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Contributed Mini Review

4-1BB (CD137), an inducible costimulatory receptor, as a specific target for cancer therapy

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Although considerable progress has been made in understanding how tumors evade immune surveillance, measures to counter the same have not kept pace with the advances made in designing effective strategies. 4-1BB (CD137; TNFRS9), an activation-induced costimulatory molecule, is an important regulator of immune responses. Targeting 4-1BB or its natural ligand 4-1BB ligand (4-1BBL) has important implications in many clinical conditions, including cancer. In-depth analysis revealed that 4-1BB-mediated anti-cancer effects are based on its ability to induce activation of cytotoxic T lymphocytes (CTL), and among others, high amounts of IFN-γ. In this review, we will discuss the various aspects of 4-1BB-mediated anti-tumor responses, the basis of such responses, and future directions. [BMB Reports 2014; 47(3): 122-129]

INTRODUCTION

Cancer remains one of the leading causes of death in the world. Recent studies have shown an estimated 12.7 million cancer cases worldwide, which affect both sexes equally. This number is expected to increase to 21 million by 2030. Cancers are mainly caused by the rapid spread and mutation of certain cells of the body. Although considerable knowledge has accumulated of how tumors evade immune surveillance, effective cancer therapies are still a daunting task for the clinician. This is mainly due to two reasons: poorly immunogenic tumors, and evasion of immune surveillance by the tumors. Moreover, tumors use multiple mechanisms to bypass immune surveillance, and many of these mechanisms are mediated at the cellular or molecular levels.

Since most tumors are killed by cytotoxic T lymphocytes (CTL) in an antigen-specific manner, agents that propel $CD8^+$ T cell activation and impart strong cytolytic, inflammatory, immune regulating properties, and antigen-specificity are therefore ideal candidates to enhance anti-tumor immunity. Immunotherapy targeting $CD8^+$ T cells by agonistic anti-4-1BB (CD137) monoclonal antibody (mAb) aptly fits these requisites. 4-1BB, a member of the tumor necrosis factor receptor superfamily T cell costimulatory receptor, is induced, when T cells receive antigen-specific signals. Among the various animal models studied to date, 4-1BB signaling was the most investigated in tumor models (1). Recently, anti-4-1BB has entered clinical trials, and results thus far show favorable toxicity. Here, we discuss the characteristics of 4-1BB, and its role as a powerful anti-cancer agent, the mechanistic basis of such action, and future direction.

4-1BB: DISCOVERY AND PHENOTYPE

4-1BB was originally discovered in the late 80s from activated cells (2), and on account of this, it was originally referred to as induced lymphocyte activation (ILA) in humans (3), and as 4-1BB in mouse (4). However, later studies showed that 4-1BB is constitutively on a number of cells, albeit at low levels, including $Foxp3^+$ Tregs and DCs (2, 6, 7). Activation with a number of agonists, such as cytokines (IL-2, IL-4), polyclonal activators (Con A and PHA), and cell surface molecules (anti-CD3 and anti-CD28), and promoters of $Ca2^+$ induction and PKC activity (ionomycin and photbol myristate acetate), further enhance the expression of 4-1BB (2, 6, 7). 4-1BB is completely cloned, and its protein sequence is documented, revealing that it exists both as a 30kDa monomer, and as a 55 kDa dimer (5).

BIOLOGICAL EFFECTS OF 4-1BB SIGNALING

Numerous studies have established that signals via 4-1BB are costimulatory in nature (8, 9). Further analysis revealed that although both $CD4^+$ and $CD8^+$ T cells express 4-1BB at comparable levels, upon activation, signals through 4-1BB are more biased toward $CD8^+$ T cells, both *in vitro* (10), and *in vivo* (11, 12). The mechanistic basis of such $CD8^+$ T cell

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biased 4-1BB signaling is yet to be elucidated in detail. Despite this functional disparity, the 4-1BB$^{-/-}$ and 4-1BBL$^{-/-}$ develop with normal cellularity, and reveal no functional or CD8$^+$ biased defects (13, 14).

Interestingly, *in vivo* administration of agonistic anti-4-1BB leads to the deletion of a number of cells, including B, NK, and CD4$^+$ T cells, while promoting CD8$^+$ T cell expansion, yet offering protection against several pathological conditions, including autoimmunity, cancer, and transplantation (11, 15-18) (Fig. 1). The reasons underlying the *in vitro* vs. *in vivo* functions of anti-4-1BB are currently unknown. Despite this, 4-1BB has emerged as a strong activator of immune cells, and as an important candidate against various diseases (3, 8, 9).

EXPRESSION OF 4-1BB ON TUMOR CELLS, AND IN THE SERA OF CANCER PATIENTS

A number of studies have shown that 4-1BB is also expressed on a wide range of tumor cells, including SPC-A-1, H446, H460, and H1299 (19). Expression of 4-1BB in cancer patients was found also on tumor vessel walls, on the endothelial wall, on vascular smooth muscles (20), and in liver tissue (21). Interestingly, constitutive and functional expression of 4-1BB was also noted on leukemic cell lines (22), and in some cases, such expression has been known to curb T cell activation (23), and is therefore considered as a possible anti-tumor agent (24). Like certain members of the TNFR superfamily, 4-1BB and 4-1BBL also exist in soluble forms in the sera of patients suffering from certain clinical conditions, including cancer. There are reports to suggest that soluble 4-1BBL possesses activation potential (25).

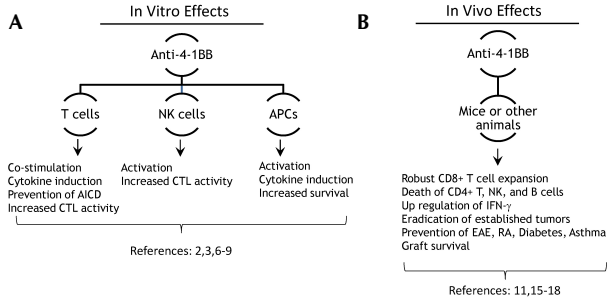

Fig. 1. Biological effects of 4-1BB signaling. 4-1BB signaling is well established in a number of cell types, including T and NK cells, and APCs. Signaling via 4-1BB by anti-4-1BB activates various immune competent cells, leading to activation, cytokine induction, prevention of activation-induced cells death (AICD), upregulation of CTL activity, and increased survival (A). *In vivo* administration of 4-1BB into mice or other animals has various consequences, including a robust activation of CD8$^+$ T cells, eradication of established tumors, prevention of autoimmune diseases, and increased graft survival (B). The numbers in parentheses denote the references highlighting the individual action of 4-1BB signaling.

ANTI-4-1BB mAbs AS ANTI-CANCER AGENTS

Since the pioneering study of Melero *et al*. (26), who first showed that *in vivo* administration of agonistic anti-4-1BB mAb has potent anti-tumor properties against both poorly immunogenic Ag104 A sarcoma and highly immunogenic P815 mastocytoma, several investigators have since corroborated the anti-tumor effects of 4-1BB. It is interesting to note that studies of 4-1BB are far more investigated in cancer, than in other pathological conditions (1). 4-1BB therapy alone, or in combination with other agents, gained widespread recognition, due to its/their strong anti-tumor properties. For example, anti-4-1BB mAb, co-injected with semi-allogenic DCs in MC38-bearing mice, resulted in the regression of these poorly immunogenic MC38 tumors (27). Likewise, the combination of anti-4-1BB and IL-12, screened for their anti-tumor properties against B16-F10 melanoma, as well as pulmonary metastatic models, revealed a 50% survival rate in tumor-bearing mice (28); while elimination of NK cells, but not others, reversed the anti-tumor effect of anti-4-1BB/IL-12 (28), highlighting the importance of the anti-4-1BB/IL-12/NK cell axis in this pulmonary metastatic model. Although most of the anti-tumor effects of anti-4-1BB are dictated by CD8$^+$ T cells (1), a connection between 4-1BB and NK and NKT cells was proposed in P815-bearing mice, where *in vivo* depletion of NK or NKT cells completely removed the tumor suppressing ability of anti-4-1BB. Although addition of anti-hu4-1BB supported the proliferation of allo-stimulated cells *in vitro*, treatment with the same Ab failed to inhibit human xenografts in SCID mice (29). To understand which component was the target of *in vivo* anti-4-1BB therapy of cancer, several investigators conducted in-depth experiments in tumor-bearing lymphocyte-deficient mice. It was found that T cells (both CD4$^+$ and CD8$^+$ T cells) are critical for *in vivo* anti-tumor effects against MCA 205 sarcoma or GL261 glioma cells, as their depletion in wild-type mice, or experiments in SCID mice, reversed the anti-tumor effects of 4-1BB (30). Others have also confirmed the above finding, where depletion of Ag-specific CTLs failed to stop the growth of C3 tumors, TC-1 lung carcinoma, and B16.F10 melanoma, despite anti-4-1BB therapy (31). Further analysis revealed that anergy, but not deletion of tumor Ag-specific CTLs, was responsible for the failure of anti-4-1BB anti-tumor effects (31). That CD8$^+$ T cells are required for anti-4-1BB-mediated suppression of P1A-expressing J558 cells in RAG2$^{-/-}$ mice was revealed, when these tumor bearing mice were infused with CD8$^+$ T cells and anti-4-1BB, and showed delayed tumor growth and enhanced survival (32). DCs have been shown to play a vital role in anti-4-1BB-mediated anti-tumor immunity (33), as their removal eliminated the efficacy of anti-4-1BB (34). The powerful anti-cancer agent 5-flurouracil (5-FU), which works through inhibiting thymidylate synthase, when combined with anti-4-1BB, but nor individually, led to the inhibition of established tumors in more than 70% of mice (35). A role for adhesion molecules was implicated in the anti-tu-

mor effects of 4-1BB. Palazzo *et al*. (36) have demonstrated that upregulation of ICAM-1 and VCAM-1 on tumor endothelial cells, following anti-4-1BB administration, increased T cell migration into tumor site. These results were further verified by treating mice with inhibiting Abs to ICAM-1 and VCAM-1, which showed reversal of anti-4-1BB anti-tumor effects (36), indicating that *in vivo* anti-4-1BB-mediated anti-tumor effects stretch beyond T and non-T cells.

Cell-based immunotherapy, also called adoptive T cell therapy (ACT), continues to be an important avenue for cancer patients who fail standard treatments. A number of reasons account for this. First, the specificity, avidity, and effector functions of infused cells can be precisely defined. Although significantly improved and streamlined, ACT still faces significant hurdles (37). Loss of important cell surface bound activation molecules, the longevity and durability of transferred cells, and activation-induced cell death (AICD) are some of the factors that are known to affect the success of ACT. ACT, when combined with anti-4-1BB therapy, has been shown to overcome some of these hurdles. It was observed that *ex vivo* amplification of isolated tumor infiltrating lymphocytes (TILs) results in the down-regulation of certain key surface molecules, and upregulation of 4-1BB (38). Interestingly, ligation of 4-1BB in these TILs by anti-4-1BB not only prevented AICD, but also augmented their proliferative potential, and enhanced their cytolytic activity against melanoma cells (38). Many studies have shown that anti-4-1BB-mediated tumor suppressive effects are not limited to $CD4^+$ T or $CD8^+$ T cells. There is enough evidence to suggest that anti-4-1BB also stimulates the cytolytic function of NK cells, as revealed by their ability to kill A549 tumor cells (39). Likewise, the cytokine-induced killer (CIK) cells, developed *in vitro* with a combination of anti-CD3, IL-2, and IFN-γ, acquire potent anti-cancer properties (40, 41); and when co-administered with anti-4-1BB into A549 tumor cells-bearing SCID mice, showed reduced mortality (39). Further studies revealed that the success of anti-4-1BB therapy against A549 tumor cells, in the above study, was due to the increased expression of IL-2, IFN-γ and TNF-α in the activated CIK cells (39). These data strongly suggest that anti-4-1BB has many potential targets *in vivo*, whose stimulation results in augmented tumor eradication.

VARIANTS OF ANTI-4-1BB AS ANTI-TUMOR AGENTS

In addition to the anti-cancer effects of anti-4-1BB Abs, targeting the 4-1BB receptor with variants of the 4-1BB molecule has also shown promise. A large proportion of carcinomas express surface mesothelin (42), and T cells engineered to express a single chain variable fragment that binds mesothelin fused with the T cell zeta (ζ) chain, CD28, and 4-1BB, showed enhanced persistence, and decreased the tumor burden, when transferred into NOD/SCID/IL-2γ-/- mice carrying pre-established human primary M108 tumors (43). T cells from umbilical cord blood (UCB) could kill leukemia and lymphoma cells *in vitro*, when equipped with a single chain chimeric antigen-receptor (CAR), carrying the intracellular domain of the CD3ζ chain and 4-1BB (44). On the other hand, *in vivo* infusion of these engineered UCB T cells into human Daudi lymphoma tumor-bearing SCID mice showed only marginal (but not significant) survival rates over control group (44). Human T cells engineered to express a chimeric immune receptor (CIR) specific for folate receptor-alpha (FRα), had strong anti-tumor activity against epithelial cancers *in vitro*, but not *in vivo*, due mainly to their short lifetimes, and inability to migrate to tumor sites. Song *et al*. (45) devised a strategy to overcome this problem: they modified the CIR containing a FRα-specific scFV (MOv19), by coupling it to the TCR CD3ζ chain signaling molecule, either alone (MOv19-ζ), or in combination with the CD137 (4-1BB) costimulatory motif (MOv19-BBζ). Although both modified CIRs induced *in vitro* tumor activity, only MOv19-BBζ elicited robust *in vivo* anti-tumor activity, when transferred into immune-deficient mice bearing established FRα human cancers (45). Careful examination revealed that the MOv19-BBζ -expressing human T cells survived longer, and were present within the tumors, suggesting that they homed efficiently. When a vector encoding a cell-bound single-chain Fv fragment from the anti-4-1BB mAb clone, 1D8, was transduced into mice harboring K1735 melanoma cells, and these were implanted into mice, they induced robust Th1 responses in a $CD4^+$ T- and NK cell-dependent manner (46). Collectively, these findings indicate that alternative ways of targeting 4-1BB for cancer treatment are available.

ANTI-4-1BB COMBINATION THERAPY WITH OTHER ANTI-CANCER AGENTS

A number of studies have demonstrated that anti-4-1BB Ab, when combined with other anti-cancer agents, can enhance anti-tumor activity. The B16.F10 melanoma-bearing mice, when treated with IL-12 gene transfer, or with anti-4-1BB alone, had no effect (47); however, when the two treatments were combined and administered, tumor reduction was observed in about 50% of the tumor-bearing mice, and their survival increased in a T- and NK cell-dependent manner, as cell depletion studies showed that elimination of $CD8^+$ T or NK cells, but not $CD4^+$ T cells, inhibited the anti-tumor activity of the combination therapy (47). Interestingly, repeated injections, as opposed to single injections, of DC engineered to secrete IL-12, resulted in significant suppression of CT26 colon carcinomas (48). Importantly, when this treatment for both spontaneous and established tumors was combined with anti-4-1BB mAb, the therapeutic effect was increased further (48). Ito *et al*. (49) showed that anti-4-1BB, when combined with vaccination with tumor cell lysate-pulsed dendritic cells (TP-DC), increased tumor regression, and enhanced the survival of tumor-bearing mice. Further studies showed that the combined therapy also resulted in improved local control of subcutaneous tumors, following surgical resection. Cell depletion

studies have revealed that $CD8^+$, $CD4^+$, and NK cells are involved in tumor regression, in the combination TP-DC/anti-4-1BB therapy (50). When mice with preexisting MC38 (murine adenocarcinoma), but not B16 melanoma, tumors were administered with antibodies to CTLA-4 and anti-4-1BB, significant $CD8^+$ T cell-dependent tumor regression was observed, together with long-lasting immunity to these tumors (50). Sin *et al*. (51) have recently demonstrated that a combination of anti-4-1BB antibodies with Trp2 peptides, in the presence of TLR9 agonists, increased the antitumor effect from 0% to 75%. In an orthotropic model of metastatic colon carcinoma established in the liver of mice, combination therapy with IL-12 and stimulatory anti-4-1BB led to $CD8^+$ T and NK cell-dependent tumor regression (52). Treatment with anti-4-1BB (Bristol-Myers Squibb (BMS)-469492) led to only modest regression of M109 tumors, but significantly delayed the growth of EMT6 tumors. On the other hand, BMS-469492, an anti-mouse agonist, therapy, combined with irradiation (single or multiple exposures), resulted in enhanced anti-tumor activity against the EMT6 tumors (53).

Pathological angiogenesis is an important aspect of cancers (54). Therefore, treatments directed against tumor-initiated angiogenesis have attracted attention in recent years, and anti-tumor strategies using endothelial cells (EC) to overcome tumor-induced angiogenesis have shown promise (55). Ko *et al*. (56) observed that significant inhibition of both B16.F10 and MC38 colon adenocarcinomas occurred, when EC therapy was combined with hybrid cells (DC), and with anti-4-1BB. Subsequent experiments revealed that the anti-tumor effects of EC/DC/anti-4-1BB were mediated by EC-specific $CD4^+$ and $CD8^+$ T cells (56). Li *et al*. (57) confirmed that anti-4-1BB, when complexed with other anti-tumor agents, was more effective, than when administered individually. These authors demonstrated that irradiated tumors engineered to produce granulocyte-macrophage colony-stimulating factor (GM-CSF), enhanced robust anti-tumor activity against established B16 tumors, when complexed with agonistic anti-4-1BB. Subsequent studies showed the existence of an elevated proportion of tumor antigen-specific $CD8^+$ T cells, in the GM-CSF/anti-4-1BB-treated mice. Interestingly, virotherapy, using a genetically engineered strain of oncolytic vaccinia virus, in conjunction with agonistic anti-4-1BB, significantly reduced the growth of established subcutaneous tumors, but not when injected individually (58). Chronic anti-4-1BB therapy is known to deplete $CD4^+$ T cells (14). Choi *et al*. (59) have shown a useful effect of $CD4^+$ T cell elimination on anti-4-1BB-mediated anti-tumor activity. These authors uncovered that treatment of B16.F10-bearing C57BL/6 mice, with either agnostic anti-4-1BB, or depletion with anti-CD4 mAbs, had no effect on tumor regression. However, when the two treatments were combined, significant tumor regression was observed (59). Examination of the mechanism involved revealed that massive expansion of novel IFN-γ producing $NKGD2^+KLRG1^+$ $CD11c^+CD8^+$ T cells was a key element of the anti-4-1BB/ anti-CD4-mediated tumor regression. In agreement with this, blockade of NKGD2 reduced the therapeutic effect by 20%-26% (59). Treatment of renal cell carcinomas (RCC) with agonistic anti-4-1BB or 5-FU had a negligible effect on tumor regression (35); whereas, the combination of 5-FU and anti-4-1BB eradicated established tumors, in more than 70% of mice. Further analysis revealed that this tumor regression in mice receiving the above combination therapy was correlated with increased numbers of lymphocytes in their spleens, and tumor-draining lymph nodes, and enhanced proportions of apoptotic cells (35). Furthermore, mice that had received the combination therapy rapidly rejected re-challenge with the same tumors, suggesting that long-lasting tumor-specific memory had been established (35). A recent study indicated that treatment of mice bearing B16 melanomas, which are poorly immunogenic (60), with cyclophosphamide (CTX) or anti-4-1BB was ineffective (61); whereas, the combined treatment resulted in significant anti-cancer effects. Further analysis showed that the efficacy of the combined therapy involved the production of large numbers of effector IFN-γ^+CD11c^+ $CD8^+$ T cells, which in turn were responsible for tumor suppression (61).

TUMOR DEVELOPMENT IN 4-1BB$^{-/-}$ MICE

The importance of the 4-1BB-4-1BBL pathway in cancer is further underscored, by studies with 4-1BB$^{-/-}$ mice. Treatment with B16.F10 melanoma cells increased the mortality of 4-1BB$^{-/-}$, but not 4-1BB$^{+/+}$ mice, and treatment of B16.F10-bearing 4-1BB$^{+/+}$ mice with agonistic anti-4-1BB Ab prolonged their survival, in a $CD8^+$ T cell- and IFN-γ-dependent manner (62). 4-1BB expression has been reported on follicular dendritic cells (63), and anti-4-1BB treatment affects FDC networks inhibiting T-dependent humoral responses (64), suggesting a role for this molecule in germinal center (GC) formation. Consistent with this, about 60% of 4-1BBL$^{-/-}$ mice develop B cell lymphomas by age 12 months (65). Further analysis revealed that this effect was associated with increased expression of, among others, Bcl-10, and the GC response regulators, Bcl-6, spi B, Elf-1, Bach-2, and activation-induced cytidine deaminase (65). Vinay *et al*. (66) have demonstrated that 4-1BB$^{-/-}$ mice have reduced NK cell numbers and activity. As a result, co-culture of spleen cells and tumor cells failed to lyse the latter. However, when the residual NK cells in 4-1BB$^{-/-}$ mice were isolated, pooled, and co-cultured with tumor cells, the latter were efficiently lysed, suggesting that the cytolytic activity of the residual NK cells in 4-1BB$^{-/-}$ mice is intact, and their inability to cause tumor lysis is attributable to suboptimal NK numbers (66). In an analogous study, Choi *et al*. (67) have examined the tumor reactivity of 4-1BB$^{-/-}$ mice, but in a $CD8^+$ T cell setting. These authors found that when 4-1BB$^{+/+}$ and 4-1BB$^{-/-}$ mice were treated with $CD8^+$ T cell sensitive tumors like MC38, EL4, CT26, and RENCA, the 4-1BB$^{-/-}$, but not the littermate wild type controls, showed significant suppression of tumors (67). To understand the under-

lying mechanisms of enhanced tumor suppression in 4-1BB$^{-/-}$ mice, Choi *et al*. (67) have depleted CD8$^+$ or NK cells, and found that tumor protection is significantly lost in both CD8$^+$ T and NK cell-depleted 4-1BB$^{-/-}$ mice, suggesting that NK cells play an important anti-tumor supporting role in CD8$^+$ T cell-mediated tumor suppression. These authors further pointed out that the enhanced NK numbers in the bone marrows of 4-1BB$^{-/-}$ may support the CD8$^+$ T cell function (67).

Taken together, several of the 4-1BB agonists show great potential for human cancer application. For example, BMS-666513, fully humanized mAb against 4-1BB, has completed phase I and II trials for its anti-cancer properties in patients with melanoma, renal cell carcinoma, and ovarian cancer patients (68). Results thus far suggest that the Ab therapy is well tolerated across various dose ranges (0.3 mg/kg-15 mg/kg body weight, given every three weeks). Biomarker analysis revealed elevated expression of IFN-inducible genes in peripheral blood, and in post-treatment biopsies of patients receiving BMS-666513 (68). However, 6%-15% of the patients developed grade 3 or higher neutropenia and liver enzymes, mild fatigue, rash, pruritis, diarrhea, and fever (68). It appears to be important to determine in future trials dose ranges of the anti-4-1BB that are safe and effective. Meseck *et al*. (69) have recently reported a recombinant human 4-1BB ligand fusion protein (hIg-hu4-1BBs) that showed the ability to activate human, as well as monkey T cells, *in vitro*. These results are encouraging, and future studies on human cancer subjects will determine its efficacy as a potent anti-cancer agent. Porter *et al*. (70) have recently shown that in patients with chronic lymphocytic leukemia (CLL), treatment with a low dose (1.5×10^5 cells/ kg body weight) of lentiviral vector, expressing a chimeric antigen receptor (CAR) specific for B-cell antigen CD19 linked to 4-1BB and CD3ζ chain, corresponded with a delayed occurrence of tumor lysis syndrome, and a complete remission of CLL (70). It is noteworthy that inclusion of endodomain of 4-1BB sequence enhanced the anti-tumor activities of the CAR, which might result in prolonging the functions of the T cells in the tumor microenvironment (43). The infused engineered cells not only self-replicated over 1000-fold, but persisted in higher numbers for six months in the blood and bone marrow, after the initial transfusion. However, hypogammaglobulinemia was routinely observed as a chronic side effect in these patients (70). The latter results (70) are promising, and thus far appear to have an edge over mAb therapy, as the infused cells self-replicated, survived for long periods, and more importantly, retained their CARs intact.

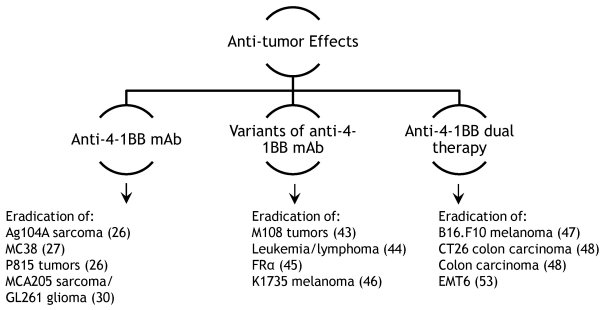

Fig. 2. Anti-tumor effects of 4-1BB. Targeting 4-1BB either by anti-4-1BB alone, or its variants, or in combination with other agents, has powerful anticancer properties. The numbers in parentheses are relevant literature highlighting the action of anti-4-1BB-mediated anti-cancer effects.

CONCLUSION

Taken together, the findings we have summarized clearly support the therapeutic potential of targeting the 4-1BB pathway in cancer treatment (Fig. 2). More importantly, the observation that targeting the 4-1BB pathway eliminates established tumors is an added advantage; and the fact that anti-4-1BB therapy acts in concert with other anti-cancer agents and/or radiation therapy, to eradicate non-immunogenic and weakly immunogenic tumors, is a further benefit. Furthermore, the anti-tumor activity of *ex vivo* anti-4-1BB-stimulated leukocytes in adoptive cell therapy has tremendous potential. Future studies should be directed at translating anti-4-1BB therapy into clinical trials, in various forms of cancer.

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REFERENCES

1. Vinay, D. S. and Kwon, B. S. (2012) Immunotherapy of cancer by 4-1BB. *Mol. Cancer Ther.* **11**, 1062-1070.
2. Kwon, B. S. and Weissman, S. M. (1989) cDNA sequences of two inducible T-cell genes. *Proc. Nat. Acad. Sci. U.S.A.* **86**, 1963-1967.
3. Schwarz, H., Tuckwell, J. and Lotz, M. (1983) A receptor induced by lymphocyte activation (ILA): a new member of the human nerve-growth-factor/tumor-necrosis-factor receptor family. *Gene* **134**, 295-298.
4. Vinay, D. S. and Kwon, B. S. (2009) TNF superfamily: costimulation and clinical applications. *Cell Biol. Int.* **33**, 453-465.
5. Vinay, D. S. and Kwon, B. S. (2006) Genes, transcripts and proteins of CD137 receptor and ligand. In: CD137 pathway: immunology and diseases (Ed Chen L). Springer, New York, pp. 1-14.
6. Pollok, K. E., Kim, Y. J., Zhou, Z., Hurtado, J., Kim, K. K., Pickard, R. T. and Kwon, B. S. (1993) Inducible T cell antigen 4-1BB. Analysis of expression and function. *J. Immunol.* **150**, 771-781.
7. Garni-Wagner, B. A., Lee, Z. H., Kim, Y. J., Wilde, C., Kang, C. Y. and Kwon, B. S. (1996) 4-1BB is expressed on

CD45RAhiROhi transitional T cell in humans. *Cell Immunol*. **169**, 91-98.
8. Watts, T. H. (2005) TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol*. **23**, 23-68.
9. Croft, M. (2003) Co-stimulatory members of TNFR family: keys to effective T-cell immunity. *Nature Rev. Immunol*. **3**, 609-620.
10. Shuford, W. W., Klussman, K., Tritchler, D. D., Loo, D. T., Chalupny, J., Siadak, A. W., Brown, T. J., Emswiler, J., Raecho, H., Larsen, C. P., Pearson, T. C., Ledbetter, J. A., Aruffo, A. and Mittler, R. S. (1997) 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification of in vivo cytotoxic T cell response. *J. Exp. Med*. **186**, 47-55.
11. Seo, S. K., Choi, J. H., Kim, Y. H., Kang, W. J., Park, H. Y., Suh, J. H., Choi, B. K., Vinay, D. S. and Kwon, B. S. (2004) 4-1BB-mediated immunotherapy of rheumatoid arthritis. *Nature Med*. **10**, 1088-1094.
12. Takahashi, C., Mittler, R. S. and Vella, A. T. (1999) 4-1BB is a bona-fide CD8 T cell survival signal. *J. Immunol*. **162**, 5037-5040.
13. DeBenedette, M. A., Wen, T., Bachmann, M. F., Ohashi, P. S., Barber, B. H., Stocking K. L., Peschon, J. J. and Watts, T. H. (1999) Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J. Immunol*. **163**, 4833-4841.
14. Kwon, B. S., Hurtado, J. C., Lee, Z. H., Kwack, K. B., Seo, S. K., Choi, B. K., Koller, B. H., Wolisi, G., Broxmeyer, H. E. and Vinay, D. S. (2002) Immune responses in 4-1BB (CD137)-deficient mice. *J. Immunol*. **168**, 5483-5490.
15. Sun, Y., Lin, X., Chen, H. M., Wu, Q., Subudhi, S. K., Chen, L. and Fu, Y. X. (2002) Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis. *J. Immunol*. **168**, 1457-1465.
16. Foell, J., Strahotin, S., O'Neil, S. P., McCausland, M. M., Suwyn, C., Haber, M., Chander, P. N., Bapat, A. S., Yan, X. J., Chiorazzi, N., Hoffmann, M. K. and Mittler, R. S. (2003) CD137 costimulatory T cell receptor engagement reverses acute disease in lupus-prone NZB x NZWF1 mice. *J. Clin. Invest*. **111**, 1505-1518.
17. Sun, Y., Chen, H. M., Subudhi, S. K., Chen, J., Koka, R., Chen, L. and Fu, Y. X. (2003) Costimulatory molecule-targeted antibody therapy of a spontaneous autoimmune disease. *Nature Med*. **8**, 1405-1413.
18. Agarwal, A. and Newell, K. A. (2008) The role of positive costimulatory molecules in transplantation and tolerance. *Curr. Opin. Organ. Transplant* **13**, 366-372.
19. Zhang, G. B., Dong, Q. M., Hou, J. Q., Ge, Y., Ju, S. G., Lu, B. F. and Zhang, X. G. (2007) Characterization and application of three novel monoclonal antibodies against human 4-1BB: distinct epitopes of human 4-1BB on lung tumor cells and immune cells. *Tissue Antigens*. **70**, 470-479.
20. Broll, K., Richter, G., Pauly, S., Hofstaedter, F. and Schwarz, H. (2001) CD137 expression in tumor vessel walls. High correlation with malignant tumors. *Am. J. Clin. Pathol*. **115**, 543-549.
21. Wan, Y. L., Zheng, S. S., Zhao, Z. C., Li, M. W., Jia, C. K. and Zhang, H. (2004) Expression of co-stimulator 4-1BB molecule in hepatocellular carcinoma and adjacent non-tumor liver tissue, and its possible role in tumor immunity. *World J. Gasteroenterol*. **10**, 195-199.
22. Palma, C., Binaschi, M., Bigioni, M., Maggi, C. A. and Goso, C. (2004) CD137 and CD137 ligand constitutively expressed on human T and B leukemia cells signal proliferation and survival. *Int. J. Cancer* **108**, 390-398.
23. Ho, W. T., Pang, W. L., Chong, S. M., Castella, A., Al-Salam, S., Tan, T. E., Moh, M. C., Koh, L. K., Gan, S. U., Cheng, C. K. and Schwarz, H. (2013) Expression of CD137 on Hodgkin and Reed-Sternberg cells inhibits T-cell activation by eliminating CD137 ligand expression. *Cancer Res*. **73**, 652-661.
24. Anderson, M. W., Zhao, S., Freud, A. G., Czerwinski, D. K., Kohrt, H., Alizadeh, A. A., Houot, R., Azambuja, D., Biasoli, I., Morais, J. C., Spector, N., Molina-Kirsch, H. F., Warnke, R. A., Levy, R. and Natkunam, Y. (2012) CD137 is expressed in follicular dendritic cell tumors and in classical Hodgkin and T-cell lymphomas: diagnostic and therapeutic implications. *Am. J. Pathol*. **181**, 795-803.
25. Shao, Z. and Schwarz, H. (2010) CD137 ligand, a member of the tumor necrosis factor family, regulated immune responses via reverse signal transduction. *J. Leukobiol*. **89**, 21-29.
26. Melero, I., Shuford, W. W., Newby, S. A., Aruffo, A., Ledbetter, J. A., Hellström, K. E., Mittler, R. S. and Chen, L. (1997) Monoclonal antibodies against the 4-1BB T-cell activating molecule eradicate established tumors. *Nature Med*. **3**, 682-685.
27. Tirapu, I., Arina, A., Mazzolini, G., Duarte, M., Alfaro, C., Feijoo, E., Qian, C., Chen, L., Prieto, J. and Melero, I. (2004) Improving efficacy of interleukin-12-transfected dendritic cells injected into murine colon cancer with anti-CD137 monoclonal antibodies and alloantigens. *Int. J. Cancer* **110**, 51-60.
28. Xu, D., Gu, P., Pan, P. Y., Li, Q., Sato, A. I. and Chen, S. H. (2004) NK and CD8+ T cell-mediated eradication of poorly immunogenic B16-F10 melanoma by the combined action of IL-12 gene therapy and 4-1BB costimulation. *Int. J. Cancer* **109**, 499-506.
29. Sabel, M. S., Conway, T. F., Chen, F. A. and Bankert, R. B. (2000) Monoclonal antibodies directed against the T cell activation molecule CD137 (interleukin-A or 4-1BB) block human lymphocyte-mediated suppression of tumor xenograft in severe combined immunodeficient mice. *J. Immunother*. **23**, 362-369.
30. Kim, J. A., Averbook, B. J., Chambers, K., Rothchild, K., Kjaergaard, J., Papay, R. and Shu, S. (2001) Divergent effects of 4-1BB antibodies on anti-tumor immunity and on tumor-reactive T-cell generation. *Cancer Res*. **61**, 2031-2037.
31. Wilcox, R. A., Flies, D. B., Zhu, G., Johnson, A. J., Tamada, K., Chapoval, A. I., Strome, S. E., Pease, L. R. and Chen, L. (2002) Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. *J. Clin. Invest*. **109**, 651-659.
32. May, K. F., Chen, L., Zheng, P. and Lui, Y. (2002)

Anti-4-1BB monoclonal antibody enhances rejection of large burden by promoting survival but not clonal expansion of tumor-specific CD8+ T cells. *Cancer Res.* **62**, 3459-3465.
33. Miller, R. E., Jones, J., Le, T., Whitmore, J., Boiani, N., Gliniak, B. and Lynch, D. H. (2002) 4-1BB-specific monoclonal antibody promotes the generation of tumor-specific immune responses by direct activation of CD8 T cells in a CD40-dependent manner. *J. Immunol.* **169**, 1792-1800.
34. Murillo, O., Dubrot, J., Palazón, A., Arina, A., Azpilikueta, A., Alfaro, C., Solano, S., Ochoa, M. C., Berasain, C., Gabari, I., Pérez-Gracia, J. L., Berraondo, P., Hervás-Stubbs, S. and Melero, I. (2009) In vivo depletion of DC impairs the anti-tumor effect of agonistic anti-CD137 mAb. *Eur. J. Immunol.* **39**, 2424-2436.
35. Ju, S. A., Cheon, S. H., Park, S. M., Tam, N. Q., Kim, Y. M., An, W. G. and Kim, B. S. (2008) Eradication of established renal cell carcinoma by a combination of 5-fluorouracil and anti-4-1BB monoclonal antibody in mice. *Int. J. Cancer* **122**, 2784-2790.
36. Palazón, A., Teijeira, A., Martínez-Forero, I., Hervás-Stubbs, S., Roncal, C., Peñuelas, I., Dubrot, J., Morales-Kastresana, A., Pérez-Gracia, J. L., Ochoa, M. C., Ochoa-Callejero, L., Martínez, A., Luque, A., Dinchuk, J., Rouzaut, A., Jure-Kunkel, M. and Melero, I. (2011) Agonist anti-CD137 mAb act on tumor endothelial cells to enhance recruitment of activated T lymphocytes. *Cancer Res.* **71**, 801-811.
37. June, C. H. (2007) Adoptive T cell therapy for cancer in clinic. *J. Clin. Invest.* **117**, 1466-1476.
38. Hernandez-Chacon, J. A., Li, Y., Wu, R. C., Bernatchez, C., Wang, Y., Weber, J. S., Hwu, P. and Radvanyi, L. G. (2011) Costimulation through the CD137/4-1BB pathway protects human melanoma tumor-infiltrating lymphocytes from activation-induced cell death and enhances antitumor effector function. *J. Immunother.* **34**, 236-250.
39. Zhu, B. Q., Ju, S. W. and Shu, Y. Q. (2009) CD137 enhances cytotoxicity of CD3(+)CD56(+) cells and their capacities to induce CD4 (+) Th1 responses. *Biomed. Pharmacother.* **63**, 509-516.
40. Lu, P. H. and Negrin, R. S. (1994) A novel population of expanded human CD3+CD56+ cell derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J. Immunol.* **153**, 1687-1696.
41. Verneris, M. R., Arshi, A., Edinger, M., Kornacker, M., Natkunam, Y., Karami, M., Cao, Y. A., Marina, N., Contag, C. H. and Negrin, R. S. (2005) Low levels of Her2/neu expressed by Ewing's family tumor cell lines can redirect cytokine-induced killer cells. Clin. *Cancer Res.* **11**, 4561-4570.
42. Hassan, R. and Ho, M. (2008) Mesothelin targeted cancer immunotherapy. *Eur. J. Cancer* **44**, 46-53.
43. Carpenito, C., Milone, M. C., Hassan, R., Simonet, J. C., Lakhal, M., Suhoski, M. M., Varela-Rohena, A., Haines, K. M., Heitjan, D. F., Albelda, S. M., Carroll, R. G., Riley, J. L., Pastan, I. and June, C. H. (2009). Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 3360-3365.
44. Tammana, S., Huang, X., Wong, M., Milone, M. C., Ma, L., Levine, B. L., June, C. H., Wagner, J. E., Blazar, B. R. and Zhou, X. (2010). 4-1BB and CD28 signaling plays a synergistic role in redirecting umbilical cord blood T cells against B-cell malignancies. *Human Gene Ther.* **21**, 75-86.
45. Degang, S., Qunrui, Ye., Carmine, C., Mathilde, P., Li, P. W., Chunyan, J., Mariangela, F., Carl, H. J., George, C. S. and Daniel J. P. J. (2011) Improved survival provided by CD137 costimulatory signaling to folate receptor-redirected T cells results in tumor localization and eradication. *Cancer Res.* **171**, 4617-4627.
46. Ye, Z., Hellstrom, I., Hayden-Ledbetter, M., Dahlin, A., Ledbetter, J. A. and Hellstrom, K. E. (2002) Gene therapy of cancer using single-chain Fv fragments specific for 4-1BB. *Nature Med.* **8**, 343-348.
47. Xu, D., Gu, P., Pan, P. Y., Li, Q., Sato, A. I. and Chen, S. H. (2004) NK and CD8+ T cell-mediated eradication of poorly immunogenic B16-F10 melanoma by the combined action of IL-12 gene therapy and 4-1BB costimulation. *Int. J. Cancer* **109**, 499-506.
48. Tirapu, I., Arina, A., Mazzolini, G., Duarte, M., Alfaro, C., Feijoo, E., Qian, C., Chen, L., Prieto, J. and Melero, I. (2004) Improving efficacy of interleukin-12-transfected dendritic cells injected into murine colon cancer with anti-CD137 monoclonal antibodies and alloantigens. *Int. J. Cancer* **110**, 51-60.
49. Ito, F., Li, Q., Shreiner, A. B., Okuyama, R., Jure-Kunkel, M. N., Teitz-Tennenbaum, S. and Chang, A. E. (2004) Anti-CD137 monoclonal antibody administration augments the antitumor efficacy of dendritic cell-based vaccines. *Cancer Res.* **64**, 8411-8419.
50. Kocak, E., Lute, K., Chang, X., May, K. F., Exten, K. R., Zhang, H., Abdessalam, S. F., Lehman, A. M., Jarjoura, D., Zheng, P. and Liu, Y. (2006) Combination therapy with anti-CTL antigen-4 and anti-4-1BB antibodies enhances cancer immunity and reduces autoimmunity. *Cancer Res.* **66**, 7276-7284.
51. Sin, J. I., Kim, H., Ahn, E., Jeon, Y. H., Park, W. S., Lee, S. Y. and Kwon, B. (2013) Combined stimulation of TLR9 and 4-1BB augments Trp2 peptide vaccine-mediated melanoma rejection by increasing Ag-specific CTL activity and infiltration into tumor sites. *Cancer Lett.* **3330**, 190-199.
52. Chen, S. H., Pham-Nguyen, K. B., Martinet, O., Huang, Y., Yang, W., Thung, S. N., Chen, L., Mittler, R. and Woo, S. L. (2000) Rejection of disseminated metastases of colon carcinoma by synergism of IL-12 gene therapy and 4-1BB costimulation. *Mol. Ther.* **2**, 39-46.
53. Shi, W. and Siemann, D. W. (2006) Augmented antitumor effects of radiation therapy by 4-1BB antibody (BMS-469492) treatment. *Anticancer Res.* **26**, 3445-3453.
54. Carmeliet, P. and Jain, R. K. (2000) Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257.
55. Wu, H. C. and Li, P. C. (2008) Proteins expressed on tumor endothelial cells as potential targets for anti-angiogenic therapy. *J. Cancer Mol.* **4**, 17-22.
56. Ko, E., Luo, W., Peng, L., Wang, X. and Ferrone, S. (2007) Mouse dendritic-endothelial cell hybrids and 4-1BB costimulation elicit antitumor effects mediated by broad anti angiogenic immunity. *Cancer Res.* **67**, 7875-7884.
57. Li, B., Lin, J., Vanroey, M., Jure-Kunkel, M. and Jooss, K.

(2007) Established B16 tumors are rejected following treatment with GM-CSF-secreting tumor cell immunotherapy in combination with anti-4-1BB mAb. *Clin. Immunol.* **125**, 76-87.
58. John, L. B., Howland, L. J., Flynn, J. K., West, A. C., Devaud, C., Duong, C. P., Stewart, T. J., Westwood, J. A., Guo, Z. S., Bartlett, D. L., Smyth, M. J., Kershaw, M. H. and Darcy, P. K. (2012) Oncolytic virus and anti-4-1BB combination therapy elicits strong anti-tumor immunity against established cancer. *Cancer Res.* **72**, 1651-1660.
59. Choi, B. K., Kim, Y. H., Kang, W. J., Lee, S. K., Kim, K. H. and Shin, S. M. (2007) Mechanisms involved in synergistic anticancer immunity of anti-4-1BB and anti-CD4 therapy. *Cancer Res.* **67**, 8891-8899.
60. Geiger, J. D., Wagner, P. D., Camerom, M. J., Shu, S. and Chang, A. E. (1993) Generation of T-cell reactive to the poorly immunogenic B16-BL6 melanoma with efficacy in the treatment of spontaneous metastases. *J. Immunother. Emphasis Tumor Immunol.* **13**, 153-165.
61. Kim, Y. H., Choi, B. K., Kang, W. J., Mittler, R. S. and Kwon, B. S. (2009) Mechanisms involved in synergistic anti-cancer effects of anti-4-1BB and cyclophosphamide therapy. *Mol. Cancer Ther.* **8**, 469-478.
62. Ju, S. A., Lee, S. C., Kwon, T. H., Heo, S. K., Park, S. M. and Park, S. M. (2005) Immunity to melanoma mediated by 4-1BB is associated with enhanced activity of tumor-infiltrating lymphocytes. *Immunol. Cell Biol.* **83**, 344-351.
63. Lindsedt, M., Johansson-Lindbom, B. and Borrebaeck, C. A. (2003) Expression of CD137 (4-1BB) on human follicular dendritic cells. *Scand. J. Immunol.* **57**, 305-310.
64. Sun, Y., Blink, S. E., Chen, J. H. and Fu, Y. X. (2005) Regulation of follicular dendritic cell networks by activated T cells: the role of CD137 signaling. *J. Immunol.* **175**, 884-890.
65. Middendorp, S., Xiao, Y., Song, J. Y., Peperzak, V., Krijger, P. H., Jacobs, H. and Borst, J. (2009) Mice deficient in CD137 ligand are predisposed to develop germinal center-derived B-cell lymphoma. *Blood* **114**, 2280-2289.
66. Vinay, D. S., Choi, B. K., Bae, J. S., Kim, W. Y., Gebhardt, B. M. and Kwon, B. S. (2004) CD137-deficient mice have reduced NK/NKT cell numbers and function, are resistant to lipopolysaccharide-induced shock syndromes, and have lower IL-4 responses. *J. Immunol.* **173**, 4218-4229.
67. Choi, B. K., Kim, Y. H., Kim, C. H., Kim, M. S., Kim, K. H., Oh, H. S., Lee, M. J., Lee, D. K., Vinay, D. S. and Kwon, B. S. (2010) Peripheral 4-1BB signaling negatively regulates NK cell development through IFN-γ. *J. Immunol.* **185**, 1404-1411.
68. Zhang, N., Sadun, R. E., Arias, R. S., Flanagan, M. L., Sachsman, S. M., Nien, Y. C., Khawli, L. A., Hu, P. and Epstein, A. L. (2007) Targeted and untargeted CD137L fusion proteins for the immunotherapy of experimental solid tumors. *Clin. Cancer Res.* **13**, 2758-2767.
69. Meseck, M., Huang, T., Ma, G., Wang, G., Chen, S. H. and Woo, S. L. C. (2011) A functional recombinant human 4-1BB ligand for immune costimulatory therapy of cancer. *J. Immunother.* **34**, 175-182.
70. Porter, D. L., Levine, B. L., Kalos, M., Bagg, A., June, C. H. (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *New. England. J. Med.* **365**, 725-733.