Combination of 4-1BB Agonist and PD-1 Antagonist Promotes Antitumor Effector/Memory CD8 T cells in a Poorly Immunogenic Tumor Model

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Abstract

Immunotherapies targeting Programmed death 1 (PD-1) co-inhibitory receptor have shown great promise for a subset of cancer patients. However, robust and safe combination therapies are still needed to bring the benefit of cancer immunotherapy to broader patient populations. To search for an optimal strategy of combinatorial immunotherapy, we have compared the antitumor activity of anti-4-1BB/anti-PD-1 combination with that of anti-PD-1/anti-LAG-3 combination in the poorly immunogenic B16F10 melanoma model. Pronounced tumor inhibition occurred only in animals receiving anti–PD-1 and anti–4-1BB concomitantly, while combining anti-PD-1 with anti-LAG-3 led to a modest degree of tumor suppression. The activity of anti-4-1BB/anti-PD-1 combination was dependent on IFNγ and CD8+ T cells. Both 4-1BB and PD-1 proteins were elevated on the surface of CD8+ T cells by anti-4-1BB/anti-PD-1 co-treatment. In the tumor microenvironment, an effective antitumor immune response was induced as indicated by increased CD8+/Treg ratio and the enrichment of genes such as CD3ε, CD8α, IFNγ and Eomesodermin. In the spleen, the combination treatment shaped the immune system to an effector/memory phenotype and increased the overall activity of tumor-specific CD8+ CTLs, reflecting a long-lasting systemic antitumor response. Furthermore, combination treatment in C57BL/6 mice showed no additional safety signals, and only minimally increased severity of the known toxicity relative to 4-1BB agonist alone. Therefore, in the absence of any cancer vaccine, anti-4-1BB/anti-PD-1 combination therapy is sufficient to elicit a robust antitumor effector/memory T-cell response in an aggressive tumor model and is therefore a candidate for combination trials in patients.
Introduction

Immunotherapy has emerged as a promising treatment for cancer in recent years, showing durable responses in subsets of patients, and thus was selected as the Breakthrough of Year 2013 by the journal Science (1). For example, ipilimumab, an anti-Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) antagonistic antibody, showed an objective response rate (~10%) in patients with unresectable or metastatic melanoma in a phase 3 trial, while severe or life-threatening toxicity was seen in 10-15% of the patients (2). Targeting the PD-1/Programmed cell death ligand 1 (PD-L1) axis resulted in a higher response rate of up to 37% while treatment-related grade ≥ 3 toxicity rates were 9-33% in the melanoma cohorts of multiple phase 1 trials (3). The combination of two checkpoint inhibitors, anti-CTLA-4 and anti-PD-1, increased the response rate up to 53% in a phase 1 trial with advanced melanoma; however, severe treatment-related adverse events (grade ≥ 3) were also reported in 53% of cases and treatment was discontinued in 21% of the patients (3, 4). While these results are promising, it is clear that increasing the frequency of response while limiting toxicities will be crucial for the success of combinatorial cancer immunotherapy in a wider population of cancer patients.

4-1BB (CD137, TNFRSF9) belongs to the Tumor Necrosis Factor (TNF) receptor superfamily (TNFRSF) and its expression is generally activation-dependent on a subset of immune cells including T cells and natural killer (NK) cells (5). The ligand that stimulates 4-1BB (4-1BBL) is expressed on antigen-presenting cells (APC) including dendritic cells (DC), B cells, and macrophages (6). Ligation of 4-1BB receptors elicits a variety of immune cell-specific biological responses. For instance, 4-1BB activation promotes cell proliferation, survival, and cytokine production in T cells. In several tumor models, 4-1BB agonists have demonstrated antitumor efficacy (6, 7). Interestingly, in some autoimmunity models, 4-1BB agonist also
inhibited autoimmune reactions partly by antagonizing Th17 cell polarization (8, 9). This dual immunoregulatory activity of 4-1BB offers the possibility to enhance antitumor activity while simultaneously dampening autoimmune side effects associated with immunotherapy approaches that break immune tolerance. Agonistic antibodies to 4-1BB (Urelumab, BMS and PF-05082566, Pfizer) are currently being tested in humans with advanced cancer, and PF-05082566 was found well tolerated with evidence of disease stabilization in multiple patients (10, 11).

PD-1 is an inhibitory receptor expressed on activated T cells as well as other immune cells, and its expression can be associated with T-cell exhaustion (12). PD-1 binds to two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) that are often over-expressed on tumor cells. Such interactions deliver a negative signal to T cells to dampen the immune response. Antibody blockade of PD-1 was shown to enhance effector T-cell responses and induce T cell–mediated tumor rejection in some mouse models (12). Additive or synergistic antitumor effects were also reported when PD-1 blockade was combined with other immune checkpoint blockade such as T-cell Immunoglobulin Mucin 3 (TIM-3) or Lymphocyte Activation Gene 3 (LAG-3) in different tumor models (13, 14).

Anti-4-1BB agonist has been investigated extensively in pre-clinical studies to treat cancer when combined with other immunomodulatory antibodies. Synergistic antitumor effects have been observed for combinations of anti-4-1BB with anti-TIM-3 (15) or with anti-PD-1 (16, 17) in the ID8 murine ovarian cancer model. Studies on ID8 ovarian carcinoma also indicated that the anti-4-1BB/anti-PD-L1 combination had moderate impact on survival which could be further improved with additional vaccination (18). The anti-4-1BB/anti-CTLA-4 combination significantly delayed MC38 colon carcinoma growth over anti-4-1BB or anti-CTLA-4 alone; however, the same regimen failed to control B16F1 melanoma growth (19). The anti-4-1BB/anti-
CTLA-4 combination also failed to prolong the survival of B16F10 tumor-bearing mice (20). Further studies showed that active vaccination was required for anti-4-1BB/anti-CTLA-4 combination to effectively control the growth of B16-BL6 melanoma (21). These studies suggested that not all combinations are therapeutically equivalent and different models might respond to treatment differently. Here, we report that the anti-4-1BB/anti-PD-1 combination is more efficacious than that of anti-PD-1/anti-LAG-3 in suppressing B16F10 melanoma growth without adjuvant or vaccination. We investigated the cellular mechanism underlying this synergy. Finally we showed that the combination was well tolerated in naive or tumor-bearing mice, indicative of a potential therapeutic index for the combination.

Materials and Methods

Mice

Six- to 8-week old female C57BL/6 mice and IFNγ-deficient mice were purchased from The Jackson Laboratories. Mice were maintained and all animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of Rinat, South San Francisco and Worldwide Research and Development (WRD), La Jolla, Pfizer Inc.

Cell lines

The B16F10 melanoma cell line was purchased from American Type Culture Collection (ATCC) in 2010. MC38 colon carcinoma cell line was kindly provided by Dr. Antoni Ribas at UCLA, California in 2011. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and IMPACT-tested for pathogens at Research Animal...
Diagnostic Laboratory (RADIL) (Columbia, MO). No other authentication assay was performed. Pathogen-free cells growing in an exponential growth phase were harvested and used for tumor inoculation.

**Antibodies for immunotherapy and flow cytometry**

Therapeutic rat anti-mouse 4-1BB mAb (clone MAB9371) was purchased from R&D systems. Rat anti-mouse PD-1 mAb (clone RMP1-14) and rat IgG2a isotype control were purchased from BioXcell. Rat anti-mouse LAG-3 mAb (clone eBioC9B7W) was purchased from eBioscience. mAbs used for cell surface or intracellular stains were purchased from BD Biosciences or eBioscience. They were hamster anti-mouse CD3ε (clone 145-2C11), rat anti-mouse CD4 (Clone RM4-5), rat anti-mouse CD8α (clone 53-6.7), rat anti-mouse CD25 (clone PC61), hamster anti-mouse CD137 (4-1BB) (clone 17B5), hamster anti-mouse CD279 (PD-1) (clone J43), rat anti-mouse FoxP3 (clone FJK-16s), rat anti-mouse Eomes (Clone: Dan1mag), hamster anti-mouse KLRG1 (clone 2F1), and rat anti-mouse NK-1.1 (Clone PK136). Live cells were separated from dead cells using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen).

**Immune-cell phenotyping using flow cytometry**

Spleens from tumor-bearing mice were harvested and dissociated mechanically into single-cell suspension in ice-cold PBS. Splenocytes were treated with Red Blood Cell Lysing Buffer Hybrid-Max (Sigma-Aldrich), washed twice with PBS, and resuspended in PBS supplemented with 2% FBS and 0.9% NaN₃. An aliquot of ~1 x 10⁶ splenocytes was pre-incubated with 10 µg/mL of mouse BD Fc Block (BD Biosciences) for 10 minutes before phenotyping mAb cocktail was added to specifically stain immune cells.
Tumor-infiltrating lymphocytes (TIL) were prepared using mouse tumor dissociation kit and the GentleMACS Dissociator according to manufacturer's instructions (Miltenyi Biotec).

Cell surface antigens were labeled by incubating cells at 4°C for 30 minutes. Intracellular staining was carried out using FoxP3/Transcription Factor Staining Buffer set according to the manufacturer’s protocol (eBioscience). Flow cytometry data were acquired using LSR Fortessa (BD Biosciences) and analyzed using FlowJo (TreeStar Inc.).

**In vivo tumor efficacy studies**

C57BL/6 mice were inoculated subcutaneously at the right flank with 1 x 10^6 B16F10 or MC38 cells in 0.1 ml of serum-free DMEM medium or PBS. Treatment was started when tumors reached 50 – 154 mm^3 in size. Antibodies to 4-1BB (1 mg/kg), PD-1 (10 mg/kg) and LAG-3 (10 mg/kg), alone or in combination, were administrated twice intraperitoneally (i.p.) 5 days apart. In the study of treating larger tumors (range of 126-350 mm^3), antibodies were given four times i.p. 3 days apart. To treat IFNγ-deficient mice, antibodies were given 3 times 3 days apart. To deplete CD8+ T cells, anti-CD8 (53.5.8, BioXcell) was given as described previously (22).

Briefly, depleting antibody (100 μg) was administrated one day prior to anti-4-1BB/anti-PD-1 administration and every 5 days thereafter for a total of 3 doses. Tumor size was measured in two dimensions using calipers, and the volume was expressed in mm^3 using the formula: V = 0.5 L x W^2 where L and W are the long and short diameters of the tumor, respectively.

**Antigen-specific immune response assay**

Frozen splenocytes from MC38 tumor-bearing mice after antibody treatment were thawed and rested for 12 hours. Viable cells were plated at 1 x 10^6 / well in duplicate in the presence of 0.5 μg/mL of CD8-restricted MC38 immunodominant peptide KSTPFTTL (Proimmune) or the
positive activation control ConA at 5 μg/mL (Sigma Aldrich) for 48 hours. The assay was developed according to the manufacturer's instructions and read on a CTL ELISpot reader followed by spot enumeration using Immunospot 5.0.3. Upper sensitivity limit of the assay was 500 spots. Samples with no detectable ConA response were removed from the analysis.

Quantitative RT-PCR

B16F10 or MC38 tumor samples (~2 mm³) were collected into RNAlater stabilization reagent at the end of efficacy studies. Tumor pieces were homogenized into RLT buffer containing 1% 2-mercaptoethanol (ME) using a Qiagen TissueLyser and 5 mm steel beads. RNA was isolated from the homogenate using RNeasy mini spin columns, and quantified via UV absorbance using a Nanodrop 8000 spectrophotometer. One (1) μg of total RNA per sample was used as template to prepare cDNA used as template for TaqMan primer probe sets targeting mouse CD3ε, CD8α, IFNγ, Eomes, PD-1, PD-L1, 4-1BB, CD4, FoxP3, GAPDH, and Actin. Samples were amplified in duplicate in 384 well plates using an Applied Biosystems ViiA7 Real Time PCR system. Relative quantity (RQ) was calculated via the ddCt method relative to the average of the PBS control group.

Mouse toxicology study

Naïve C57BL/6 (n = 5 per group) were given a single subcutaneous administration of test articles or control (PBS) and clinical and anatomic pathology was analyzed 10 days later. At necropsy, the following tissues were collected for histology: cecum, colon, duodenum, epididymides, heart, ileum, jejunum, kidneys, liver, lung, spleen, pancreas, stomach, and testes. The testes and epididymides were preserved in modified Davidson’s solution, while all other tissues were fixed in 10% neutral buffered formalin. All tissues were routinely processed, embedded in paraffin and
sectioned, stained with hematoxylin and eosin (H&E), and analyzed by a board certified veterinary pathologist. In addition, blood and serum were collected for hematology using the Advia 2120 (Bayer, Leverkusen, Germany) and clinical chemistry analysis using the Advair 1200 (Siemens, Munich, Germany).

Potential toxicity was also assessed in B16F10 tumor-bearing mice. Mice with average tumors of ~100 mm³ (n = 5 per group) received a single s.c. dose of antibodies and clinical and anatomic pathology was analyzed 10 days later. Spleen and liver were processed and evaluated as described above for the tumor-free mice. Whole blood was analyzed for complete blood count and liver enzymes by the Diagnostic lab, Department of Comparative Medicine, Stanford University.

**Statistical analysis**

Results were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 one-way or two-way ANOVA to compare the differences among multiple groups. P < 0.05 was considered as significant difference.

**Results**

4-1BB activation and PD-1 blockade but not LAG-3 and PD-1 dual blockade resulted in synergistic antitumor effects in murine B16F10 melanoma

To evaluate the antitumor efficacy of combination immunotherapy against established B16F10 tumors, an anti-4-1BB dosing regimen was selected based on prior studies that showed efficacy in the CT26 model (23) and anti-PD-1 and anti-LAG-3 dosing regimens as previously reported (14, 21). Consistent with published results, anti-4-1BB, anti-PD-1 or anti-LAG-3 alone did not
consistently inhibit B16F10 tumor growth when treatments were started on tumors of 50 – 154 mm$^3$ in size (Figs. 1A-B). Combining anti-PD-1 with anti-LAG-3 resulted in 54% of tumor growth inhibition (TGI) relative to the isotype control ($p < 0.001$), but no mice were tumor free. In contrast, when animals were concurrently administered anti-4-1BB and anti-PD-1 antibodies, a dramatic efficacy of 85% TGI relative to the isotype control ($p < 0.0001$) was observed and 7 of 10 treated animals were tumor-free (Figs. 1A-B). Combinatory efficacy was reproducible in an independent study by different experimenters (Supplementary Fig. S1). Furthermore, significant TGI (51% relative to the control group, $p < 0.0001$) was observed for the anti-4-1BB/anti-PD-1 combination when treatment was applied to very large tumors (size between 126-350 mm$^3$), whereas the anti-PD-1/anti-LAG-3 combination was ineffective (Fig. 1C).

Likewise, a robust antitumor effect of the anti-4-1BB/anti-PD-1 combination was observed in the MC38 colon cancer model (Fig. 1D). At the end of the study (day 21 post tumor implant), the TGI for the combination treatment was 63% relative to the PBS control ($p < 0.0001$). The suppression was also significant when compared to single agent alone ($p < 0.05$ vs. anti-4-1BB alone and $p < 0.001$ vs. anti-PD-1 alone).

The efficacy of anti-4-1BB/anti-PD-1 combination was dependent on IFN$\gamma$ and CD8$^+$ T cells

Given the antitumor efficacy of anti-4-1BB/anti-PD-1 combination, it is important to understand how the synergy was achieved. First, we compared tumor growth in response to anti-4-1BB/anti-PD-1 co-treatment in wild-type and IFN$\gamma$-deficient mice. The combination suppressed tumor growth in wild-type animals, while such antitumor effect was completely abolished in the IFN$\gamma$-deficient mice (Fig. 2A). To determine the immune-cell subset that is required for the anti-4-1BB/anti-PD-1 combination, we depleted CD8$^+$ T cells in B16F10-bearing mice and compared
tumor growth after treatment. In the absence of CD8⁺ T cells, combination treatment-related tumor suppression was completely abrogated (Fig. 2B).

**Anti-4-1BB/anti-PD-1 combination increased the availability of target molecules for therapeutic antibodies on CD8⁺ T cells**

To further understand the mechanisms underlying the synergistic effects achieved by the anti-4-1BB/anti-PD-1 combination, we examined the expression of 4-1BB and PD-1 on CD8⁺ T cells in response to antibody treatment. Splenocytes or TILs from B16F10-bearing mice after treatment were isolated, immunostained and analyzed by flow cytometry. A representative flow cytometric gating strategy is shown in Supplementary Fig. S2. The anti-PD-1 (J43) used for detection does not compete with the therapeutic anti-PD-1 (RMP1-14) for the binding to PD-1, as determined by BIAcore analysis (data not shown). In PBS- and isotype-treated animals, PD-1 expression was barely detected (<1%) on splenic CD8⁺ T cells (Fig. 2C). Anti-PD-1 treatment did not affect its own expression or that of 4-1BB. However, anti-4-1BB treatment induced the expression of PD-1 on 34% of splenic CD8⁺ T cells when administrated alone (p < 0.0001 vs. isotype control) and on 29% of CD8⁺ T cells when co-administered with anti-PD-1 (p < 0.001 vs. isotype control). Combination therapy also induced PD-1 expression on 74% of TIL CD8⁺ T cells (p < 0.001 vs. isotype control) (Fig. 2D).

Therapeutic 4-1BB agonist relies on 4-1BB expressed on primed T cells for efficacy (24, 25). Anti-4-1BB treatment induced its own expression, with the cell surface expression detectable on 15% of splenic CD8⁺ T cells in the anti-4-1BB-treated group and 10% of the combination treatment group, compared to 0.2% in PBS- or isotype-treated groups (p < 0.0001 anti-4-1BB treatment vs. isotype control and p < 0.01 combination treatment vs. isotype control) (Fig. 2E).
There was a trend that the combination treatment up-regulated 4-1BB expression on TIL CD8\(^+\) T cells, although the increase was not statistically significant (Fig. 2F). These results indicated more abundant targets were available for therapeutic antibodies both in the tumor and in the spleen after combination treatment.

**Anti-4-1BB/anti-PD-1 treatment promoted antitumor immune response in the tumor microenvironment**

In order to provide further evidence of a localized antitumor immune response, TILs were isolated from B16F10 tumors 3 days after the 2\(^{nd}\) dose of antibodies and an immune-cell subset analysis was performed by flow cytometry. The combination treatment enriched CD8\(^+\) T cells with an 8-fold increase in the CD8\(^+/\)CD4\(^+\) ratio over the isotype group (p < 0.0001) (Fig. 3A). Treg abundance was not altered (Fig. 3B). However, the ratio of CD8\(^+\)/Treg was increased by 7-fold in response to the combination over the isotype treatment (p < 0.0001) (Fig. 3C). In agreement with published results (9), we observed the induction of KLRG1 on approximately 30% of the CD8\(^+\) T cells in the anti-4-1BB alone and combination groups (p < 0.001 vs. isotype) (Fig. 3D).

In addition, we evaluated the genes associated with antitumor immune responses in B16F10 tumor samples by quantitative PCR. The relative quantity (RQ) of each target gene was related to the average of the PBS control samples and calculated by the ddCt method using GAPDH and actin housekeeping genes. Compared to the PBS group, combination treatment led to an enrichment of signals for CD3\(\varepsilon\) (p < 0.001), CD8\(\alpha\) (p < 0.001), IFN\(\gamma\) (p < 0.001), Eomes (p < 0.001), 4-1BB (p < 0.05), PD-1 (p = 0.0001), PD-L1 (p < 0.001), and FoxP3 (p < 0.05) (Fig. 4). No significant changes were noted for CD4 expression. The profile of mRNA enrichment
observed here and in MC38 tumors (Supplementary Fig. S3) is largely congruent with the flow cytometric findings in the B16F10 tumors, supporting a strong enhancement of CD8 effector/memory cell phenotype induction by the anti-4-1BB/anti-PD-1 combination.

**Anti-4-1BB/anti-PD-1 combination induced T-cell effector/memory differentiation**

To explore modulation of the immune system systemically following anti-4-1BB/anti-PD-1 treatment, splenocytes were isolated from tumor-bearing mice and detailed phenotypic analysis of the immune cells was performed by flow cytometry. One of the characteristics of 4-1BB activation is to induce the expression of eomesodermin (Eomes), a transcription factor associated with T-cell effector/memory differentiation (9, 26). We detected Eomes expression on 64% of CD8⁺ T cells in the anti-4-1BB-treated animals and on 69% of CD8⁺ T cells in the anti-4-1BB/anti-PD-1 combination-treated animals, but only on 19% of CD8⁺ T cells in the isotype-treated animals (p < 0.0001 vs. isotype control) (Fig. 5A). Anti-PD-1 treatment alone did not alter Eomes expression (18% Eomes⁺CD8⁺ T cells). CD4⁺ T cells from control groups (PBS or isotype) did not express Eomes (< 1%), while expression was increased to 2.8% of CD4⁺ T cells in the anti-4-1BB-treated group, and further increased to 8.3% of CD4⁺ T cells in the anti-4-1BB/anti-PD-1 combination group (p < 0.0001 vs. isotype group) (Fig. 5B).

Memory is the hallmark of the acquired immune system. Thus, we characterized the memory phenotype of T cells at the end of a B16F10 efficacy study. The combination treatment significantly increased both the central memory (Tcm, CD44⁺CD62L⁺) (p < 0.0001 vs. PBS control) and effector memory (Tem, CD44⁺CD62L⁻) (p < 0.0001 vs. PBS control) CD8⁺ T cells in the spleen (Figs. 5C-D). CD8⁺ Tem subset increase was also observed in the MC38 model (Supplementary Figure S4).
Anti-4-1BB/anti-PD-1 treatment generated antigen-specific CTL response

To determine if anti-4-1BB/anti-PD-1 treatment induced an antigen-specific immune response, we isolated splenocytes from MC38 tumor-bearing mice after treatment and stimulated with the CD8-restricted MC38 immunodominant peptide KSTPFTTL ex vivo. IFNγ production assayed by ELISpot was evaluated as a measurement of response. Anti-4-1BB treatment increased the number of IFNγ-producing cells (250 ± 36) compared to that of the PBS group (117 ± 28) (p < 0.05) (Fig. 6). Anti-PD-1 treatment also increased IFNγ production (242 ± 46, p < 0.05 vs. PBS group). The combination of anti-4-1BB and anti-PD-1 further increased the numbers of peptide/tumor-specific T cells to 377 ± 24 and the increase was statistically significant as compared to those treated with either PBS (p < 0.0001), anti-4-1BB alone (p < 0.05), or anti-PD-1 alone (p < 0.05). The data indicated that the combination elicited a greater antigen-specific CTL response than either single agent alone.

Anti-4-1BB/anti-PD-1 combination immunotherapy resulted in moderate liver enzyme elevation in mice compared to anti-4-1BB alone

To understand the safety profile of the anti-4-1BB/anti-PD-1 combination therapy, we tested these antibodies either within or above their respective therapeutic dose range in non-tumor-bearing mice. C57BL/6 mice were administered a single subcutaneous injection of PBS, anti-4-1BB alone (at 0.1 mg/kg, 1 mg/kg or 5 mg/kg), anti-PD-1 alone (at 20 mg/kg), and three combination treatment groups consisting of 20 mg/kg anti-PD-1 with each of the three dose levels of anti-4-1BB listed above. Ten days after dosing, the study was terminated and tissue, blood, and serum collected for clinical and anatomic pathology assessment.
Of the clinical chemistry pathology measured, only those associated primarily with liver toxicity and certain peripheral blood subsets were significantly affected. The groups treated with 1 or 5 mg/kg anti-4-1BB alone demonstrated increases in ALT of 6- (p < 0.01) and 5-fold (p < 0.05), respectively, compared to the PBS group (Fig. 7A). The combination groups with anti-PD-1 had slightly greater increases in ALT of 10- (p < 0.0001) and 8-fold (p < 0.0001), respectively, compared to the PBS group. GLDH potentially offers differential diagnostic potential in the investigation of liver disease, particularly when interpreted in conjunction with other hepatic enzyme test results. It is more concentrated in the central areas of the liver lobules than in the periportal zones and is usually only released from necrotic cells due to its exclusive mitochondrial localization (27). The results of the GLDH measurements were similar to those of ALT, with increases in the 1, and 5 mg/kg anti-4-1BB single agent and combination groups of 4- (p < 0.001), 3- (p < 0.01), 6- (p < 0.0001), and 5-fold (p < 0.0001), respectively, relative to the PBS group (Fig. 7B). While the difference between the combination and the corresponding anti-4-1BB alone groups was statistically significant (Figs. 7A-B), the magnitude of difference was small (~50 % increase in combination versus single agent for either ALT or GLDH). No effects on ALT or GLDH were observed in the other groups. Aspartate aminotransferase (AST) followed a similar pattern as the other liver enzyme markers (Fig. 7C).

Several hematology parameters were affected by the study treatments. There were decreases in platelet counts in the 1 and 5 mg/kg anti-4-1BB alone (p < 0.001 and p < 0.0001 respectively vs. PBS) and combination with anti-PD-1 groups (p < 0.0001 vs. PBS) (Fig. 7D). While there was a statistically significant decrease in platelets in the combination groups relative to the corresponding anti-4-1BB single agent groups (p < 0.05 1 mg/kg anti-4-1BB/anti-PD-1 vs. 1 mg/kg anti-4-1BB alone, and p < 0.01 5 mg/kg anti-4-1BB/anti-PD-1 vs. 5 mg/kg anti-4-1BB
alone), the magnitude of the additional increase was quite modest (roughly 15%). No effects on platelets were observed in the other treatment groups. The 5 mg/kg anti-4-1BB alone and anti-PD-1 combination at 1 and 5 mg/kg anti-4-1BB showed dramatic lymphocyte decreases (~75%) (p < 0.0001 vs. PBS), while the decrease in other groups ranged from 18-35% relative to the PBS group (Fig. 7E). Not only is the difference between the combination at 1 mg/kg anti-4-1BB and the 1 mg/kg anti-4-1BB alone statistically significant (p < 0.0001), but further decrease of lymphocyte count by 55% (combination vs. 4-1BB alone) may be biologically significant. Only the 5 mg/kg anti-4-1BB alone group showed a statistically significant decrease in neutrophils (p < 0.001 vs. PBS), and addition of anti-PD-1 appeared to block the effects of anti-4-1BB on neutrophil decrease (Fig. 7F). Total white blood cells were also affected by the treatments, but this change was dominated by the lymphocyte effects (data not shown).

Among the tissues examined histopathologically, treatment-related findings were only observed in the liver and spleen. In the liver (Supplementary Table S1A), treatment with anti-4-1BB at 1 and 5 mg/kg led to an increased incidence and severity of mixed cell infiltrate and caused single cell necrosis that ranged from minimal to moderate in severity. Addition of anti-PD-1 did not cause additional effects. Mixed cell infiltration was present with minimal severity and single cell necrosis was absent in all other treatment groups. In the spleen (Supplementary Table S1B), the incidence of lymphoid hyperplasia was increased in all antibody-treated groups. The severity increased from minimal in the PBS group to minimal to mild in all other groups except the 5 mg/kg anti-4-1BB/anti-PD-1 combination group that was mild to moderate. The incidence and severity of extramedullary hematopoiesis was increased in the 1 and 5 mg/kg anti-4-1BB combination groups, but not significantly altered in all other groups.
In order to assess potential toxicity in tumor-bearing animals, mice with established B16F10 tumors were treated with isotype (20 mg/kg), anti-4-1BB (1 mg/kg), anti-PD-1 (20 mg/kg), or the anti-4-1BB (1 mg/kg) / anti-PD-1 (20 mg/kg) combination. Antibody treatment, tissue collection and data analysis were performed similarly to that of tumor-free mice. There were no statistically significant changes in the absolute number of peripheral platelets, lymphocytes and neutrophils among all groups. ALT was elevated in the combination group (p < 0.05 vs isotype) (Supplementary Figure S5). However, the AST levels were highly variable and greatest in the control group, which prevented us to evaluate its changes in response to treatments (data not shown). The histopathology of the spleen and liver after treatments displayed similar patterns as described in the tumor-free mice (data not shown).

**Discussion**

T-cell activation is regulated through effective coordination of costimulatory signals provided by cell surface co-regulatory receptors in addition to the antigen-specific TCR signaling (28). Agonistic antibodies directed against activating receptors and blocking antibodies to inhibitory receptors may enhance T-cell stimulation and are under active investigation for cancer therapy (7, 29). Blockade of the suppressive signals via anti-CTLA-4, anti-PD-1, and anti-PD-L1 agents has shown clinical benefit (3). Dual blockade of immunosuppressive pathways via anti-CTLA-4 and anti-PD-1 or via anti-PD-1 and anti-LAG-3 has entered clinical trials (3, 4). Modulation of stimulatory signals by 4-1BB, OX40 or GITR pathway is still in the early stages of clinical evaluation (30). In preclinical studies, combinations of immunomodulators as well as in combination with chemotherapy, targeted agents, vaccination or irradiation, have been evaluated in various models (15-18, 31-33). Here we showed that the combination of anti-4-1BB with anti-PD-1 synergistically inhibited the growth of B16F10 melanoma and MC38 colon carcinoma in
syngeneic C57BL/6 mice, and that the combination was reasonably well tolerated, supporting the clinical development of an anti-4-1BB/anti-PD-1 combination immunotherapy.

Anti-4-1BB therapy demonstrated promising but limited clinical benefits in early clinical trials (34). Potential mechanisms of 4-1BB agonistic antibody-mediated tumor regressions include breaking of immunologic ignorance and prevention of T-cell deletion and anergy (5, 35, 36). Given that 4-1BB is specifically expressed on antigen-experienced T cells (7, 37), the observed increase in 4-1BB+ T cells may implicate an enhanced immune response to tumor challenge in anti-4-1BB-treated animals. We and others have shown that 4-1BB activation up-regulated PD-1 expression on effector T cells, and the PD-1/PD-L1 axis has been reported to confer tumor resistance to 4-1BB co-stimulatory therapy (38). In addition, PD-L1 was induced in anti-4-1BB/anti-PD-1-treated tumors in vivo (Fig. 4 and Supplementary Fig. S3) as well as IFNγ-treated cells in vitro (data not shown and (38)). Therefore co-treatment of anti-4-1BB with anti-PD-1 represents a mechanistically rational combination to maximize the antitumor potential. This hypothesis is further supported by directly comparing different combinations in the same model. Here we showed that anti-4-1BB/anti-PD-1 combination was more potent than anti-PD-1/anti-LAG-3 combination in suppression of both small and large B16F10 tumors (Fig. 1). The combination of anti-4-1BB with anti-PD-1 was also more efficacious than dual combinations of anti-CLTA-4/anti-PD-1 and anti-4-1BB/anti-CTLA-4 in the ID8 model (16, 17). Therefore, in at least two of the most aggressive and poorly immunogenic tumor models (ID8 and B16), anti-4-1BB/anti-PD-1 combination appears to exhibit the strongest antitumor effect among the dual-agent combinations of several widely studied pathways (CTLA-4, PD-1, LAG-3, and 4-1BB).

Previous work has implicated that the T-box transcription factors Eomes and T-bet act as master regulators in CD8+ T-cell effector and memory differentiation and function (26, 39-41). Recently,
Eomes has been shown to be required for the antitumor immunity mediated by 4-1BB agonist immunotherapy (42). 4-1BB activation was able to induce Eomes expression in tumor-infiltrating T cells without affecting T-bet expression, and the high expression of Eomes promoted the development of novel KLRG1+ Eomes+ T-cell subsets characterized by enhanced, multipotent cytotoxicity (9). We also showed here that 4-1BB stimulation increased Eomes on splenic CD8+ T cells and to a lesser degree on CD4+ T cells, while anti-PD-1 alone had no effect. Consistent with the role of Eomes in T-cell polarization, we found the accumulation of CD8+ effector and central memory T cells in the spleen of tumor-bearing mice after anti-4-1BB/anti-PD-1 treatment. Furthermore, these CD8+ T cells were functionally active, and produced large amounts of IFNγ upon tumor antigen-specific re-stimulation ex vivo. The loss of antitumor protection when CD8+ T cells were depleted further supported their pivotal role in antitumor immunity. In agreement with the essential role of IFNγ for 4-1BB agonistic activity (43), the combination also required IFNγ as the antitumor activity was abolished in IFNγ-deficient mice (Fig. 2A). Durable efficacy is likely to occur given that the combination treatment induced tumor antigen-specific and memory phenotype T cells. In the tumors, we observed signals associated with antitumor activity, such as increased CD8+/Treg ratio and induced KLRG1 on CD8+ T cells. By gene expression analysis, we were able to demonstrate that an active antitumor immune response was induced with increases in the expression of mRNA encoding CD8α, IFNγ and Eomes. Unlike published data (15, 17), we did not observe changes in myeloid-derived suppressor cells (MDSCs) in response to treatment (data not shown).

Finally, the combination of anti-4-1BB and anti-PD-1 antibodies was well tolerated in mice. There were no clinical signs of toxicity or mortalities from either of the single agents or the combination; and anti-PD-1 alone caused only minimal decreases in peripheral lymphocytes and
splenic lymphoid hyperplasia. The effects in the combination arms, which included liver mixed
 cell infiltration and single cell necrosis with associated increases in plasma liver enzymes,
 splenic lymphoid hyperplasia and extramedulary hematopoiesis, and decreases in peripheral
 white blood cell subsets and platelets, were driven predominantly by the anti-4-1BB antibody.
 These findings were observed with similar severity in the anti-4-1BB-alone treatment arms and
 have been described previously with another mouse-reactive anti-4-1BB (44). The human
 relevance of the mouse findings is apparent in a previous report listing neutropenia and
 transaminitis as two of the most common adverse events in a Phase 1 trial of a 4-1BB agonist
 antibody in cancer patients (45). The most notable combination effect in the mouse study was the
 synergistic decrease in peripheral lymphocytes observed at the 1 mg/kg dose of anti-4-1BB in
 combination with anti-PD-1. This observation likely represents the desired combined
 pharmacologic effect of lymphocyte activation and tissue sequestration.

 Taken together, the pharmacologic effects of the anti-4-1BB/anti-PD-1 combination demonstrate
 a much greater synergy with respect to the antitumor efficacy than toxicity in syngeneic models
 with an intact immune system. This preclinical study provides a strong rationale for developing
 anti-4-1BB/anti-PD-1 combination immunotherapy in human patients. A study of 4-1BB agonist
 PF-05082566 plus PD-1 inhibitor MK-3475 in patients with solid tumors
 (B1641003/KEYNOTE-0036) has been proposed (clinicaltrials.gov). The cellular phenotypes
 and immune mechanisms underlying the synergism as we delineated here also provide a
 framework for patient biomarker investigation in combination clinical trials.
References

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Figures

Figure 1. 4-1BB activation and PD-1 blockade synergistically amplify the antitumor effects in B16F10 melanoma and MC38 colon carcinoma models.

C57BL/6 mice were inoculated s.c. (day 0) with (A-C) $1 \times 10^6$ B16F10 or (D) $1 \times 10^6$ MC38 cells. (A-B) After tumors were established, mice were randomized into groups of 10 animals per group with an average tumor volume of $\sim 85 \text{ mm}^3$ (range 50 – 154 mm$^3$) on day 9. Animals were administered intraperitoneally rat IgG2a (isotype control, 10 mg/kg), anti-4-1BB (1 mg/kg), anti-PD-1 (10 mg/kg) or anti-LAG-3 (10 mg/kg) mAbs in a volume of 200 $\mu$L, alone or in combinations as indicated in the figure, on days 9 and 14 (indicated by arrows). (C) When B16F10 tumor volume reached 126-350 mm$^3$ (day 10), mice were randomized into groups of 10 animals per group and treated with rat IgG2a, anti-PD-1/anti-LAG-3 or anti-4-1BB/anti-PD-1 mAbs every 3 days for a total of 4 doses (indicated by arrows). (D) MC38 tumor-bearing animals (8 mice per group) with an average tumor volume of $\sim 140 \text{ mm}^3$ (day 10) were treated with anti-4-1BB and anti-PD-1 mAbs i.p. on days 10 and 15 (indicated by arrows). Tumor size was measured 2-3 times a week. Mean ± SEM of each treatment group is shown in (A), (C) and (D). (B) represents the individual tumor growth over time for plot (A). Statistics were generated using two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ when comparing groups as indicated by the vertical lines.

Figure 2. CD8$^+$ T cells were immunomodulated and required for the efficacy of anti-4-1BB/anti-PD-1 combination treatment.

Wild-type C57BL/6 or IFN$\gamma$-deficient mice were inoculated s.c. (day 0) with $1 \times 10^6$ B16F10. Mice were randomized into groups of 10 animals per group with an average tumor volume of
~80 mm³. (A) Mice were randomized on day 8, and anti-4-1BB (1 mg/kg)/anti-PD-1 (10 mg/kg) mAbs were dosed on days 8, 11 and 14 (indicated by arrows). (B) Mice were randomized on day 10. One hundred (100) µg of isotype control IgG or anti-CD8 mAb was administered i.p. on days 11, 16, and 21 (indicated by blue arrows). Anti-4-1BB (1 mg/kg)/anti-PD-1 (10 mg/kg) mAbs were dosed on days 12, 17 and 22 (indicated by black arrows). Tumor size was measured 2-3 times a week. Mean ± SEM of each treatment group is shown. (C-F) Spleens and tumor fragments from B16F10 tumor-bearing mice after antibody treatment (Fig.1A and supplementary Fig. S1) were harvested and disaggregated into a single-cell suspension. After immunostaining, cells were analyzed by flow cytometry for the expression of CD3, CD4, CD8, PD-1 and 4-1BB. The expression of PD-1 on splenic and TIL CD8⁺ T cells is shown in (C) and (D), respectively. The expression of 4-1BB on splenic and TIL CD8⁺ T cells is shown in (E) and (F), respectively. Each symbol represents an individual animal within the same treatment group. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001 when comparing groups as indicated by the horizontal lines.

**Figure 3. Anti-4-1BB/PD-1 combination enriched CD8⁺ T cells in TILs.**

Tumor fragments were harvested from B16F10 tumor-bearing mice after antibody treatment and disaggregated into a singlecell suspension. After surface staining, cells were fixed and permeabilized for intracellular marker staining, and finally analyzed by flow cytometry for the expression of CD3, CD4, CD8, CD25, KLRG1, and Foxp3. (A) the ratio of CD8⁺ to CD4⁺ T cell; (B) the frequency of Treg (CD4⁺CD25⁺Foxp3⁺); (C) the ratio of CD8⁺ T cells to Treg; and (D) the expression of KLRG1 on CD8⁺ T cells. *** p < 0.001 and **** p < 0.0001 when comparing groups as indicated by the horizontal lines.
Figure 4. Anti-4-1BB/PD-1 combination enriched genes associated with antitumor immune response.

B16F10 tumor samples were collected after antibody treatment and total RNA was isolated. Gene expression from each treatment group was calculated using real-time PCR analysis with GAPDH and Actin as the endogenous controls. Column dot plots are shown for the genes of CD3ε, CD8α, IFNγ, Eomes, PD-1, PD-L1, 4-1BB, CD4, and FoxP3. Except for 4-1BB expression, the relative expression for all other genes is shown in log scale due to the great variance within the group. Each symbol represents an individual animal within the same treatment group. * p < 0.05, ** p < 0.01 and *** p < 0.001 when comparing groups as indicated by the horizontal lines.

Figure 5. Anti-4-1BB/PD-1 combination induced T-cell effector/memory differentiation.

At the end of efficacy studies, spleens from B16F10 tumor-bearing mice were harvested and disaggregated into a single-cell suspension. After surface staining, cells were fixed and permeabilized for intracellular marker staining, and finally analyzed by flow cytometry for the expression of CD3, CD4, CD8, CD44, CD62L, and Eomes. The expression of Eomes in CD8^+ and CD4^+ T cells are shown in (A) and (B), respectively. The percentages of central memory (CD44^+ CD62L^+) and effector memory (CD44^+ CD62L^-) CD8^+ T cells are shown in (C) and (D), respectively. Each symbol represents an individual animal within the same treatment group. ** p < 0.01, *** p < 0.001 and **** p < 0.0001 vs. PBS or rat IgG2a.

Figure 6. Anti-4-1BB/PD-1 combination enhanced antigen-specific T-cell response.

At the end of the efficacy study as described in Figure 1D, splenocytes were isolated and fresh frozen. Viable cells after thaw were rested for 12 hr and challenged with CD8-restricted MC38
immunodominant peptide KSTPFTTL ex vivo. IFNγ production was assessed by ELISpot. The number of spots, an indication of the number of IFNγ-producing cells, was reported. Each symbol represents an individual animal within the same treatment group. * p < 0.05 and **** p < 0.0001 when comparing groups as indicated by the horizontal lines.

Figure 7. Serum liver enzyme levels and hematology alterations in response to 4-1BB activation therapy in normal mice.

Serum samples collected 10 days following a single administration of treatments to C57BL/6 mice (n = 5/group) were analyzed for the amount of (A) alanine aminotransferase (ALT), (B) glutamate dehydrogenase (GLDH) or (C) aspartate aminotransferase (AST). Whole blood samples were subjected for (D) platelet counts, (E) lymphocyte counts, or (F) neutrophil counts. The anti-4-1BB-0.1, anti-4-1BB-1 and anti-4-1BB-5 represent the 0.1, 1 and 5 mg/kg of anti-4-1BB alone groups, respectively. Anti-PD-1-20 represents the group of anti-PD-1 treatment at 20 mg/kg. The combo-0.1, combo-1 and combo-5 represent the groups of 0.1, 1 and 5 mg/kg anti-4-1BB mAb in combination with 20 mg/kg anti-PD-1 mAb. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001 when comparing groups as indicated by the horizontal lines.
Figure 1
Figure 2
Figure 3

(A) Ratio CD8^+ / CD4^+

(B) % CD25^+Foxp3^+ / CD4^+

(C) Ratio CD8^+ / T_reg

(D) % KLRG1^+ / CD8^-

Legend:
- Rat IgG2a
- Anti-4-1BB
- Anti-PD-1
- Anti-4-1BB/anti-PD-1

Significance levels:
- **** p < 0.0001
- *** p < 0.001
Figure 5
Figure 6

Number of spots

PBS
Anti-4-1BB
Anti-PD-1
Anti-4-1BB/Anti-PD-1

0 100 200 300 400 500
Figure 7
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Combination of 4-1BB Agonist and PD-1 Antagonist Promotes Anti-tumor Effector / Memory CD8 T cells in a Poorly Immunogenic Tumor Model

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