Targeting 4-1BB Costimulation to the Tumor Stroma with Bispecific Aptamer Conjugates Enhances the Therapeutic Index of Tumor Immunotherapy

Brett Schrand1, Alexey Berezhnoy1, Randall Brenneman1, Anthony Williams2, Agata Levay1, Ling-Yuan Kong3, Ganesh Rao3, Shouhao Zhou4, Amy B. Heimberger3, and Eli Gilboa1

Abstract

Despite the recent successes of using immune modulatory Abs in patients with cancer, autoimmune pathologies resulting from the activation of self-reactive T cells preclude the dose escalations necessary to fully exploit their therapeutic potential. To reduce the observed and expected toxicities associated with immune modulation, here we describe a clinically feasible and broadly applicable approach to limit immune costimulation to the disseminated tumor lesions of the patient, whereby an agonistic 4-1BB oligonucleotide aptamer is targeted to the tumor stroma by conjugation to an aptamer that binds to a broadly expressed stromal product, VEGF. This approach was predicated on the premise that by targeting the costimulatory ligands to products secreted into the tumor stroma, the T cells will be costimulated before their engagement of the MHC–peptide complex on the tumor cell, thereby obviating the need to target the costimulatory ligands to noninternalizing cell surface products expressed on the tumor cells. Underscoring the potency of stroma-targeted costimulation and the broad spectrum of tumors secreting VEGF, in preclinical murine tumor models, systemic administration of the VEGF-targeted 4-1BB aptamer conjugates engendered potent antitumor immunity against multiple unrelated tumors in subcutaneous, postsurgical lung metastasis, methylcholangi-trene-induced fibrosarcoma, and oncogene-induced autochthonous glioma models, and exhibited a superior therapeutic index compared with nontargeted administration of an agonistic 4-1BB Ab or 4-1BB aptamer.

Cancer Immunol Res; 2(9); 867–77. ©2014 AACR.

Introduction

Antigen-activated T cells express stimulatory and inhibitory receptors that regulate their fate and ultimately control the outcome of the immune response. 4-1BB (CD137) is a major costimulatory receptor promoting the survival and expansion of activated CD8+ T cells and their differentiation into memory cells (1). Underscoring the lack of optimal 4-1BB costimulation at the tumor site, intratumoral administration of 4-1BB ligands (4-1BBL) as 4-1BB-Fc fusion protein (2) or adenoviral vector-encoded 4-1BBL (3), as well as systemic administration of agonistic anti-4-1BB Abs (4) or soluble 4-1BB ligands (5), enhanced tumor immunity and inhibited tumor growth. Systemic anti-4-1BB Ab therapy synergized with vaccination (6, 7) and other immune modalities (3, 8–12) to inhibit tumor growth in mice. Enhancing 4-1BB costimulation, therefore, represents a potentially useful modality to potentiate protective immunity in patients with cancer. Notwithstanding, systemic administration of agonistic 4-1BB Abs to mice was accompanied by immune anomalies, notably polyclonal activation of CD8+ T cells and a "cytokine storm" consisting predominantly of IFNγ, TNF, and type-I IFNs that affected the function of organs such as liver, spleen, and bone marrow (9, 13, 14).

The recent FDA approval of ipilimumab, a blocking anti-CTLA4 Ab, for the treatment of advanced melanoma has provided formal validation for using immune-potentiating drugs in the treatment of cancer. Nonetheless, treatment of patients with cancer with ipilimumab, and with other immune-potentiating Abs such as anti–PD-L1 or anti–PD-1 that have shown promise in clinical trials, was associated with autoimmune pathologies, including grade III or IV toxicities (15). Clinical trials using an agonistic anti–4-1BB Ab in advanced cancers were associated with high frequencies of objective responses, yet adverse effects became significant at the highest dose used, causing liver toxicity that resulted in two fatalities (16). Arguably, reducing the toxicity of immune-potentiating drugs without compromising their antitumor activity will be paramount for exploiting their clinical potential to the fullest extent.
We have shown previously that an agonistic 4-1BB oligonucleotide aptamer conjugated to a prostate-specific membrane antigen (PSMA)–binding aptamer is efficiently targeted to PSMA-expressing tumors in vivo and potentiates vaccine-induced protective immunity (17). Given that most receptors engaged by their ligand, including PSMA, are internalized (18), and because the tumor-targeted 4-1BB costimulatory ligands need to be displayed on the cell surface to engage the 4-1BB–expressing tumor-infiltrating T cells, tumor cells were engineered to express a mutant PSMA containing a small deletion in the cytoplasmic domain to prevent its internalization upon aptamer engagement. Because this is not clinically feasible, the need to identify tumor-specific surface products that do not internalize upon interaction with the bispecific aptamers significantly reduces the clinical applicability of this approach.

In general, the professional or nonprofessional (i.e., tumor) cell is costimulated concurrently with antigen presentation by the same cell expressing both the costimulatory ligand and the MHC–peptide complex. In this study, we tested the hypothesis that by targeting the costimulatory ligands to products secreted into the tumor stroma, the tumor-infiltrating T cells will be costimulated before their engagement and presentation of the MHC–peptide complex by the tumor cell, thereby obviating the need to target the costimulatory ligands to noninternalizing cell surface products expressed on the tumor cells. In addition, because unlike tumor cell–expressed products such as PSMA, Her2, or EGFR, stroma-secreted products such as VEGF, osteopontin (OPN), or metalloproteases are secreted by many tumors of distinct origins, tumor stroma–targeted costimulation would be more broadly applicable.

Materials and Methods
Construction of aptamer conjugates
A 2′-fluoro-pyrimidine–modified dimeric 4-IBB RNA aptamer transcribed in vitro from a DNA template described in ref. (19) extended at the 3′ end with a linker sequence 5′-UCCGCUAUAAGUGUUGCAAGAAGC-3′ was annealed to either a VEGF (20) or OPN (21) chemically synthesized (IDT) aptamer via a complementary linker sequence engineered at their 3 ends. Equimolar amounts of 4-IBB and either VEGF or OPN aptamers were mixed, heated to 75°C, and cooled to room temperature. Annealing efficiency, monitored by agarose gel electrophoresis, was >80%. To prevent conjugation of the two aptamers, they were annealed separately with a 2-fold excess of complementary linker sequence before tail vein injection. 32P-labeled 4-IBB dimer was generated by transcription in the presence of αP32-ATP (PerkinElmer).

In vitro costimulation assay
CD8⁺ T cells were isolated from the spleens of Balb/C mice using a Miltenyi CD8⁺ T-cell isolation kit. Briefly, 10⁶ cells/mL were plated in a 96-well plate at 200 μL per well in the presence or absence of suboptimal concentration of CD3ε Ab (250 ng/mL). Eighteen hours later, Ab (1 μg/mL) or aptamer (100 pmol/mL) was added. Forty-eight hours later, media were supplemented with 1 μCi/mL of [³H]-thymidine. Six hours later, cells were harvested and counted using a scintillation counter.

Histology and IHC
4T1 and 4T07 subcutaneously established tumors were resected and embedded in paraffin. Nonspecific immunoreactivity in slide-mounted tissue sections was blocked with Serum Blocker Reagent D (R&D Systems), and incubated with goat polyclonal anti-mouse VEGF at 1:20 (R&D Systems) at 4°C overnight, washed with PBS, and incubated with biotinylated anti-goat secondary Ab (R&D Systems) for 40 minutes, followed by HSS (high-sensitivity streptavidin)–HRP (horseradish peroxidase; R&D Systems) for 30 minutes. Slides were washed with PBS and then incubated with an HRP-reactive DAB chromogen (R&D Systems) for 7 minutes. Slides were rinsed in H₂O, incubated in CAT hematoxylin (Biocare Medical) at 1:5 for 3 minutes and Tacha’s Bluing Agent (Biocare Medical) at 1:5 for 3 minutes, followed by rinses with H₂O. Slides were dehydrated in increasing concentrations of alcohol (70%, 90%, 100%; 3 minutes each), briefly washed in xylene, and coverslipped using mounting medium (Richard-Allan Scientific). Negative control slides for VEGF IHC were prepared by completing the above protocol in the absence of primary Ab.

Tumor immunotherapy studies
The facilities at the University of Miami’s Division of Veterinary Resources are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and U.S. Drug Administration. An Office of Laboratory Animal Welfare assurance is on file, ensuring that humane animal care and use practices, as outlined in the Guide for the Care and Use of Laboratory Animals, are followed. Of note, 5- to 6-week-old female C57BL/6 (H-2b) and Balb/c (H-2d) mice were purchased from The Jackson Laboratory and used within 1 to 3 weeks.

Subcutaneous tumor models. 4T1 breast carcinoma model
Balb/c mice were injected subcutaneously in the right flank with 1.0 × 10⁴ 4T1 tumor cells and immunized with a mixture of irradiated (6,000 rad) B7-1 and MHC class II–expressing 4T1 cells (22) injected into the opposite flanks at days 7, 10, and 13. Eighteen hours after each injection, 100 pmol of aptamer conjugates were administered by tail vein injection.

B16.F10 melanoma model
C57BL/6 mice were injected subcutaneously in the right flank with 1.0 × 10⁶ B16.F10 cells and immunized with 10⁵ irradiated (6,000 rad) GM-CSF–expressing B16.F10 cells (GVAX; ref. 23) injected into the opposite flanks at days 4, 7, and 10. Eighteen hours after each injection, 100 pmol of aptamer conjugates were administered by tail vein injection.

Mice were sacrificed when tumor diameter exceeded 1.2 cm or mice exhibited signs of morbidity. Experiment was terminated when 2 or more mice were sacrificed in the “no treatment” group. 4T1, 4T07, and B16.F10 parental, B7-1-, and MHC class II–expressing cells were tested and validated to be mycoplasma-free. B7-1 and MHC class II expressions were validated by IHC; no other authentication assays were performed.

4T1 breast carcinoma postsurgical metastasis model
Balb/c mice were injected with 1.0 × 10⁴ 4T1 cells into the abdominal mammary fat pad. At day 11, 2 days after local tumors became palpable, mice were anesthetized and primary tumors were surgically removed (24). Mice were vaccinated with the
irradiated B7-1 and MHC class II–expressing 4T1 cells at days 13, 16, and 19. Eighteen hours after each vaccination, 100 pmol of aptamer conjugates were administered as described above. Mice were sacrificed when they showed signs of morbidity.

Methylcholanthrene carcinogenesis model. Balb/c mice were injected subcutaneously with 400 µg of 3-methylcholanthrene (MCA) in castor oil. As indicated, 100 pmol of VEGF–4-1BB conjugates were administered once weekly for a total of 4 injections. Mice were sacrificed when tumor diameter exceeded 1.2 cm or mice exhibited signs of morbidity.

Oncogene-induced high-grade glioma model. The immune competent RCAS/Ntv-a murine model in which intracerebral high-grade gliomas are induced by expressing PDGFβ and STAT3 from glioneuronal precursors has been described previously (25, 26). Briefly, to transfer genes via RCAS vectors, DF-1 producer cells transfected with a particular RCAS vector (1 × 10^7 DF-1 cells in 2 µL of PBS) were injected into the frontal lobes of day 1 or 2 Ntv-a mice at the coronal suture. Twenty-one days after introducing the glioma-inducing transgenes, mice were randomly assigned to the treatment or control group. Mice were sacrificed 90 days after injection or sooner if they demonstrated morbidity related to tumor burden. DF-1 parental and transfected cells (25, 26) were tested and validated to be mycoplasma-free. Transgene expression was validated by IHC/flow cytometry; no other authentication was performed.

Statistical analysis
Mouse survival, defined as the time (in days) the mouse was born to the date of death, was estimated using the method of Kaplan and Meier. Cox regression model was used to explore the survival difference between individual treatment groups using statistical software R V-3.0.2. For other continuous measurements, unpaired, two-tailed Student t tests were performed between individual treatment groups using GraphPad Prism V-5.00 (GraphPad Software Inc.). P values less than or equal to 0.05 were considered statistically significant.

Results
Potentiation of vaccine-induced antitumor immunity in subcutaneously implanted tumor-bearing mice with VEGF–4-1BB aptamer conjugates
To target costimulation to the tumor stroma, we conjugated an agonistic dimeric 4-1BB aptamer (27) to a VEGF aptamer selected against human VEGF (20; Supplementary Fig. S1). VEGF is secreted by progressing tumor lesions in many types of cancers (28). We have confirmed that the VEGF aptamer also binds to murine VEGF and the VEGF–4-1BB conjugate binds to both 4-1BB and VEGF (Fig. 1A). Figure 1B shows that the VEGF–4-1BB aptamer conjugates retained 4-1BB costimulatory activity.

To test whether the VEGF–4-1BB aptamer conjugate can enhance vaccine-induced antitumor immunity, mice were implanted subcutaneously with either 4T1 breast carcinoma cells and 7 days later vaccinated with a mixture of irradiated MHC class II- and B7-1–transfected 4T1 cells (22; Fig. 2A) or with B16.F10 melanoma cells and 5 days later vaccinated with GM-CSF–expressing irradiated B16.

Figure 1. Characterization of VEGF–4-1BB aptamer conjugates. A, binding of VEGF–4-1BB aptamer conjugates to filter-immobilized targets was measured using a double-filter binding assay. 32P-labeled 4-1BB aptamer, VEGF aptamer, or VEGF–4-1BB aptamer conjugates were passed through nitrocellulose filters immobilized with decreasing amounts of murine 4-1BB protein (m4-1BB), murine VEGF protein (mVEGF), or IgG, except for the rightmost lane that contained no protein. Bound radioactivity was visualized by exposure to X-ray–sensitive film. B, 4-1BB costimulation of polyclonally activated CD8+ T cells. CD8+ T cells were activated with anti-CD3 Ab and incubated with an agonistic 4-1BB Ab or IgG control, 4-1BB aptamer, VEGF–4-1BB, or control aptamers in which the 4-1BB aptamer was replaced with a scrambled aptamer (Scram). Proliferation was measured 48 hours later by 3H-thymidine incorporation. Statistical analysis: 4-1BB Ab versus isotype Ab, P = 0.01; 4-1BB aptamer versus scrambled aptamer, P = 0.028; VEGF–4-1BB aptamer conjugate versus VEGF-scrambled aptamer conjugate, P = 0.069.
tumor vaccination as monotherapy is largely ineffective—no impact on tumor growth—simulating a scenario in which tumor vaccination as monotherapy is largely ineffective. As shown in Fig. 2, using experimental conditions whereby vaccination of tumor-bearing mice had minimal to no impact on tumor growth—simulating a scenario in which tumor vaccination as monotherapy is largely ineffective—cotreatment with the VEGF–4-1BB aptamer conjugates inhibited tumor growth treatment. Treatment with conjugates alone had a small effect that did not reach statistical significance in either model. Vaccination with a mixture of VEGF and 4-1BB aptamers had no effect, ruling out the possibility that tumor inhibition was an additive contribution of the two aptamers. This was to be expected given that the mice were administered with 300 pmol of (monovalent) aptamer conjugates that were 5–10-fold less than unconjugated 4-1BB aptamers (27) and 20–50-fold less than (bivalent) VEGF Ab (29–31) used to inhibit tumor growth in mice.

**VEGF-dependent tumor-targeted 4-1BB costimulation**

To determine whether inhibition of tumor growth was a result of VEGF-dependent tumor-targeted 4-1BB costimulation, we compared the homing and antitumor activities of the VEGF–4-1BB aptamer conjugates in 4T1 and 4T07 tumor-bearing mice. 4T1 and 4T07 are two subcell lines derived from a thioguanine-resistant breast carcinoma tumor (32). VEGF expression in subcutaneously implanted tumors was measured by HIC. As shown in Fig. 3A, the metastatic 4T1 tumors expressed higher levels of VEGF than the nonmetastatic 4T07 tumors. Mice were then implanted contralaterally in opposite flanks with 4T1 and 4T07 cells, and 21 days later 32P-labeled VEGF–4-1BB aptamer conjugates were administered via the tail vein. As shown in Fig. 3B, the VEGF–4-1BB aptamer conjugates accumulated preferentially in the VEGFhigh 4T1 tumors. Consistent with the preferential homing of the VEGF–4-1BB conjugates to 4T1 cells, the VEGF–4-1BB aptamer conjugates inhibited 4T1 (Fig. 3C), but not, or to a much lesser extent, 4T07 (Fig. 3D) tumor growth.

Unlike the experiment shown in Fig. 2A, in this experiment, mice were not vaccinated, yet treatment with conjugates alone inhibited 4T1 tumor growth. The reason is that conditions used in this experiment were less stringent, starting treatment at day 4 rather than day 7, after tumor implantation. The lack of inhibition of 4T07 tumor growth was not due to its resistance to 4-1BB costimulation because a 5-fold higher dose of unconjugated 4-1BB aptamer inhibited both 4T07 and 4T1 tumor growth to a similar extent. The small inhibition of 4T07 tumor growth in mice treated with the aptamer conjugate, which did not reach statistical significance, could therefore be due to the nontargeted 4-1BB costimulatory effect of the conjugates administered at a 5-fold lower dose, and/or the low level of VEGF expressed in the 4T07 tumors (Fig. 3A). Supplementary Figure S2 shows the biodistribution of the systemically administered VEGF–4-1BB aptamer conjugates in various tissues and organs of the mouse. Because the VEGF-targeted aptamer conjugates do not become cell associated, the tested tissues could not be perfused. Thus, the relatively high levels of aptamer conjugates in the various tissues and organs, especially in the blood-rich spleen and liver, are likely a reflection of their presence in the circulation.

To assess the generality of tumor stroma–targeted costimulation, the dimeric 4-1BB aptamers were conjugated to an OPN-binding aptamer that was selected for binding to both murine and human orthologs (21; Supplementary Fig. S1). OPN, like VEGF, is secreted by progressing tumor lesions in many types of cancer (33). As shown in Supplementary Fig. S3A, 4T1 tumors secrete higher levels of OPN than 4T07 tumors. 32P-labeled OPN–4-1BB aptamer conjugates injected into mice that were coimplanted with both 4T1 and 4T07 tumor cells accumulated preferentially in the OPNhigh 4T1 tumors (Supplementary Fig. S3B), and inhibited the growth of 4T1 tumor but not, or to a lesser extent, the growth of 4T07 tumor cells (Supplementary Fig. S3C). In a more stringent experimental setting, the OPN–4-1BB aptamer conjugates potentiated an
otherwise ineffective vaccine-induced immune response resulting in reduced tumor growth (Supplementary Fig. S3D).

**Superior therapeutic index of VEGF–4-1BB aptamer conjugates compared with 4-1BB Ab**

To determine whether tumor stroma targeting of 4-1BB aptamers enhances the therapeutic index of 4-1BB costimulation, we compared treatment with VEGF–4-1BB aptamer conjugates with that of unconjugated 4-1BB aptamer and with anti–4-1BB Ab. As shown in Fig. 4A, on a molar basis, the VEGF–4-1BB aptamer conjugates were as effective as a 6-fold higher dose of unconjugated 4-1BB aptamer or anti–4-1BB Ab at inhibiting tumor growth. Ab, but not unconjugated 4-1BB aptamer or VEGF–4-1BB conjugate, treatment was associated with increased spleen, lymph node, lung, and liver weights (Fig. 4B and data not shown), resulting mostly from increased CD8⁺ T-cell numbers (Fig. 4C) and notably, extensive infiltration of leukocytes in the lung and liver (Fig. 4D), as described previously (9, 13, 14). Interestingly, as we have shown (17), treatment with an equimolar dose of 4-1BB aptamer that elicits a comparable antitumor response to that of treatment with 4-1BB Ab did not elicit CD8⁺ T-cell hyperplasia (Fig. 4C) or leukocytic infiltration (Fig. 4D). The experiments shown in Fig. 4, therefore, suggest that the therapeutic index of the VEGF-targeted 4-1BB aptamer could be significantly higher than that of the nontargeted anti–4-1BB Ab, and that nontargeted 4-1BB aptamer exhibits an intermediate therapeutic index.

**Inhibition of tumor growth by VEGF–4-1BB aptamer conjugates in a postsurgical metastasis model**

Given the limitations of subcutaneously implanted tumor models, we evaluated the therapeutic potential of stroma-targeted 4-1BB costimulation using increasingly relevant murine tumor models. In the experiment shown in Fig. 5, we used a postsurgical metastasis model to simulate treatment of residual metastatic disease. In this model, 4T1 breast carcinoma cells were implanted orthotopically into the abdominal mammary fat pad, and when they became palpable, 10 to 11 days later, tumors were surgically resected. 4T1 growth parallels highly invasive human metastatic stage IV breast cancer metastasizing sequentially to the lung, lymph nodes, liver, and brain (34, 35). Two days after surgical resections, mice were
Figure 4. Therapeutic index of VEGF–4-1BB aptamer conjugates. A, 4T1 tumor cells were injected s.c. into Balb/c mice and 4 days later administered via the tail vein with 150 pmol of VEGF–4-1BB aptamer conjugates or with 800 pmol of either unconjugated 4-1BB aptamer, anti–4-1BB Ab, or IgG control Ab. Aptamer and Ab administration was repeated two additional times at 3-day intervals as described in Materials and Methods, and tumor growth was monitored (5 mice per group). B, mice shown in A were sacrificed at day 17, and spleens and lymph node weights were measured. C, CD8+ T cells were isolated from the spleens, lymph nodes, and livers, and were counted. The difference between the 4-1BB Ab group and all other treated groups in B and C was statistically significant (P < 0.001). There was no statistical difference between the control IgG, 4-1BB aptamer, and VEGF–4-1BB aptamer conjugate groups. D, tissue sections from the lung and liver were stained with hematoxylin and eosin and visualized by light microscopy.
subjected to three vaccinations and conjugate treatment as described in the legend to Fig. 2, and monitored for survival. As shown in Fig. 5A, vaccination or administration of VEGF–4-1BB aptamer conjugates did not improve the survival of treated mice, which presented extensive metastasis in the lung at the time of sacrifice. On the other hand, mice that were vaccinated and treated with the VEGF–4-1BB aptamer conjugates survived longer, with 3 of 5 mice surviving long term and showing no signs of metastasis in the lung at the time of sacrifice (day 90). The targeted nature of the VEGF–4-1BB aptamer conjugates mechanism of action is consistent with the observation that a mixture of VEGF and 4-1BB aptamers was ineffective (Fig. 5A), and that accumulation of 32P-labeled 4-1BB aptamers in the lung was enhanced by conjugation to the VEGF aptamer (Fig. 5B).

**Inhibition of tumor growth by VEGF–4-1BB aptamer conjugates in nontransplanted autochthonous tumor models**

Given the limitations of modeling tumorigenesis with tissue culture–adapted tumor cell lines, we determined whether VEGF-targeted 4-1BB costimulation can control the growth of tumors that develop in situ from normal precursors. To this end, we used a carcinogen-induced tumor model in which mice injected with 3-MCA develop fibrosarcomas over the course of several months, by and large recapitulating the naturally occurring multistep carcinogenesis process (36). Starting at about day 70 when tumors are readily palpable, mice were treated with either VEGF–4-1BB conjugates or a mixture of VEGF and 4-1BB aptamers. As shown in Fig. 6A, mice treated with the conjugates, but not with the nonconjugated aptamers,
exhibited a significant survival advantage compared with untreated mice, with 2 of 9 mice surviving long term.

As a second model, we used an oncogene-induced high-grade glioma model whereby tumors are induced endogenously by the expression of PDGF-B and STAT3 in glioneuronal precursor cells of newborn mice (25, 26). This model was also used for assessing the ability of the aptamer conjugates to cross the blood–brain barrier that may exist in tumor-bearing mice. As shown in Fig. 6B, treatment of the tumor-bearing mice with the VEGF-targeted 4-1BB aptamer conjugates, but not with a mixture of VEGF and 4-1BB aptamers, led to the enhanced survival of the aptamer conjugate–treated mice. The therapeutic effect of the VEGF–4-1BB conjugates in this model was comparable with that of the previously reported treatment with an immune modulatory microRNA, miR124 (26).

Discussion

Dose-limiting toxicities of cancer drugs, reflecting their limited tumor specificity, are a major hurdle in developing effective treatments for cancer. Predicted by animal studies, systemic administration of immune modulatory Abs to patients with cancer was also associated with dose-limiting autoimmune pathologies (15, 16). Arguably, enhancing the therapeutic index of immune-potentiating drugs, namely reducing their toxicity without compromising their antitumor activity, is paramount for exploiting their clinical potential to the fullest extent.

A major step toward addressing dose-limiting drug toxicity was heralded with the introduction of molecule-targeted therapies. Targeted therapies act by blocking essential biochemical pathways or mutant proteins that are required for tumor cells to grow and survive. The success of imatinib, a BCR–ABL kinase inhibitor that has shown striking responses in patients with chronic myelogenous leukemia, an inhibitor that blocks a specific mutant of the B-RAF serine/threonine kinase (V600E-B-RAF) and demonstrated dramatic efficacy in the treatment of metastatic melanoma, as well as other inhibitors targeting EGFR, KIT, HER2, and ALK, has by and large validated this novel paradigm of cancer therapeutics (37). Nonetheless, the Achilles’ heel of targeted therapy is that the target is often a cancer-driving mutation in chronic myelogenous leukemia, an inhibitor that blocks a specific mutant of the B-RAF serine/threonine kinase (V600E-B-RAF) and demonstrated dramatic efficacy in the treatment of metastatic melanoma, as well as other inhibitors targeting EGFR, KIT, HER2, and ALK, has by and large validated this novel paradigm of cancer therapeutics (37). Nonetheless, the Achilles’ heel of targeted therapy is that the target is often a cancer-driving mutation in a product that normally regulates cell behavior, which leads to increasing fragmentation of drug treatments among subtypes of cancer, and more importantly, the development of resistance even to the most dramatically effective therapies.

An alternative, mutually nonexclusive, way to reduce drug toxicity is to limit drug exposure to normal tissues by targeting the drug to the tumor cells of the patients. mAbs are currently the main platform for targeting ligands. For example, phase I clinical trials in patients with cancer with a CD19-CD3 bispecific Ab have underscored the therapeutic benefit of targeting T cells to cancer cells using low-dose therapy (38). Nonetheless, despite a growing list of therapeutic Abs that have been approved for cancer therapy, only two Ab-targeted cytotoxic drug conjugates are currently licensed for cancer therapy (39).

The development of therapeutic Ab-based targeting ligands is hindered by several factors. Abs are cell-based products that increase the complexity and cost of manufacturing, and the regulatory approval process. Conjugation of Abs to their therapeutic drug requires special skill sets and expensive instrumentation, and mAbs, even when fully humanized, run the risk of eliciting neutralizing Ab response upon repeated administrations (40, 41). Unlike Abs, the short oligonucleotide aptamers can be synthesized in a cell-free chemical process that is more cost-effective and requires a less complex regulatory approval process. Conjugation to nucleic acid–based therapeutic agents by hybridization between short complementary sequences is straightforward, and the short oligonucleotides are less likely to stimulate neutralizing immunity. Recent studies have underscored the therapeutic potential of using aptamers as targeting ligands to deliver therapeutic siRNAs and aptamers to tumor cells and T cells to eradicate tumors (42), to sensitize tumor cells to radiation therapy (43), to inhibit HIV replication (44, 45), and to potentiate tumor immunity (17, 19, 46).

In addition to activated CD8+ T cells, 4-1BB is also upregulated, though to a lesser extent, on activated CD4+ T cells, natural killer (NK) cells, endothelial cells, mature dendritic cells, and foxp3+ CD4+ regulatory T cells (Treg; refs. 47, 48). Administration of anti–4-1BB Ab to mice led not only to CD8+ T-cell hyperplasia (14) but also to the deletion or inactivation of activated CD4+ T cells, NK cells, and B cells (14, 49) that could compromise protective immunity against tumors and infectious agents, and may have contributed to the autoimmune pathologies in the Ab-treated mice (9, 13, 14). The risks of nontargeted Ab administration have been underscored by the severe liver toxicities in patients treated with an agonistic 4-1BB Ab (16). In this study, we used aptamers to target 4-1BB costimulation to tumor lesions to reduce its systemic and potentially harmful effects. We have shown that on a molar basis tumor stroma-targeted 4-1BB costimulation with bispecific aptamer was as effective at inhibiting tumor growth as a 6-fold higher dose of an agonistic 4-1BB Ab (Fig. 4A), and that the Ab, but not the aptamer conjugate, elicited CD8+ T-cell hyperplasia (Fig. 4C) and immune pathology (Fig. 4D). The therapeutic index of tumor-targeted 4-1BB costimulation was, therefore, significantly higher than that of the nontargeted Ab, the current gold standard of therapeutic costimulation. Whereas the observed VEGF-dependent accumulation of the VEGF-conjugated 4-1BB aptamer in the tumor lesions is likely to be the underlying mechanism for the enhanced therapeutic index, given that VEGF concentrations can be elevated in tumor-bearing mice and patients with cancer, an additional mechanism contributing to the enhanced bioactivity of the VEGF–4-1BB aptamer conjugates could be due to stabilization of the 4-1BB aptamer in the circulation.

Interestingly, as we have shown previously (17), treatment with an equimolar dose of 4-1BB aptamer that elicits a comparable antitumor response to that of treatment with the 4-1BB Ab did not elicit either CD8+ T-cell hyperplasia or immune pathology, suggesting that even the nontargeted agonistic 4-1BB aptamer exhibits a superior therapeutic index compared with Ab. A possible explanation that will require additional studies is that the half-life of aptamers in the circulation, from a few hours to 1 to 2 days, is considerably less than that of Abs that can persist for weeks and thereby contribute more extensively to the activation of autoreactive T cells. Given the severe hepatic toxicities in patients treated with a 4-1BB Ab, predicted by preclinical studies showing extensive inflammatory foci in the liver of Ab-treated mice.
(Fig. 4D; refs. 9, 13, 14), the observations summarized in Fig. 4 suggest that tumor-targeted, and even nontargeted, aptamer-based immune modulatory drugs, not limited to 4-1BB costimulation, could offer certain advantages over Ab-based immune modulatory drugs.

Targeting the 4-1BB costimulatory ligand to the tumor stroma, instead of to the tumor cell, obviates the limitation imposed by the internalization of most receptors upon ligation (18). Using the aptamer platform, we have shown that VEGF or OPN aptamer conjugated 4-1BB aptamers accumulated preferentially in tumors that express higher levels of VEGF or OPN and inhibit their growth but not that of tumors expressing low levels of VEGF or OPN (Fig. 3 and Supplementary Fig. S3). Another important advantage of targeting costimulation to the tumor stroma is that unlike tumor-specific receptors that exhibit restricted expression patterns, for example, PSMA expressed mostly on prostate tumors or Her2 expressed on a subset of breast tumors, many stroma-secreted products, such as metalloproteases, VEGF, or OPN, are secreted by most, if not all, tumor lesions regardless of their origin. The generality of this approach was indicated in this study by showing that the VEGF-targeted 4-1BB ligand inhibited tumor growth in four unrelated tumor models, B16 melanoma, 4T1 breast carcinoma (Figs. 2A and 5A), MCA-induced fibrosarcoma (Fig. 6A), and Nvt-a glioma (Fig. 6B).

To assess the therapeutic potential of stroma-targeted costimulation, we measured the survival of tumor-bearing mice using increasingly relevant models for human cancer. The postsurgical lung metastasis model (Fig. 5), except for using an established tumor cell line, simulates a common scenario and critical aspect of cancer therapy, the treatment of minimal or residual metastic disease. Complementing the postsurgical metastasis model, in the carcinogen- and oncogene-induced models (Fig. 6), tumors develop in situ from normal cells recapitulating the multistep carcinogenesis process. The MCA-treated (Fig. 6A) and the Ntv-a (Fig. 6B) mice are excellent models for carcinogen-induced skin cancer and high-grade glioma, respectively. The therapeutic impact of VEGF-targeted 4-1BB costimulation in the 4T1 postsurgical (Fig. 5A) and MCA-induced (Fig. 6A) tumor models seems to be significantly more pronounced than what has been reported in the literature. In the 4T1 postsurgical model, vaccination with irradiated cells ectopically expressing syngeneic MHC class II, B7-1 (used in our study), and the staphylococcal enterotoxin B was shown to prolong survival, but eventually all mice succumbed to disease (24). In the MCA model, a proportion of mice could be cured only when treated with a combination of three or four immune-stimulatory Abs (12, 50), whereas no cure was observed when a combination of two Abs, anti-4-1BB and either anti-CD40 or anti-DR5 Abs, was used (12). By contrast, VEGF–4-1BB aptamer conjugates potentiated vaccination in the 4T1 postsurgical metastasis model, leading to long-term survival of over 50% of the treated mice (Fig. 5A), whereas in the MCA model, monotherapy with the VEGF–4-1BB aptamer conjugates was able to extend the survival of the treated mice, with 2 of the 9 mice surviving long term (Fig. 6A). In the high-grade glioma model, the therapeutic effect of the VEGF-targeted 4-1BB aptamer conjugates (Fig. 6B) was comparable with that of treatment with the previously described immune modulatory mR124 (26). In both the MCA fibrosarcoma (Fig. 6A) and the high-grade glioma (Fig. 6B) models, the therapeutic effect of monotherapy with the VEGF–4-1BB aptamer conjugates reflects the potentiation of a naturally occurring antitumor immune response. It is conceivable that combination with other forms of immune therapy, such as vaccination or checkpoint blockade, would further potentiate the antitumor effects shown in Fig. 6.

In summary, this study suggests that tumor stroma–targeted costimulation with bispecific aptamer ligands, not limited to 4-1BB costimulation, is a clinically feasible, broadly applicable approach to modulate immune response expressed on tumor-infiltrating immune cells that obviates the current limitations of using nontargeted administration of mAbs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B. Schrand, A. Berezhnoy, R. Brenneman, A.B. Heimberger, E. Gilboa
Development of methodology: B. Schrand, A. Berezhnoy, R. Brenneman, A.B. Heimberger
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Schrand, A. Berezhnoy, R. Brenneman, A. Williams, L.-Y. Kong, G. Rao, A.B. Heimberger
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): B. Schrand, R. Brenneman, A. Williams, S. Zhou, A.B. Heimberger
Writing, review, and or revision of the manuscript: B. Schrand, A. Williams, S. Zhou, A.B. Heimberger, E. Gilboa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.-Y. Kong, G. Rao, A.B. Heimberger
Study supervision: A.B. Heimberger, E. Gilboa

Grant Support
This study was funded by a bequest from the Dodson Estate and the Sylvester Comprehensive Cancer Center (Miller School of Medicine, University of Miami), the Susan G. Komen for the Cure of Breast Cancer Foundation (KG090348), the Dr. Marine Rose Foundation (to A.B. Heimberger), and the NIH CA120813, P50 CA127001 (to A.B. Heimberger), and K08 NS079928 (to G. Rao). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 12, 2014; revised June 5, 2014; accepted June 5, 2014; published OnlineFirst June 17, 2014.

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### Cancer Immunology Research

**Targeting 4-1BB Costimulation to the Tumor Stroma with Bispecific Aptamer Conjugates Enhances the Therapeutic Index of Tumor Immunotherapy**

Brett Schrand, Alexey Berezhnoy, Randall Brenneman, et al.


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