

Reovirus Virotherapy Overrides Tumor Antigen Presentation Evasion and Promotes Protective Antitumor Immunity

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Abstract

Tumor-associated immunosuppressive strategies, such as lack of tumor antigen recognition and failure of lymphocyte activation and homing, resist the development of tumor-specific immunity and hamper the immune response-mediated elimination of cancerous cells. In this report, we show that reovirus virotherapy overrides such a tumor immune evasion and establishes clinically meaningful antitumor immunity capable of protecting against subsequent tumor challenge. Reovirus-mediated destruction of tumor cells facilitates the recognition of tumor antigens by promoting the display of otherwise inaccessible tumor-specific immunogenic peptides on the surface of dendritic cells (DC). Furthermore, on exposure to reovirus, DCs produce IL-1 α , IL-1 β , IL-6, IL-12p40/70, IL-17, CD30L, eotaxin, GM-CSF, KC, MCP-1, MCP-5, M-CSF, MIG, MIP-1 α , RANTES, TNF- α , VCAM-1, VSGF, CXCL-16, AXL, and MCP-2; undergo maturation; and migrate into the tumor microenvironment along with CD8 T cells. These reovirus-activated DCs also acquire the capacity to prime tumor antigen-specific transgenic T cells *in vitro* and intrinsic antitumor T-cell response *in vivo*. Further, reovirus virotherapy augments the efficacy of DC- or T cell-based anticancer immunotherapies and synergistically enhances the survival in tumor-bearing mice. Most importantly, antitumor cellular immune responses initiated during reovirus oncotherapy protect the host against subsequent tumor challenge in a reovirus-independent but antigen-dependent manner. These reovirus oncotherapy-initiated antitumor immune responses represent an anticancer therapeutic entity that can maintain a long-term cancer-free health even after discontinuation of therapy. *Mol Cancer Ther*; 9(11); 2924–33. ©2010 AACR.

Introduction

Tumors use various evasion strategies to inhibit the development of antitumor immunity and avoid subsequent immune response-mediated elimination (1–3). Some of the well-recognized tumor immune evasion mechanisms include failure of tumor antigen recognition (4–6), modulation of MHC molecule expression (7, 8), improper activation of antigen-presenting cells (APC; refs. 1, 9), failure of lymphocyte homing (1, 10), and production of immunosuppressive cytokines (11, 12). These immunologic abnormalities associated with the tumor microenvironment either inhibit the priming of antitumor adaptive immunity (1) or tolerize tumor-specific CD4 (13, 14) and

CD8 (15, 16) T cells. However, recent reports show that synchronized blockade or overriding of tumor immune evasion strategies with appropriate immunomodulatory intervention may overcome immunologic tolerance and promote tumor regression (1, 17, 18). It is believed that immunologic events associated with unrelated but stronger antigenic stimuli (19–21) or an infectious pathogen (e.g., hepatitis B virus infection; ref. 22) can alter the immunosuppressive milieu and aid in the initiation of antigen-specific immune responses (17, 18).

Reovirus, a naturally occurring benign human pathogen, preferentially targets cancerous cells of many origins and is currently under clinical trials as a novel anticancer therapeutic agent (23, 24). Reovirus efficiently destroys local as well as distant metastatic tumors in immunocompromised animals even after a single injection administered through intravenous, intraperitoneal, or intratumoral routes (25–28). Tumor regression achieved during reovirus anticancer therapy is thought to be primarily mediated by direct destruction of cancer cells. However, recent findings suggest that combination of reovirus-mediated oncolysis and host immune responses is necessary to achieve efficient tumor regression associated with this virotherapy (29). We hypothesize that antiviral immune responses generated during oncoviral therapy assist the immune system in overcoming the immune evasive strategies used by tumors and induce

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antitumor immune responses. Thus, the achievement of optimum anticancer effects during oncotherapy hinges on a harmonious exploitation of reovirus-mediated direct oncolysis and antitumor immune activities. However, reovirus-mediated antitumor immunologic events during and after oncotherapy as well as their specific therapeutic capabilities have not been thoroughly explored.

In the present study, we investigated if immunologic events initiated on reovirus exposure could overcome the immunosuppressive activities associated with the tumor microenvironment. We show that therapeutic administration of reovirus overrides tumor-associated antigen presentation abnormalities before initiating tumor-specific adaptive immune responses. Additionally, reovirus oncotherapy synergistically enhances the efficacy of tumor-specific immunotherapies and, most importantly, promotes the establishment of protective antitumor immunity. These findings should advance our current knowledge in devising antitumor immunotherapeutic remedies to complement virotherapy-mediated tumor destruction and restrict cancer remission in clinical settings.

Materials and Methods

Reovirus, cell lines, and reagents

Reovirus (serotype 3, Dearing strain) was grown and purified as described (1). Native B16 melanoma (B16) and Lewis lung carcinoma (LLC) were purchased from the American Type Culture Collection, whereas ovalbumin (ova)-expressing B16 (B16-ova; ref. 30) and LLC (LLC-ova; ref. 31) cell lines were kindly provided by Dr. J. Xiang (Saskatchewan Cancer Agency, Saskatoon, Saskatchewan, Canada) and Dr. E. Podack (University of Miami School of Medicine, Miami, FL), respectively.

Antibodies and reagents used in this study were purchased from different manufacturers as follows: eBioscience: Alexa 488-anti-CD11c, PE-anti-CD86, APC-anti-CD40, APC-anti-CD80, PE-anti-mouse MHC class I molecule Kb bound to the peptide SIINFEKL (25-D1.16), unconjugated anti-mouse CD16/32, and unconjugated anti-mouse CD49d; Invitrogen: PE-anti-CD3, Alexa 488-IFN- γ , APC-anti-CD107a, unconjugated anti-mouse CD28, and 5-(and-6)-carboxyfluorescein diacetate (CFSE); BioLegend: PerCp-anti-MHC class II (H-2A/E) and PerCp-anti-CD90.2; BD Biosciences: PerCp-anti-CD8; GenScript: SIINFEKL (ova₂₅₇₋₂₆₄) and KAVYNFATM (LCMV gp₃₃₋₄₁) peptides.

In vivo experimental manipulations

Six- to 8-week-old female wild-type (WT) C57BL/6 (thy1.2) mice were obtained from Charles River Laboratory. WT B6.PL-Thy1^a/CYJ (thy1.1; congenic to WT C57BL/6) and transgenic OT-1 mice bearing T-cell receptor (TCR) V α 2 β 5 specific for the ova₂₅₇₋₂₆₄ peptide (SIINFEKL) were purchased from The Jackson Laboratory. Tumors were generated according to protocols described for respective experiments, and dimensions were calculated as follows: longest diameter \times smallest diameter of tumor. The animals

were sacrificed when tumor dimensions reached 150 mm². The experimental procedures were governed by the approval of Ethics Committee at the Dalhousie University.

Isolation of lymphocytes, enrichment of dendritic cells, and generation of BMDCs

Lymphocytes were isolated from single-cell suspensions of spleens, inguinal lymph nodes (LN), or tumors using Ficoll-Paque Plus (Amersham Pharmacia) density gradient as described (32). These lymphoid cells were incubated with negative selection MACS beads (Invitrogen) containing anti-CD3, anti-CD14, and anti-B220 antibodies and then passed through magnetic column to obtain enriched dendritic cells (DC) with a purity of \geq 95% as determined by subsequent flow cytometry analysis.

Bone marrow-derived DCs (BMDC) were generated by culturing bone marrow cells in complete RPMI 1640 containing 10% (v/v) FCS, 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 50 units/mL penicillin/streptomycin, and 0.1 mmol/L 2-mercaptoethanol and supplemented with 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL interleukin-4 (IL-4) for 6 to 8 days (33).

Antibody-based cytokine array

Quantitative (and Qualitative) Cytokine Arrays (Raybiotech, Inc.) were used as per the manufacturer's instructions to analyze selected 60 cytokines. Culture supernatants of PBS/reovirus-stimulated BMDCs, splenocytes, or B16 tumor cells or sera from PBS/reovirus-injected WT and severe combined immunodeficient (SCID) mice were quantitatively analyzed by Raybiotech's Quantibody Array Testing Service. The increase or decrease in the level of cytokine was considered significant when the difference between the mean of cytokine concentrations from stimulated and unstimulated samples was \pm 2.1-fold.

B3Z antigen presentation assay

B3Z is a genetically engineered T-cell hybridoma that bears ova-specific TCR and expresses β -galactosidase (β -gal) on recognizing SIINFEKL ova peptide in the context of H2-Kb. B3Z cells (1×10^5 ; a gift from Dr. N. Shastri, University of California, Berkeley, CA; ref. 34) were added to 1×10^5 BMDCs cocultured with 1×10^5 reovirus-treated tumor cells or directly enriched DCs from tumor, spleens, or LNs; incubated for 18 to 24 hours; and then supplemented with 0.15 mmol/L chlorophenolred- β -D-galactopyranoside. After 4 hours, the breakdown of chlorophenolred- β -D-galactopyranoside was read at 570 nm and defined as CD8 T-cell response.

T-cell proliferation and IFN- γ /CD107a assay

Lymphocytes were labeled with 1 μ mol/L CFSE (32) and then stimulated with either SIINFEKL, tumor lysate (35), and control LCMV gp₃₃₋₄₁ peptide (5 μ g/mL)– or UV reovirus [1 multiplicity of infection (MOI)]–pulsed BMDCs or concanavalin A (5 μ g/mL), or left unstimulated. After 5 days of incubation, cells were harvested,

stained with PE-anti-CD3 antibodies and analyzed. The intracellular production of IFN- γ and degranulation of CD107a [lysosomal-associated membrane protein 1 (LAMP-1)] in CD3⁺, CD8⁺ lymphocytes were detected as described (36). Briefly, lymphocytes were stimulated with 5 μ g/mL of SIINFEKL or control peptide in the presence of 1 μ g/mL each of unconjugated CD28 and CD49d antibodies, 2 μ g/mL of brefeldin A, and FITC-anti-CD107a antibodies. After 6 hours, cells were washed, fixed, permeabilized, stained with APC-anti-IFN- γ antibodies, and analyzed.

Adoptive transfer experiments

CFSE-labeled naive OT-1 splenocytes were transferred in B6.PL-Thy1^a/CYJ mice to study reovirus therapy-induced activation of antitumor T cells *in vivo*. On the other hand, OT-1 lymphocytes were cultured in complete RPMI 1640 supplemented with 50 IU/mL of IL-2 and 5 μ g/mL of SIINFEKL peptide for 5 to 7 days, whereas BMDCs were stimulated with IL-1 β (10 ng/mL), tumor necrosis factor- α (TNF- α ; 10 ng/mL), IL-6 (1,000 units/mL), and prostaglandin E₂ (1 μ g/mL) in the presence of SIINFEKL peptide for 2 days before using them as immunotherapeutic agents.

Passive transfer of reovirus-induced antitumor immunity

Splenocytes from PBS- or reovirus-treated B16-ova tumor-bearing and nontumor-bearing (naive) C57BL/6 mice were transferred in naive WT C57BL/6 mice. After 7 days, recipient mice were challenged with 5×10^5 cells of B16-ova, native B16, or LLC cells and monitored for the development of tumors.

Flow cytometry and statistical analysis

Data acquisition was done with BD FACSCalibur flow cytometer, whereas data analysis was done using BD CellQuest Pro, FCS Express V3, and/or ModFit LT softwares. The statistical analysis was done using two-tailed Student's *t* test or Kaplan-Meier survival analysis coupled with log-

rank test (both with 95% confidence interval). *P* values of <0.05 were considered to be statistically significant.

Results

Reovirus enhances the capacity of DCs to present tumor antigen *in vitro*

Tumors resist the generation of antitumor immune responses by avoiding the presentation of immunogenic tumor peptides by APCs. Hence, we first evaluated if reovirus modifies the ability of DCs to process tumor antigens using the B3Z antigen presentation assay *in vitro* (35, 37). For this purpose, BMDCs were first cultured in the presence of native or ova-expressing B16 and LLC tumor cells, which were exposed to reovirus or PBS control, and then incubated with B3Z cells. The capacity of BMDCs to present surrogate tumor antigen to CD8 T cells was evaluated by analyzing the B3Z cell activation-induced breakdown of β -gal substrate. As shown in Fig. 1, BMDCs incubated with B16-ova or LLC-ova and live reovirus initiated significantly higher (*P* < 0001) β -gal activity than that of BMDCs cultured with B16-ova or LLC-ova and PBS. Importantly, such an activation of β -gal was absent when UV-inactivated reovirus was used in place of live reovirus. These results suggest that oncolytic actions of live reovirus are necessary to expose otherwise inaccessible tumor antigen for the processing by DCs. BMDCs incubated with native (non-ova-expressing) B16 or LLC cells and live or UV-inactivated reovirus failed to induce activation of B3Z CD8 T cells due to the absence of ova expression on native tumors. Collectively, our results suggest that reovirus oncolysis enhances the capacity of APCs to process and present tumor antigens to CD8 T cells.

Reovirus induces proinflammation, lymphoid cell migration, DC activation, and enhanced tumor antigen presentation *in vivo*

Tumors persist in a milieu containing immunosuppressive cytokines that resist appropriate activation and homing of lymphoid cells. Reovirus-initiated cytokine

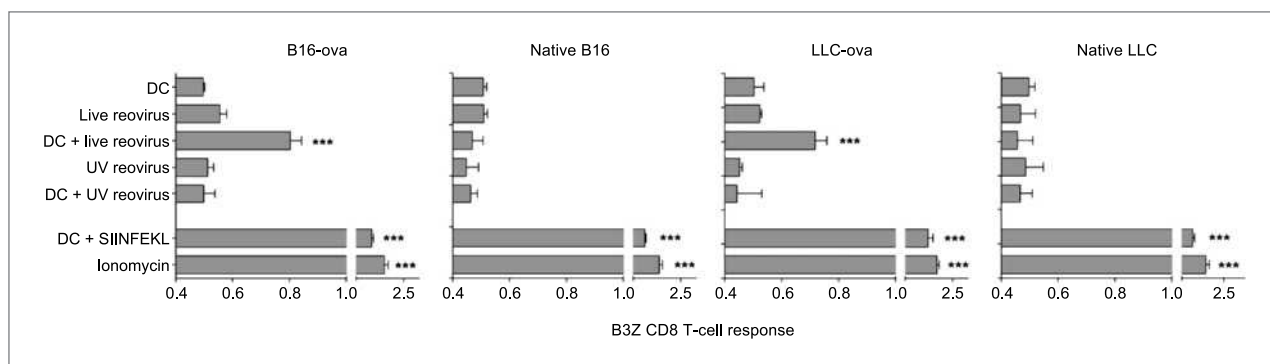


Figure 1. Reovirus-induced enhanced tumor antigen presentation by DCs *in vitro*. B3Z cells were added to the BMDCs cultured with B16-ova, native B16, LLC-ova, or native LLC tumor cells and 1 MOI of live or UV-inactivated reovirus and then analyzed for β -gal activity in B3Z assay. The data are combined from three independent experiments.

response *in vitro* has previously been reported (38, 39); however, the comprehensive profile of such a response in different immune cell subsets or *in vivo* is still obscure. Using Antibody-based Quantitative (and Qualitative) Cytokine Array, we evaluated the expression of 60 cytokines following reovirus exposure of BMDCs, lymphocytes, and tumor cells *in vitro* or immunocompetent and immunocompromised mice *in vivo*. First, BMDCs were stimulated with PBS or reovirus *in vitro* and their respective supernatants were then analyzed for the expression of cytokines. As summarized in Table 1 (and shown in Supplementary Fig. S1 and Supplementary Table S1), the expression of IL-6 in reovirus-induced BMDC cultures was increased by 95 times over that of PBS-stimulated ones (from 394.9 to 37,522.4 pg/mL). Reovirus exposure also enhanced the expression of GM-CSF, IL-1 α , IL-1 β , TNF- α , and IL-12, all of which have the potential to induce the differentiation and maturation of DCs. Additionally, reovirus-stimulated BMDC supernatants displayed elevated expression of KC, MCP-1, MCP-5, M-CSF, MIG, MIP-1 α , RANTES, CXCL-16, AXL, and MCP-2 along with decreased expression of IL-3, IL-5, IL-15, and TCA-3. This reovirus-induced proinflammatory response collectively represents a characteristic profile of cytokines and chemokines capable of initiating lymphocytic migration and antigen-specific T-cell response.

Additionally, we also evaluated if reovirus exposure initiates similar proinflammatory cytokines from lymphoid or tumor cells. For this, C57BL/6 splenocytes or B16 cells were stimulated with PBS or reovirus and then analyzed with cytokine array as described above. Similar to BMDCs, reovirus exposure also initiated higher expression of IL-6 and RANTES in both splenocyte (20.2 to 104.6 pg/mL and below level of detection to 295.9 pg/mL, respectively) and B16 tumor cell cultures (107.1 to 2,876.1 pg/mL and 1,180.3 to 3,997.9 pg/mL). Additionally, reovirus-stimulated splenocytes produced greater amounts of IFN- γ , eotaxin, KC, MCP-5, and MIP-1 α than that of PBS-stimulated control. These data

show the ability of reovirus to stimulate proinflammatory cytokine response from lymphoid as well as tumor cells.

We then evaluated the expression profile of these cytokines after reovirus injection *in vivo*. For this, WT C57BL/6 or SCID mice were injected with PBS or reovirus. After 24 hours, serum samples were collected and directly evaluated as described above. Similar to *in vitro*-activated lymphoid cells, sera from reovirus-injected WT and SCID mice showed increased expression of IL-6, RANTES, KC, MCP-1, MCP-5, and MIG (Table 1; also see Supplementary Table S1). Following reovirus injection, decreased levels of eotaxin-2, FasL, IL-7, IL-15, IL-17, leptin, M-CSF, TARC, and TCA-3 were detectable only in the serum collected from WT but not SCID mice. This analysis shows that reovirus administration initiates systemic proinflammatory response *in vivo*.

Next, the effect of reovirus oncotherapy on lymphoid cell homing *in vivo* was assessed using the B16 tumor model. For this purpose, WT C57BL/6 mice were s.c. injected with B16 cells and allowed to develop tumors (Fig. 2A). After 10 days, mice were administered with therapeutic regimen of PBS or reovirus and sacrificed on day 2 or day 5 after the last injection to obtain spleen and tumor samples. The trafficking of lymphoid cells into tumors was evaluated by directly staining tumor-infiltrating lymphoid cells (TIL). As shown in Fig. 2B, reovirus-treated mice showed significantly higher numbers of tumor-infiltrating CD3⁺, CD8⁺ T cells and CD11c⁺-expressing DCs and natural killer cells at both days 2 and 5 when compared with PBS-injected mice. These observations are compatible with the above demonstration that reovirus exposure enhances the expression of chemotactic IL-6 and RANTES from BMDCs, splenocytes, and B16 tumor cells in WT C57BL/6 mice. These findings suggest that reovirus exposure facilitates the migration of lymphoid cells in tumor milieu.

We further analyzed the ability of reovirus to induce the maturation of DCs *in vivo*. DCs collected from spleen and tumors of PBS- or reovirus-treated tumor-bearing

Table 1. Summary of reovirus-induced proinflammatory cytokine protein expression

	Increased expression	Decreased expression
BMDC	CD30L, eotaxin, GM-CSF, IL-1 α , IL-1 β , IL-6, IL-17, KC, MCP-1, MCP-5, M-CSF, MIG, MIP-1 α , RANTES, TNF- α (IL-12p40/70, VCAM-1, VSGF, CXCL-16, AXL, MCP-2)	IL-3, IL-5, IL-15, TCA-3
Splenocytes	Eotaxin, IFN- γ , IL-6, KC, MCP-5, MIP-1 α , RANTES	ICAM-1, IL-7, IL-10, IL-12p70, IL-21, LIX, M-CSF, MIG, TARC
B16 tumor	IL-6, IL-17, MCP-1, MIG, RANTES	IL-1 α , MIP-1 γ , PF-4, TIMP-1
WT mouse serum	BLC, eotaxin, G-CSF, IL-3, IL-6, KC, MCP-1, MCP-5, MIG, RANTES, TIMP-1	Eotaxin-2, FasL, IL-7, IL-15, IL-17, leptin, M-CSF, TARC, TCA-3
SCID mouse serum	ICAM-1, IL-1 α , IL-4, IL-6, IL-7, IL-13, IL-15, MCP-1, MCP-5, M-CSF, RANTES	

NOTE: Cytokines represented in bracket were additionally evaluated using Qualitative Cytokine Array.

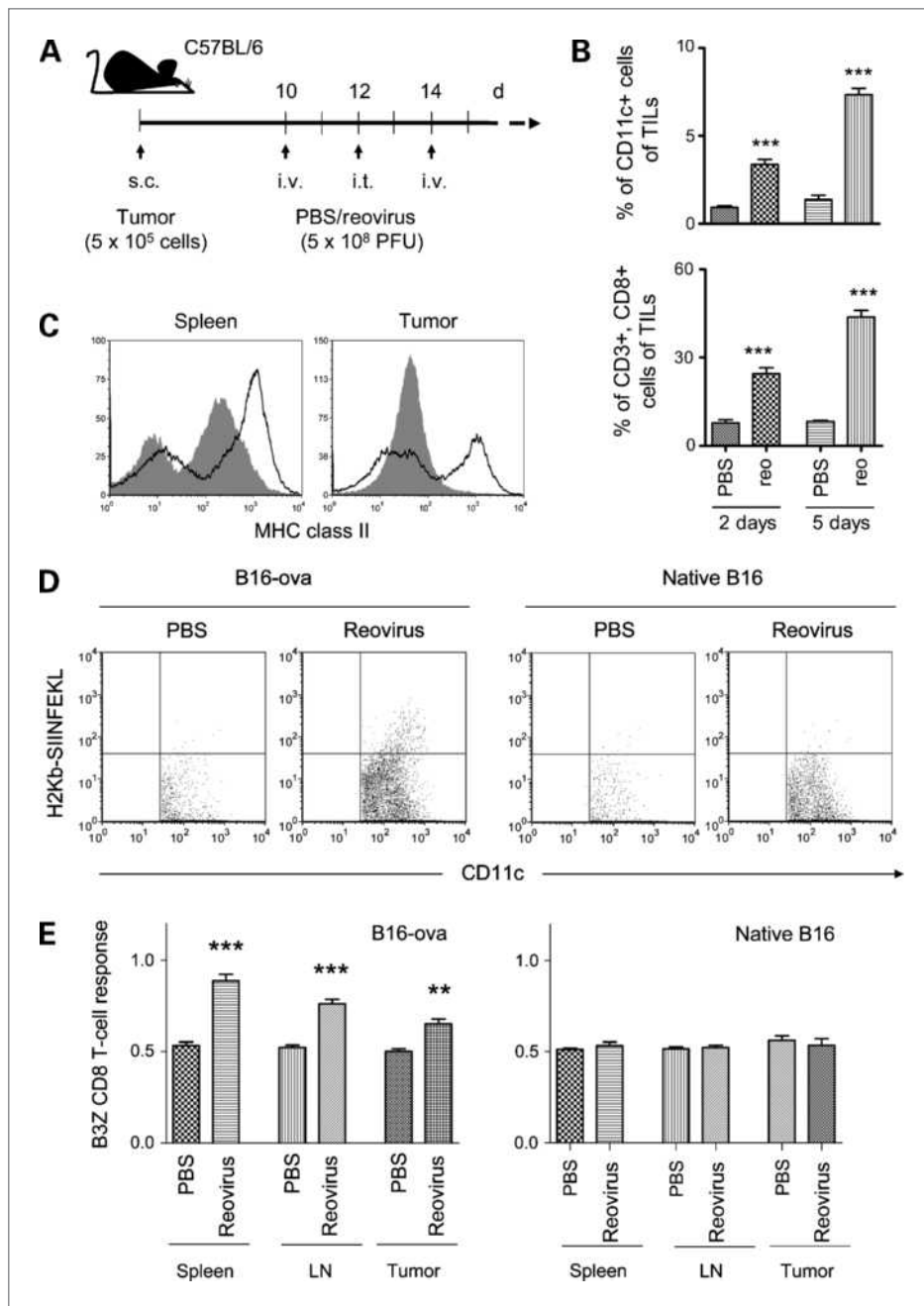


Figure 2. Reovirus-induced tumor infiltration, DC activation, and tumor antigen presentation *in vivo*. **A**, WT C57BL/6 mice were grafted with native B16 or B16-ova cells s.c. and then injected with PBS or reovirus i.v. or i.t. at indicated time points. PFU, plaque-forming units. **B**, TILs were obtained on days 2 and 5 after the last reovirus injection and directly analyzed in flow cytometry to measure the frequency of infiltrating CD11c⁺ and CD3⁺, CD8⁺ lymphoid cells. **C**, on day 7 after the last reovirus injection, DCs collected from spleen and tumor were stained for MHC class II and analyzed in flow cytometry. Histograms are represented as follows: filled, PBS treated; solid line, reovirus treated. **D**, TILs were directly stained with anti-CD11c antibody and 25.D1.16 hybridoma (40) and analyzed in flow cytometry. The expression of H2-Kb-SIINFEKL complexes on CD11c⁺ cells has been shown in dot plots. **E**, DCs were enriched from the spleens, LNs, and tumors and then evaluated for their capacity to activate CD8 T cells using B3Z assay. Data are representative of four independent experiments.

mice were harvested and directly stained. We found that CD11c⁺ DCs collected from reovirus-treated mice displayed higher expression of MHC class II (Fig. 2C) and CD86 (Supplementary Fig. S2A) molecules compared with PBS-treated mice. Of note, CD11c⁺ BMDCs also display the higher expression of costimulatory molecules (CD86, CD80, CD40, and MHC class II) on reovirus exposure *in vitro* compared with PBS-stimulated control (Supplementary Fig. S2B). These results show that reovirus induces the expression of appropriate costimulatory molecules on APCs.

Considering the ability of reovirus to enhance tumor antigen presentation *in vitro*, we further investigated if reovirus modifies tumor antigen display *in vivo*. To this end, native B16 or B16-ova tumor-bearing C57BL/6 mice were injected with PBS or reovirus (Fig. 2A) and sacrificed on day 7 after the last injection. First, the status of surrogate tumor antigen on DC surface was analyzed by using the 25-D1.16 hybridoma that exclusively detects SIINFEKL-presenting H2-Kb MHC class I molecules (40, 41). As shown in Fig. 2D, reovirus-treated B16-ova tumor showed the presence of higher number

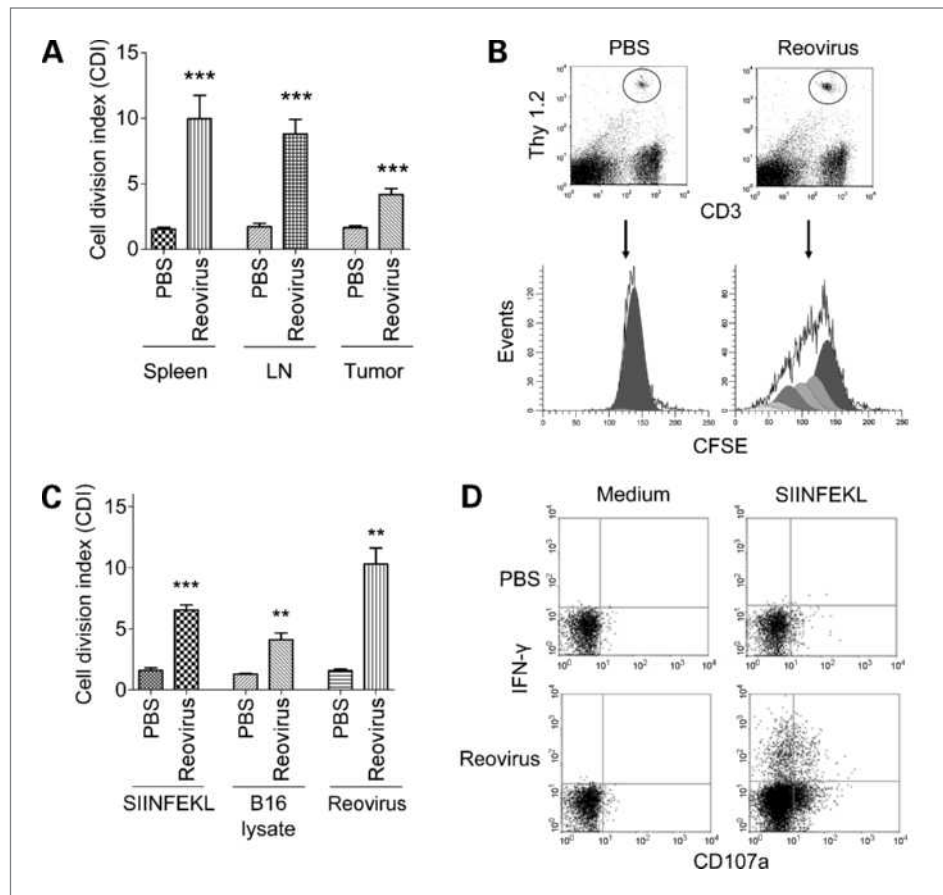
of H2-Kb-SIINFEKL-expressing CD11c⁺ cells compared with PBS-treated B16-ova tumor. As anticipated, CD11c⁺ cells collected from reovirus- or PBS-treated control native B16 tumor-bearing mice lacked the expression of H2-Kb-SIINFEKL complexes due to the absence of ova expression on tumor cells. These results show that during reovirus virotherapy, CD11c⁺ DCs successfully acquire antigens from the tumor cells and display it on their surface.

Next, the functional capacity of these DCs collected from tumor-bearing, PBS- or reovirus-treated mice was analyzed in B3Z assay. As shown in Fig. 2E, DCs isolated from reovirus-treated B16-ova tumor-bearing mice initiated significantly higher activation of B3Z cells (spleen, $P = 0.001$; LN, $P = 0.0009$; tumor, $P = 0.008$) than that of DCs obtained from PBS-treated B16-ova tumor-bearing mice. As anticipated, DCs obtained from PBS- or reovirus-treated native B16 tumor-bearing mice failed to activate B3Z cells. Similar results were also obtained with DCs enriched from PBS- or reovirus-treated LLC-ova or native LLC tumor-bearing C57BL/6 mice (Supplementary Fig. S3). Collectively, our data suggest that reovirus oncotherapy exposes otherwise inaccessible tumor antigen for processing by DCs and augments its presentation to CD8 T cells *in vivo*.

Antitumor T-cell responses follow the immunologic events that override tumor evasion

We then evaluated whether reovirus-enhanced costimulation and antigen presentation on DC surface is sufficient to prime antitumor T-cell response. For this purpose, WT B6.PL-Thy1^a/CYJ mice were first injected with B16-ova tumor cells and then administered with therapeutic regimen of PBS or reovirus (as per protocol shown in Fig. 2A). On day 7 after the last injection, DCs from spleen, LN, and tumor were directly incubated with CFSE-labeled naive splenocytes isolated from OT-1 mice (42). After 5 days, cells were labeled with anti-CD3 and anti-thy1.2 antibodies and evaluated for T-cell proliferation. As shown in Fig. 3A, DCs from different organs of reovirus-treated B16-ova tumor-bearing mice initiated significantly higher proliferation of OT-1 T cells (spleen, $P = 0.0009$; LN, $P = 0.0001$; tumor, $P = 0.0004$) than that of DCs obtained from PBS-treated B16-ova tumor-bearing mice. However, DCs obtained from PBS- or reovirus-treated native B16 tumor-bearing mice failed to activate OT-1 T cells (Supplementary Fig. S4). To visualize such a tumor-specific T-cell response *in vivo*, CFSE-labeled, naive OT-1 lymphocytes were adoptively transferred into PBS- or reovirus-treated B16-ova

Figure 3. Characterization of antitumor T-cell responses initiated during reovirus virotherapy. **A**, naive OT-1 splenocytes were labeled with CFSE; cultured in the presence of enriched DCs collected from the spleens, LNs, and tumors of PBS- or reovirus-treated B16-ova tumor-bearing B6.PL-Thy1^a/CYJ mice; and then analyzed in proliferation assay. The data are combined from at least three independent experiments. **B**, B16-ova tumor-bearing B6.PL-Thy1^a/CYJ mice were treated with PBS or reovirus and then adoptively transferred with CFSE-labeled naive OT-1 splenocytes. Five days after cell transfer, lymphocytes were isolated from LN, stained with anti-CD3 and anti-thy1.2 antibodies, and analyzed in flow cytometry. **C** and **D**, lymphocytes from the spleens, LNs, and tumors of PBS- or reovirus-treated B16-ova tumor-bearing mice were labeled with CFSE and then analyzed in proliferation assay (**C**) or IFN- γ /CD107a assay (**D**). Cumulative data from four independent experiments ($n = 5-7$ mice in each group) are shown.



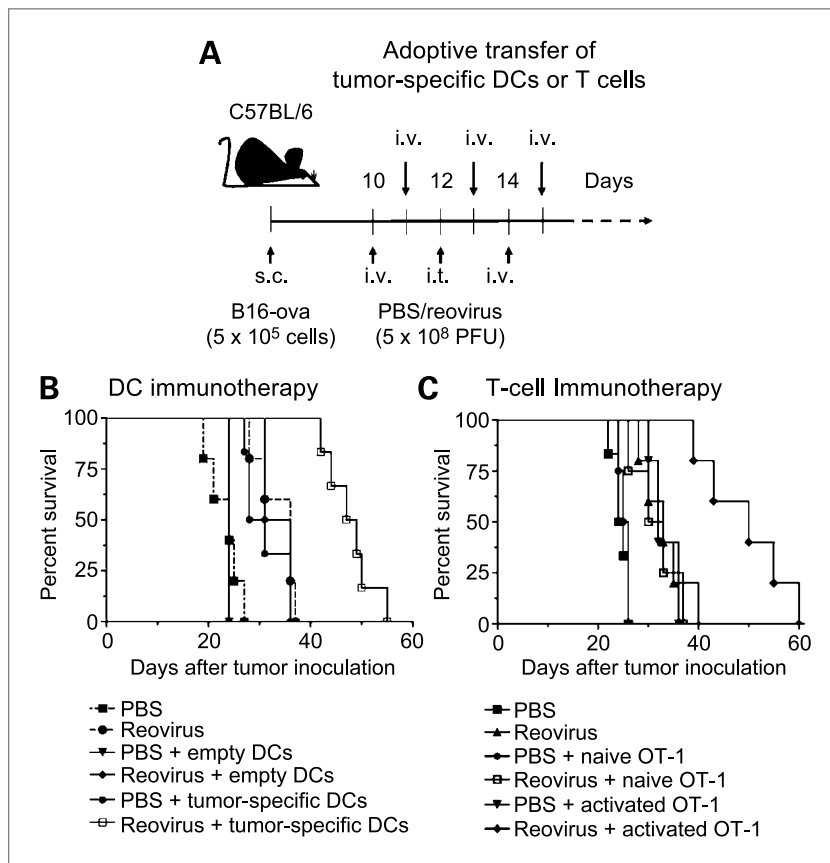


Figure 4. Reovirus oncotherapy enhances the efficacy of tumor-specific DC- and T cell-based immunotherapy. A, B16-ova tumor-bearing WT C57BL/6 mice were administered with SIINFEKL-pulsed BMDCs (B) or activated OT-1 T cells (C) along with a regimen of PBS or reovirus at the indicated time points and then monitored for survival. Data are representative of three independent experiments ($n = 5-6$ mice per group).

tumor-bearing B6.PL-Thy1^a/CYJ WT mice on day 7 after the last injection and then tracked in different organs. As shown in Fig. 3B, thy1.2⁺, CD3⁺ T cells from LNs of reovirus-treated, but not PBS-treated, animals showed sequential halving of CFSE fluorescence. Similar results were also obtained with OT-1 T cells collected from spleen and tumor of these animals (data not shown), conclusively showing the ability of reovirus oncotherapy to prime tumor-specific T cells *in vivo*.

We also evaluated whether DC activation during oncotherapy leads to the development of intrinsic T-cell response against different tumor and reoviral antigens. For this purpose, WT C57BL/6 mice were injected with B16 or B16-ova tumors and PBS or reovirus (as per Fig. 2A). Seven days after the last injection, lymphocytes were isolated from different organs; labeled with CFSE; stimulated with BMDCs pulsed with SIINFEKL, native B16 lysate, or inactivated reovirus; and then analyzed in proliferation assay. As shown in Fig. 3C, CD3⁺ T cells from spleens of reovirus-treated mice showed significantly higher proliferation than those from PBS-treated mice after stimulation with surrogate and native tumor- or reovirus-specific antigens. Similar results were also observed for CD3⁺ T cells collected from LNs and tumors of these mice (Supplementary Fig. S5A). Of note, T cells from both PBS- and reovirus-treated mice showed com-

parable generalized immunocompetence as assessed by their ability to proliferate after mitogenic (concanavalin A) stimulation (Supplementary Fig. S5B).

LNs of reovirus-treated mice also showed significantly higher number of IFN- γ - and CD107a-producing CD3⁺, CD8⁺ T cells after SIINFEKL ($P = 0.0001$ for IFN- γ and 0.0011 for CD107a) and reoviral antigen ($P = 0.0001$ for both IFN- γ and CD107a) stimulation *in vitro* than those from PBS-treated mice (Fig. 3D; Supplementary Fig. S5C). As anticipated, the magnitude of antiviral proliferative and IFN- γ /CD107a T-cell responses was higher than that of antitumor response. These tumor-specific proliferative and IFN- γ /CD107a-producing capacities of CD3⁺ T cells were evident even 40 to 50 days after the last reovirus injection (data not shown). Together, our data show that reovirus oncotherapy overcomes the immune evasive strategies used by tumors and primes antitumor proliferative and cytotoxic antitumor T-cell responses.

Reovirus oncotherapy synergistically enhances the efficacy of tumor-specific immunotherapies

Although promising, the anticancer immunotherapies fail to achieve optimum anticancer effects (43–46). When administered alone, the efficacy of these immunotherapies is compromised in the presence of tumor-associated suppressive microenvironment. However, because reovirus

overrides such immune evasive milieu, it was hypothesized that the simultaneous administration of reovirus also potentiates the efficiency of immunotherapies. To test this hypothesis, reovirus oncotherapy was administered during DC- and T cell-based antitumor therapies. To this end, B16-ova tumor-bearing mice were administered with therapeutic regimen of *in vitro*-activated, tumor-specific DCs or OT-1 T cells and PBS or reovirus injections (as per protocol shown in Fig. 4A). The transfer of optimally matured SIINFEKL-presenting DCs (Supplementary Fig. S6) in B16-ova tumor-bearing mice along with reovirus treatment resulted in significantly higher survival rates than that of similar DC preparation administered along with PBS ($P = 0.0009$; Fig. 4B). Further, survival rate initiated by SIINFEKL-DC + reovirus treatment combination was also significantly greater than that of PBS alone ($P = 0.0007$), reovirus alone ($P = 0.0008$), empty DC + PBS ($P = 0.0007$), or empty DC + reovirus ($P = 0.0008$) treatments. Similarly, administration of activated OT-1 T cells along with reovirus injections in B16-ova tumor-bearing mice resulted in significantly higher survival rates compared with PBS alone ($P = 0.0018$) and reovirus alone ($P = 0.006$) or OT-1 + PBS ($P = 0.006$) treatments (Fig. 4C). Thus, our results show that complementation of tumor-specific immunotherapies with reovirus oncotherapy enhances their efficacy, leading to a higher survival rate in cancer-bearing hosts.

Antitumor immunity initiated during virotherapy protects against subsequent tumor challenge in an antigen-dependent but reovirus-independent manner

Finally, the ability of oncotherapy-initiated antitumor immune responses in conferring protection against a subsequent tumor challenge was evaluated. For this pur-

pose, splenocytes isolated from PBS- or reovirus-treated B16-ova tumor-bearing C57BL/6 mice were transferred into naive C57BL/6 mice (Fig. 5A). These recipient mice were subsequently challenged with B16-ova or native B16 and LLC tumors and monitored for tumor growth. As shown in Fig. 5B, the transfer of splenocytes from a reovirus-treated B16-ova tumor-bearing mice (B16-ova + reovirus) produced significantly higher survival in recipient C57BL/6 mice compared with that of splenocytes obtained from mice in PBS + PBS ($P = 0.0007$), PBS + reovirus ($P = 0.0007$), or B16-ova + PBS ($P = 0.0005$) treatment groups. Thus, once primed, reovirus oncotherapy-initiated antitumor immune responses restricted the growth of secondary tumors even after discontinuation of reovirus therapy.

Most importantly, splenocytes from reovirus-treated B16-ova tumor-bearing mice also restricted the growth of native B16 tumors (Fig. 5B) compared with those obtained from mice treated with PBS + PBS ($P = 0.0005$), PBS + reovirus ($P = 0.0005$), or B16-ova + PBS ($P = 0.0005$). These results showed that antitumor immune responses generated during reovirus oncotherapy target not only surrogate but also native tumor antigens. However, such splenocytes from reovirus-treated B16-ova tumor-bearing mice failed to prolong the survival in control native LLC tumor-bearing animals and suggested that these antitumor immune responses confer protection against tumor challenge in an antigen-specific fashion. Collectively, our results conclusively show the capacity of reovirus oncotherapy-initiated antitumor immunity to protect against subsequent tumor challenge in an antigen-dependent but reovirus-independent manner. These findings have great significance in terms of restricting tumor remission and maintaining tumor-free life following discontinuation of the oncolytic virus-based anticancer therapies.

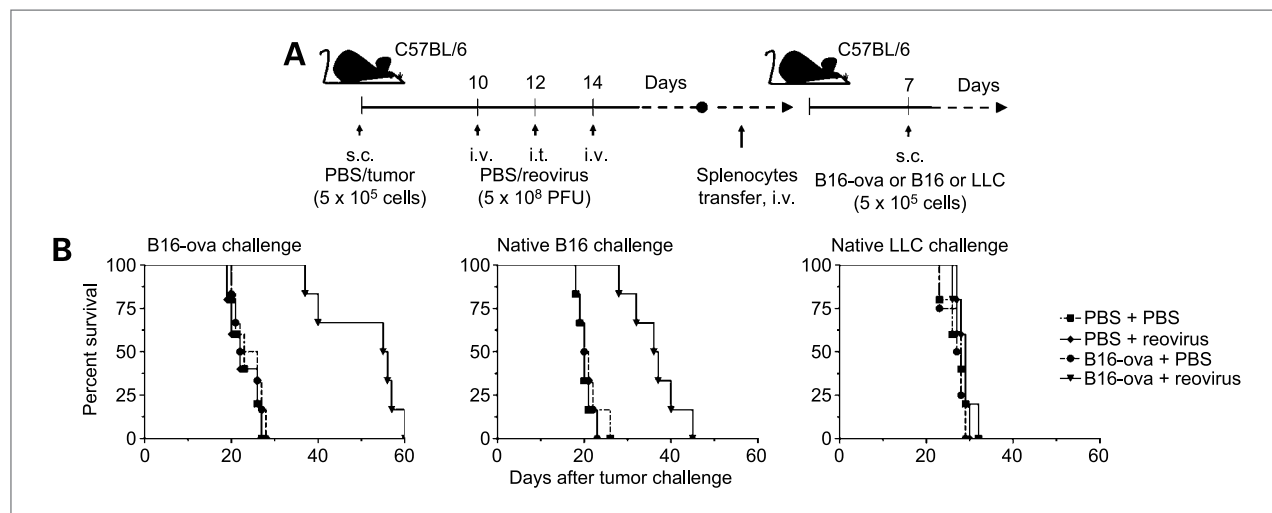


Figure 5. Reovirus oncotherapy initiated protective antitumor immunity. A, WT C57BL/6 mice were first injected with PBS or B16-ova tumor cells and then administered with a therapeutic regimen of PBS/reovirus. Seven days after the last injection, splenocytes were isolated and adoptively transferred in naive C57BL/6 mice. B, these recipient mice were then challenged with 5×10^5 B16-ova, native B16, or LLC cells and monitored for tumor growth and survival. Data are representative of three independent experiments ($n = 5$ mice per group).

Discussion

Appropriate antitumor immune responses can eliminate established tumors and restrict tumor recurrence (43–47). However, the development of such an antitumor immunity is thwarted by varied immune evasion strategies used by tumors. Immune system fails to recognize tumor antigens as “danger signals” because most tumors arise from the alterations of “self” tissue. Consequently, potentially immunogenic tumor peptides remain inaccessible to APCs. Other anomalies associated with tumor immune evasion include production of immunosuppressive cytokines, failure of lymphocyte homing and absence of costimulation on the surface of DCs (1, 3). Here, we show that reovirus-mediated immunologic events override such immune evasion strategies. Reovirus induces the proinflammatory cytokines such as IL-6, IL-1, TNF- α , GM-CSF, and IL-12 that have a capacity to enhance maturation and tumor antigen presentation capacity of APCs. On reovirus exposure, DCs are able to process otherwise sequestered tumor antigens and successfully present it to interacting T cells. It is interesting to note that reovirus-induced production of IL-6, RANTES, M-CSF, BLC, MIP-1 α , and MCP, along with decreased ICAM-1, can direct the trafficking of T and B lymphocytes, DCs, and macrophages to the tumor site and increase the chance of tumor antigen procurement and presentation.

Ironically, antireovirus immune responses influence the efficacy of reovirus oncotherapy in a positive as well as negative manner. The host immune system regards reovirus as an invading pathogen and induces strong antiviral innate and adaptive immune responses. These antireovirus immune responses can inhibit replication and spread of reovirus into tumor cells, and thus impair efficacy of oncolysis. On the other hand, when reovirus-specific T cells mount an attack on reovirus-infected cells, they essentially target tumor cells as reovirus preferentially infects cancerous cells (48). This inadvertent antireovirus T cell–mediated destruction of tumor cells can additionally contribute to the oncolytic activities of reovirus anticancer treatment.

Our study shows that antitumor T-cell proliferative and IFN- γ /CD107a responses are readily detectable during reovirus oncotherapy. This type of antitumor T-cell responses can directly kill tumor cells or restrict their expansion in a cytokine-dependent manner (49). The administration of tumor-specific T cells (or DCs capable of activating these T cells) during reovirus oncotherapy significantly enhances the survival in tumor-bearing hosts, suggesting that antitumor immune responses can coexist with reovirus-mediated oncolysis and synergistically contribute in tumor regression.

Most importantly, reovirus-activated antitumor immune response confirms the protection against subsequent tumor challenge. However, this capacity of reovirus-induced antitumor immune responses cannot be shown by challenging reovirus-treated primary tumor-bearing mice, as reovirus present in circulation may target freshly implanted secondary tumor cells and influence the outcome of challenge experiment. In this situation, a contribution of antitumor immunity in governing protective immunity against tumor challenge would be indistinguishable. Hence, reovirus oncotherapy–activated antitumor immune cells were transferred in reovirus-naive animals and then analyzed for their ability to restrict the growth of freshly implanted tumors. Further, recent reports have shown that reovirus loaded on immune cells can initiate the tumor regression on transfer of these cells (50). Thus, it can be argued that donor immune cells can acquire reovirus during virotherapy of primary tumors and carry it in naive host during transfer, and then restrict the development of secondary tumors after challenge. However, T cells isolated from reovirus-treated naive (nontumor-bearing) mice failed to inhibit the growth of secondary tumor, suggesting that such possible remnants of reovirus in immune cells do not contribute toward tumor growth inhibition in recipient mice. We also allowed 7-day time gap between adoptive transfer of donor lymphocytes and tumor challenge so that immune responses of recipient animal can purge residual reovirus present in transferred cells. It is noteworthy that most of the current oncotherapy protocols use immunosuppressive agents to promote direct cytolysis by reovirus, and inadvertently prevent the priming of antitumor immunity. Our study emphasizes that reovirus oncotherapy should be complimented with immunomodulatory interventions that will facilitate the priming and subsequent existence of antitumor immunity alongside with virus-mediated oncolysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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