

Host Lymphodepletion Enhances the Therapeutic Activity of an Oncolytic Vaccinia Virus Expressing 4-1BB Ligand

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Abstract

Oncolytic viral vectors have shown promise as antitumor therapeutic agents but their effectiveness is complicated by induction of antiviral antibody responses and rapid host clearance of recombinant vectors. We developed a recombinant oncolytic vaccinia virus expressing the 4-1BBL T-cell costimulatory molecule (rV-4-1BBL) and showed modest tumor regression in the poorly immunogenic B16 murine melanoma model. To improve the therapeutic potential of this vector, we tested the antitumor activity of local intratumoral injection in the setting of host lymphodepletion, which has been shown to augment vaccination and adoptive T-cell therapy. In this model, rV-4-1BBL injection in the setting of lymphodepletion promoted MHC class I expression, reduced antiviral antibody titers, promoted viral persistence, and rescued effector-memory CD8⁺ T cells, significantly improving the therapeutic effectiveness of the oncolytic vector. These data suggest that vaccination with rV-4-1BBL in the setting of host nonmyeloablative lymphodepletion represents a logical strategy for improving oncolytic vaccination in melanoma, and perhaps other cancers as well. [Cancer Res 2009;69(21):8516–25]

Introduction

Recombinant vaccinia virus has been used with the goal of inducing systemic host antitumor immunity in preclinical and early phase clinical trials (1–4). The direct oncolytic potential of vaccinia virus has only recently been used to improve the overall therapeutic effectiveness of these agents. Vaccinia virus is an ideal oncolytic vector because the virus has a well-established safety profile, has attenuated pathogenicity, selectively replicates in tumor cells, exhibits broad tumor tropism, and accommodates large eukaryotic transgenes (5–8). Further advantages of vaccinia include a life cycle limited to the cytoplasm, rapid tumor lysis, induction of danger signals, and local tumor vasculature shutdown (9, 10). We have previously shown that oncolytic vaccinia viruses expressing T-cell costimulatory molecules, such as B7.1, intercellular adhesion molecule-1, and lymphocyte function-associated antigen-3, were safe, induced tumor-specific T-cell responses, and resulted in objective tumor regression in metastatic melanoma patients (11, 12). The induction of strong antivaccinia antibody titers, however, can limit clinical effectiveness and result in more rapid viral clearance.

One strategy for improving therapeutic responses is to express more potent T-cell costimulatory molecules into the vector. 4-1BBL is a T-cell costimulatory molecule and binds to 4-1BB on activated T cells. 4-1BBL is particularly important for expansion of T cells and induction of long-term memory CD8⁺ T-cell responses, likely to be critical for tumor rejection (13–15). Consistent with this hypothesis, murine tumor cells engineered to express 4-1BBL exhibited enhanced immunogenicity (16), and adoptive transfer of T cells expressing 4-1BBL resulted in potent tumor rejection (17, 18). Systemic administration of agonistic anti-4-1BB monoclonal antibodies enhanced tumor immunity and tumor rejection in a murine melanoma tumor model (19, 20). Finally, we have previously shown that an oncolytic vaccinia virus expressing 4-1BBL had significant antitumor activity in a carcinoembryonic antigen transgenic murine carcinoma model. Furthermore, the rV-4-1BBL resulted in antigen spreading and a significant increase in tumor antigen-specific T cells within the tumor microenvironment of regressing tumors (21).

Another strategy that has been reported to increase the effectiveness of immunotherapy is the use of host nonmyeloablative lymphodepletion before vaccination or adoptive T-cell transfer. The potential role for nonmyeloablative lymphodepletion has been evaluated in patients with non-small cell lung carcinoma and melanoma (22–24). Four patients were treated with an adjuvant irradiated, autologous tumor cell vaccine with recombinant granulocyte macrophage colony-stimulating factor after nonmyeloablative lymphodepletion using cyclophosphamide and fludarabine. All patients tolerated the treatment well, and there was evidence of immune system activation although clinical outcomes were not reported (22). A similar lymphodepletion preparative regimen was combined with adoptive transfer of MART-1-specific T cells in patients with metastatic melanoma, and objective clinical responses were seen in 6 of 13 patients (23, 24). The mechanisms through which lymphodepletion improves immunotherapy are not defined, but they may be related to the removal of endogenous cellular elements that act as sinks for cytokines (25), the elimination of CD4⁺FoxP3⁺ regulatory (Treg) cells (26), the activation of innate immune responses through toll-like receptor-4, or some combination of these pathways (27, 28). The use of host lymphodepletion has not yet been reported as an adjunct for oncolytic viral therapy.

Materials and Methods

Mice. Six- to eight-week-old female C57BL/6 mice were purchased from Charles River Laboratory. Mice were housed and maintained under pathogen-free conditions and treated according to approved institutional protocols for animal care.

Cell lines. B16F10 and BS-C-1 cell lines were obtained from the American Type Culture Collection. Cell line verification was done by checking the morphology by routine microscope and growth curve analysis. The cell lines were also checked for IMPACT IV PCR Profile (MHV, MPV, MVM, *Mycoplasma* sp., PVM, Sendai, TMEV GDVII by MU Research

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Animal Diagnostic lab) and all test results were negative. BS-C-1 cells were used for viral titer assays and virus propagation. All cell lines were grown in MEM containing 10% fetal bovine serum, 10 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin.

Recombinant viruses. rV-4-1BBL and rV-LacZ were constructed as described previously (21). All viruses were propagated in BS-C-1 cells and purified on a sucrose gradient by ultracentrifugation. Viral titers were determined on BS-C-1 cells using standard plaque assays (7).

Characterization of rV-4-1BBL. B16F10 cells were infected with rV-4-1BBL *in vitro*, stained with 4-1BBL monoclonal antibody (mAb; eBioscience), and analyzed by flow cytometry. To identify *in vivo* 4-1BBL expression, mice bearing 7-d-old B16F10 tumor were vaccinated with rV-4-1BBL [1×10^8 plaque-forming units (pfu)] for 1 or 5 d, and 4-1BBL expression in tumor cells was analyzed by fluorescence-activated cell sorting (FACS). To identify cell death patterns *in vitro*, B16F10 cells were infected with rV-4-1BBL and stained using the TACS Annexin V-FITC Apoptosis kit (R&D) and then analyzed by flow cytometry.

Tumor treatment studies and statistical analysis. B16F10 cells (3×10^5) in 100- μ L PBS were implanted s.c. on the right flank of C57BL/6 mice. Mice were injected intratumorally with 100 μ L of PBS, rV-LacZ (10^8 pfu), or rV-4-1BBL (10^8 pfu) on days 4, 7, and 10. For host lymphodepletion studies, tumor-bearing mice were exposed to 5-Gy sublethal total body irradiation (TBI) on day 4. Tumors were measured in two dimensions using calipers as follows: tumor area (mm^2) = length \times width. Survival was analyzed using Kaplan-Meier log-rank test. Differences in tumor growth were analyzed by a one-way ANOVA test. $P < 0.05$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***) were considered significant.

MHC class I expression. B16F10 cells were stimulated with recombinant murine IFN- γ at different doses (Invitrogen), stained with phycoerythrin-conjugated MHC class I antibody (eBioscience), and analyzed by flow cytometry. For *in vivo* MHC class I expression, tumors were collected on day 21, and then tumor single-cell suspension was labeled with phycoerythrin-conjugated MHC class I antibody and analyzed by flow cytometry.

Determination of antivaccinia antibody titers. Sera were collected from inferior vena cava puncture 14 and 21 d after tumor challenge. Antibody titers were determined by standard ELISA. Briefly, immunoplates were coated with 10^6 pfu of purified UV-inactivated vaccinia virus followed by addition of mouse sera. A_{450} was determined using a plate reader.

Determination of viral titers. Tumor tissue and peripheral blood were collected 21 d after tumor injection. One milliliter of frozen tumor lysate was treated with three cycles of freeze-thawing followed by 1-min sonication. Individual lysates were diluted and viral titers determined by plaque assay on BS-C-1 cells as described previously (7).

Evaluation of systemic and local immune responses. Mice were sacrificed, and spleen and tumor were harvested and homogenized into single-cell suspensions. Erythrocytes were removed and cells labeled with the following mAbs: allophycocyanin-conjugated CD4, F4/80, and phycoerythrin-conjugated CD8, CD25, CD44, Gr-1, and FITC-conjugated CD11b, CD62L, and FoxP3. All antibodies were purchased from eBiosciences. For FoxP3 staining, splenocytes were treated by using FoxP3 staining buffer kit (eBioscience) according to the manufacturer's instructions. Samples were acquired using a FACSCalibur flow cytometer and CELLquest software.

Results

rV-4-1BBL expresses 4-1BBL *in vitro* and *in vivo*. We have previously constructed recombinant vaccinia viruses expressing different T-cell costimulatory molecules, and direct comparison of these vectors as oncolytic agents in the B16 melanoma model showed rV-4-1BBL to have the most potent therapeutic effects (see Supplementary Fig. S1). To confirm that rV-4-1BBL can infect and express 4-1BBL in B16F10 cells, a nearly confluent cell monolayer was infected with rV-4-1BBL at various multiplicities of infection (MOI). 4-1BBL expression was increased in a dose-dependent manner and became saturated at 2 MOI (Fig. 1A, *top*) within

24 hours. To determine the length of 4-1BBL expression time following infection, 2 MOI of rV-4-1BBL was used to infect B16F10 cells. As shown in Fig. 1A (*bottom*), 4-1BBL expression was maximal at 24 hours and was sustained for up to 72 hours when cell lysis was complete and cells detached from the plate (data not shown).

To confirm 4-1BBL expression *in vivo*, B16F10 cells were inoculated into C57BL/6 mice. After 7 days, rV-4-1BBL or rV-LacZ was injected intratumorally and tumors were collected at different time points. Flow analysis of tumor single-cell suspension shows that at 24 hours of infection, 40% of tumor cells expressed 4-1BBL, which declined slowly until day 5 when $\sim 23\%$ of the tumor cells maintained 4-1BBL expression (Fig. 1B). Because vaccinia virus can infect other cells in the tumor microenvironment, we tested the efficiency of infection in other trafficking immune cells (Supplementary Fig. S2). We observed 4-1BBL expression to some degree in almost all immune cells tested following exposure to rV-4-1BBL, including CD8 $^+$ (16%) and CD4 $^+$ T cells (19%), natural killer cells (28%), CD11b $^+$ macrophages (44%), and CD11c $^+$ dendritic cells (32%); however, the cells with the highest 4-1BBL expression were the B16F10 tumor cells, which maintained 80% expression at 48 hours (see Supplementary Fig. S2). These data show that rV-4-1BBL can preferentially and efficiently infect B16F10 melanoma cells *in vitro* and *in vivo* where expression is maintained for up to 5 days.

4-1BBL does not inhibit the oncolytic effect of vaccinia virus. Because the therapeutic goals of rV-4-1BBL vaccination are to induce an oncolytic effect in established tumors and to prime local T-cell immune responses through local 4-1BBL expression, we determined the oncolytic activity and cell death pattern of rV-4-1BBL infection in B16F10 cells. The degree of B16F10 cell lysis observed with rV-4-1BBL was comparable to that with rV-LacZ at the same dose and exhibited comparable kinetics (Supplementary Fig. S3). Because the mechanism of cell death can influence the type and magnitude of immune response, we also examined the cell death pattern of rV-4-1BBL by Annexin V-propidium iodide (PI) staining. rV-4-1BBL induced apoptotic cell death in 25%, 33%, 48%, and 70% of infected cells after 24, 36, 48, and 72 hours, respectively. Some cells showed signs of early apoptosis (Annexin V $^+$, PI $^-$), but this represented only 8%, 12%, and 16% of infected cells at 36, 48, and 72 hours, respectively (Fig. 1C and D). Similar results were obtained for rV-LacZ-infected cells (data not shown). These data show that rV-4-1BBL has comparable oncolytic activity to that of control vaccinia vectors and induces a largely apoptotic cell death beginning 24 hours after infection.

Oncolytic rV-4-1BBL induces therapeutic activity against established B16F10 melanoma. To determine if rV-4-1BBL could be used as an oncolytic therapy against established melanoma, we inoculated B16F10 murine melanoma cells into the flank of C57BL/6 mice. A small palpable tumor was generally present within 4 days and the tumor was injected with PBS (viral control), rV-LacZ (4-1BBL control), or rV-4-1BBL on days 4, 7, and 10. As expected, rV-LacZ showed significant tumor growth inhibition when compared with PBS-treated mice ($P < 0.0001$; Fig. 2A) due to the potent oncolytic effects of the virus. However, mice treated with rV-4-1BBL had a significant increase in tumor growth inhibition compared with mice treated with rV-LacZ ($P < 0.05$; Fig. 2A). Furthermore, rV-4-1BBL mice had smaller tumors, were more likely to have complete regression (Fig. 2A), and showed a survival advantage (Fig. 2B).

To determine if rV-4-1BBL induced local and/or systemic immunity, we collected spleen and tumor from vaccinated mice on day

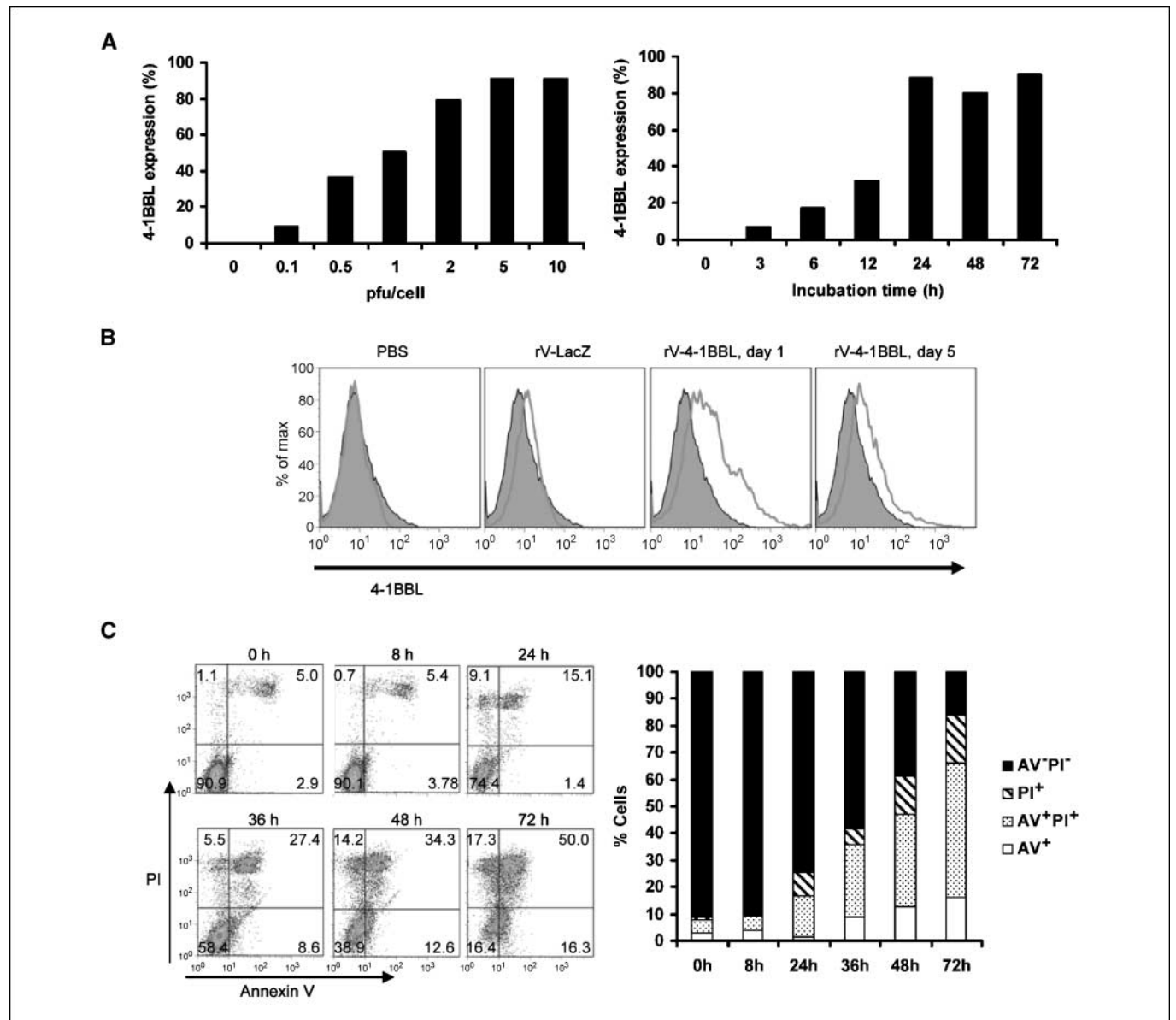


Figure 1. rV-4-1BBL induces 4-1BBL expression in B16F10 melanoma *in vitro* and *in vivo* and results in apoptotic cell death. **A**, flow analysis of 4-1BBL expression on B16F10 cells infected with rV-4-1BBL at 24 h (*left*). 4-1BBL expression on B16F10 cells infected with rV-4-1BBL (MOI = 2) at the indicated time points (*right*). **B**, 4-1BBL expression determined *in vivo* by FACS. IgG isotype controls shown as shaded area. **C**, cell death pattern of B16F10 cells infected with rV-4-1BBL (MOI = 1), determined by flow cytometry using Annexin V-PI (AV-PI) staining. Summary of cell populations based on Annexin V-PI expression over time after infection (*bar graph, right*).

14 to determine the frequency of various immune cell populations. In the spleen, rV-4-1BBL significantly increased the percentage of CD8⁺CD44⁺ T cells compared with rV-LacZ, although the frequency of total CD4⁺ and CD8⁺ T cells was similar in both groups (Supplementary Fig. S4A). Treatment with rV-4-1BBL also induced a shift in the CD8⁺ T-cell population from CD8⁺CD44⁻ (naïve) to CD8⁺CD44⁺ (effector) compared with mice treated with PBS or rV-LacZ (Supplementary Fig. S4B). In the tumor microenvironment, rV-4-1BBL significantly increased the frequency of CD8⁺ and CD8⁺CD44⁺ T cells compared with rV-LacZ ($P < 0.05$; Fig. 2C). The frequency of CD8⁺CD44⁺ T cells was similar between rV-LacZ- and rV-4-1BBL-treated mice, but the absolute numbers of CD8⁺CD44⁺ T cells were significantly increased in rV-4-1BBL-treated mice ($P < 0.05$; Fig. 2D).

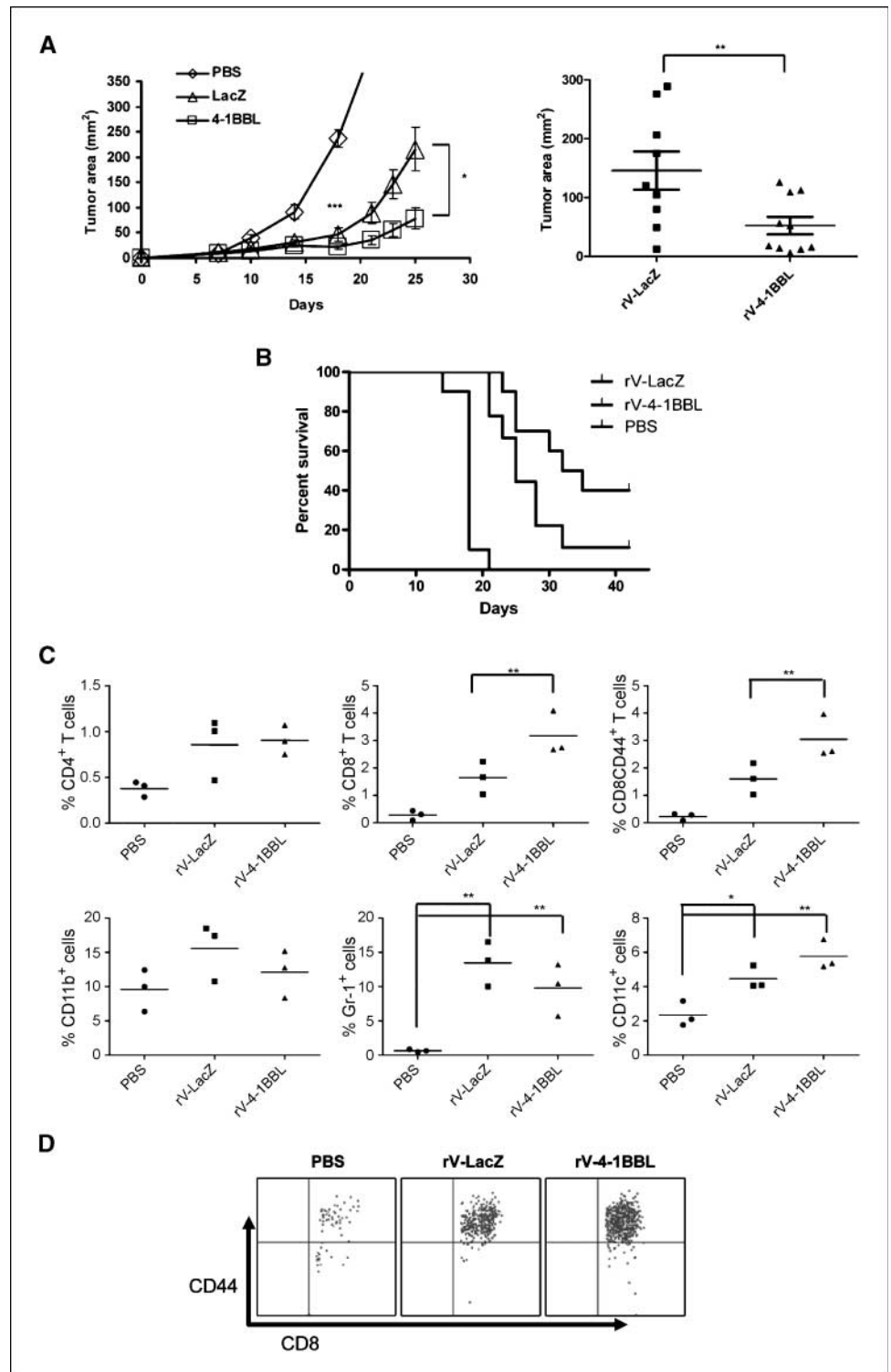
Both rV-4-1BBL and rV-LacZ increased the frequency of innate (CD11b⁺, Gr-1⁺, and CD11c⁺) immune cells in both the spleen and the tumor compared with PBS-treated mice without a significant difference between rV-LacZ and rV-4-1BBL (Fig. 2C; Supplementary Fig. S4A). These data show that oncolytic vaccinia virus induces innate immune responses in the local and systemic compartments, and rV-4-1BBL enhances therapeutic activity by promoting the expansion of CD8⁺CD44⁺ effector cells.

Lymphodepletion enhances the therapeutic effects of oncolytic vaccinia virus. To identify whether host lymphodepletion could augment the therapeutic response of rV-4-1BBL, mice bearing B16F10 for 4 days were treated with 5-Gy TBI and vaccinated with PBS, rV-LacZ, or rV-4-1BBL on days 4, 7, and 10. TBI alone showed significant tumor growth inhibition and had additive

antitumor effects with rV-LacZ or rV-4-1BBL (Fig. 3A and B). TBI in combination with rV-4-1BBL ($P < 0.01$) showed maximum anti-tumor effects. Whereas some tumors still escaped complete rejection, host lymphodepletion clearly affected the kinetics of tumor growth (Fig. 3B). When the number of mice completely rejecting their tumors was evaluated, we found that 2 of 24 (12.5%) mice treated with rV-LacZ alone, 5 of 25 (20%) mice treated with rV-

4-1BBL alone, 2 of 22 (18.2%) mice treated with TBI and rV-LacZ, and 8 of 27 (29.6%) mice treated with TBI and rV-4-1BBL had complete rejection (Fig. 3C). Vaccination induced vaccinia-specific T-cell responses as shown by IFN- γ ELISPOT assay using whole splenocytes from day 14 treated mice exposed to autologous antigen-presenting cells pulsed with vaccinia lysate (Fig. 3D, left). Mice treated with rV-LacZ and rV-4-1BBL in the setting of TBI also

Figure 2. Local injection of rV-4-1BBL induces therapeutic responses in a B16F10 melanoma model by increasing the effector CD8⁺ T cells in the tumor microenvironment. B16F10 cells were inoculated into the mice and treated with PBS, rV-LacZ, or rV-4-1BBL on days 4, 7, and 10 after tumor implantation. *A, left*, tumor growth curves for each experimental group [points, mean ($n = 9-10$); bars, SE]. *Right*, tumor size in individual mice on day 24. *B*, survival curves of PBS-, rV-LacZ-, and rV-4-1BBL-treated mice. *C*, tumor tissues were collected on day 14 after tumor implantation and the frequency of individual immune cells was analyzed by FACS analysis. *D*, representative dot plot of CD8⁺ T cells in the tumor microenvironment for each treatment group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



showed an increase in tumor-reactive CD4⁺ and CD8⁺ T cells, with a slight increase in CD8⁺ T cells in mice receiving the rV-4-1BBL compared with rV-LacZ (Fig. 3D, right). These data show that host lymphodepletion has an additive effect with oncolytic vaccinia therapy and achieves the greatest effect in combination with rV-4-1BBL. The data also support the notion that whereas some mice

exhibit dramatic complete tumor regression that is associated with an increase in CD4⁺ and especially CD8⁺ T cells, in some mice, tumors escape rejection.

B16F10 melanomas escape rejection through loss of MHC class I expression. Melanoma may escape immune-mediated rejection through loss of MHC class I expression (29, 30). Thus, we

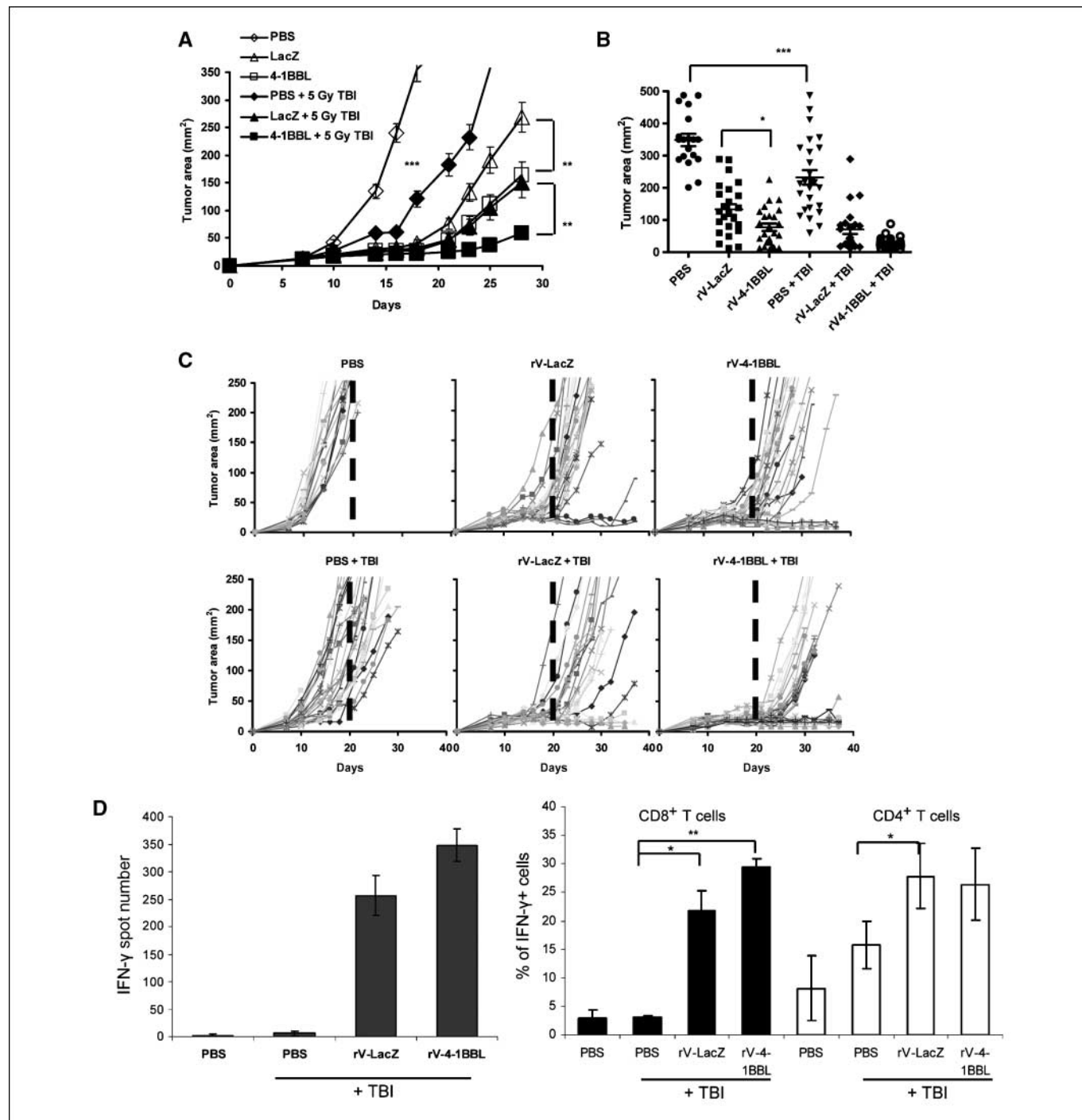


Figure 3. Host lymphodepletion enhances the therapeutic effect of oncolytic vaccinia vectors. B16F10 cells were injected in C57BL/6 mice followed by TBI (5 Gy) on day 4. Mice were then vaccinated with PBS, rV-LacZ, or rV-4-1BBL on days 4, 7, and 10. *A*, tumor growth curves for each treatment group (points, mean; bars, SE). *B*, tumor size of individual mice on day 24. *C*, tumor growth curves of individual mice in each treatment group. All mice in the PBS group died by day 20 (dotted line). *D*, IFN-γ ELISPOT of day 14 splenocytes exposed to vaccinia lysate-pulsed antigen-presenting cells (left) or B16 (right). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

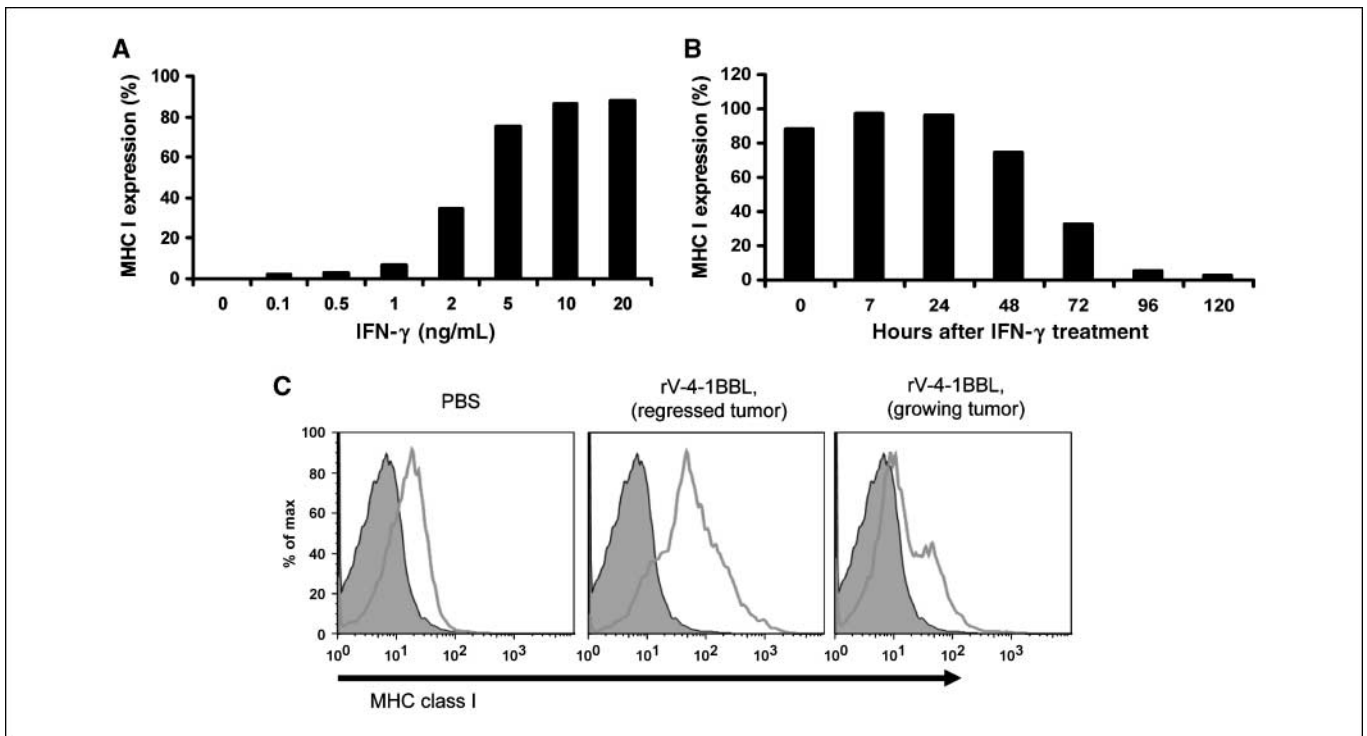


Figure 4. rV-4-1BBL restores MHC class I expression in B16F10 melanoma. *A*, B16F10 cells were treated with increasing doses of IFN- γ *in vitro*. After 24 h, MHC class I expression was determined. *B*, B16F10 cells were treated with IFN- γ (20 ng/mL) for 24 h, medium was replaced, and MHC class I expression was determined at the indicated times. *C*, mice were treated as described in Fig. 3. MHC class I expression was evaluated from tumors excised on day 21. Representative histograms are shown, with IgG isotype control shaded.

sought to determine if this could be playing a role in preventing rejection with TBI and rV-4-1BBL treatment. To evaluate this possibility, we first evaluated MHC class I expression in our parental B16F10 cell line because variations in this cell line have been reported (31). Figure 4 shows that the B16F10 melanoma cell line did not exhibit MHC class I expression *in vitro*, but class I could be restored in a dose-dependent manner on exposure to exogenous IFN- γ , up to >90% expression with 10 ng/mL IFN- γ (Fig. 4*A*). MHC class I expression was sustained for 48 hours *in vitro* and was then decreased, suggesting that this might be a major obstacle to CD8⁺ T-cell rejection *in vivo* (Fig. 4*B*). To further explore the role of MHC class I loss *in vivo*, we harvested tumor tissue 21 days after treatment with rV-4-1BBL, a time at which tumors are clearly regressing or progressing. Mice with regressing small tumors showed high levels of MHC class I expression *in vivo* (Fig. 4*C*). In contrast, mice with progressing large tumors showed no MHC class I expression. These data suggest that oncolytic vaccinia virus can restore MHC class I expression on poorly immunogenic B16F10 melanoma in some mice and is associated with a tumor regressor phenotype.

Host lymphodepletion blocks antiviral antibody responses and promotes viral persistence in the tumor microenvironment. Because a major obstacle to successful gene therapy is the induction of vector-specific antibody titers limiting viral persistence and replication, we sought to determine the effect of host lymphodepletion on vaccinia persistence and antivaccinia viral titers. First, tumor tissue from rV-LacZ- or rV-4-1BBL-treated mice was collected 21 days after tumor challenge and vaccinia titers were determined by standard plaque assay. In both rV-LacZ- and rV-4-1BBL-treated mice, TBI resulted in a 3- to 4-fold increase in viral titers within tumor samples compared with non-TBI vac-

cine-treated groups (Fig. 5*A*). There were no detectable viral titers in progressing tumors regardless of the treatment conditions (data not shown).

Next, host antivaccinia antibody responses were detected using sera collected on days 14 and 21 following tumor challenge and treatment. Mice treated with rV-LacZ or rV-4-1BBL alone showed significant antivaccinia antibody titers by day 14. In contrast, mice treated with TBI before vaccinia injection showed no antibody titers at day 14 (Fig. 5*B*). Vaccinia-specific antibody titer at day 21 in TBI-treated mice was significantly lower than that in non-TBI-treated mice. There was no difference in vaccinia-specific antibody responses between rV-LacZ and rV-4-1BBL (Fig. 5*B*). These data suggest that host lymphodepletion increases viral persistence in the tumor microenvironment by reducing antiviral antibody responses.

Oncolytic rV-4-1BBL rescues CD8⁺ T-cell responses following lymphodepletion. On day 14 after tumor injection, the frequency of CD4⁺ T cells in the tumor of TBI-treated mice was significantly decreased compared with non-TBI-treated animals ($P < 0.01$). Further, exposure to rV-4-1BBL did not affect the frequency of total CD4⁺ T cells (Supplementary Fig. S5*A*). When we examined the frequency of CD8⁺ T cells, we found that mice treated with TBI and rV-LacZ showed a trend toward fewer CD8⁺ T cells compared with immunocompetent mice (Fig. 6*A*). The frequency of CD8⁺ T cells in mice treated with TBI and rV-4-1BBL, however, was significantly increased compared with mice treated with rV-4-1BBL alone ($P < 0.01$). The CD8⁺ T cells recovered were largely CD44⁺ (Fig. 6*A*; Supplementary Fig. S5*A*) and CD62L⁻ (Supplementary Fig. S4*B*), consistent with an activated effector-memory phenotype. These data suggest that 4-1BBL expression rescues

effector-memory CD8⁺ T cells that are otherwise lost following host lymphodepletion.

Local rV-4-1BBL does not affect Tregs but does influence the CD8/Treg T-cell ratio following lymphodepletion. Because CD4⁺FoxP3⁺ Tregs appear in melanoma patients and inhibit T-cell immunity, we also determined the frequency of Tregs. In the spleen, oncolytic vaccinia virus–treated groups had significantly fewer Tregs compared with PBS-treated mice (Fig. 6B). The Treg frequency in the spleen, however, was similar among all groups following TBI. When the ratio of effector CD8⁺ T cells to Tregs was determined in non-TBI-treated mice, an increase was seen in both the rV-LacZ–treated and the rV-4-1BBL–treated groups compared with the PBS control group. However, following TBI exposure, only the rV-4-1BBL–treated mice showed a trend toward increased effector CD8⁺/Treg ratio (Fig. 6B). In the tumor microenvironment, the Treg frequency was similar in all treatment groups, but the ratio of effector CD8⁺ to Tregs was significantly increased in rV-4-1BBL–treated mice compared with both PBS-treated and rV-LacZ–treated animals. In the setting of host lymphodepletion, the effect of rV-4-1BBL on CD8⁺ to Treg ratio was even more significant (Fig. 6C). These data suggest that the ratio of effector CD8⁺ T-cell to Tregs may be a critical determinant of tumor growth inhibition and suggests that 4-1BBL contributes to tumor rejection through expansion of effector CD8⁺ T cells with minimal direct effect on Tregs.

Discussion

In this report, we showed the feasibility and enhanced therapeutic effectiveness of an oncolytic vaccinia virus administered in the setting of host lymphodepletion. The oncolytic effect of vaccinia

virus results in apoptotic tumor cell death (Fig. 1C), which likely promotes therapeutic benefit both directly through tumor cell killing and indirectly through stimulation of host T-cell responses induced by cross-presentation of tumor antigens derived from apoptotic melanoma cells. The oncolytic effect depends on the ability of the virus to enter tumor cells, and this has been a challenge with other viral vectors, such as adenovirus and herpes virus, which access cells through highly specific cell surface receptors (32, 33). Tumors that lack viral entry receptors may not be amenable to infection. This is less problematic with vaccinia virus, which uses a membrane fusion process allowing entry to many different cell types (34). In this report, we found that whereas vaccinia does infect a wide range of cells often present in the tumor microenvironment, melanoma cells were the most efficiently infected. This is consistent with previous reports suggesting that vaccinia virus exhibits particular tropism for tumor cells (7).

The recombinant vaccinia vector used in this report also resulted in local expression of the 4-1BBL T-cell costimulatory molecule, which improved therapeutic responses significantly. We also showed that host lymphodepletion can improve therapeutic responses to oncolytic viral therapy, an effect previously reported for both systemic vaccination and adoptive T-cell therapy. The best therapeutic effects in this study were observed when all three strategies—oncolytic virus, 4-1BBL expression, and prior host lymphodepletion—were combined. The use of combination approaches to optimize the type and intensity of immune response is an increasingly important theme for tumor immunotherapy (35, 36). The vaccinia virus seems to contribute significantly to this effect, as we observed an increase in tumor-specific CD4⁺ and CD8⁺ T cells in the spleen of mice. 4-1BBL

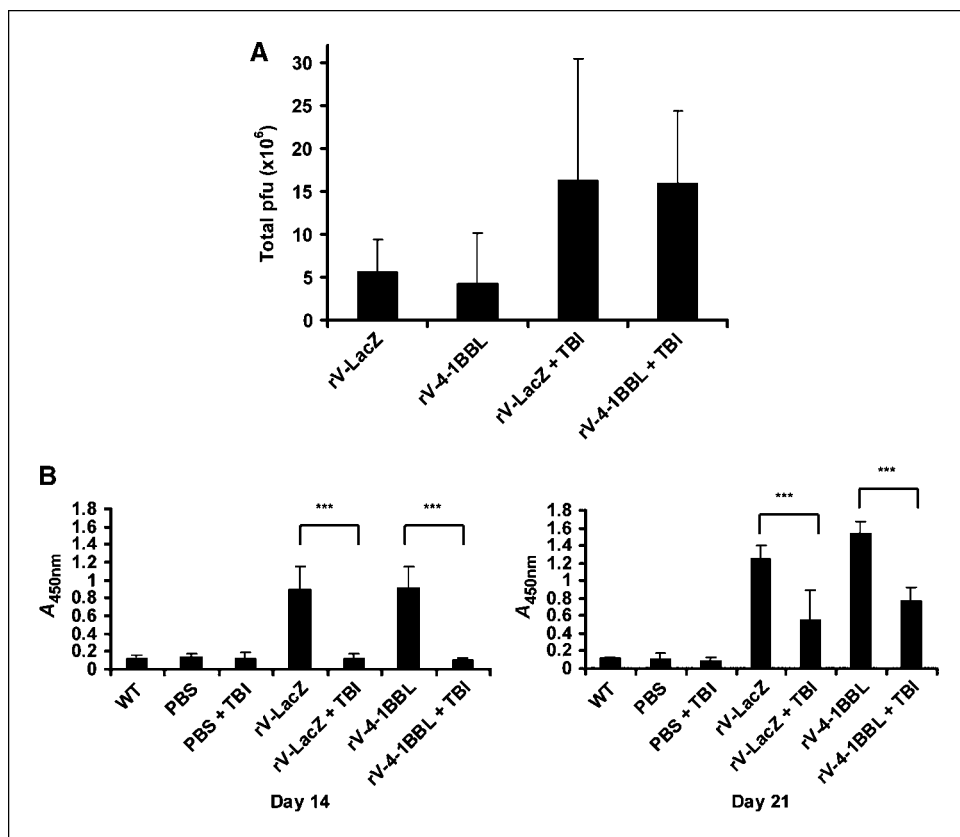


Figure 5. Host lymphodepletion promotes viral persistence by blocking antiviral antibody responses. Mice were treated as described in Fig. 3. *A*, B16F10 tumors were harvested at day 21 and vaccinia titers determined by plaque assay. *B*, antivaccinia antibody titers in sera collected on days 14 and 21 were determined by ELISA. Columns, mean of triplicate samples; bars, SD.

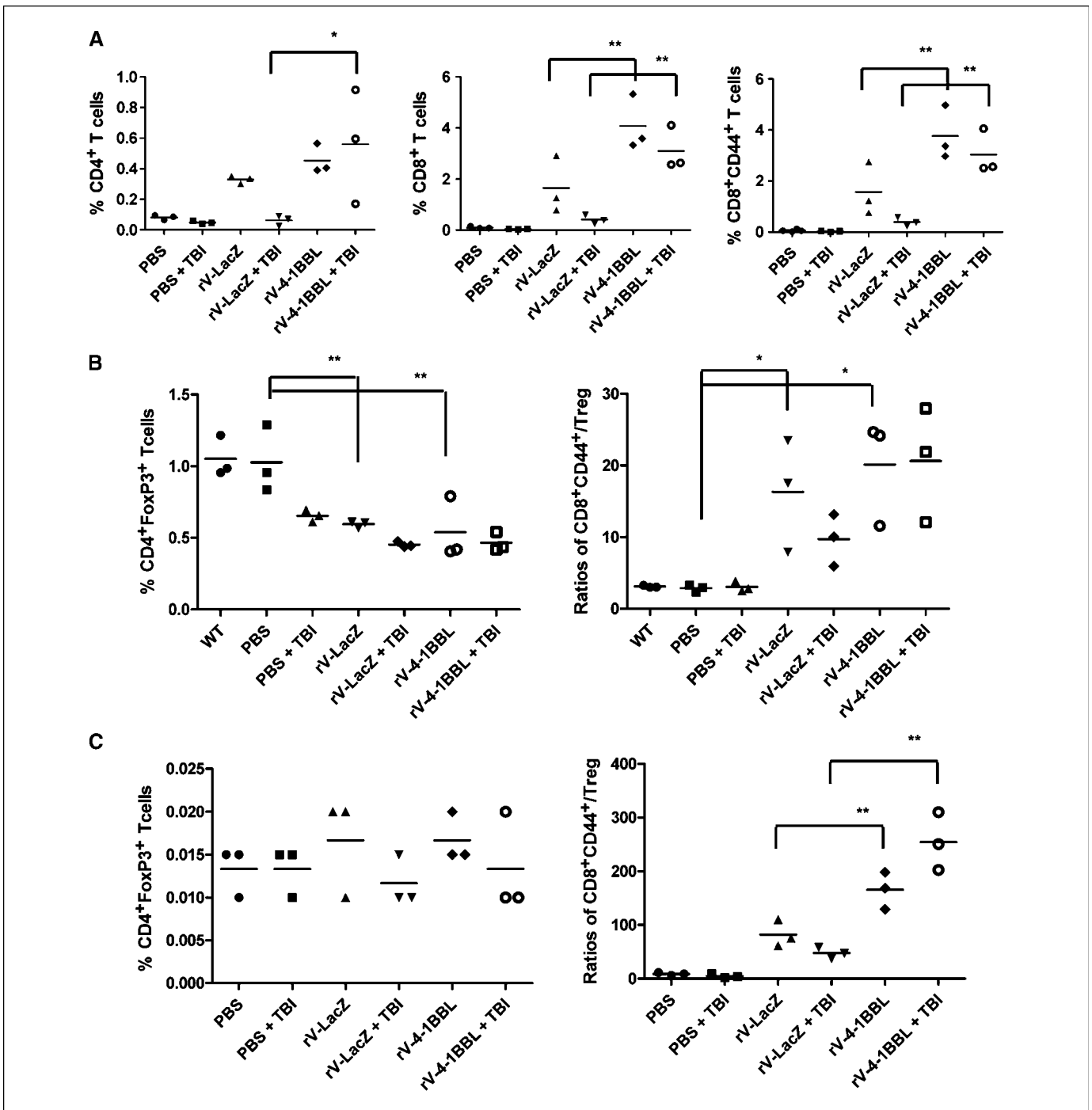


Figure 6. Oncolytic rv-4-1BBL rescues CD8⁺ effector-memory T cells and increases the CD8⁺ T-cell to CD4⁺FoxP3⁺ Treg ratio in the systemic and tumor microenvironments. Mice were treated as described in Fig. 3. Tumor tissue was collected on day 14. *A*, frequency of CD4⁺, CD8⁺, and CD8⁺CD44⁺ T cells within the tumor microenvironment. *B* and *C*, CD4⁺FoxP3⁺ Treg frequency (*left*) and the effector CD8 to Treg ratio (*right*) in the spleen (*B*) and tumor (*C*) microenvironment.

may add to the therapeutic effect by promoting cross-presentation of melanoma antigens during initial encounter with viral-infected tumor cells or maintenance of reactive CD8⁺ T cells, and in fact, we did observe an increase in tumor-specific CD8⁺ T cells in the spleen at day 14 in vaccinated mice. An important caveat of our model was that even under the optimal treatment conditions, we observed only a 30% complete regression of established tumors (Fig. 3). Although the combination treatment clearly delayed tu-

mor growth in all mice, the majority of tumors eventually escaped and continued to grow. This is highly analogous to the clinical situation where immunotherapy has been remarkably effective but only in a small subset of patients (11, 12). The data presented in our model of melanoma provided some insight into how each component of the treatment regimen augmented host antitumor immunity and suggested potential mechanisms of tumor escape.

A potential reason for failure of tumor immunotherapy is the loss of antigen or MHC class I expression on tumor cells. There is evidence that human melanoma and other tumors frequently lose class I or β_2 -microglobulin expression (29, 30). We found that the B16F10 cell line used in our studies was deficient in class I expression but that expression could be restored by exposure to IFN- γ (Fig. 4). To determine if loss of MHC I expression was a mechanism of tumor escape in our model, we evaluated established B16 tumors *in vivo* for evidence of MHC class I expression following treatment. We found that mice with regressing tumors in virus-treated groups had increased class I expression compared with mice treated with the same regimen but experiencing tumor progression. This is consistent with a CD8⁺ T-cell-mediated mechanism of tumor rejection, and suggests that melanoma may escape immunotherapy through loss of MHC class I and that strategies aimed at increasing or restoring class I expression might be useful. The host response to vaccinia infection typically results in a potent IFN- γ response and likely explains the increased class I expression seen in responding tumors (37).

Another major challenge to oncolytic gene therapy with viral vectors is the development of neutralizing antibodies against the viral vector and rapid clearance of viral particles. Whereas host lymphodepletion has been used in preclinical and early phase clinical studies (38–40), the mechanisms by which tumor immunity is enhanced are not fully defined (41, 42). One possibility is the elimination of B lymphocytes by TBI, resulting in decreased antibody production and prolonged viral persistence in the host. Our data would support such a hypothesis, as we observed that mice treated with TBI showed a significant lack of antivaccinia antibodies on day 14, although these slowly recovered with detectable titers by day 21. The lack of antiviral antibodies was also associated with a significant increase in the number of viral particles found within the tumor microenvironment on day 21, supporting a relationship between antibody response and viral clearance (Fig. 5).

Host lymphodepletion has also been suggested to benefit cancer patients by eliminating Tregs from the system (26, 43). The depletion of naïve CD8⁺ T cells, however, may be deleterious to generating antitumor CD8⁺ T cells during lymphodepletion. In the present study, we found that host lymphodepletion resulted in a decrease in CD4⁺ T cells, although vaccination had no effect on this population. In contrast, the CD8⁺ T cells were similarly depleted following TBI in all groups except the rV-4-1BBL-treated mice, suggesting that exposure to 4-1BBL early after lymphodepletion can rescue CD8⁺ T cells (Fig. 6A). In this system, the antigens responsible for tumor rejection are not known, and thus we could not define specific monoclonal T cells responsible for tumor rejection. There are studies that the balance of effector CD8⁺ T cells to regulatory CD4⁺FoxP3⁺ T cells is the critical determinant of tumor rejection (44, 45). Consistent with these studies, we found that there was a significant increase in the CD8⁺ T-cell to Treg ratio in mice treated with TBI and rV-4-1BBL. This is consistent with a predominantly CD8⁺ T-cell-mediated mechanism of tumor rejection in this model (Fig. 6B and C).

In summary, host lymphodepletion seems to be a useful adjuvant for oncolytic viral therapy resulting in decreased antiviral antibody responses and prolonged viral persistence *in vivo*. Further studies to better understand the antigenic basis of rejection and the mechanism of tumor escape in this model are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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