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SA-4-1BBL as a Novel Adjuvant for the Development of Therapeutic Cancer Vaccines

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Abstract

Tumor associated antigen (TAA)-based therapeutic vaccines have great potential as a safe, practical, and cost-efficient alternative to standard treatments for cancer. Clinical efficacy of TAA-based vaccines, however, has yet to be realized and will require adjuvants with pleiotropic functions on immune cells. Such adjuvants need not only to generate/boost T cell responses, but also reverse intrinsic/extrinsic tumor immune evasion mechanisms for therapeutic efficacy. This review focuses on a novel agonistic ligand, SA-4-1BBL, for 4-1BB costimulatory receptor as an adjuvant of choice because of its ability to: i) serve as a vehicle to deliver TAAs to dendritic cells (DCs) for antigen uptake and cross-presentation to $CD8^+$ T cells; ii) augment adaptive Th1 and innate immune responses; and iii) overcome various immune evasion mechanisms, cumulatively translating into therapeutic efficacy in preclinical tumor models.

Keywords

Costimulation; Adjuvants; 4-1BB; CD137; SA-4-1BBL; Cancer vaccines

Introduction

Therapeutic vaccines against tumor represent a practical and potentially effective alternative to standard treatments due to their specificity for tumor and ability to establish long-term immune memory critical for the control of recurrences [1,2]. T cells play a critical role in driving immune effectors to destroy cancer [3,4,5]. The most relevant evidence for the importance of T cells in cancer immunotherapy come from recent clinical trials demonstrating the benefit of using antibodies to immune checkpoint blockers cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) receptors [6,7]. T cells require a minimum of three signals (1, 2, and 3) for activation, acquisition of effector function, and establishment of long-term recall responses [8]. Signal 1 is antigen-specific

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Competing interests disclosure

The SA-4-1BBL described in this manuscript is developed at the University of Louisville, Louisville, KY, and licensed by ApoVax, Inc., Louisville, KY, for which HS serves as CSO and member of the Board of Directors, and HS and ESY have significant equity interest in the Company. The other author, R.K.S. disclosed no potential conflict of interest.

and delivered by the interaction of T-cell receptor (TCR) with major histocompatibility complex (MHC)-peptide complex on the surface of DCs. This signal is qualified by the delivery of costimulation, Signal 2, through various cell surface receptors and ligands belonging to the CD28 and tumor necrosis factor receptor (TNFR) superfamilies. Acquisition of Signal 3 via various cytokines enables T cells to undergo a series of metabolic changes that culminate into productive T cell activation and the generation of effective immune responses, which combat infections and tumors. The lack of costimulation during T cell activation by MHC-peptide complexes results in T cell anergy and abortive immune responses [9]. Therefore, costimulation is critical for the generation of productive adaptive immune responses, and as such may serve as an important target for the development of novel adjuvants.

This review focuses on a novel recombinant agonist, SA-4-1BBL, of the 4-1BB costimulatory molecule of the TNFR superfamily as an immune adjuvant for TAA-based subunit vaccine with demonstrated ability to drive T cell activation, expansion, survival, and acquisition of effector function, while overcoming various cancer immune evasion mechanisms with therapeutic efficacy in various preclinical tumor models [3,4,10,11,12,13,14]. The key qualitative/quantitative differences between SA-4-1BBL and agonistic 4-1BB monoclonal antibodies (mAbs) in regulating the function of T cells and other immune cells will also be highlighted. An argument based on these differences as well as safety profile of SA-4-1BBL over agonistic 4-1BB mAbs will be made for the preferential choice of SA-4-1BBL for the development of therapeutic vaccines against cancer. Finally, the prospect of using SA-4-1BBL in combinatorial approaches, including established immune adjuvants and standard/targeted cancer treatment regimens, will be discussed.

Problems and prospects for cancer vaccine design

Preventive vaccines against infections have been the miracle of advanced medicine with significant health and economic impact globally. In marked contrast, the development of effective therapeutic vaccines, particularly against cancer, has been a major challenge in spite of extensive effort over the past several decades. The lack of progress is primarily due to our incomplete understanding of immune responses against cancer as well as intrinsic and extrinsic immune evasion mechanisms employed by the progressing tumor. The major challenge and requirement for prophylactic vaccines against infections is to prime, in most cases, a naïve immune response in a healthy individual to generate protective T helper 2 (Th2)-driven humoral immune responses. In marked contrast, therapeutic vaccines against cancer need to achieve much more than prophylactic vaccines to attain therapeutic efficacy. First, therapeutic vaccines need to generate Th1 cellular responses in a host whose immune system not only has seen cancer and failed to control it, but also has been compromised by standard cancer treatment regimens. Second, therapeutic vaccines need to overcome various immune evasion mechanisms, which are most likely the reason for tumor progression. Third, in case of subunit vaccines, self TAAs are generally not immunogenic and in most cases immune responses to such antigens have been tolerized. Other contributing challenges to the failure of TAA-based therapeutic vaccines include antigenic drift, antigen heterogeneity, and selection of appropriate antigens. Therefore, immune adjuvants that are not only effective in

generating anti-tumor immune responses, but also are able to overcome various immune evasion mechanisms without causing toxicity stand a good chance for delivering the promise of therapeutic cancer vaccines.

The subunit cancer vaccines based on TAA proteins, peptides, or DNA present an attractive choice due to their ease of production, storage, off-the-shelf availability, and probable safety profiles. However, despite considerable efficacy in rodent tumor models, such vaccines have shown limited immune responses and/or therapeutic efficacy in the clinic [1,2]. Although complete mechanisms underlying vaccine inefficacy are not fully understood and include those enumerated above, the immune status of cancer patients, extent of tumor burden, and most importantly the design of vaccine formulation may provide an explanation. The perceived key elements of vaccine formulations include one and/or multiple TAA(s) and an adjuvant to enhance breadth and magnitude of the immune response against the antigen(s). The choice of TAA is critical to vaccine efficacy as it distinctly marks the tumor for destruction by $CD8^+$ T as well as $CD4^+$ T killer cells [15]. A TAA that is not only mutated/overexpressed by the tumor, but is also actively involved in tumor survival, progression and, most importantly, in immune evasion may be an optimal choice. Such a TAA is expected to show persistent expression by the tumor and be refractive to antigenic drift and escape caused by vaccine induced immune pressure.

Immune adjuvants

Adjuvants, the second component of vaccine formulations, are of utmost importance to the enhancement of TAA-generated immune responses and their long-term persistence. Clinically approved adjuvants and most adjuvants under development achieve such effects by igniting innate immune responses to bridge over and stimulate adaptive immunity. For example, most toll like receptor (TLR) agonist-based adjuvants activate innate immunity by targeting antigen-presenting cells (APCs), such as DCs, positively impacting their migration, maturation, antigen presentation, and expression of costimulatory molecules [16]. Adjuvants shape or polarize the nature of immune responses, such as T helper 1 (Th1) *vs*. Th2, and T *vs.* B cells. Adjuvants may also play a decisive role in the generation and maintenance of immunological memory as well as reversal of immunoregulatory mechanisms, such as $CD4^+CD25^+FoxP3^+$ T regulatory (Treg) cells, myeloid derived suppressor cells (MDSC), and T cell anergy [4,10,17,18,19]. Therefore, adjuvants that modulate innate, adaptive, and regulatory immunity in favor of effective anti-tumor immune responses with safety profile may have the best chance for achieving therapeutic efficacy in the clinic. Given the importance of DCs in the generation of robust T cell responses, adjuvants that also serve as a vehicle to deliver TAAs to DCs for accelerated antigen uptake, processing, and cross-presentation to $CD8^+$ T cells will have added benefits for generating timely and robust immune responses. This may also overcome the danger of reported immune tolerization when the antigen is encountered by DCs without adjuvant in an immunosuppressive microenvironment [20], such as within the tumor and tumor-draining lymph nodes.

Alum, the only Food and Drug Administration (FDA) approved adjuvant for human vaccines, induces effective Th2 responses with minimal efficacy in eliciting Th1 immunity [21] necessary for the eradication of tumors. Emulsion adjuvants are also often used in

experimental animals and are paving their way towards clinical trials in humans. TLR agonists have recently been the subject of intense preclinical and clinical investigations as the most promising vaccine adjuvants. Most TLR agonists activate APCs for maturation and improved antigen uptake and presentation, which in turn lead to the generation/augmentation of acquired immunity. One such TLR4 agonist is the monophosphoryl lipid A (MPL), a detoxified derivative of bacterial lipopolysaccharide. MPL has recently been approved for human use in the context of a prophylactic vaccines against human papilloma virus (HPV) [22]. However, several studies demonstrated that TLR signaling also generates regulatory immunity that may counterbalance productive immune responses against tumors and infections [23]. For example, various TLR agonists, such as MPL, CpG ODN, and Poly I:C, generate T effector cell responses. However, they concomitantly expand T regulatory cells [23,24,25], which may negatively impact the overall anti-tumor productive immune responses. Moreover, TLRs are expressed on various immune and non-immune cells, such epithelial cells [16]. As such, stimulation through these receptors may generate a wide-range of responses at therapeutic doses that result in intolerable toxicity.

The use of immune modulating cytokines, such as IL-2 and GM-CSF, as potential adjuvants is also associated with the generation of mixed effector and regulatory immune reposes against tumors. IL-2 not only expand T effector cells, but also is a critical growth factor for immunosuppressive Treg cells [26]. Similarly, GM-CSF, which enhances DC maturation, activation, and function, can act as a double edge sword for the generation of effector vs. tolerogenic anti-tumor responses depending on the timing and dose of administration [27,28]. Incomplete Freund's adjuvant, another well characterized adjuvant extensively used in preclinical and clinical settings, in a peptide-based vaccine formulation induced tumor specific $CD8^+$ T cell sequestration, dysfunction, and deletion at the vaccination site, leading to poor antitumor immunity [29]. Therefore, the development of novel adjuvants that specifically or preferentially generate effector innate and adaptive immune responses and inhibit/minimize regulatory immune responses in favor of heightened therapeutic efficacy against tumor in the absence and/or tolerable toxicity will be key to the success of therapeutic vaccines.

Finally, therapeutic vaccines may benefit from adjuvants that also serve as a vehicle to deliver TAAs to DCs for the most desired immune outcome. Targeted delivery of TAAs to DCs using various approaches has proven effective for the generation of immune responses at low antigen doses [30,31]. For example, the targeted delivery of human survivin as xenogeneic TAA to DCs using a mAb to the DEC205 receptor expressed on these cells resulted in robust survivin-specific $CD4^+$ T cell responses as characterized by the production of IFN-γ, TNF-α, and IL-2 cytokines [30]. An agonistic mAb to CD40 and poly I:C were used as adjuvants. This approach also induced lytic MHC class II-restricted T cell effector and memory responses without a significant effect on the generation of $CD8^+$ T cell responses [30]. Similarly, mAbs to human (h)DEC205 molecule engineered to include human immunodeficiency virus (HIV) Gag p24 protein when used to vaccinate hDEC205 transgenic mice along with poly I:C as an adjuvant induced production of high titers of Abs as well as IFN-γ and IL-2 by $CD4^+$ T cells [31]. Anti-hDEC205 also improved cross-presentation of Gag antigen to $CD8^+$ T cells from HIV-infected individuals [31]. Therefore, it is envisioned that an immune adjuvant having pleiotropic effects on various immune

effector cells, required for the generation of therapeutic tumor-specific immune responses, that also serve as a vehicle to deliver TAAs to DCs may serve an ideal choice. In this context, SA-4-1BBL has potential as a preferred adjuvant because of its i) pleiotropic positive effects on the immune effectors for tumor eradication, ii) potential to overcome various immune evasion mechanisms, and iii) ability to serve as a vehicle to target TAAs to DCs for a heightened immune response against tumor.

Costimulatory pathways and their role in the regulation of immune responses

Costimulatory molecules can be broadly categorized into four superfamilies that include CD28, TNFR, adhesion molecules/integrins, and T cell Ig domain and mucin domain. In particular costimulation delivered by members of CD28 and TNFR superfamilies is of critical importance to T cell primary and long-term recall responses. The CD28 family members include stimulatory CD28, ICOS, and inhibitory, PD1, CTLA-4, B and T lymphocyte attenuator receptors. The prototype CD28 receptor is constitutively expressed on T cells and stimulation through this receptor by CD80/86 ligands expressed on APCs is critical to T cell activation and initiation of adaptive immune responses [9,32,33]. In contrast, most of the TNFR costimulatory molecules are inducibly expressed on activated T effector cells [34], and as such targeting these costimulatory receptors provide a unique opportunity to expand the ongoing immune responses in an antigen-specific fashion, thereby preventing non-specific immune responses and associated potential collateral damage.

The TNFR superfamily consists of more than 27 members that can be divided into two subgroups based on the presence or absence of a death domain in the cytoplasmic tail of the receptors. The members of TNFR costimulatory superfamily playing important roles in adaptive immunity include, but not limited to, 4-1BB (CD137), OX-40 (CD134), CD27, glucocorticoid-induced TNFR-related protein (GITR), and CD40L (Table 1). The 4-1BB receptor and its ligand, 4-1BBL, have emerged as the pair with robust effects on the expansion of activated $CD8^+$ T cells, their survival, acquisition of effector function, and establishment of long-term memory [19,35,36,37,38]. As such, targeting this receptor/ligand pair for immune modulation presents a great opportunity for devising therapeutic interventions that require $CD8^+$ T cells as the primary immune effectors. Indeed, this pathway has extensively been targeted for cancer immunotherapy in various animal models with demonstrated therapeutic efficacy and is presently being pursued in clinical trials [4,39,40].

4-1BB costimulatory pathway and its role in the regulation of immune responses

The 4-1BB (CD137) receptor was originally discovered in activated, but not resting, $CD4^+$ and $CD8^+$ T cells [41]. The expression of this receptor was subsequently demonstrated on various cells of innate, adaptive, and regulatory immunity. 4-1BB is inducibly expressed on natural killer (NK) cells, NKT cells, monocytes, macrophages, neutrophils, mast cells, and eosinophils [42,43]. A subpopulation of DCs and Treg cells are the only known immune

cells to express 4-1BB constitutively [43,44]. The expression of 4-1BB is not restricted to hematopoietic derived cells as various parenchymal cells also express 4-1BB under inflammatory conditions. For example, 4-1BB receptor was shown to be expressed on endothelial cells in blood capillaries in tumor beds, but not in healthy vasculature, in response to hypoxia with significant positive impact on the therapeutic efficacy of agonists Abs to the 4-1BB receptor [45]. The expression of the only known ligand, 4-1BBL, is inducible and restricted to APCs, such as DCs, macrophages, and B cells [33,43,44].

Ligation of 4-1BB by its agonists results in aggregation and recruitment of TNFR-associated factors (TRAFs) adopter proteins, TRAF-1 and TRAF-2, to its cytoplasmic tail, initiating a series of signaling events that culminate in NF-κB and MAPK activation [46,47,48]. The final outcome of the signaling pathway is better cell survival due to up regulation of anti-apoptotic genes, such as *bcl-X_L* and *bfl*, and down-regulation of apoptotic genes, such as Bcl-2-interacting mediator of cell death [47,49]. 4-1BB/4-1BBL interaction transduces Signal 2 to $CD8^+$ T cells in a CD28-independent manner and stimulates these cells to produce cytokines, expand, and acquire effector functions. Similarly, in addition to its role in promoting the expansion of antigen-specific T cells, 4-1BB signaling is critical for T cell survival as it prevents activation-induced cell death due to high expression levels of anti-apoptotic genes and the establishment of long-term immunological memory. The 4-1BB/4-1BBL interaction selectively promotes type 1 cytokines, such as IL-2, IFN-γ, IL-6, TNF-α, and IL-12, suggesting that 4-1BB may be specific for type 1 effector T cells, which are critical to eradication of chronic infections and malignant cells. CD28 receptor on T cells is down-regulated following stimulation by CD80/86 and 4-1BB costimulation on such cells restores its expression, resulting in T cell proliferative capacity [35]. This aspect of 4-1BB signaling is important as it serves as a positive feedback loop to maintain CD28 signaling for sustained T cell responses.

Signaling through 4-1BB also plays critical roles in DC and NK cell activation, proliferation, survival, and function. 4-1BB signaling in DCs is shown to be important to their survival in vivo by up regulating Bcl-2 and Bcl-x_L, migration to T cell zone in lymph nodes, and activation of T cells [50]. Signaling via 4-1BB in combination with IL-15 expands human NK cells *in vitro* [51]. NK cells play a pivotal role in the prevention and control of tumor growth and metastasis without prior sensitization. Oncogenic transformation is often associated with the expression of stress-induced proteins. Recognition of these proteins by NK cells may result in stimulatory cytokine release, which in turn initiates an immune response, including DC antigen uptake and cross-presentation [52]. DCs and NK cells reciprocally regulate each other in contact dependent manner, and such cross-talk contributes to adaptive immune responses. Although the molecular mediators of the DC-NK-cell crosstalk are yet to be fully elucidated, the interaction of the membranous form of TNF on DCs with TNFR2 on NK cells plays a critical role for DC-mediated NK-cell expansion and manifestation of lytic function [53]. Given that activated NK cells express 4-1BB, targeting this receptor provide a great opportunity to exploit the DC-NK cell axis for cancer immunotherapy. Consistent with this notion, the depletion of NK cells in a DC-based therapy negates the Th1 polarization effect of anti-4-1BB mAbs [54].

The major focus of studies related to the 4-1BB/4-1BBL costimulatory system has been on the immune consequences of unidirectional signaling via the receptor. However, a recent study has demonstrated reverse signaling through the ligand [55]. Engagement of 4-1BBL with an anti-4-1BBL mAb on human monocytes was shown to result in their proliferation and differentiation into DCs with upregulated expression of costimulatory molecules. Such DCs were shown to have improved stimulatory capacity for T cells and generate robust Th1 responses. Although the importance of 4-1BBL reverse signaling in the generation/regulation of immune responses in vivo needs to be assessed, it is plausible that the engagement of 4-1BBL on APCs with 4-1BB on T effector cells culminates into simultaneous forward and reverse signaling that drives activation, expansion, and acquisition of effector functions for both T cells and DCs. Inasmuch as activated DCs express both the receptor and ligand on their surface, they may be the target of both forward and reverse signaling, via paracrine or autocrine 4-1BB/4-1BBL interactions, as another self-perpetuating mechanism of expansion, survival, and improved function for the generation of robust T cell responses. These two positive feedback loops, if proven effective in vivo, are quite unique and potentially powerful, which is consistent with the demonstrated roles of the 4-1BB/4-1BBL pathway in the regulation of immune responses and the robust therapeutic efficacy of 4-1BB agonists in various tumor models [37,40].

$CD4^+CD25^+FoxP3^+$ Treg cells constitutively express 4-1BB receptor on their surface and signaling through 4-1BB receptor is associated with their expansion both in vitro and in vivo [17,56,57]. However, Treg cells are unable to suppress T effector cells simultaneously costimulated by 4-1BBL, providing an important immunoregulatory mechanism that ensures the generation of productive T cell responses and control of excessive inflammation. Taken together, these studies show multifaceted functions of 4-1BB signaling on a wide range of immune cells, some with opposing functions. Although the complexity of 4-1BB signaling and the wide range of immune cells being affected provide an important opportunity for its exploitation for cancer immunotherapy, it also presents challenges that will require a careful calibration of immune positive and negative effects for the net benefit of enhanced effector responses against cancer.

4-1BB signaling for the development of cancer vaccines

4-1BBL is a type II membrane protein inducibly expressed on APCs that delivers a costimulatory signal to target cells by engaging as a trimer with the 4-1BB receptor [58]. The costimulatory function of the ligand is restricted to its membranous form as trimeric form of soluble ligand has no detectable activity on T cells [58]. As such, agonistic 4-1BB Abs have been used preclinically and clinically for the investigation of 4-1BB biology and its potential for immunotherapy. Administration of agonistic Abs to 4-1BB alone or in combination with other immunotherapies have been shown to generate potent anti-tumor immune responses that translate into tumor eradication in various preclinical animal models, including colon carcinoma, P815 mastocytoma, Ag104A sarcoma, and lymphomas [37,40]. Mechanistic studies have shown an absolute requirement for $CD8^+$ T cells for agonistic Ab-mediated tumor eradication, while $CD4^+$ T cells, NK cells, and NKT cells play secondary, but non-requisite roles [19,37,40,43]. Agonistic Abs to 4-1BB also induced expansion of Th1 biased $CD11c^+$ $CD8^+$ T cells in B16F10 melanoma bearing mice, implicating their

possible role in therapy [59]. Agonist 4-1BB Ab appear not to require IL-15 for the maintenance of tumor-specific effector cells [60], despite documented role of IL-15 in clonal expansion of $CD8^+$ T cells with a memory phenotype [61].

Although showing potent therapeutic efficacy in various tumor models, the use of agonistic 4-1BB Abs has been associated with opposing immunoregulatory functions at therapeutic doses. While agonistic Abs positively affect the activation, survival, and establishment of long-term memory of $CD8^+$ T cells, their use is associated with various immune distortions, which include apoptosis of $CD4^+$ T cells, decrease in B cell numbers and function, and depletion of NK cells [11,62,63,64]. The effect of agonistic Abs on B cells may be direct or indirect through the ablation of $CD4^+$ T cells, which together result in poor B cell mediated humoral responses [62]. Depletion of $CD4^+$ T cells may also result in defective immune memory formation [5,65], which is important for the control of cancer recurrences. Despite the depletion effect on $CD4^+$ T effector cells, the use of agonistic 4-1BB mAbs for immunotherapy is associated with impressive therapeutic efficacy in various mouse tumor models [37,40]. The depletion effect of anti-4-1BB mAbs on $CD4^+$ T cells is not immediate and occurs over a period of time post Ab therapy. Therefore, it is plausible that $CD4^+$ T cells provide the necessary help for the generation of effective anti-tumor immune responses before being eliminated. Furthermore, agonistic 4-1BB Abs appear to preferentially target $CD8^+$ T cells for expansion, survival, and acquisition of effector function [19,37]. Inasmuch as $CD8^+$ T cells are critical for tumor eradication in most settings, the delayed $CD4^+$ T cell depletion by agonistic 4-1BB Abs may not impact their therapeutic efficacy. This notion is supported by our recently published observations that the depletion of $CD4^+$ T cells one day before vaccination with SA-4-1BBL does not negate the therapeutic efficacy in various tumor settings [5].

Preclinical success of agonistic 4-1BB Abs led to the development of humanized agonistic-4-1BB mAbs that are presently being tested in various phase I/II clinical trials (National Institutes of Health Clinical trials database NCT00309023). The use of agonistic Abs to costimulatory molecules in general appear to cause some levels of toxicity, arising from nonspecific, systemic activation of lymphocytes [66,67,68]. For example, a single intravenous dose of an anti-CD28 mAb in 6 healthy volunteers resulted in life threatening toxicity due to systemic inflammatory responses in a Phase I clinical trial [69]. Several recent studies also reported toxicity associated with the use of agonistic 4-1BB Abs manifested as cytokine-mediated disruption of lymphocyte trafficking, lymphodenopathy, splenomegaly, and multifocal hepatitis [11,62]. We have also demonstrated that agonistic 4-1BB Abs cause the activation of naïve T cells leading to cytokine-mediated disruption of lymphocyte trafficking, lymphodenopathy, splenomegaly that was not dependent on FcγRs or complement [11]. It is unclear at the present if the reported toxicities and perturbation of the immune system are the inherent features of costimulation through 4-1BB receptor or the byproducts of stimulation with agonistic Abs. Therefore, the utility of the 4-1BB costimulation pathway as an effective therapeutic target for cancer immunotherapy will depend on the development of new classes of agonists with safety profiles at therapeutic doses and elucidation of mechanistic basis of their immune efficacy, which may ultimately facilitate their probable use with other immune potentiators with synergistic functions for a better clinical outcome.

SA-4-1BBL as a novel adjuvant

We hypothesized that 4-1BBL may have better efficacy and safety as compared to agonistic Abs to 4-1BB receptor due to its quantitative and/or qualitative differences in signal delivery. The ligand may also lack agonistic Ab associated toxicities, plausibly precipitated by Fc-mediated immune responses and/or other unknown factors. Inasmuch as 4-1BBL functions as a membrane-bound protein and has no costimulatory activity in soluble form [48,58], we thus generated a chimeric form of 4-1BBL, SA-4-1BBL, by fusing the extracellular portion of 4-1BBL to the C-terminus of a modified form of core streptavidin (SA). The SA-4-1BBL molecule exists as tetramers/oligomers, owing to the structural features of SA, with robust costimulatory activity. SA-4-1BBL in its soluble form drives the proliferation of both $CD4^+$ T and $CD8^+$ T cells in vitro in a standard anti-CD3-based proliferation assay [4,17]. This costimulatory effect was not limited to in vitro systems, as SA-4-1BBL as the adjuvant component of an ovalbumin-based vaccine generated robust proliferation in vivo in both ovalbumin-specific OT-I $CD8^+$ T and OT-II $CD4^+$ T cells [11]. Importantly, an agonistic 4-1BB mAb (3H3) when used at equimolar doses generated a rather subdued response in $CD8^+$ T cells as compared with SA-4-1BBL without appreciable proliferative effect on $CD4^+$ T cells in vitro. Similarly, the costimulatory effect of the agonistic mAb on OT-I and OT-II cells in vivo was inferior to that of SA-4-1BBL used at equivalent doses [11]. The observed better proliferative efficacy of SA-4-1BBL as compared with the agonistic mAb was also translated into a better CTL response as assessed by an *in vivo* antigen-specific cytotoxicity assay [11]. Immunoregulatory function of SA-4-1BBL is not restricted to only $CD4^+$ T and $CD8^+$ T cells as stimulation of bone marrow-derived DCs with this molecule resulted in upregulation of MHC and costimulatory molecules and enhanced antigen uptake and cross-presentation [4,14]. Modulation with soluble agonists, mAbs or SA-4-1BBL, targets unidirectional forward signaling via the 4-1BB receptor on immune effector cells, such as T cells, NK cells. However, it is conceivable that modulation with 4-1BB agonists may also facilitate reverse signaling through 4-1BBL by upregulating the expression of endogenous molecule on APCs for subsequent bidirectional signaling. Forward and reverse signaling is then expected to result in the expansion of both DCs and T cells and generation of a robust immune effector response.

Costimulation of naïve $CD4^+$ T cells with SA-4-1BBL renders them refractory to suppression by Treg cells, without a direct effect on the suppressive function of Treg cells [4]. In contrast, an agonistic 4-1BB Ab was reported to overcome Treg mediated immune suppression by directly targeting T effector cells, making them resistant to suppression, as well as inhibiting the suppressive function of Treg cells. [70]. It is unclear at this point if these reported key differential effects on Treg cell function between SA-4-1BBL and agonistic 4-1BB Ab are real or due to the experimental models/conditions used in these studies. Furthermore, vaccination with agonistic 4-1BB Ab in tumor bearing or naïve mice increased the numbers of T regulatory cells, whereas SA-4-1BBL based vaccines lacked such in vivo effects [14,57]. Although the mechanistic basis of these observations is unknown, we speculate that agonistic mAbs act as super agonists and cause nonspecific activation of both naïve conventional T cells and Treg cells [11,57], whereas SA-4-1BBL selectively and preferentially activates and expends antigen primed effector $CD4^+$ and $CD8^+$

T cells in a more physiological manner. This differential response may be due to the differential signaling through 4-1BB in these two cells types or kinetics/quality of downstream events. The key qualitative and quantitative differences between the signaling outcomes by the agonistic 4-1BB mAb used in our studies and SA-4-1BBL are summarized in Table 2.

SA-4-1BBL blocks antigen and TGF-β-mediated conversion of T effector into induced Treg cells *in vitro* [10]. SA-4-1BBL also blocks tumor-mediated conversion of T effector cells into induced Treg cells in a lymphoma model [10]; an important observation with significant implication in cancer immunotherapy where Treg cells serve as a major culprit of tumor immune evasion and contribute to reported inefficacy of various vaccines [71]. However, we have also demonstrated that SA-4-1BBL can expand naturally occurring Treg cells, which constitutively express 4-1BB receptor, in the presence of exogenous IL-2 [17]. Although the implication of these opposing functions of SA-4-1BBL on natural Treg cells, expansion vs. overcoming inhibition of Teff cells, is unknown, it may serve as a forward negative feedback loop where 4-1BBL expands both T cell populations in response to infections while rendering T effector cells immune to suppression by Treg cells. Once the infection clears, APCs down-regulate 4-1BBL expression, thereby allowing Treg cells to control the function of T effector cells to avoid potential collateral damage triggered by excessive inflammation.

The increased immune stimulation of SA-4-1BBL compared to the agonistic 4-1BB mAb is achieved in the absence of detectable autoimmunity or toxicity (Table 1). Unlike SA-4-1BBL, the agonistic Abs to 4-1BB cause severe toxicity as assessed by enlarged spleen and peripheral LNs, non-specific T cell proliferation, hepatitis, and systemic inflammatory cytokine production [11]. Although mechanisms of the differential effects of agonistic Abs and natural ligands *vis-a-vis* toxicity *vs.* efficacy are unknown, FcγR receptors and complement system do not play a role. Treatment of mice lacking activating FcγRs or complement component *C1q* and *C3* with the agonistic 4-1BB mAb resulted in full toxicity [11]. However, we cannot rule out the role of inhibitory FcγRIIB, which could create cognate interactions between T cells, monocytes, or potentially other cell types, in the observed mAb-induced toxicity.

SA-4-1BBL as adjuvant for cancer vaccines

The importance of costimulation in the generation of primary and long-term immune responses combined with the defined roles of 4-1BB signaling in the generation, maintenance, and establishment of long-term immune memory of $CD8^+$ T cells and the critical roles of these cells in tumor eradication led us to assess the efficacy of SA-4-1BBL as adjuvant component of TAA-based vaccines in various preclinical cancer models. Using the HPV-16 E7-expressing TC-1 as a preclinical cervical cancer mouse model, we demonstrated that a single vaccination with SA-4-1BBL in combination with a peptide ($E7_{49–57}$) representing the dominant $CD8^+$ T cell epitope for E7 as TAA resulted in better therapeutic efficacy than vaccine formulations containing TLR agonists, LPS, MPL, CpG, or the agonistic 4-1BB mAb [4]. Similar results were also obtained when a complete E7 protein was used as TAA [12]. The observed therapeutic efficacy was not limited to the E7 TAA

and TC-1 model, as adjuvant component of survivin as a *bona fide* self-TAA, SA-4-1BBL demonstrated robust therapeutic efficacy against 3LL lung carcinoma expressing survivin [3,14]. The therapeutic efficacy of SA-4-1BBL-based vaccines was further improved by multiple vaccinations as demonstrated in both the 3LL and TC-1 tumor models [3,5]. Importantly, the therapeutic efficacy of SA-4-1BBL was achieved without acute toxicity or detectable systemic autoimmunity.

DCs play a critical role in the generation of adaptive immune responses and as such have been targeted for the development of cancer vaccines [72]. Indeed, a DC-based cancer vaccine was the first to be approved by FDA for immunotherapy [73]. Simultaneous antigen delivery and maturation/activation of DCs are critical to the generation of effective antitumor immune responses [74]. Since ex vivo manipulation of DCs for antigen loading and maturation represents an expansive and patient customized approach, we assessed the capacity of SA-4-1BBL as an adjuvant as well as antigen delivery vehicle to DCs in vivo. Inasmuch as the streptavidin portion of SA-4-1BBL allows for rapid conjugation of biotinylated antigens of interest and a population of resting DCs constitutively express 4-1BB [14,44], we tested the feasibility of generating conjugate vaccines and using 4-1BBL as vehicle to deliver TAAs into DCs for improved therapeutic efficacy. A single immunization with SA-4-1BBL/E7 or survivin conjugate vaccines resulted in eradication of TC-1 and 3LL primary and metastatic tumors, respectively, to a significantly higher extent as compared with non-conjugate vaccine formulations [14]. This study highlights the rationale that vaccine formulations employing immunomodulators that not only regulate the function of DCs, T cells, and other critical cells of innate, adaptive, and regulatory immunity, but also serve as vehicles to deliver TAA to DCs in vivo may have increased utility and potential success in the clinic.

A host of mechanisms are associated with the therapeutic efficacy of SA-4-1BBL-based vaccines. These include i) activation/maturation of DCs for augmented antigen uptake and presentation [4,14], ii) $CD4^+$ T cell priming [11], iii) $CD8^+$ T cell expansion, acquisition of effector function, and long-term survival [4,14], iv) reversal of $CD8^+$ T cell anergy [4], v) inhibition of Treg mediated suppression [4], and vi) blockade of conversion of $CD4^+$ T effector cells into induced Treg cells [10]. These pleiotropic immune effects of SA-4-1BBL culminate in robust $CD8^+$ T and NK effector responses that are critical to tumor destruction. $CD8^+$ T cell effector function is required for tumor elimination, while NK cells play a secondary, but important role [3,12]. Therapeutic efficacy of SA-4-1BBL-based vaccines is also associated with enhanced infiltration of $CD8^+$ T cells into the tumor and reduced presence of Treg cells within the tumor, leading to a favorable T effector/Treg cell ratio [14]. Robust pleiotropic immunomodulatory activities of SA-4-1BBL and its ability to serve as a vehicle to deliver TAAs to DCs combined with lack of detectable toxicity at therapeutic doses rationalize the extensive evaluation of this novel adjuvant as a platform for the development of therapeutic vaccines against cancer and chronic infections.

Prospect of SA-4-1BBL use in combinatorial cancer therapies

Emerging evidences from preclinical and clinical cancer immunotherapy studies provide compelling rationale for a multifaceted approach to combat cancer that has evolved multiple

layers of immune evasion mechanisms for progression. In particular, adjuvant/treatment modalities with diverse, yet synergistic mechanisms of action may yield the best therapeutic outcome against cancer. Eradication of established tumors by vaccination may present a significant challenge if tumor mass is too extensive. Therefore, tumor debulking by surgery, which may not only reduce tumor burden but also the associated intratumoral and potentially distant immune evasion mechanisms, may significantly improve vaccine efficacy. Similarly radiotherapy, chemotherapy, and targeted therapies may precondition the patient for immunotherapy by creating an environment conducive to homeostatic lymphoproliferation and elimination of some, if not all of the suppressive immune networks, thereby contributing to the efficacy of cancer vaccines. Chemotherapy, such as cyclophosphamide, and radiotherapy in combination with agonistic 4-1BB mAbs have already shown synergy in preclinical tumor models [75]. The use of SA-4-1BBL in combination with targeted therapies, such as sunitinib, which blocks suppressive functions of both MDSCs and Tregs, presents another attractive treatment modality. Consistent with this notion, sunitinib has been shown to work in synergy with an agonistic 4-1BB mAb for the treatment of gastrointestinal stromal tumors [76] and metastatic renal cell carcinoma [77] in preclinical tumor models.

Molecules from the same class of immunomodulators, but with complimentary and/or synergistic functions on immune effectors, such as $CD8^+$ T and $CD4^+$ T cells, or immune evaders, such as Treg cells and MDSCs, may have significant potential for cancer immunotherapy. In this context, combinatorial use of SA-4-1BBL with OX40L, another member of the TNFL costimulatory family, is of interest. Although both molecules have costimulatory activity on $CD4^+$ T and $CD8^+$ T cells, 4-1BB signaling appears to primarily target $CD8^+$ T cells, whereas OX40L signaling targets $CD4^+$ T cells [78]. Moreover, OX-40 signaling directly inhibits Treg cell function, while 4-1BB signaling renders T effector cells refractory to inhibition by Treg cells [78]. Given the robust immune efficacy of mAbs against immune checkpoint blockers, CTLA-4 and PD-1, in preclinical and clinical settings [6,7], it will be highly desirable to use such immune blockers with SA-4-1BBL and other 4-1BB agonists. The principle here is rather obvious as costimulation via 4-1BB is expected to generate adaptive T cell responses, while immune check point blockers will unleash the breaks to further drive this response. Indeed, a recent study has shown that an anti-CTLA-4 mAb worked in synergy with an agonistic 4-1BB mAb for eradication of B16 melanoma in a preclinical animal model [79].

Combinatorial use of immunomodulators targeting the innate and adaptive immunity presents another important treatment scheme. For example, alpha-gal-ceramide, which has shown therapeutic efficacy in tumor models by NKT cell priming [80], may be combined with SA-4-1BBL to generate both T and NKT cell responses for improved vaccine efficacy. Agents that activate DCs may work in synergy with SA-4-1BBL by specifically activating T cells, which in turn upregulate 4-1BB and serve as the direct target of SA-4-1BBL. Mice immunized with either GM-CSF-transduced tumor cells or a DC-based vaccine with an agonistic 4-1BB mAb resulted in augmentation of anti-tumor immune responses [28]. Inasmuch as agonists of pattern recognition receptors, such as TLRs, activate APCs for enhanced antigen uptake and cross-presentation to T cells, which in turn upregulate TNFR costimulatory molecules, including 4-1BB, a combinatorial use of SA-4-1BBL and TLR

agonists as component of vaccine formulations is attractive. Indeed, we have shown robust therapeutic synergy between SA-4-1BBL and MPL in various preclinical tumor models (Srivastava *et al.*; unpublished observations). SA-4-1BBL, as an adjuvant with pleiotropic immune functions, has potential to work in synergy with selected cancer conventional and immune therapy modalities for a better therapeutic outcome. Target engagement on a variety of immune cell types such as T, NK, NKT, and DCs as well as noncancerous parenchymal cells within the tumor microenvironment may switch on multiple mechanisms of action which could ultimately eradicate tumors and establish long-term immune memory for control of recurrences.

Given pleiotropic and potent biological activities of costimulatory receptor signaling, an inherent risk of immune toxicity associated with the use of agonists cannot be ruled out. Therefore, careful optimization and evaluation of such adjuvants stand alone or in combination with other therapies for dose, vaccination regimen, and toxicity are required before entering into human clinical trials. Nevertheless, such potent immune stimulators may have a chance to show clinical efficacy long awaited for the treatment of cancer patients.

Expert commentary & five-year view

Subunit vaccines based on TAAs present an attractive choice for cancer immunotherapy due to their cost-effectiveness, ease of production, off-the-shelf availability, distribution, and potential applicability to a wide range of tumor types. However, the clinical utility of such vaccines will depend on the use of adjuvants that not only generate/amplify TAA-specific effector immune responses, but also simultaneously overcome intrinsic/extrinsic tumor-mediated immune evasion mechanisms. In this context, the development/discovery of immune modulators with pleiotropic effects on a broad range of immune cells with defined mechanisms of action and lack of toxicity in humans will be an important step forward. Our findings support the notion that SA-4-1BBL may have the potential to serve as an effective adjuvant for therapeutic vaccines against cancer and chronic viral infections due to its robust pleotropic functions in modulating innate, adaptive, and regulatory immunity in the absence of detectable toxicity. SA-4-1BBL as an adjuvant with pleiotropic immune functions and effective vehicle to deliver TAAs to DCs may benefit from combinatorial use with other adjuvants, immune potentiators, and/or targeted therapies. Robust immune and therapeutic efficacy combined with its safety profile at therapeutic doses differentiate SA-4-1BBL from agonistic 4-1BB mAbs with significant clinical potential. If the immune and therapeutic efficacies observed in preclinical tumor models translate to clinical cancer settings, this vaccine concept may have broad application not only for cancer types with well-defined TAAs, but also chronic viral infections where $CD8^+$ T cells play a defining role. We expect accelerated development of SA-4-1BBL as an adjuvant stand alone or in combination with other adjuvants in the next five years. In particular, combined use of SA-4-1BBL with TLR agonists and/or immune checkpoint blockers has great potential in tumor setting. Such a combinatorial use is expected to target cells of innate, adaptive, and regulatory immunity, culminating in the generation of robust effector immune responses with potential clinical efficacy against cancer.

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Key issues

- Subunit cancer vaccines require potent adjuvants to enhance TAA-specific adaptive Th1 (mainly $CD8^+$ T cell) biased responses to control and eradicate existing tumors.
- An adjuvant with pleiotropic effects on immune effectors that can also overcome various tumor employed immune evasion mechanisms is likely to generate better therapeutic efficacy in vaccine settings.
- CD28 and TNFR co-stimulatory molecules, such as 4-1BB, regulate innate adaptive and regulatory immunity and act as intrinsic adjuvant for qualifying T cell responses commonly known as signal 2.
- Targeting the 4-1BB pathway by agonistic 4-1BB mAbs has shown encouraging results in preclinical and limited clinical settings, however enthusiasm is somewhat dampened by the reported toxicity at therapeutic doses.
- There is need for the development of new forms of non-toxic agonists to 4-1BB for effective exploitation of this pathway in cancer immunotherapy and SA-4-1BBL may be such an agonist.
- Targeting antigens to DCs enhances vaccine efficacy; therefore, adjuvants, such as SA-4-1BBL, that can also simultaneously serve as a vehicle to deliver TAAs to DCs stand a better chance to succeed in the clinic.
- SA-4-1BBL appears to deliver qualitatively and quantitatively different signals than an agonistic 4-1BB mAb, which result in better immune efficacy in the absence of mAb precipitated immune abnormalities and toxicity.
- SA-4-1BBL enhances and tailors immune responses for effective and long-lasting protection.
- Generation of appropriate types of immune responses that culminate in therapeutic efficacy in settings of cancer and chronic infections may be difficult with a single adjuvant; hence, adjuvant systems including immune potentiators, such as TLR agonists and/or immune-checkpoint blockers, that act through diverse yet synergistic mechanisms are likely to be more effective.
- Combinatorial use of adjuvants provides new opportunities to tailor a cancer vaccine to treat a specific type of cancer; however, safety and regulatory issues will represent some of the challenges.

Table 1

TNFR Costimulatory molecules, expression patterns, and signaling outcomes for immune modulation

TNFR costimulatory receptor/ligand	Receptor expressing cells	Ligand expressing cells	Receptor signaling outcome
4-1BB/4-1BBL	Inducible expression on $CD4^+$ T cells, $CD8^+$ T cells, NK, NKT cells, mast cells, neutrophils Constitutive expression on $CD4^+CD25^+Foxp3^+$ Treg cells, and a sub-population of DCs	Inducible expression on APCs and endothelial cells Constitutive expression on hematopoietic progenitor cells	Activation, proliferation and survival effects in T and NK cells, overcome anergy in $CD8^+$ T cells, activation and survival of DCs, renders Teff cells refractory to suppression by Treg cells, blocks the conversion of Teff into Treg cells Reverse signaling through ligand in monocytes converts them to DCs expressing IL-6 and IL-12 cytokines
OX-40/OX-40L	Inducible expression on $CD4^+$ T cells, $CD8^+$ T cells, NK, NKT cells Constitutive expression on mouse $CD4^+CD25^+Foxp3^+$ Treg cells and inducible expression on human $CD4^+CD25^+Foxp3^+$ Treg cells	Constitutive/inducible expression on various APCs (B cells, DCs, macrophages, Langerhans cells), NK cells, endothelial cells, smooth muscle cells, and mast cells	Activation, proliferation, and survival effects in T cells (preferentially $CD4^+ > CD8^+$ T cells), activation of NK and NKT cells, inhibits Treg suppressive function, renders Teff cells refractory to suppression by Treg cells, blocks the conversion of Teff into Treg cells
GITR/GITRL	Inducible expression on $CD4^+$ T cells, $CD8^+$ T cells, NK, NKT cells, B cells, macrophages, DCs Constitutive expression on $CD4^+CD25^+Foxp3^+$ Treg cells	Constitutive expression on APCs and endothelial cells	Activation, proliferation and survival effects in T cells
CD40/CD40L	Constitutive and inducible expression on APCs, epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, and basophils	Inducible expression on T and B cells, eosinophils, monocytes, macrophages, endothelial cells, epithelial cells, and muscle cells	Activation of APCs, indirect activation, proliferation and survival effects on T cells
CD27/CD70	Constitutive expression on $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+CD25^+Foxp3^+$ Treg cells Inducible expression on B cells	Both receptor and ligand are constitutively expressed on APCs and inducibly expressed on T and B cells	Activation, proliferation, and survival effects in T cells, activation of NK cells

Table 2

Key qualitative and quantitative differences between SA-4-1BBL and agonistic 4-1BB antibodies

Immune responses altered	SA-4-1BBL	Agonistic 4-1BB Abs	References
Generation of primary T cell responses	++	+	4,11,12,17
$CD8^+$ T cell proliferation	++	+	11
$CD4^+$ T cell proliferation	++	+	11
Super agonist for naive T cells	−	+	11, 62
Generation of $CD8^+CD11c^+$ T cells	−	+	59, unpublished observations
$CD4^+CD25^+$FoxP3 Treg cell expansion in vitro	+	+	17, 57
$CD4^+CD25^+$FoxP3 Treg cell expansion in vivo	−/+	++	11, 56
Inhibition of $CD4^+CD25^+FoxP3^+$ Treg cell suppression	Endows $CD4^+$ T effectors resistant to suppression by Treg cells	Endows $CD4^+$ T effectors resistant to suppression by Treg cells, direct inhibition of Treg function	4, 17, 70
Inhibition of T effector conversion into $CD4^+CD25^+FoxP3^+$ Treg cells	+	?	10
Immune abnormalities	None observed	Splenomegaly, lymphadenopathy, hepatomegaly, multifocal hepatitis, lymphopenia, thrombocytopenia, anemia, systemic cytokine release, excessive T cell proliferation, altered trafficking of B cells and $CD8^+$ T cells, loss of NK and B cells, inhibition of Ab response	11, 62, 63,64
Efficacy for tumor eradication	++	+	4,5,12,14