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Original Article

Oncolytic Vaccinia Virus Expressing 4-1BBL Inhibits Tumor Growth by Increasing CD8+ T Cells in B16F10 Tumor Model

Na Kyung Lee[1] and Hong Sung Kim[2,†]

[1]Department of Biomedical Laboratory Science, Soon Chun Hyang University, Asan 336-745, Korea
[2]Department of Biomedical Laboratory Science, Korea Nazarene University, Cheonan 331-718, Korea

Oncolytic viral vectors have shown good candidates for cancer treatment but have many limitations. To improve the therapeutic potential of oncolytic vaccinia virus, we developed a recombinant vaccinia virus expressing the 4-1BBL co-stimulatory molecule or CCL21. 4-1BBL and CCL21 expression was identified by FACS analysis and immunoblotting. rV-4-1BBL vaccination shows significant tumor regression compared to rV-LacZ, but rV-CCL21 shows rapid tumor growth compared to rV-LacZ in the poorly immunogenic B16 murine melanoma model. 4-1BBL expression resulted in the increase of the number of CD8+ T cells and especially the increase of effector (CD62L-CD44+) CD8+ T cells. These data suggest 4-1BBL may be the potential target for enhancement of tumor immunotherapy.

Key Words: 4-1BBL, Gene therapy, Vaccine, Vaccinia virus

INTRODUCTION

Recombinant vaccinia virus has been used to express tumor-associated antigens, cytokines and co-stimulatory molecules with the goal of inducing systemic host anti-tumor immunity in pre-clinical and early phase clinical trials (van der Bruggen et al., 1991; Applebaum et al., 1998). The clinical studies established an acceptable safety profile for these viral vectors and also demonstrated induction of tumor-specific T cell responses in most reports (John et al., 2012; Kaufman et al., 2006; Kober et al., 2008). The direct oncolytic potential of vaccinia virus has only recently been utilized to improve the therapeutic effectiveness of these agents. Vaccinia virus is an ideal oncolytic vector since the virus has a well established toxicity profile, deletion of viral genes results in attenuated pathogenicity and selective replication in tumor cells. Also, the virus has broad tumor tropism since it utilizes a membrane fusion cell entry mechanism, and recombinant vectors can accommodate large eukaryotic gene insertions (Schmidt et al., 2012).

Further advantages of vaccinia include a life cycle limited to the cytoplasm avoiding nuclear integration and cell transformation, early lytic activity within 7~8 hours after infection allowing rapid tumor lysis and induction of potent humoral and cell-mediated immunity through necrotic tumor cell death and cross presentation of tumor antigens and release of pro-inflammatory cytokines and chemokines (Parato et al., 2005; McCart et al., 2001; Thorne et al., 2007; Kirn and Thorne, 2009). It has previously been shown that oncolytic vaccinia virus expressing the T cell co-stimulatory molecule B7.1 alone or with ICAM-1 and LFA-3 was safe, inducing systemic gp100- and MART-1-specific T cell responses and resulting in objective tumor regression in approximately 30% of metastatic melanoma patients including two durable complete responses of injected and non-injected tumor(s) (Kaufman et al., 2005; Kaufman et al., 2006).

One strategy for improving therapeutic responses is to express more potent immune cell co-stimulatory molecules

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†Corresponding author: Hong Sung Kim. Department of Biomedical Laboratory Science 456 Ssangyong, Seobuk, Chung Nam 331-718 Korea.
Tel: +82-41-570-4165, Fax: +82-41-570-4258
e-mail: hskim@kornu.ac.kr

into the vector. CCL21 is chemokine expressed in T cell zones of spleen and lymph nodes, and recruits both naïve lymphocytes and antigen stimulated DC into T cell zones of secondary lymphoid organs, co-localizing these early immune response constituents. The capacity to facilitate the co-localization of both DC and T cells forms a strong rationale for the use of SLC/CCL21 in cancer therapy. (Sharma et al., 2003). 4-1BBL is a T cell co-stimulatory molecule expressed on activated antigen-presenting cells and binds to 4-1BB on T cell, which is transiently induced during primary and recall responses. 4-1BB-4-1BBL interactions promote the survival and expansion of activated T cells. Recent data has suggested that 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 (Serghides et al., 2005; Kober et al., 2008). Consistent with this hypothesis, murine tumor cells engineered to express 4-1BBL exhibited enhanced immunogenicity following tumor challenge (Melero et al., 1998) and adoptive transfer of T cells expressing 4-1BBL resulted in potent tumor rejection (Stephan et al., 2007; Yi et al., 2007).

In this report, we established the baseline effectiveness of an oncolytic recombinant rV-4-1BBL or rV-CCL21 in the murine B16F10 melanoma model. In order to further identify the therapeutic potential of rV-4-1BBL or rV-CCL21 immunotherapy, we evaluated T cell populations in B16F10 melanoma tumor models.

MATERIALS AND METHODS

Mice

6~8 week old female C57BL/6 mice were purchased from OrientBio (SungNam, Korea). The mice were housed and maintained under pathogen-free conditions and treated according to approved institutional protocols for animal care.

Cell lines

B16F10 (murine melanoma), BS-C-1 (African green monkey kidney), and HeLaS3 (human cervical carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). BS-C-1 and HeLaS3 cells were used for vaccinia virus titer assays and virus propagation. All cell lines were grown in MEM containing 10% FBS, 10 mM L-glutamine, 100 U/ml Penicillin/streptomycin, and 0.1% Gentamicin.

Recombinant viruses

rV-4-1BBL or rV-CCL21 was constructed as described previously (Kudo-Saito, Hodge et al., 2006). Briefly, murine 4-1BBL was cloned from a cDNA library and inserted into the vaccinia plasmid, pSC65 (a kind gift from Dr. Bernard Moss, NIAID, Bethesda, MD), which places the 4-1BBL into the viral thymidine kinase (TK) gene under control of a vaccinia synthetic early-late promoter. Recombinant rV-4-1BBL was generated by homologous recombination during co-transfection of BS-C-1 cells with wild-type vaccinia virus (Western Reserve) and the recombinant pSC65-4-1BBL plasmid. rV-LacZ contains the *E. coli* β-galactosidase gene in the viral TK region and was used as a negative control. All viruses were propagated in HeLaS3 cells and purified on a sucrose gradient by ultracentrifugation. Viral titers were determined on BS-C-1 cells using standard plaque assays, as described elsewhere.

Characterization of rV-4-1BBL and rV-CCL21

Confluent B16F10 cell monolayers in 6 well plates were infected with rV-4-1BBL, rV-CCL21 at 5 pfu/cell and collected at 48 h. Cells were harvested from the plates and stained with PE conjugated 4-1BBL (eBioscience, San Diego, CA) and analyzed by flow cytometry. CCL21 expression is identified by immunoblotting.

Tumor treatment studies and statistical analysis

B16F10 melanoma cells (3×10^5) in 100 μl PBS were implanted s.c. on the right flank of C57BL/6 mice. The mice were injected i.t. with 100 μl of PBS, rV-LacZ (10^8 pfu), or rV-4-1BBL (10^8 pfu), or rV-CCL21 (10^8 pfu) on day 4, 7, and 10 after tumor inoculation. Before injection, all viruses were sonicated three times for 1 min at maximum power. Tumors were measured in two dimensions in a blinded fashion using calipers as follows: tumor area (mm^2) = length × width. Difference in tumor growth was analyzed by a one-way ANOVA test. *P* values were considered

significant at $P<0.05$: *.

Evaluation of systemic and local immune responses

Mice were sacrificed at the indicated times spleens and tumors were harvested and homogenized into single-cell suspensions using the rubber end of a 10-cc syringe and a 70 μm filter cup. Erythrocytes were removed by hypotonic lysis and cells were labeled with the following mAbs: APC-conjugated CD8 and PE-conjugated CD8, CD44, and FITC-conjugated CD4, CD62L. All antibodies were purchased from eBiosciences. Samples were acquired using a FACScalibur™ flow cytometer and CELLquest™ software. FACS data were analyzed by Flowjo software.

RESULTS

Identification of 4-1BBL and CCL21 expression

To confirm the 4-1BBL or CCL21 expression following infection of melanoma cells *in vitro*, rV-4-1BBL or rV-CCL21 was used to infect nearly confluent plated B16F10 cells. Melanoma cells were collected at 48 h following infection and 4-1BBL expression was evaluated by FACS analysis (Fig. 1), and CCL21 expression was identified by immunoblotting (data not shown).

Therapeutic activity of rV-4-1BBL or rV-CCL21 against established B16F10 melanoma

To determine if rV-4-1BBL or rV-CCL21 could be used as an oncolytic treatment against established melanoma, we inoculated B16F10 murine melanoma cells into the left flank of C57BL/6 mice. A small palpable tumor was generally present within four days and the tumor was injected with PBS (viral control), 1×10^8 pfu of rV-LacZ (virus control) or 1×10^8 pfu of rV-4-1BBL or 1×10^8 pfu of rV-CCL21 on days 4, 7 and 10. As expected, rV-LacZ vaccination demonstrated significant tumor growth inhibition compared to PBS treated mice ($P<0.05$, Fig. 2) due to the potent oncolytic effects of vaccinia virus. However, mice treated with rV-4-1BBL had a significant tumor growth inhibition compared to mice treated with

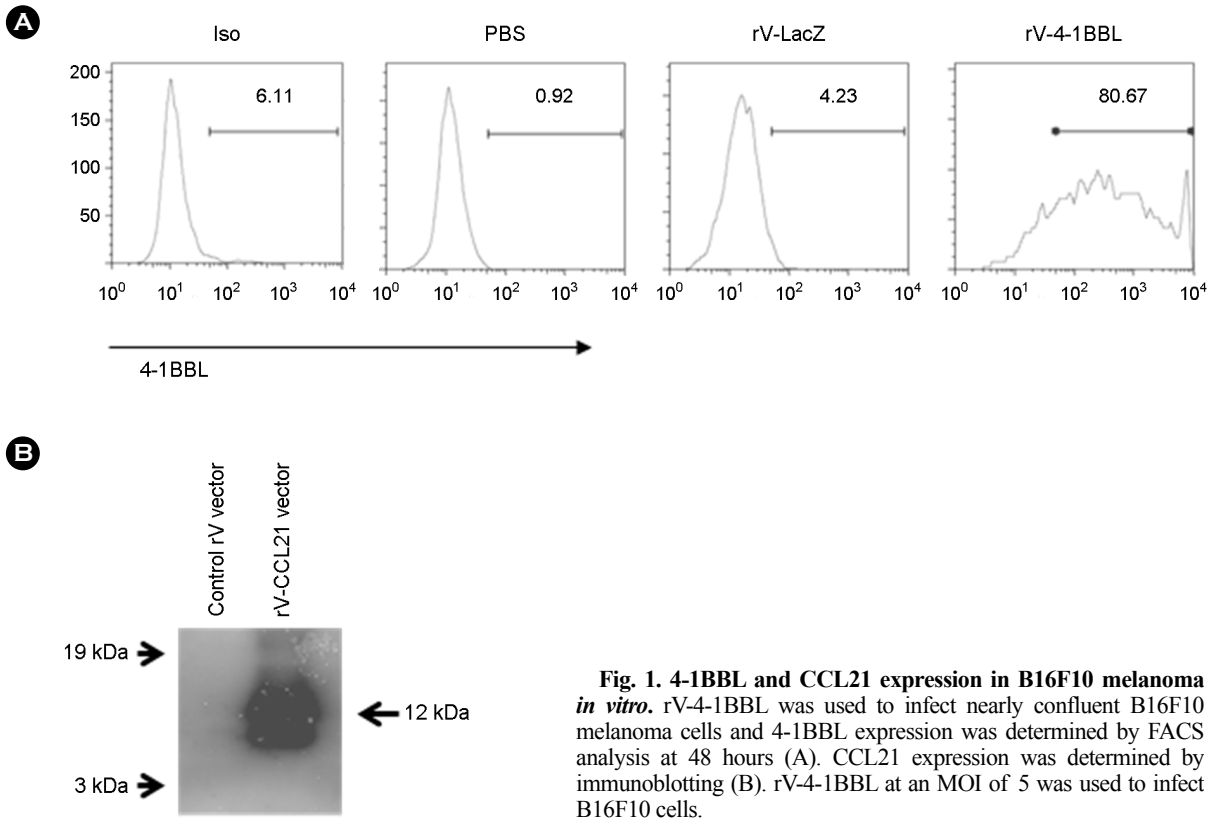

Fig. 1. 4-1BBL and CCL21 expression in B16F10 melanoma *in vitro*. rV-4-1BBL was used to infect nearly confluent B16F10 melanoma cells and 4-1BBL expression was determined by FACS analysis at 48 hours (A). CCL21 expression was determined by immunoblotting (B). rV-4-1BBL at an MOI of 5 was used to infect B16F10 cells.

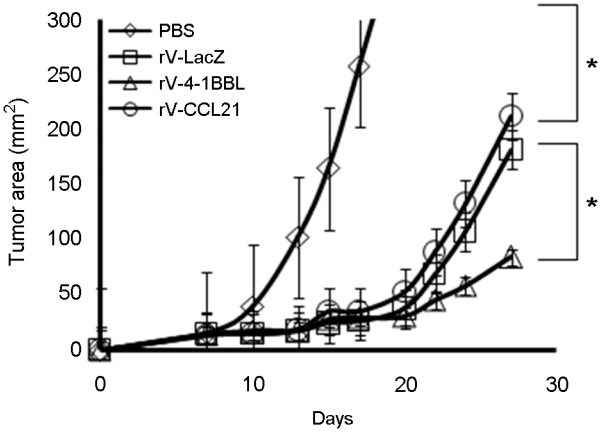

Fig. 2. Local injection of rV-4-1BBL significantly inhibits tumor growth in a B16F10 melanoma model. B16F10 cells (3×10^5) were inoculated into the right flank of C57BL/6 mice subcutaneously and treated with PBS, 10^8 pfu rV-LacZ or 10^8 pfu rV-4-1BBL or 10^8 pfu rV-CCL21 by intratumorally injection on days 4, 7, and 10 after tumor implantation. Tumor growth curves represented as mean ± SEM for each experimental group (n= 9~10). Data shown is from one of three independent experiments. *; $P<0.05$

rV-LacZ or rV-CCL21 ($P<0.05$, Fig. 2). Furthermore, rV-4-1BBL treated mice that had smaller tumors were more likely to have complete regression. rV-CCL21 treated mice showed rapid tumor growth compared with rV-LacZ treated mice.

Immune cell phenotype of rV-4-1BBL vaccination

To identify whether the rV-4-1BBL induced local or systemic immunity, we collected spleen and tumor tissue from vaccinated mice on day 14 to determine the frequency of various naïve and activated immune cell populations. In the spleen, rV-4-1BBL treated mice significantly increased the percentage of CD8+ T cells and decreased the percentage of CD4+ T cells compared to mice treated with PBS. And the frequency of total CD4+ T cells were not significant between rV-LacZ and rV-4-1BBL treated mice, but the frequency of the total CD8+ T cells were increased in rV-4-1BBL treated groups compared to rV-LacZ treated mice (Fig. 3A). Treatment with rV-4-1BBL induced a shift in the CD8+ T cell population from CD8+ CD62L+CD44- (naïve) to CD8+ CD62L-CD44+ (effector) T cells compared to PBS treated mice (Fig. 3B).

In the tumor microenvironment, rV-4-1BBL treated groups significantly increased the frequency of CD8+ T cells compared to mice treated with rV-LacZ or PBS treated mice ($P<0.05$, Fig. 4A).Specifically, the percentage of CD8+ CD62L-CD44+ (effector) T cells was significantly increased compared to rV-LacZ or PBS treated mice (Fig. 4B).

Both rV-4-1BBL and rV-LacZ increased the frequency of innate (CD11b+, Gr-1+, and CD11c+) immune cells in both the spleen and tumor microenvironment compared to PBS treated mice. There was no significant difference between the innate immune cells in mice treated with rV-LacZ compared to mice treated with rV-4-1BBL. These data show that oncolytic vaccinia virus induces T cell immune responses in the local and systemic environment and rV-4-1BBL further enhances therapeutic activity of oncolytic vaccinia virus by promoting the expansion of CD8+ CD62L-CD44+ effector T cells.

DISCUSSION

In this report we demonstrated the enhanced therapeutic effectiveness of an oncolytic vaccinia virus containing the immunomodulatory gene. We utilized the vaccinia vector to deliver local expression of the 4-1BBL co-stimulatory molecule or CCL21 chemokine. The best therapeutic effects were identified when rV-4-1BBL were treated (Fig. 2). The use of various approaches to optimize immune response is a very important strategy for cancer immunotherapy. Under the optimal treatment conditions, we observed about a 30% complete regression of established tumors in rV-4-1BBL treated mice. Although the vaccinia virus treatment delayed tumor growth in all mice, the majority of tumors eventually escaped the oncolytic effect of vaccinia virus and continued to grow. The reasons why some mice responded well and others did not are not clear. We identified that rV-CCL21 vaccination induces rapid tumor growth (Fig. 2). These data show some immune activating molecules have negative effects for cancer immunotherapy.

Kinetic studies have shown that 4-1BB expression is appearing late in the activation process and confers a survival advantage to T cells by blocking apoptosis and has been implicated in the generation of long-lived effector and memory CD8+ T cells (Zhao Y and Croft M, 2012).

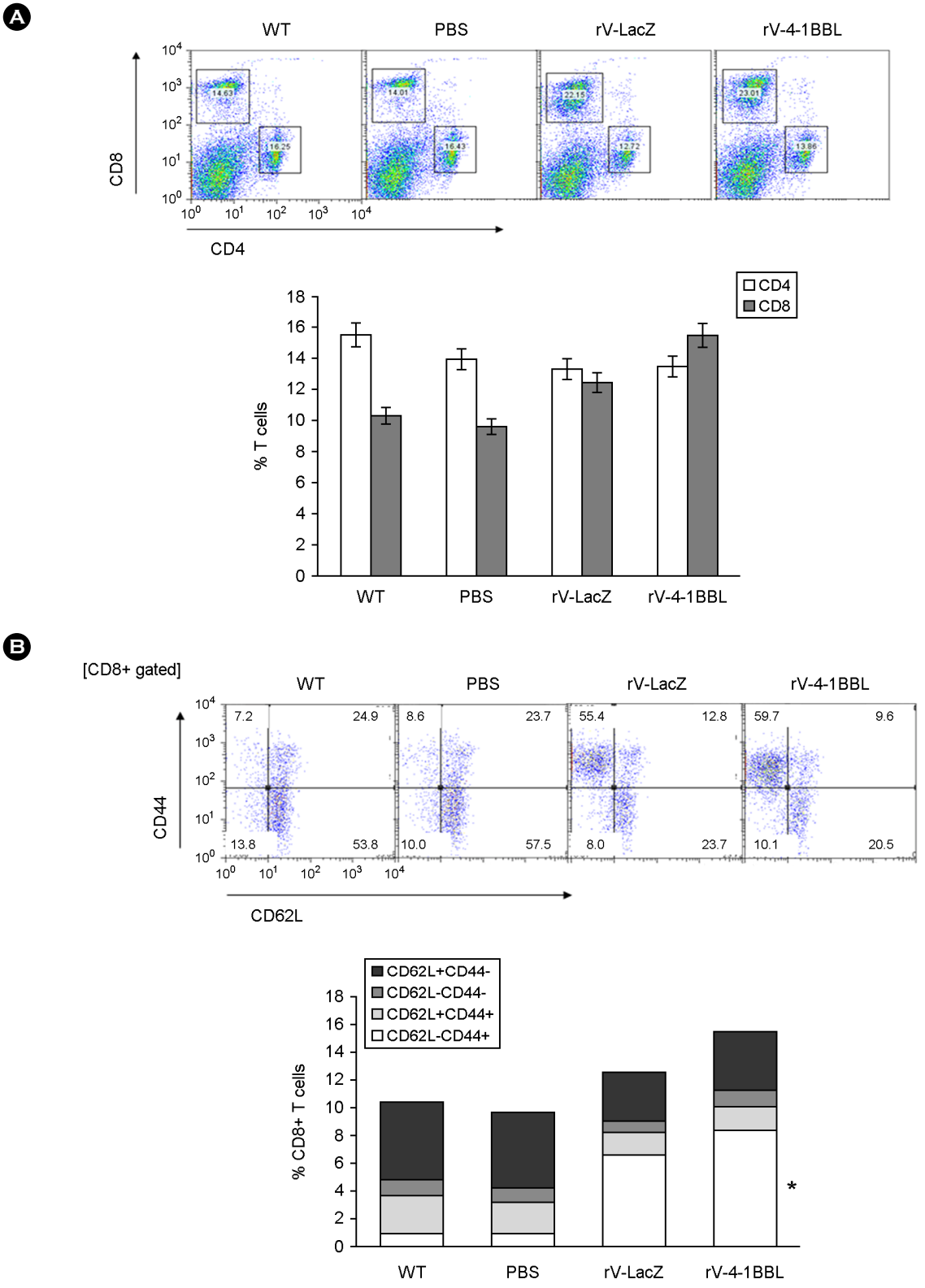

Fig. 3. Local injection of rV-4-1BBL induces therapeutic responses in a B16F10 melanoma model by increasing the effector CD8+ T cells in the systemic environments. Mice were treated as described in Fig. 2. Spleen tissue was collected on day 14 and single cell suspensions were subjected to FACS analysis (A) A representative dot plot of CD4 and CD8 T cells in the spleen (B) A representative dot plot of CD62LCD44 surface marker of CD8+ T cells in the spleen. Data shown is from one of three independent experiments. CD4-FITC: CD8-PE, CD62L-FITC: CD44-PE: CD8-APC *, $P<0.05$; compare to rV-LacZ and rV-4-1BBL.

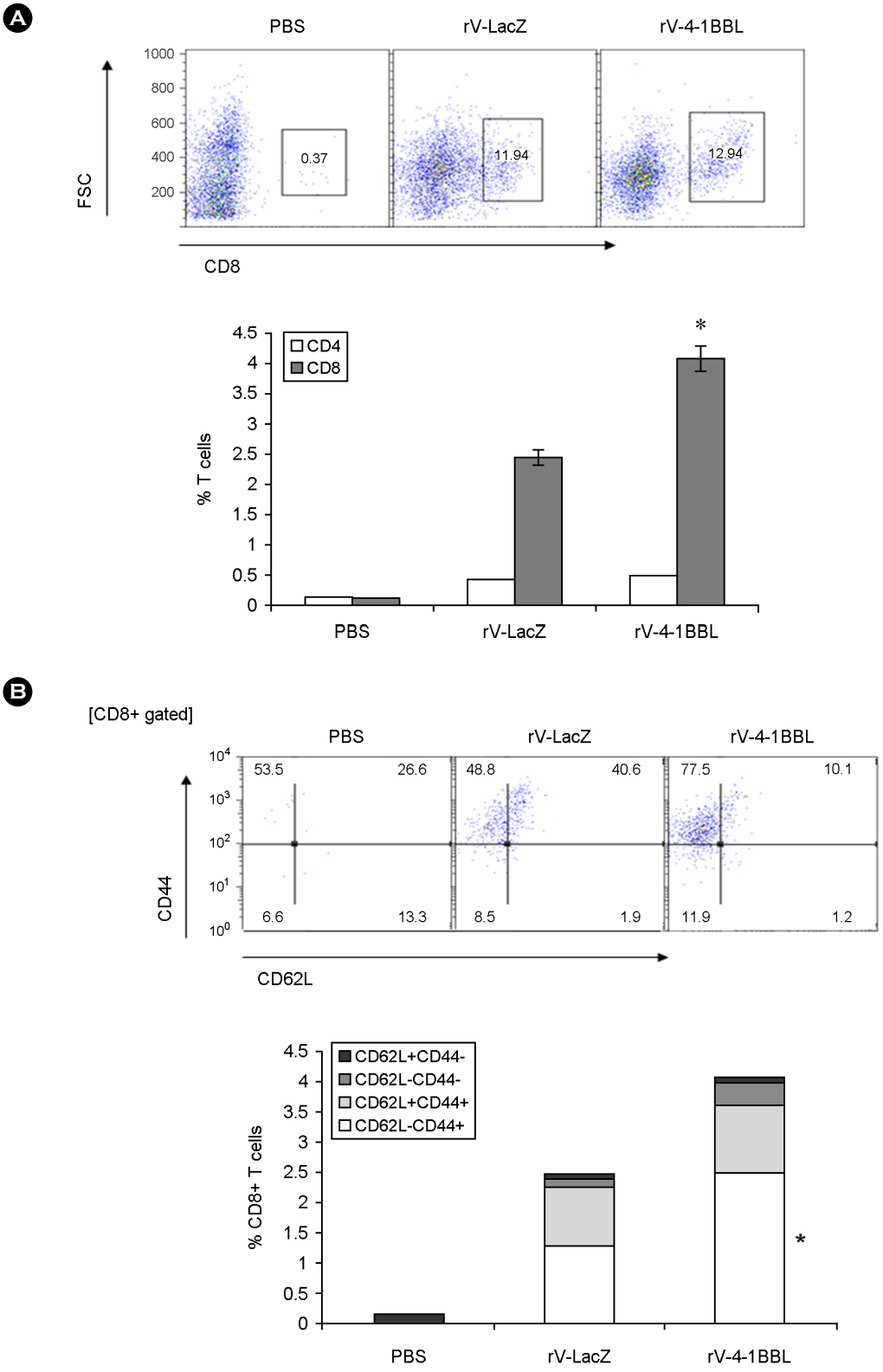

Fig. 4. Local injection of rV-4-1BBL induces therapeutic responses in a B16F10 melanoma model by increasing the effector CD8+ T cells in the tumor microenvironments. Mice were treated as described in Fig. 2. Tumor tissue was collected on day 14 and single cell suspensions were subjected to FACS analysis (A) A representative dot plot of CD4 and CD8 T cells in the tumor (B) A representative dot plot of CD62LCD44 surface marker of CD8+ T cells in the tumor. Data shown is from one of three independent experiments. CD4-FITC: CD8-PE, CD62L-FITC: CD44-PE: CD8-APC *, $P<0.05$; compare to rV-LacZ and rV-4-1BBL.

This may be critically important in establishing long-term tumor-specific CD8+ T cells, an important goal in tumor immunotherapy (Wilcox et al., 2002; Xu et al., 2005). In the present study we found that oncolytic vaccinia virus vaccination resulted in a little decrease of CD4+ T cells in the spleen (Fig. 3A) but a little increase in the tumor (Fig. 4A). In contrast, the CD8+ T cells were significantly increased in rV-4-1BBL or rV-LacZ treated mice in both spleen and tumor (Figs. 3A and 4A). These data are suggesting that oncolytic vaccinia virus itself induces a strong immune response in addition to oncolytic effect. The oncolytic effect of vaccinia virus results in immediate necrotic tumor cell death (Kalbacova et al., 2008). The induction of necrosis likely allows cross presentation of putative tumor antigens found in melanoma cells. So 4-1BBL expression may enhance the immune response by necrotic tumor cell death.

rV-4-1BBL treated mice significantly increase CD8+ T cell compared to rV-LacZ treated mice in spleen and tumor. This means that 4-1BBL expression can stimulate and expand CD8+ T cells above oncolytic vaccinia virus itself. The expanded T cells were largely CD62L-CD44+ phenotype consistent with an effector-memory phenotype (Fig. 3B, 4B). These data show 4-1BBL expression induces conversion naïve (CD62L+CD44-) CD8+ T cells to effector (CD62L-CD44+) CD8+ T cells.

In summary, we have shown that 4-1BBL enhances the therapeutic effectiveness of an oncolytic vaccinia virus and that local expression of 4-1BBL enhances the therapeutic effects against a B16 melanoma tumor. Inhibition or eradication of established tumors depends on generation of effector-memory CD8+ T cells. This suggests that 4-1BBL appears to be an important T cell co-stimulatory molecule for generating long-term tumor-specific T cell responses for effective cancer immunotherapy.

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REFERENCES

Applebaum J, Reynolds S, Knispel J, Oratz R, Shapiro R, Bystryn JC. Identification of melanoma antigens that are immunogenic in humans and expressed *in vivo*. J Natl Cancer Inst. 1998. 90: 146-149.

John LB, Howland LJ, Flynn JK, West AC, Devaud C, Duong CP, Stewart TJ, Westwood JA, Guo ZS, Bartlett DL, Smyth MJ, Kershaw MH, Darcy PK. Oncolytic virus and anti-4-1BB combination therapy elicits strong antitumor immunity against established cancer. Cancer Res. 2012. 72: 1651-1660.

Kalbacova M, Spisakova M, Liskova J, Melkova Z. Lytic infection with vaccinia virus activates caspases in a Bcl-2-inhibitable manner. Virus Res. 2008. 135: 53-63.

Kaufman HL, Cohen S, Cheung K, DeRaffele G, Mitcham J, Moroziewicz D, Schlom J, Hesdorffer C. Local delivery of vaccinia virus expressing multiple costimulatory molecules for the treatment of established tumors. Hum Gene Ther. 2006. 17: 239-244.

Kaufman HL, Deraffele D, Mitcham J, Moroziewicz D, Cohen SM, Hurst-Wicker KS, Cheung K, Lee DS, Divito J, Voulo M, Donovan J, Dolan K, Manson K, Panicali D, Wang E, Horig H, Marincola FM. Targeting the local tumor microenvironment with vaccinia virus expressing B7.1 for the treatment of melanoma. J Clin Invest. 2005. 115: 1903-1912.

Kirn DH, Thorne SH. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. Nat Rev Cancer. 2009. 9: 64-71.

Kober J, Leitner J, Klauser C, Woitek R, Majdic O, Stockl J, Herndler-Brandstetter D, Grubeck-Loebenstein B, Reipert BM, Pickl WF, Pfistershammer K, Steinberger P. The capacity of the TNF family members 4-1BBL, OX40L, CD70, GITRL, CD30L and LIGHT to costimulate human T cells. Eur J Immunol. 2008. 38: 2678-2688.

Kudo-Saito C, Hodge JW, Kwak H, Kim-Schulze S, Schlom J, Kaufman HL. 4-1BB ligand enhances tumor-specific immunity of poxvirus vaccines. Vaccine 2006. 24: 4975-4986.

McCart JA, Ward JM, Lee J, Hu Y, Alexander HR, Libutti SK, Moss B, Bartlett DL. Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. Cancer Res. 2001. 61: 8751 -8757.

Melero I, Bach N, Hellstrom KE, Aruffo A, Mittler RS, Chen L.

Amplification of tumor immunity by gene transfer of the co-stimulatory 4-1BB ligand: synergy with the CD28 co-stimulatory pathway. Eur J Immunol. 1998. 28: 1116-1121.

Parato KA, Senger D, Forsyth PA, Bell JC. Recent progress in the battle between oncolytic viruses and tumors. Nat Rev Cancer. 2005. 5: 965-976.

Schmidt FI, Bleck CK, Mercer J. Poxvirus host cell entry. Curr Opin Virol. 2012. 2: 20-27.

Serghides L, Bukczynski J, Wen T, Wang C, Routy JP, Boulassel MR, Sekaly RP, Ostrowski M, Bernard NF, Watts TH. Evaluation of OX40 ligand as a costimulator of human antiviral memory CD8 T cell responses: comparison with B7.1 and 4-1BBL. J Immunol. 2005. 175: 6368-6377.

Sharma S, Yang SK, Hillinger S, Zhu LX, Huang M, Batra RK, Lin JF, Burdick MD, Strieter RM, Dubinett SM. SLC/CCL21-mediated anti-tumor responses require IFN, MIG/CXCL9 and IP-10/CXCL10. Mol Cancer. 2003. 2: 22-27.

Stephan MT, Ponomarev V, Brentjens RJ, Chang AH, Dobrenkov KV, Heller G, Sadelain M. T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection. Nat Med. 2007. 13: 1440-1449.

Thorne SH, Hwang TH, O'Gorman WE, Bartlett DL, Sei S, Kanji F, Brown C, Werier J, Cho JH, Lee DE, Wang Y, Bell J, Kirn DH. Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. J Clin Invest. 2007. 117: 3350-3358.

van der Bruggen P, Traversari C, Chomez P, Lurguin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991. 254: 1643-1647.

Wilcox RA, Flies DB, Zhu G, Johnson AJ, Tamada K, Chapoval AI, Strome SE, Pease LR, Chen L. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. J Clin Invest. 2002. 109: 651-659.

Xu DP, Sauter BV, Huang TG, Meseck M, Woo SL, Chen SH. The systemic administration of Ig-4-1BB ligand in combination with IL-12 gene transfer eradicates hepatic colon carcinoma. Gene Ther. 2005. 12: 1526-1533.

Yi KH, Nechushtan H, Bowers WJ, Walker GR, Zhang Y, Pham DG, Podack ER, Federoff HJ, Tolba KA, Rosenblatt JD. Adoptively transferred tumor-specific T cells stimulated ex vivo using herpes simplex virus amplicons encoding 4-1BBL persist in the host and show antitumor activity *in vivo*. Cancer Res. 2007. 67: 10027-10037.

Zhao Y, Croft M. Dispensable role for 4-1BB and 4-1BBL in development of vaccinia virus-specific CD8 T cells. Immunol Lett. 2012. 141: 220-226.