Fludarabine as an Adjuvant Improves Newcastle Disease Virus-Mediated Antitumor Immunity in Hepatocellular Carcinoma

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INTRODUCTION

Oncolytic virotherapy is a highly promising treatment modality that uses replication-competent viruses to destroy cancers. The approved oncolytic virus T-VEC, a genetically engineered herpes simplex virus-1 (HSV-1) expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as OncoVEXGM-CSF, is a milestone in viro-immunotherapy.1 Therapeutic oncolytic viruses (OVs) activate antitumor immune responses by inducing immunogenic cell death and type I interferon (IFN)-mediated lymphocyte infiltration.2–5 Clinical trials of various OVs such as adenovirus, vaccinia, herpesvirus, reovirus, and paramyxovirus (measles virus and Newcastle disease virus [NDV]) in advanced cancer patients have shown promise.6

NDV is a member of the Avulavirus genus of the Paramyxoviridae family. In both preclinical and clinical studies, NDV induced the production of type I IFNs and is an effective oncolytic agent with a good safety record.7–15 On one hand, NDV induces both extrinsic and intrinsic apoptosis of malignant cells,10 and on the other hand, NDV infection elicits both innate and adaptive antiviral immunity, resulting in cross-activated antitumor immune responses.11,12 Localized therapy with oncolytic NDV induces an inflammatory response, leading to lymphocyte (NK1.1+, CD3−CD8−, and CD11b+ lymphocytes, and monocytes) infiltration and an antitumor effect in distant (nonvirally injected) tumors without dissemination of the virus. The therapeutic efficacy of NDV depends on CD8+ T cells, natural killer (NK) cells and type I IFNs, but not CD4+ lymphocytes.13,14

Limited viral replication and immune-negative feedback in the tumor microenvironment (TME) limit the efficacy of viro-immunotherapy for cancer. NDV is an enveloped negative-sense single-strand RNA virus7 that elicits an antiviral innate immune response via retinoic acid-induced gene I (RIG-I) signaling, which induces the production of type I IFNs and pro-inflammatory cytokines. Signal transducer and activator of transcription 1 (STAT1) amplifies the RIG-I-mediated IFN response to RNA viruses. Several viruses (e.g., hepatitis C virus, simian virus 5, and measles virus) interfere with STAT1 phosphorylation, thereby reducing type I IFN production, which benefits viral replication.15–17

Antitumor immune activation is often accompanied by immunenegative feedback, including the production of immunosuppressive cytokines, cell types, and negative co-stimulators. STAT3 plays a key role in generating an immunosuppressive TME by regulating inflammation and various immune cell types including myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and...
T helper 17 cells (Th17). Hyperactivation of STAT3 signaling occurs in the majority of human cancers and is correlated with a poor prognosis. STAT3 activation is mediated by various cytokines (e.g., interleukin-6 [IL-6], IL-10, and IFNs). Viral infection with, for example, hepatitis C virus, Epstein-Barr virus, and varicella-zoster virus, can activate the STAT3 signaling pathway in host cells. Whether NDV activates STAT3 in hepatocellular carcinoma (HCC) cells is unknown.

Metabolic changes in the TME determine the fate of immune cells such as survival, proliferation, polarization, and activities. Indoleamine 2,3-dioxygenase 1 (IDO1) is highly expressed by dendritic cells, macrophages, MDMCs, and tumor cells and catabolizes the essential amino acid tryptophan into kynurenine. Deprivation of tryptophan and accumulation of kynurenine and its metabolic product 3-hydroxyanthranilic acid lead to apoptosis or dysfunction of effecter T cells and induction of Tregs, respectively. IDO1 effector T cells and induction of Tregs, respectively. IDO1 expression therefore enhances the antitumor activity of adoptive T cells. It is yet unknown if fludarabine downregulates STAT3 signaling. Effective antitumor immunotherapy requires both immune activation and prevention of immunosuppression. Given its multiple functions, we hypothesized that fludarabine would be a powerful adjuvant for oncolytic viro-immunotherapy. In this study, we employed oncolytic NDV to activate antitumor immunity and used fludarabine as an adjuvant to enhance NDV replication and prevent concomitant immunosuppression in HCC.

**RESULTS**

**Fludarabine Enhances NDV-Mediated Oncolyis in HCC Cells** Fludarabine and NDV had dose-dependent cytotoxic effects on human and murine HCC cells (Figures 1A and 1B). Indeed, 200 nM fludarabine together with NDV at an MOI of 10 resulted in slight cytotoxic effects. Fludarabine dramatically enhanced the NDV-induced oncolysis of HCC cells (Figures 1C and 1D). Moreover, fludarabine and NDV significantly increased the number of annexin V-positive (apoptotic) cells (Figure S1), which indicates that fludarabine markedly enhances NDV-induced apoptotic cell death. Therefore, fludarabine significantly enhances the NDV-mediated oncolysis of HCC cells.

**Fludarabine Promotes NDV Replication by Inhibiting STAT1** Next, we quantified NDV replication by determining the viral production and the expression levels of Ndv-hn...
(hemagglutinin-neuraminidase) and NdV-m (matrix) genes. Fludarabine (200 nM) increased NDV replication 2- to 6-fold in HCC cells at 24 h after infection (Figures 2A and 2B). We then evaluated the expression of Ifnb (IFN-β) and Cxcl10 (C-X-C motif chemokine 10), key cellular innate antiviral factors. Viral infection was unaffected by fludarabine (Figure S2), however, Ifnb and Cxcl10 expression were significantly reduced by fludarabine at 6 h after infection (Figures 2C and 2D), suggesting that fludarabine may promote viral replication by mitigating antiviral innate immunity.

To further clarify how fludarabine downregulated Ifnb and Cxcl10 expression in cells, we assessed the activation of STAT1. In
HCC cells, STAT1 was activated by NDV at 12 and 24 h after infection (Figure S3), and it was significantly reduced by fludarabine (Figure 2E). We further found that STAT1 silencing by siRNA (Figure 2F) increased viral replication in HCC cells similar to fludarabine. Moreover, fludarabine failed to increase viral replication in STAT1-silenced HCC cells (Figures 2G and 2H). Consistently, both STAT1 silencing and fludarabine reduced Ifnb and Cxcl10 expression in NDV-infected HCC cells, and fludarabine did not suppress innate antiviral immunity in STAT1-silenced cells (Figures 2I and 2J). Taken together, fludarabine increases NDV replication in HCC cells by inhibiting the activation of STAT1.

Fludarabine Mitigates NDV-Induced Activation of STAT3 by Accelerating Ubiquitin-Proteasomal Degradation

STAT3 participates in cancer-related inflammation, cellular transformation, and immune suppression. NDV infection activated STAT3 (phosphorylated STAT3, p-STAT3) at 12, 24, and 48 h after infection (Figure 3A). Interestingly, NDV-induced STAT3 activation was markedly reduced by fludarabine (Figure 3B). In addition, the expression of IL-6, a pro-tumor inflammatory cytokine and downstream target of STAT3, was markedly upregulated by NDV but was reduced by fludarabine to ca. 50% to 70% (Figure 3C). These results suggest that fludarabine ameliorates NDV-induced inflammation by targeting STAT3 signaling in HCC cells. Next, we evaluated the mechanism by which fludarabine promotes degradation of p-STAT3. First, we investigated the role of STAT1. In STAT1-silenced HCC cells, the p-STAT3 level was not reduced by viral infection, and the fludarabine-induced decrease in p-STAT3 level was not recovered (Figure 3D). These data suggest that STAT1 signaling is not involved in the fludarabine-mediated inhibition of p-STAT3 in HCC cells. Second, we evaluated the role of autophagy in fludarabine-induced p-STAT3 degradation. At 2, 6, 12, and 24 h, we determined the ratio of microtubule-associated proteins 1A/1B light chain 3B (LC3-II/I). However, fludarabine did not increase the LC3-II/I ratio (a marker of autophagosome formation) in HCC cells (Figure 3G, lower), which indicates that p-STAT3 is not degraded in the autophagosome. However, in NDV infected HCC cells, the fludarabine-induced reduction in p-STAT3 level was almost completely blocked by the proteasome inhibitor MG132 (Figure 3E), which indicates that proteasomal degradation is involved in the elimination of p-STAT3. Moreover, cellular proteasomal activity was not altered by fludarabine in infected or uninfected HCC cells (Figure 3F). However, the levels of polyubiquitinated proteins, particularly those of similar size to p-STAT3 (75–100 kDa; Figure 3G) were affected by fludarabine. Following fludarabine treatment, the levels of polyubiquitinated proteins increased at 2 and 6 h and decreased at 12 and 24 h; the p-STAT3 level was also decreased at the latter two time points (Figure 3G, left). Therefore, fludarabine induces ubiquitin-proteasomal degradation of p-STAT3.

Fludarabine Antagonizes NDV-Induced IDO1 by Promoting Proteasomal Degradation

IDO1 plays a crucial role in tumor-associated immunosuppression and is upregulated during an immune response. IDO1 protein and mRNA levels were significantly increased in NDV-infected HCC cells (Figures 4A and 4B). Fludarabine dramatically decreased the NDV-induced increase in IDO1 level (Figure 4C). Interestingly, fludarabine significantly increased Ifnb mRNA expression in NDV-infected LM3 and Hepa1-6 HCC cells (Figure 4D). Moreover, in HCC cells treated with fludarabine and MG132, IDO1 expression was almost completely recovered (Figure 4E). Therefore, fludarabine promotes proteasomal degradation of IDO1 in NDV-infected HCC cells.

Fludarabine Increases NDV Replication In Vivo and Enhances NDV-Mediated Antitumor Immunity

In a mouse model of ascitic HCC (Figure 5A), fludarabine increased NDV replication 2- to 13-fold in ascitic cells on days 12 and 16 after tumor transplantation (Figures 5B and 5C) and markedly promoted p-STAT3 and IDO1 degradation (Figures 5D). Moreover, NDV induced an overall immune response in ascites, which was significantly increased by fludarabine (Figure 5E). Consistently, Ifnb, Cxcl10, and Ifnb expression was significantly upregulated by NDV and further increased by fludarabine (Figure 5F). Moreover, CD8+ T and NK1.1+ cell infiltration in ascites was increased almost 2-fold by NDV (Figures 5G and 5H). Interestingly, infiltration of NK cells, but not CD8+ T cells, was further significantly increased by fludarabine (Figures 5G and 5H). Notably, fludarabine at 7.5 mg/mouse did not have significant cytotoxic effects on CD8+ T and NK cells (Figures 5G and 5H). Surprisingly, NDV increased the number of MDSCs in ascites (CD11b+Gr-1int); importantly, fludarabine antagonized this effect (Figure 5I). Thus, fludarabine increases the oncolytic effects of NDV by upregulating viral replication, promoting degradation of p-STAT3 and IDO1, increasing NK cell infiltration, and reducing the number of MDSCs.

Fludarabine Enhances the Antitumor Efficacy of NDV

Next, we investigated the antitumor efficacy of NDV plus fludarabine in vivo. In the ascitic HCC model (Figure 6A), fludarabine enhanced the antitumor efficacy of NDV (Figures 6B and 5B) resulting in prolonged survival (Figure 6C). Of note, the abdomen circumference was markedly reduced in two of seven mice (Figure 5A), and one mouse acquired a complete response to the combination treatment (Figure 6C). In a subcutaneous HCC model (Figure 6D), fludarabine significantly enhanced the therapeutic efficacy of NDV (Figure 6E) and prolonged the survival. Three out of seven mice obtained completed responses to the combination treatment (Figure 6F). No obvious therapy-associated side effect or body-weight loss was recorded (Figures S5C and S5D). These results indicate that fludarabine improves the antitumor efficacy of NDV.

Fludarabine Antagonizes IFN-γ-Induced STAT3 Activation and Upregulation of IDO1

Negative feedback plays a crucial role in immune homeostasis and cancer-associated immune suppression. We further determined the influence of fludarabine on p-STAT3 and IDO1 in the presence of IFN-γ, which was upregulated by NDV (Figure 5E). While STAT3 was activated by IFN-γ at 2 and 12 h (Figure 7A), it was massively reduced by fludarabine (Figure 7B). Furthermore, MG132 abrogated the fludarabine-mediated reduction in p-STAT3 level, which
indicates promotion of its proteasomal degradation in the presence of IFN-γ. Similarly, IFN-γ-mediated upregulation of IDO1 was blocked by fludarabine (Figure 7C). The results indicate that fludarabine counters IFN-γ-induced immune-negative feedback such as activation of STAT3 and upregulation of IDO1.

**DISCUSSION**

OV-mediated viro-immunotherapy for cancer shows much promise; however, its clinical use is hampered by limited viral replication and immune-negative feedback. Here, we report that fludarabine is an effective adjuvant for oncolytic viro-immunotherapy. Fludarabine...
enhanced NDV-mediated viro-immunotherapy by promoting viral replication by targeting STAT1, which enhanced oncolysis, and promoting NDV-induced antitumor immune responses by accelerating the ubiquitin-proteasomal degradation of p-STAT3 and IDO1, increasing NK cell infiltration and reducing the number of MDSCs in the TME (Figure 8). Thus, fludarabine and NDV represent a promising oncolytic viro-immunotherapy.

Efficient replication in cancer cells is a crucial determinant for oncolytic virotherapy. Viral replication is mainly dependent on cell viability and host cellular antiviral innate immunity. Replication of oncolytic measles virus in lymphoma cells was increased by fludarabine; however, the mechanism is unknown. In this study, fludarabine at a low dose had little impact on cell viability but significantly enhanced NDV replication. We previously reported that autophagic flux favored oncolytic NDV replication by preventing virus-induced apoptosis. Fludarabine did not induce de novo formation of autophagosomes. Type I IFNs induce STAT1 expression as a part of positive feedback loop to augment the extent and duration of IFN responses, which may limit viral replication. Thus, as an inhibitor of STAT1, fludarabine promoted NDV replication by suppressing the production of type I IFNs at the early stage (Figure 2C). However, when more viral yields were generated, the production of type I IFNs was increased accordingly (Figures 5F and S4), which may further participate in immune activation.

![Figure 4. Fludarabine Accelerates the Proteasomal Degradation of IDO1](image-url)

(A and B) LM3, HepG2, Hepa1-6, and H22 cells were infected with NDV (MOI = 10) for 0, 6, 12, 24, or 48 h, harvested, and the IDO1 (A) protein level was quantified by western blotting and (B) mRNA level by qPCR. (C and D) Cells were infected with NDV (MOI = 10) in the presence or absence of fludarabine (200 nM) for 24 h and lysed, and the IDO1 (C) protein level was determined by western blotting and (D) the mRNA level by qPCR. Means ± SDs of quadruplicates are shown. (E) LM3 cells were infected with NDV (MOI = 10) in the presence or absence of fludarabine (200 nM) or MG132 (10 μM) for 24 h, and the IDO1 protein level was determined by western blotting. Representative blots (left) and IDO1/GAPDH intensity ratios of three independent experiments (right) are shown. GAPDH was used as a loading control. *p < 0.05; **p < 0.01; ***p < 0.001; #not significant.
Figure 5. Fludarabine Enhances NDV-Induced Antitumor Immunity In Vivo
(A) Mice received an ip. injection of 2 x 10^6 H22 cells followed by 1 x 10^7 pfu NDV with or without fludarabine (30 mg/kg, ip.) at the indicated time points (n = 4 per group).
(B and C) Ascitic cells were harvested at days 12 and 16, Ndv-hn and Ndv-m expression was quantified by qPCR (B), and NDV titers were measured by plaque assay (C).

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Activated STAT3 and IDO1 participate in cancer-associated immunosuppression, and their expression in cancer cells is induced by immune stimulation such as viral infection, IFNs, and adoptive immune cell therapy. Elevated levels of p-STAT3 and IDO1 are detrimental to viro-immunotherapy. Fludarabine is suitable for oncolytic viro-immunotherapy, as it suppressed NDV-activated IL-6/STAT3 signaling and IDO1 upregulation. Moreover, fludarabine significantly reduced p-STAT3 and IDO1 expression in HCC cells treated with IFN-γ, the level of which is markedly elevated during STAT3 activation, this decrease in the number of MDSCs was most suppressive. NDV infection induced polarization of monocytes into proinflammatory M1 macrophages and MDSCs in the TME. Indeed, fludarabine enhanced overall immune response induced by NDV in the ascitic fluid. Direct activation of NK cells contributes to the antitumor effects of NDV. NDV-induced NK-cell infiltration was further increased by fludarabine, possibly by increasing NDV replication.

Immunosuppressive MDSCs comprise granulocytic CD11b+Gr-1high cells and monocytic CD11b+Gr-1int cells, the latter subset of which is most suppressive. NDV infection induced polarization of monocytes to CD11b+Gr-1int MDSCs, which may repress antigen presentation. Surprisingly, fludarabine significantly decreased the number of MDSCs in the TME. Because the differentiation of MDSCs depends on STAT3 activation, this decrease in the number of MDSCs was likely due to accelerated degradation of p-STAT3. Furthermore, as IDO1 is correlated with the expansion, recruitment, and activation of these cells, the downregulation of IDO1 observed in vivo may contribute to the antitumor effects of NDV.

(D) Ascitic cells were harvested at day 16, and IDO1 and phosphorylated STAT3 protein levels (left) and IDO1/GAPDH and p-STAT3/GAPDH intensity ratios (right) were evaluated. (E) Ascitic cells were harvested on days 12 and 16, washed, counted, and subjected to the IFN-γ ELISPOT assay. (F) Ascitic cells were harvested on days 12 and 16, and IgG, Cxcl10, and Ifng expression was quantified by qPCR. (G–I) Ascitic cells were harvested on day 16 and subjected to flow cytometry analyses of (G) CD3+CD8+ cells, (H) NK1.1+ cells, and (I) CD11b+Gr-1int cells. *p < 0.05; **p < 0.01; ***p < 0.001; #not significant.
of MDSCs in tumors, the fludarabine-induced decrease in IDO1 expression might also have led to a reduction in the number of MDSCs. The ascitic HCC tumor model used in this study facilitates dynamic monitoring of viral replication, oncolysis, immune activation, and cell infiltration. Finally, the antitumor potency of fludarabine with NDV was validated in a subcutaneous HCC tumor model.

In conclusion, NDV induced an antitumor immune response in the TME as well as negative feedback, such as STAT3 activation, and increased IDO1 and MDSCs. Fludarabine not only promotes viral replication by targeting STAT1 but also accelerates proteasomal degradation of p-STAT3 and IDO1 and reduces the number of infiltrating MDSCs. These effects of fludarabine significantly prolong the survival of HCC-bearing mice. Our findings suggest the potential of fludarabine as an adjuvant for oncolytic viro-immunotherapy, but further work is required before it can be used clinically.

MATERIALS AND METHODS

Cell Culture and Reagents

The human HCC cell line HCCLM3 and mouse HCC cell line H22 were obtained from the China Center for Type Culture Collection; the human HCC cell line HepG2 and mouse HCC cell line Hepa1-6 were obtained from the Cell Bank of Type Culture Collection Chinese Academy of Sciences. These cell lines were authenticated by short tandem repeat (STR) analysis and tested for mycoplasma contamination. HCCLM3 (abbreviated LM3), HepG2, and Hepa1-6 cells were cultured in DMEM, whereas H22 cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (all from Thermo Fisher Scientific, Gibco, Grand Island, NY, USA), and maintained in a humidified incubator with atmosphere containing 5% CO2 at 37°C.

The following reagents were used in this study: fludarabine monophosphate (#F0913; Tokyo Chemical Industry, Tokyo, Japan), MG132 (#M8699; Sigma-Aldrich, St. Louis, MO, USA), human IFN-γ (#Z02915; GenScript, Piscataway, NJ, USA), trypsin blue (#ST798; Beyotime Biotechnology, Shanghai, China), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (#M2128; Sigma-Aldrich).

NDV Propagation, Viral Titers, and Infection

The NDV La Sota strain was a gift from Prof. Y. Wang (Jiangsu Academy of Agricultural Sciences, China), propagated in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs from seed virus, harvested from the allantoic fluid, and purified by centrifugation at 3,000 rpm for 10 min. The viral particles in the supernatant were harvested and cryopreserved at −80°C. Viral titers were determined by plaque assay. In brief, samples were serially diluted, and 100 μL of each dilution was added per well to Vero cells in 12-well plates. 2 h later, DMEM (containing 2 μg/mL N-tosyl-L-phenylalanine chloromethyl ketone [TPCK]-treated trypsin, 2% FBS, and 0.8% agar) was added and cells were incubated at 37°C for 4 days. Then cells were fixed with 4% paraformaldehyde solution and stained with 0.5% neutral red solution for observation of plaques.

Tumor cells were washed once with PBS and infected with NDV in OptiMEM (Thermo Fisher Scientific, Gibco) at the indicated MOI for 2 h, and complete medium was added to each well.

Cytotoxic Effect Assay

MTT Cell Viability Assay

Cells were seeded in 96-well plates and treated with fludarabine, NDV, or both at the indicated doses. Cell viability was determined after 48 h of incubation by adding 100 μL MTT solution (1 mg/mL). Following 4 h incubation at 37°C, the MTT solution was aspirated, 50 μL L-methionine was added per well, and the absorbance at 570 nm was recorded using the SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Trypan Blue Exclusion

Cells were harvested using trypsin-EDTA (0.25%) solution (Thermo Fisher Scientific, Gibco) and stained with trypan blue solution; viability was determined by trypan blue exclusion using the Countstar Automated Cell Counter (Inno-Alliance Biotech, Wilmington, DE, USA). The cell death (%) was calculated as number of dead cells/total number of cells × 100%. The cell activity (%) was calculated as number of live cells/total number of cells × 100%.
Cells were lysed in RIPA buffer (#P0013C; Beyotime) containing protease inhibitor tablets (#05892791001; Roche, Indianapolis, IN, USA). Protein concentration was determined using an enhanced BCA protein assay kit (#P0010, Beyotime). Equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane (#03010040001; Roche). After blocking in 5% nonfat milk in Tris-buffered saline, the membrane was incubated with specific primary antibodies, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence reagent (#WBKLS0500, Millipore, Billerica, MA, USA) and subjected to chemiluminescent imaging system (ChampChemi 610, Sage Creation Science, Beijing, China). The following antibodies were used:

- RIG-I
- MAVS
- IRF3
- IRF7
- IFNAR1
- IFNAR2
- JAK
- STAT1
- STAT3
- IL-6R
- IDO1
- Trp
- Kyn
- IL-6
- IFNs
- Proteasomal Degradation

**Abbreviations:** RIG-I, retinoic acid-inducible gene I; MAVS, mitochondrial antiviral-signaling protein; IRF3/7, interferon regulatory factor 3/7; IFNAR, interferon-α/β receptor; JAK, Janus kinase; IL-6R, interleukin-6 receptor; Ub, ubiquitin; Trp, tryptophan; Kyn, kynurenine.

**Western Blot**

Figure 8. Fludarabine Improves NDV-Mediated Oncolytic Viro-immunotherapy

On the one hand, the viral RNA activates the cytoplasmic RIG-I signaling pathway in the host cells to produce type I IFNs or other antiviral molecules. Autocrine or paracrine of IFNs activate STAT1 signaling pathway to amplify the antiviral loop. Fludarabine blocks this feedforward loop and favors viral replication at an early stage after infection. Increased viral replication in the host cells results in more IFN production at the late stage of infection, which further activates antitumor immune responses (left). On the other hand, fludarabine increases proteasomal degradation of p-STAT3 and IDO1 by accelerating ubiquitination of target proteins, in turn, blocks immune suppression (right).

**Figure 8. Fludarabine Improves NDV-Mediated Oncolytic Viro-immunotherapy**

Abbreviations: RIG-I, retinoic acid-inducible gene I; MAVS, mitochondrial antiviral-signaling protein; IRF3/7, interferon regulatory factor 3/7; IFNAR, interferon-α/β receptor; JAK, Janus kinase; IL-6R, interleukin-6 receptor; Ub, ubiquitin; Trp, tryptophan; Kyn, kynurenine.
For oncolysis and survival experiments, tumors were implanted by spots (ELISpots), IDO1 expression, and STAT3 phosphorylation. On days 12 and 16, ascites samples (500 μL) were removed for determination of NDV replication, antiviral gene expression, and immune cell infiltration. The Ascitic HCC Tumor Model 6- to 8-week-old male C57/BL6 mice received subcutaneous injection of 5 × 10⁶ Hepa1-6 cells of each. On days 7, 10, and 13, the mice received intratumoral (i.t.) injection of 1 × 10⁷ pfu NDV per mouse, respectively. On days 7 and 13, the mice received intraperitoneal injection of 0.75 mg/mouse fludarabine monophosphate solution in a total volume of 100 μL. The mice received equal volume of PBS as untreated control. Tumor volume was monitored every 2–3 days by caliper measurement and calculated by length × width × width/2.

Flow Cytometry For immune activation experiments in vivo, ascitic cells were harvested and washed twice with PBS and incubated with the following antibodies: anti-NK1.1 (clone PK136, #553164), CD11b (clone M1/70, #557396), Gr-1 (clone RB6-8C5, #553128) (all from BD Biosciences, Franklin Lakes, NJ, USA); and anti-CD3e (clone 17A2, #17-0032-82), anti-CD8a (clone 53-6.7, #45-0081-82), and isotype antibodies (#45-4321-80, 11-4714-41, 17-4031-81, and 12-4321-82) (all from Thermo Fisher Scientific, Waltham, MA, USA). Samples were subjected to flow cytometry using a FACS Calibur instrument (BD Biosciences), and data were analyzed using FlowJo software (v. 7.6.5, Tree Star, Ashland, OR, USA).

Proteasome Activity Assay Proteasome activity was monitored based on the release of fluorescent 7-amido–4-methylcoumarin (AMC) from its tagged peptide substrate using a proteasome activity fluorometric assay kit (#K245-100; BioVision, Milpitas, CA, USA). The activation status of immune cells in ascites was evaluated by the mouse IFN-γ ELISpotPLUS kit (3321-2AW-Plus; Mabtech, Nacka Strand, Sweden) according to the manufacturer’s protocols. Treatment was determined based on the standard curve.

IFN-γ Enzyme-Linked Immunosorbent Spot Assay The activation status of immune cells in ascites was evaluated by the mouse IFN-γ ELISpotPLUS kit (3321-2AW-Plus; Mabtech, Nacka Strand, Sweden) according to the manufacturer’s protocols. In brief, cells were seeded in a 96-well plate coated with an IFN-γ capture antibody at a density of 2 × 10⁶ cells/well at 37°C for 24 h in a humidified incubator with an atmosphere containing 5% CO₂. The
cells were removed, the plate was washed five times with PBS, a biotinylated anti-IFN-γ antibody was added, and the plate was incubated at room temperature for 2 h. Next, the plate was washed with PBS, streptavidin-ALP was added, and the plate was incubated at room temperature for 1 h. Finally, the BCIP/NBT-plus substrate was added until spots emerged, and the plate was washed with tap water to stop the reaction. The plate was analyzed using an enzyme-linked immunosorbent spot (ELISpot) reader (Autoimmun Diagnostika, Strassberg, Germany) to enumerate spots, and spot activity was characterized as the weighted average of the spot size and intensity in a well.

Statistical Analyses
Data were analyzed by the two-tailed unpaired Student’s t test (for comparisons of two groups). Survival data were analyzed by the log rank (Mantel-Cox) test. Statistical analyses were conducted using Microsoft Excel (Microsoft, Redmond, WA, USA) or Prism software (v. 6.01; GraphPad).

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Conception and Design, J.W. and G.M.; Development of Methodology, G.M., B.L., C.X., and M.X.; Acquisition of Data (providing animals, acquiring and managing patients, providing facilities, etc.), G.M., Z.F., B.L., M.F., A.C., and D.Y.; Analysis and Interpretation of Data (e.g., statistical analysis, biostatistics, computational analysis), G.M., Z.F., and B.L.; Writing, Review, and/or Revision of the Manuscript, G.M. and J.W.; Administrative, Technical, or Material Support (i.e., reporting or organizing data, constructing databases), J.W., D.Y., and G.M.; Study Supervision, J.W.

CONFLICTS OF INTEREST
No potential conflicts of interest were disclosed.

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