brain tumor sections by exposing the slides to 10 mM citric acid (pH 6.0) inside a steamer for 30 min. The slides were then incubated in 3% hydrogen peroxide in 100% methanol for 10 min at RT to quench endogenous peroxidases. The sections were then subjected to blocking with 5% goat serum in PBS for 1 h at RT followed by incubation with a primary rabbit monoclonal anti-CD3 antibody overnight and then incubation with a biotinylated anti-rabbit IgG secondary antibody diluted in 1% goat serum. The Vector Vectastain ABC kit (Vector Biolabs, PK-4000) and ImmPACT and DAB Peroxidase Substrate Kit (Vector Biolabs, SK-4105-Reagent 1) were utilized to visualize positive cells. Images were captured using an Aperio ScanScope slide scanner, which was also used to measure high-grade tumor areas. Specific antibody information is given in Supplementary Table 1.

**Statistical analyses.** Experiments involving the groups included in the quantitative analyses were performed at least in triplicate. GraphPad Prism 9 was used to perform all statistical analyses and generate all graphs for the *in vitro* and *in vivo* experiments. To determine statistical differences between 2 groups, we performed a two-tailed Student t-test; to determine statistical differences among 3 or more groups, we performed an ordinary one-way ANOVA. The animal survival curves were plotted according to the Kaplan–Meier method. Survival rates in the different treatment groups were compared using the log-rank test, and to account for the presence of long-term survivors, we calculated the difference in restricted mean survival Qme (RMST) of the two groups, Delta-24-RGDOX (arm 0) and Delta-24-RGDOX+IDOi (arm 1), using the R package survRM2 version 1.0-4. At the truncation time tau we used the last point examined as default $^{15,16}$. The statistical analyses of RNA sequencing data are described in the “RNA sequencing and data analysis” section above. *P*-values < 0.05 were considered significant.
References


Supplemental Figure S1—IFN-γ induces IDO expression.

**A**

Human Cancer Cells

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**B**

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**Supplemental Figure S1. IFN-γ induces IDO expression.** (A and B) Relative IDO expression of an array of human (A) and murine (B) cancer cells in response to IFN-γ. Cells were mock-treated or treated with IFN-γ (200 u/ml) for 24-48 h. RNA was extracted, and relative levels of IDO were measured using qRT-PCR: GAPDH or β-Actin was used as a housekeeping gene control. The column graphs show $2^{ΔΔCt}$ normalized to the control. Data are means ± SDs. P-values were derived from a two-tailed Student t-test.
Supplemental Figure S2—Delta-24-RGDOX activity is preserved in the context of IDO inhibition.

A

B

C

D

E

F

G

H

I

J Immunother Cancer


Supplemental Figure S2. 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 ± SDs. P-values were derived by using a two-tailed Student’s t-test.
Supplemental Figure S3—Delta-24-RGDOX elicits anti-cancer effect in an IDO-KO genetic background glioma murine model.

Supplemental Figure S3. Delta-24-RGDOX elicits anti-cancer effect in an IDO-KO genetic background glioma murine model. (A) Treatment schedule of intracranial GL261-5 tumor–bearing IDO-KO C57BL/6 mice receiving PBS or Delta-24-RGDOX (n=10 or 11 per group, respectively). Survival was monitored for up to 100 days. (B) Kaplan-Meier survival plot of IDO-KO GL261-5 tumor–bearing mice treated as explained in (A). P-value derived from a log-rank test.
Supplemental Figure S4—Combination of Delta-24-RGDOX and IDO inhibition induces anticancer effect in the intracranial GL261-5 glioma model.

A  GL261-5, Day 15

B  GL261-5, Day 24
Supplemental Figure S4. Combined Delta-24-RGDOX infection and IDO inhibition induces anticancer effect in the intracranial GL261-5 glioma model. (A and B) Representative images of H&E-stained coronal brain sections of intracranial GL261-5 tumor-bearing C57BL/6 mice. Mice were implanted with GL261-5 cells and intratumorally treated with Delta-24-RGDOX or PBS with or without the IDO inhibitor (IDOi) indoximod. Brains were collected on day 15 (A) or day 24 (B). Coronal brain sections were subjected to H&E staining and scanned using Aperio ImageScope pathology slide viewing software. Brains from individual mice are shown. Scale bars, 2 mm.
**Supplemental Figure S5**: Combination of Delta-24-RGDOX and IDO inhibition induces an anticancer effect in the intracranial GSC-005 glioma murine model.

(A) Representative images of H&E-stained brain sections from intracranial GSC-005 tumor-bearing mice receiving Delta-24-RGDOX alone or in combination with an IDO inhibitor (IDOi; BGB-7204). Mice were intracranially implanted with GSC-005 cells and randomly assigned to the indicated treatment groups 7 days later. On day 24, brains were collected, and brain sections were stained with H&E. Aperio ImageScope software was used to acquire images. Scale bars, 2 mm.
Supplemental Figure S6—Delta-24-RGDOX and IDO inhibitor combined treatment did not cause significant weight loss in mice.

(A) Change in Weight: GL261-5

(B) Change in Weight: GSC-005

Supplemental Figure S6. Combined Delta-24-RGDOX and IDO inhibitor treatment did not cause significant weight loss in mice. (A and B) XY plots showing the change in weight of mice bearing intracranial GL261-5 (A) or GSC-005 (B) tumors during indicated treatments over 12-15 days. Mice were implanted with GL261-5 or GSC-005 cells and administered the indicated treatments. (GL261-5 tumor–bearing mice received the IDO inhibitor [IDOi] indoximod; GSC-005 tumor–bearing mice received the IDOi BGB-7204). Weights of mice were measured on indicated day and were normalized to the baseline weight. P-values were derived from an ordinary one-way ANOVA.
Supplemental Figure S7—CD3+ T-cell infiltration increases upon treatment with IDO inhibitors in combination with Delta-24-RGDOX.

A

GL261-5

PBS

IDOi

D24-RGDOX

Combination

B

GSC-005

PBS

IDOi

D24-RGDOX

Combination
Supplemental Figure S7. CD3⁺ T-cell infiltration increases upon treatment with IDO inhibitors in combination with Delta-24-RGDOX. (A and B) Representative images of CD3 immunohistochemistry of brain sections from individual mice bearing intracranial GL261-5 (A) or GSC-005 (B) tumors. Mice were implanted with GL261-5 or GSC-005 cells and administered the indicated treatments. (GL261-5 tumor–bearing mice received the IDO inhibitor [IDOi] indoximod; GSC-005 tumor–bearing mice received the IDOi BGB-7204). Mice were humanely killed on day 24. Slides of tumor-bearing coronal brain sections were stained for CD3 expression using immunohistochemistry. Slides were scanned and images were acquired using Aperio ImageScope pathology slide viewing software. Scale bars, 100 μm.
Supplemental Figure S8. Analysis of the presence of CD4+CD25+FOXP3- cells after treatment

Supplemental Figure S8. Column graphs show the absolute numbers of CD4+CD25+FOXP3- cells per hemisphere. Data are shown as the means ± SDs (n = 3). P-values were derived with a two-tailed Student’s t-test.
Supplemental Figure S9. Combined Delta-24-RGDOX infection and IDO inhibition results in anti-tumor immune activation. (A) Timeline of treatment for the functional splenocyte assays. C57BL/6 mice were intracranially (ic) implanted with GL261-5 cells and randomly assigned to receive PBS (control), the IDO inhibitor (IDOi) indoximod, Delta-24-RGDOX, or indoximod plus Delta-24-RGDOX, it, intratumorally; OG, oral gavage. (B) Immune activity was assessed by co-culturing splenocytes from the treated GL261-5 glioma–bearing mice with prefixed GL261-5 cells for 48 h and then using ELISA to assess the concentration of secreted IFNγ. Column graphs show means ± SDs (n=3). P-values were derived from a two-tailed Student t-test.
Graphical Abstract

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

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In Brief
While the infection of gliomas with oncolytic viruses, such as Delta-24-RGDOX, induces a Th1 phenotype with increased production of IFNγ, viral infection also activates the IFNγ-driven IDO-Kyn-AhR cascade generating immunosuppression via the recruitment of MDSC and CD4+ Treg populations of cells. The addition of IDO inhibitors to virotherapy counteract this immunosuppressive effect by reinforcing the skew from Th1 to Th2 and CD8+ cytotoxic T cells in the tumor microenvironment, leading to glioma eradication.
## Supplemental Table S1: Antibody Information

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