SA-4-1BBL and monophosphoryl lipid A constitute an efficacious combination adjuvant for cancer vaccines

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This work was funded in parts by grants from the NIH (R41 CA121665, R44 AI071618, and R43AI074176), KLCRP, W.M. Keck Foundation, and the Commonwealth of Kentucky Research Challenge Trust Fund to HS and ESY.

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Conflict-of-interest disclosure: HS and ESY serve as inventor for several patents on the SA-4-1BBL. The other authors disclosed no potential conflict of interest.

Running title: SA-4-1BBL/MPL as a potent adjuvant system for cancer vaccines

Keywords: Costimulation, SA-4-1BBL, 4-1BBL, CD137, MPL, Adjuvant, Cancer Vaccines
Abstract

Vaccines based on tumor-associated antigens (TAA) have limited therapeutic efficacy due to their weak immunogenic nature and the various immune evasion mechanisms active in advanced tumors. In an effort to overcome these limitations, we evaluated a combination of the T cell co-stimulatory molecule SA-4-1BBL with the TLR4 agonist monophosphoryl lipid A (MPL) as a novel vaccine adjuvant system. In the TC-1 mouse allograft model of HPV-induced cancer, a single administration of this combination adjuvant with HPV E7 protein caused tumor rejection in all tumor-bearing mice. On its own, SA-4-1BBL outperformed MPL in this setting. Against established tumors, two vaccinations were sufficient to elicit rejection in the majority of mice. In the metastatic model of Lewis lung carcinoma, vaccination of the TAA survivin with SA-4-1BBL/MPL yielded superior efficacy against pulmonary metastases. Therapeutic efficacy of SA-4-1BBL/MPL was achieved in the absence of detectable toxicity, correlating with enhanced DC activation, CD8+ T cell function and an increased intratumoral ratio of CD8+ T effector cells to CD4+FoxP3+ T regulatory cells. Unexpectedly, use of MPL on its own was associated with unfavorable intratumoral ratios of these T cell populations resulting in suboptimal efficacy. The efficacy of MPL monotherapy was restored by depletion of T regulatory cells, whereas eliminating CD8+ T cells abolished the efficacy of its combination with SA-4-1BBL. Mechanistic investigations showed that IFN-γ played a critical role in supporting the therapeutic effect of SA-4-1BBL/MPL. Taken together, our results offer a preclinical proof of concept for the use of a powerful new adjuvant system for TAA-based cancer vaccines.
Introduction

Therapeutic vaccines are preferred alternatives to conventional treatments for cancer primarily because of their safety profile, specificity and generation of long-term immunological memory critical for the control of recurrences, which are the main cause of death from cancer. Therapeutic vaccines based on tumor associated antigens (TAAs) are particularly attractive because of their ease of production, scale-up, storage, and administration to a broad patient population. The efficacy of such vaccines, however, is curtailed by the weak antigenic nature of self TAAs due to both central and peripheral tolerogenic mechanisms (1, 2). These limitations can potentially be overcome by developing vaccine formulations containing adjuvants that not only generate potent immune responses against TAAs with long-term immunological memory, but also overcome various immune evasion mechanisms. There remains an active interest in identifying adjuvants and adjuvant combinations, not only towards suitable therapy for cancers, but also towards developing a theoretical framework to improve cancer vaccine therapies.

Recent advances in our understanding of the immune system, mechanistic basis of immune activation, immune response, and establishment of long-term immunological memory, and key molecules involved in regulating such responses have provided an unparalleled opportunity to design adjuvants with known molecular actions and desired activities for the development of effective and safe therapeutic vaccines. Critical to the activation and maintenance of an immune response are the signals transduced by toll-like receptor (TLR) and costimulatory receptor pathways (3, 4). As such, agonistic ligands to receptors of these two pathways have significant potential as adjuvants for therapeutic vaccines. Consistent with this notion is the approval of TLR-4 agonist MPL, a nontoxic version of lipopolysaccharide, by FDA to be used as the adjuvant component of a preventive vaccine against HPV infection (5).
However, the efficacy of MPL as the adjuvant component of therapeutic vaccines against cancer remains to be realized.

We have recently proposed costimulatory ligands of TNF family as potential adjuvants of choice and particularly focused on 4-1BBL because of the critical role this molecule plays in the generation and maintenance of CD8$^+$ T cell responses (6, 7) and the importance of CD8$^+$ T cells in eradication of tumors (8, 9). Inasmuch as 4-1BBL is a cell surface membranous protein and has no function in soluble form, we fused the extracellular functional domain of this molecule to a modified form of core streptavidin (SA) to generate a chimeric molecule (SA-4-1BBL) that exists as tetramers and oligomers owing to the structural features of SA (10). SA-4-1BBL has potent immune activity in soluble form and targets T effector (Teff) cells for activation, acquisition of effector functions, and establishment of long-term memory (10-14). Most importantly, SA-4-1BBL also modulates regulatory immunity by reversing tumor induced clonal anergy, rendering Teff cells resistant to suppression by CD4$^+$CD25$^+$FoxP3$^+$ T regulatory (Treg) cells (13), and inhibiting the conversion of Teff cells into Treg cells through the production of IFN-$\gamma$ (15). These combined effects translate into significant therapeutic efficacy in various preclinical models (10-14), establishing SA-4-1BBL as an important new class of adjuvant for the development of cancer vaccines.

We herein tested whether a combination of SA-4-1BBL, as a new class of adjuvant under development, and MPL, as an FDA approved adjuvant, has therapeutic utility in preclinical cancer settings, and if the combination provides mechanistic insights that aid in further improvement of adjuvant formulations. We demonstrate that a combination of dual adjuvant SA-4-1BBL/MPL gives 100% therapeutic efficacy in murine cancer models without evidence of adverse effects due to acute toxicity or autoimmune activation. A single vaccination with SA-4-
1BBL/MPL and E7 TAA resulted in effective eradication of E7 expressing TC-1 tumor in all mice. This effect was extendable to the 3LL pulmonary lung carcinoma model where survivin (SVN) was used as a bona fide self-TAA. The efficacy of SA-4-1BBL/MPL is mainly dependent on CD8+ T cells/IFN-γ responses and associated with increased intratumoral CD8+ Teff/Treg cell ratio, which played a definitive role in vaccine therapeutic efficacy as confirmed by the depletion or blocking of these critical components. Taken together, these data demonstrate the utility of SA-4-1BBL/MPL as a novel adjuvant system for the development of therapeutic TAA-based subunit cancer vaccines with significant clinical potential. Given that MPL primarily targets APCs, such as DCs and macrophages, for the initiation of adaptive immunity (16) and 4-1BBL targets antigen-primed CD8+ T cells for activation, expansion, acquisition of effector function, survival, and long-term memory (17-19), and the critical role of CD8+ T cells for tumor eradication (8, 9), this study suggests that stimulation of both pathways is key to peak vaccine effectiveness.

Materials and Methods

Mice and cell lines

C57BL/6 and C57BL/6.SJL mice were bred in our barrier animal facility at the University of Louisville. All animals were cared for in accordance with institutional and NIH guidelines. TC-1 and 3LL cell lines were purchased from ATCC and not authenticated. Cell lines were regularly tested for mycoplasma. The anti-IFN-γ producing hybridoma XMG1.2 was kindly provided by Dr. A.T. Vella of University of Connecticut, Farmington, CT.

Antibodies and other reagents
All fluorochrome-conjugated antibodies against various immune cell markers and isotype controls were purchased from BD Bioscience, eBioscience, Invitrogen, or BioLegend. MPL was purchased from InvivoGen (San Diego, CA). The HPV16 RAHYNIVTF E7 peptide (E7\textsubscript{49-57}), SA-4-1BBL, E7 and mouse SVN proteins were reported previously (13).

**Tumor models, vaccination and cell depletion**

For TC-1 tumor therapy, mice were challenged s.c. with 1x10\textsuperscript{5} TC-1 cells and vaccinated s.c. on day 6 post-tumor challenge. For established tumor study, mice with ~9mm\textsuperscript{2} established tumors were vaccinated twice at 10 days interval. For the pulmonary tumor model, 2x10\textsuperscript{5} live 3LL cells were injected i.v. into the tail vein of mice. Mice were vaccinated s.c. once on day 6 post-tumor challenge and euthanized 27 days post-tumor challenge for analysis of lung tumor burden as described (10).

CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells were depleted using Abs against CD8 (clone 53.6.72) and CD4 (clone GK 1.5) at 500 µg/mice via i.p. once one day before vaccination while IFN-γ blockade was performed by injecting the anti-IFN-γ Ab (XMG1.2; 500µg/mouse) 6 hrs before tumor inoculation, followed by 3 more doses every 3 days post-tumor challenge.

**Cytotoxicity assay**

Splenocytes were cultured with 10 µg of E7\textsubscript{49-57} peptide/mL in complete MLR medium supplemented with 50 IU/mL of IL-2 for 5 days. Viable lymphocytes were harvested and used as effectors against TC-1 target cells in a JAM assay as published (14).

**Intracellular cytokine and confocal microscopy analyses**
Lymphocytes (1x10^6 cells/mL) were stimulated with either 10 µg/mL E749-57 peptide for 2 hrs followed by overnight incubation with GolgiPlug (1 µl/mL, BD PharMingen) or stimulated with PMA (5 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) for 2 hrs followed by an additional 4 hrs incubation with GolgiPlug. Cells were first stained with anti-CD44-APC and anti-CD8-APC-Cy7, fixed with 4% paraformaldehyde, and then stained with anti-IFN-γ-PE-Cy7, anti-IL-2-Percp-Cy5.5, anti-TNF-PE, or isotype controls as previously reported (10).

Intratumoral CD8^+ T cells and CD4^+Foxp3^+ Treg cells were analyzed using confocal microscopy as previously described (10).

Analysis of autoantibody to ssDNA

A ssDNA ELISA was performed to assess the presence of auto-Abs in treated mice as described (20) with modifications detailed in Supplementary Materials and Methods.

Acute toxicity analysis

Mice were vaccinated and serum was analyzed for aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine (CREA) levels 18 hrs post vaccination. Liver tissues were also collected from these mice, fixed in 3.7% formaldehyde, embedded in paraffin, sliced and stained with hematoxylin and eosin for pathological changes.

Statistics

Statistical analyses were performed using the Student's t test, one-way ANOVA-Tukey HSD test, Mann-Whitney U test, or log-rank test using the SPSS software. For each
test, \( P \) values of <0.05 and 0.001 were considered significant (*) and very significant (***) respectively.

**Results**

**SA-4-1BBL/MPL as the adjuvant component of E7 TAA-based vaccine has robust efficacy in eradicating established TC-1 tumors**

We recently demonstrated that a single vaccination with SA-4-1BBL and E7 protein was effective in eradicating E7 expressing TC-1 tumors in > 70% of mice (10). Although impressive, we sought to test whether the therapeutic efficacy of this vaccine can further be improved by modifying the formulation to include MPL as the second adjuvant with primary effect on the innate immunity (16, 21). A single vaccination of SA-4-1BBL/MPL with E7 protein resulted in complete eradication of TC-1 tumors in all mice for an observation period of 90 days (Fig. 1A). In contrast, monotherapy with SA-4-1BBL and MPL resulted in eradication of tumor in only 80% and 50% of mice, respectively. However, mice that expired from tumor burden in monotherapy groups had slow kinetics of tumor progression as compared with both PBS and E7 protein control groups where all mice expired within 50 days (Fig. S1A).

The therapeutic efficacy of the combined adjuvants was next tested in a more stringent and clinically relevant setting. Mice with ~9mm\(^2\) established tumors treated with a prime-boost vaccination 10 day apart. Combined adjuvant treatment resulted in complete eradication of established tumors in 75% of mice, whereas SA-4-1BBL and MPL monotherapies resulted in ~40% and 25% tumor eradication, respectively (Fig. 1B). Apart from inducing moderate therapeutic benefit, SA-4-1BBL monotherapy also slows the tumor growth compared to MPL monotherapy (Fig. S1B). Taken together, these data demonstrate that SA-4-1BBL/MPL is
effective in eradicating the established TC-1 tumors with better therapeutic efficacy than the individual agents, and that SA-4-1BBL has better efficacy than MPL.

The therapeutic efficacy of SA-4-1BBL/MPL is associated with a robust effect of SA-4-1BBL and MPL on the generation of peripheral CD8$^+$ T cell responses

CD8$^+$ T cell effector and memory responses are critical to the elimination of primary tumor and control of recurrences, respectively, in various tumor settings, including the TC-1 model (8, 9, 11, 13). We, therefore, assessed the CD8$^+$ T cell effector and long-term memory responses elicited by SA-4-1BBL/MPL. Mice that had eradicated the tumor in response to various vaccine formulations (from Fig. 1A) were boosted with the same formulations and then euthanized one week later to test the intracellular cytokine response of CD8$^+$ T cells to the dominant E7$_{49-57}$ epitope (8). Consistent with the therapeutic efficacy, SA-4-1BBL/MPL generated a better antigen-specific IFN-$\gamma$, IL-2, and TNF-$\alpha$ triple cytokine response than SA-4-1BBL and MPL monotherapies as assessed by percentage (Fig. 2A-C) and absolute number (Fig.S2) of CD8$^+$ T cells. Additionally, SA-4-1BBL/MPL induced higher E7 TAA-specific CD8$^+$ T cell killing responses as compared with monotherapies at 20:1 effector/target ratio (Fig. 2D). Similar to therapeutic responses, SA-4-1BBL monotherapy generated better IFN-$\gamma$ and E7 TAA-specific CD8$^+$ T cell killing responses than MPL monotherapy (Fig. 2A, 2D). Importantly, SA-4-1BBL/MPL also generated the most effective CD8$^+$ T cell memory recall responses as compared to SA-4-1BBL and MPL monotherapies (Fig. S3). Collectively, these data demonstrate the robust effect of SA-4-1BBL and MPL adjuvants in the generation of potent CD8$^+$ T cell effector and memory responses that correlate with impressive therapeutic efficacy against TC-1 tumor.
Vaccination with the SA-4-1BBL/MPL results in a favorable intratumoral CD8⁺ Teff/CD4⁺Foxp3⁺ Treg cell ratio

Elevated levels of intratumoral CD4⁺Foxp3⁺ Treg cells along with a decline in CD8⁺ Teff cells is associated with a clinically unfavorable prognosis of cancer patients (22, 23) and depletion of Treg cells results in better immune efficacy of therapeutic vaccines (24, 25). Therefore, we evaluated the effect of SA-4-1BBL/MPL on the status of intratumoral Treg and Teff cells. Mice bearing ~9mm² TC-1 tumor were vaccinated with various vaccine formulations. One week post-vaccination, tumors were harvested and analyzed for the presence of intratumoral CD8⁺ Teff cells and CD4⁺FoxP3⁺ Treg cells using confocal microscopy. The frequency of intratumoral Treg cells was significantly reduced following the vaccination of SA-4-1BBL and SA-4-1BBL/MPL when compared with PBS or E7 alone controls (Fig. 3A right panel, B). Surprisingly, MPL monotherapy did not have detectable effect on the number of intratumoral Treg cells as compared with PBS control, and indeed performed worse than E7 protein alone that appreciably, but not statistically significant, reduced the intratumoral number of Treg cells.

We next tested whether decrease in the number of Treg cells caused by SA-4-1BBL/MPL or SA-4-1BBL as monotherapy inversely correlates with the number of intratumoral CD8⁺ T cells, a hallmark of successful immunotherapeutic approach against cancer (26). Vaccination with SA-4-1BBL/MPL had the most pronounced effect on the number of intratumoral CD8⁺ T cell infiltration followed by SA-4-1BBL, whereas MPL had a moderate effect that was similar to the E7 protein alone (Fig. 3A left panel, C). This increased intratumoral CD8⁺ T cells by SA-4-1BBL/MPL resulted into the most favorable intratumoral Teff/Treg cell ratio followed by SA-4-1BBL as monotherapy (Fig. 3D). In marked contrast, MPL monotherapy had no effect on the intratumoral Teff/Treg cell ratio as compared with both PBS and E7 protein controls. Taken
together, these findings demonstrate that SA-4-1BBL and MPL work together to increase the intratumoral Teff/Treg cell ratio that correlates with the potent efficacy in eliminating established tumors.

**CD8⁺ T cells and IFN-γ are critical to the therapeutic efficacy of SA-4-1BBL/MPL while Treg cells are detrimental to the efficacy of MPL monotherapy**

To test if a high CD8⁺ Teff/Treg cell ratio can serve as a predictor of SA-4-1BBL/MPL therapeutic efficacy, we next depleted CD8⁺ Teff and Treg cells one day before vaccination using Abs against CD8 and CD4 molecules, respectively. As shown in Fig. 4A, depletion of CD8⁺ T cells completely abrogated the therapeutic efficacy of SA-4-1BBL/MPL adjuvant system with a measurable (~15%) but not statistically significant negative effect on the efficacy of MPL, while depletion of CD4⁺ T cells, including Treg cells, improved the therapeutic efficacy of MPL from 50 to 100%. Blockade of IFN-γ using a neutralizing Ab significantly abrogated the therapeutic efficacy of SA-4-1BBL/MPL (20% tumor eradication vs 100% without blocking; Fig. 4B). Taken together, these data establish a critical role for CD8⁺ T cells and IFN-γ driven immune responses in therapeutic efficacy achieved by the combined adjuvant system and point to the importance of Teff/Treg cell ratio as a predictor of vaccine success/failure in this model.

**SA-4-1BBL/MPL controls 3LL pulmonary metastasis progression**

The robust efficacy of SA-4-1BBL/MPL with xenogeneic E7 TAA in complete rejection of TC-1 tumors in all mice led us to test if this efficacy is translatable to SVN, a weak and potentially tolerant self-TAA, using the 3LL pulmonary metastasis model. Mice were
challenged i.v. with a lethal dose of 3LL cells followed by s.c. vaccination on day 6 with various formulations containing SVN recombinant protein and SA-4-1BBL and/or MPL as adjuvants. SA-4-1BBL/MPL had the most therapeutic efficacy over single adjuvants in controlling tumor growth as demonstrated by both lung weight and presence of tumor nodules (Fig. 5A). Similar to the TC-1 model, SA-4-1BBL demonstrated better efficacy in controlling tumor growth than MPL, which also showed moderate yet statistically significant effect in controlling tumor growth over PBS and SVN alone controls. The therapeutic efficacy of SA-4-1BBL/MPL and SA-4-1BBL, but not MPL, as monotherapy correlated with significantly higher number of CD8+ T cells expressing IFN-γ as compared with PBS and SVN alone controls (Fig. 5B).

Although lungs of SA-4-1BBL/MPL vaccinated mice had similar weights as compared with lungs of naïve mice, some of the lungs had microscopically detectable tumor nodules. We therefore, tested the efficacy of a booster injection 7 days after the first vaccination. Additional SA-4-1BBL/MPL booster resulted in complete rejection of lung tumor in all mice (Fig. S4). Booster vaccination with SA-4-1BBL and MPL monotherapies also improved the rejection of tumor burden (Fig. S4). Collectively, these findings further confirm the utility of SA-4-1BBL/MPL as a powerful adjuvant system to elicit potent immune responses to a self-TAA that translates into effective immunotherapy in a stringent pulmonary preclinical metastasis model.

**Therapeutic efficacy of the SA-4-1BBL/MPL is achieved in the absence of autoimmunity and detectable clinical toxicity**

Autoimmunity is a potential setback to effective self-TAA-based therapeutic vaccine formulations using potent adjuvants to induce immune responses to such antigens (27). Given the potent therapeutic activity of the adjuvant system used in this study, we tested serum from
mice with successful immunotherapy for both the TC-1 (Fig. 6A) and 3LL (Fig. 6B) models for
the presence of Abs against ssDNA as a sign of systemic autoimmunity. There was lack of
significant amount of auto-Abs to ssDNA in all the groups tested compared to auto-Abs from
mice with full blown lupus.

To further evaluate the toxicity profile, we examined the effect of SA-4-1BBL/MPL on
total number of various cell populations (T cells, B cells, NK cells, NK T cells, DCs and
macrophages) in both spleen and dLNs and vaccine-induced organ damage by measuring serum
levels of ALT and AST as a means of assessing liver while BUN and creatinine (CREA) as renal
function. As shown in Fig. 6C, there was no significant difference in these enzymes levels in all
the vaccinated mice compared to naïve mice. Pathological analysis of liver tissue from SA-4-
1BBL/MPL vaccinated mice (Fig. 6D) or enumeration of various cell populations harvested from
the draining lymph nodes or spleen (Table S1) did not show any significant changes as compared
to naïve mice. Taken together, these data demonstrate that the use of adjuvant system is not
associated with detectable acute toxicity or chronic autoimmunity in mice.

Discussion

In the present study, we tested if the costimulatory ligand SA-4-1BBL and TLR-4 agonist
MPL with distinct mechanisms of action can serve as a novel adjuvant system for the
development of therapeutic TAA-based subunit cancer vaccines. SA-4-1BBL worked in concert
with MPL for the generation of robust therapeutic efficacy against established large TC-1 tumor.
The therapeutic efficacy of SA-4-1BBL/MPL was primarily dependent on CD8⁺ T cells and
IFN-γ and associated with a favorable intratumoral CD8⁺ Teff/CD4⁺Foxp3⁺ Treg cell ratio.
Importantly, this effect was not limited to the xenogeneic E7 TAA and was equally effective with SVN as bona fide self-TAA in controlling 3LL pulmonary metastasis progression.

The robust therapeutic efficacy of the combined adjuvants could be due to their distinct mechanisms of action and targeting different immune cells for activation and acquisition of effector function. MPL primarily targets innate immunity by interacting with the constitutively expressed TLR-4 on DCs and macrophages, leading to the production of various proinflammatory cytokines and upregulation of various costimulatory and MHC molecules that altogether regulate adaptive immune responses (16). SA-4-1BBL, on the other hand, interacts with the inducibly expressed 4-1BB receptor on both CD4$^+$ and CD8$^+$ T cells, leading to their survival, expansion, acquisition of effector function, and long-term immune memory (17-19). Importantly, 4-1BB signaling preferentially targets CD8$^+$ T cells critical for anti-tumor efficacy (8, 9, 11, 13). Therefore, MPL in SA-4-1BBL/MPL combination is envisioned to enhance SA-4-1BBL for the activation of effector CD8$^+$ T cells through the activation of DCs and antigen cross-presentation (28), resulting in the upregulation of 4-1BB receptor on the surface of CD8$^+$ T cells that in turn become the direct target of SA-4-1BBL. The combined adjuvant may also work in concert at the level of antigen presenting cells, such as monocytes and DCs. It has been shown that 4-1BB/4-1BBL signaling is critical for the activation and survival of dendritic cells (29) and the conversion of monocytes into dendritic cells (30). Interesting in this context, it was shown that TLR signaling induces 4-1BBL expression on the surface of macrophages and the physical interaction of 4-1BBL with TLR-4 is necessary for sustained TNF production (31, 32).

We did not observe synergy between MPL and SA-4-1BBL on bone marrow derived DCs for the induction of costimulatory molecules or elaboration of various proinflammatory cytokines (Table S2). However, SA-4-1BBL may augment the effect of MPL on DCs in vivo by improving their
antigen uptake, cross-presentation, and survival. This notion is supported by observations that a subpopulation of DCs constitutively express 4-1BB receptor (33, 34), and vaccination with SA-4-1BBL enhances their antigen uptake and cross-presentation (10, 13). Furthermore, it has been demonstrated that monocyte/macrophage proliferation and survival was only achieved by 4-1BB signaling, but not LPS as TLR-4 agonist (35).

One of the main outcomes of using SA-4-1BBL and MPL adjuvants together was the generation of potent CD8$^+$ T cell effector response that translated into effective therapy in both TC-1 cervical and 3LL pulmonary carcinoma tumor models. Depletion of CD8$^+$ T cells totally negated the therapeutic efficacy of the SA-4-1BBL/MPL adjuvant system in the TC-1 model. The critical role of SA-4-1BBL/MPL generated CD8$^+$ T cells in anti-tumor efficacy is perhaps due to its ability to produce polyfunctional effector cytokines, such as IFN-γ, IL-2 and TNF-α, found in this study. It is well documented that polyfunctional effector T cells that simultaneously produce multiple effector cytokines are more effective in killing tumors than cells that secret single cytokine (36). The blockade of IFNγ almost completely negated the therapeutic efficacy of the combined adjuvant against the TC-1 model. This observation taken together with significantly increased numbers of IFN-γ$^{+}$IL-2$^{+}$TNF-α$^{+}$CD8$^+$ T cells in mice receiving the most effective vaccine formulation (SA-4-1BBL/MPL) are suggestive of the importance of this polyfunctional T cell population in the vaccine therapeutic efficacy. However, further studies will be needed to provide direct evidence for this contention.

The role of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells in mediating immune suppression and as an important barrier for the vaccines efficacy has been well documented (2, 24, 37-39). Therefore, vaccine that reverses the Treg-mediated immune suppression should have desired anti-tumor efficacy. Consistent with this notion are studies demonstrating that the physical depletion of
Treg cells or modulation of their regulatory function have protective and therapeutic effects against various tumors in preclinical models (39-43). A recent study using mice transgenically expressing the diphtheria toxin receptor only in Treg cells demonstrated that specific and conditional depletion of these cells protected mice from carcinogenesis induced spontaneous tumors via innate immunity and eradicated established tumor via CD8+ T cell- and IFN-γ-dependent responses (42). In clinic, Treg cells were shown to accumulate in various progressing cancers in patients and a high intratumoral Teff/Treg cells ratio is considered the hallmark of a favorable prognosis (22-24). Important in this context, we found a robust increase in the ratio of intratumoral CD8+ Teff/Treg cells in response to vaccination with the SA-4-1BBL/MPL, which was correlated with therapeutic efficacy in the cancer model. Vaccination with SA-4-1BBL as monotherapy also significantly improved the intratumoral CD8+ Teff/Treg cell ratio, which is consistent with our recently published data (10). Surprisingly, MPL as monotherapy was not only inefficient in significantly increasing the frequency of intratumoral CD8+ T cell infiltration, but also failed to decrease the intratumoral number of Treg cells, resulting in an unfavorable CD8+ Teff/Treg cell ratio. The Treg cells played a detrimental role in the efficacy of MPL monotherapy since their depletion resulted in eradication of all tumors. Although, primary target of TLR-4 agonists are cells of innate immunity, their role on Teff and Treg cells is also critical due to the expression of TLR-4 on these cells (44, 45). TLR-4 signaling plays an inhibitory role on CD4+ Teff cells in an experimental colitis model (45), while promotes the survival, expansion, and improved regulatory function of Treg cells (44), which may account for the unfavorable intratumoral CD8+ Teff/Treg cell ratio found in our study in the MPL monotherapy group. This finding, to our knowledge first to be reported, that MPL efficacy is compromised by
Treg cells is significant and provides better understanding of the immunobiology of this FDA approved adjuvant for the development of therapeutic cancer vaccines.

Although the exact mechanistic basis of the combined effect of SA-4-1BBL and MPL on the intratumoral CD8^+ T/Treg cell ratio observed in our model is unknown, it is possible that SA-4-1BBL may i) preferentially induce apoptosis in Treg cells as reported for the agonists of OX-40 pathway (46), another close member of TNFR costimulatory family, and/or ii) block the tumor-mediated conversion of Teff cells into induced Treg cells, while iii) both agents increasing the intratumoral frequency of CD8^+ Teff cells, thereby favorably influencing the CD8^+ Teff/Treg cell ratio. This notion is supported by our recent data demonstrating that SA-4-1BBL blocks antigen- and TGF-β-induced conversion of Teff cells into induced Treg cells through IFN-γ (15). The increased expression of IFN-γ in response to SA-4-1BBL/MPL in the present study may further provide an explanation for this observation. Blockade of IFN-γ almost completely negated the therapeutic efficacy achieved by the adjuvant system as tested in the TC-1 model. Interestingly, although we observed enhanced frequency of CD8^+ T cells expressing IFN-γ in the periphery of mice treated with MPL monotherapy, this effect did not result in increased intratumoral CD8^+ T cells. Furthermore, the depletion of CD8^+ T cells in the MPL monotherapy group did not significantly negated vaccine efficacy, suggesting their inability to traffic into the tumor. In contrast, mice treated with the SA-4-1BBL/MPL had higher numbers of peripheral and intratumoral CD8^+ Teff cells, indicating that both adjuvants in combination may affect trafficking/entry of CD8^+ Teff into the tumor resulting in survival benefit. Consistent with the notion, it has been shown that under hypoxic conditions tumor stroma expresses 4-1BB and signaling through this receptor generates various cytokines and chemokines that facilitates lymphocyte trafficking into the tumor (47). Although not investigated, we think that the
therapeutic efficacy of combined adjuvant system relies on similar immunological mechanisms in the 3LL and TC-1 models given the demonstrated role of CD8$^+$ Teff cells as immune effectors and Treg cells as immune evaders in these tumor models (14, 48-50).

Importantly, the therapeutic efficacy of SA-4-1BBL/MPL adjuvant system was achieved in the absence of chronic autoimmunity and detectable acute toxicity as assessed by various indicators of toxicity, including vaccine-induced organ damage and significant alteration in the number of various cell populations in the dLNs and spleen. The lack of acute toxicity is consistent with our previously published studies demonstrating that treatment of mice with 4-fold higher SA-4-1BBL over the therapeutic dose used in this study did not result in detectable toxicity as assessed by systemic cytokine response, non-specific lymphoproliferation, altered lymphocyte trafficking, generalized lymphomegaly and splenomegaly, and hepatitis, all of which were observed with similar doses of an agonistic Ab to 4-1BB receptor (12). The safety of MPL has already been demonstrated both in preclinical and clinical settings (5, 16, 21). Nevertheless, in view of the widespread concern that autoimmune responses may results from the use of potent adjuvants, the lack of any such pathology despite a highly effective immune response is positive.

In conclusion, the studies presented in this communication demonstrate the robust efficacy of the SA-4-1BBL/MPL in inducing potent CD8$^+$ Teff primary and long-term memory responses against TAAs and a favorable intratumoral CD8$^+$ Teff/Treg cell ratio that translate into potent therapeutic efficacy in two different tumor models. The better immune and therapeutic efficacy of SA-4-1BBL/MPL over MPL as monotherapy along with MPL being a clinically approved adjuvant (5) emphasizes the importance of further developing this adjuvant system and
assessing its efficacy as component of subunit therapeutic vaccines against cancer and chronic infections.

Acknowledgments

We thank Orlando Grimany-Nuño and Vahap Ulker for their excellent technical help with the production and characterization of recombinant proteins.
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infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome.


Figure Legends

**Figure 1.** SA-4-1BBL/MPL adjuvant system eradicates established TC-1 tumors. (A), C57BL/6 mice were challenged s.c. with \(1 \times 10^5\) TC-1 cells and left unvaccinated (PBS) or vaccinated once s.c. on day 6 post-tumor challenge with E7 (50 μg) alone or mixed with SA-4-1BBL (25 μg), MPL (25 μg), or the combination of both agents (25 μg/agent). (B), C57BL/6 mice with ~9mm² established tumors were vaccinated twice at 10 days interval. The log-rank test and Kaplan-Meier method were used for analyses. ***P < 0.001 compared to all the other groups, except SA-4-1BBL. *P < 0.05 SA-4-1BBL vs. MPL group. Data are pooled from two independent experiments.

**Figure 2.** Vaccination with the SA-4-1BBL/MPL adjuvant system induces strong anti-tumor CD8⁺ T cell effector responses that correlate with vaccine efficacy. Long-term (> 90 days) surviving mice were boosted with the same vaccine formulations used for primary immunization as indicated. Lymph node cells were harvested 7 days later and assessed for E7_{49-57} peptide-specific CD8⁺ T cells expressing intracellular IFN-γ mono (A), IFN-γTNF-α double (B), and IFN-γTNF-αIL-2 triple (C) cytokines. Lymph node cells from naïve mice stimulated with E7 peptide were used as background control with minimal to undetectable cytokine response (0-1% cells). Data is expressed as percentage of total CD8⁺ T cells (D), Splenocytes from mice from above groups were stimulated with E7_{49-57} peptide and IL-2 for 5 days and used as effectors against TC-1 tumor targets. 3LL tumor cells were used as irrelevant targets. Data shown are the mean ± SEM of 3-4 mice per group and representative of two independent experiments. * P < 0.05, *** P < 0.001, ns > 0.05.
**Figure 3.** Vaccination with the SA-4-1BBL/MPL adjuvant system results in an increase in the intratumoral Teff/Treg cells ratio. Mice bearing TC-1 tumor (~9mm²) were vaccinated s.c. with E7 protein (50 µg) alone or with SA-4-1BBL (25 µg), MPL (25 µg), or a combination of both agents (25 µg/agent). One week post-vaccination, tumors were harvested and stained for intratumoral CD8⁺ T cells and CD4⁺Foxp3⁺ Treg cells followed by analysis using confocal microscopy. (A), Confocal pictures of tumor sections showing CD4⁺Foxp3⁺ Treg cells (right panel) stained with anti-CD4 Ab (red), anti-Foxp3 Ab (green), and Hoechst (blue), and CD8⁺ T cells (left panel) stained with anti-CD8 Ab (red) and Hoechst (blue). Quantitative analysis of intratumoral CD4⁺Foxp3⁺ Treg cells (B), CD8⁺ T cells (C), and CD8⁺ Teff/Treg cell ratio (D). Four areas per tissue section and three tissue sections per tumor per animal were analyzed and compiled for represented quantitative data. The data represents 4 animals per group (each dot represents single animal). * P< 0.05, *** P< 0.001, ns> 0.05.

**Figure 4.** CD8⁺ T cells and IFN-γ play critical roles in the therapeutic efficacy of SA-4-1BBL/MPL adjuvant system while Treg cells compromise the efficacy of MPL monotherapy. (A), CD8⁺ T cells and CD4⁺ T cells, including Treg cells, were depleted using Abs against CD8 and CD4 molecules, respectively, one day before indicated vaccination using the TC-1 established tumor model. (B), IFN-γ blockade was performed by i.p. injection of anti-IFN-γ Ab 6 hrs before tumor challenge.

**Figure 5.** Vaccination with the SA-4-1BBL/MPL adjuvant system generates potent therapeutic response in the 3LL lung metastasis model. Mice (n = 4-5/group) were challenged with 2x10⁵ 3LL cells by i.v. tail injection and vaccinated once s.c. on day 6 post-tumor challenge with SVN.
(50 µg) alone or with SA-4-1BBL (25 µg), MPL (25 µg), or a combination of both agents (25 µg/agent). (A), Lungs were harvested 27 days post tumor challenge and assessed for tumor growth by weight and macroscopic presence of tumor nodules. (B), Intracellular IFN-γ response of CD8⁺ T cells was assessed after PMA and ionomycin stimulation of lymphocytes harvested from mice in (A). * P< 0.05, *** P< 0.001, ns> 0.05.

**Figure 6.** Vaccination with the SA-4-1BBL/MPL adjuvant system does not promote chronic autoimmunity or acute toxicity. Sera were harvested from mice from Fig. 1(A) and Fig. 5(A) at the experimental endpoints and tested for the presence of autoantibodies against ssDNA in TC-1 (A) and 3LL (B) tumor models. Serum pooled from a minimum of 3 naïve and 3 lupus mice were used as negative and positive controls, respectively. Acute toxicity was assessed in naïve C57BL/6 mice treated once with SA-4-1BBL/MPL adjuvant system and euthanized after 18 hrs for (C), vaccine-induced organ damage by measuring serum levels of ALT and AST for liver; BUN and CREA for renal function. (D), H&E staining of liver tissues, showing lack of pathology.
A

- PBS (n=10)
- E7 (n=8)
- E7+MPL (n=8)
- E7+SA-4-1BBL (n=10)
- E7+SA-4-1BBL/MPL (n=12)

B

- PBS (n=10)
- E7+SA-4-1BBL(n=8)
- E7+MPL(n=8)
- E7+SA-4-1BBL/MPL(n=12)

Survival (%) vs Days post tumor challenge

Srivastava et al., Fig. 1
Srivastava et al., Fig. 2
Figure 4

A

- PBS (n=7)
- E7+SA-4-1BBL/MPL+anti-CD8 (n=8)
- E7+MPL+anti-CD8 (n=10)
- E7+MPL+anti-CD4 (n=5)

B

- E7+SA-4-1BBL/MPL (n=5)
- PBS (n=11)
- E7+SA-4-1BBL/MPL+IFN-γ Ab (n=5)

Survival (%) vs Days post tumor challenge
One vaccination therapy

A

Lung weight (mg)

PBS  SVN  SVN+MPL  SVN+SA-4-1BBL  SVN+SA-4-1BBL/MPL  Naive

B

CD8^IFN-γ^+ cells (%)

PBS  SVN  SVN+MPL  SVN+SA-4-1BBL  SVN+SA-4-1BBL/MPL

Srivastava et al., Fig.5
Srivastava et al., Fig.6
SA-4-1BBL and monophosphoryl lipid A constitute an efficacious combination adjuvant for cancer vaccines

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Cancer Res Published OnlineFirst September 24, 2014.