A Novel Form of 4-1BBL Has Better Immunomodulatory Activity than an Agonistic Anti-4-1BB Ab without Ab Associated Severe Toxicity

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Abstract

Agonistic Abs to select costimulatory members of CD28 and TNFR family have shown efficacy in various preclinical cancer immunotherapeutic settings. However, the use of agonistic Abs is often associated with severe toxicity due to nonspecific activation of lymphocytes. We hypothesized that natural costimulatory ligands may serve as more potent and safer alternative to agonistic Abs for immunotherapy. In this communication, we focused on 4-1BBL as the molecule of choice because of the pleiotropic effects of 4-1BB signaling in the immune system and the demonstrated therapeutic efficacy of 4-1BB agonistic Abs in preclinical cancer and infection models. We report that a novel form of soluble ligand, SA-4-1BBL, delivered more potent and qualitatively different signals to T cells than an agonistic Ab. Importantly, while treatment of naïve mice with the agonistic Ab resulted in severe toxicity, as assessed by enlarged spleen and peripheral LNs, non-specific T cell proliferation, hepatitis, and systemic inflammatory cytokine production, treatment with SA-4-1BBL lacked these immune anomalies. Agonistic Ab treatment produced full toxicity in $FcγR^{−/−}$ or complement $C1q^{−/−}$ or $C3^{−/−}$ knockout mice, suggesting lack of involvement of stimulatory FcγRs or complement system in the observed toxicity. Naïve and memory T cells served as direct targets of anti-4-1BB Ab mediated toxicity. Potent immunostimulatory activity combined with lack of toxicity rationalizes further development of soluble SA-4-1BBL as an immunomodulatory component of therapeutic vaccines against cancer and chronic infections.

Keywords

Costimulation; 4-1BB; SA-4-1BBL; Vaccines; Toxicity; Adjuvant

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1. Introduction

Costimulatory molecules of the CD28 and TNFR superfamilies play critical roles in modulating innate, adaptive, and regulatory immune responses [1]. As such, agonistic ligands to these receptors have the potential to serve as effective immunomodulatory components of vaccines. Consistent with this notion are a series of studies demonstrating that agonistic Abs to the CD28 and TNFR superfamilies were effective in generating potent immune responses with therapeutic potential in settings of chronic infections and cancer [2–5]. However, the use of agonistic Abs can be associated with severe toxicity arising from nonspecific, systemic activation of lymphocytes [6–12]. For example, a single intravenous dose of a superagonist anti-CD28 mAb in 6 healthy volunteers enrolled in a Phase I clinical trial resulted in a systemic inflammatory response and life threatening toxicity [8]. Therefore, the potential use of costimulatory agonists for therapeutic vaccines in humans requires the generation of agonists that transduce the appropriate stimulatory signals without toxic side effects *in vivo*.

Costimulatory receptors that are inducibly expressed or upregulated on activated T cells may serve as preferred targets for immunomodulation due to their potential to selectively target antigen-experienced T cells for expansion, survival, and establishment of long-term immune memory. Using the appropriate form of agonists would ideally lead to the generation of a highly specific immune response, and importantly, potentially avoid toxic side effects often associated with systemic immune activation. 4-1BB, a costimulatory receptor belonging to the TNF receptor family, is inducibly expressed to high levels on activated $CD4^+$ and $CD8^+$ T cells, and as such represents a promising target for immunomodulation [13,14]. Engagement of 4-1BB with its ligand, 4-1BBL, or agonistic Abs results in T cell activation, clonal expansion, survival, and the establishment and maintenance of long-term immune memory [1,15–18]. Agonistic Abs to 4-1BB have demonstrated impressive therapeutic efficacy in various preclinical cancer and viral models [5,16,18,19]. As a result, a humanized agonistic mAb was developed and tested in phase I and II clinical trials with metastatic or locally advanced solid tumors (National Institutes of Health Clinical trials database NCT00309023). However, we have recently reported that multiple injections of anti-4-1BB mAbs at therapeutic doses in naïve mice resulted in transient toxicity and the development of a series of immunological anomalies [7]. We hypothesized that natural 4-1BBL may have better efficacy and safety as compared with agonistic Abs by delivering signals quantitatively and/or qualitatively different from those transduced by Abs. Inasmuch as 4-1BBL functions as a membranous protein and has no costimulatory activity in soluble form [20,21], we recently generated a novel chimeric form of 4-1BBL, SA-4-1BBL, by fusing the extracellular domains of 4-1BBL to the C-terminus of a modified form of core streptavidin (SA). The SA-4-1BBL molecule exists as tetramers/oligomers, owing to the structural features of SA, and has the ability to cross-link 4-1BB receptors for potent costimulatory activity on T cells [22]. In addition, we have recently demonstrated that SA-4-1BBL served as a potent immunomodulatory component of a peptide-based vaccine and had better efficacy than an agonist 4-1BB Ab (3H3) as well as TLR agonists LPS, MPL, and CpG in eradicating established tumors in an animal model of cervical cancer [23].

In this study, we directly compared the immunomodulatory activity and toxicity of SA-4-1BBL to the 3H3 agonistic Ab, which has shown therapeutic efficacy in various experimental settings [16,24,25]. SA-4-1BBL demonstrated more potent and qualitatively different immunostimulatory activity as compared with the agonistic mAb. Importantly, the better efficacy of SA-4-1BBL was achieved in the absence of Ab-associated severe systemic toxicity that did not involve FcγR or complement activation. Both naïve and memory $CD4^+$ and $CD8^+$ T cells served as direct targets for Ab-associated toxicity. Potent immunostimulatory

activity and lack of toxicity support our perception that SA-4-1BBL may serve an effective immunomodulatory component of prophylactic and therapeutic vaccine formulations.

2. Materials and Methods

2.1 Mice

C57BL/6.SJL, C57BL/6, C57BL/6 *Fcer1g*$^{-/-}$, C57BL/6 OT-I*rag*$^{-/-}$, and C57BL/6 OT-II*rag*$^{-/-}$ mice were purchased from The Jackson Laboratory or bred in our barrier animal facility at the University of Louisville. C57BL/6 *4-1BB*$^{-/-}$ mice were kindly provided by Dr. A.T. Vella of University of Connecticut, Farmington, CT, with permission from Dr. B.S. Kwon of University of Ulsan, Korea. C57BL/6 *C3*$^{-/-}$ and C57BL/6 *C1q*$^{-/-}$ mice were generously provided by Dr. M. Ratajczak of University of Louisville. All animals were cared for in accordance with institutional and National Institutes of Health guidelines.

2.2 Reagents

Construction, expression, purification, and characterization of SA-4-1BBL and core streptavidin (SA) were recently described [22] and endotoxin levels were nill. Anti-41BB agonistic Ab, 3H3, was previously described [26]. The preparation of Ab contained 0.6 EU/mg or 0.06 EU/100 µg endotoxin per injection. Rat IgG2a was purchased from Sigma-Aldrich. Fluorochrome-conjugated Abs to various immune cell surface markers or cytokines and isotype controls were purchased from BD Bioscience (San Jose, CA), eBioscience (San Diego, CA), and BioLegend (San Diego, CA). Chicken ovalbumin (OVA) was purchased from Pierce, and OVA SIINFEKL peptide (aa 257–264) was purchased from CPC Scientific Inc, San Jose, CA.

2.3 Flow cytometry

For phenotyping and sorting, spleens and LNs were processed into single-cell suspensions, treated with ACK solution to lyse RBC, Fc blocked (BD Pharmingen), and labeled with saturating concentrations of fluorochrome-conjugated Abs. Isotype matched Abs with the same fluorochrome were used as controls. For T cell sorting, lymphocytes were stained with CD4-FITC, CD25-PE, and CD8-APC Abs. CD4$^+$CD25$^-$ (CD4$^+$ Teff) and CD8$^+$ (CD8$^+$ Teff) T cells were sorted to > 95% purity using a FACSVantage cell sorter (BD Bioscience). Intracellular FoxP3 staining was performed according to the manufacturer's protocol (eBiosciences). Intracellular cytokine staining on PMA (5 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) stimulated cells was performed as previously described [23]. Serum cytokine levels were determined using the inflammatory CBA kit (BD Pharmingen) according to the manufacturer's protocol. FACS Caliber or LSR-II (BD Bioscience) was used for FACS acquisition. Data was analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

2.4 In vitro T cell proliferation

Sorted CD8$^+$ and CD4$^+$ T cells were cultured (2.5×10^4/well) with 0.25 µg/ml of soluble anti-CD3 Ab and irradiated (2000 cGy) syngeneic splenocytes (1×10^5/well) in the presence of varying concentrations of soluble SA-4-1BBL, equimolar quantity of control SA protein, or agonistic anti-4-1BB Ab (3H3) for 3 days in complete MLR medium. Cultures were pulsed with [^{3}H]-thymidine during the last 16 hrs of the culture, and harvested on a Tomtec Harvester 96 (Tomtec Inc., Hamden, CT) for quantification of incorporated radioactivity. Results expressed as mean ±SD cpm of triplicate wells.

2.5 In vivo OT-I and OT-II proliferation

Flow cytometry-sorted OT-I CD8$^+$ T cells and OT-II CD4$^+$ T cells (*CD45.2*$^+$) were labeled with 2.5 µM CFSE and 1×10^6 OT-I and OT-II T cells were transferred by i.v. injection into naïve C57BL/6 mice (SJL, *CD45.1*$^+$). After 24 hrs, mice were immunized s.c. with OVA (10

µg) in conjunction with various doses of SA-4-1BBL, anti-4-1BB Ab, or equimolar concentration of SA control protein. After 3 days, peripheral LNs and spleens were harvested and OT-I and OT-II proliferation was assessed by analyzing the CFSE dilution of CD45.2+CD8+ and CD45.2+CD4+ cells using flow cytometry.

2.6 In vivo cytotoxicity

Naïve C57BL/6 mice were immunized i.v. with OVA (50 µg) in conjunction with various doses of SA-4-1BBL, anti-4-1BB Ab, or equimolar concentration of SA control protein. One week later, mice received CFSE labeled target cells pulsed with SIINFEKL as previously described [23].

2.7 Toxicity studies

Naïve mice were injected s.c. with 100 µg of 3H3 mAb, rat isotype IgG2a, SA-4-1BBL, or equimolar SA control protein either once or once weekly for 3 wks. One week following the last treatment, mice were euthanized and subjected to various analyses to determine toxicity.

2.8 Adoptive transfer model

C57BL/6 *4-1BB*−/− mice, which do not develop toxicity from agonistic Ab treatment [7], were adoptively transferred with various cell populations, including total splenocytes, DCs, and T cells, of WT mice. One day after adoptive transfer, mice were treated with 100 µg of the 3H3 Ab and analyzed for signs of toxicity one week later. C57BL/6 *4-1BB*−/− mice adoptively transferred with highly purified cell populations and treated with agonistic Ab were compared to C57BL/6 *4-1BB*−/− mice that underwent the same agonistic Ab treatment but were not adoptively transferred with WT cells.

2.9 Statistics

Data were compared using the Student's *t* test or ANOVA and a $p < 0.05$ was considered significant.

3. Results

3.1 SA-4-1BBL has better immunostimulatory activity than an agonistic anti-4-1BB Ab

We compared the immunostimulatory activity of SA-4-1BBL on T cells to that of an agonistic 4-1BB Ab (3H3) with demonstrated therapeutic efficacy in various experimental settings [16,24,25]. SA-4-1BBL was more effective than the 3H3 Ab for driving the proliferation of CD8+ T cells in a CD3 Ab-based *in vitro* stimulation assay (Fig. 1A, left). Interestingly, while SA-4-1BBL induced proliferation of CD4+ T cells in a dose dependent manner, the 3H3 Ab showed little to no effect on the proliferation of CD4+ T cells *in vitro* (Fig. 1A, right). The better efficacy of SA-4-1BBL in driving T cell proliferation was not dependent on the dose of soluble CD3 Ab as SA-4-1BBL induced higher levels of proliferation of both CD8+ and CD4+ T cells compared to the agonistic anti-4-1BB Ab at all CD3 Ab concentrations tested (Supporting Fig. 1). Importantly, SA-4-1BBL showed no effect on the proliferation of both CD8+ and CD4+ T cells from C57BL/6 *4-1BB*−/− mice (Supporting Fig. 2), demonstrating that SA-4-1BBL acts directly through 4-1BB.

The immunostimulatory activity of SA-4-1BBL was also compared to 3H3 Ab in an *in vivo* OT-I and OT-II adoptive transfer model. Vaccination with ovalbumin (OVA) and SA-4-1BBL resulted in greater proliferation of OT-I CD8+ T cells in both the peripheral LNs (Fig. 1B, left) and spleen (Supporting Fig. 3) at lower doses (1 and 5 µg) and similar proliferation at higher doses (10 and 25 µg) as compared with the 3H3 Ab. Similar to the *in vitro* proliferation assays, vaccination with SA-4-1BBL resulted in higher levels of proliferation of OT-II CD4+ T cells

in both the peripheral LNs (Fig. 1B right) and spleen (Supporting Fig. 3) at all concentrations tested as compared to vaccination with the agonistic 4-1BB Ab. The enhanced efficacy of the SA-4-1BBL over the Ab was even more pronounced in an *in vivo* killing assay using OVA as a cognate antigen for vaccination (Fig. 1C). Importantly, animals treated with the agonistic anti-4-1BB Ab, but not SA-4-1BBL, had enlarged spleen and lymph nodes (Fig. 1D), suggesting possible toxicity.

3.2 Treatment of naïve mice with agonistic anti-4-1BB Ab, but not SA-4-1BBL, results in nonspecific naïve and memory T cell proliferation, altered lymphocyte trafficking, and enlarged lymphoid organs

Our observation that treatment with one dose of agonistic anti-4-1BB Ab resulted in enlarged spleen and peripheral LNs is consistent with our recent study reporting severe toxicity from multiple high-dose therapeutic injections (200 µg) of various agonistic anti-4-1BB Abs, including 3H3 Ab.[7] We therefore compared the toxicity profile of SA-4-1BBL directly with the 3H3 Ab under similar conditions. Three weekly treatments of naïve mice with 100 µg of 3H3 Ab, a therapeutic dose used in various settings [27,28], resulted in gross enlargement of spleen and LNs as demonstrated by weight (Fig. 2A) and total cell numbers (Fig. 2B). In contrast, the same treatment with SA-4-1BBL did not result in lymphadenopathy, splenomegaly, and increased cell numbers (Fig. 2A,B). Treatment with 3H3 Ab resulted in > 2-fold increase in $CD4^+$ T cells and > 3-fold increase in $CD8^+$ T cells in the peripheral LNs, as well as significant increases in both T cell subsets in the spleen. However, the same treatment resulted in > 3-fold increase in $CD19^+$ B cells in the peripheral LNs, but a dramatic decrease in $CD19^+$ B cells in the spleen (Table I). We found similar toxicity results using only one injection of anti-4-1BB Ab (Fig. 2A–C) with the exception of an increase, rather than decrease, in the numbers of $CD19^+$ B cells in the spleen.

Anti-4-1BB Ab treatment also led to a dramatic increase in $CD8^+$ T cells and decrease in $CD19^+$ B cells in the bone marrow (data not shown). Unlike other lymphocytes, treatment with 3H3 Ab did not significantly alter the absolute number of NK cells ($NK1.1^+CD3\varepsilon^-$) in both the peripheral LNs and spleen. In addition, anti-4-1BB Ab treatment resulted in increased percentage as well as absolute number (2-fold) of $CD4^+FoxP3^+$ regulatory T cells (Table I). Importantly, these immune abnormalities were absent in SA-4-1BBL treated animals (Supporting Fig. 4 and Table I).

The enlargement in the spleen and LNs of Ab-treated mice was primarily due to non specific proliferation of T cells as demonstrated by adoptive transfer of CFSE-labeled congenic T cells one day before Ab treatment. While SA-4-1BBL showed no effect on the *in vivo* proliferation of cells compared to control animals, anti-4-1BB Ab acted as a superagonist *in vivo* and resulted in striking non-specific proliferation of the majority of $CD4^+CD45.1^+$ and $CD8^+CD45.1^+$ congenic T cells in both the spleen (Fig. 2D) and peripheral LNs of treated mice (Supporting Fig. 5). More than 85% and 55% of transferred T cells proliferated in response to multiple and single injections of anti-4-1BB Ab, respectively, as compared with < 40% and 15% for treatment groups receiving SA-4-1BBL or control reagents. Antibody treatment caused the proliferation of both newly activated naive ($CD43^{high}CD44^{high}$) and memory ($CD43^{low/-}CD44^{high}$) $CD8^+$ T cells [29–31] (Supporting Fig. 6). Thus, although T cells could still be trafficking to the spleen and peripheral LNs, nonspecific proliferation of both memory and naïve T cells plays a major role in the increased absolute number of T cells. On the other hand, anti-4-1BB Ab treatment had no effect on the proliferation of $CD19^+$ B cells (data not shown), suggesting that the increase or decrease in $CD19^+$ B cell numbers in lymphoid organs and BM of these mice was due to cell trafficking.

3.3 Agonistic anti-4-1BB Ab, but not SA-4-1BBL, results in systemic cytokine response

Toxic effects of agonistic Abs to CD28 and CD40 are associated with a systemic inflammatory cytokine response [6,8–12,32]. Therefore, we compared the effects of treatment with SA-4-1BBL and anti-4-1BB Ab on the production of inflammatory cytokines. Whereas treatment of naïve mice with SA-4-1BBL had no effect on the production of inflammatory cytokines, anti-4-1BB Ab resulted in a significant increase in the percentage (Fig. 3A) and absolute number (Fig. 3B) of total $CD8^+$ T cells (left) and $CD4^+$ T cells (right) producing IFN-γ and $CD8^+$ T cells producing IL-2 and TNF-α (data not shown). Mice treated with multiple injections of anti-4-1BB Ab demonstrated approximately a 2-fold increase in percentage and a 4-fold increase in absolute number of splenic $CD8^+IFN$-γ^+ and $CD4^+IFN$-γ^+ T cells compared to all other groups. This effect was also observed with a single Ab treatment, but it was not as drastic as that obtained using multiple treatments. Importantly, repeated anti-4-1BB Ab treatment resulted in significant increases in the serum cytokine levels for IFN-γ, TNF-α, and MCP-1 (Fig. 3C). Of note, treatment with either SA-4-1BBL or anti-4-1BB Ab demonstrated no effect on the production of IL-4, IL-6 or IL-10 cytokines as compared to control groups (data not shown).

Treatment with the agonistic anti-4-1BB Ab resulted in massive infiltration of $CD8^+$ T cells into the liver as determined by confocal microscopy (Supporting Fig. 7) and H&E staining (data not shown), while SA-4-1BBL treated mice showed little evidence of any hepatitis. Taken together, these data demonstrate that in spite of its more potent antigen-specific immunostimulatory activity, SA-4-1BBL lacks toxicity caused by the agonistic anti-4-1BB Ab .

3.4 Toxic effects of anti-4-1BB Ab are FcγR and complement independent

We next tested if the systemic inflammatory responses and the associated toxicity seen in this model is manifested by 3H3 Ab interaction with FcγRs expressed on various immune cell types, resulting in cellular activation and the production of inflammatory cytokines [33]. C57BL/6 mice lacking the expression of FcR γ-chain (*Fcer1g*$^{-/-}$), and as a result surface expression and function of stimulatory Fc-γRI, Fc-γRIII, and Fc-γRIV [33,34], were injected with 100 µg of anti-4-1BB Ab or isotype control and analyzed for toxicity. Similar to wild type C57BL/6 mice, a single treatment with 100 µg of anti-4-1BB Ab resulted in severe toxicity in *Fcer1g*$^{-/-}$ mice as assessed by enlarged spleen and peripheral LNs (Fig. 4A), increased total cell numbers (Fig. 4B), and increased $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cell populations (Fig. 4C) in these organs. We further eliminated the role of complement in 3H3 Ab associated toxicity by treating C57BL/6 mice deficient in C1q (*C1q*$^{-/-}$) or complement factor C3 (*C3*$^{-/-}$). A single treatment with 100 µg anti-4-1BB Ab resulted in full toxicity in *C1q*$^{-/-}$ and *C3*$^{-/-}$ mice, as demonstrated by enlarged spleen and peripheral LNs (Fig. 4A) and increased total leukocyte (Fig. 4B) and $CD4^+$ and $CD8^+$ T cell and $CD19^+$ B cell populations (Fig. 4C). Taken together, these data strongly suggest that activating FcγRs and complement mediated mechanisms do not play a role in the anti-4-1BB Ab associated toxicity.

3.5 T cells are critical targets of anti-4-1BB Ab mediated toxicity

We next tested if T cells are the direct target of 3H3 Ab-induced toxicity. The cross-linking of 4-1BB receptor is essential for the induction of toxicity as all anti-4-1BB Ab associated immune anomalies are absent in C57BL/6 *4-1BB*$^{-/-}$ mice [7]. Therefore, we established an adoptive transfer model where highly purified lymphocyte populations from WT mice were transferred into naïve *4-1BB*$^{-/-}$ mice that were subjected to Ab treatment. The adoptive transfer of 2×10^6 WT whole splenocytes into *4-1BB*$^{-/-}$ mice followed by a single injection of 100 µg of anti-4-1BB Ab resulted in full toxicity as demonstrated by greatly enlarged spleen and peripheral LNs (Fig. 5A) and increased numbers of total leukocytes (Fig. 5B), $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells (Supporting Fig. 8).

To determine the cell population(s) that serves as the direct target for the agonistic anti-4-1BB Ab-mediated toxicity, we performed adoptive transfer experiments using various purified cell populations. Inasmuch as 4-1BB is constitutively expressed on a subpopulation of DCs [1; 35], we first tested if these cells serve as initial targets for the anti-4-1BB Ab-mediated toxicity. The transfer of 2×10^6 highly purified bone marrow-derived WT DCs (purity >97%) into $4\text{-}1BB^{-/-}$ mice followed by treatment with anti-4-1BB Ab did not result in detectable toxicity (data not shown). In marked contrast, adoptive transfer of flow cytometry sorted WT CD8$^+$ or CD4$^+$ T cells (both purities >95%) into $4\text{-}1BB^{-/-}$ mice one day prior to anti-4-1BB Ab treatment resulted in full toxicity in a dose dependent manner as manifested by enlarged spleens and peripheral LNs (Fig. 5A), and increased total (Fig. 5B) and individual CD4$^+$ T cells, CD8$^+$ T cells, and CD19$^+$ B cells in these organs (Supporting Fig. 8). This effect was not limited to polyclonal T cell populations as flow sorted monoclonal naïve OT-I $Rag^{-/-}$ CD8$^+$ T cells and OT-II $Rag^{-/-}$ CD4$^+$ T cells (1×10^6 of each cell type, both purities >95%) resulted in full blown toxicity when adoptively transferred into $4\text{-}1BB^{-/-}$ mice treated with the anti-4-1BB Ab (Fig. 5A,B). In addition, over 95% of the transferred OT-I and OT-II T cells underwent proliferation as determined by CFSE dilution assay (data not shown). Furthermore, treatment of naïve OT-I $Rag^{-/-}$ or OT-II $Rag^{-/-}$ mice with one injection of anti-4-1BB Ab resulted in significant toxicity (Fig. 5C–F). Taken together, these data demonstrate that both CD8$^+$ and CD4$^+$ T cells serve as targets for the anti-4-1BB induced toxicity irrespective of their TCR antigen specificity or TCR repertoire diversity.

4. Discussion

The development of immunomodulators that invoke desired immune responses without adverse toxicity is crucial to the success of vaccines against cancer and infections. As costimulatory molecules of the CD28 and TNFR superfamilies play critical roles in the modulation of innate, adaptive, and regulatory immunity[1], they may serve effective immunomodulatory components of vaccines. Consistent with this notion are the demonstrated efficacy of agonistic Abs to costimulatory receptors in various virus and cancer immunotherapy settings [2–5]. However, systemic toxicity appears to be a common side effect of treatment with agonistic Abs to the members of CD28 and TNFR superfamilies [6–12,32]. The failed Phase I clinical trial resulting from a single intravenous dose of a superagonist anti-CD28 monoclonal Ab that led to multi-organ failure and a systemic inflammatory response [8] highlights the importance of developing costimulatory agonists that transduce the appropriate stimulatory signals without toxic side effects *in vivo*. We hypothesized that toxicity reported using agonistic mAbs to costimulatory receptors may be inherent to Abs, rather than being a generalized feature of the costimulatory system. Natural ligands may lack such toxicity and have better efficacy due to their potential ability to transduce signals having quantitative and/ or qualitative differences with those elicited by agonistic Abs.

This hypothesis was tested using a novel form of 4-1BBL with potent immunomodulatory activity in soluble form [22,23] as a test case. The rationale for using 4-1BBL is several-fold. First, 4-1BB receptor is constitutively expressed by a subpopulation of DCs, which receive a stimulatory signal via 4-1BB resulting in up-regulation of costimulatory molecules and increased secretion of cytokines [35,36]. Second, 4-1BB is transiently expressed to high levels on activated CD4$^+$ and CD8$^+$ T cells, but not naïve T cells, thereby potentially allowing for selected modulation of antigen-specific T cells. Third, signaling via 4-1BB endows T effector cells refractory to suppression by CD4$^+$CD25$^+$FoxP3$^+$ Treg cells [22,23,27] as well as reverses anergy in CD8$^+$ T cells [3]. Therefore, 4-1BB/4-1BBL system has the potential to serve as an effective component of therapeutic vaccines against chronic infections and cancer due to its pleiotropic effects on innate, adaptive, and regulatory immunity. Consistent with this notion is our recent publication demonstrating that SA-4-1BBL as the immunomodulatory component

of a peptide-based vaccine had better therapeutic efficacy in a mouse cervical cancer model than TLR agonists MPL and CpG [23].

We herein report that SA-4-1BBL had better costimulatory activity on both CD4$^+$ and CD8$^+$ T cells as compared with the agonistic 3H3 Ab at various doses tested *in vitro* and *in vivo*. Indeed, the agonistic Ab to 4-1BB had no detectable costimulatory effect on CD4$^+$ T cells *in vitro* while such an effect *in vivo* was moderate. This observation is consistent with various studies demonstrating the preferential induction of CD8$^+$ T cells by agonistic Abs to 4-1BB [3,17,26]. The potent costimulatory activity of SA-4-1BBL on CD8$^+$ T cells and its differential effect on CD4$^+$ T cells as compared with 3H3 Ab is consistent with the better efficacy of the ligand in generating *in vivo* killing responses and eradicating established tumors as the immunomodulatory component of a therapeutic cancer vaccine [23]. With potent costimulatory activity on CD4$^+$ T cells, SA-4-1BBL may serve as a powerful adjuvant in subunit vaccines targeting CD4$^+$ T cells, such as those against HIV and tuberculosis. The better efficacy of natural ligands as compared with agonistic Abs may be due to the kinetics of on/off rates with their respective receptors, the better ability of natural ligands to crosslink their respective receptors on the cell surface, and/or the quality/quantity of downstream signaling. Most importantly, the better immunostimulatory activity of SA-4-1BBL was achieved in the absence of severe toxicity associated with therapeutic doses of agonistic 3H3 Ab in naïve mice that caused enlarged spleen and LNs, non-specific T cell proliferation and expansion, systemic proinflammatory cytokine response, altered lymphocyte trafficking, and hepatitis. The toxicity associated with the use of 3H3 Ab in this study is consistent with our recently published study using various agonistic Abs to 4-1BB for immunomodulation [7]. However, lack of toxicity with SA-4-1BBL treatment is inconsistent with previous reports demonstrating toxicity in transgenic mice over-expressing 4-1BBL under the control of MHC class II promoter [37, 38]. This may be due to 4-1BBL reverse signaling in the case of the transgenic mice [39–42]. Reverse signaling through 4-1BBL can lead to APC maturation, upregulation of T cell costimulatory molecules, production of proinflammatory TNF-α and IL-6, and inhibition of IL-10 production [39–42].

Our observation that 3H3 Ab causes nonspecific proliferation of T cells in naïve mice is consistent with a recent report by Zhu *et al.* using a different agonistic anti-4-1BB Ab (clone 2A) [38]. Unlike the studies of Zhu *et al.* that demonstrated the proliferative effect of the 2A Ab on memory, but not naïve, T cells, we observed the proliferative effect of 3H3 Ab on both naive and memory T cells. First, using Abs to the CD43 (mAb 1B11) and CD44 to distinguish between newly activated naïve CD8$^+$ T cells (CD43^{high}CD44^{high}) and memory CD8$^+$ T cells (CD43$^{low/-}$ CD44^{high}) cells [29–31], we demonstrated that both T cell populations undergo proliferation in response to 3H3 Ab treatment. Second, treatment of 4-1BB$^{-/-}$ mice adoptively transferred with CFSE labeled sorted naïve OT-I *Rag*$^{-/-}$ or OT-II *Rag*$^{-/-}$ T cells (both purities >95%) resulted in excessive T cell proliferation and full toxicity. Third, direct treatment of naïve OT-I *Rag*$^{-/-}$ or OT-II *Rag*$^{-/-}$ mice with one injection of 3H3 Ab resulted in significant toxicity. Although the source of discrepancy between our data and those of Zhu *et al.* is not known, differences in experimental conditions and nature of agonistic Abs used may provide an explanation. Consistent with this notion are reports that certain CD28 superagonist Abs directly induce the proliferation of both naïve and T memory cells [43,44], while others act strictly on T memory cells [45].

However, unlike CD28 receptor that is constitutively expressed on T cells, 4-1BB is selectively expressed at high levels in activated, but not naive, T lymphocytes as assessed by both mRNA as well as protein expression [1;13;14]. Therefore, our observations that naïve T cells serve as a direct target of the agonistic anti-4-1BB Ab is surprising. A plausible explanation is that Ab-mediated toxicity could be initiated by a subpopulation of newly activated or memory T cells specific for environmental antigens with upregulated expression of 4-1BB on their surface.

This contention, however, is inconsistent with Ab-mediated toxicity observed in OT-I $Rag^{-/-}$ and OT-II $Rag^{-/-}$ mice or $4-1BB^{-/-}$ mice adoptively transferred with sorted naïve OT-I CD8$^+$ and OT-II CD4$^+$ T cells as these cells are not expected to react to environmental antigens due to their strict specificities for OVA, and as such represent naïve T cells. Alternatively, all naïve or a subpopulation of T cells could express undetectable levels of 4-1BB that upon supraphysiological agonistic 4-1BB Ab stimulation initiates immune responses resulting in full blown Ab-mediated toxicity.

The mechanisms of T-cell activation by agonistic mAbs to CD28 and 4-1BB Ab in the absence of antigen are unclear, but in both cases involve receptor cross linking. The signaling ability of CD28 superagonist Abs depends on a functional TCR [44;46]. Therefore, it is hypothesized that CD28 superagonist Abs activate T cells by amplifying low level tonic signals from unligated TCR. A similar mechanism may operate in anti-4-1BB Ab activation of antigen inexperienced T cells. Similar to CD28 superagonist Abs, treatment with anti-4-1BB Ab resulted in *in vivo* expansion of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells. In rodents, CD28 superagonist Ab administration resulted in almost immediate severe lymphopenia similar to that seen in humans, and induced strong naïve and effector T cell activation and increased mRNA levels of proinflammatory cytokines and chemokines. This initial wave of effector T cell activation was followed by a second wave of activation that resulted in the preferential expansion of Treg cells, and there was no detectable increase in secreted pro-inflammatory cytokines [47]. It was hypothesized that expanded Treg cells were able to suppress conventional T cell inflammatory cytokine secretion before it reached harmful levels. In support of this hypothesis, Treg depleted mice treated with a CD28 superagonist Ab resulted in the rapid, systemic release of high levels of inflammatory cytokines [48]. However, anti-4-1BB Ab administration resulted in increases in both serum and intracellular pro-inflammatory cytokines, irrespective of Treg expansion. This may be due to the ability of 4-1BB signaling to endow T effector cells refractory to the suppression function of Treg cells [22,27,49].

Mechanism(s) responsible for the differential effects of agonistic Abs and natural ligands *vis-a-vis* toxicity *vs*. efficacy are not known. Engagement of the activating FcγR receptors on immune cells by anti-4-1BB Ab or activation of the complement system do not play a role as treatment of mice lacking activating FcγRs or complement *C1q* and *C3* with the agonistic anti-4-1BB Ab resulted in full blown toxicity. However, it is conceivable that the inhibitory FcγRIIB, which does not require FcR γ-chain expression for cell surface expression or function [34], could create cognates between T cells and monocytes or other cell types and could trigger toxicity. Such cognates could increase low level tonic signals to T cells leading to T cell activation.

In summary, we demonstrated that a soluble form of 4-1BBL chimeric with core streptavidin has better immunostimulatory activity and safety profile than an agonistic Ab to 4-1BB receptor. These observations support our notion that active forms of natural ligands, such as 4-1BBL, to costimulatory molecules have the potential to serve as more powerful and safer alternatives to agonistic mAbs to the receptors as immunomodulatory components of vaccine formulations. Generation of additional costimulatory proteins having potent immunomodulatory functions in soluble form and testing their efficacy in various prophylactic and therapeutic vaccine settings will be critical to the development of costimulatory ligands as a new class of adjuvants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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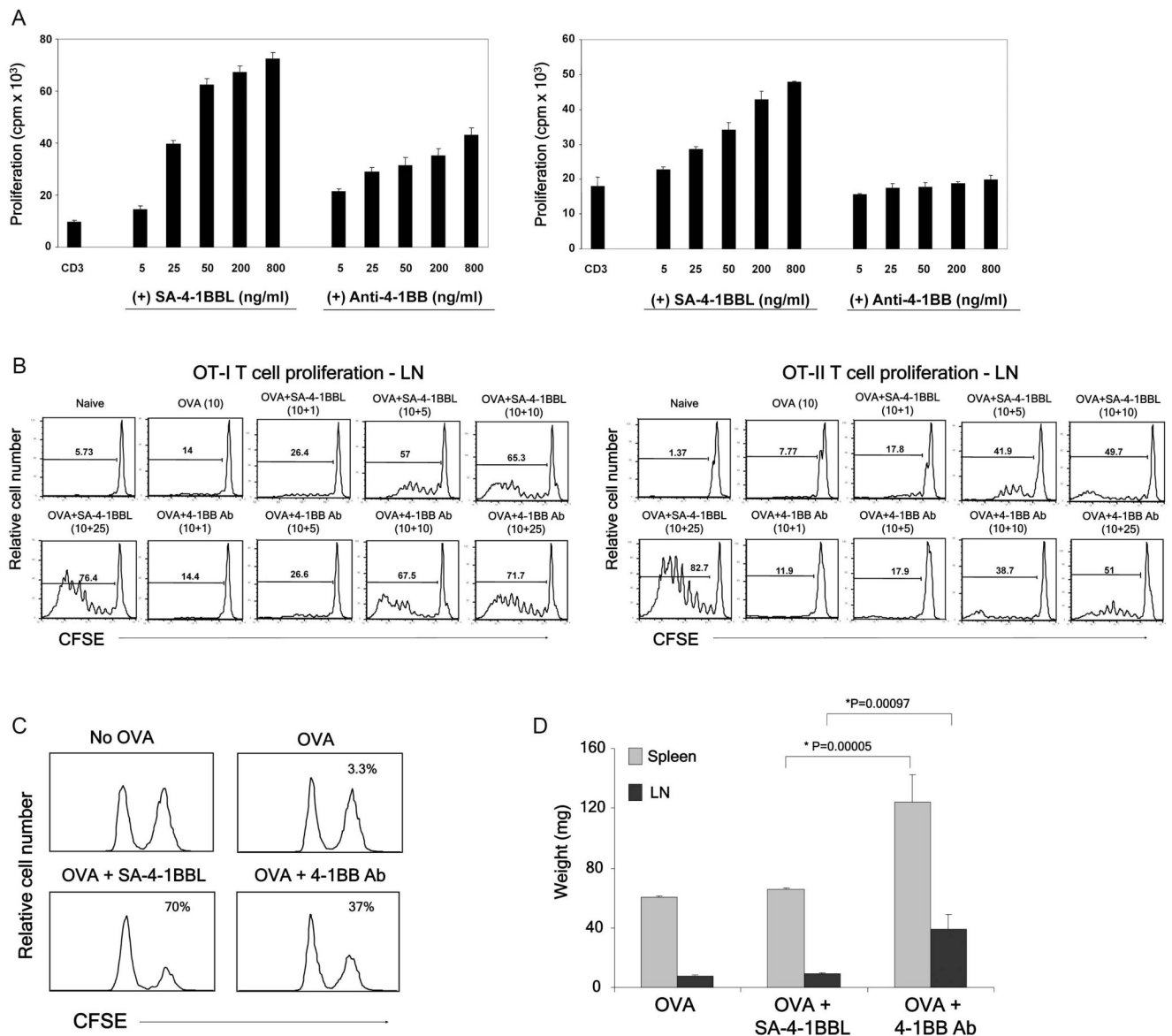
Fig 1.
SA-4-1BBL demonstrates better immune activity than an agonistic anti-4-1BB Ab without Ab-associated toxicity. (A) *In vitro* CD8^{+} T cell (left) and CD4^{+} T cell (right) proliferation. Flow sorted CD8^{+} and CD4^{+} T cells were stimulated for 3 days with a suboptimal dose of an agonistic anti-CD3 Ab in conjugation with various doses of SA-4-1BBL or 3H3 Ab in the presence of irradiated syngeneic splenocytes. Cells were pulsed with [^{3}H]-thymidine for the last 16 hrs of culture. (B) *In vivo* OT-I and OT-II T cell proliferation. One million flow sorted OT-I T cells and OT-II (CD45.2[+]) were labeled with CFSE and injected i.v. into naïve congenic C57BL/6.SJL (CD45.1[+]) mice. Mice were vaccinated i.v. with OVA (10 µg) in combination with various doses (µg) of SA-4-1BBL or 3H3 Ab as indicated. Proliferation was assessed using multiparameter flow cytometry 3 days after vaccination. (C) *In vivo* killing response. C57BL/6 mice were immunized i.v. with OVA (50 µg) in combination with 3H3 Ab (25 µg) or SA-41BBL (25 µg). Seven days post vaccination, mice received SIINFEKL-pulsed syngeneic splenocytes and peptide-specific killing was assessed 2 days later and expressed as percent

lysis for each histogram. (D) *In vivo* toxicity as assessed by the weight of spleen and lymph nodes harvested from mice vaccinated in panel C. *p* values determined by Student's *t* test. Data are representative of a minimum of three independent experiments for Panels A–C.
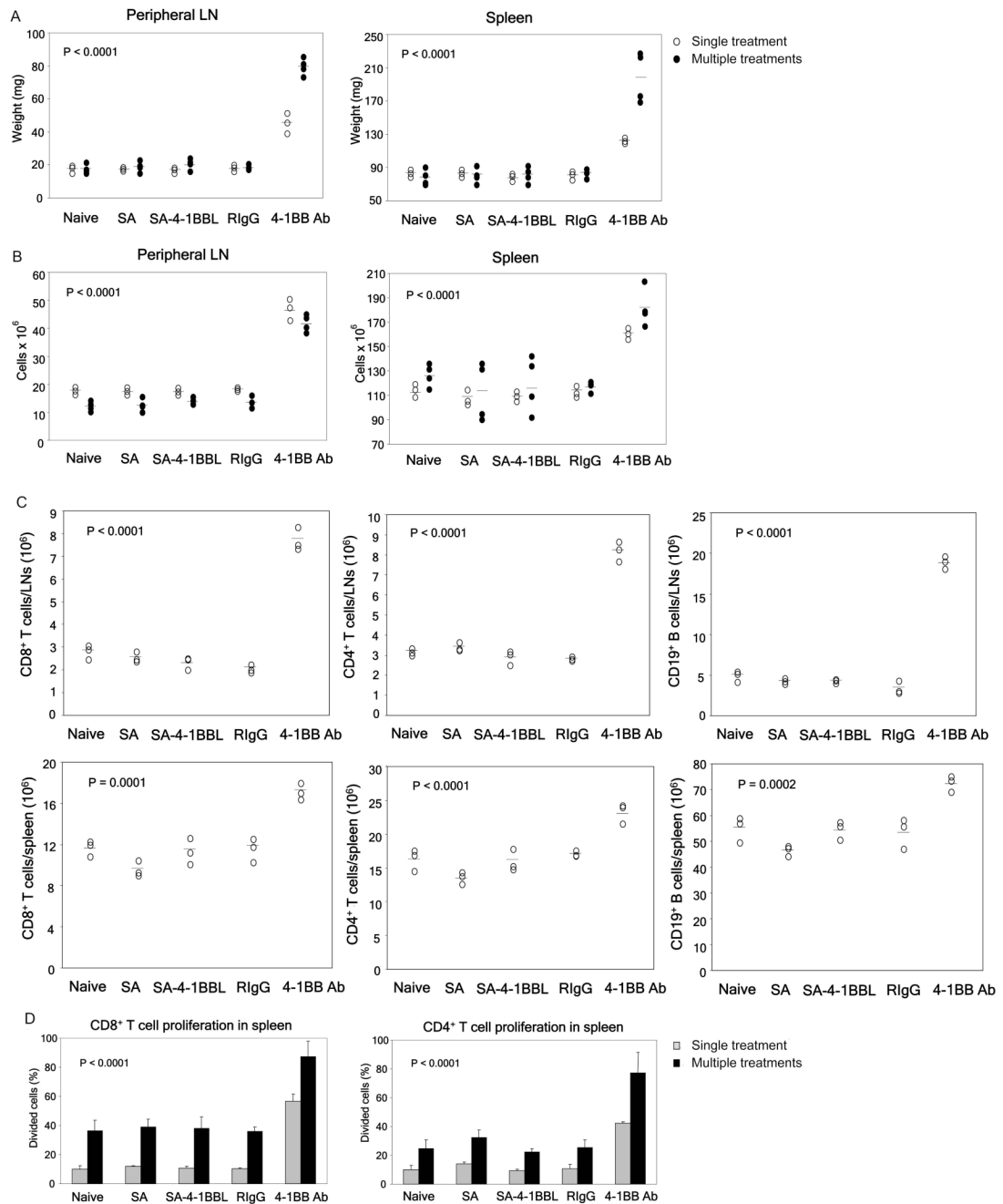
Fig 2.
SA-4-1BBL treatment lacks Ab associated toxicity. Naïve C57BL/6 mice were injected s.c. once (○) or once weekly over a 3-wk period (●) with 100 µg of SA-4-1BBL, equivalent doses of anti-4-1BB Ab, or other appropriate controls. Animals were euthanized one week after the final treatment and assessed for various indicators of toxicity, including peripheral LN and spleen weights (A) and total peripheral LN and spleen cell numbers (B). (C) Anti-4-1BB Ab demonstrates super-agonistic activity *in vivo*. *In vivo* proliferation of spleen CD8^{+} and CD4^{+} T cells was determined by adoptively transferring 2×10^6 CFSE-labeled CD45.1^{+} congenic splenocytes into naïve C56BL/6 (CD45.2^{+}) mice one day prior to the indicated treatments. Percentage of proliferating cells was determined by CFSE dilution using FACS analysis. *p*

values determined by ANOVA, anti-4-1BB *vs.* all other groups within same treatment schedule. Data were obtained from a minimum of 3 mice per group.
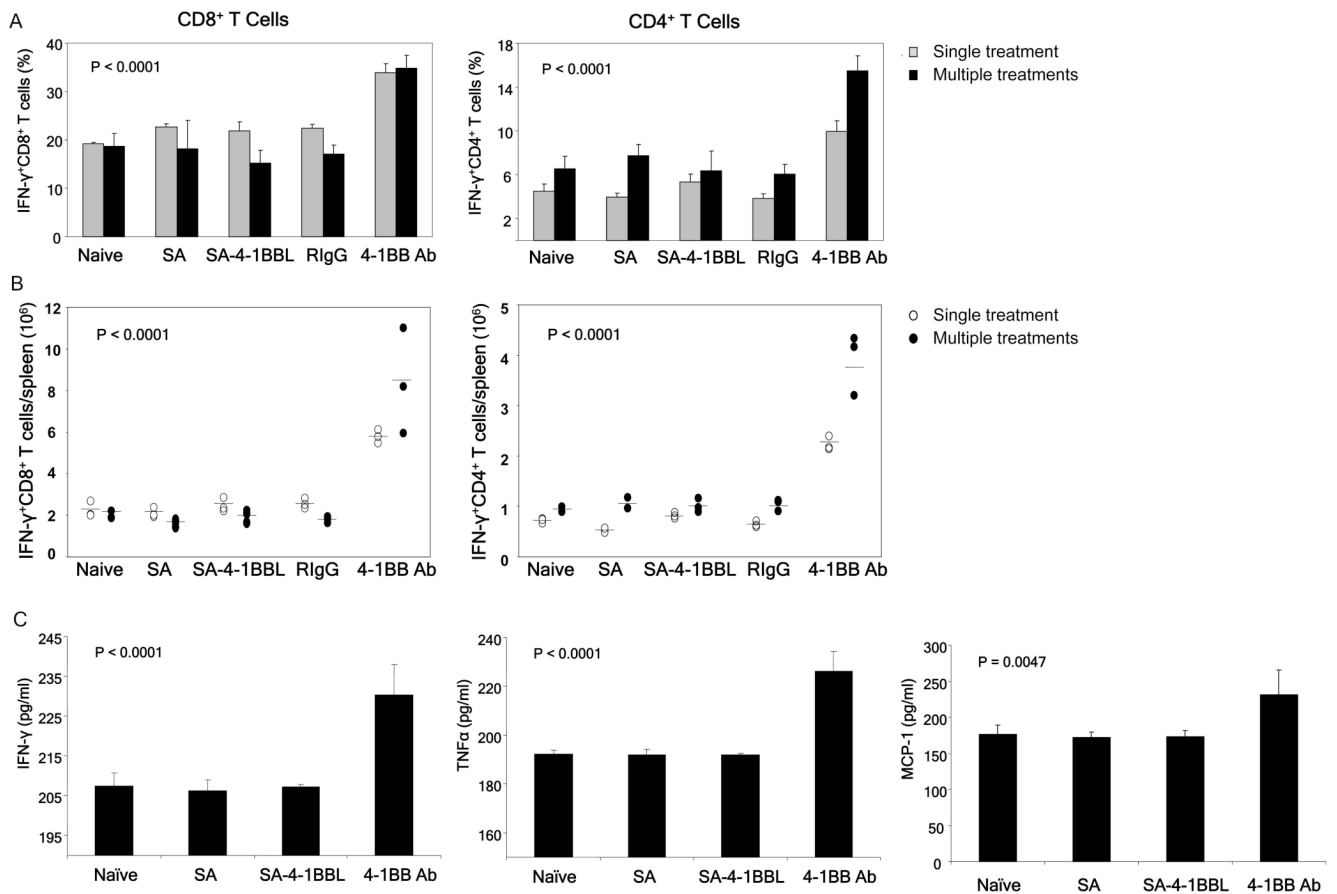
Fig 3.
Treatment with anti-4-1BB Ab, but not SA-4-1BBL, results in a drastic increase in the production of inflammatory cytokines. Lymphocytes and sera harvested from mice that underwent treatment as described in Figure 2 were analyzed for percentage (A) and absolute numbers (B) of splenic CD8+ and CD4+ T cells expressing IFN-γ. Spleen cells were stimulated with PMA and ionomycin for 6 hours and then surface stained with anti-CD8 and anti-CD4 Abs followed by intracellular staining for IFN-γ. (C) Cytokine levels in the serum of various treatment group, as determined using a CBA kit and flow cytometry. *p* values determined by ANOVA, anti-4-1BB *vs*. all other groups within same treatment schedule. Data were obtained from a minimum of 3 mice per group.
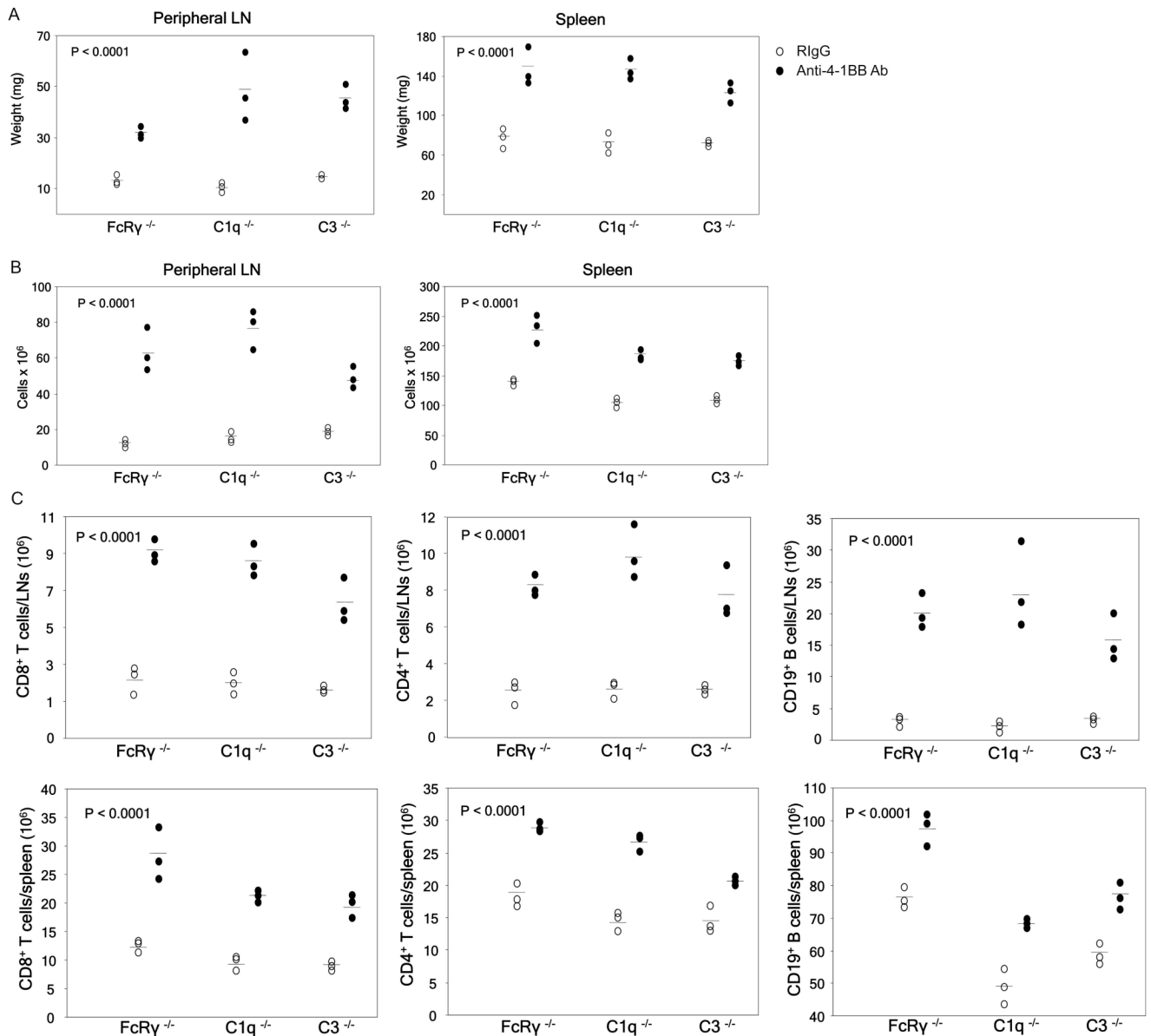
Fig 4.
Anti-4-1BB Ab associated toxicity is FcR and complement independent. Naïve C57BL/6
Fcer1g^{−/−}, C57BL/6 C3^{−/−}, and C57BL/6 C1q^{−/−} male mice were injected once s.c. with 100
µg of anti-4-1BB Ab or control rat IgG, sacrificed one week after treatment, and assessed for
various indicators of toxicity. Peripheral LN and spleen weights of treated mice (A), total
peripheral LN and spleen cell numbers (B), as well as total T cell and B cell numbers as
determined by FACS analysis (C). *p* values for anti-4-1BB *vs*. rat IgG for each type of KO
mice was determined by Student's *t* test. Data were obtained from a minimum of 3 mice per
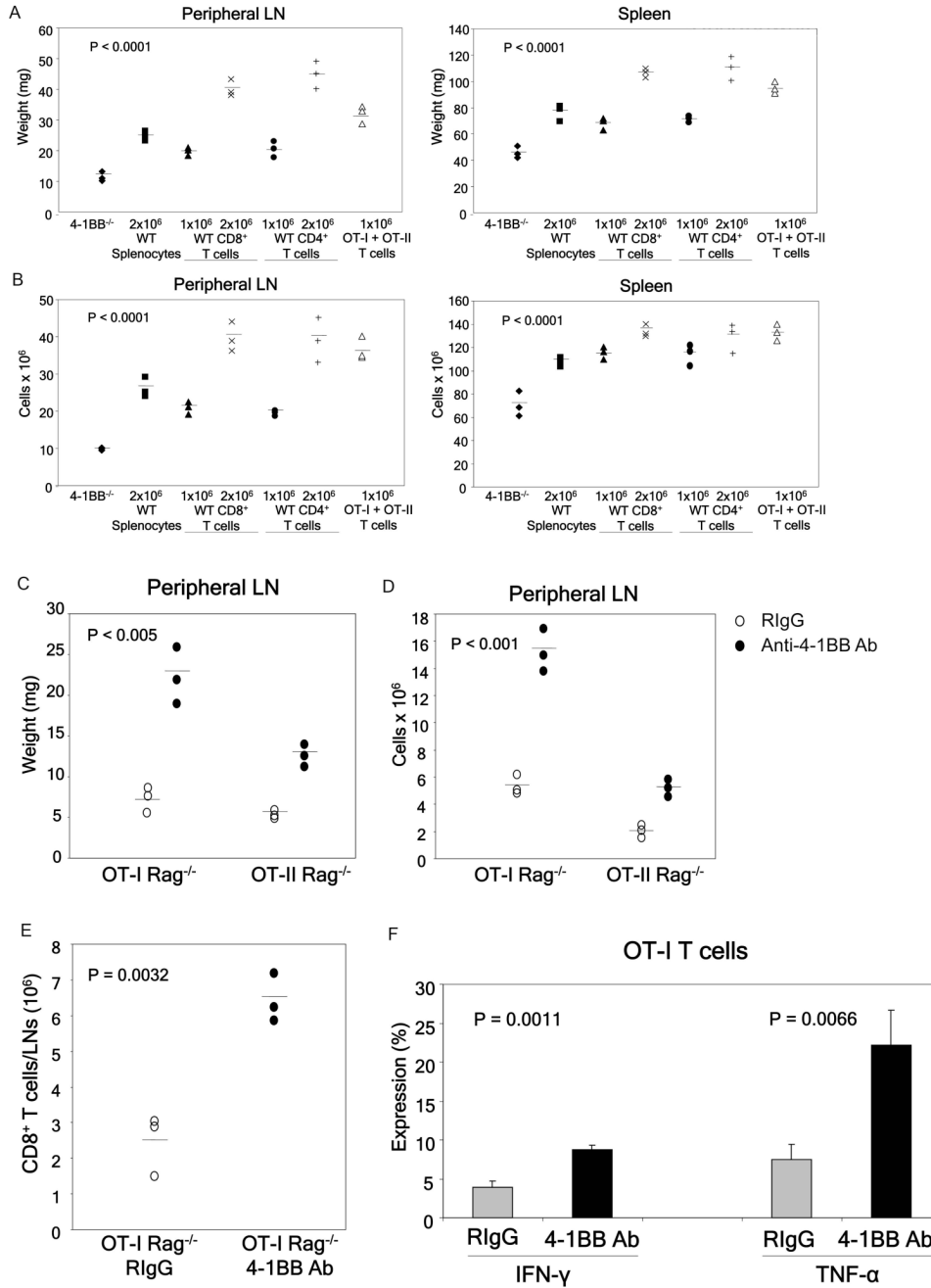group.

Fig 5.
T cells are the direct target of anti-4-1BB Ab mediated toxicity. C57BL/6 4-1BB$^{-/-}$ mice were adoptively transferred with the indicated numbers and types of lymphocytes and treated one day later using 100 µg of anti-4-1BB Ab. Mice were sacrificed one week after treatment, and assessed for various indicators of toxicity. All the cells, except splenocytes, were sorted by flow cytometry to ensure purity before adoptive transfer. Peripheral LN and spleen weights (A) and total peripheral LN and spleen cell numbers (B) of the treated mice. *p* values determined by ANOVA, 4-1BB$^{-/-}$ without adoptive transfer *vs*. all other groups. (C–F) Anti-4-1BB Ab directly activates monoclonal T cells resulting in toxicity. A single treatment of OT-I Rag$^{-/-}$ and OT-II Rag$^{-/-}$ mice with 100 µg of anti-4-1BB Ab results in enlarged peripheral lymph

nodes (C), total cell numbers (D), and CD8$^+$ T cell numbers (E) as determined by flow cytometry. (F) As compared to controls, more CD8$^+$ T cells from anti-4-1BB Ab treated OT-I Rag$^{-/-}$ mice produce IFN-γ and TNF-α as determined by intracellular cytokine staining. p values determined by Student's t test. Data were obtained from a minimum of 3 mice per group.

Table 1

Repeated injections with anti-4-1BB Ab, but not the SA-4-1BBL, results in significant alterations in absolute number of peripheral lymphocytes

Treatment	LN				Spleen			
	CD8+ T cells[a]	CD4+ T cells	CD19+ B cells	CD4+Foxp3+ T cells	CD8+ T cells	CD4+ T cells	CD19+ B cells	CD4+Foxp3+ T cells
None	1.89 ± 0.6	2.09 ± 0.7	2.45 ± 0.6	3.80 ± 0.8	10.39 ± 2.1	14.21 ± 1.4	56.99 ± 14.0	2.48 ± 0.5
SA	1.99 ± 0.3	2.25 ± 0.2	3.20 ± 0.5	3.67 ± 0.6	10.79 ± 2.6	15.89 ± 3.8	60.84 ± 13.8	2.53 ± 0.6
SA-4-1BBL	1.83 ± 0.7	2.21 ± 0.8	3.32 ± 1.2	3.38 ± 1.3	12.37 ± 1.9	17.38 ± 2.8	61.80 ± 9.4	2.75 ± 0.7
R-IgG	2.21 ± 0.5	2.66 ± 0.5	4.23 ± 0.6	4.01 ± 0.7	10.78 ± 0.5	17.36 ± 0.5	64.14 ± 7.4	2.45 ± 0.1
4-1BB Ab	$8.20 \pm 1.8^*$	$6.59 \pm 1.4^*$	$13.14 \pm 1.84^*$	$14.86 \pm 3.7^*$	$23.43 \pm 5.5^*$	$23.84 \pm 3.4^*$	$35.49 \pm 6.5^*$	$5.05 \pm 1.7^*$

[a] All numbers, except Treg cells in LN $\times 10^5$, are shown as $\times 10^6 \pm$ SD.

* $p < 0.05$, ANOVA, anti-4-1BB Ab vs. all other groups.