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SA-4-1BBL as a modulator of innate, adaptive, and regulatory immunity : implications for cancer prevention and treatment.

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IMMUNITY: IMPLICATIONS FOR CANCER PREVENTION AND TREATMENT

By

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DEDICATION

This dissertation is dedicated to my parents

Mr. Berj Barsoumian

and

Mrs. Armine Akinian Barsoumian

who have illuminated and generously supported my educational journey through science

and life.

ACKNOWLEDGMENTS

ABSTRACT

SA-4-1BBL AS A MODULATOR OF INNATE, ADAPTIVE, AND REGULATORY
IMMUNITY: IMPLICATIONS FOR CANCER PREVENTION AND TREATMENT

Hampartsoum Barsoumian

April 14, 2016

SA-4-1BBL is a recombinant costimulatory molecule that is active in its soluble

form and has pleiotropic effects on the functions of innate, adaptive, and regulatory

immune cells. We have previously shown that SA-4-1BBL makes $CD4^+$ T conventional

cells (Tconvs) resistant to suppression by $CD4^+CD25^+Foxp3^+$ T regulatory cells (Tregs).

The mechanistic basis of this observation is the subject of this study. We demonstrate that

Tconvs, but not Tregs, are the direct targets of SA-4-1BBL-mediated evasion of Treg

suppression without contribution from antigen presenting cells (APCs). SA-4-1BBL

engagement with its receptor, 4-1BB, on Tconvs resulted in the production of high levels

of IL-2 cytokine that was necessary and sufficient in overcoming Treg suppression.

Removal of IL-2 from culture supernatant restored Treg suppression and repletion of

Tconv:Treg cocultures with exogenous recombinant IL-2 overcame suppression.

In a series of studies our laboratory demonstrated the therapeutic efficacy of SA-

4-1BBL as an adjuvant component of subunit cancer vaccines in various preclinical

rodent cancer models. Inasmuch as SA-4-1BBL contains streptavidin (SA) as a foreign

bacterial protein, repeated vaccinations may generate anti-SA antibodies. Such antibodies

may have positive or negative effect on the efficacy of the vaccines. Therefore, a series of

studies were conducted to assess the potential impact of SA's immunogenicity on the immuno-stimulatory function of SA-4-1BBL molecule. We demonstrated that repeated treatments with SA-4-1BBL generate both cellular and humoral immune responses against the SA portion of the molecule. Antibodies against SA neither blocked nor improved the costimulatory function of SA-4-1BBL on T cells. Importantly, pre-immunization with SA-4-1BBL protein alone did not interfere with the therapeutic efficacy of a subunit vaccine consisting of the human papillomavirs (HPV) E7 tumor associated antigen (TAA) + SA-4-1BBL in the TC-1 cervical cancer tumor model. Surprisingly, pretreatment with SA-4-1BBL alone conferred protection regardless of the type of tumor tested.

The robust anti-tumor function of SA-4-1BBL involved a communication bridge between CD4 and NK cells without significant contribution from CD8 cells or B cells. NK cells were found to be critical to the observed tumor suppression and required $CD4^+$ T cell help for protective efficacy at both priming and effector stages. Depletion of either NK or CD4 cells negated SA-4-1BBL's anti-tumor protection. Moreover, the tumor suppressive effect of SA-4-1BBL was shown to be a bona fide property of this molecule, as immunizing mice with an agonistic antibody to 4-1BB receptor did not confer any protection against TC-1 tumors.

The significance of understating the mechanisms underlying SA-4-1BBL's mode of action provides new tools in the evolving field of cancer immunotherapy and prevention. The implications also extend to transplantation settings as well as infectious diseases where tipping the balance between Tregs and T effector cells (Teffs) plays a critical role in achieving desired outcomes.

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CHAPTER 1: INTRODUCTION

Costimulation as a critical signal for productive T cell responses

T cells are the critical cell type in adaptive immunity. T cells require 3 standard signals for activation, proliferation, acquisition of effector function, and establishment of long-term memory. Signal 1 is usually provided through T cell receptor (TCR) interacting with Major Histocompatibility Complex (MHC) class I or II molecules on antigen presenting cells (APCs) loaded with processed antigens. Costimulation provides signal 2, while soluble factors and cytokines constitute signal 3. All signals together decide on the fate and differentiation of the T cell in concern (1). $CD8^+$ T cells for example interact with MHC-I during viral infections or in the presence of intracellular pathogens and acquire effector functions capable of clearing the infection of concern. $CD4^+$ T cells on the other hand interact with MHC-II molecules and convert from their T helper Th0 state into either Th1, Th2, Th17, or $CD4^+CD25^+Foxp3^+$ T regulatory cells (Tregs) , based on the antigen and type of costimulation engaged (Illustration 1). More importantly, the lack of costimulation leads to T cell anergy and unresponsiveness followed by cell death and inefficient tumor or infection clearance.
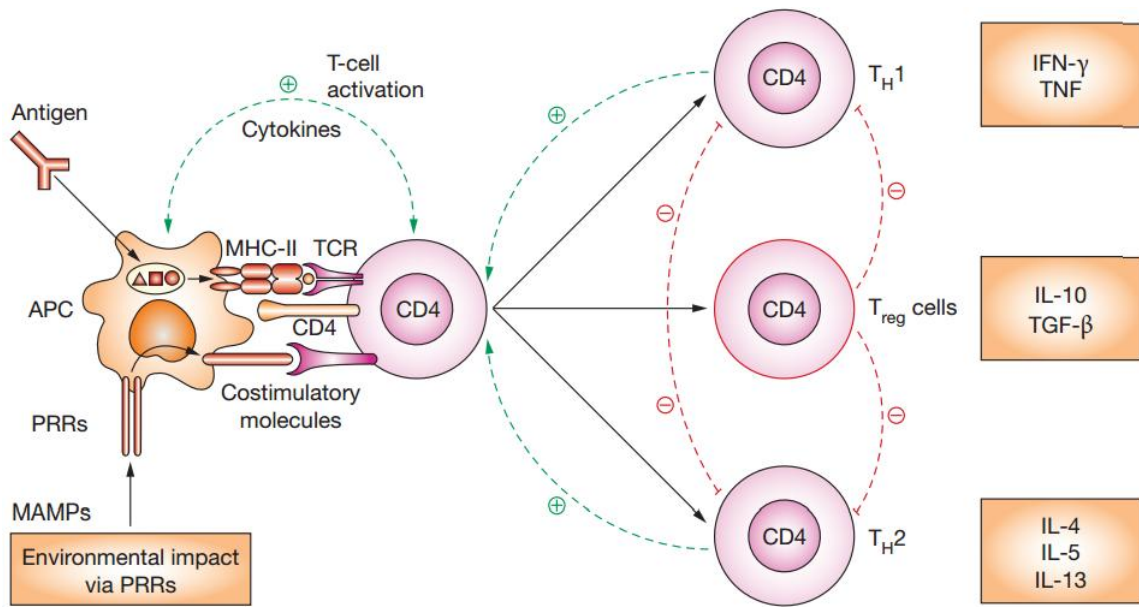
Illustration 1. Guarner et al. *Nature Clinical Practice Gastroenterology & Hepatology* (2006) **3**, 275-284.

Costimulatory molecules in general are categorized into two wider families:

CD28 and tumor necrosis factor receptor (TNFR) superfamily. CD28 for example is

constitutively expressed on T cells and interacts with B7 molecules on APCs to drive

activation of T cells. CD28 action is usually maintained under control by its competitor

cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also expressed on T cells and

delivers inhibitory signals when interacting with B7 at higher affinity (2). TNFR family

members on the other hand, such as 4-1BB, OX40, and CD27 (Illustration 2), are

inducibly expressed on activated T cells (CD4^{+} and CD8^{+}), Tregs, NK cells, mast cells,

and neutrophils (3). The inducible expression on antigen-experienced T cells is

advantageous for the expansion of antigen-specific immune responses in therapeutic

settings and provides a better on/off switch.
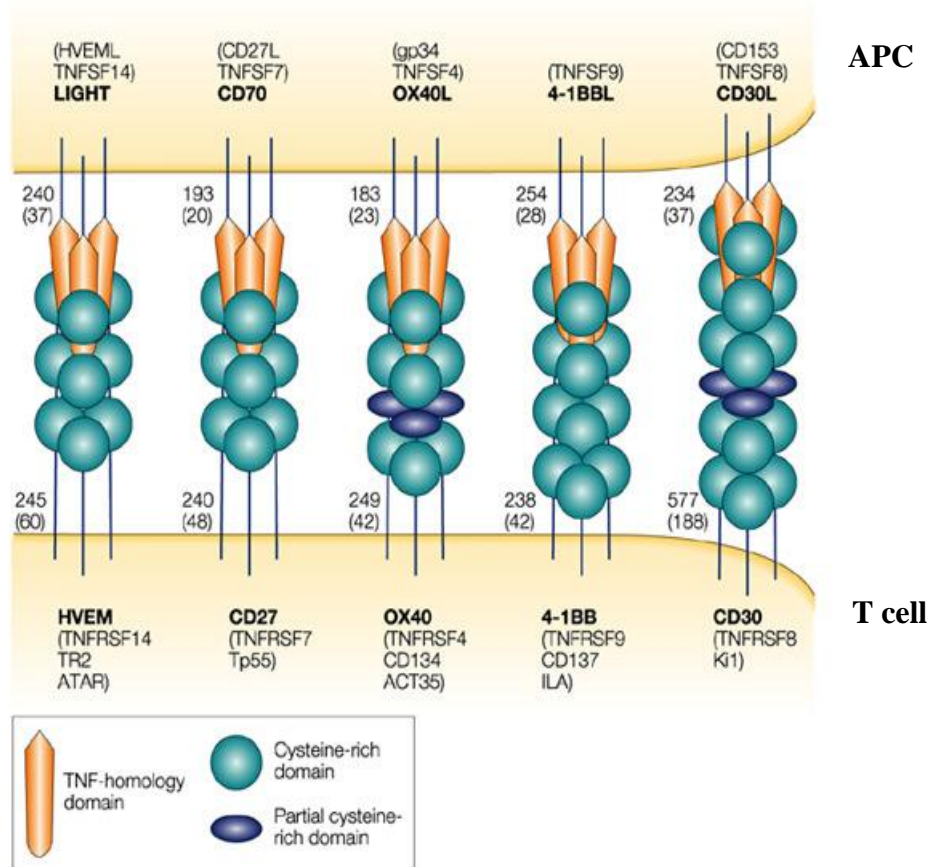
Illustration 2. Michael Croft, *Nature Reviews Immunology* (2003) **3**. 609-620.

TNFR family members in costimulation

There are 27 TNF-ligands identified so far with biological functions. Ligand/receptor pairs with costimulatory signaling capacity include CD40L (CD154)/CD40, CD70/CD27, 4-1BBL/4-1BB (CD137), OX40L/OX40 (CD 134), GITRL/ GITR (CD 357), and HVEMLs/ HVEM (CD 270), that have showed promise as therapeutic modulators of T cell immunity (4).

CD40L/CD40: CD40L expression has been identified on activated B cells, epithelial cells, endothelial cells, platelets, smooth muscle cells, and DCs. Expression of its receptor CD40 is found on B-cells, monocytes, macrophages, platelets, DCs, eosinophils, and activated CD8^{+} T cells (4). The main function of CD40L/CD40 is to activate and

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"license" DCs to prime effective cytotoxic CD8$^+$ T-cell responses. In its absence "unlicensed" DCs induce T-cell anergy or T-cell deletion and generate regulatory T cells (5, 6).

CD70/CD27: The expression of CD70 is restricted to activated T and B lymphocytes and mature DCs. CD27 is a type I transmembrane receptor expressed on naive T cells, mature T cells, memory B cells, and NK cells (4). The key biological function of CD70 is to prime CD4$^+$ and CD8$^+$ T cell responses and enhance T-cell survival and effector functions. CD70/CD27 signaling promotes the development of CD4$^+$ T cells that are able to produce Th1 and Th2 cytokines (7).

OX40L/OX40: OX40 is majorly expressed by activated CD4$^+$ and CD8$^+$ T cells, NK cells, NKT cells, and neutrophils. OX40L is primarily expressed by APCs such as DCs, B-cells, and macrophages. OX40 provides a co-stimulatory signal to TCR-stimulated T cells and enhances clonal expansion, proinflammatory cytokine production, and generation of memory CD4 T cells. Furthermore, OX40 promotes CD8 T cells survival and expansion directly or indirectly via the induction of CD4 T-helper responses (8).

GITRL/GITR: GITR is expressed at low levels on resting CD4$^+$ and CD8$^+$ T cells, DCs, monocytes, and NK cells. GITR ligand (GITRL) is highly expressed on activated APCs and endothelial cells (ECs), and it promotes leukocyte adhesion and transmigration (9). Moreover, GITR ligation has been shown to break self-tolerance and abrogate T-cell suppression by Tregs, which could be utilized as a target for cancer immunotherapy (10).

HVEMLs/HVEM: The Herpesvirus entry mediator (HVEM) expression is in fact opposite to 4-1BB, OX40, and GITR expression patterns. HVEM is highly expressed on naive and memory B cells as well as resting T cells, but down regulates upon B and T

cell activation. There are four ligands for HVEM: CD160 (a member of the Ig superfamily); B- and T-lymphocyte attenuator (BTLA); LIGHT; and lymphotoxin-alpha. Interaction of CD160 and BTLA with HVEM inhibits B and T cell activation, while interaction of LIGHT or lymphotoxin-alpha with HVEM triggers T and B cell activation and APC maturation and differentiation (11).

4-1BB costimulation and signaling background

4-1BB, also known as CD137/TNFSF9, is a member of TNFR superfamily (12). It is a costimulatory molecule that is upregulated on the surface of lymphoid cells such as T cells, B cells, and NK cells when activated and non-lymphoid cells such as dendritic cells (DCs), macrophages, neutrophils, and malignant tumor endothelial cells (13). Upon 4-1BB receptor clustering, a heterotrimer is formed consisting of TNF-receptor associated factors (TRAFs) where two molecules of TRAF2 combine with one molecule of TRAF1. TRAF2 initiates a signaling cascade of mitogen-activated protein (MAP) kinases and NF-κB activation, which in turn leads to the production of more TRAF1 that sustains the effect of 4-1BB costimulation. TRAF1 favors extracellular signal-regulated kinase (ERK) pathways (Illustration 3). The end result is the expansion and survival of T cells, acquisition of effector functions, and establishment of long-term memory (14, 15). Key cytokines produced also include TNF-α, IL-2, IL-12, and IFN-γ.
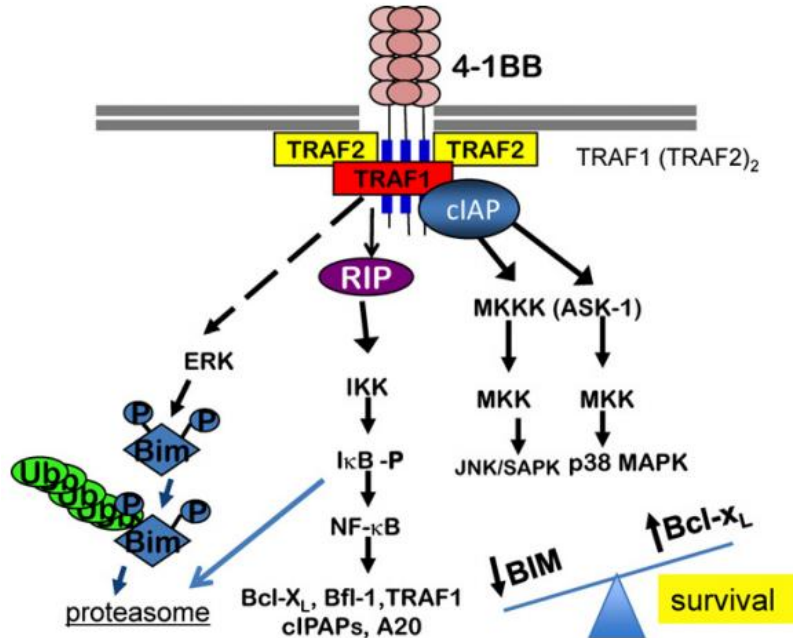
Illustration 3. Chao Wang, Tania H. Watts. *Cytokine & Growth Factor Reviews* (2012) **23**, 245–254.

More recently 4-1BB costimulation has been shown to reduce T cell exhaustion in

ex vivo settings where there is persistent antigen exposure. The authors in the study used

genetically engineered T cells that express chimeric antigen receptors (CARs) capable of

recognizing specific antigens and activating T cells independent of MHCs. Contrary to 4-

1BB, CD28 costimulation in the CAR model has been shown to enhance exhaustion (16).

In a transplanted melanoma model, Curran et al. have shown that mice treated with anti-

4-1BB antibodies generate novel forms of CD4^{+} killer and CD8^{+} killer tumor infiltrating

T cells characterized by the expression of killer-cell lectin like receptor G1 (KLRG1)

and the transcription factor Eomesodermin (Eomes) independent of the T-box

transcription factor (T-bet) expression. These cells were shown to produce granzymes

capable of killing melanoma cells. In addition to that, amongst the members of TNFR

family agonists, only 4-1BB signaling has generated the KLRG1^{+}Eomes[+] killer

phenotype, while anti-OX40, anti-GITR, and anti-CD27 could not (17).

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It is important here to also shed light on the regulatory aspects of 4-1BB signaling as it plays a double edged sword by generating and expanding effector immunity as well as expanding and promoting Treg functions and redirecting T cell polarization. In mice for example, 4-1BB is constitutively expressed on Tregs and can respond to 4-1BBL + IL-2 + TGF-β stimuli to induce their proliferation. The duality of 4-1BB function seems to be at least partially controlled by Galectin-9, a member of the β-galactoside–binding family of lectins (18). Upon addition of anti-4-1BB antibody (clone 3H3), Gal-9 binds to 4-1BB on the cell surface without competing with the binding sites of the ligand nor 3H3. Gal-9 binding is demonstrated to be required for the ability of 4-1BB signaling to suppress experimental autoimmune encephalomyelitis (EAE) and allergic asthma *in vivo* (18). In another study, 4-1BB ligand was shown to play a regulatory role in T cells that are weakly activated under suboptimal conditions below the threshold of 4-1BB expression and activation. Once the activation threshold was met, cell intrinsic 4-1BB was shown to downregulate the expression of 4-1BBL by endocytosis and internalization (19). This balance between the ligand and the receptor is necessary for maintaining immune system homeostasis.

4-1BB in Cancer Vaccines

Cancer vaccines usually consist of two major categories: Prophylactic/preventative such as Gardasil® and Cervarix® against Human Papillomavirus (HPV) 16 and 18, or therapeutic such as the dendritic cell based vaccine (*Sipuleucel-T)* approved by the FDA in the USA on 2010. The down side of DC vaccines is that they are relatively expensive, need to be subject customized, and are technically challenging to produce. Subunit vaccines consisting of tumor associated or specific

antigens and immune-adjuvants, are more economically feasible and present as effective alternatives to cell-based vaccines with ease of production and marketing. The culprit in the subunit vaccines is that the immunogenicity of most antigens is low, most of them being self antigens, as well as the lack of potent and safe vaccine adjuvants capable of overcoming tumor immune evasion mechanisms. As such, in the absence of predefined tumor specific antigens and alongside conventional chemotherapy, scientists have started incorporating alternative immune modulators in clinical cancer therapies, such as the use of monoclonal antibodies targeted towards tumors and/or cells of the immune system. Some of the clinically approved candidates include i) the monoclonal antibody Herceptin (Trastuzumab) directed towards breast cancer cells expressing the human epidermal growth factor receptor 2 (Her2) protein (20). ii) Ipilimumab, an anti-CTLA antibody, which blocks the action of Tregs giving more room for Teffs and NK cells to carry out their killer functions (21). iii) Antibodies blocking the programmed death receptor-1 (PD-1) that is usually expressed by activated lymphocytes and delivers an inhibitory signal when associated with its ligand expressed by APCs as well as tumors (22). iv) Agonistic antibodies to 4-1BB costimulatory receptor, especially picked from the tumor necrosis factor superfamily due to its promising pre-clinical and clinical outcomes in fighting tumors and activating a wide array of effector immune cells.

The major drawback of anti-4-1BB antibodies is the high level of associated toxicity as reported by several studies in rodents and humans (15). Toxicity profiles are usually characterized by non-specific activation of immunity resulting in systemic inflammation, splenomegaly, lymphadenopathy, increased liver enzymes AST/ALT, as well as hepatitis. The observed toxicity is shown to be independent of FcγR or

complement system activation as inflammation was persistent in $Fc\gamma R^{-/-}$ and

complement $C1q^{-/-}$ or $C3^{-/-}$ knockout mice (23). Upregulation of non-specific CD4^{+} and

CD8^{+} T cells was noted upon immunizing naïve C57BL/6 mice with anti-4-1BB antibody

clone 3H3 accompanied with high levels of IFN-γ and TNF-α production. Higher doses

of 3H3 also abrogated T helper cell dependent humoral immunity, caused more

hepatomegaly, as well as anemia and disturbance in bone marrow's cellular compartment

(24).

SA-4-1BBL as a potent adjuvant

Taking into account the various toxic facets of agonistic 4-1BB antibody

treatments and the fact that naturally occurring 4-1BBL is inactive in its soluble form

unless bound to a cell surface membrane, our lab has generated and produced a novel

soluble form of 4-1BBL adjuvant by fusing the extracellular domain of murine 4-1BBL

to the C-terminus of a modified core streptavidin (SA). SA forms stable tetramers and

oligomers which is important to cluster the 4-1BB receptors on corresponding cells and

enhance signal transduction (Illustration 4). Side by side comparison studies between

soluble SA-4-1BBL and 3H3 have shown SA-4-1BBL to be more potent activator than

3H3 while lacking the antibody associated toxicity (23). Our lab has conducted a series of

extensive studies demonstrating the *in vitro* and *in vivo* pleiotropic functions of SA-4-

1BBL on adaptive, innate, and regulatory immune cells.
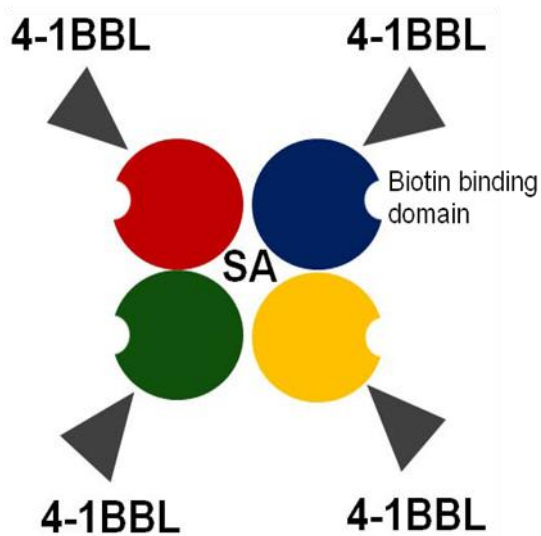
Illustration 4. SA-4-1BBL structural representation. Shirwan's Lab, 2016.

SA-4-1BBL adjuvant effect on adaptive immunity

In our attempt to develop subunit based therapeutic cancer vaccines using defined TAAs, SA-4-1BBL was shown to act on $CD4^+$ as well as $CD8^+$ T cells to fulfill its potent adjuvant role in the designed vaccine formulations. Depletion of $CD4^+$ T cells 1 day prior tumor inoculation in two transplantable models (TC-1 cervical cancer expressing Human Papillomavirus antigen E7; and 3LL lung carcinoma overexpressing the self antigen survivin) was shown to significantly reduce the therapeutic vaccine efficacy administered 6 days later from tumor challenge (25). Upon rechallenge of tumor free mice, long term immunological memory was also lost in the absence SA-4-1BBL effect on $CD4^+$ T cells. In another study using survivin + SA-4-1BBL vaccine in 3LL model, 100% of tumor eradication was achieved in a prime-boost vaccination strategy. Depletion of $CD8^+$ T cells producing IFN-γ 1 day prior vaccinations completely compromised therapeutic efficacy while depletion of NK cells had moderate yet noticeable effect on tumor growth. NK cells isolated from immunized mice showed improved killing against YAC-1 target cells *in vitro* (26).

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SA-4-1BBL adjuvant effect on innate immunity

Switching gears towards innate immunity, SA-4-1BBL's direct or indirect effect on NK cells has been shown to be indispensable for HPV-16 E7 recombinant protein-based therapeutic vaccine in the TC-1 murine tumor model. Eradication of established tumors was mainly dependent on 4-1BB signaling as C57BL/6.4-1BB knockout (C57BL/6.4-1BB$^{-/-}$) mice failed to clear the tumors and expired accordingly (27). Activated NK cells have been shown in the literature to express 4-1BB receptor themselves and are involved in the eradication of P815 mastocytoma *in vivo* although they lack the *in vitro* lysis function upon stimulation with anti-4-1BB monoclonal antibody, suggesting an immuno-regulatory role of NK cells in this case as compared to direct killing and effector functions (28). NK cells isolated from human peripheral blood, activated with IL-2/IL-15 cytokines for 48 hours to upregulate 4-1BB, and incubated with the MCF-7 human breast cancer cell line overexpressing 4-1BBL for additional 12 hours, have been shown to upregulate perforin, granzyme B, and FasL genes without enhancing cellular degranulation nor IFN-γ production upon engagement of 4-1BB with 4-1BBL on MCF-7 cells (29).

Dendritic cells (DCs) of the innate immune system on the other hand, produce a variety of cytokines such as TNF-α, IL-6, and IL-12 upon 4-1BB stimulation (30, 31). SA-4-1BBL not only provides robust immune costimulatory activity in DCs in therapeutic cancer vaccines but also contributes to the delivery of TAAs conjugated through biotin to the streptavidin portion of the molecule (32). DCs in turn engulf the SA-4-1BBL-TAA conjugate and enhance antigen presentation to T cells. TC-1 or 3LL tumor bearing mice showed significant improvement in survival upon administering SA-4-

1BBL-TAA conjugate vaccine on day 6 post-tumor challenge versus administering SA-4-1BBL + TAA without conjugation. The therapeutic efficacy of the vaccine was associated with higher tumor infiltrating $CD8^+$ T cells to Tregs ratio (32).

Another study conducted in our lab has recently aimed at simultaneous stimulation of innate and adaptive immunity in fighting tumors using a combination of the Toll-like receptor 4 (TLR4) agonist called monophosphoryl lipid A (MPL) plus SA-4-1BBL. One vaccination with the combination of both adjuvants and HPV E7 protein as TAA resulted in 100% survival in murine TC-1 model. Vaccine formulation containing SA-4-1BBL had better efficacy (~80%) than formulation with MPL alone (~50%) (33).

SA-4-1BBL's role in regulatory immunity

SA-4-1BBL has a major impact on regulatory immunity. SA-4-1BBL drives the proliferation of activated Tregs when combined with the cytokine IL-2 while maintaining Tregs' suppressive function (34). Tregs constitutively express the IL-2 and 4-1BB receptors and SA-4-1BBL costimulation further upregulates the expression of both receptors. This regulatory aspect of SA-4-1BBL was shown to overcome autoimmune diseases and prevent graft rejection (34). On the other hand, we showed that SA-4-1BBL without exogenous IL-2 blocks the conversion of conventional $CD4^+$ T cells into induced Tregs through the production of IFN-γ (35). One of the important mechanisms by which tumors evade suppression is the induction of iTregs in the tumor microenvironment and SA-4-1BBL has the ability to block the accumulation of iTregs even in the presence of tumor antigens and TGF-β pressure. In the process of exploring the effects of SA-4-1BBL on Tregs, we have observed that Teffs themselves become resistant and refractory

to suppression by Tregs (34, 36), the mechanism of which is yet unknown and will be elucidated in the subsequent chapter of this dissertation.

In summary, 4-1BB signaling has pleiotropic effects in modulating the immune system by acting on all 3 branches: innate, adaptive, and regulatory immunity. These effects are content- and model- dependent and need to be carefully considered when exploiting 4-1BB signaling for the generation of desired immune responses for therapeutic purposes.

CHAPTER 2: SA-4-1BBL LIBERATES CONVENTIONAL T CELLS FROM
NATURAL T REGULATORY CELLS SUPPRESSION VIA IL-2

<u>Introduction</u>

CD4$^+$CD25$^+$Foxp3$^+$ Tregs comprise a distinct and important cell type that confers

peripheral tolerance. Deficiencies in Tregs or their regulatory function are associated with

severe autoimmunity in rodents and humans. Their presence or absence has high impact

in transplantation as well as tumor settings. They can mediate graft survival, but can also

abrogate effector T cells from carrying their killing function in tumor microenvironments

(37). Subtypes of Tregs include naturally occurring thymic Tregs (nTregs) that are

CD4$^+$CD25$^+$Foxp3$^+$, and induced Tregs (iTregs) that are CD4$^+$ Foxp3$^-$ but convert into

Foxp3$^+$ upon induction in the periphery. Other subtypes include Tr1 and Th3 iTregs that

are Foxp3$^-$ but are specialized in IL-10 and TGF-β production (38). Although 4-1BB

receptor is constitutively expressed on Tregs in mice, our lab has previously shown that

in Teff:Treg co-culture experiments, SA-4-1BBL drives the proliferation of Teffs without

altering the suppressive functions of Tregs. Tregs pre-incubated with SA-4-1BBL prior

addition to suppression assays did not cease their suppressive function (34). Moreover,

cultures with Tregs sorted from C57BL/6. 4-1BB$^{-/-}$ mice and incubated with sorted Teffs

from wild type C57BL/6 mice in the presence of SA-4-1BBL showed high proliferation

of Teffs proving that evasion of suppression is not due to direct engagement of SA-4-

1BBL with its receptor on Tregs (36). Combining our

observations together, we hereby shifted our focus to study the direct mode of action of

SA-4-1BBL on conventional T cells (Tconvs) and the mechanism(s) underlying the

escape from suppression.

Materials and Methods

Mice and reagents

Transgenic C57BL/6.Foxp3^{GFP} and wild type C57BL/6 mice were purchased from The

Jackson Laboratory. C57BL/6.Foxp3^{hCD2} mice (39) and 4-1BB knockout mice (40) were

bred and cared for in University of Louisville's SPF-free vivarium in accordance with

NIH guidelines. All animal procedures were approved by the University of Louisville's

Institutional Animal Care and Use Committee. Antibodies used include anti-CD3

agonistic Ab (clone 145-2C11, BD Pharmingen, Cat# 553058), anti-TGF-β (1,2,3)

neutralizing Ab (clone 1D11, homemade), anti-IFN-γ blocking Ab (clone XMG 1.2,

homemade), and anti-IL-2 neutralizing Ab (clone S4B6, BD Pharmingen, Cat# 554375).

Fluorescein-conjugated Abs to various cell surface markers were obtained commercially:

anti-CD4-APC (BD Pharmingen, 553051); anti-CD25-PE (BD Pharmingen, 553866);

anti-CD25-PE-Cy7 (eBioscience, 25-0251-82); anti-hCD2-PE (BioLegend, 300208).

Where indicated Tconvs or Tregs were stained with 2.5µM CFSE (Invitrogen, C1157).

CTLL-2 cell line was obtained from ATCC (TIB-214). Human IL-2, SA-4-1BBL, and

SA proteins were produced in our lab according to standard protocols as previously

reported (34, 41, 42).

Cell sorting

Both Tconvs and Tregs were sorted from splenocytes of naive C57BL/6.Foxp3^{GFP} mice using BD FACSAria by gating on CD4$^+$ CD25$^-$ GFP$^-$ cells to collect Tconvs and on CD4$^+$ CD25$^+$ GFP$^+$ population to collect Tregs. Tconvs from WT C57BL/6 or Tregs from C57BL/6.Foxp3^{hCD2} mice were sorted by gating on CD4$^+$ CD25$^-$ to collect Tconvs and CD4$^+$ CD25$^+$ to collect Tregs. Sorted populations purity was $\geq$ 99%.

Ex-vivo expansion of Tregs

In select experiments, Tregs were expanded using our previously described protocol (34). Briefly, 1 x 10^5 to 5 x 10^5 sorted Tregs were cultured in a 6 well plate in addition to 1 x 10^6 irradiated C57BL/6 splenocytes (2000 cGy); anti-CD3 antibody (0.5 µg/ml); SA-4-1BBL (1 µg/ml); IL-2 cytokine (25 IU/ml). Cells were cultured for 4 days at 37°C, 5% CO$_2$ after which 1 ml of culture media was displaced with 1 ml fresh MLR media and supplemented with IL-2 (25 IU/ml). On day 7, cells were collected, centrifuged, washed and resuspended in fresh MLR media for additional 3 days with IL-2 only (25 IU/ml). On day 10, cells were washed and rested without IL-2 for two to three days (while checking viability) prior addition to suppression assays. Expanded Tregs maintain their Foxp3 expression as tested by flow cytometry (data not shown) and retain their suppressive functionality.

Suppression assays with and without APCs

Freshly sorted Tconvs (2.5 x 10^4) were cocultured in 96-well U-bottom plates with freshly sorted or expanded Tregs (2.5 x 10^4) plus irradiated naive C57BL/6 splenocytes

as source of APCs (1 x 10^5, 2000 cGy). Cultures were supplemented with anti-CD3 Ab

(0.5 µg/ml), SA-4-1BBL (1 µg/ml), and IL-2 (3 IU/ml) where indicated. In absence of

APCs, Tconvs and Tregs were cultured with plate bound anti-CD3 (5 µg/ml), while

maintaining the rest of the conditions constant. Coculture plates were incubated for 48 h

(37° C, 5% CO_2) and pulsed with 1 µCi/well of [^{3}H]-thymidine (PerkinElmer, Cat#

NET027X001MC) for an additional 16 h. Plates were harvested using Tomtec cell

harvester and counts per minute (CPMs) were recorded using Wallac Betaplate liquid

scintillation counter as a measure of cell proliferation.

Cytometric bead array

Th1/Th2/Th17 panel of cytokine beads (BD Biosciences, Cat# 560485) were used to

measure IL-2, IL-4, IL-6, IFN-γ, TNF-α, and IL-17a levels in cell culture supernatants

following manufacturer's instructions. Concentrations of the various cytokines were

calculated against a standard that runs from 0-5000 pg/ml.

RT-PCR for IL-2 expression

Tconvs and Tregs were sorted from splenocytes of naive C57BL/6.Foxp3^{GFP} mice using

BD FACSAria by gating on CD4$^+$ CD25$^-$ GFP$^-$ cells to collect Tconvs and on CD4$^+$

CD25$^+$ GFP$^+$ population to collect Tregs. Cocultures were stimulated with SA-4-1BBL (1

µg/ml) and plate-bound anti-CD3 Ab (5 µg/ml). Cultures were harvested for total RNA

isolation 24 and 48 hours post-stimulation. RNA was converted into cDNA and used for

real-time PCR amplification using primers for housekeeping gene glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) and IL-2. Primer sequences (GenScript) were as

follows: IL-2 forward 5'-TGA GCA GGA TGG AGA ATT ACA GG-3', reverse 5'-GTC

CAA GTT CAT CTT CTA GGC AC-3'; GAPDH forward 5'-CCA TCA CCA TCT TCC AGG AGC GAG-3', reverse 5'-CAC AGT CTT CTG GGT GGC AGT GAT-3'. Delta-Delta CT values were calculated and normalized to GAPDH.

Removal of SA-4-1BBL and IL-2 from Tconv culture supernatant

Supernatants were generated from 96 well cultures of (Tconvs + 5 µg/ml plate bound anti-CD3 + 1 µg/ml SA-4-1BBL) incubated for 48 hours at 37° C, 5% CO_2. Taking advantage of the 6xHis tag engineered into the SA-4-1BBL molecule, culture supernatants were incubated with $CuSO_4$-charged sepharose beads for 15 minutes at room temperature followed by centrifugation and aspiration of the SA-4-1BBL-cleaned supernatant.

IL-2 was removed by incubating supernatants with anti-IL-2 neutralizing antibody (20µg/ml, clone S4B6) to form complexes at 37° C for 15-30 minutes. Ab/IL-2 complexes were then removed by incubating with Immobilized Protein G high affinity beads (Thermo Scientific, Cat# 20398). IL-2-cleaned supernatant was tested on CTLL-2 cells that require IL-2 cytokine as a limiting factor for their growth. CTLL-2 cells were plated in U-bottom 96 well plates (5000 cells/50µl + 50µl supernatant cleaned from SA-4-1BBL only as positive control, or cleaned from SA-4-1BBL and IL-2). Cultures were incubated for 28 hours total at 37° C, 5% CO_2 with addition of [^{3}H]-thymidine for the last 8 hours. Cells were harvested using Tomtec cell harvester and proliferation of CTLL-2 cells was quantified.

Statistical analysis

Each data point represents the mean ± SEM of triplicate wells unless otherwise stated. The statistical significance between data points was estimated with two-tailed Student's *t* test using GraphPad software. A *p* value ≤ 0.05 was considered to be statistically significant.

Results

APCs do not contribute to SA-4-1BBL-mediated evasion of Treg suppression

In previously published studies, we have demonstrated that SA-4-1BBL costimulation allows Tconvs to overcome Treg suppression (36). These studies were conducted using Tregs and Tconvs cocultured with APCs from C57BL/6 wild type mice. The 4-1BB receptor is expressed on a subpopulation of DCs and stimulation via this receptor results in the secretion of various cytokines, such as IL-6, IL-12, and TNF-α (30, 31). To assess the potential contribution of signaling in APCs to the observed Tconv evasion of Treg suppression, suppression assays were performed with irradiated splenocytes from wild type as well as 4-1BB KO mice. SA-4-1BBL overcame the suppression of sorted Tconvs cultured by Tregs. More importantly, Tconvs cocultured with 41BB$^{-/-}$ APCs showed robust proliferation responses when costimulated with SA-4-1BBL in the absence or presence of Tregs (Fig. 1). These responses were comparable to those generated with wild type APCs.

To further confirm that APCs do not play a role in the Tconv evasion of Treg suppression, the coculture studies were performed in the absence of APCs. Stimulation of Tconvs with plate bound anti-CD3 Ab alone under these culture conditions did not

generate a significant proliferative response (Fig. 2). However, costimulation with SA-4-1BBL resulted in robust Tconv proliferation that was refractory to suppression by Tregs. Taken together these data demonstrate that costimulation via 4-1BB receptor expressed on APCs is not involved in SA-4-1BBL-mediated Tconv resistance to suppression by Treg cells.

TGF-β and IFNγ do not contribute to SA-4-1BBL-mediated evasion from Treg suppression

TGF-β has been shown to play an important role in the generation of Tregs and their suppressor function (43-45). We therefore envisioned that SA-4-1BBL stimulation may overcome the Treg mediated suppression by modulating the function of TGF-β. A neutralizing Ab against TGF-β at two different doses (8 and 50 μg/ml) did not neutralize Treg suppression nor did it further improve the observed SA-4-1BBL-mediated evasion of Treg suppression (Fig. 3).

It has been demonstrated that T cells in response to T-cell receptor signaling produce an active form of TGF-β, which is adsorbed to the cell surface immediate post-secretion (46). Membrane-bound TGF-β could act as a mediator for Treg suppression (47). Therefore, to eliminate the possibility that SA-4-1BBL may overcome Treg suppression by modulating the function of membrane-bound TGF-β, we sorted Tregs and pre-incubated them with high dose anti-TGF-β Ab (50 μg/ml) for 1 hour prior addition to coculture suppression assays. These cells were able to suppress Tconvs and costimulation with SA-4-1BBL was still able to overcome suppression (Fig. 4).

SA-4-1BBL costimulation of Teffs results in their secretion of IFN-γ, which blocks TGF-β and tumor-mediated conversion of these cells into induced Tregs (35).

However, opposite findings show that IFN-γ may also contribute to the conversion of Tconvs into peripheral Tregs and improve their suppressive function by upregulating Foxp3 gene expression (48). To study the potential role of this cytokine in the observed SA-4-1BBL-mediated evasion of Treg suppression, we performed suppression assays with a blocking Ab against IFN-γ (25 µg/ml). The antibody added to the cocultures did not block the suppression of Tconvs by Tregs, also it provided evidence that IFN-γ is not responsible for the evasion of suppression in the presence of SA-4-1BBL (Fig. 5). These observations are consistent with previous findings that IFN-γ has no direct effect on natural thymic Treg development and function (49).

IL-2 is the major cytokine produced in the supernatant of Tconv:Treg cocultures costimulated with SA-4-1BBL

In an attempt to identify potential soluble factors that contribute to the Tconv evasion of Treg suppression, we assessed cytokines generated in coculture supernatants in response to SA-4-1BBL costimulation using cytokine bead array analysis. As shown in (Fig. 6), costimulation of Tconvs with SA-4-1BBL resulted in upregulated secretion of all cytokines tested except IL-17. IL-2 and TNF-α production levels were particularly increased in the 48 hour culture supernatants of Tconvs + SA-4-1BBL. However, the levels of these two cytokines were similar in supernatants harvested from Tconv:Treg cocultures with or without SA-4-1BBL costimulation. This cytokine pattern was not time dependent as 24 hour culture supernatants showed a similar pattern (data not shown).

The presence of APCs and particularly their irradiation may impact overall cytokine production and secretion into the coculture supernatants. Therefore, these studies were performed with sorted Tconvs and Tregs in the absence of APCs. IL-2 stood

out as the highlight cytokine with significant and robust production in supernatants

harvested from Tconvs, as well as Tconv:Treg cocultures costimulated with SA-4-1BBL,

but not in culture supernatants without SA-4-1BBL costimulation (Fig. 7). The lack of

high levels of IL-2 in cultures with irradiated APCs may be due to its non-specific

binding or consumption by such cells, or other unknown mechanisms.

We also noticed a significant drop in the levels of IL-2 in Tconv:Treg cocultures

stimulated with SA-4-1BBL (Fig. 6 and 7) versus Tconvs + SA-4-1BBL cultures. To test

if this is due to IL-2 produced by Tconvs being consumed by Tregs that do not produce

significant levels of this cytokine, we performed quantitative RT-PCR to assess the level

of IL-2 mRNA under various culture conditions. As shown in Figures 8A and 8B, IL-2

transcript levels were comparable between Tconv and Tonv:Treg cocultures stimulated

with SA-41BBL examined at 24 and 48 h time points. Taken together, these data suggest

that the reduced levels of IL-2 protein observed in supernatant of Tconv:Treg coculture

stimulated with SA-4-1BBL is not due to the diminished IL-2 transcript levels, but

possibly because of the consumption of this cytokine by Tregs.

IL-2 is necessary and sufficient for Tconvs to overcome Treg suppression

The high levels of IL-2 production in Tconv:Treg cocultures costimulated with

SA-4-1BBL has captured our interest to test the role of this cytokine in the evasion of

Treg suppression. Culture supernatants from Tconvs + plate-bound anti-CD3 + SA-4-

1BBL for 48 h were harvested and depleted for SA-4-1BBL protein using $CuSO_4$ charged

sepharose beads, taking advantage of the 6xHis Tag engineered into the protein for

purification purposes. The complete removal of SA-4-1BBL was confirmed by Western

blots (Fig. 9). We next subjected this supernatant for removal of IL-2 by first incubating

with an excessive amount of an anti-IL-2 Ab followed by incubation and removal using protein G beads. The lack of IL-2 in culture supernatant was confirmed in a CTLL-2 proliferation assay (Fig. 10). Tconv culture supernatants lacking SA-4-1BBL or both SA-4-1BBL and IL-2 were tested for their ability to overcome Treg suppression. Addition of SA-4-1BBL-free culture supernatant resulted in effective evasion of suppression in Tconv:Treg coculture experiments with (Fig. 11A) or without APCs (Fig. 11B). On the other hand, culture supernatant lacking both SA-4-1BBL and IL-2 was ineffective in overcoming Treg suppression. To further confirm that IL-2 is required for overcoming Treg suppression, Tconv:Treg cocultures were supplemented with recombinant IL-2. Exogenous IL-2 resulted in robust proliferative responses in both Tconv and Tconv:Treg cocultures (Fig. 11A, 11B).

IL-2-mediated Tconvs evasion of Tregs suppression is independent of Tregs proliferative state

In environments where IL-2 is not a limiting factor, both Teffs and Tregs are known to proliferate upon primary stimulation such as during inflammatory insults (50). A previous publication by our group also indicates that SA-4-1BBL causes the proliferation of both Teffs and to a lower extent Tregs (34). Putting one and two together we have stained either Tconvs sorted from C57BL/6 or Tregs sorted from C57BL/6.Foxp3^{hCD2} splenocytes with CFSE prior addition to suppression assays. The CFSE labeled Tconvs proliferated by 67.5% with SA-4-1BBL and by 64.1% in the presence of Tregs and SA-4-1BBL. The CFSE labeled Tregs on the other hand proliferated by 11.1% with SA-4-1BBL and by 27.3% in the presence of Teffs and SA-4-1BBL (Fig. 12). One might claim here that when Tregs are in an active proliferative state

they might lose their suppressive capacity and the Tconvs can evade and over-proliferate. In fact, Tregs at their anergic non-proliferative state are shown to be better suppressors (51).

To eliminate the contribution of Treg proliferation to the observed evasion of suppression achieved by SA-4-1BBL, we irradiated sorted-expanded Tregs with 2000 cGy before utilizing them in suppression assays. The irradiated Tregs did not proliferate upon addition of exogenous IL-2 or SA-4-1BBL and they still maintained their suppressive function (Fig. 13). More interestingly, when we added SA-4-1BBL to the cocultures in the presence of irradiated Tregs, Tconvs were still able to proliferate significantly and escape the suppressive non-proliferative Tregs, which gives us further confirmation that the mechanism is through IL-2 acting directly on Tconvs in an autocrine fashion.

Discussion

Our first experiment aimed at reproducing the evasion of suppression data from previous studies in the lab in the presence and absence of SA-4-1BBL in coculture suppression assays (36), only this time we have used splenocytes from $41BB^{-/-}$ mice as source of irradiated APCs along with soluble anti-CD3. The reason was to eliminate any interference of 4-1BB receptor on the surface of APCs binding with SA-4-1BBL and causing the leakage of cytokines such as IL-6, TNF-α, and IL-15, despite irradiation. Sorted Tconvs cultured with $41BB^{-/-}$ APCs proliferated successfully in the presence of SA-4-1BBL and were able to escape suppression when co-cultured with Tregs. The finding further emphasizes the direct effect of SA-4-1BBL on Tconvs. More Importantly,

SA-4-1BBL conferred Tconv resistant to Treg suppression in the complete absence of APCs.

In an attempt to understand the mechanism of evasion, we initially hypothesized that if Tregs suppress primarily through TGF-β, SA-4-1BBL might be downregulating TGF-β receptor II on Tconvs, hence desensitizing them to suppression (52). Adding anti-TGF-β neutralizing antibody to the specified cultures (8 µg/ml and 50 µg/ml) did not block the suppression of Tregs and did not improve the evasion levels noticeably. These findings are in accordance with published studies demonstrating that thymically derived (t)Tregs suppress through cell-to-cell contact without a significant contribution by TGF-β (51, 53). Signaling via 4-1BB receptor on T cells results in IFN-γ production that has the ability to block TGF-β and tumor-mediated conversion of Tconvs into iTregs (35). On the other hand, IFN-γ contributes to the conversion of Tconvs into iTregs and enhances their suppressive function through upregulation of the forkhead box P3 (48). However, these divergent observations are conducted under different experimental setups as the first study has used TGF-β and antigen stimulation-dependent conversion, while the second study has utilized antigen- and a TGF-β-independent conversion setting. In the present study, a blocking Ab to IFN-γ (25 µg/ml) neither prevented Treg suppression in the absence of SA-4-1BBL, nor altered SA-4-1BBL-mediated evasion of suppression. Our data supports previous findings stating that IFN-γ has no direct effect on (t)Tregs development and function (49).

IL-4 may be another cytokine yet that regulates the balance between Teffs and Tregs in a twofold fashion: acting on Teffs and activating STAT 6 pathway on one hand making Teffs resistant to suppression (54, 55), or on the other hand IL-4 may act on

Tregs and activate GATA 3 that binds in turn to Foxp3 and blocks it (56, 57). The same studies have reported that the effect of IL-4 is pronounced in iTregs and not nTregs in general, which dramatically drops the odds of IL-4 involvement in our study since we have sorted nTregs from naïve mice that are Foxp3$^+$.

IL-2 expressed by Tconvs is required and sufficient for the ability of these cells to evade Treg suppression under the tested coculture conditions. First, IL-2 was the major cytokine upregulated in cocultures stimulated with SA-4-BBL in the absence of APCs. Second, supernatant from SA-4-1BBL-stimulated cultures was effective in overcoming Treg suppression, and removal of IL-2 nullified this effect. Third, exogenous recombinant IL-2 alone was effective in overcoming Treg suppression. Competition for IL-2 has been shown to be an important mechanism that dictates the functional balance between Tconvs and Tregs. The receptor for IL-2 is a trimeric complex consisting of IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132). The high affinity receptor CD25 is constitutively expressed on nTregs while it is upregulated upon induction on Teffs. IL-2 is also known to be a growth factor for Tregs (58), more specifically IL-2 stabilizes the expression of Foxp3 in iTregs in the presence of TGF-β (59). Our observation is explained by the fact that in the presence of weak signals such as anti-CD3 and low levels of IL-2, Tregs that constitutively express CD25 drain all the IL-2 and deprive the Tconvs from surviving and proliferating (60, 61). On the other hand, a strong costimulatory molecule such as SA-4-1BBL that provides a potent secondary signal, generates an abundance of IL-2 and upregulates CD25 on Tconvs allowing them to break from suppression. Indeed, a recent study using an *in vivo* imaging approach has demonstrated that autoantigen-driven Treg clustering with Tconvs on the surface of DCs and

competition for IL-2 expressed by Tconvs is a major mechanism of autoimmune suppression (62). A similar IL-2 competition-based suppressive mechanism has recently been reported for NK cells (63).

The present study demonstrating that SA-4-1BBL targets Tconvs directly for evasion of Treg suppression is in conflict with another study conducted with agonistic 4-1BB antibody (64). The authors in the latter state that anti-4-1BB Ab impedes the suppressive function of Tregs by significantly downregulating the expression of IL-9 in these cells. They also demonstrate that the combination treatment with CpG-ODN adjuvant plus anti-IL-9 neutralizing antibody *in vivo* causes tumor rejection in the BALBneuT and MUC-1 models (64). However, this study did not provide direct evidence demonstrating that Tregs are the direct target of agonistic Abs to 4-1BB. The in vitro evasion of suppression data was obtained using Tconv:Treg cocultures similar to ours. Also, this study failed to demonstrate the direct role of IL-9 on Treg suppressive function in vitro. In contrast, our previous study using Tconvs and Tregs from wild type and 4-1BB KO mice provided direct evidence for Tconvs, but not Tregs, being the target of SA-4-1BBL for the observed evasion of suppression (36).

In conclusion, our findings that excessive production of IL-2 by Tconvs in response to SA-4-1BBL costimulation is both necessary and sufficient in overcoming Treg suppression may have significant implications for treatment of diseases associated with an imbalance between Tconvs and Tregs, such as cancer. High dose IL-2 immunotherapy against tumors as well as low dose in combination with chemotherapy or IFN-α treatment has been the subject of several clinical studies since 1980s. The most pronounced effects were noticed in melanoma and renal cell carcinoma patients (65). The

toxicity caveat associated with the treatment regimen and administration of exogenous IL-2 has still yet to be overcome (66). SA-4-1BBL that safely drives the endogenous production of IL-2 from Teffs could serve as a great alternative without overdosing the system with excessive amounts of the cytokine.

CHAPTER 3: SA-4-1BBL AS AN IMMUNE ADJUVANT FOR THE GENERATION
OF PROTECTIVE INNATE AND ADAPTIVE IMMUNE RESPONSES AGAINST
TUMORS

<u>Introduction</u>

Previous studies in our laboratory have demonstrated that SA-4-1BBL as an

adjuvant component of TAA-based subunit vaccine generates robust $CD8^+$ T cell

responses with therapeutic efficacy in various tumor models (27, 36). The SA component

of the molecule is a bacterial antigen extracted from *Streptomyces avidinii* and has the

potential to generate antibodies following repeated injections. The presence of anti-SA

antibodies (Abs) may play a dual role by enhancing the efficacy of the vaccine through

crosslinking the SA-4-1BBL molecules, hence leading to better receptor clustering and

signaling. On the other hand, anti-SA Abs may bind and neutralize the effect of the SA-4-

1BBL molecule by blocking its interaction with 4-1BB or accelerating its *in vivo*

clearance.

In order to understand the positive or negative side effects of antibodies produced

against vaccine immunogens, it is best to refer to studies concerning anti-drug antibodies

(ADAs) against persistent biologics and protein treatments. For example, patients on

adalimumab treatment for rheumatoid arthritis showed lower response rates in the

presence of anti-adalimumab Abs, which directly correlated to lower levels of the

adalimumab drug in their sera (67). Yet another study explored the effect of ADAs

against infliximab, etanercept, adalimumab and ustekinumab in treated patients with

psoriasis (68). The presence of anti-infliximab and anti- adalimumab Abs was associated

with lower serum drug concentrations, while anti-etanercept Abs had no apparent effect. Anti-ustekinumab antibodies played both positive and negative roles against psoriasis in separate studies. Therefore, a review of data shows that not all cases positive for ADAs have a loss of drug efficacy or cause hypersensitivity reactions.

In this particular chapter, we explore the solo role of SA-4-1BBL costimulatory molecule and its impact on the immune system. The SA portion is a biological agent that carries several epitopes that can be recognized by T and B cells. In some studies it is suggested to reduce the immunogenicity of SA through the concomitant administration of methotrexate, or the use of mutated forms of the de-immunized protein with less antigenic epitopes, while maintaining tetramer forming and biotin binding capacities of the molecule (69, 70). Other studies exploit the use of immunogenic SA to the advantage of vaccine development and treatment. For example, vaccination with soluble tumor proteins conjugated to SA has been shown to be effective in the 9L rat glioma model (71). In that study, antibodies against SA were detected in the immunized rats. However, Ab levels did not correlate with the anti-tumor response. Similarly, we show below that pre-treating mice with SA-4-1BBL protects against subsequent tumor challenges without interfering in post-tumor vaccine outcomes and without contribution from anti-SA antibodies. An insight into the mechanism of action of SA-4-1BBL and its role in anti-tumor immunity is further demonstrated and discussed in this chapter.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6.4-1BB knockout mice (40) and 4C mice (72) were bred and cared for in a University of Louisville specific pathogen-free animal facility in accordance with NIH guidelines. All animal procedures were approved by the University of Louisville's IACUC.

Antibodies and Reagents

Antibodies used include anti-CD3 agonistic Ab (clone 145-2C11, BD Pharmingen, Cat# 553058). Fluorescent-conjugated Abs to various cell surface markers were obtained commercially: anti-CD3-V500 (BD Horizon 560771); anti-CD4-Alexa700 (BD Pharmingen 557956); anti-CD8-APC-Cy7 (BD Pharmingen 557654); anti-NK1.1-PE (BD Pharmingen 553165); anti-CD19-APC (BD Pharmingen 550992); anti-CD44-APC (eBioscience 17-0441-83); anti-CD62L-PerCp-Cy5.5 (eBioscience 45-0621-82). Anti-4-1BB agonistic Ab (clone 3H3) was produced and purified in our Lab. 500 µg/mouse of depletion antibodies were injected i.p. in specified experiments including anti-CD4 Ab (clone GK1.5); anti-NK1.1 Ab (clone PK 136); anti-CD8 Ab (clone 53.6.72); and anti-CD20 Ab 200 µg/mouse (clone 5D2).

SA-4-1BBL and SA proteins were produced in our lab according to standard protocols as previously reported (34, 41, 42). TC-1 tumor cell line was obtained from American Type Culture Collection (ATCC).

Immunization strategy and tumor challenge

Several groups of mice were challenged subcutaneously (s.c.) with 2 shots of SA-4-1BBL

(25 µg) on days 0 and 14. Unless specified, mice were injected two weeks later s.c. with

1×10^5 cells of TC-1 tumor cell line. TC-1 is originally derived from lung epithelial cells

of C57BL/6 mice and transfected to express the human papillomavirus HPV-E6 and E7

proteins. In certain groups, mice also received post tumor challenge vaccination with 50

µg E7-P1 peptide (RAHYNIVTF) + 25 µg SA-4-1BBL (Fig. 14). Tumors were

monitored and measured twice a week using calipers over a period of 60 days or until

tumor average diameter reaches its endpoint (12 mm). In select experiments, Lewis Lung

Carcinoma (LLC) cell line was used instead of TC-1. For long term memory studies,

tumor free mice at the experimental endpoint were re-challenged with 1×10^5 cells of TC-

1 and monitored for at least additional 60 days.

Enzyme-linked immunosorbent assay (ELISA) for serum antibodies and subtyping

Blood was collected and serum isolated from different groups of mice 1 week after the

2nd immunization with SA-4-1BBL and at experimental end points. ELISA plates were

coated overnight with 50 ng of either SA-4-1BBL or SA proteins. Next day plates were

washed, blocked with milk, and incubated with serial dilutions of specified sera for 1.5

hours at room temperature. After that, secondary antibodies conjugated to horseradish

peroxidase enzyme (HRP) were added for 1 hour to measure for total IgG titers as well as

several isotypes including IgG1, IgG2b, IgG2c, IgG3, IgM, and IgA. Plates were

incubated for 30 min with TMB substrate (BD Biosciences, Cat#555214), reaction was

stopped with 2N H_2SO_4 and plates were read for colorimetric reaction at 450 nm.

Adoptive serum transfer

Sera collected on Day 27 from SA-4-1BBL-immunized mice (25 µg on days 0 and 14 s.c.) were adoptively transferred to naïve C57BL/6 mice (200 µl i.v.) 24 hours prior to TC-1 tumor challenge (1×10^5 cells s.c.). Sera antibody titers were tested by ELISA against SA prior to transfer. Tumor diameters were measured twice a week using calipers over a period of 60 days or until tumor average diameter reaches its endpoint (12 mm).

Anti-CD3 proliferation assay

C57BL/6 splenocytes (2×10^5 cells/well) were cultured in 96-well U-bottom plates and stimulated with anti-CD3 agonistic antibody (0.025 µg/ml) and serially diluted doses of SA-4-1BBL in the presence or absence of mice serum pre-immunized twice with 25 µg of SA-4-1BBL. Cultures were incubated for 48 h and pulsed with [^{3}H]-thymidine for an additional 16 h. Plates were harvested with Tomtec Cell Harvester and proliferative counts were measured with a Beta plate counter and reported as CPM.

Flow cytometry and phenotyping

Spleens and draining lymph nodes from naïve, 12.5 µg SA + 100 µg 3H3 immunized, or 25 µg SA-4-1BBL immunized C57BL/6 mice were harvested at different time points and processed into single cell suspensions. One million cells of each sample were stained with fluorescent-conjugated antibodies with corresponding compensation and isotype controls for flow cytometry. CD4$^+$ T cells and CD8$^+$ T cells were gated on CD3$^+$ population. B cells were gated on CD19$^+$, NK cells were gated on CD3$^-$ and NK1.1$^+$. NKT cells were gated on CD3$^+$ and NK1.1$^+$. Cell percentages as well as absolute cell numbers were calculated and reported. For memory phenotyping, CD3$^+$ CD62L^{lo} CD44^{hi}

T cells were characterized as T-effector memory, while $CD3^+$ $CD62L^{hi}$ $CD44^{hi}$ were characterized as T-central memory.

Statistics

At experimental end points, Kaplan-Meier survival curves were graphed and log-rank statistical method was applied between groups using GraphPad software, with $p \leq 0.05$ considered significant.

<u>**Results**</u>

Streptavidin portion of SA-4-1BBL is immunogenic

To assess the immunogenicity of SA-4-1BBL, a group of C57BL/6 mice (Group 1) was immunized twice with 25 µg of SA-4-1BBL via s.c. injection on days 0 and 14. Blood was withdrawn and isolated serum was tested by ELISA for humoral responses one week before TC-1 tumor challenge, one week after tumor challenge, and at experimental endpoint (Fig. 14). ELISA plates pre-coated with SA-4-1BBL (Fig. 15) or SA (data not shown) had comparable total IgG titers. Serum antibody titers were positive on day 21 (4 out of 5 animals), slightly elevated on day 34 (5 out of 5), then slightly reduced on day 90 (Fig. 15). A second group of mice (Group 2) was treated with the same regimen, except that this group also received a subunit vaccine consisting of a peptide (P1) representing the dominant $CD8^+$ T cell epitope for HPV E7 antigen and SA-4-1BBL on day 36. Antibody titers were tested positive on day 21 (2 out of 5 animals), elevated on day 34 (5 out of 5), then slightly reduced on day 90 (Fig. 15).

We next subtyped the Abs to assess the nature of immune responses generated. IgG1 isotype is usually indicative of Th2 responses while IgG2a is associated with Th1

responses (73). IgM is mainly upregulated during primary immune responses and undergoes class switching during secondary responses. T-helper independent antibody production on the other hand favors the IgG3 subtype. Subtyping ELISA for group 1 on days 21 and 90 (Fig. 16) revealed IgG1 and IgM to be the major isotypes upregulated upon SA-4-1BBL immunization.

The positive humoral immune response led us to test if SA also generates CD4$^+$ T cell responses. Mice were immunized once with SA-4-1BBL. Seven days later spleens from naïve as well as immunized mice were harvested, processed, and cultured in vitro for 48 hours in the presence or absence of SA stimulation. Cultures were pulsed with ^{3}[H]-thymidine for additional 16 hours and CPMs were recorded. SA was capable of generating more than 3 fold T cell proliferative responses in SA-4-1BBL immunized cultures as compared to naïve background (Fig. 17).

Anti-SA Abs do not negatively impact the costimulatory function of SA-4-1BBL *in vitro* and *in vivo*

To test if anti-SA antibodies hinder or augment the costimulatory function of the SA-4-1BBL molecule, we conducted an *in vitro* proliferation assay with SA-4-1BBL costimulation-driven expansion of T cells as a read out. In brief, naïve C57BL/6 splenocytes were stimulated with the agonistic anti-CD3 Ab in the presence of SA-4-1BBL incubated with naïve (set 1) or pre-immunized (set 2) serum (Fig. 18). The pre-immunized serum containing anti-SA antibodies did not alter the ability of SA-4-1BBL to deliver its costimulatory signal, as set 2 splenocytes proliferated as efficiently as set 1 *in vitro.*

Following the experimental design demonstrated in Figure 14, we assessed if the *in vivo*-generated anti-SA antibodies, produced by repeated injections with SA-4-1BBL, might modify the post-tumor vaccination outcome with the E7 peptide-based vaccine in the transplantable TC-1 tumor model. As depicted in the survival curve in Figure 19, administering SA-4-1BBL twice not only left the post-tumor vaccine efficacy intact, but also induced 100% tumor protection by itself up to 70 days after tumor challenge.

Pretreatment with SA-4-1BBL generates a prolonged window of immune responses against TC-1 tumor

In order to understand the dynamics of the SA-4-1BBL anti-tumor immune response observed in Figure 19, we next performed extensive time-course pre-immunization studies in C57BL/6 mice receiving TC-1 tumors at different time points after prime or prime/boost immunizations with SA-4-1BBL. As shown in Fig. 20A and 20B, 1 shot of SA-4-1BBL, 1 day later or 1 week later TC-1 challenge protocols did not confer any tumor protectivity, suggesting that immediate innate immune response alone is not sufficient. On the other hand, administering TC-1 two weeks after SA-4-1BBL immunization, showed around 40% survival (Fig. 20C), which provides enough time for adaptive immunity to kick in.

In a second set of studies, we observed 40% survival with 2 shots of SA-4-1BBL, 1 day later TC-1 challenge (Fig. 21A); 100% survival with 2 shots of SA-4-1BBL, 1 week later TC-1 (Fig. 21B); 100% survival and 80% survival were recorded with 2 shots of SA-4-1BBL, 4 weeks and 8 weeks later TC-1 injection respectively (Fig. 22A); but we noticed a significant drop in survival for the 2 shots SA-4-1BBL, 12 weeks later TC-1 challenged group (Fig. 22B).

SA-4-1BBL generates long-term immune memory against TC-1

Our study design to test for protection against TC-1 recurrences was as follows: naïve C57BL/6 mice were immunized twice with SA-4-1BBL (2 weeks apart), followed by 1st TC-1 tumor cells challenge 2 weeks after the second immunization. Mice were monitored for a period of 60 days after 1st tumor injection. At the end of the 60 days, tumor free mice were rechallenged with a 2nd shot of TC-1 tumor cells and monitored for another 80 days (Fig. 23). As shown, pre-immunization with SA-4-1BBL alone significantly delayed the onset and growth rate of tumors after 2nd TC-1 challenge, hence establishing a long term memory effect. Pre-immunization + post-tumor vaccination group establishes a different type of memory that is specifically directed towards E7-P1 tumor antigen and is mediated through IFN-γ and CD8 cells (27) where we clearly obtain 60% survival even after 80 days from 2nd tumor challenge (Fig. 23).

Phenotyping of spleens (spls) and draining lymph nodes (dLNs) for the SA-4-1BBL pre-immunized group 60 days post-tumor injection revealed a significant increase in the CD4$^+$ T effector memory compartment both in spls and dLNs. In addition, the CD4$^+$ T central memory compartment was elevated in the draining lymph nodes of these mice (Fig. 24).

The protective effect of pretreatment with SA-4-1BBL is tumor-type independent

We hypothesized that since the SA-4-1BBL-generated immune response was independent from tumor specific antigens, then the type of the transplantable tumor used would not affect the outcome. To test this, C57BL/6 mice were immunized twice with 25 µg SA-4-1BBL (2 weeks apart), followed by Lewis Lung Carcinoma (LLC) or TC-1

tumor challenge 2 weeks after the 2nd immunization. Indeed, pre-immunization with SA-4-1BBL alone showed significant protection against LLC relative to TC-1 (Fig. 25).

Anti-SA Abs do not play a role in the protective effect of pretreatment with SA-4-1BBL against TC-1 tumor

Humoral immunity has been shown to play a role in the efficacy of various cancer immunotherapies. Antibodies can have a direct effect on the tumor by recognizing and binding to surface antigens, or by helping antigen-presentation and processing by APCs through opsonization, thereby augmenting downstream adaptive immune responses. Given the positive titers of SA-Abs in the pretreatment setting, we asked if such Abs can contribute to the protective effect we observe against TC-1 tumors. Towards this goal, sera were collected on day 27 from SA-4-1BBL immunized mice (25µg on days 0 and 14) and adoptively transferred into naïve C57BL/6 mice (200 µl/mouse) 24 hours prior to TC-1 tumor challenge. Anti-SA Abs in adoptively transferred sera did not show any significant protection and all the recipient mice expired similar to TC-1 control (Fig. 26).

Protective effect of SA-4-1BBL against tumor involves a cross-talk between NK and CD4^{+} T cells

The lack of a positive effect of anti-SA Abs on tumor protection led us to hypothesize that protection involves cellular immunity. In search for target effector cells, and in the light of our previous findings about the role of NK cells in tumor protection (27), we conducted 2 sets of NK depletion studies with reference to Figure 14 design. In the first set, NK cells were depleted with anti-mouse NK1.1 Ab 1 day prior to each immunization with SA-4-1BBL (days -1 and 13). In the second set, NK cells were depleted once 1 day before TC-1 tumor challenge (day 27). Mice were monitored for

additional 60 days after tumor injection and survival assessed at the experimental

endpoints (Fig. 27). In both sets of experiments, depletion of NK cells significantly

compromised SA-4-1BBL efficacy against TC-1 tumors.

In the same fashion, CD4 cells were depleted using anti-CD4 Ab during 2 distinct

phases. The initiation phase covers the first 27 days before TC-1 challenge where CD4

cells were depleted 1 day prior to immunizations with SA-4-1BBL (Fig. 28), and the

effector phase covers the 60 day period after TC-1 challenge where CD4 cells were

depleted 1 day before TC-1 injection (Fig. 29). In both phases, the presence of CD4 cells

was critical in driving and maintaining the SA-4-1BBL-mediated anti-tumor effect.

Surprisingly, depletion of CD8 cells 1 day prior to SA-4-1BBL immunizations did not

abrogate the anti-tumor effect (Fig. 28).

In order to discount any role of B cells or contribution from antibodies in the

observed SA-4-1BBL-mediated anti-tumor immunity, we depleted B cells with an anti-

CD20 antibody during the initiation phase (1 day before SA-4-1BBL immunizations). In

the absence of B cells, SA-4-1BBL still induced 100% survival against TC-1 (Fig. 30),

showing the lack of dependence on B cells and anti-SA antibodies.

CD4 cells should recognize SA for optimal anti-tumor protectivity

To further confirm the importance of the CD4$^+$ T cell responses to the observed

protective effect of SA-4-1BBL pretreatment, we used the 4C T-cell receptor transgenic

mice where 99% of the CD4 T cells express the vβ13 chain and exclusively recognize

allogeneic Balb/c MHC class II antigen. Due to this specificity, these mice should not

recognize SA upon SA-4-1BBL immunization. Indeed, pre-immunization with SA-4-

1BBL was futile in these mice and tumor protectivity against TC-1 was lost (Fig. 31),

although we noticed a non-significant delay in tumors expiration as compared to 4C

controls injected with TC-1 alone. We also noticed attenuated T cell proliferation in 4C

mice first immunized with SA-4-1BBL for 7 days, followed by stimulation *in vitro* with

SA antigen, as compared to wild type C57BL/6 mice (Fig. 32). On the other hand, this

minimal recognition of SA in 4C mice was sufficient to generate anti-SA antibodies (Fig.

33), which further confirmed our previous observation that antibodies themselves are not

protective, as all mice expired from tumor burden. Sera collected from SA-4-1BBL-

immunized 4C mice was subjected to subtyping ELISA resulting in detection of high

levels of IgM and IgG1 isotypes and low levels of IgG2b and IgG2c as compared to

immunized B6 control (Fig. 34).

The anti-tumor effect of SA-4-1BBL is a unique property of this molecule and is not achieved using agonistic Ab to the receptor

We have previously shown qualitative and quantitative difference between SA-

41BBL and agonistic Ab to 4-1BB (23). We therefore tested if the anti-tumor protection

of SA-4-1BBL is a unique property of this molecule or it can be extended to any agonist

of 4-1BB receptor. Different groups of C57BL/6 mice were immunized twice with either

SA alone (12.5 µg), 3H3 alone (100 µg), SA + 3H3, or SA-4-1BBL (25 µg), followed by

TC-1 challenge 2 weeks after the 2nd immunization. Pre-immunization with 3H3 alone,

SA alone, or with SA + 3H3 did not result in any tumor protection compared to the TC-1

control group (Fig. 35). On the other hand, pre-immunization with SA-4-1BBL has

demonstrated a unique attribute in rejecting subsequently challenged tumors by reaching

80 to 100% survival.

Lymphocyte percentages and absolute cell number analysis in spleens of mice immunized twice with SA + 3H3 vs. SA-4-1BBL showed a significant reduction in NK cells in the SA + 3H3 group (Figs. 36, 37). This could be a direct effect of 3H3 on NK cells since they express the 4-1BB receptor, or it could be the result of 3H3 blocking the function of a helper cell population providing assistance to NK cells such as CD4$^+$ T cells. On the other hand, analysis of draining lymph nodes of the immunized mice showed reduced percentages of NK cells but not absolute cell numbers in the SA + 3H3 treated group (Figs. 38, 39). This observation could be attributed to the toxicity and lymphadenopathy associated with 3H3 treatment, since the lymph nodes harvested were significantly larger in size and had greater total cell counts as compared to the SA-4-1BBL treated group (data not shown).

Moreover, we noticed a shutdown in anti-SA antibody production in the group immunized with SA + 3H3 twice (Fig. 40). Sera was collected on day 21 (1 week after 2nd immunization) and at tumor endpoints, and was subjected to ELISA. We have previously demonstrated in this chapter that the lack of anti-SA antibodies has no effect on tumor rejection, which suggests a direct negative role of 3H3 on CD4$^+$ T cells in this case.

Discussion

Our hypothesis was that, since SA portion of SA-4-1BBL is a foreign bacterial antigen, repeated immunizations with SA-4-1BBL might generate desirable or non-desirable immune responses affecting the overall efficacy of the therapeutic anti-tumor vaccine. Indeed, immunizations with SA-4-1BBL generated anti-SA antibodies detected in mice sera over a period of 90 days. We initially thought that these antibodies might be able to activate NK cells through antibody-mediated cell cytotoxicity (ADCC) leading to tumor eradication. Ideally, for that to take place the tumors should express SA antigen. Upon testing TC-1 tumor lysates by Western blotting, SA protein was not detected (data not shown), although we cannot exclude the possibility of cross-reactivity occurring with another tumor-associated protein that resembles SA. Antibodies on the other hand may also play a negative role, since they may be recruited by the tumor to mask itself and evade immunity. Anti-SA antibodies may also potentially bind to the SA-4-1BBL molecule hindering its efficacy or neutralizing its effect. Our data here show that anti-SA Abs do not have an effect on the costimulatory function of the SA-4-1BBL molecule either *in vitro* or *in vivo*.

Based on our previous work, vaccination with E7 + SA-4-1BBL had ~70% therapeutic efficacy in the TC-1 model (27). Therefore, the observed 100% therapeutic efficacy against TC-1 tumor with the subunit vaccine in the SA-4-1BBL pretreatment setting was surprising (Fig. 19), and led us to further test the immune responses generated with SA-4-1BBL treatment alone. One shot of SA-4-1BBL, one day or one week later TC-1 challenge, did not show any anti-tumor effect suggesting that initial innate immune responses alone are not sufficient. Two shots of SA-4-1BBL treatment regimen, followed

by TC-1 challenge one, two, four, or eight weeks afterwards showed full anti-tumor protection. This prolonged SA-4-1BBL effect suggests the involvement of adaptive immunity which can further sustain innate immune activity.

Mechanistic studies aiming at identifying effector immune cells involved in the observed SA-4-1BBL protection were conducted. Inasmuch as our previous studies have demonstrated the critical role of CD8^{+} T cells in vaccine efficacy (26), we depleted these cells one day before pretreatments with SA-4-1BBL followed by TC-1 tumor challenge after two weeks. Immunized mice suppressed tumor growth, discounting the potential involvement of CD8 cells. The interplay between NK cells and CD4 cells was proven critical for maintaining a prolonged effect against tumors as shown by the depletion studies. During the initiation phase, SA-4-1BBL may be acting on NK cells that express 4-1BB receptor on their cell surface (28), CD4^{+} T conventional cells, or on both cell types simultaneously. After tumor challenge and during effector phase, the presence of CD4 cells proved essential for maintaining the anti-tumor effect observed. In addition to that, SA-4-1BBL's effect was not directed towards B cells, as depletion of such cells before immunizations did not interfere in protection from tumor. The later observation further confirmed that anti-SA antibodies generated by plasma cells do not play a role. Adoptive serum transfer of SA-4-1BBL-immunized sera to naïve mice prior to tumor challenge strongly suggests a lack of antibody protectivity.

Our data indicate that pretreatment of mice with SA-4-1BBL chimeric protein establishes a communication bridge between NK and CD4^{+} T cells. To further test the hypothesis, we used 4C TCR transgenic mice pre-immunized twice with SA-4-1BBL prior to TC-1 tumor challenge. CD4^{+} T cells in these mice did not recognize, or at least

did not optimally recognize SA as antigen; hence all mice expired due to tumor burden despite the presence of NK cells and treatment with SA-4-1BBL. It is important here to recall that 4-1BB expression on CD4$^+$ T cells is inducible and requires an initial signal, apparently provided by SA in this case, to be upregulated and to deliver effective costimulatory signals. The importance of CD4$^+$ T cells shown here is in agreement with our therapeutic vaccine data published earlier emphasizing the effect of SA-4-1BBL on CD4$^+$ T cells (25). Depletion of CD4 cells one day before tumor challenge resulted in compromised vaccine efficacy delivered after six days, and depletion of CD4 cells one day before vaccine led to loss of long-term immunological memory against Lewis lung carcinoma (25).

Several studies in the literature suggest IL-2 cytokine to be fundamental for CD4-NK communication in overcoming tumors and fighting infectious microbes. HIV-1 viral infection provides a good example, where CD4$^+$ T cell numbers are compromised along with their IL-2 production, leading to NK cells anergy and irresponsiveness to infection (74). In the same study, subjects immunized with HIV-1 subunit vaccine showed increased IL-2 production by Gp120-specific CD4$^+$ T cells associated with higher IFN-γ levels produced by NK cells (74). In another study conducted in CD8 α-chain KO mice, collaboration between CD4$^+$ T cells and NK cells was critical in fighting B16 melanoma tumors upon delivery of non-antigen-loaded immature dendritic cell vaccine (75). Another clinically relevant report demonstrated that CD56^{bright} NK cells isolated from human subjects constitutively express the high-affinity IL-2 receptor. These NK cells produced higher levels of IFN-γ when cocultured with CD4$^+$ T cells producing IL-2 cytokine upon activation with tetanus toxoid (76). Moreover, human NK cells activated

by IL-2 and NKG2D receptor or CD16 were shown to upregulate OX40L expression on their surface, costimulating CD4$^+$ T cells expressing OX40 to produce IFN-γ (77). In the dLNs of mice newly infected with *Leishmania major* parasite, CD4$^+$ T cells were shown to directly activate NK cells through the production of IL-2 or indirectly through the regulation of IL-12 secretion by dendritic cells (78). The cross-talk between innate and adaptive immunity through IL-2 was also emphasized in a malaria infection setting with special focus on NK and CD4$^+$ T cells interacting together in an MHC class II-dependent manner (79).

Killer Eomes$^+$ KLRG1$^+$ CD4$^+$ T cells (17), capable of producing granzymes and fighting melanoma cells, don't seem to be involved in SA-4-1BBL's anti-tumor effect. While conducting our pre-immunization mechanistic studies, we depleted NK cells 1 day before TC-1 while leaving CD4 cells intact and subject to activation by SA-4-1BBL. Regardless of the presence of any killer CD4$^+$ T cells during priming and effector phases, the lack of NK cells voided SA-4-1BBL's effect and all mice expired.

In this study we recognized and established the unique anti-tumor property of the SA-4-1BBL molecule, not achieved otherwise using agonistic Ab to the receptor. In our hands, anti-tumor protection was lost when mice were immunized with SA + 3H3. We also observed the shutdown of antibody production against SA in the presence of 3H3. Our observation is in accordance with published literature suggesting that monoclonal agonistic antibodies against 4-1BB can block antibody production by B cells, a method used in the treatment of autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and Graft-versus-host disease (GVHD) (80, 81). The mechanism seems to be mediated by inducing anergy of helper T cells, which in turn cease to provide help to B

cells, halting their antibody production (82). Moreover, we have also noticed a reduction in the percentages and absolute cell numbers of NK cells in mice pretreated with SA + 3H3, but not SA-4-1BBL. This may be due to the indirect deleterious effect of 3H3 on CD4$^+$ T helper functions, or it may be due to direct engagement of 3H3 with its receptor on NK cells. The effect of agonistic antibodies to 4-1BB on NK cells is controversial in the literature. Some of the studies reporting positive aspects include human NK cells activated by trastuzumab in breast cancer treatment which upregulated 4-1BB, and further stimulation with anti-4-1BB Ab killed breast cancer cells (83). Similarly, the anti-lymphoma activity of rituximab was improved by stimulation of NK cells with anti-4-1BB monoclonal antibody (84). Others also reported regulatory aspects of NK cells in anti-tumor settings without having cytolytic activity (85). In that study, CD137-stimulated helper NK cells successfully proliferated and produced IFN-γ to augment CD8$^+$ cytotoxic T lymphocytes, but did not directly kill p815 mastocytoma tumor cells. On the other hand, several studies report the negative role of anti-4-1BB stimulation on NK cells including toxicity and excessive systemic inflammation in mice and humans (15, 23). Overproduction of IFN-gamma by CD8$^+$ T cells and NK cells upon stimulation with anti-4-1BB Ab was shown to downregulate the frequency and number of NK cells in the spleens and bone marrows of tumor-bearing mice. NK numbers were otherwise restored in 4-1BB knockout mice (86).

In conclusion, our data collectively confirm SA-4-1BBL to be a bona fide immune modulator possessing anti-tumor properties, successfully bridging innate and adaptive immunities with vast clinical applications. SA-4-1BBL immunotherapy provides a better and safer alternative for clinically applied agonistic TNFR antibodies, and/or

standard treatment regimens such as chemotherapy or radiotherapy that bear several side

effects. Deciphering the detailed mechanism(s) of communication between CD4$^+$ T cells,

NK cells, and other accessory immune cells in pre-clinical settings, will definitely help in

future development of preventative as well as therapeutic anti-tumor vaccines. Towards

that end, our future experiments will aim at monitoring and studying the nature and levels

of cytokines produced upon SA-4-1BBL versus 3H3 and SA + 3H3 pre-immunizations as

well as the cellular phenotypes associated (successfully activated and functional versus

anergic and irresponsive). We will also explore the possibility of combining innate

immune adjuvants such as monophosphoryl lipid A (MPL) and/or other TNF

costimulatory molecules such as OX40L with SA-4-1BBL in pre-immunization settings

in order to reach ultimate preventative anti-tumor protection with prolongation in

immune responses against metastatic tumor recurrences.

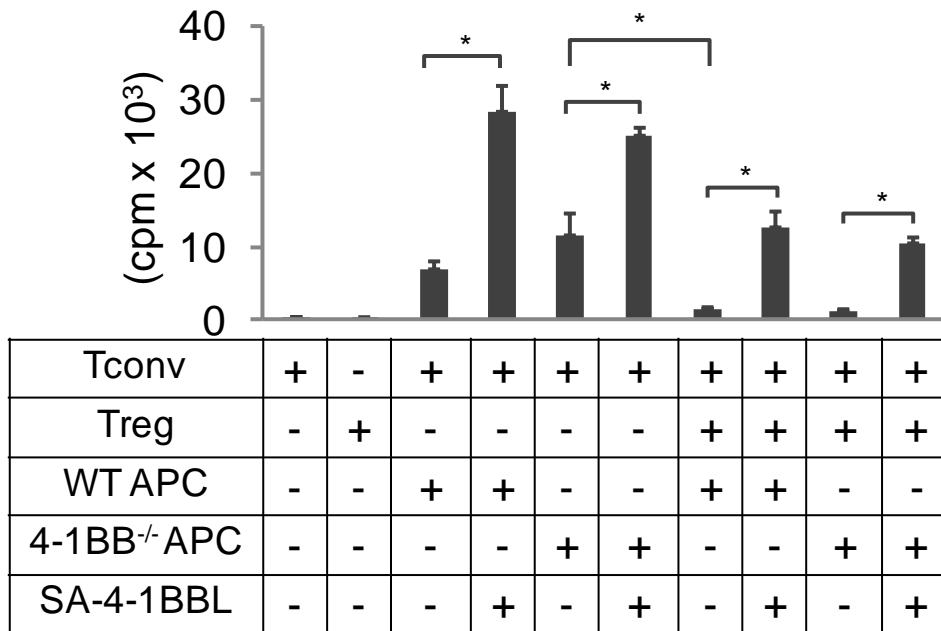| Tconv	+	-	+	+	+	+	+	+	+	+
Treg	-	+	-	-	-	-	+	+	+	+
WT APC	-	-	+	+	-	-	+	+	-	-
4-1BB$^{-/-}$ APC	-	-	-	-	+	+	-	-	+	+
SA-4-1BBL	-	-	-	+	-	+	-	+	-	+

Figure 1. Costimulation by SA-4-1BBL overcomes suppression of Tconvs by Tregs independent of APCs. Flow-sorted Tconvs were cocultured with freshly sorted or expanded Tregs at 1:1 ratio in the presence of irradiated splenocytes from wild type or 4-1BB$^{-/-}$ mice as APCs and anti-CD3 Ab (0.5 µg/ml). SA-4-1BBL (1 µg/ml) was added to cultures where indicated. Cultures were incubated for 48 h, pulsed with [^{3}H]-thymidine for an additional 16 h, and proliferation was measured and graphed as cpm. Each data point is indicative of mean ± SEM of triplicate wells and representative of 2 separate experiments. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq$ 0.05 being significant.
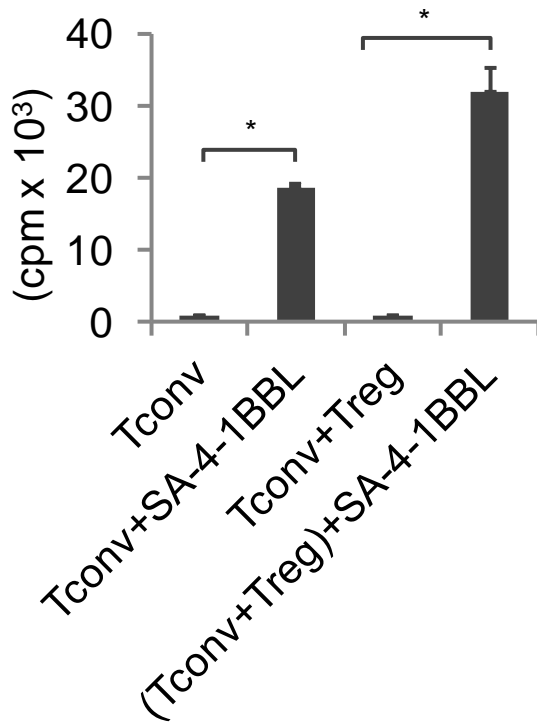
Figure 2. Costimulation by SA-4-1BBL overcomes suppression of Tregs to Tconvs in absence of APCs. Suppression assay was conducted with sorted Tconvs and Tregs without APCs using plate bound anti-CD3 Ab (5 µg/ml) and soluble SA-4-1BBL (1 µg/ml) where indicated. Each data point is indicative of mean ± SEM of triplicate wells and representative of 2 separate experiments. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq 0.05$ being significant.
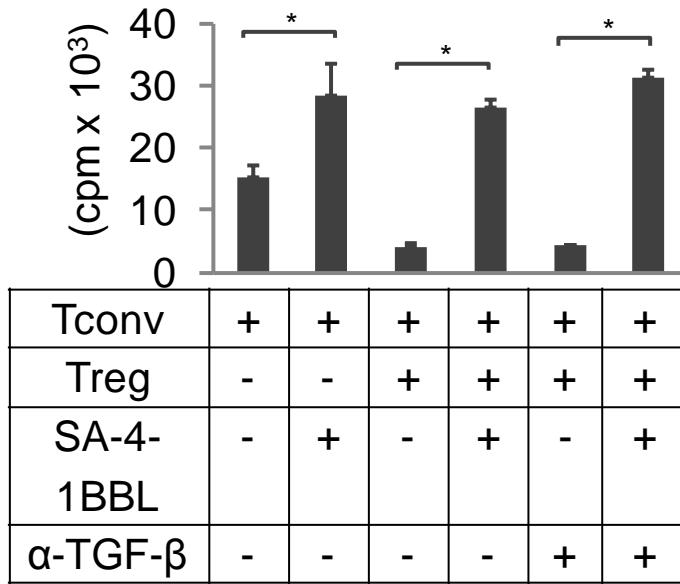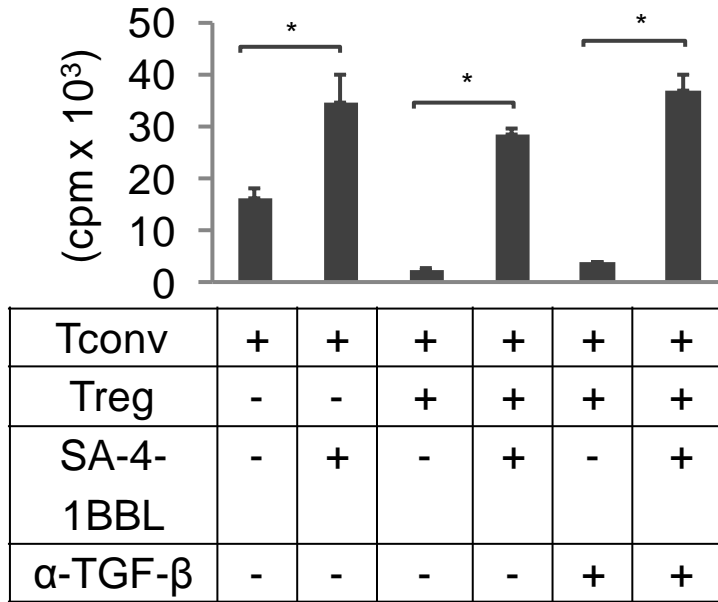
Figure 3. TGF-β cytokine is not involved in Tconv evasion of Treg suppression. Sorted

Tconvs and Tregs were cocultured at 1:1 ratio in the presence of irradiated APCs and

anti-CD3 Ab (0.5 µg/ml). Cultures were also supplied with SA-4-1BBL (1 µg/ml) and

blocking Ab to TGF-β (either 8 or 50 µg/ml) at the time of incubation. Each data point is

indicative of mean ± SEM of triplicate wells and representative of 2 independent

experiments. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq$

0.05 being significant.

Tconv	+	+	+	+	+	+
Treg	-	-	+	+	+	+
SA-4-1BBL	-	+	-	+	-	+
α-TGF-β	-	-	-	-	+	+

Figure 4. Cell surface-bound TGF-β is not involved in Tconv evasion of Treg suppression. Sorted Tconvs and Tregs were cocultured at 1:1 ratio in the presence of irradiated APCs and anti-CD3 Ab (0.5 µg/ml). Cultures were supplemented with Tregs pre-incubated with anti-TGF-β Ab (50 µg/ml), then stimulated with SA-4-1BBL (1 µg/ml) wherever indicated. Each data point is indicative of mean ± SEM of triplicate wells and representative of 2 independent experiments. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq 0.05$ being significant.

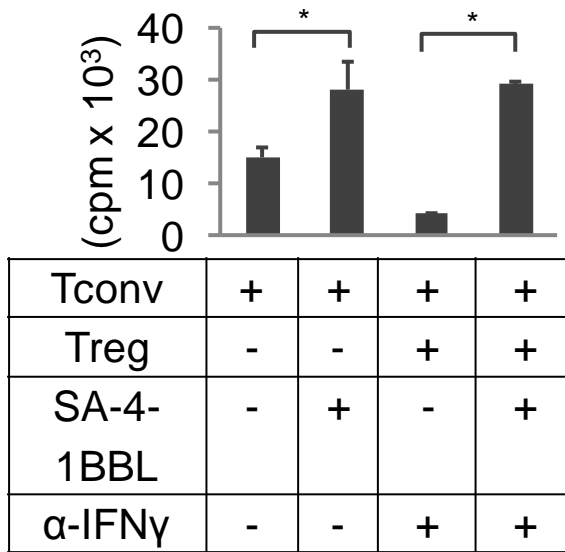|				
Tconv	+	+	+	+
Treg	-	-	+	+
SA-4-1BBL	-	+	-	+
α-IFNγ	-	-	+	+

Figure 5. IFN-γ cytokine is not involved in Tconv evasion of Treg suppression. Sorted Tconvs and Tregs were cocultured at 1:1 ratio in the presence of irradiated APCs and anti-CD3 Ab (0.5 µg/ml). Cultures were also supplied with SA-4-1BBL (1 µg/ml) and anti-IFN-γ blocking antibody (25 µg/ml). Each data point is indicative of mean ± SEM of triplicate wells and representative of 2 independent experiments. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq 0.05$ being significant.
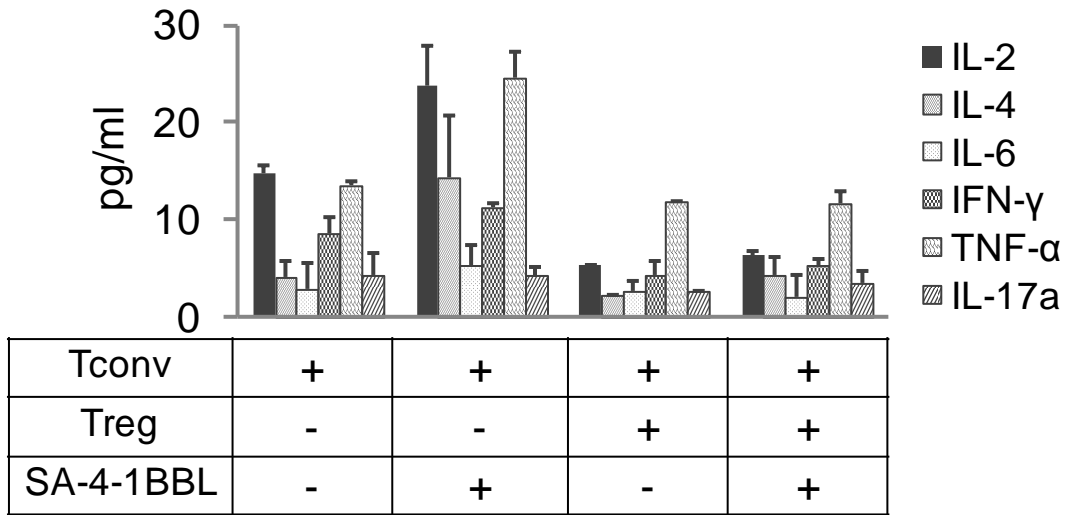
Figure 6. IL-2 is a predominant cytokine upregulated in Tconv and Tregs cocultures costimulated with SA-4-1BBL. Sorted Tconvs and Tregs were cocultured at 1:1 ratio and 48 h later supernatants were collected and subjected to cytometric bead array analysis. Cultures contain irradiated APCs, SA-4-1BBL (1 µg/ml) as shown, and soluble anti-CD3 Ab (0.5 µg/ml). Data shown is combination of two independent experiments with mean ± SEM reported.
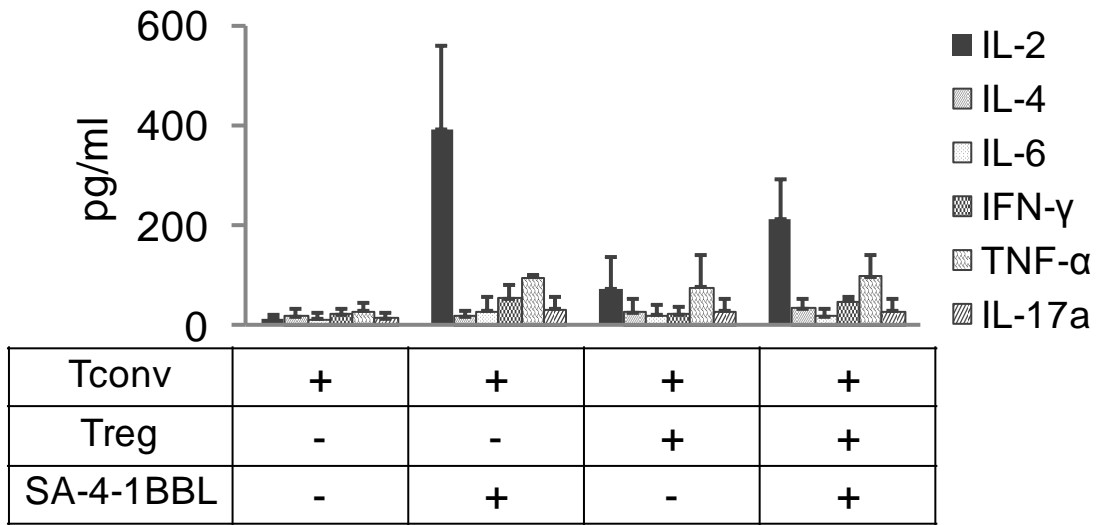
Figure 7. IL-2 is the predominant cytokine upregulated in Tconv and Tregs cocultures costimulated with SA-4-1BBL without APCs. Sorted Tconvs and Tregs were cocultured at 1:1 ratio and 48 h later supernatants were collected and subjected to cytometric bead array analysis. Cultures were stimulated with plate bound anti-CD3 Ab (5 µg/ml) and SA-4-1BBL (1 µg/ml) as indicated. Data shown is combination of two independent experiments with mean ± SEM reported.
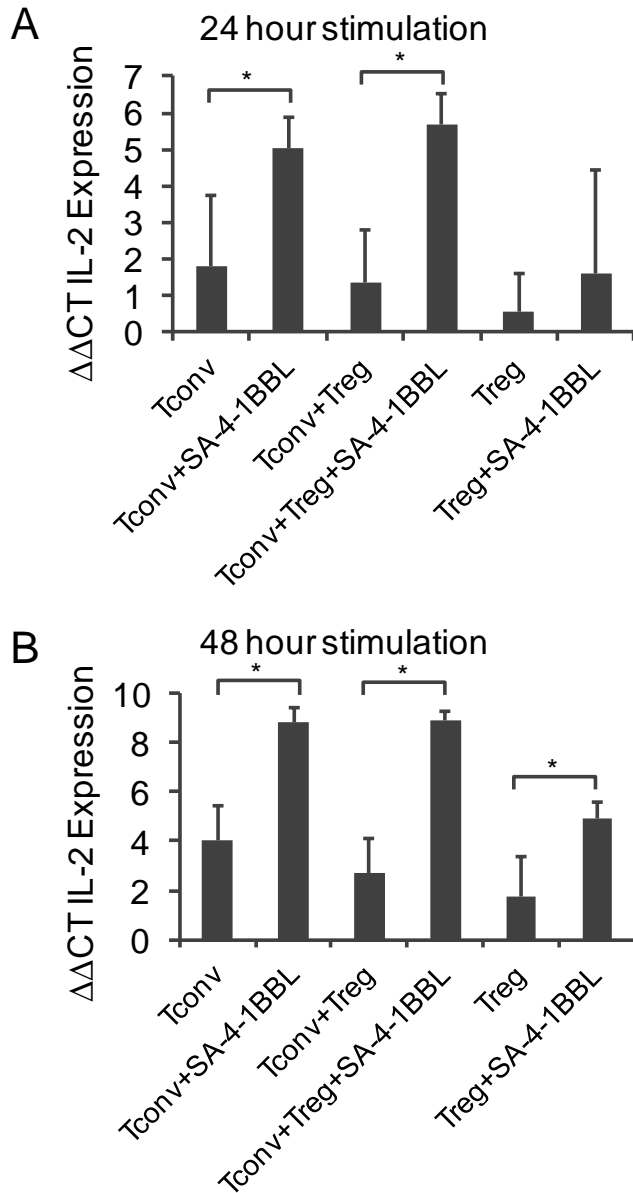
Figure 8. RT-PCR ΔΔCT values for relative IL-2 mRNA expression with respect to GAPDH gene in indicated cultures stimulated with SA-4-1BBL (1 µg/ml) and plate-bound anti-CD3 Ab (5 µg/ml). IL-2 expression was assessed at 24 **(A)** and 48 **(B)** hours post-stimulation. Each data point represents the mean of triplicate wells ± SD, with *$p \leq$ 0.05 being significant.
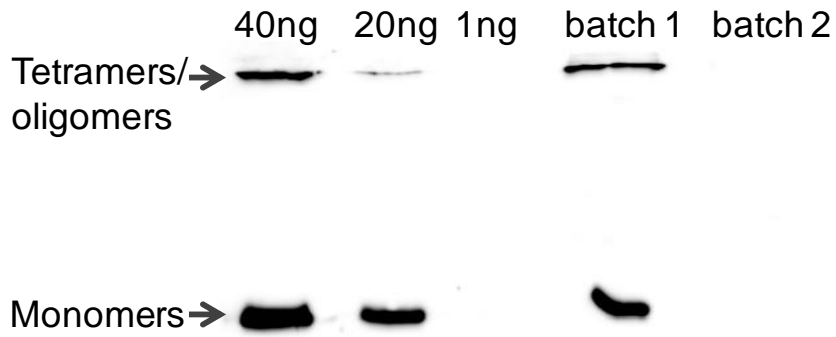
Figure 9. Removal of SA-4-1BBL from Tconv culture supernatants. Supernatant (sup) of Tconvs stimulated with an agonistic anti-CD3 Ab (5 μg/ml) and SA-4-1BBL (1 μg/ml) were incubated with $CuSO_4$-charged sepharose beads to remove SA-4-1BBL carrying a 6xHis tag. Regular sup (batch 1) and SA-4-1BBL-depleted sup (batch 2) were analyzed using anti-SA antibodies for detection in Western blots. The indicated amounts of SA-4-1BBL protein were used as detection controls.
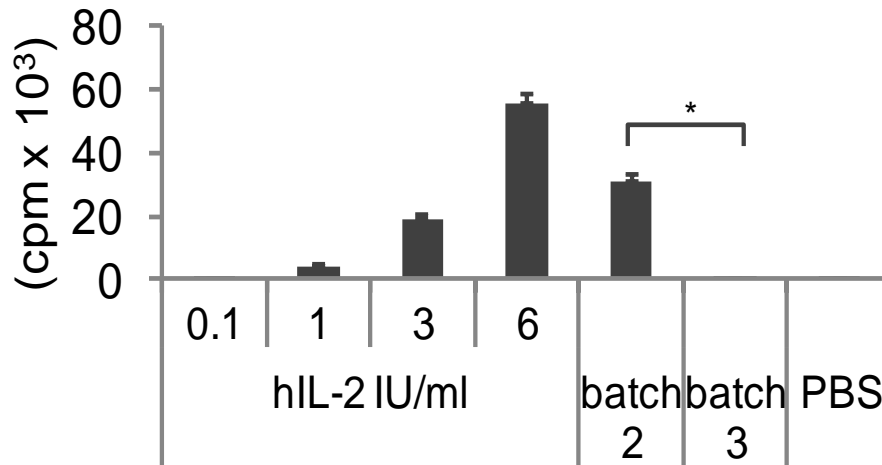
Figure 10. Removal of IL-2 from Tconv culture supernatants. Batch 2 supernatant,

cleaned from SA-4-1BBL as described in Figure 9, was incubated with an anti-IL-2 Ab

(20 µg/ml) followed by the removal of Ab/IL-2 complexes using immobilized protein G

beads to generate batch 3. Batch 2 as well as batch 3 supernatants (50 µl/each) were

tested on the IL-2-dependent CTTL-2 cell line with the indicated commercial

recombinant IL-2 doses (IU/ml) as positive and PBS as negative controls. Cultures were

incubated for 28 h with [^{3}H]-thymidine added for the last 8 h. Proliferation was measured

and graphed as cpm. Each data point is indicative of mean ± SEM of triplicate wells.

Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq 0.05$ being

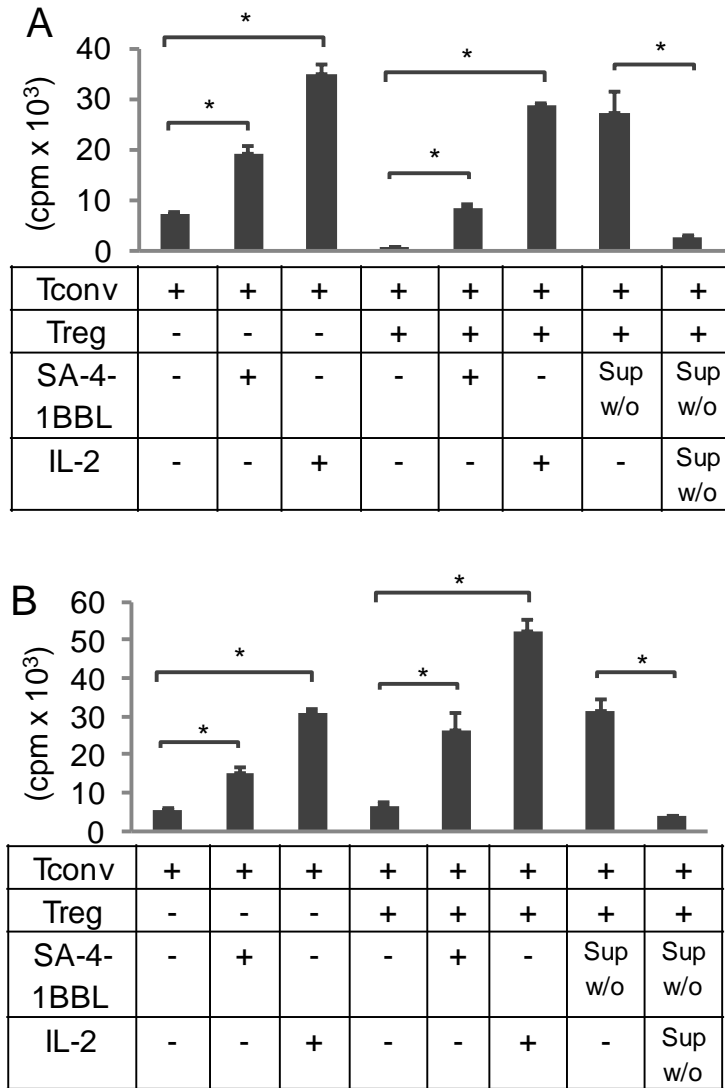significant. The data is representative for two independent experiments.

A

Tconv	+	+	+	+	+	+	+	+
Treg	-	-	-	+	+	+	+	+
SA-4-1BBL	-	+	-	-	+	-	Sup w/o	Sup w/o
IL-2	-	-	+	-	-	+	-	Sup w/o

B

Tconv	+	+	+	+	+	+	+	+
Treg	-	-	-	+	+	+	+	+
SA-4-1BBL	-	+	-	-	+	-	Sup w/o	Sup w/o
IL-2	-	-	+	-	-	+	-	Sup w/o

Figure 11. SA-4-1BBL costimulation-mediated IL-2 production by Tconvs is both necessary and sufficient in overcoming Treg suppression. Sorted Tconvs and Tregs were cocultured at 1:1 ratio in the presence **(A)** or absence **(B)** of irradiated APCs and anti-CD3 Ab. Cultures were also supplemented with SA-4-1BBL (1 μg/ml), IL-2 (3 IU/ml), SA-4-1BBL-depleted supernatants (100 μl/well), or SA-4-1BBL- and IL-2-depleted supernatants (100 μl/well) where indicated. Each data point is indicative of mean ± SEM of triplicate wells. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq 0.05$ being significant.
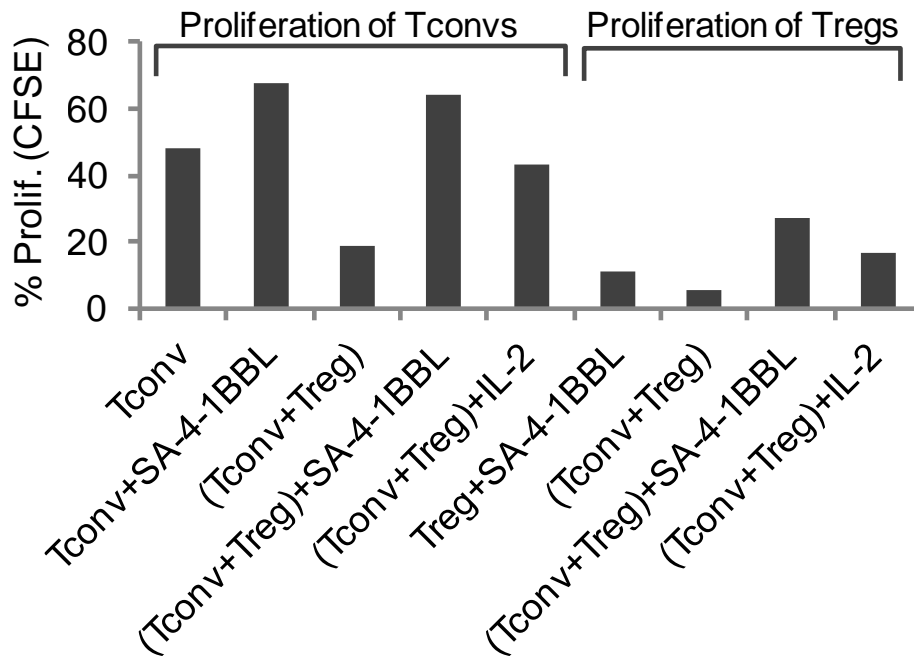
Figure 12. Tconvs proliferate to a higher extent upon SA-4-1BBL stimulation as compared to Tregs. Tconvs sorted from WT C57BL/6 or Tregs sorted from C57BL/6.Foxp3^{hCD2} mice were labeled with 2.5µM CFSE and used in coculture suppression assays with irradiated APCs. Cultures were incubated for 64 h and cells were analyzed for proliferation using LSRII flow cytometer.
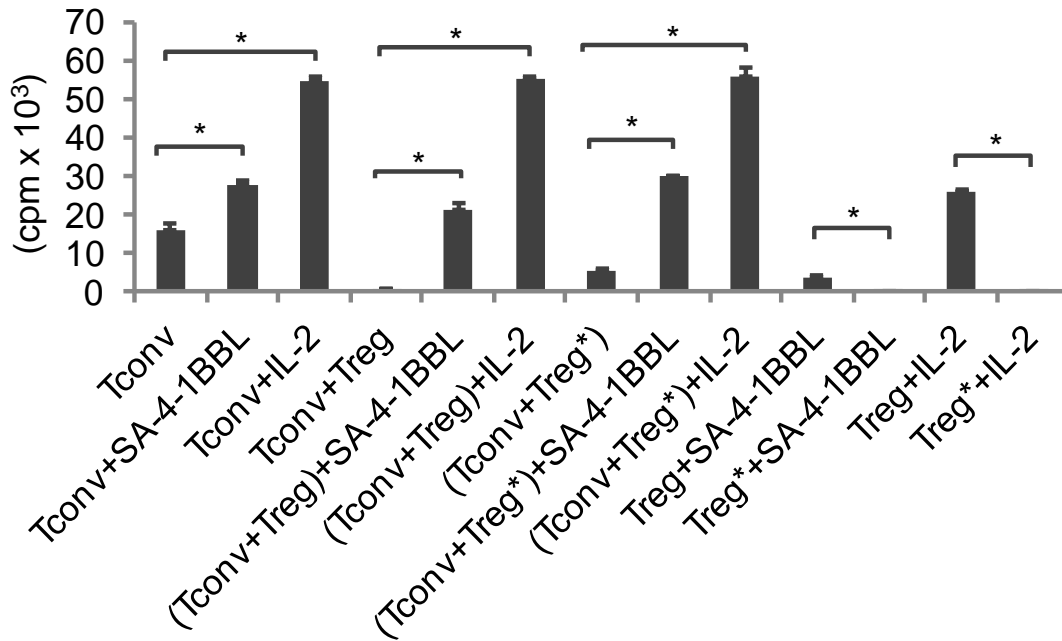
Figure 13. IL-2-mediated Tconvs evasion of Tregs suppression is independent of Tregs proliferative state. Sorted Tconvs and Tregs were cocultured at 1:1 ratio in the presence of APCs and anti-CD3 Ab (0.5 μg/ml). Cultures were also supplemented with SA-4-1BBL (1 μg/ml) or IL-2 (3 IU/ml) wherever indicated. In select cultures, Tregs were irradiated at 2000 cGy (Tregs*) prior use. Each data point is indicative of mean ± SEM of triplicate wells. Student's t-test (two-tailed) was performed for statistical analysis with *p ≤ 0.05 being significant. Experiment repeated twice with similar pattern detected.
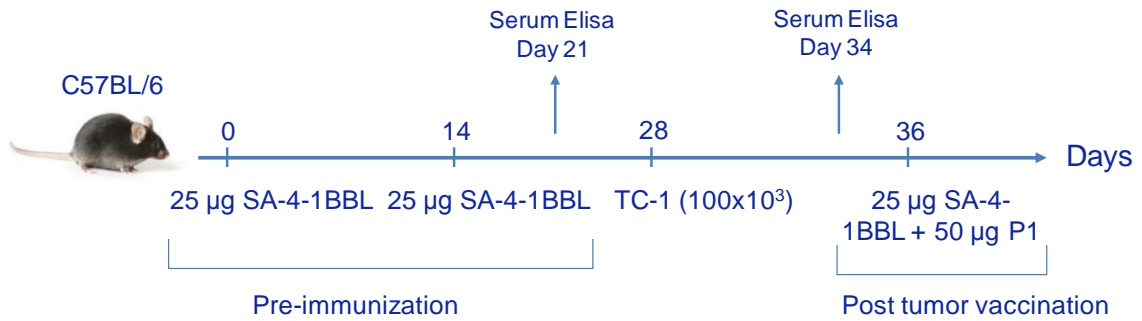
Figure 14. Schematic time line to study SA-4-1BBL's immunogenicity. C57BL/6 mice were immunized twice with 25 µg of SA-4-1BBL s.c. on days 0 and 14, followed by TC-1 tumor challenge (1×10^5) s.c. two weeks after the second immunization. Post-tumor vaccination (50 µg E7-P1 peptide + 25 µg SA-4-1BBL) was administered s.c. 8 days after tumor challenge. Mice were monitored for a period of 60 days after tumor injection and measurements were taken twice a week using calipers. Mice were euthanized when average tumor size $\geq$ 12 mm or on day 60 post tumor challenge to be considered the experimental endpoint.
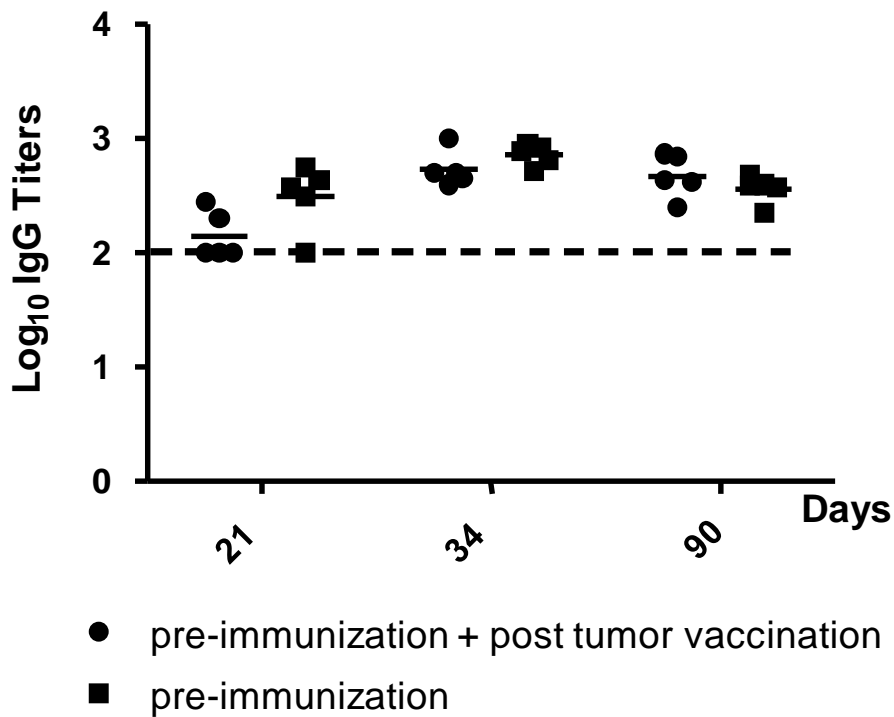
Figure 15. Anti-SA antibodies generated *in vivo* upon SA-4-1BBL immunization. ELISA

plates were coated overnight with 50 ng of SA-4-1BBL protein. Next day plates were

washed and incubated with serial dilutions of specified sera for 1.5 hours, after which

goat-anti-mouse secondary antibody conjugated to HRP was added for 1 hour.

Colorimetric reaction was generated using TMB substrate for 30 minutes and plates were

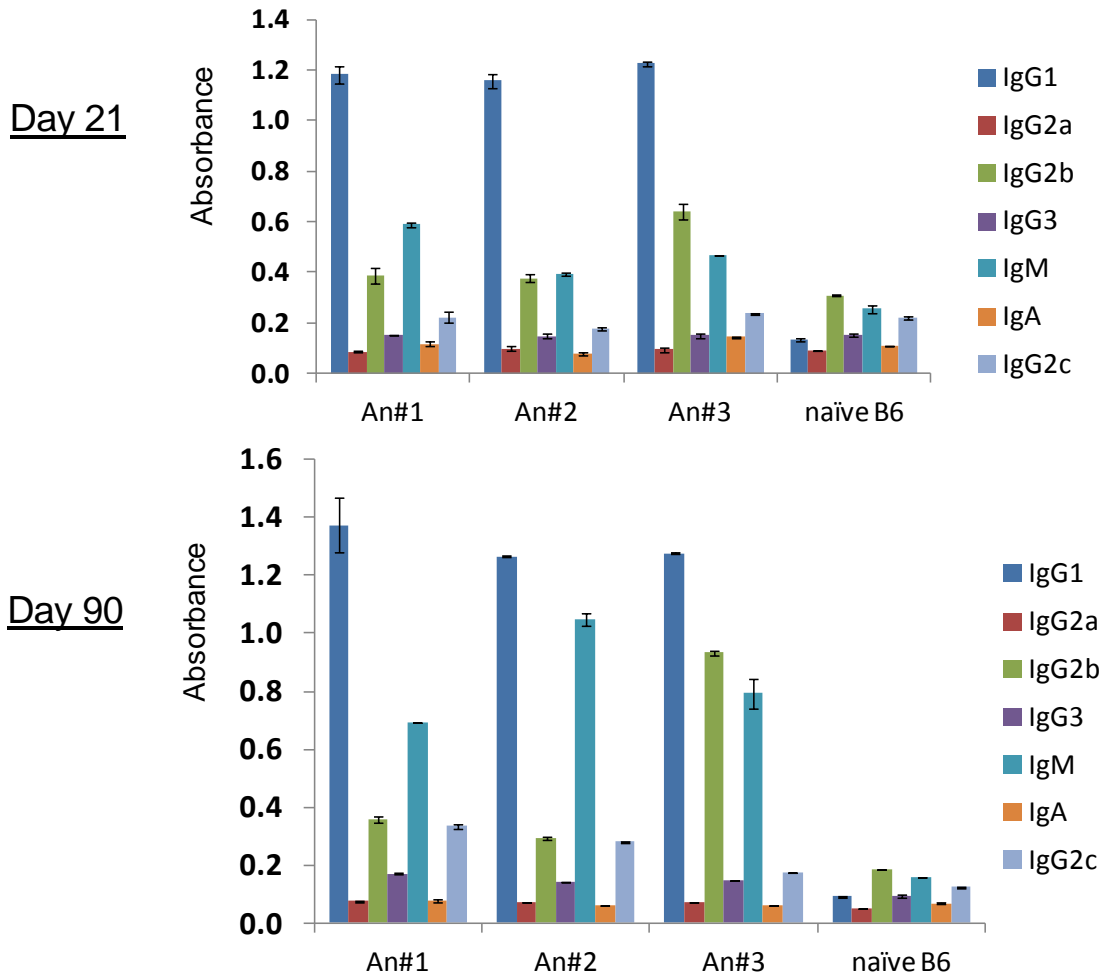read at 450 nm. Experiment repeated twice with similar pattern detected.

Figure 16. Subtyping serum ELISA for pre-immunization group on days 21 and 90.

ELISA plates pre-coated with 100 ng of SA-4-1BBL overnight. 1/25 dilution of positive

sera is used the next day to test the IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, and IgG2c

antibody titers. Each data point is indicative of the mean ± SD of duplicate wells.
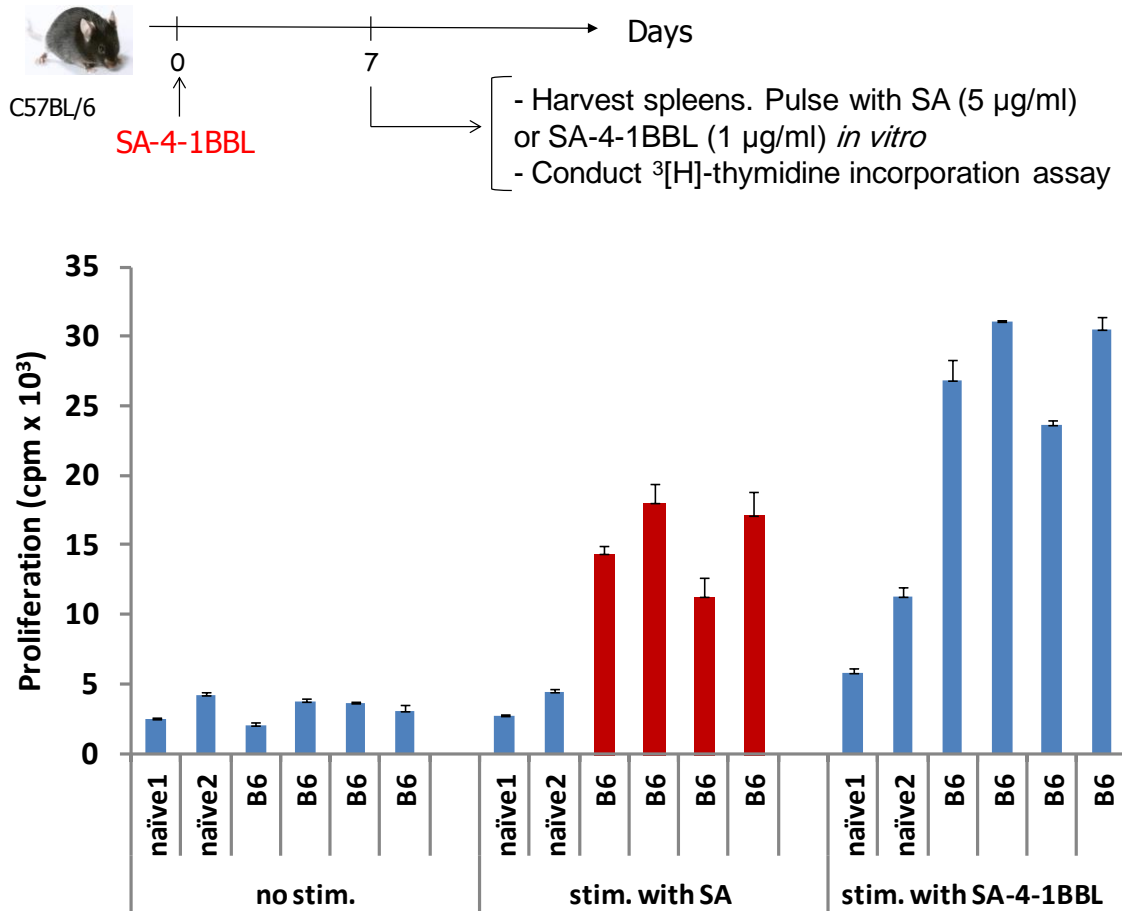
Figure 17. SA is immunogenic and drives the proliferation of T cells *in vitro*. Two naïve and four immunized C57BL/6 mice with 1 shot of SA-4-1BBL (25 µg s.c.) were used. Seven days later, spleens were harvested, processed, and cultured *in vitro* for 48 hours in the presence or absence of SA stimulation (5 µg/ml). Cultures were pulsed with ^{3}H-thymidine for additional 16 hours and CPMs were recorded. Each data point represents the mean ± SEM of triplicate wells.
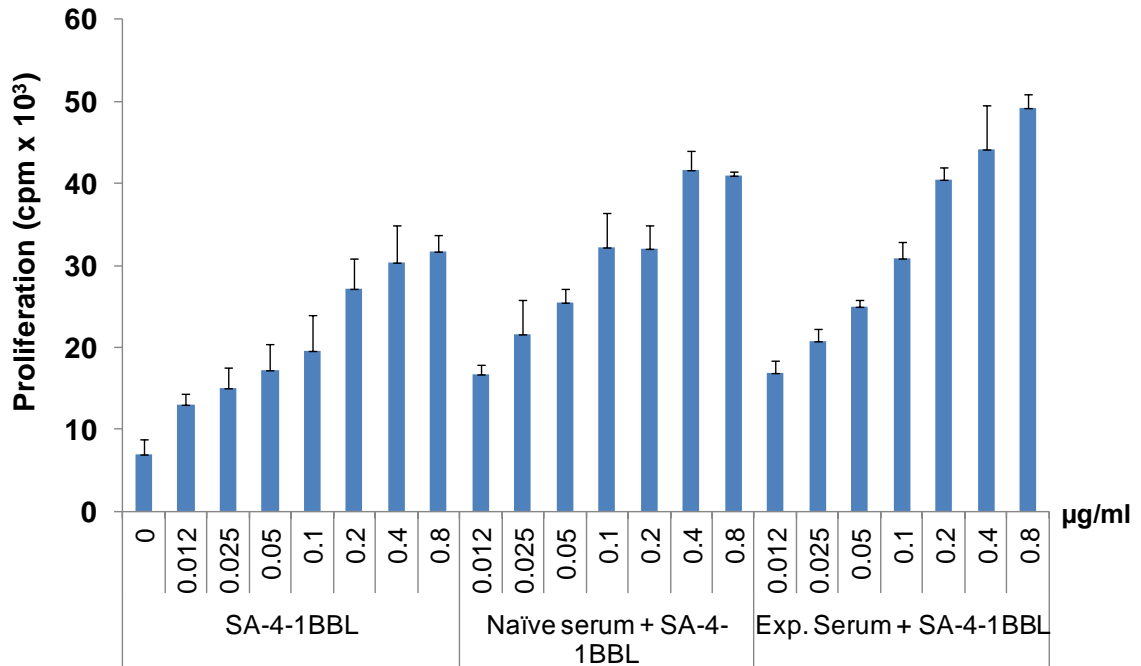
Figure 18. Anti-SA antibodies generated *in vivo* do not help the *in vitro* proliferative function of SA-4-1BBL molecule. Sera were collected on day 21 from SA-4-1BBL pre-immunized C57BL/6 mice (25µg on days 0 and 14 s.c.). SA-4-1BBL at different doses (µg/ml) was incubated *in vitro* with naïve or experimental sera ($\log_{10} = 2.8$) for 1 hour at room temperature prior addition to splenocytes stimulated with agonistic anti-CD3 (0.025 µg/ml). Cultures were incubated for 48 hours at 37° / 5% CO_2 followed by additional 16 hours with [3][H]-thymidine. Plates were harvested and counts were recorded as CPM. Each data point represents the mean ± SEM of triplicate wells. Experiment was repeated twice with same pattern detected.
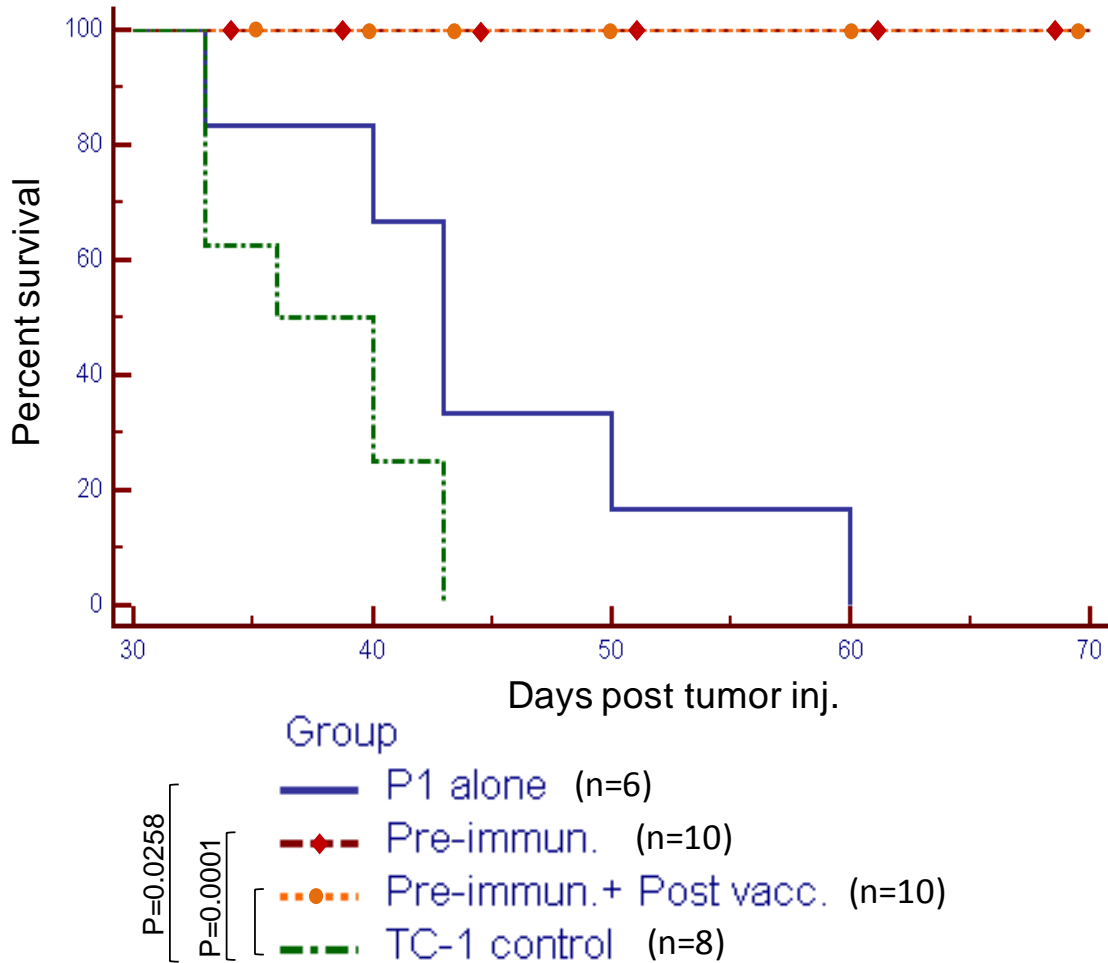
Figure 19. Pre-immunization with SA-4-1BBL does not alter the post-tumor vaccination outcome, but scores 100% survival against TC-1 tumors. Naïve C57BL/6 mice were immunized twice with 25 μg SA-4-1BBL (days 0 and 14 s.c.). Two weeks later (day 28) mice were challenged with TC-1 tumors (1 x 10^5 cells s.c.). On day 36, the pre-immunization + post vaccination group received a post-tumor vaccine challenge (50 μg E7-P1 peptide + 25 μg SA-4-1BBL s.c.). Mice were monitored for 70 days post tumor injection as shown above. Tumors were measured twice a week using calipers. Mice were euthanized when average tumor diameters had reached ≥ 12 mm or when experimental endpoints were met. Experiment repeated twice with mice survival data pooled.
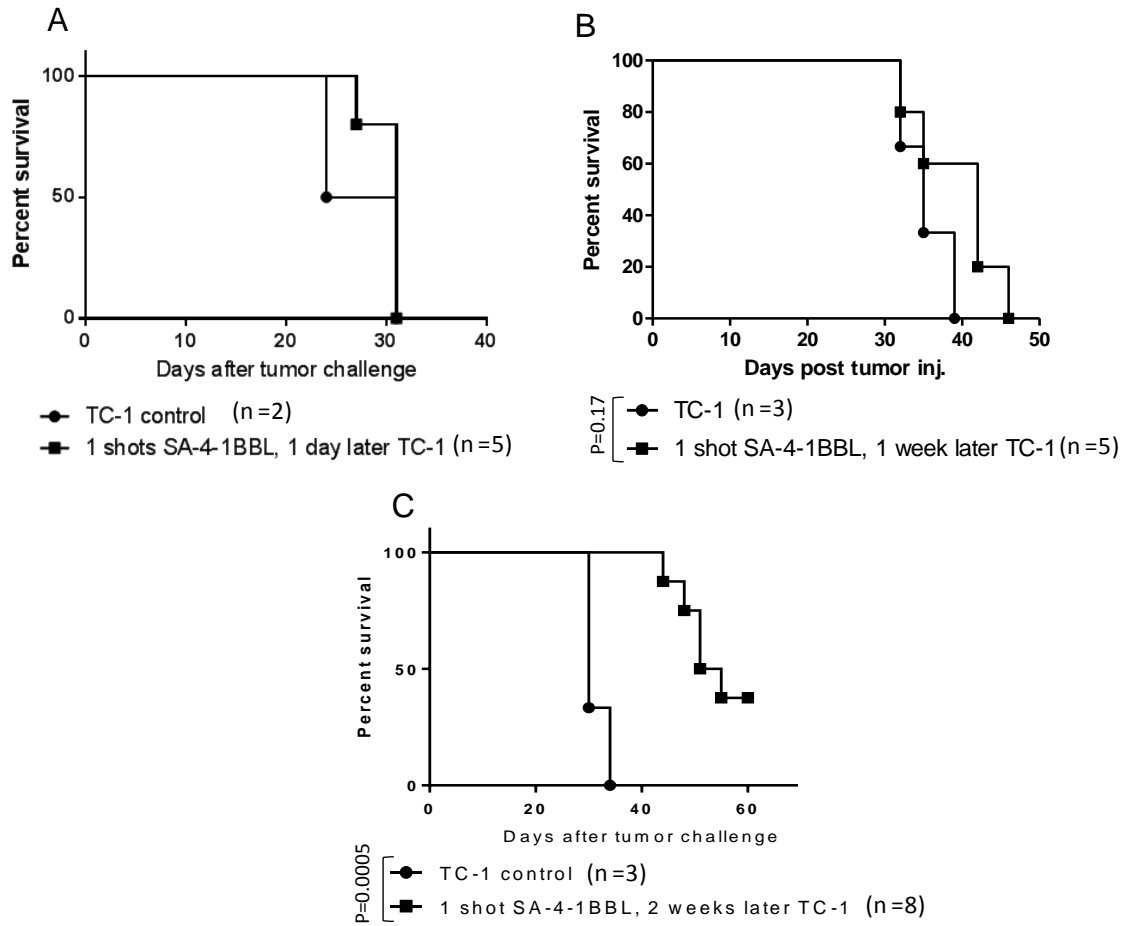
Figure 20. Time-course pre-immunization studies with 1 shot SA-4-1BBL against TC-1.

C57BL/6 mice were immunized with 1 shot SA-4-1BBL (25 μg s.c.) followed by TC-1

challenge (1 x 10^5 cells s.c.) 1 day (**A**), 1 week (**B**), or 2 weeks (**C**) after immunization.

Tumor measurements were recorded twice a week using calipers and mice were

euthanized when tumor average diameter ≥ 12 mm. Survival curves were graphed and

log-rank tests were applied between groups using GraphPad software, with p ≤ 0.05 to be

significant.

Figure 21. Time-course pre-immunization studies with 2 shots SA-4-1BBL against TC-1. C57BL/6 mice were immunized twice with SA-4-1BBL (25 µg s.c., 2 weeks apart), followed by TC-1 challenge (1 x 10^5 cells s.c.) 1 day (**A**) or 1 week (**B**), after the 2nd immunization. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter $\geq$ 12 mm. Survival curves were graphed and log-rank tests were applied between groups using GraphPad software, with $p \leq 0.05$ to be significant.
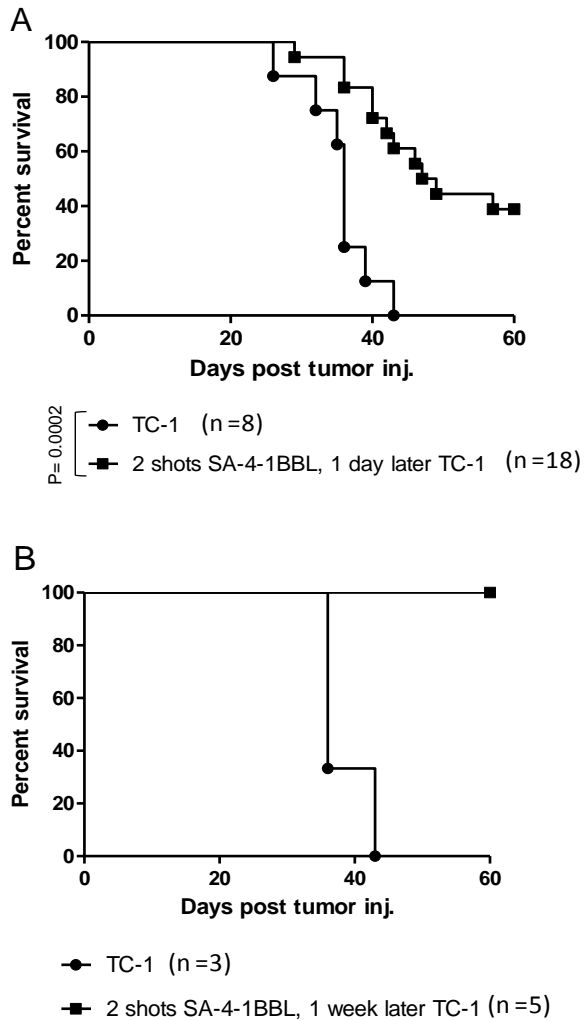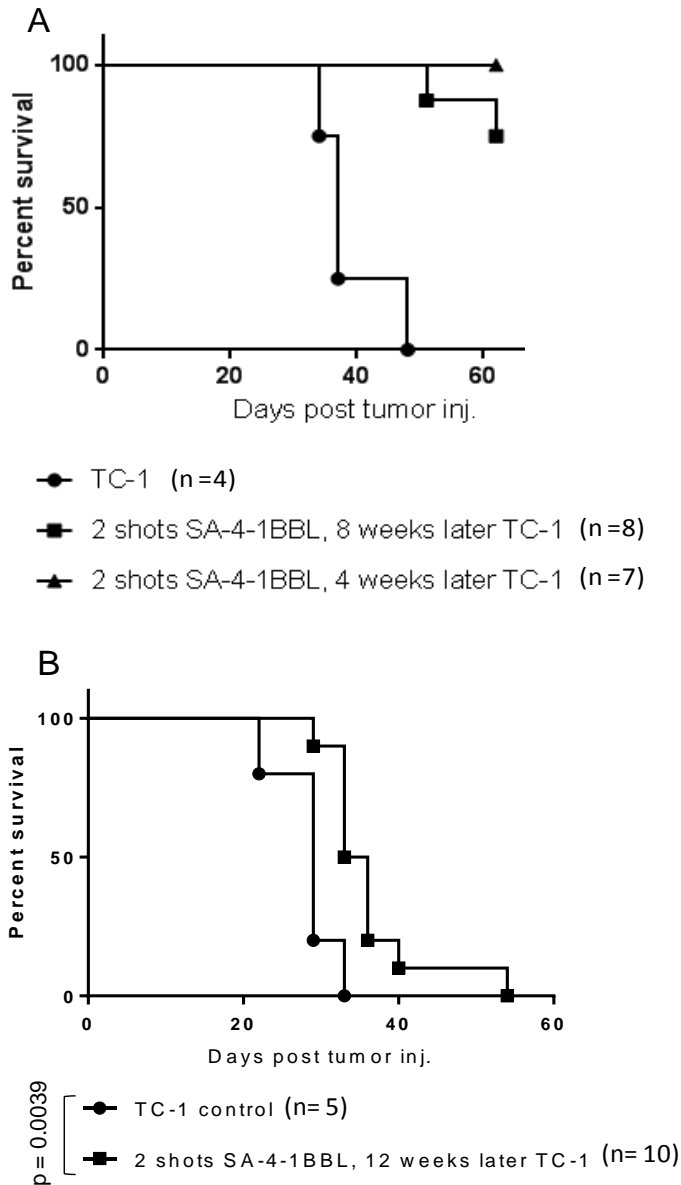
Figure 22. Time-course pre-immunization studies continuation with 2 shots SA-4-1BBL against TC-1. C57BL/6 mice were immunized twice with SA-4-1BBL (25 μg s.c., 2 weeks apart), followed by TC-1 challenge (1×10^5 s.c.) 4 weeks and 8 weeks (**A**) or 12 weeks (**B**), after the 2nd immunization. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter $\geq$ 12 mm. Survival curves were graphed and log-rank tests were applied between groups using GraphPad software, with p $\leq$ 0.05 to be significant.
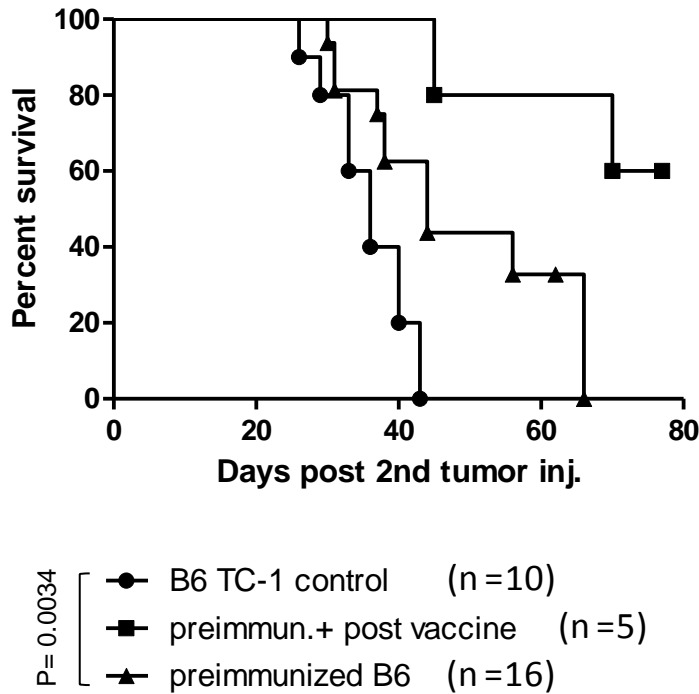
Figure 23. SA-4-1BBL establishes significant long-term immune memory against TC-1. To study TC-1 recurrences, a group of naïve C57BL/6 mice were immunized twice with SA-4-1BBL (2 weeks apart), followed by 1st TC-1 challenge 2 weeks after the second immunization. A second group of mice was treated exactly the same with additional vaccination (50 µg E7-P1 peptide + 25 µg SA-4-1BBL) 8 days after 1st TC-1 challenge. Mice in both groups were monitored for a period of 60 days after 1st tumor injection. At the end of day 60, tumor free mice were rechallenged with a 2nd shot of TC-1 and monitored for additional 80 days. Tumors were measured twice a week using calipers and mice were euthanized when average tumor diameter $\geq$ 12 mm. Survival curves were graphed and Log-rank test was applied to compare groups using GraphPad software, with $p \leq 0.05$ to be significant.
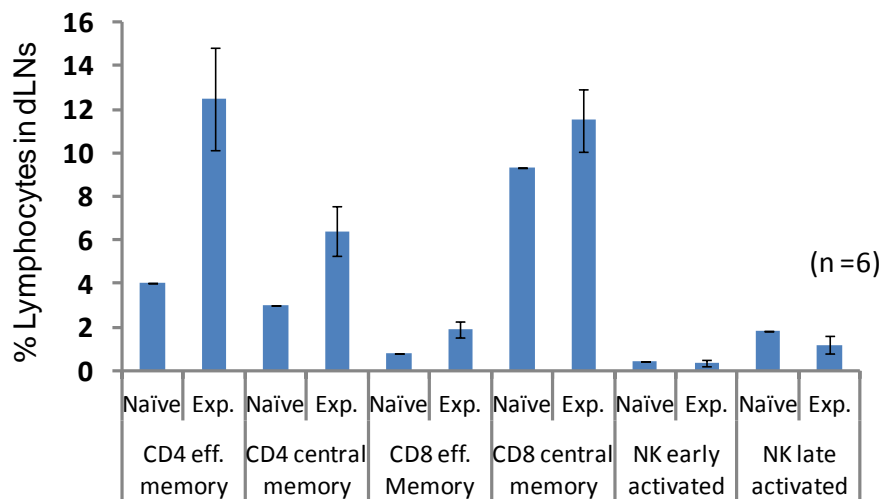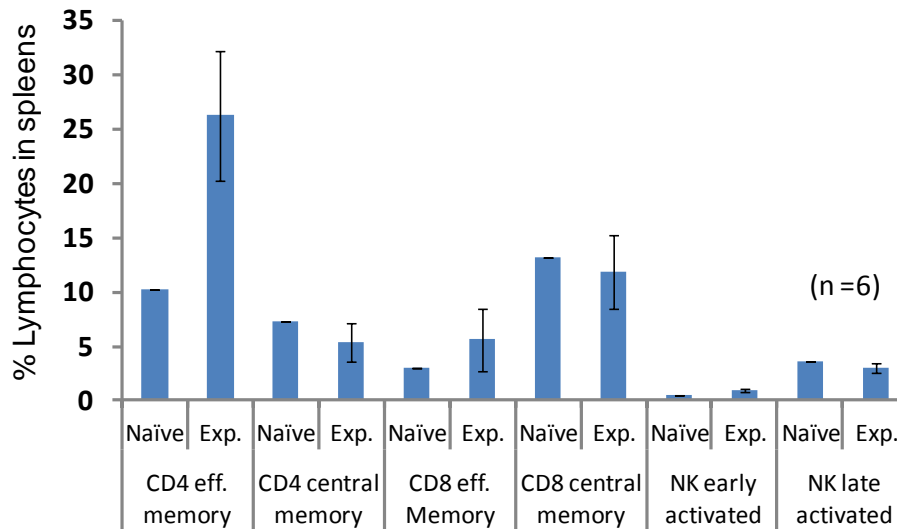
Figure 24. SA-4-1BBL primarily generates long-term CD4 effector memory.
C57BL/6 mice were immunized twice with SA-4-1BBL (2 weeks apart), followed by
TC-1 challenge 8 weeks after 2nd immunization. Mice were monitored 60 days post-
tumor challenge, after which tumor free mice were euthanized, spleens and draining
lymph nodes harvested and stained for flow-cytometric phenotyping. For T-effector
memory cells were gated on $CD3^+CD62L^{lo}CD44^{hi}$; for T-central memory
$CD3^+CD62L^{hi}CD44^{hi}$; for NK early activation $CD3^-$ $NK1.1^+$ $CD69^+$; for NK late
activation $CD3^-$ $NK1.1^+$ $CD44^+$.
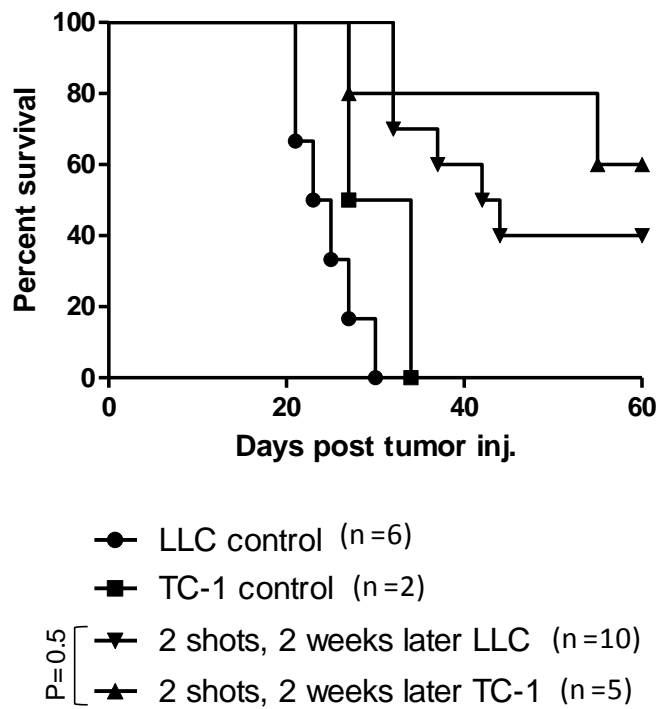
Figure 25. SA-4-1BBL rejects tumors in a non-specific fashion. C57BL/6 mice were immunized twice with SA-4-1BBL (25 μg s.c., 2 weeks apart), followed by LLC (1 x 10^5 s.c.), or TC-1 (1 x 10^5 s.c.) challenge 2 weeks after the 2nd immunization. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter ≥ 12 mm. Survival curves were graphed and log-rank stats were calculated between groups using GraphPad software, with p ≤ 0.05 to be significant.

Figure 26. Anti-SA humoral immune response did not contribute to the SA-4-1-BBL-mediated rejection of tumors. Sera collected on Day 27 from SA-4-1BBL immunized mice (25μg on days 0 and 14 s.c.) were adoptively transferred to naïve C57BL/6 mice (200 μl i.v.) 24 hours prior TC-1 tumor challenge (1 x 10^5 s.c.). Tumor diameters were measured with calipers twice a week. Animals were terminated when average tumor size ≥ 12mm.

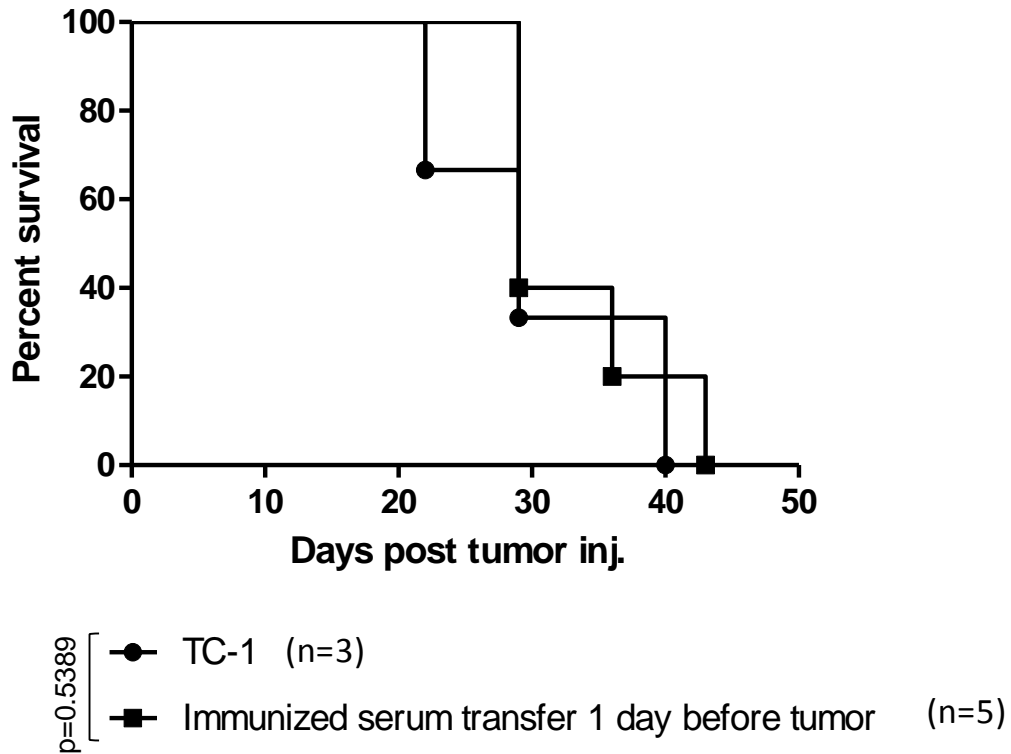**Figure 27.** Depletion of NK cells negates SA-4-1BBL's protective effect. Different groups of C57BL/6 mice were immunized twice with 25 µg SA-4-1BBL (2 weeks apart), followed by TC-1 (1 x 10^5 s.c.) challenge 2 weeks after the 2nd immunization. NK cells were depleted with anti-NK1.1 Ab clone PK136 (500 µg i.p.) 1 day before SA-4-1BBL immunizations or 1 day before TC-1 challenge. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter $\geq$ 12 mm or when experimental endpoints were reached. Survival curves were graphed and Log-rank test was applied to compare groups using GraphPad software, with $p \leq 0.05$ to be significant.
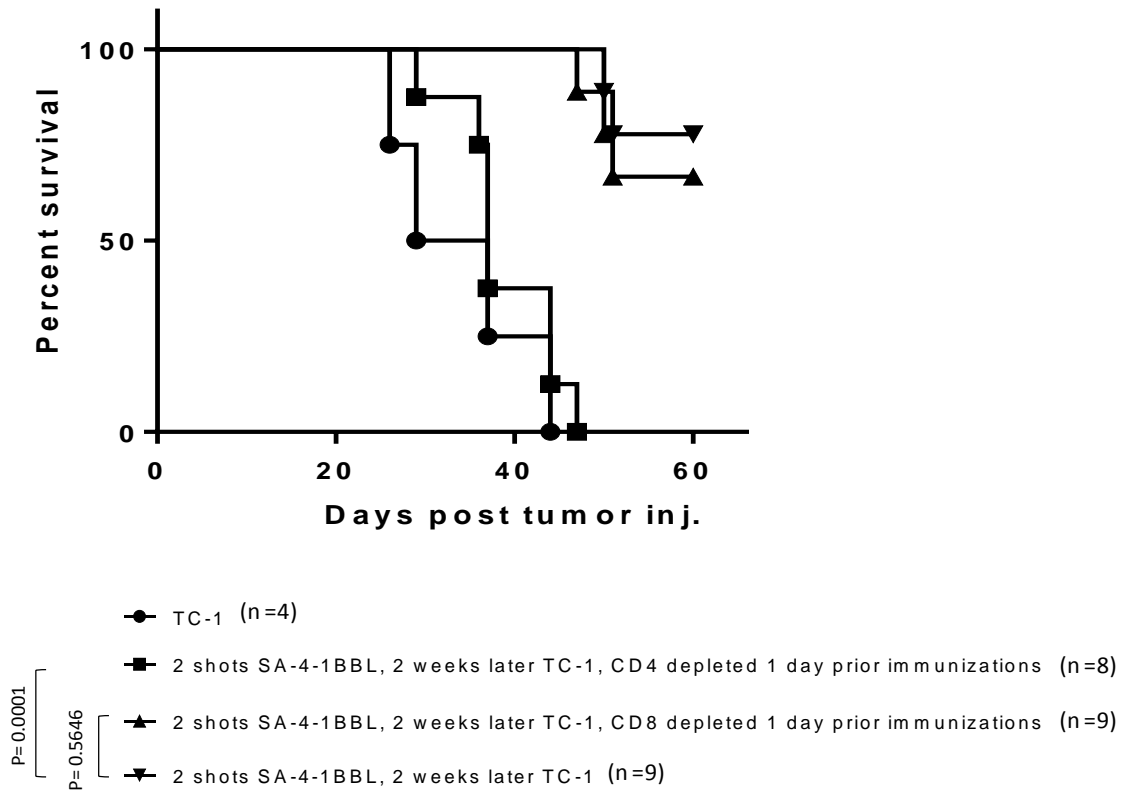
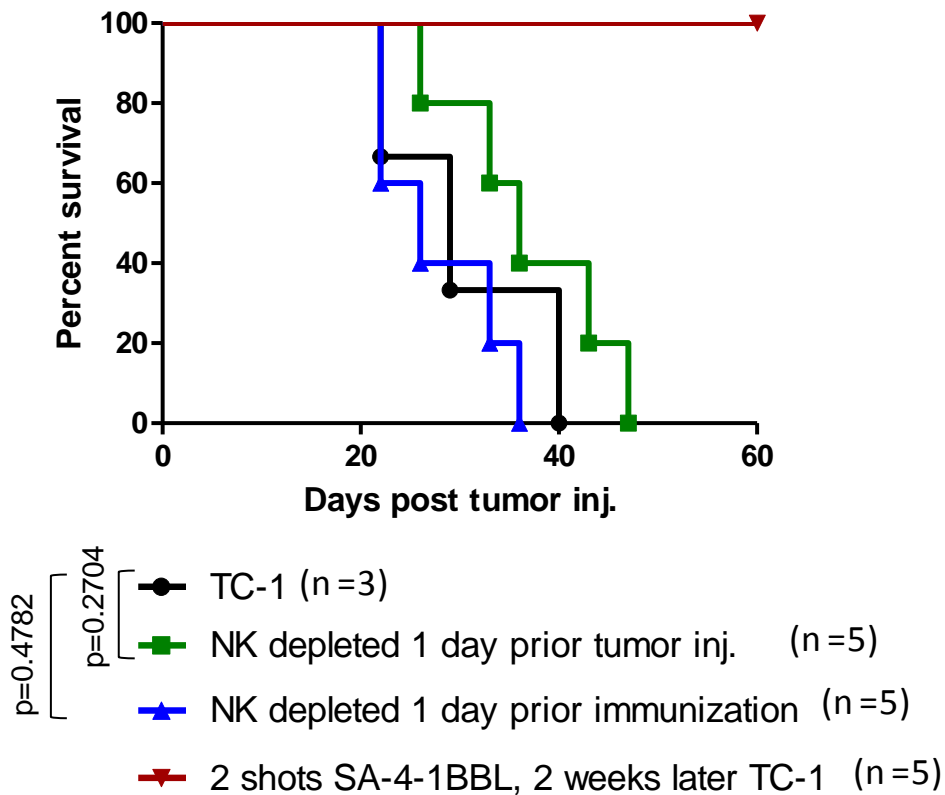**Figure 28.** Depletion of CD4 cells but not CD8 cells negates SA-4-1BBL's protective effect. Different groups of C57BL/6 mice were immunized twice with 25 μg SA-4-1BBL (2 weeks apart), followed by TC-1 (1×10^5 s.c.) challenge 2 weeks after the 2nd immunization. CD4 cells were depleted with anti-CD4 Ab clone GK1.5 (500 μg i.p.), while CD8 cells were depleted with anti-CD8 Ab clone 53.6.72 (500 μg i.p.) 1 day before SA-4-1BBL immunizations. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter $\geq$ 12 mm or when experimental endpoints were reached. Survival curves were graphed and Log-rank test was applied to compare groups using GraphPad software, with $p \leq 0.05$ to be significant.
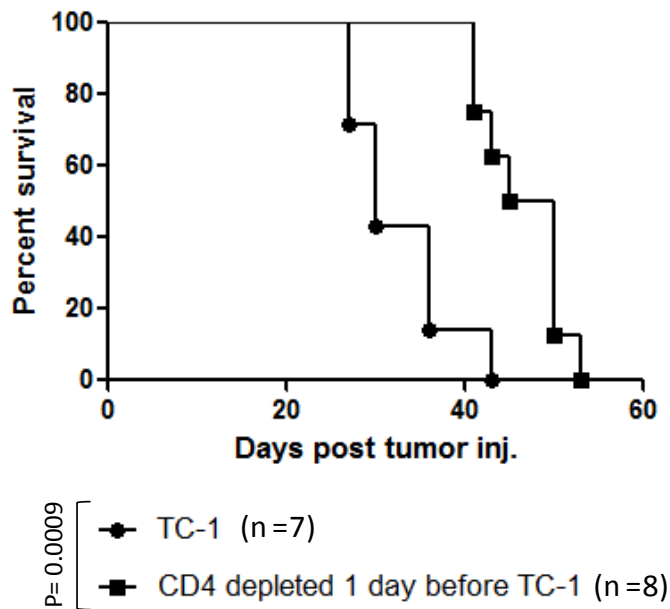
Figure 29. CD4 cells are required during effector phase after TC-1 challenge. C57BL/6 mice were immunized twice with 25 μg SA-4-1BBL (2 weeks apart), followed by TC-1 (1 x 10^5 s.c.) challenge 2 weeks after the 2nd immunization. CD4 cells were depleted with anti-CD4 Ab clone GK1.5 (500 μg i.p.) 1 day before TC-1 injection. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter ≥ 12 mm.
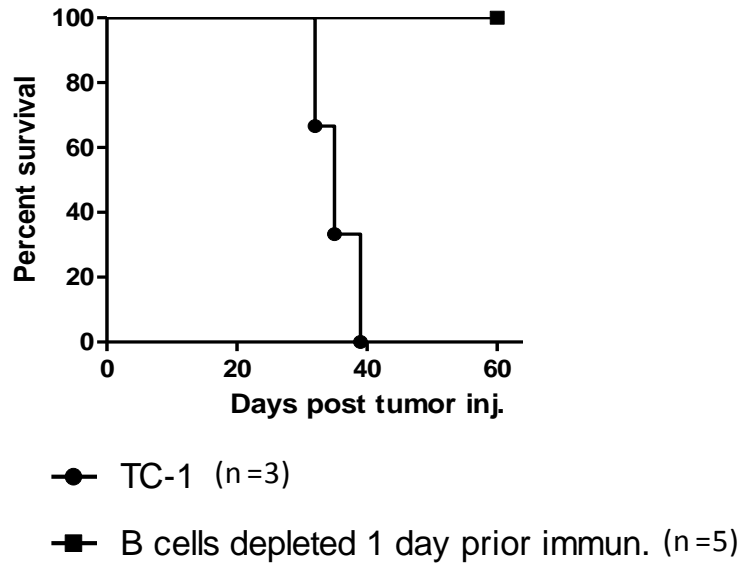
Figure 30. B cells and antibodies are not involved in SA-4-1BBL-mediated tumor protection. C57BL/6 mice were immunized twice with 25 µg SA-4-1BBL (2 weeks apart), followed by TC-1 (1 x 10^5 s.c.) challenge 2 weeks after the 2nd immunization. B cells were depleted with anti-CD20 Ab clone 5D2 (200 µg i.p.) 1 day before SA-4-1BBL immunizations. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter $\geq$ 12 mm or when experimental endpoint was reached.
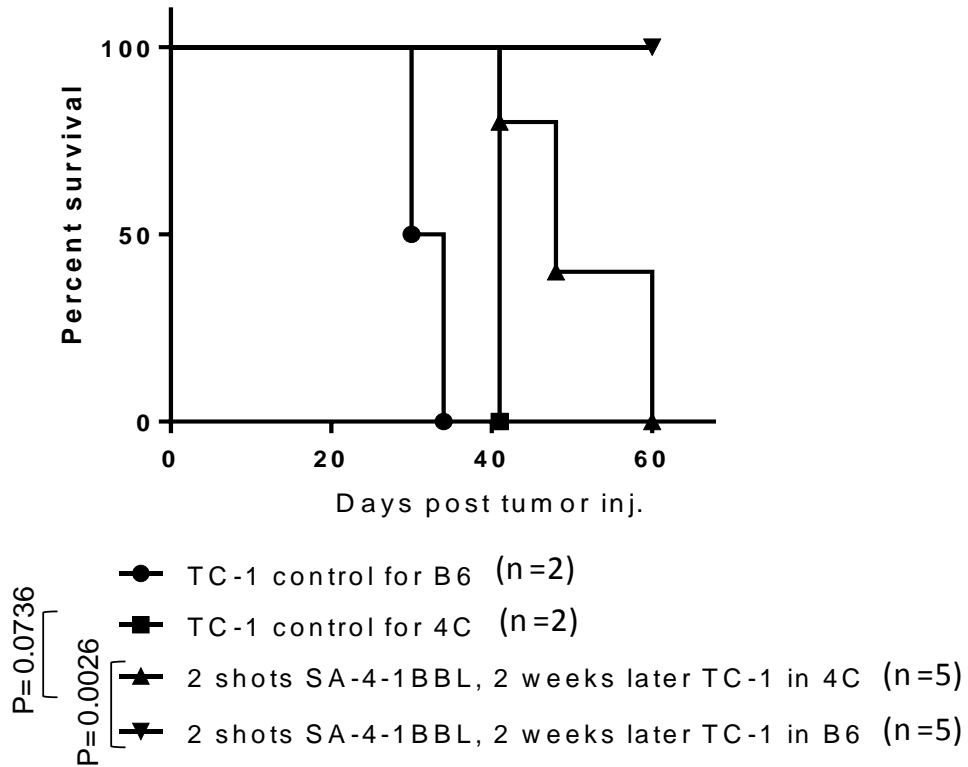
Figure 31. SA-4-1BBL immunization is ineffective against TC-1 in 4C mice. Either 4C or wild type C57BL/6 mice were immunized twice with SA-4-1BBL (25 μg s.c.) followed by TC-1 challenge (1 x 10^5 s.c.) 2 weeks later from 2nd immunization. Tumor measurements were recorded twice a week using calipers and mice were euthanized when tumor average diameter ≥ 12 mm or when experimental endpoints were reached. Survival curves were graphed and Log-rank test was applied to compare groups using GraphPad software, with p ≤ 0.05 to be significant.
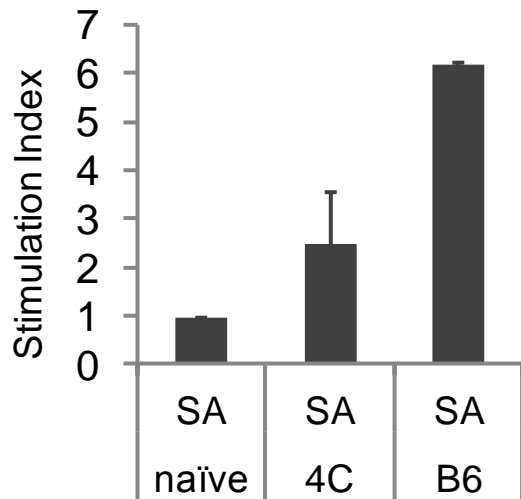
**Figure 32.** 4C splenocytes stimulated with SA have compromised proliferation.

Four 4C and two C57BL/6 mice were immunized with 1 shot of SA-4-1BBL (25 µg s.c.).

Seven days later, spleens were harvested, processed, and cultured *in vitro* for 48 hours in

the presence or absence of SA stimulation (5 µg/ml). Cultures were pulsed with ^{3}[H]-

thymidine for additional 16 hours and CPMs were recorded. Stimulation index (SI) =

average cpm on assessment day with stimulation / average cpm on assessment day

without stimulation. Experiment repeated twice with same pattern detected.
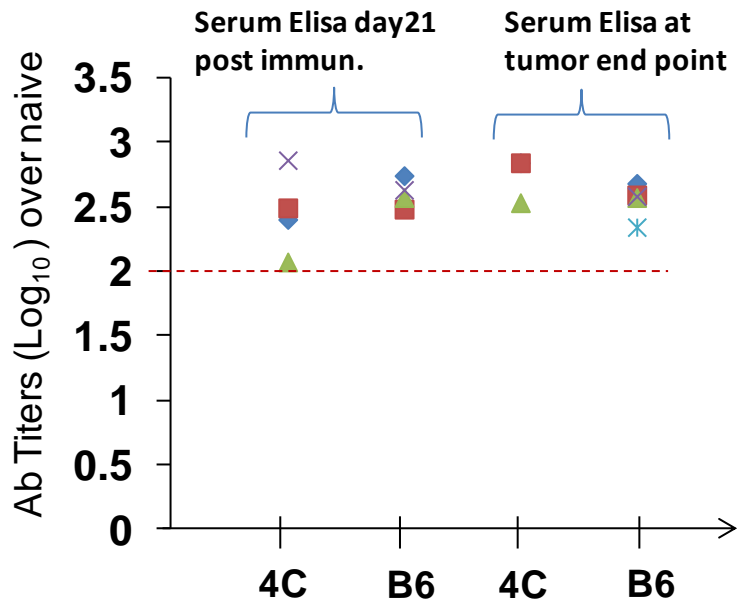
Figure 33. SA-4-1BBL immunization is capable of generating anti-SA antibodies in 4C

mice. 4C or wild type C57BL/6 mice were immunized twice with SA-4-1BBL (2 weeks

apart), and sera were collected 1 week after 2nd immunization (day 21), and at

experimental endpoint when tumors expire. ELISA plates were coated overnight with 50

ng of SA-4-1BBL protein. Next day plates were washed and incubated with serial

dilutions of specified sera for 1.5 hours, after which goat-anti-mouse secondary antibody

conjugated to HRP was added for 1 hour. Colorimetric reaction was generated using

TMB substrate for 30 minutes and plates were read at 450 nm.
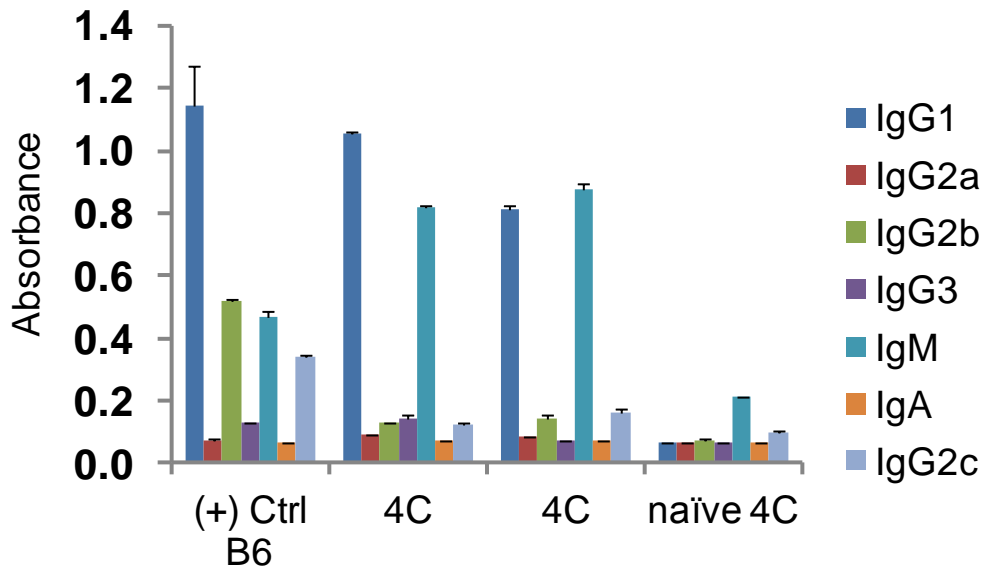
Figure 34. Subtyping ELISA at tumor endpoints for 4C mice pre-immunized twice with

SA-4-1BBL. ELISA plates were pre-coated with 100 ng of SA-4-1BBL overnight. 1/25

dilution of positive sera is used the next day to test the IgG1, IgG2a, IgG2b, IgG3, IgM,

IgA, and IgG2c antibody titers. Each data point is indicative of the mean ± SD of

duplicate wells.
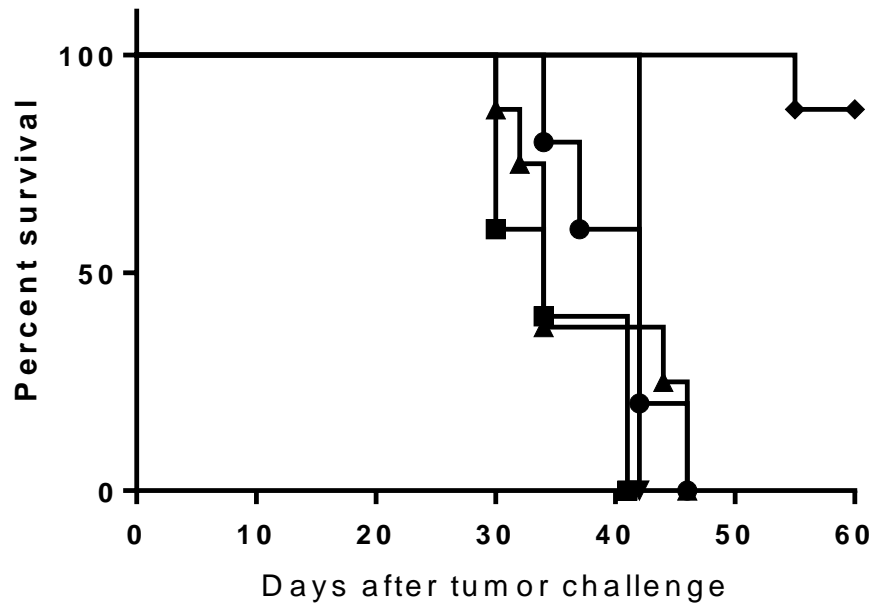
Figure 35. SA-4-1BBL vs. SA+3H3: SA-4-1BBL as a *bona fide* novel

immunomodulatory molecule. Different groups of C57BL/6 mice were immunized twice

with either SA alone (12.5 μg s.c.), 3H3 alone (100 μg s.c.), SA + 3H3, or SA-4-1BBL

(25 μg s.c.), followed by TC-1 (1 x 10^5 s.c.) challenge 2 weeks after the 2nd

immunization. Tumor measurements were recorded twice a week using calipers and mice

were euthanized when tumor average diameter $\geq$ 12 mm or when experimental endpoints

were reached.
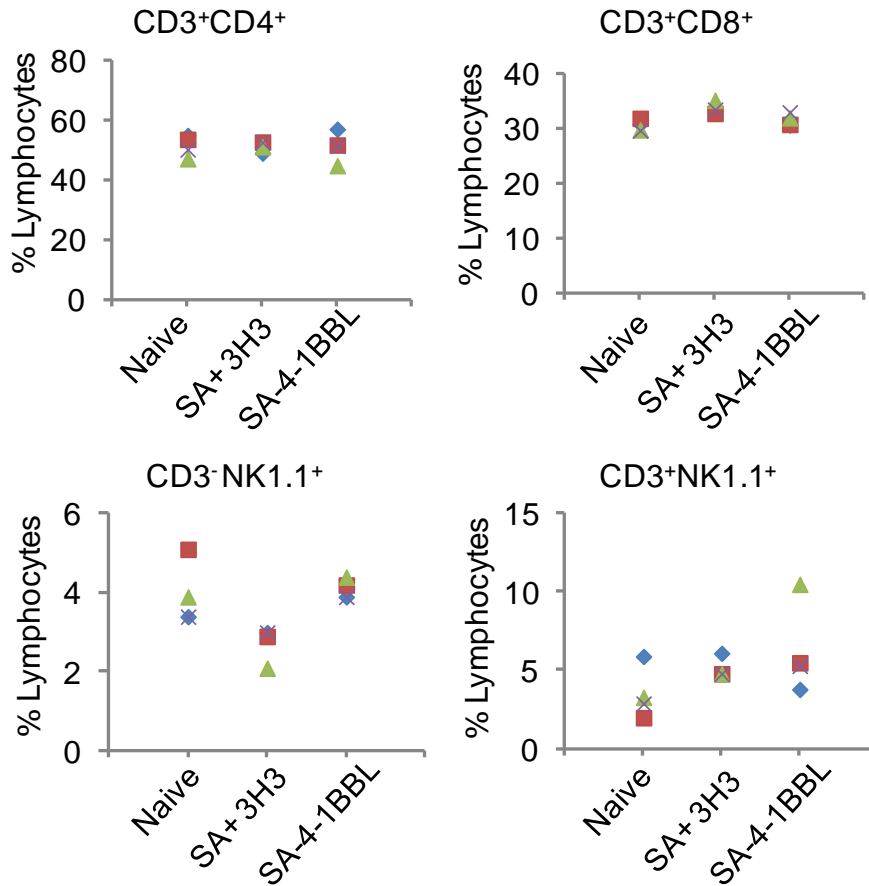
Figure 36. Immunization with 3H3 reduces NK cell percentages in spleen lymphocyte population. C57BL/6 mice were immunized twice (2 weeks apart) with either (12.5 µg SA + 100 µg 3H3 n=4) or (25 µg SA-4-1BBL n=4). Three days later, spleens were harvested, processed, and stained for flow cytometric phenoytping. CD4 T cells were gated on $CD3^+CD4^+$; CD8 T cells were gated on $CD3^+CD8^+$; NK cells were gated on $CD3^-NK1.1^+$; and NKT cells were gated on $CD3^+NK1.1^+$.
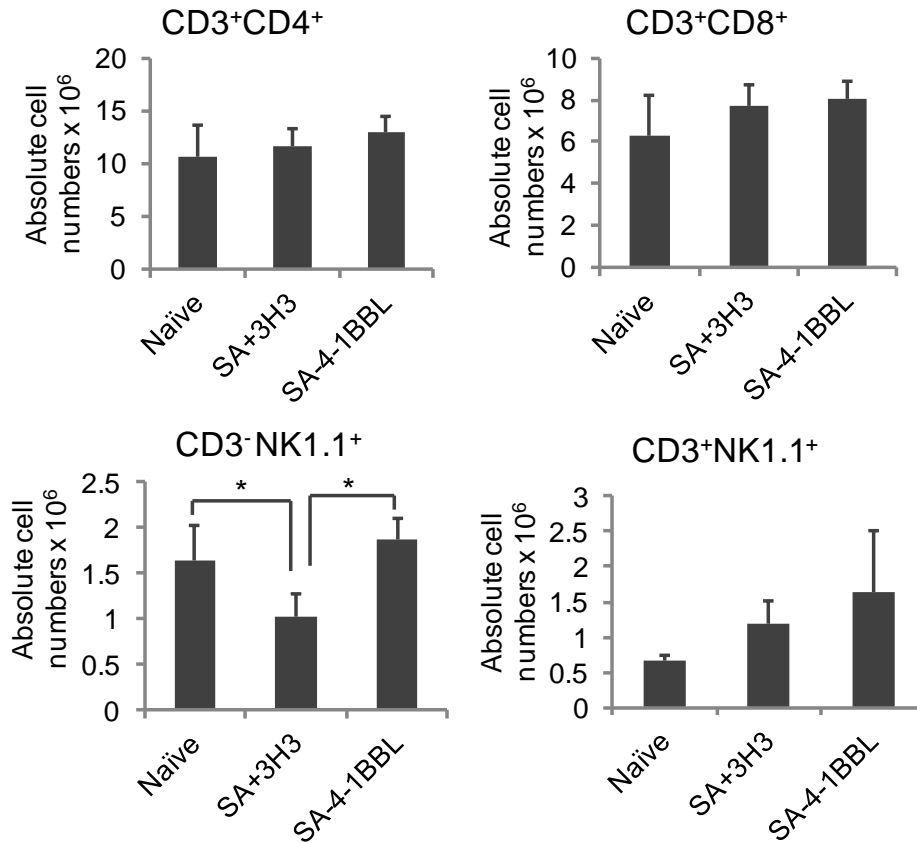
Figure 37. Immunization with 3H3 reduces NK absolute cell numbers in spleen lymphocyte population. C57BL/6 mice were immunized twice (2 weeks apart) with either (12.5 µg SA + 100 µg 3H3 n=4) or (25 µg SA-4-1BBL n=4). Three days later, spleens were harvested, processed, and stained for flow cytometric phenoytping. CD4 T cells were gated on $CD3^+CD4^+$; CD8 T cells were gated on $CD3^+CD8^+$; NK cells were gated on $CD3^-NK1.1^+$; and NKT cells were gated on $CD3^+NK1.1^+$. Student's t-test (two-tailed) was performed for statistical analysis with *$p \leq 0.05$ being significant.
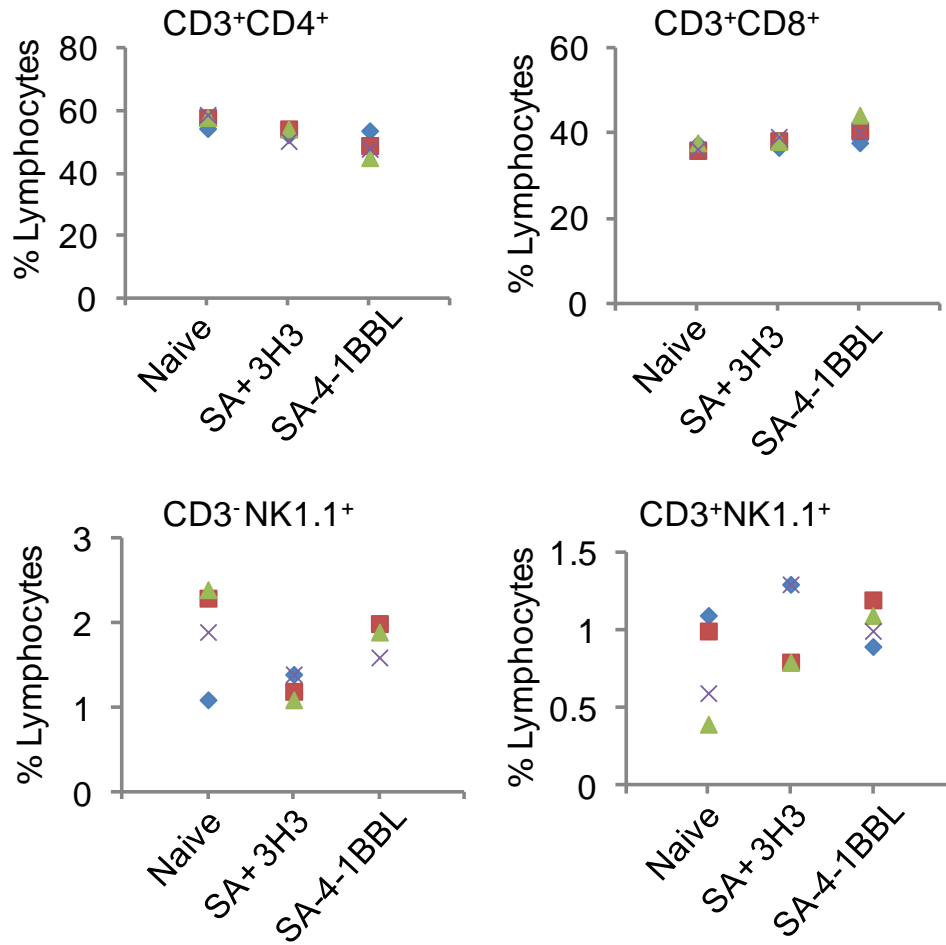
Figure 38. Immunization with 3H3 reduces NK cell percentages in draining lymph nodes' lymphocyte population. C57BL/6 mice were immunized twice (2 weeks apart) with either (12.5 µg SA + 100 µg 3H3 n=4) or (25 µg SA-4-1BBL n=4). Three days later, draining lymph nodes were harvested, processed, and stained for flow cytometric phenoytping. CD4 T cells were gated on $CD3^+CD4^+$; CD8 T cells were gated on $CD3^+CD8^+$; NK cells were gated on $CD3^-NK1.1^+$; and NKT cells were gated on $CD3^+NK1.1^+$.
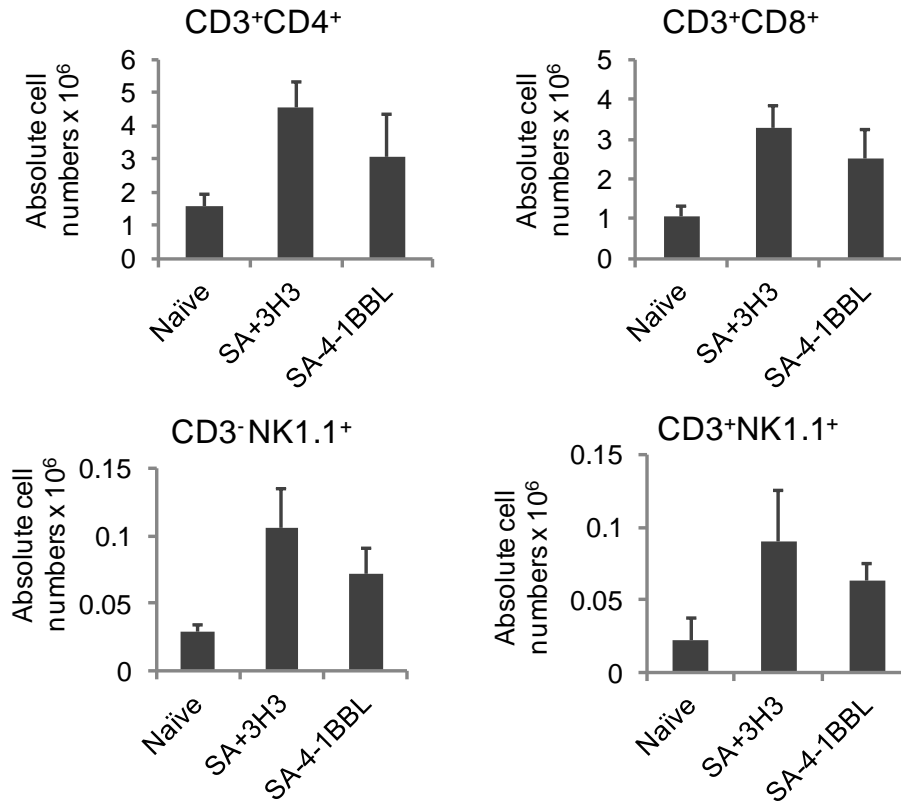
Figure 39. Immunization with SA+3H3 vs. SA-4-1BBL absolute cell numbers profile in draining lymph nodes. C57BL/6 mice were immunized twice (2 weeks apart) with either (12.5 μg SA + 100 μg 3H3 n=4) or (25 μg SA-4-1BBL n=4). Three days later, draining lymph nodes were harvested, processed, and stained for flow cytometric phenoytping. CD4 T cells were gated on CD3^{+}CD4^{+}; CD8 T cells were gated on CD3^{+}CD8^{+}; NK cells were gated on CD3^{-}NK1.1^{+}; and NKT cells were gated on CD3^{+}NK1.1^{+}.
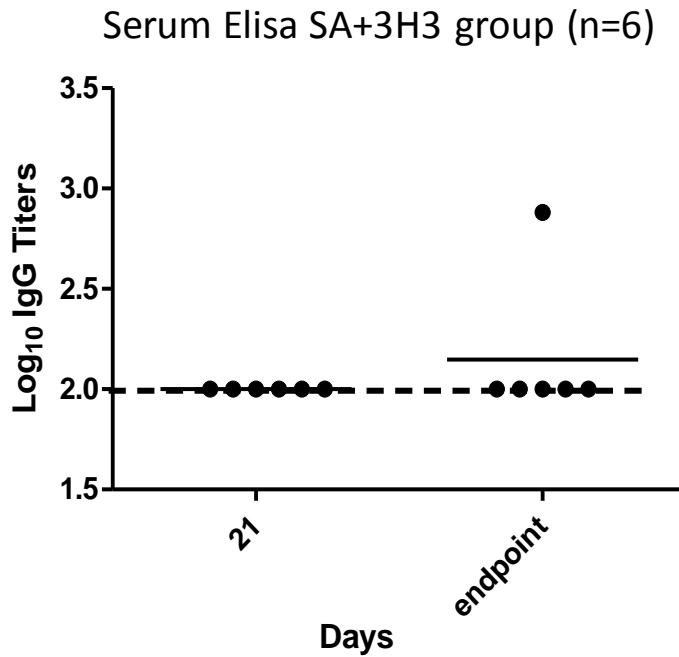
Figure 40. SA+ 3H3 immunization shuts down antibody production. C57BL/6 mice were immunized twice with 12.5 µg SA + 100 µg 3H3 Ab. Sera were collected 1 week after the 2nd immunization and again at tumor endpoints and subjected to anti-SA ELISA.

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I. Education & Training

University of Louisville, Department of Microbiology and Immunology – May 2016

Doctor of Philosophy in Microbiology and Immunology

American University of Beirut, Faculty of Medicine, Department of Microbiology and Immunology – May 2010

Masters of Science in Immunology and Graduate Assistant

American University of Beirut – May 2008

Bachelors of Science in Biology and Pre-med

- Took the Medical College Admission Test (MCAT) - 2008
- Cambridge University AS level Certificate in 5 subjects - 2007
- Cambridge University IGCSE O-Level Certificate in 7 subjects - 2005
- SAT and TOEFL certificates – 2005

II. Research Background

1st author on the research article titled: 4-1BB signaling in conventional T cells drives IL-2 production that overcomes $CD4^+CD25^+FoxP3^+$ T regulatory cell suppression.

1st author on the research article titled: The Effect of Antibacterial agents on Nitric Oxide Levels Induced by Lipopolysaccharide in Mice.

2nd author on the research article titled: The effect of nebivolol on the production of nitric oxide induced by bacterial lipopolysaccharide and peptidoglycan in mice.

Research assistant on the project titled: Presence of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, Serum CRP Levels and HLA Associations in Chronic and Aggressive Periodontitis in a Selected Lebanese Group.

III.   Laboratory Acquired Techniques

- Tumor injections, measurements, and handling mice
- Tissue processing and culturing
- PCR and RT-PCR
- Western Blotting
- ELISA and Elispot
- Flow Cytometry
- *In vitro* Killing Assays
- *In vivo* killing assays
- Immune cells proliferation assays
- Suppression coculture assays
- Cytokine analysis
- Synthesis and purification of proteins from bacterial or insect cell lines
- Histological staining

IV.   Awards and Achievements

- Tuition Match Award from University of Louisville, School of Interdisciplinary and Graduate Studies (Spring 2014 to Spring 2015)
- Center of Predictive Medicine at University of Louisville Retreat presentation (Nov. 20th 2015). Titled: SA-4-1BBL in Cancer and Immunity
- Research Louisville poster presentation finalist (2014)
- International Student Leadership award 2013, University of Louisville
- Certificate of participation in the 7th Arab Conference for Antimicrobial Agents, Beirut, Lebanon
- Research award from the company of "Initiative for Biodiversity Studies in Arid Regions" (IBSAR) in Lebanon
- Gold medal holder in national piano contest

V.   Extracurricular Activities

- American International Relations Club at University of Louisville
  - Member (2010)
  - Co-president (2011)
  - President (2012, 2013)
- Admissions committee representative for 2 years, Department of Microbiology and Immunology, University of Louisville
- Member at the Peer mentoring program, Microbiology and Immunology Student Organization, University of Louisville

- Volunteer at the University of Louisville Hospital Emergency Medicine Department (2013 – 2015)
- Poster Presentation Judge – Manual High School, Louisville, KY
- Member of the Biology Society at the American University of Beirut
- Treasurer and Vice President at the Latino Dance Club at the American University of Beirut
- Member of the Lebanese Armenian Heritage Club
- Member of the Armenian General Benevolent Union's Young Professionals

Spoken Languages

- Excellent English
- Excellent Armenian
- Excellent Arabic

Supporting information and recommendations are available upon request