Chapter 8

4-1BB as a Therapeutic Target for Human Disease

Seung-Woo Lee and Michael Croft*

Abstract

4-1BB (CD137) is being thought of as an attractive target for immunotherapy of many human immune diseases based on encouraging results with 4-1BB agonistic antibody treatment in mouse models of cancer, autoimmune disease, asthma and additionally as a means to improve vaccination. In this review, we will summarize the results of basic research on 4-1BB and 4-1BB immunotherapy of disease and provide some potential mechanistic insights into the many stimulatory and regulatory functions of 4-1BB.

Introduction to Basic Research

4-1BB (CD137, ILA, TNFRSF9), a member of the tumor-necrosis factor receptor (TNFR) superfamily, was originally identified as an inducible costimulatory molecule on activated T-cells.[1-4] The ligand of 4-1BB (4-1BBL, TNFSF9), a member of the TNF super-family, was later found expressed on activated antigen-presenting cells (APC) such as B-cells, macrophages and dendritic cells (DC).[3,5-7] Based on these expression characteristics and early functional data, it was thought that 4-1BBL expressed on activated APC binds to 4-1BB that is induced on T-cells, generating positive signals inside T-cells to help them function and to augment various aspects of immunity. Many in vitro studies have supported this concept showing that ligation of 4-1BB by either agonistic antibody, a soluble 4-1BBL molecule, or 4-1BBL-expressed on fibroblast cells can costimulate both CD4 and CD8 T-cells, leading to enhanced proliferation and cytokine secretion.[9-11] In line with this positive regulation of T-cells, similar to other members of the TNFR family, the ligation of 4-1BB can recruit TNFR-associated factor (TRAF) adaptor molecules,[12-14] and activate pro-inflammatory signaling pathways involving phosphatidylinositol-3-kinase (PI3K), protein kinase B (PKB, also known as Akt) and nuclear factor κB (NF-κB) pathways, as well as up-regulate expression of anti-apoptotic Bcl-2 family molecules that aid in survival.[15,16]

4-1BB$^{-/-}$ and 4-1BBL$^{-/-}$ mice show no obvious defects in the development of lymphocytes and lymphoid organs.[17,18] The in vivo role of 4-1BB and 4-1BBL in T-cell immunity has largely been addressed in 4-1BBL$^{-/-}$ mice in various infectious model systems such as monitoring response to Listeria,[19] LCMV,[20] and influenza virus.[21,22] Overall, 4-1BBL$^{-/-}$ mice were observed to generate decreased CD8 T-cell responses, although some variations in the deficiency were seen between models, ranging from moderate to pronounced, with the deficiency tending to manifest late after infection. On the other hand, 4-1BBL$^{-/-}$ mice have been observed to generate normal CD4 T-cell responses to viruses, prompting the suggestion that 4-1BB/4-1BBL preferentially influences CD8 T-cell responses. Arguing against this strict dichotomy are the in vitro results demonstrating costimulation of CD4 cells,[8,9] and adoptive transfer experiments with CD4 T-cells showing

*Corresponding Authors: Michael Croft—Molecular Immunology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, California 92037, USA. Email: mick@liai.org

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normal expansion to protein Ag in LPS in 4-1BBL$^{-/-}$ mice, but defects in the late primary phase and in secondary responses.[23]

In contrast to these results, other data suggest the biology of 4-1BB is much more complicated than positively regulating the interactions between T-cells and APC. A number of studies that will be described in more detail below have shown apparent negative effects of agonistic antibodies to 4-1BB when administered in vivo, particularly in autoimmune situations and other inflammatory responses where these reagents surprisingly suppressed T-cell responsiveness and inflammation. Furthermore, splenocytes from 4-1BB$^{-/-}$ mice displayed hyper-, not hypo-, proliferation to mitogens[17] and adoptive transfer experiments with antigen-specific T-cells that could not express 4-1BB clearly showed enhanced rather than suppressed initial CD4 and CD8 T-cell responses in vivo.[24,25] These results have promoted the idea that in the physiological setting, 4-1BB can play both a negative regulatory role in immunity in addition to its apparent positive role. Here, we will review some of the more recent literature on 4-1BB and discuss the implications of these findings with regard to potential targeting of 4-1BB and 4-1BBL for therapy of immune disease.

Expression of 4-1BB and 4-1BBL

The duration of 4-1BB expression on activated CD4 and CD8 T-cells is variable depending on experimental conditions. It can be seen several hours after T-cell activation and peak within 2 days,[4] but can be long-lasting in some cases and be expressed for as long as a 7 day period following antigen challenge.[25] Exceptions to inducible expression of 4-1BB on T-cell subsets are now recognized, with CD4+CD25+ regulatory T-cells (Treg)[26-28] and natural killer T-cells (NKT), including invariant Vα14 NKT (Lee, S.-W. and Croft, M. unpublished results), showing constitutive expression of 4-1BB without further stimulation. Some T-lymphoma cell lines express 4-1BBL[6] but so far there are no reports of 4-1BBL being expressed on primary T-cells.

Although originally thought to be exclusive to T-cells, recent studies have shown that the expression of 4-1BB in vivo is very promiscuous. Natural killer (NK) cells express 4-1BB whose ligation can induce proliferation and secretion of IFN-γ.[29,30] Some NK-cells express 4-1BB constitutively and others might up-regulate it after stimulation. It is interesting that some myeloid lineage cells, such as DC,[6,31,32] granulocytes[33] and mast cells,[34] also express 4-1BB. DC express both 4-1BB and 4-1BBL upon activation. So far it has been reported that follicular DC,[31] spleen DC,[6,32] and GM-CSF-induced bone marrow (BM) DC[32] express 4-1BB and 4-1BBL. It is not clear why DC express both molecules, possibly at the same time, but the cross-linking of 4-1BB or 4-1BBL on these cells can induce cytokines such as IL-12 and IL-6.[6,31,35] 4-1BB and 4-1BBL can also be expressed on BM-derived mast cells after stimulation through the high-affinity receptor for IgE (FcεRI).[34] Both 4-1BB$^{-/-}$ and 4-1BBL$^{-/-}$ mast cells have defects in cellular function such as degranulation and cytokine production upon FcεRI stimulation, suggesting that 4-1BB/4-1BBL interactions can costimulate mast cells perhaps analogous to the action on T-cells. Collectively, the expression profiles of 4-1BB and 4-1BBL on various subsets of immune cells raise the interesting notion that both molecules might control innate and adaptive immunity by bridging multiple cell-to-cell interactions.

Therapeutic Effects of Targeting 4-1BB or 4-1BBL

Cancer Immunology

Following the initial observation that the ligation of 4-1BB with agonistic antibody augmented the activity of T-cells, 4-1BB has been thought of as an attractive target for many diseases in which augmentation of the numbers and reactivity of antigen-specific T-cells might be desirable. In particular, the efficacy of targeting 4-1BB has been investigated in many murine tumor models (Table 1). Ligation of 4-1BB by either agonistic antibody treatment in vivo, or by transfecting tumor cells directly with 4-1BBL, can lead to expansion of tumor-reactive T-cells and suppression of tumor growth and in some cases regression of established tumors.[36-38] Moreover, anti-4-1BB has also been found effective for regression of poorly immunogenic tumors, such as C3 and

Table 1. Immunotherapy of tumors in mice through targeting 4-1BB

Method	Tumor Type	Immune Cells Involved	Ref.
Agonist 4-1BB-specific antibody	Sarcoma, mastocytoma	CD8 and CD4	36
Tumor transfected with 4-1BBL	Sarcoma, mastocytoma	CD8	37
Tumor transfected with 4-1BBL	Lymphoma	CD8	38
Agonist 4-1BB-specific antibody	Fibrosarcoma	CD8	62
Agonist 4-1BB-specific antibody	Lung carcinoma, melanoma	CD8	39
Tumor transfection with Fv of 4-1BB-specific antibody	Melanoma	CD4 and NK	41
Agonist 4-1BB-specific antibody with adenovirus expressing IL-12	Colon carcinoma	CD8 and NK	43
Agonist 4-1BB-specific antibody with dendritic cell vaccine	Fibrosarcoma	CD8, NK, CD4	42
Adenovirus expressing Ig-4-1BBL	Hepatic colon carcinoma	CD8	44

melanoma, when given in combination with peptide immunization.[39] Similarly, anti-4-1BB was also shown to be capable of breaking tolerance of CD4 T-cells in more experimental settings and reversing poor T-cell responsiveness that is characteristic of growing old.[40] These results suggest that the strong costimulatory signal imparted by agonistic antibody can transform the immune status of T-cells from being inert (i.e., where ignorance and tolerance are operative) into being active. The majority of tumor studies have shown that antigen-specific CD8 cells are crucial for tumor immunotherapy mediated by anti-4-1BB, with the assumption that the T-cell is the direct recipient of 4-1BB signals. However, roles for CD4 and NK-cells in these processes have been highlighted,[41-43] implying multiple targets of action. Furthermore, another approach was recently developed for systemic delivery of 4-1BBL through recombinant adenovirus, which could be easily translated into immunotherapy of human cancer with human 4-1BBL.[44]

Viral Immunology

Based partially on results showing that 4-1BBL$^{-/-}$ mice generate reduced CD8 T-cell responses against certain viruses, 4-1BB stimulation has also been exploited for boosting anti-viral immunity. 4-1BB agonistic antibody was shown to enhance the numbers and reactivity of viral antigen-specific CD8 cells following intranasal infection with influenza and interestingly, it was shown to result in strongly enhanced CD8 cell responses to subdominant peptide epitopes of influenza that might also be useful for protection.[45] More recently, several groups have additionally used anti-4-1BB to boost immune responses following vaccination. Codelivery of an agonistic antibody with DNA[46] or recombinant adenovirus[47] vaccination strategies augmented T-cell immunity to human immunodeficiency virus (HIV) and hepatitis C virus (HCV) proteins, respectively. Another protocol used a recombinant poxvirus encoding 4-1BBL and viral antigen to enhance anti-viral immunity.[48] Although more studies are needed to evaluate 4-1BB as a realistic target for augmenting the efficacy of anti-viral vaccination, these studies do suggest that 4-1BB may be a promising candidate as an adjuvant for developing vaccines against human viruses.

Autoimmunity

Another avenue of immunotherapy that has recently emerged as a potential application of targeting 4-1BB is in autoimmune disease. The most logical route to therapy of these diseases, based on the idea that 4-1BB binding to 4-1BBL augments T-cell and APC activity, would be to suppress these interactions using blocking reagents to 4-1BBL. This has been examined in some animal models but with largely unimpressive results. Treatment with anti-4-1BBL blocking antibody reduced the development of collagen-induced arthritis (CIA) only moderately with no

evident suppressive action against established CIA.[49] Furthermore, anti-4-1BBL could not inhibit the induction of experimental allergic conjunctivitis (EAC).[50]

In contrast to these results, it was very surprising when the group of Fu and colleagues found that the same agonistic 4-1BB antibody used to promote anti-tumor immunity also resulted in ameliorating both the incidence and severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). Interestingly, anti-4-1BB also inhibited the relapse that occurs in EAE that is characteristic of the human disease.[51] Since this result was published, many other studies have followed and also injected anti-4-1BB into various autoimmune disease models with the majority of data demonstrating strong inhibition of the development and progression of these diseases (Table 2).

Agonistic 4-1BB antibody successfully ameliorated acute and established lupus-like symptoms in MRL/lpr[52] and NZB × NZW F1[53] mouse strains that develop spontaneous disease resembling human systemic lupus erythematosus (SLE). In both animal strains, 4-1BB targeting reduced autoantibody production and renal disease, which suggests that anti-4-1BB ultimately inhibited pathogenic B-cell responses. In MRL/lpr mice, anti-4-1BB depleted B-cells in the periphery through an unclear mechanism involving apoptosis and IFN-γ.[52] In contrast, B-cell loss was not seen in the NZB × NZW F1 strain, however, germinal center (GC) formation, which is central to the development of functional class-switched B-cells, was abolished.[53] Correlating with these studies, a recent report also showed that anti-4-1BB inhibited GC formation apparently by diminishing FDC networks in B-cell follicles.[54]

Agonistic 4-1BB antibody also inhibited the development of CIA in DBA/1 mice which is a common animal model reminiscent of human rheumatoid arthritis.[49] Similar to the EAE study, anti-4-1BB suppressed antigen (collagen)-specific CD4 T-cell responses. In this case, it was proposed that IFN-γ secretion was also involved in the suppressive activity along with indoleamine-2,3-dioxygenase (IDO), an enzyme that regulates tryptophan metabolism.[55] The source of these molecules was not clear but anti-4-1BB treatment resulted in expansion of a novel population of cells expressing CD8 and CD11c that were proposed to directly or indirectly mediate the suppression.[49] More recently, anti-4-1BB immunotherapy was also applied to Type-I diabetes that arises spontaneously in non-obese diabetic (NOD) mice. 4-1BB is a candidate gene thought to be involved in autoimmune diabetes and was mapped in the Idd (insulin dependent diabetes) loci known to be associated with susceptibility to this disease.[56] Similar to the other autoimmune studies, anti-4-1BB treatment in NOD mice strongly prevented diabetes.[28] Although the mechanism of action was again not clear, of note antibody treatment significantly increased the numbers of CD4+CD25+FOXP-3+ regulatory T-cells (Treg), which may aid in dampening the action of pathogenic T-cells.

Inflammation and Transplantation

Anti-4-1BB treatment has also been used in other disease scenarios in mice, such as in models of allergic asthma[57,58] and chronic graft-versus-host disease (cGVHD).[59] Quite remarkably, this antibody was again shown to prevent the development of the symptoms associated with these immune reactions and also reversed established disease. Anti-4-1BB in these cases was described to down-regulate the activity of CD4 T-cells, although suggested mechanisms of action varied between the reports. In the asthma model, CD4 T-cells from 4-1BB antibody-treated mice did not proliferate when restimulated with antigen in vitro, but did proliferate to IL-2, leading to the suggestion that targeting 4-1BB induced an anergic state in CD4 T-cells.[57] In contrast, in the cGVHD model, it was suggested that the antibody accelerated activation-induced cell death (AICD) of donor CD4 T-cells, whose expansion and survival was critical for inducing disease.[59] Furthermore, the therapeutic effect of anti-4-1BB in the asthma model was partly dependent on CD8 T-cells and IFN-γ but in the cGVHD model CD8 cells and IFN-γ had no apparent roles.

Thus, although a common theme has been that anti-4-1BB suppresses immune disease, the mechanism by which it has done so has not been clear in many cases and might have involved both similar and dissimilar processes depending on the target disease.

Table 2. Amelioration of autoimmune disease in mice through agonistic 4-1BB antibody therapy

Disease Model	Effect on B Cell Number	Effect on Ig Response	Effect on T-Cell Response*	Effect of IFN-γ Neutralization**	Reference
Lupus in NZB x NZW F1	↔	↓↓↓ (IgG)	ND	ND	53
Inflammatory bowl disease in Balb/c	ND	ND	CD4 ↓, CD8 ↑ CD4+CD25+Treg ↑	ND	61
Lupus in MRL/lpr	↓↓↓	↓↓ (IgG)	CD4 ↓↓, CD8 ↑	Blocking of pathogenic B-cell depletion	52
Experimental allergic conjunctivitis in Balb/c	↑	↓↓ (IgE)	CD4 ↑, CD8 ↑↑	No effect##	50
Rheumatoid arthritis in DBA	ND	↓↓ (IgG)	CD4# ↓↓↓, CD8 ↑↑	Reversed antibody effect	49
Hg-autoimmunity in A.SW	↓↓↓	↓↓↓ (IgE, IgG)	CD4 ↑↑, CD8 ↑↑	Partial blocking of B-cell loss	63
Experimental autoimmune encephalomyelitis in C57BL/6	ND	ND	CD4# ↓↓↓	ND	51
Autoimmune diabetes in NOD	ND	ND	CD4+CD25+ Treg ↑	ND	28

*Represents effect on total T-cells in spleen except #indicating antigen-specific recall responses.
**IFN-γ was neutralized by anti-IFN-γ treatment except ##indicating IFN-$\gamma^{-/-}$ mice.
ND, not done.
Note, in all cases, disease symptoms and pathology were strongly suppressed by anti-4-1BB.

Possible Mechanisms of Action of 4-1BB Agonistic Antibodies

A challenging feature of agonistic 4-1BB antibody-mediated therapy, particularly if this type of reagent is to be considered for the clinic, **is how it modulates immune responses in completely contrasting disease situations.** The same agonistic antibody operates positively to augment immunity against tumors and infectious pathogens, while it functions negatively to suppress immunity and reduce pathology in autoimmune diseases and inflammatory situations. One of the reasons might be the promiscuous expression of 4-1BB on many kinds of immune cells that have specific and perhaps opposing roles in various patho-physiological circumstances. As discussed earlier 4-1BB is expressed on T-cells including Treg and NKT, as well as NK-cells, DC, granulocytes and mast cells, leading to one hypothesis that the cellular target of anti-4-1BB dictates whether its action becomes stimulatory or suppressive. Whether this is truly a factor is not clear, as the expression of 4-1BB in most disease situations has not been characterized.

A positive role of anti-4-1BB in vivo is perhaps straightforward in that the antibody most likely directly stimulates T-cells, NK-cells, or mast cells, leading to augmented activities of these cell types that could participate in anti-tumor or anti-viral activity (Fig. 1). The majority of in vitro data clearly support this especially in T-cells, showing biochemical changes in pro-inflammatory intracellular signaling pathways after 4-1BB stimulation.[3,4] On the other hand, a regulatory role, or resultant suppressive activity, of anti-4-1BB is likely quite complicated. For example, it has been suggested that the negative effects of administering anti-4-1BB could be due to promoting apoptosis of CD4 cells[59] or inducing a state of unresponsiveness or anergy in CD4 cells.[57] Alternatively, 4-1BB antibody treatment has been proposed to directly induce regulatory cells in the CD8 lineage. In one study, such regulatory CD8 cells were suggested to suppress other T-cells through TGF-β in an IFN-γ dependent manner.[60] Whereas in another study, a population of CD8 cells expressing CD11c were found to expand after targeting 4-1BB. Through secretion of IFN-γ these CD8 cells directly or indirectly were reported to regulate IDO production, a powerful suppressor of T-cell proliferation, from macrophages and/or DC.[49] Interestingly in these settings, it can still be argued that the action of anti-4-1BB was again positive and stimulatory, even though the ultimate outcome was negative and suppressive for the immune response. In terms of augmenting CD8

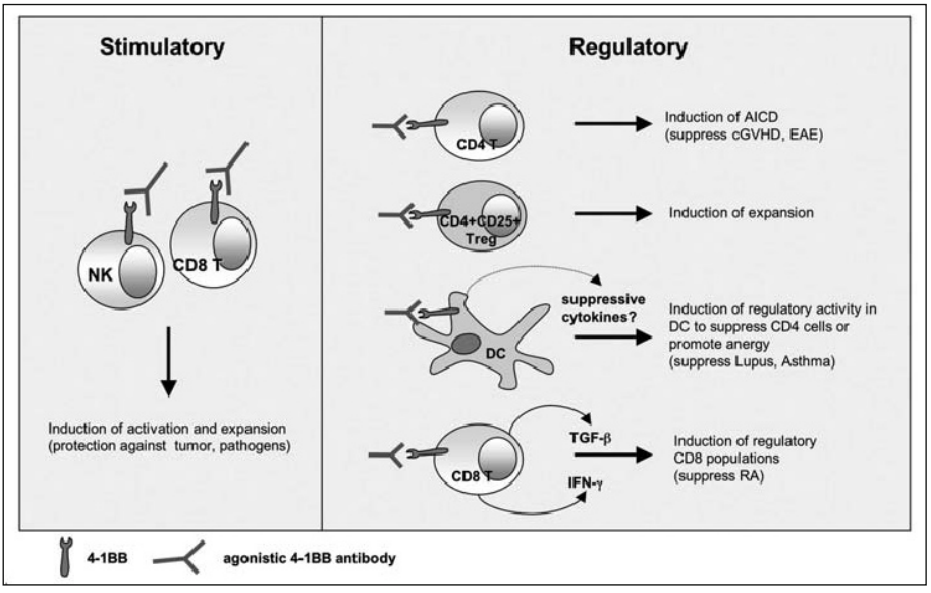

Figure 1. Possible mechanisms underlying the dual function of anti-41BB in augmenting or suppressing immunity.

activity associated with anti-tumor immunity or anti-viral responses, as opposed to augmenting CD8 regulatory activity, the issue then becomes whether the CD8 cells in each individual situation were really different. The CD8 populations that were characterized to be regulatory appeared to represent alternate subsets of cells, since one population expressed CD11c and did not produce TGF-β, whereas the other did not express CD11c but did produce TGF-β. Despite these apparent differences, both populations exerted suppressive activity that was dependent on IFN-γ, a feature that was shared with CD8 cells elicited by anti-4-1BB that protected against tumor growth and viral replication. Further studies will be required to understand whether 4-1BB signaling can differentially induce alternate subsets of T-cells and if so what will dictate whether they become regulatory or nonregulatory.

Possibly related to the latter point, but relevant to potential effects on CD4 cells as opposed to CD8 cells, is the finding that 4-1BB is also constitutively expressed on CD4+CD25+Treg cells. In one report, agonistic 4-1BB antibody had no effect on expansion of these Treg cells but it did neutralize their suppressive function.[27] In contrast, 4-1BB ligation by 4-1BBL was shown to expand Treg cell numbers in vitro and in vivo without a loss in their suppressive activity.[26] Furthermore, anti-4-1BB enhanced the number of CD4+CD25+ Treg in NOD mice[28] and in mice undergoing colitis,[61] situations in which the antibody suppressed these diseases. These data then also raise the possibility that 4-1BB stimulation may preferentially modulate the number and/or function of several types of Treg and the nature of these regulatory populations could vary in specific disease situations.

Another possible target of anti-4-1BB was highlighted in the lupus studies with NZB × NZW F1 mice. Here, anti-4-1BB mediated suppression of disease was abolished after adoptive transfer of autoantigen-primed CD4 T-cells or bone marrow derived DC.[53] This suggested that the regulatory function of the agonistic antibody might have been transmitted in this case through DC. It is certainly plausible that 4-1BB antibodies target DC directly as many activated DC can express 4-1BB and DC are known to be capable of both stimulatory or suppressive activity, e.g., through production of pro-inflammatory cytokines such as IL-6 and IL-12, or secreting inhibitory cytokines such as IL-10 and TGF-β. As before, more studies are needed to test the hypothesis that signals to DC from 4-1BB can impact immune disease and explain some of the dramatic effects of anti-4-1BB therapy.

Concluding Remarks

Based on very promising results from many different disease models, 4-1BB agonistic antibody immunotherapy is indeed a candidate for clinical modulation of human diseases. One important issue will be potential side effects. For example, anti-4-1BB treatment dramatically reduces pathogenic T- and B-cell responses in many autoimmune disease situations (Table 2), however, it seems to globally influence nonpathogenic immune responses. The repeated injection of anti-4-1BB in naïve mice leads to development of severe immunological anomalies, including splenomegaly, lymphadenopathy, hepatomegaly, multi-focal hepatitis and anemia.[64] These adverse effects are again apparently dependent on activation of CD8 T-cells and involve production of several cytokines such as IFN-γ, TNF and Type I IFN. More studies will then be needed to determine any unwanted immunopathological consequences of administering anti-4-1BB in disease models, as well as to understand any dosage effects of the antibody in terms of pro- or anti-inflammatory activities. It does not need to be stressed that testing in preclinical animal models of disease and in nonhuman primates is essential before contemplating human clinical trials. Recent approaches with systemic delivery of soluble 4-1BBL by recombinant viruses is an alternative to the use of anti-4-1BB, although at present there is no information on whether 4-1BBL will replicate the therapeutic activity of the antibody or lead to similar side effects.

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