

Immunotherapy of Cancer with 4-1BB

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Abstract

4-1BB (CD137), a member of the TNF receptor superfamily, is an activation-induced T-cell costimulatory molecule. Signaling via 4-1BB upregulates survival genes, enhances cell division, induces cytokine production, and prevents activation-induced cell death in T cells. The importance of the 4-1BB pathway has been underscored in a number of diseases, including cancer. Growing evidence indicates that anti-4-1BB monoclonal antibodies possess strong antitumor properties, which in turn are the result of their powerful CD8+ T-cell activating, IFN- γ producing, and cytolytic marker-inducing capabilities. In addition, combination therapy of anti-4-1BB with other anticancer agents, such as radiation, has robust tumor-regressing abilities against nonimmunogenic or poorly immunogenic tumors. Furthermore, the adoptive transfer of *ex vivo* anti-4-1BB-activated CD8+ T cells from previously tumor-treated animals efficiently inhibits progression of tumors in recipient mice that have been inoculated with fresh tumors. In addition, targeting of tumors with variants of 4-1BBL directed against 4-1BB also have potent antitumor effects. Currently, a humanized anti-4-1BB is in clinical trials in patients with solid tumors, including melanoma, renal carcinoma, and ovarian cancer, and so far seems to have a favorable toxicity profile. In this review, we discuss the basis of the therapeutic potential of targeting the 4-1BB–4-1BBL pathway in cancer treatment. *Mol Cancer Ther*; 11(5); 1062–70. ©2012 AACR.

Introduction

Despite intensive research, the successful treatment of cancers is still a daunting task for clinicians. The inability of the immune system to mount responses to poorly immunogenic or nonimmunogenic tumors is a major stumbling block in the development of effective anticancer agents. Because tumor cells, among others, are killed by cytotoxic T lymphocytes (CTL) in an antigen-specific manner, agents that promote CD8+ T-cell activation and impart strong cytolytic and inflammatory properties, as well as antigen specificity, are ideal candidates for enhancing tumor-antigen-specific immunity. Immunotherapy targeting CD8+ T cells with agonistic anti-4-1BB (CD137) monoclonal antibody (mAb) fulfills these requirements because 4-1BB-mediated signals are biased toward CD8+ T cells, promoting their survival, differentiation, and acquisition of potent cytolytic properties (1).

4-1BB–4-1BB ligand

4-1BB was discovered more than 2 decades ago in screens for receptors on mouse concanavalin A (ConA)-

activated helper and cytotoxic T-cell lines (2). With few exceptions, expression of 4-1BB is activation dependent. 4-1BB is not detected (<3%) on resting T cells or T-cell lines. However, 4-1BB is stably upregulated when T cells are activated by a variety of agonists, such as plate-bound anti-CD3, ConA, phytohemagglutinin, interleukin (IL)-2, IL-4, CD28, phorbol myristoyl acetate, or ionomycin (alone or in combination) in the presence of antigen-presenting cells (APC; refs. 3–5). 4-1BB has been reported to be expressed on activated CD4+, CD8+, natural killer (NK), and NKT cells, and it is expressed in a constitutive manner on CD11c+ dendritic cells (DC) and CD4+CD25+ regulatory T cells (Treg; refs. 3–5).

Functional 4-1BB expression has also been observed on myeloid cells, such as monocytes, neutrophils, mast cells, and eosinophils (6). Once expressed, 4-1BB binds to a high-affinity 4-1BB ligand (4-1BBL) that is present on a variety of APCs, including DCs, B cells, and macrophages (3–5).

Expression and function of 4-1BB and 4-1BBL

Many studies have established that 4-1BB is a potent costimulatory molecule (3–5). Signaling via 4-1BB by agonistic mAb, soluble 4-1BBL, or cell lines expressing 4-1BBL in the presence of anti-T-cell-receptor Ab has been shown to cause T-cell expansion, cytokine induction, and upregulation of antiapoptotic genes, and to prevent activation-induced cell death (AICD; Fig. 1, left panel; refs. 3–5). Kinetic studies and expression analyses have revealed that although levels of 4-1BB expression are comparable in CD4+ and CD8+ T cells, CD8+ T cells have a considerably higher proliferative potential than CD4+

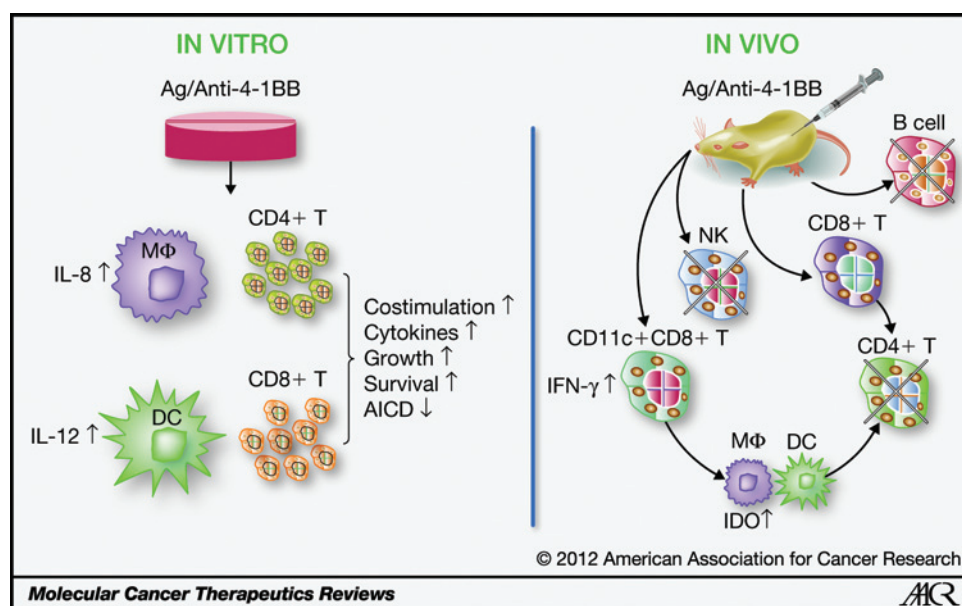
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Figure 1. Biological effects of 4-1BB signaling. *In vitro* 4-1BB signaling (left panel) costimulates CD4+ and CD8+ T cells to comparable extents with respect to cytokine production and upregulation of cell survival genes, and prevents their AICD (3–5). Ligation of the 4-1BB receptor on macrophages (M Φ) and DCs results in activation and induction of IL-8 and IL-12, respectively (6). On the other hand, *in vivo* 4-1BB stimulation (right panel) leads to biased CD8+ T-cell activation with a concomitant decline of B, NK, and CD4+ T cells in an IFN- γ , TNF- α -, TGF- β -, and IDO-dependent manner (1).



T cells (7). A similar effect was observed *in vivo* (8). The reasons for the differential costimulatory ability of anti-4-1BB are still not completely understood; nevertheless, the available data strongly suggest that 4-1BB is a *bona fide* CD8+ T-cell-activating molecule (9). In addition to its effects on T cells, ligation of the 4-1BB receptor on DCs and macrophages induces the production of IL-12 and IL-8, respectively, by these cells (3–5).

The *in vivo* effects of anti-4-1BB are more dramatic than those seen *in vitro* (summarized in Fig. 1, right panel). To date, the role of 4-1BB signaling has been studied in several autoimmune processes, including rheumatoid arthritis (8), experimental autoimmune encephalomyelitis (10), and systemic lupus erythematosus (11, 12), and the results have revealed several hitherto unknown aspects of *in vivo* 4-1BB signaling. It is clear that *in vivo* administration of anti-4-1BB mAb kills or suppresses more cell types than it costimulates, but it nonetheless offers protection against a variety of clinical disorders (3–5). The dual immunoregulatory effects of 4-1BB signaling continue to be puzzling. Anti-4-1BB therapy severely affects B-cell numbers and function, resulting in damping of overall humoral immunity such that autoantibody production is inhibited (13). The basis of this effect is not clear, but it is suspected that B cells are susceptible to stand-alone anti-4-1BB mAb treatment even in the absence of antigen stimulation (14). Sun and colleagues (12) showed that much of the loss of B-cell function during anti-4-1BB mAb treatment of lupus-prone mice is prevented if *in vivo* IFN- γ is neutralized.

Basis of anti-4-1BB-mediated immunotherapy

Several mechanisms have been proposed as the basis for anti-4-1BB immunotherapy (Fig. 1, right panel; ref. 15). Mittler and colleagues (13) were the first to make the

important observation that administration of anti-4-1BB causes loss of humoral activity against T-dependent antigens. Closer examination revealed that the loss of humoral activity was correlated with the appearance of anergic CD4+ T cells (13). On the other hand, Sun and colleagues (12) suggested that the reduction of B-cell numbers and function in lupus-prone mice was associated with loss of CD4+ T cells and upregulation of IFN- γ , because the effect was considerably reduced by anti-IFN- γ mAb. These anti-4-1BB mAb-mediated effects on B-cell function in mice were subsequently confirmed in experiments with non-human primates. Hong and colleagues (16) showed that treatment with humanized anti-4-1BB mAb suppressed humoral responses to a T-dependent antigen (ovalbumin) in nonhuman primates, and Myers and colleagues (17) suggested that anti-4-1BB administration deleted CD4+ T cells in a TGF- β -dependent manner. Seo and colleagues (8) showed that anti-4-1BB mAb treatment increases IFN- γ , which in turn upregulates indoleamine 2,3-dioxygenase (IDO) in macrophages and DCs. They proposed that the interaction between IDO+ macrophages and DCs and partnering T cells inhibits or abolishes T-cell activity (Fig. 1, right panel), as mimicked by the effect of inhibiting IDO activity with 1-methyl tryptophan (a pharmacological inhibitor of IDO).

In this work, we briefly review the potential of targeting the 4-1BB–4-1BBL pathway in cancer therapy.

Anti-4-1BB mAbs as anticancer agents

The first comprehensive evidence that anti-4-1BB antibodies have strong antitumor effects came to light through studies on the poorly immunogenic Ag104A sarcoma and the highly tumorigenic P815 mastocytoma. Administration in mice bearing the above tumors with anti-4-1BB was shown to significantly inhibit tumor growth by increasing

CTL activity (18). In the years that followed, studies substantiated the antitumor effects of 4-1BB signaling and extended our understanding of how the 4-1BB signal suppresses tumor development. Melero and colleagues (19) reported that treatment of P815-bearing mice with anti-4-1BB suppressed tumor growth, and depletion of NK or NKT cells completely abrogated this antitumor effect. The authors pointed out that, despite this finding, NK1.1+ cells could not be the effector cells because P815 cells are resistant to lysis by anti-4-1BB mAb, as revealed by the lack of P815 lysis by NK1.1 cells *in vitro*. They further suggested the involvement of regulatory functions of NK cells in redirecting the NK1.1 cytotoxic effects (19). Administration of human anti-4-1BB mAb to suppress human xenografts in severe combined immunodeficient mice (SCID) mice resulted in significant inhibition of tumor growth (20). Anti-4-1BB treatment of mice that had received MCA205 sarcoma or GL261 glioma cells 3 days previously was shown to prolong survival and cause regression of the tumors. These effects were dependent on T cells, because anti-4-1BB failed to suppress tumor growth in mice depleted of CD4+ and/or CD8+ T cells and promoted tumor growth in SCID mice (21). Although studies revealed the antitumor effects of anti-4-1BB, similar therapy of poorly immunogenic tumors, such as C3 tumors, TC-1 lung carcinoma, and B16.F10 melanoma, was shown to be ineffective (22). Careful examination revealed that immunological ignorance rather than anergy or deletion of tumor-antigen-specific CTLs was responsible for the absence of anti-4-1BB effects (22). Substantial tumor eradication, delayed tumor progression, and prolonged survival occurred in RAG2-/- mice that were given P1A-expressing J558 cells and P1A-specific CD8+ T cells and were then treated with anti-4-1BB mAb (23). Treatment of tumor-bearing mice with immune-stimulatory anti-4-1BB significantly reduced the tumor burden. Cell-depletion studies have shown that the antitumor effects of anti-4-1BB depend on CD8+ T cells and not on CD4+ T cells or NK cells. Similarly, treatment of tumor-bearing IFN- γ -/- or CD40-/- mice with anti-4-1BB is ineffective, pointing to important roles for these molecules. Of interest, DC amplification *in vivo* by repeated injections of Fms-like tyrosine kinase 3 ligand enhanced the antitumor activity of anti-4-1BB (24). Administration of agonistic anti-4-1BB or the cytotoxic drug 5-fluorouracil (5-FU) to renal cell carcinomas in mice had a negligible effect on tumor regression (25). However, combination therapy with 5-FU and anti-4-1BB eradicated established tumors in more than 70% of the mice. Further analysis revealed that this tumor regression was correlated with increased numbers of lymphocytes in the mice's spleens and tumor-draining lymph nodes, and increased numbers of apoptotic cells and tumor-infiltrating lymphocytes. Furthermore, mice that received this combination therapy rapidly rejected tumors when rechallenged, suggesting that long-lasting tumor antigen-specific memory had been established (25). 4-1BB was found on endothelial tumor cells, and its expression was upregulated upon activation (26). Exposure of tumor

endothelial cells to anti-4-1BB mAbs upregulated intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin (26). Upon adoptive transfer into Rag1-/- mice and after treatment with immune stimulatory anti-4-1BB mAb, the donor T cells displayed enhanced migration into tumor tissue (26). Inhibition studies using anti-ICAM-1 and anti-VCAM-1 Abs showed that the stimulation of ICAM-1 and VCAM-1 expression on tumor endothelial cells by anti-4-1BB was responsible for the enhanced T-cell migration into malignant tissue (26). These observations suggest that the *in vivo* anticancer effects of anti-4-1BB mAbs encompass both T and non-T cells.

Adoptive T-cell therapy (ACT) of cancers involves expanding tumor-infiltrating lymphocytes (TIL) *ex vivo* in the presence of IL-2, and reinfusing them into tumor-bearing hosts (27). One of the challenges facing ACT is AICD of the TILs and loss of key accessory molecules due to the *ex vivo* culture conditions. This can lead to reduced *in vivo* persistence after adoptive transfer (27). Hernandez-Chacon and colleagues (28) observed that *ex vivo* expansion of TILs resulted in loss of CD27 and CD28 expression but gain of 4-1BB expression and, to a lesser extent, CD134/OX40 (28). Stimulation of these cells with agonistic anti-4-1BB mAbs significantly inhibited their AICD, enhanced their cell division, and increased their cytolytic activity against melanoma cells (28). Similarly, stimulation of human CD3+CD56+ NK cells with anti-4-1BB increased their cytolytic activity, as shown by their ability to lyse A549 tumor cells (29). Cytokine-induced killer cells, generated *in vitro* by stimulation with anti-CD3/IL-2/IFN- γ , are potent anticancer agents (30, 31). When a group of SCID mice were engrafted with A549 tumor cells and received in addition anti-4-1BB and cytokine-induced killer cells, their mortality rate was reduced (29). Further analysis revealed increased expression of IFN- γ , IL-2, and TNF- α , and decreased expression of TGF- β , IL-4, and IL-10 in CD3+CD56+ cells taken from the treated mice (29). Taken together, these findings underline the important role of 4-1BB signaling in tumor development, and the therapeutic potential of anti-4-1BB mAbs in cancer therapy.

Variants of anti-4-1BB as antitumor agents

In addition to the anticancer effects of anti-4-1BB mAbs, targeting the 4-1BB receptor with variants of the 4-1BB molecule has also shown promise. A large proportion of carcinomas express surface mesothelin (32), and T cells engineered to express a single-chain variable fragment that binds mesothelin fused with the T-cell ζ chain, CD28, and 4-1BB showed enhanced persistence and decreased the tumor burden when transferred into NOD/SCID/IL-2 γ -/- mice carrying preestablished human primary M108 tumors (33). T cells from umbilical cord blood were able to kill leukemia and lymphoma cells *in vitro* when equipped with a single-chain chimeric antigen receptor carrying the intracellular domain of the CD3 ζ chain and 4-1BB (34). In addition, *in vivo* adoptive transfer of these

engineered umbilical cord blood T cells into K562 erythroleukemia tumor-bearing mice prolonged their survival, and the umbilical cord blood T cells displayed robust antitumor activity (34). Human T cells engineered to express a chimeric immune receptor specific for folate receptor α (FR α) had strong antitumor activity against epithelial cancers *in vitro* but not *in vivo*, due mainly to their short lifetimes and inability to migrate to tumor sites. Song and colleagues (35) devised a strategy to overcome this problem: they modified the chimeric immune receptor containing a FR α -specific scFv (MOv19) by coupling it to the T-cell-receptor CD3 ζ chain signaling molecule, either alone (MOv19- ζ) or in combination with the CD137 (4-1BB) costimulatory motif (MOv19-BB ζ). Although both modified chimeric immune receptors induced *in vitro* tumor activity, only MOv19-BB ζ elicited robust *in vivo* antitumor activity when transferred into immune-deficient mice bearing established FR α human cancers (35). Careful examination revealed that the MOv19-BB ζ -expressing human T cells survived longer and were present within the tumors, suggesting that they homed efficiently. In a study by Ye and colleagues (36), 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, it induced robust Th1 responses in a CD4+ T-cell- and NK-cell-dependent manner. Collectively, these findings indicate that alternative ways of targeting 4-1BB for cancer treatment are available.

Anti-4-1BB combination therapy with other anticancer agents

Studies have shown that the combination of anti-4-1BB mAb with other anticancer agents can enhance antitumor activity. With poorly immunogenic tumors such as B16.F10 melanoma, treatment by IL-12 gene transfer or with anti-4-1BB alone had no effect (37); however, when the 2 treatments were combined and administered, tumor regression was observed in ~50% of the tumor-bearing mice and their survival increased (37). The combined therapy also had a robust antitumor effect in a pulmonary metastatic model (37). Cell-depletion studies showed that elimination of CD8+ T or NK cells, but not CD4+ T cells, inhibited the antitumor activity of the combination therapy. In addition, depletion of NK cells in the tumor-bearing mice reduced CTL activity and IFN- γ production. Repeated injections, in contrast to single injections, of DCs engineered to produce IL-12 resulted in significant suppression of CT26 colon carcinomas. The efficacy of this treatment for both spontaneous and established tumors was increased further by combining it with agonistic anti-4-1BB mAb (38). To overcome the therapeutic challenges involved in treating poorly immunogenic tumors, Ito and colleagues (39) developed a strategy in which anti-4-1BB was combined with vaccination with tumor-cell lysate-pulsed DCs (TP-DC), and they reported that this strategy increased tumor regression and enhanced survival of the 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 revealed that CD8+, CD4+, and NK cells are involved in tumor regression in the combination TP-DC/anti-4-1BB therapy (39). When mice with preexisting MC38 (murine adenocarcinoma) tumors were treated 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. Of interest, a similar combination therapy was ineffective against B16 melanomas (40). 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-cell- and NK-cell-dependent tumor regression (41). Treatment with anti-4-1BB (BMS-469492; Bristol-Myers Squibb) 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 antitumor activity against the EMT6 tumors (42).

Pathological angiogenesis is an important aspect of cancers (43). Therefore, treatments directed against tumor-initiated angiogenesis have attracted attention in recent years, and antitumor strategies using endothelial cells (EC) to overcome tumor-induced angiogenesis have shown promise (44). To further refine this strategy, Ko and colleagues (45) combined EC therapy with hybrid DCs to specifically target molecules critical for angiogenesis, and with agonistic anti-4-1BB to boost tumor-specific CD8+ T-cell responses. They observed significant inhibition of both MC38 colon adenocarcinomas and B16.F10 melanomas. Subsequent experiments revealed that the antitumor effects of EC/DC/anti-4-1BB were mediated by EC-specific CD4+ and CD8+ T cells. The combination treatment had no therapy-related side effects, except for minor transient hematological alterations. Li and colleagues (46) confirmed that anti-4-1BB monotherapy was more effective when combined with other antitumor agents. These authors showed that irradiated tumors engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated robust antitumor activity against established B16 tumors when combined with agonistic anti-4-1BB. Further studies uncovered the existence of an increased proportion of tumor antigen-specific CD8+ T cells in the GM-CSF/anti-4-1BB-treated mice. Chronic anti-4-1BB therapy is known to deplete CD4+ T cells (14), and this effect was found to be useful for treating autoimmune diseases (4). Choi and colleagues (47) showed a beneficial effect of CD4+ T-cell depletion on anti-4-1BB-mediated antitumor activity. These authors found that treatment of B16.F10-bearing C57BL/6 mice with either agonistic anti-4-1BB or depletion with anti-CD4 mAbs had no effect on tumor regression. However, when the 2 treatments were combined, significant tumor regression was observed (47). An examination of the mechanism involved revealed that massive expansion of

novel IFN- γ producing NKG2D+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 NKG2D reduced the therapeutic effect by 20% to 26% (47). Treatment of renal cell carcinomas with agonistic anti-4-1BB or 5-FU had a negligible effect on tumor regression (25), whereas the combination of 5-FU and anti-4-1BB eradicated established tumors in more than 70% of the mice studied. Further analysis revealed that this tumor regression in mice that received 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 (25). Furthermore, mice that had received the combination therapy rapidly rejected rechallenge with the same tumors, suggesting that long-lasting tumor-specific memory had been established (25). A recent study indicated that treatment of mice bearing B16 melanomas, which are poorly immunogenic (48), with cyclophosphamide or anti-4-1BB was ineffective (48), whereas the combined treatment resulted in significant anticancer 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 (49).

4-1BBL and its variants as antitumor agents

The importance of targeting 4-1BB by administering 4-1BBL or its variants to inhibit tumors has been shown in a number of studies. Mice that received intratumoral adenovirus-mediated gene transfer of 4-1BB ligand (ADV/4-1BBL) to liver metastases in a syngeneic model of breast cancer underwent dramatic tumor regression. In addition, intratumoral implantation of the IL-12 gene into mice together with anti-4-1BB and ADV/4-1BBL led to markedly increased (>78%) survival (50). Recombinant E1/E3-deleted adenovirus encoding human 4-1BBL (Ad4-1BBL), which efficiently infects several human adenocarcinoma cell lines and induces surface expression of 4-1BBL, enhanced the antitumor activity of partnering 4-1BB+ T cells. This antitumor activity was further elevated upon stimulation with a bispecific Ab (BsAb: anti-MUC1/anti-CD3) and led to enhanced production of IL-2, IFN- γ , and GM-CSF (50). When Ad4-1BBL and 4-1BB+ T cells were adoptively transferred together into cholangiosarcoma-grafted SCID mice, significant tumor suppression was observed (51). Administration of 4-1BBL-expressing neuroblastoma tumor cells to mice resulted in increased CD8+ T-cell amplification (52). It was noted that the increase of CD8+ T cells in these mice was correlated with a memory phenotype, suggesting a key role for the CD8+DX5+ cell population in 4-1BBL-mediated antitumor effects (52). There is evidence that acute myelogenous leukemia (AML) blasts can differentiate into leukemia-derived DCs (AML-DC), thus allowing augmented presentation of known and unknown leukemic antigens (53). Therefore, when AML-DCs were treated

with 4-1BBL, they stimulated CD8+ T-cell proliferation, and produced high levels of IFN- γ and cytolytic activity against Wilms tumor 1 (53). 4-1BBL that was engineered by linking it to the COOH terminus of either the Fc fragment of immunoglobulin (untargeted version) or TNT-3 (targeted version) was shown to bind to necrotic regions of tumors (54). These 2 variants of 4-1BBL had enhanced tumor-binding activities, and induced survival rates of 60% (TNT/4-1BBL) and 40% (Fc/4-1BBL) in treated mice as much as 150 days after tumor implantation (54). The antitumor effects of Fc/4-1BBL and TNT-3/4-1BBL were comparable to those of agonistic anti-4-1BB (54). Tumor suppression in this model correlated with increased central necrosis and infiltration of CD8+ granzymeB+ cells into necrotic regions in the treated mice. In agreement with this finding, depletion of CD8+ T cells reversed the effect (54). Subcutaneously transplanted P815 plasmacytoma tumors regressed significantly when treated with anti-4-1BB or M12-23-4 4-1BB aptamers (55). Further analysis revealed that 4-1BB aptamers caused significant expansion of CD8+ T cells and led them to secrete IFN- γ (55). These results suggest that 4-1BB aptamers have antitumor activity on a par with anti-4-1BB mAb therapy and, therefore, are a cost-effective alternative to anti-4-1BB mAb therapy. Treatment of mice bearing P-glycoprotein-overexpressing K562/AO2 cells with anti-CD3/anti-P-glycoprotein bispecific diabody led to tumor relapse 1 week after therapy. However, when this diabody therapy was supplemented with the extracellular domain of 4-1BBL, *in vitro* cytotoxicity increased and K562/AO2 xenografts in nude mice were eradicated (56), with no tumor recurrence within 100 days of the first treatment (56).

In vitro antitumor activity of 4-1BBL has also been shown. When mouse forestomach carcinoma cells were transfected with 4-1BBL and transferred into mice, they induced strong CTL activity and caused substantial inhibition of tumor growth (57). Human NK cells express 4-1BB upon activation (58). Consistent with this, NK cells from patients with AML displayed increased 4-1BB expression (58). However, coculturing of the NK cells with 4-1BBL-transfected cells led to reduced NK tumor activity (58), suggesting that 4-1BB-4-1BBL interactions permit immune evasion by AML cells by inhibiting NK-mediated antitumor activity. Recently, 4-1BBL was discovered on human $\gamma\delta$ T cells (59). When human $\gamma\delta$ + T cells, expanded by isopentenylpyrophosphate, were cocultured with NK cells and restimulated with plate-bound human IgG1, costimulation with CD137+ $\gamma\delta$ + T cells increased the expression of NKG2D on the NK cells. This increased NKG2D expression in turn enhanced the antitumor activities of the NK cells (59). Although 4-1BBL-mediated signaling had no effect on B-cell lymphoma cell lines, it caused significant inhibition of multiple myeloma, a malignancy of terminally differentiated plasma cells, by inducing apoptosis (60). This inhibition was correlated with the expression of IL-6 and IL-8 in the multiple-myeloma cells, suggesting that 4-1BBL agonists

play an important role in multiple-myeloma-cell-mediated cancer (60).

Successful adoptive cell therapy requires efficient *ex vivo* priming of tumor-associated antigen (TAA)-specific CD8+ T cells and their reinfusion into tumor-bearing individuals. Sluijter and colleagues (61) observed that when TAA-specific CD8+ T cells isolated from seminal lymph nodes of patients with stage I-II melanoma were stimulated *ex vivo* with an artificial APC system (K562 cells) designed to express human CD32 and 4-1BBL, they caused more effective recall responses and expansion of TAA-specific CD8+ T cells than did activation of anti-CD3/anti-CD28 (61). This suggests that such activation regimens facilitate efficient monitoring of functional anti-tumor T-cell immunity in the seminal lymph nodes.

Tumor development in 4-1BB^{-/-} and 4-1BBL^{-/-} 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 mAb prolonged their survival in a CD8+ T-cell- and IFN- γ -dependent manner (62). 4-1BB expression has been observed on follicular DCs (63), suggesting a role for this molecule in germinal center formation. Consistent with this, ~60% of 4-1BBL^{-/-} mice develop B-cell lymphomas by the age of 12 months (64). Further analysis revealed that this effect was associated with increased expression of Bcl-10 and the germinal-center response regulators Bcl-6, spi B, Elf-1, Bach-2, and activation-induced cytidine deaminase, among others (64). Vinay and colleagues (65) showed that 4-1BB^{-/-} mice have reduced NK cell numbers and activity. As a result, coculture of spleen cells and tumor cells failed to lyse the latter. However, when the residual NK cells in the 4-1BB^{-/-} mice were isolated, pooled, and cocultured 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 (65).

Activation of the 4-1BB pathway in human immunotherapy trials

Expression of 4-1BB has been observed on cancer cell lines, such as SPC-A-1, H446, H460, and H1299 (54), on tumor vessel walls, on the endothelial layer, and on vascular smooth muscles (66). Increased expression of 4-1BB has also been noted close to the edges of tumors in the liver tissues of patients with cancer (67). Furthermore, constitutive expression of 4-1BB and 4-1BBL was observed on a number of human T and B leukemia cell lines (68). Elevated levels of the soluble form of 4-1BBL were detected in the sera of patients with hematological malignancies (69). In contrast, significantly reduced serum 4-1BBL concentrations were observed in patients with colon cancer. However, in the same patients, levels of

serum 4-1BB were higher than in control subjects (70). Elevated levels of 4-1BBL have also been observed in the sera of more than 40% of patients with AML and non-Hodgkin lymphoma (71), and expression of 4-1BBL has been noted on carcinomas of the liver, lung, and colon, as well as on lymphomas and leukemias (72). When 4-1BBL+ tumor cells were cocultured with 4-1BB+ T cells or with Chinese hamster ovary cells expressing 4-1BB, expression of IL-8 and IFN- γ was induced (72). The significance of the soluble forms of 4-1BB and 4-1BBL in cancer patients, and their expression on tumor cells, is not clear. It has been difficult to determine whether elevated levels of endogenously produced soluble 4-1BB and 4-1BBL correlate with tumor progression, mainly because of the lack of adequate tools to study this phenomenon. However, soluble 4-1BBL does show activating abilities *in vitro* (73).

Several of the 4-1BB agonists show great potential for application to human cancers. For example, BMS-666513, a fully humanized mAb against 4-1BB, has completed phase I and II trials for its anticancer properties in patients with melanoma, renal cell carcinoma, and ovarian cancer (74). The results thus far suggest that the Ab therapy is well tolerated across various dose ranges (0.3–15 mg/kg body weight given every 3 weeks). Biomarker analysis revealed elevated expression of IFN-inducible genes in peripheral blood and in posttreatment biopsies of patients receiving BMS-666513 (74). However, 6% to 15% of the patients developed grade 3 or higher neutropenia and liver enzymes, mild fatigue, rash, pruritus, diarrhea, and fever (74). In future trials, it will be important to determine the dose ranges of anti-4-1BB that are safe and effective. Meseck and colleagues (75) recently reported a recombinant human 4-1BB ligand fusion protein (hIg-hu4-1BBs) that showed the ability to activate both human and monkey T cells *in vitro*. These results are encouraging, and future studies involving human subjects with cancer will determine its efficacy as a potent anticancer agent. Porter and colleagues (76) recently showed that, in patients with chronic lymphocytic leukemia, treatment with a low dose (1.5×10^5 cells/kg body weight) of lentiviral vector expressing a chimeric antigen receptor specific for B-cell antigen CD19 linked to 4-1BB and CD3 ζ chain corresponded with a delayed occurrence of tumor lysis syndrome and a complete remission of chronic lymphocytic leukemia. It is noteworthy that inclusion of the endodomain of the 4-1BB sequence enhanced the antitumor activities of the chimeric antigen receptor, which may result in prolonging the functions of the T cells in tumor microenvironment (42). The infused engineered cells not only self-replicated more than 1000-fold but persisted in higher numbers for 6 months in the blood and bone marrow after the initial transfusion. However, hypogammaglobulinemia was routinely observed as a chronic side effect in these patients (76). The results of Porter and colleagues (76) are promising, and thus far their approach appears to have an edge over mAb therapy because the infused cells self-replicated, survived for long periods,

Table 1. Suppression of various tumors by targeting the 4-1BB–4-1BBL pathway

Agent	Cancer type suppressed	References
Anti-4-1BB mAb	Ag104A sarcoma	(27)
	MCA205, GL261 glioma	(30)
	C3 tumors, TC1 carcinoma	(31)
	J558 tumors	(32)
	A549 tumors	(38)
Variants of anti-4-1BB	K1735 melanoma	(45)
	M108 tumors	(42)
	K562 erythroleukemia	(43)
	FR α tumors	(44)
Anti-4-1BB combination therapy	B16 melanoma	(46, 48, 54–56, 58)
	Renal cell carcinoma	(34)
	K1735 melanoma	(45)
	CT26 colon carcinoma	(46)
	MCA205 tumors	(49)
	MC38 tumors	(49, 54)
	M109, EMT6 tumors	(51)
4-1BBL and its variants	Liver metastases	(59)
	Cholangiosarcoma	(60)
	Neuroblastoma	(61)
	AML, Wilms tumor 1	(62, 67)
	Colon 2A and 26 tumors	(18)
	P815 plasmacytoma	(64)
	K562/AO2 tumors	(65)
	Mouse forestomach carcinoma	(54)

and more importantly, retained their chimeric antigen receptor intact.

Conclusions

Taken together, the findings we have summarized clearly support the therapeutic potential of targeting the 4-1BB–4-1BBL pathway in cancer treatment. Of importance, targeting the 4-1BB pathway eliminates established tumors, and the fact that anti-4-1BB therapy acts in concert with other anticancer agents and/or radiation therapy to eradicate nonimmunogenic and weakly immunogenic tumors is an additional benefit (Table 1). Furthermore, the antitumor activity of *ex vivo* anti-4-1BB–stimulated

leukocytes in adoptive cell therapy has tremendous potential. Future studies should be directed toward translating anti-4-1BB therapy into clinical trials in various forms of cancer.

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No potential conflicts of interest were disclosed.

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