

## Augmented Antitumor Effects of Radiation Therapy by 4-1BB Antibody (BMS-469492) Treatment

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**Abstract.** *Background:* 4-1BB (CD137) is a member of the tumor necrosis factor receptor superfamily. It interacts with 4-1BB ligand (4-1BBL) and delivers a costimulatory signal for T cell activation. The immune response induced by 4-1BB monoclonal antibodies (mAbs) has been shown to have a marked augmentation of tumor-selective cytolytic T cell activity. *Materials and Methods:* The antitumor efficacy of agonistic 4-1BB mAbs (BMS-469492, Bristol-Meyer Squibb), used alone or in combination with radiation therapy, was evaluated in murine lung (M109) and breast (EMT6) carcinoma models. *Results:* Treatment with BMS-469492 led to only a modest growth retardation in M109 tumors (3 days,  $p > 0.05$ ), but a significant growth delay in EMT6 tumors (12.5 days,  $p < 0.05$ ). When BMS-469492 was administered in conjunction with single dose radiation therapy (5, 10 or 15 Gy), enhanced tumor responses were noted only at the highest evaluated radiation dose (15 Gy). In contrast, in the EMT6 model, BMS-469492 treatment resulted in enhanced antitumor effects at all radiation doses. In addition, the response of EMT6 tumors to fractionated radiotherapy also was significantly increased when BMS-469492 was included in the treatment protocol. *Conclusion:* These results suggest that monoclonal antibodies against 4-1BB may not only be efficacious in cancer immunotherapy, but may also have utility when applied in combination with conventional anticancer treatments, such as radiation therapy.

The establishment and progression of tumors are indications of a failure in the generation of effective antitumor immune responses by the host. In antitumor immunity, cell-mediated responses play a central role. In order to activate a naive T

cell, two signals are required. The primary signal occurs through the T cell receptor, major histocompatibility complex (MHC) and antigen complex, while the second signal is provided through costimulation (12). The CD28 and B7 pathway is the most widely characterized costimulatory signal (2, 13). Recently, a number of other costimulatory molecules were identified (27). One of these, 4-1BB (CD137), a member of the TNF receptor superfamily, is expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (20, 25), activated natural killer cells (16) and murine dendritic cells (5). 4-1BB binds to a high affinity ligand (4-1BBL), which is a type II surface glycoprotein expressed on several types of antigen presenting cells, such as activated B cells, macrophages (1, 19) and splenic dendritic cells (4). The interaction of 4-1BB with its ligand in addition to T cell receptor engagement co-stimulates T cell activation and clonal expansion (4, 23). 4-1BB ligation can also be accomplished with agonist monoclonal antibodies (mAbs), such as BMS-469492, directed against 4-1BB. Furthermore, administration of agonistic 4-1BB mAbs can lead to the generation of effective antitumor responses (16, 17).

Despite the encouraging results observed to date in murine tumor models, achieving tumor cures in patients with 4-1BB mAb immunotherapy alone is extremely difficult. This conclusion is based, at least in part, on the general belief that immunotherapy will be most efficient at eradicating small tumor masses. This concept is supported by animal studies showing that gene transfer of multiple costimulatory molecules, including B7.1, B7.2, ICAM-1 and VCAM-1, may lead to effective tumor therapy in small tumors (0.1 - 0.3 cm in diameter), whereas larger tumors (>0.3 cm) are typically resistant to such treatment (9). Since bulky neoplastic disease is typically the most difficult to manage in the clinic, the greatest benefit of cell-mediated tumor immunotherapy may occur when it is used in combination with conventional cytotoxic antitumor therapies. The present studies were therefore designed to evaluate the therapeutic efficacy of treatments combining agonistic anti-4-1BB mAbs (BMS-469492) with radiation therapy in murine breast (EMT6) and lung (M109) tumor models.

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## Materials and Methods

**Tumor cell lines.** EMT6 mouse breast carcinoma (26) and M109 mouse lung carcinoma (21) cells were grown in Dulbecco's modified minimum essential medium (DMEM, Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin-streptomycin (Invitrogen) and 1% 200 mmol/L L-glutamine (Invitrogen).

**Mice and tumors.** Female Balb/c mice, age 6 - 8 weeks were obtained from the National Cancer Institute (Frederick, MD, USA) and were maintained under specific-pathogen-free conditions (University of Florida Health Science Center) with food and water supplied *ad libitum*. To establish tumors *in vivo*, animals were inoculated intramuscularly in one hind limb with  $1 \times 10^5$  EMT6 or M109 tumor cells.

**Anti-4-1BB monoclonal antibodies.** Anti-4-1BB (CD137) mAbs (BMS-469492) were obtained from Bristol-Myers Squibb (Princeton, NJ, USA). BMS-469492 was diluted in sterile 0.9% saline and administered *via* tail vein at the dose of 0.1 mg per injection in a volume of 0.2 ml.

**Irradiation.** Tumors were irradiated using a 6MV Clinac 600c linear accelerator (Varian Oncology Systems, Palo Alto, CA, USA), operating at a dose rate of 4 Gy/min. Mice were not anesthetized but confined to plastic jigs, such that their tumor-bearing legs extended through openings in the sides of the jigs and allowed the tumors to be irradiated locally.

**Measurement of tumor response.** The response of tumors to treatment was determined using a tumor growth delay assay. Tumor size was measured every second day by passing the tumor-bearing leg through a series of holes in a plastic plate with increasing diameters. The diameter of the smallest hole, a tumor-bearing leg would pass through, was recorded and converted to a tumor volume using the formula: tumor volume =  $[(\pi d^3)/6] - 100$ , where  $d$  = the hole diameter and 100 represent a volume correction factor determined for a mouse leg without a tumor. The times for the tumors in the various treatment groups to grow from 200 mm<sup>3</sup> to 1000 mm<sup>3</sup> were recorded and compared (Wilcoxon rank sum test).

**4-1BBL expression.** The expression levels of 4-1BBL by the tumor cells were determined by flow cytometry. Briefly,  $1 \times 10^5$  EMT6 or M109 cells were plated in 60 mm dishes and allowed to attach overnight. The cells were either untreated or irradiated with radiation doses ranging from 2 to 20 Gy. The cells were collected 48 h later and were fixed by exposing them to 4% para-formaldehyde for 10 min on ice. The cells were then incubated with R-phycoerythrin-labeled antibody against 4-1BBL (BioLegend, San Diego, CA, USA) for 1 h at room temperature, followed by 3 washes of phosphate buffered saline. The percentage of cells expression 4-1BBL then was determined by FACS analysis (Becton Dickinson flow cytometer, University of Florida Core Facility for Flow Cytometry).

## Results

Initial experiments determined the antitumor efficacy of BMS-469492 by assessing the effect of the anti-4-1BB mAbs

on EMT6 and M109 tumor growth. Two antibody administration schedules were tested. In both cases the animals were injected with  $1 \times 10^5$  tumor (EMT6 or M109) cells on day 0. BMS-469492 (0.1 mg per animal) was administered *i.v.* either on days 1, 8, 15 or days 8, 15, 22. Neither treatment schedule resulted in statistically significant growth delays in M109 tumors (Figures 1 and 2). In contrast, both BMS-469492 treatment schedules led to significant growth inhibition in the EMT6 tumor model. The median time for EMT6 tumors to grow from 200 to 1000 mm<sup>3</sup> increased from 28.5 days in untreated control animals to 40 and 41 days in BMS-469492 treated mice ( $p < 0.05$ , Wilcoxon Rank Sum Test) (Figures 1 and 2). Although both courses of treatment resulted in similar tumor growth delays, the schedule in which BMS-469492 was given on days 8, 15, 22 resulted in several long-term survivors (3 out of the 8 animals showed no evidence of macroscopic tumors at the termination of the experiment 120 days post-initiation). Consequently, this schedule was utilized in all subsequent studies.

In order to evaluate the efficacy of combining BMS-469492 with radiation, EMT6 and M109 tumors were irradiated locally 8 days after cell inoculation at a volume of  $\sim 200$  mm<sup>3</sup>. BMS-469492 was administered *via* tail vein (0.1 mg) on days 8, 15 and 22, with the first dose of mAbs given immediately after the completion of the radiation exposure. In the M109, a tumor model in which BMS-469492 mAbs alone had no significant effect on tumor growth (Figures 1 and 2), a significantly enhanced antitumor effect was nevertheless observed when the mAbs were combined with radiation, albeit only at the highest radiation dose (15 Gy) tested (Figures 3 and 4). In the EMT6 tumor model, combining BMS-469492 with radiation resulted in significantly enhanced antitumor effects compared to those seen in animals treated with either therapy alone (Figures 5 and 6). This enhancement occurred for all radiation doses investigated. For example, the time for tumors to grow from 200 to 1000 mm<sup>3</sup> was 21 days in the control group, 30.5 days in the BMS-469492 group, 31 days in 15 Gy group and 56 days in the combination group that received both BMS-469492 and radiation ( $p < 0.05$  vs. 15 Gy alone,  $p < 0.05$  vs. BMS-469492 alone).

The combination of BMS-469492 with a more clinically relevant fractionated radiation schedule was also evaluated in the EMT6 tumor model. In this study, tumors were locally irradiated with 4 Gy per day on days 8-13 and 15-19. BMS-469492 was administered *via* tail vein at a dose of 0.1 mg per animal on days 8, 15 and 22. Significant enhancement of the radiation response was observed in this study when BMS-469492 was incorporated into the fractionated radiation treatment regimen (Figures 7 and 8). The median tumor growth delay (time for tumors to grow from  $\sim 200$  to 1000 mm<sup>3</sup>) increased from 18.5 and 12.5 days in radiation- and BMS-469492-treated mice, respectively, to

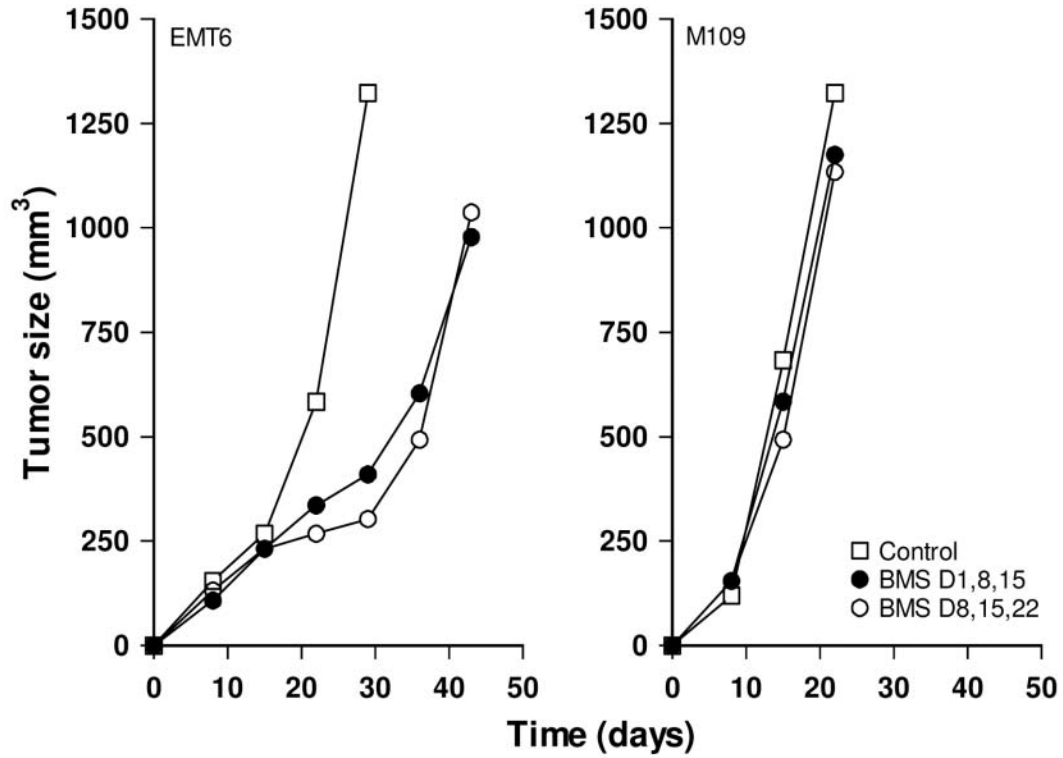


Figure 1. Median tumor growth curves of EMT6 and M109 tumors. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0, followed by 3 i.v. doses of saline (control group) or BMS-469492 on days 1, 8 15 or days 8, 15, 22.

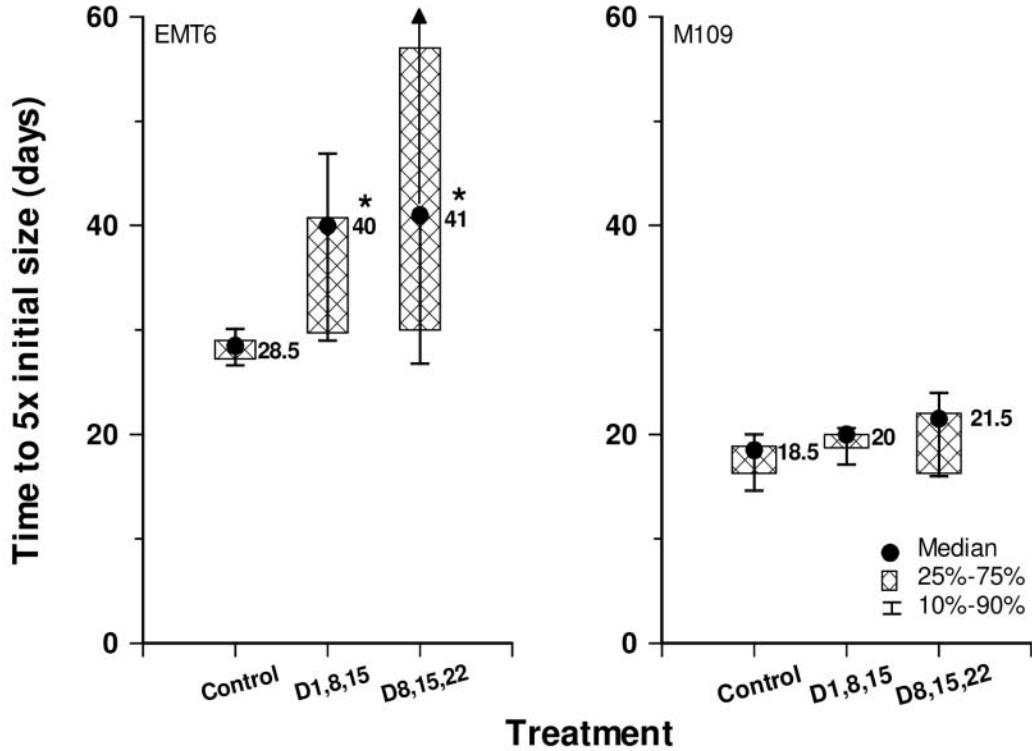


Figure 2. Time for EMT6 and M109 tumors to grow to 5 times the starting size after BMS-469492 treatment. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0 and then injected with 3 i.v. doses of saline (control group) or BMS-469492 on days 1, 8 15 or days 8, 15, 22. \*Indicates statistical significance compared to control group ( $p < 0.05$ , Wilcoxon Rank Sum Test).

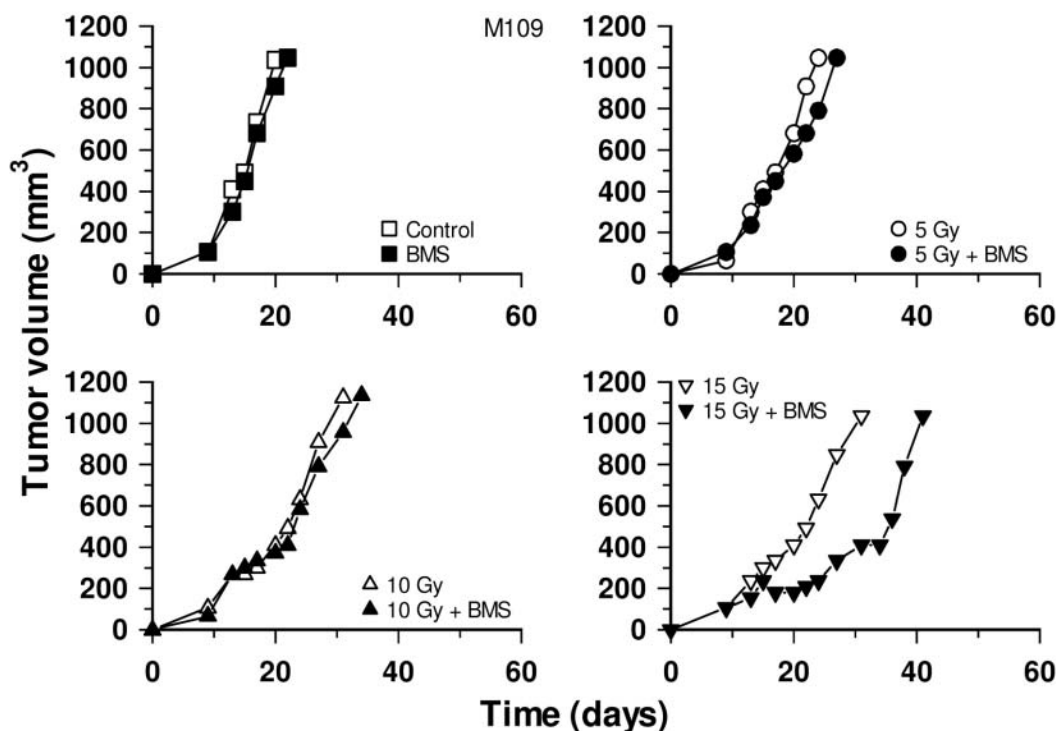


Figure 3. Median tumor response curves of M109 tumors treated with BMS-469492 and radiation either alone or combined. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0. Single doses of radiation (5, 10, 15 Gy) were administered on day 8. In the combination groups, animals were given BMS-469492 on days 8, 15 and 22 at a dose of 0.1 mg per dose.

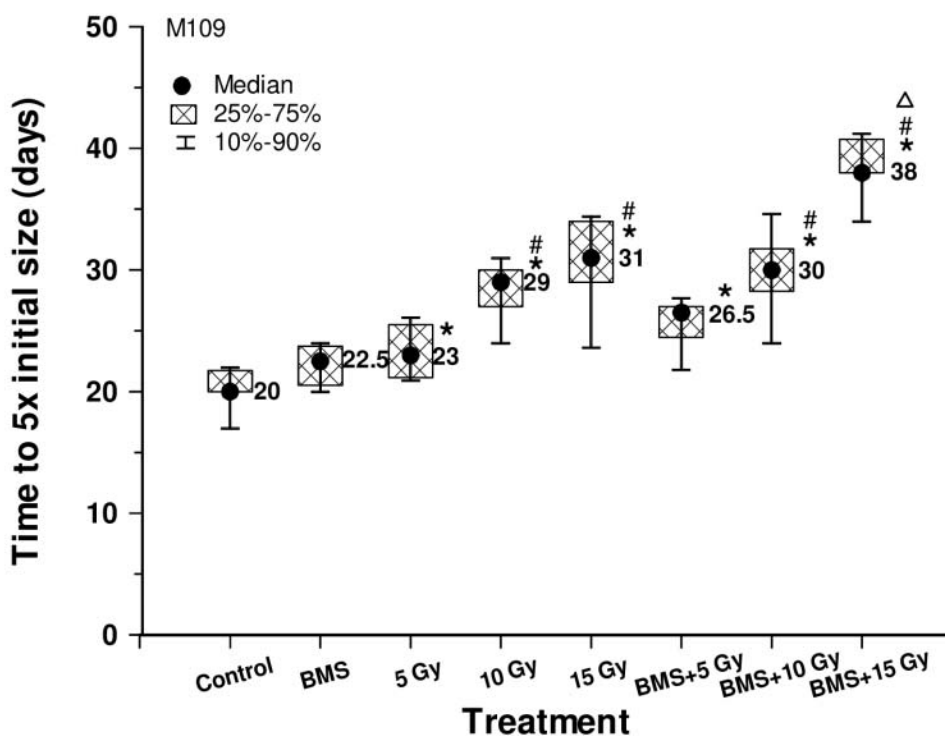


Figure 4. Time required for the M109 tumors to grow to 5 times their initial sizes after treatment. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0. Single doses of radiation (5, 10, 15 Gy) were administered on day 8. In the combination groups, animals were given BMS-469492 on days 8, 15 and 22 at a dose of 0.1 mg per dose. \*Indicates statistical significance compared to the control group ( $p < 0.05$ , Wilcoxon Rank Sum Test). #Indicates statistical significance compared to the BMS-469492 alone group ( $p < 0.05$ , Wilcoxon Rank Sum Test). Δ indicates statistical significance compared to the corresponding radiation alone group ( $p < 0.05$ , Wilcoxon Rank Sum Test).

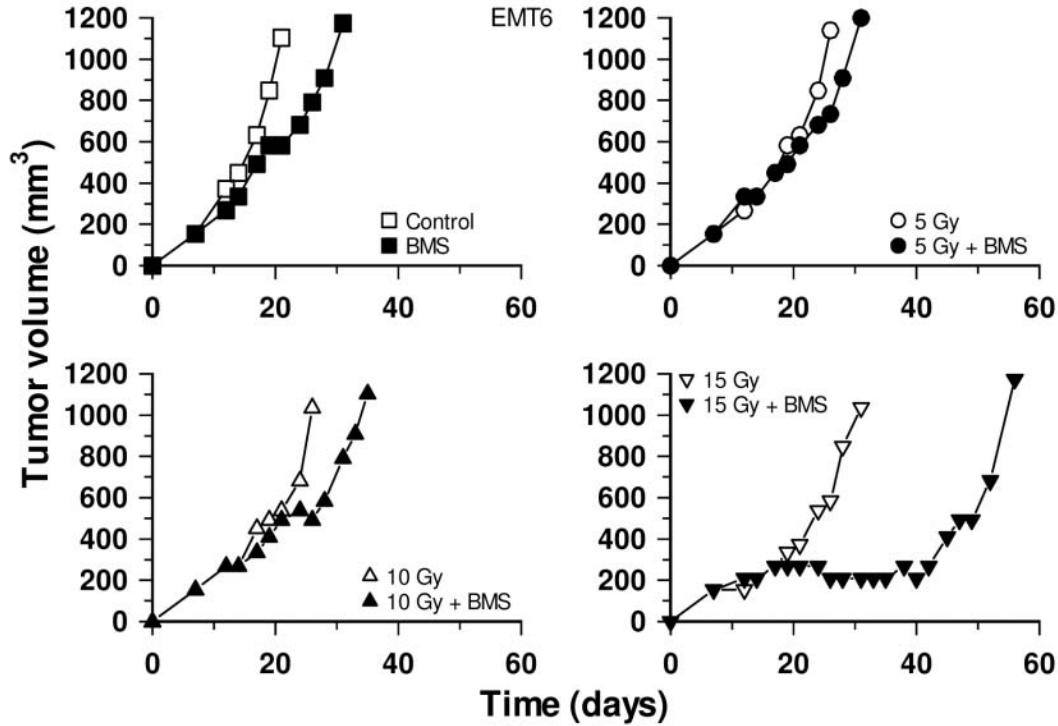


Figure 5. Median tumor response curves of EMT6 tumors exposed to various treatments. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0. Single dose of radiation (5, 10, 15 Gy) were administered on day 8. In the combination groups, animals were given BMS-469492 on days 8, 15 and 22 at a dose of 0.1 mg per dose.

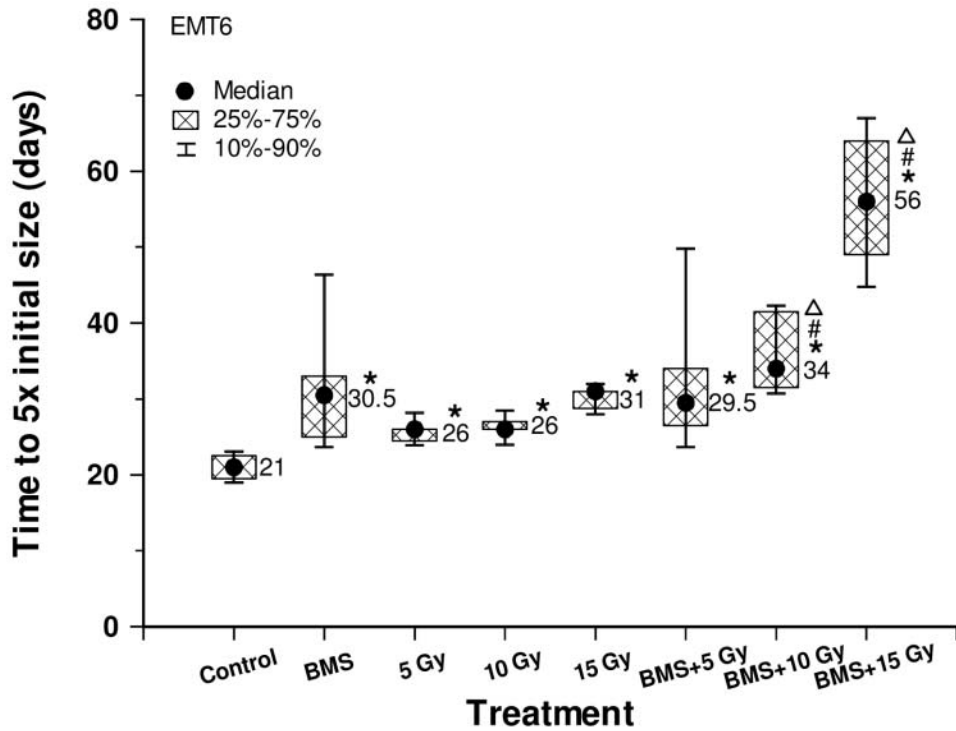


Figure 6. Time for EMT6 tumors to grow to 5 times the starting size after treatments with 4-1BB antibodies and radiation administered either alone or in combination. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0. Single doses of radiation (5, 10, 15 Gy) were administered on day 8. In the combination groups, animals were given BMS-469492 on days 8, 15 and 22 at a dose of 0.1 mg per dose. \*Indicates statistical significance compared to the control group ( $p < 0.05$ , Wilcoxon Rank Sum Test). #Indicates statistical significance compared to the BMS-469492 alone group ( $p < 0.05$ , Wilcoxon Rank Sum Test). Δ indicates statistical significance compared to the corresponding radiation alone group ( $p < 0.05$ , Wilcoxon Rank Sum Test).

43 days when the two therapies were combined ( $p < 0.05$ , Wilcoxon Rank Sum Test). Inclusion of BMS-469492 in the same fractionated radiotherapy protocol applied to M109 tumors did not, however, improve the treatment outcome in this model (data not shown).

Since the expression of 4-1BBL on tumor cells can contribute to the activation of 4-1BB, the expression levels of the ligand on both M109 and EMT6 tumor cells were determined. The results showed that M109 tumor cells have high endogenous levels of 4-1BBL expression, whereas EMT6 tumor cells have very low levels (Figure 9). In the EMT6 cells, 4-1BBL was found to be radiation inducible as indicated by the dose-dependent increase in 4-1BBL expression (Figure 9). In contrast, the high basal expression levels of 4-1BBL observed in M109 cells could not be elevated further by radiation treatment (Figure 9).

## Discussion

Interactions between co-stimulatory ligands and their receptors are crucial for the activation of T cells, T cell clonal expansion and the development of T cell immunity (2). One such co-stimulatory ligand-receptor pair is 4-1BB and 4-1BBL (1, 27). Therapies utilizing the 4-1BB:4-1BBL signaling pathway have been shown to have antitumor effects in a number of model systems (3, 6-8, 10, 14, 15, 17, 22, 29, 30). One way to target the 4-1BB:4-1BBL pathway is through the use of agonistic mAbs. The administration of anti-4-1BB mAbs has been shown to mediate antitumor responses against established weakly immunogenic tumors (17). However, in poorly-immunogenic or non-immunogenic tumors, active immunization with a peptide vaccine in conjunction of anti-4-1BB antibodies was required to cause tumor regression (28). These studies demonstrate that agonistic anti-4-1BB mAbs provide not only potent *in vivo* co-stimulation of the cellular immune responses, but also may offer a means of effective cancer immunotherapy.

In the present studies, treatment with agonistic mAbs against 4-1BB (BMS-469492) resulted in significant tumor growth retardation in one tumor model (EMT6), but not the other (M109) (Figures 1 and 2). This difference in response could be due to differences in the intrinsic immunogenicity of these two tumor models. As previously shown, 4-1BB mAb treatment alone can lead to antitumor efficacy in weakly immunogenic tumors, but requires active immunization in poorly- or non-immunogenic tumors (17, 28). In this regard, a marked antitumor effect can be demonstrated in M109 tumors when BMS-492469 is administered to animals which have been primed with lethally irradiated M109 cells 3 weeks prior to tumor inoculation (data not shown). Another factor contributing to the observed differences in response to BMS-492469 in the two tumor models studied could be the differences in

inherent levels of 4-1BBL expressed (Figure 9). A high level of 4-1BBL expression on M109 cells may provide efficient engagement of 4-1BB, thus providing little additional benefit in M109 tumors when agonistic 4-1BB mAbs are applied. In contrast, since EMT6 cells express only low levels of 4-1BBL, a weak activation of 4-1BB signaling might be significantly enhanced in EMT6 tumors through the application of 4-1BB mAbs to provide a co-stimulatory signal.

In the EMT6 tumor model, two different BMS-469492 treatment schedules were evaluated. In one, 4-1BB mAb treatment was initiated at the time of tumor cell inoculation, while in the other treatment commenced when macroscopic tumors were established. The results showed that although both treatment protocols led to similar median tumor responses (Figures 1 and 2), the later schedule (4-1BB mAbs given on days 8, 15, and 22) resulted in 3 out of 8 long-term survivors. This finding may support the notion that the triggering of 4-1BB with mAb might not be therapeutically effective against tumors until an immune response has been initiated (28).

Since definitive cancer therapy typically incorporates surgery, chemotherapy and radiotherapy, it was of interest to determine whether 4-1BB mAb therapy could modulate the efficacy of a commonly utilized conventional anticancer treatment. Such anticancer therapies could reduce the tumor mass, thereby facilitating immunotherapy, or serve to eliminate neoplastic cell populations surviving tumor immunotherapy. In addition, it was hypothesized that a cytotoxic therapy delivered to a tumor might lead to increased antigen presentation and thus greater effectiveness of the immunotherapy. For these reasons, tumor-bearing mice were irradiated with single and fractionated doses of radiation administered in the presence or absence of BMS-469492 treatment. The combination of radiation plus immunotherapy was chosen for study because these therapies have fundamentally different mechanisms of action, different cellular targets and non-overlapping toxicities (11). In addition, evidence already existed that irradiation could enhance the expression of costimulatory molecules such as B7 in tumors (18, 24). The present studies also revealed the expression levels of 4-1BBL in the inherently low expressing EMT6 tumor cells to be radiation inducible (Figure 9), thus perhaps providing the possibility of added efficiency in co-stimulation through 4-1BB in this tumor.

When BMS-469492 was included in the radiation treatment of EMT6 tumors, enhanced tumor responses were observed in regimen incorporating both single (Figures 5 and 6) and fractionated (Figures 7 and 8) doses. Since BMS-469492 had little impact on M109 tumor growth (Figures 1 and 2), little impact of 4-1BB mAb treatment on radiation response was expected in this tumor model. While this was generally the case, the combination of BMS-469492 and high single dose radiotherapy (15 Gy) did result in a significantly

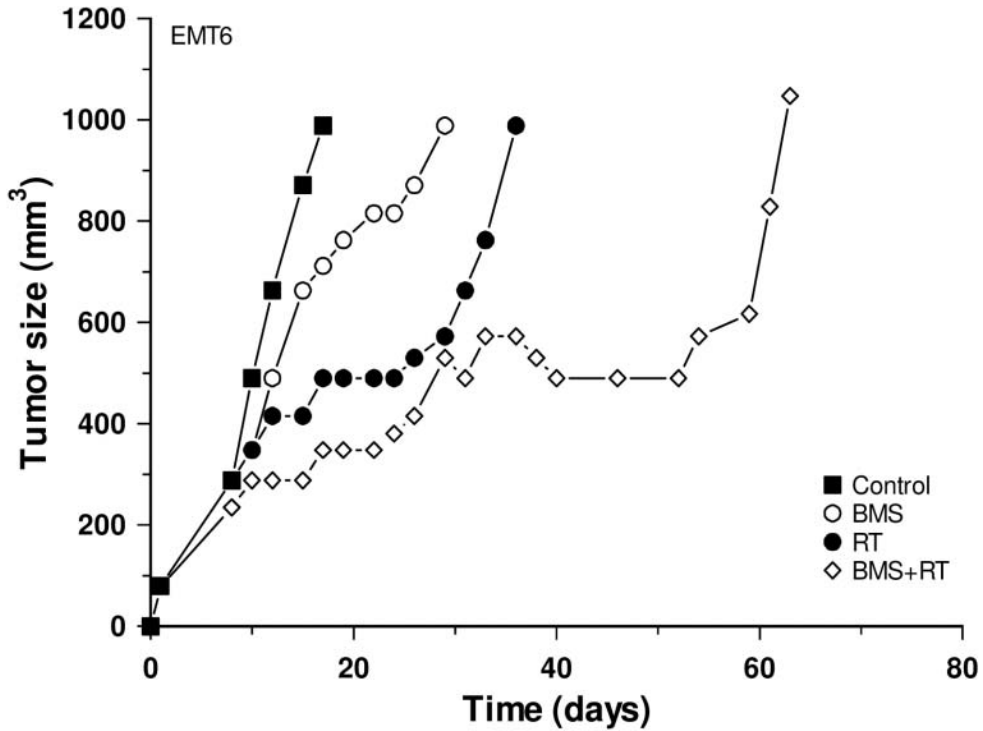


Figure 7. Median tumor response curves of EMT6 tumors exposed to 4-1BB antibodies, fractionated radiotherapy, or the combination of both treatments. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0. Radiotherapy was administered daily on days 8-12 and 15-19. Each daily dose equaled 4 Gy. In the combination groups, animals were given BMS-469492 on days 8, 15 and 22 at a dose of 0.1 mg per dose.

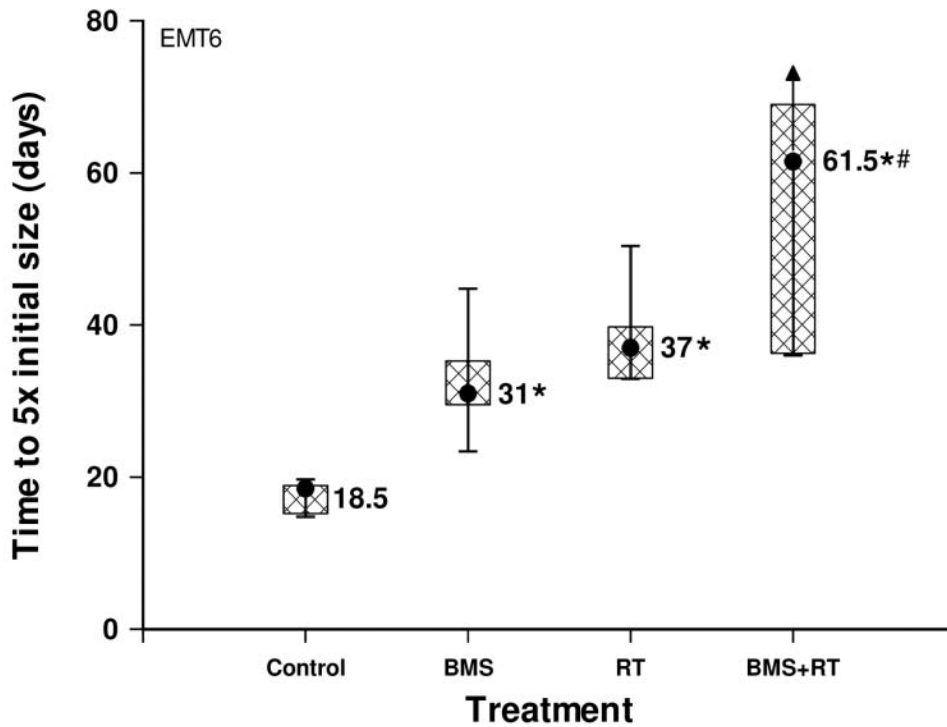


Figure 8. Time for the EMT6 tumors to grow to 5 times the starting size following treatment with BMS-469492 and fractionated radiotherapy administered either alone or in combination. Mice were injected with  $1 \times 10^5$  tumor cells in one hind limb on day 0. Fractionated dose radiation was administered daily on days 8-12 and 15-19 at a dose of 4 Gy per fraction. In the combination groups, animals were given BMS-469492 on days 8, 15 and 22 at a dose of 0.1 mg per dose. \*Indicates statistical significance compared to the control group ( $p < 0.05$ , Wilcoxon Rank Sum Test). # Indicates statistical significance compared to the radiation or BMS-469492 treatment alone groups ( $p < 0.05$ , Wilcoxon Rank Sum Test).

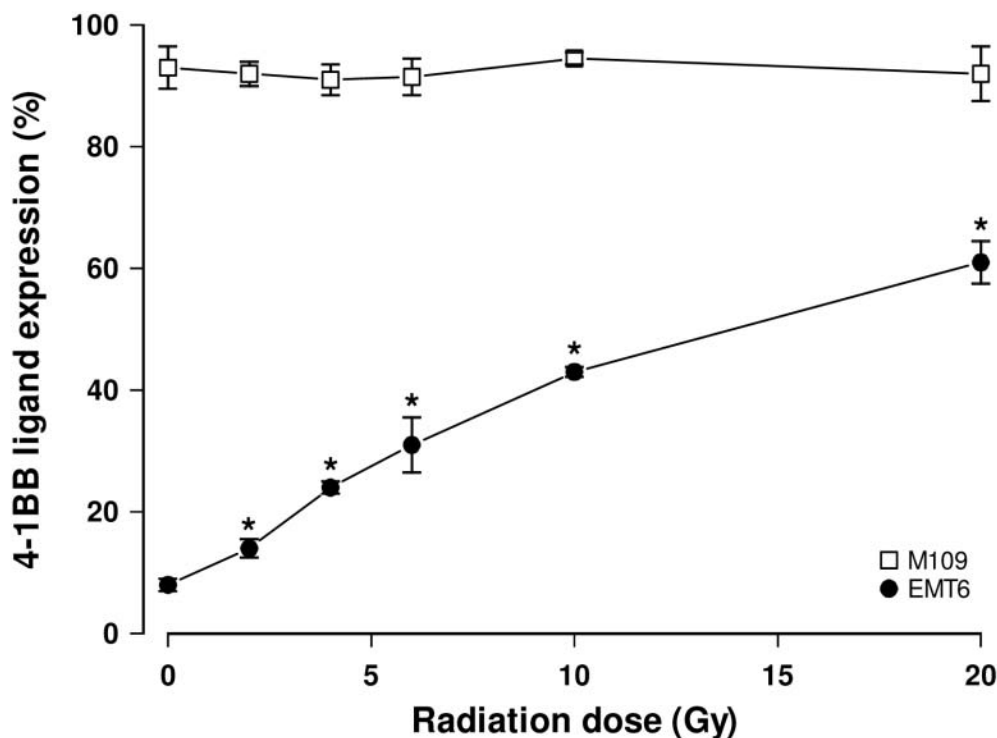


Figure 9. Expression levels of 4-1BB ligand in EMT6 and M109 tumor cells prior to and following radiotherapy. EMT6 and M109 cells were irradiated *in vitro* with doses of 2, 4, 6, 10, and 20 Gy. The expression levels of 4-1BB ligand were determined 48 h after treatment. \*Indicates statistical significance compared to the unirradiated control group ( $p < 0.05$ , Student's *t*-test).

enhanced tumor response compared to radiation alone (Figures 3 and 4). Such a dose of radiation causes extensive tumor tissue damage and cell kill. Conceivably, the extent of this damage might result in additional release or presentation of tumor antigens sufficient to elicit a 4-1BB mAb-mediated response but whether this is indeed the case remains unknown. Perhaps this observation also supports the notion that in poorly immunogenic tumors, active immunization may be needed to lead to efficacious 4-1BB mAbs treatment.

In summary, the present studies provide clear evidence that anti-4-1BB mAbs can be successfully incorporated into radiation therapy regimen. The results showed that BMS-469492 significantly enhanced the response of solid EMT6 tumors to both single and fractionated dose radiotherapy. These findings support the concept that combining tumor immunotherapy with radiation therapy can lead to significant improvements in treatment outcomes; a notion that should continue to be thoroughly explored.

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