Microenvironment and Immunology

SA-4-1BBL and Monophosphoryl Lipid A Constitute an Efficacious Combination Adjuvant for Cancer Vaccines

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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 costimulatory 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 human papilloma virus (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 dendritic cell 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 IFNγ 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. Cancer Res; 74(22): 1-11. ©2014 AACR.

Introduction

Therapeutic vaccines are preferred alternatives to conventional treatments for cancer primarily because of their safety profile, specificity, and generation of long-term immunologic memory critical for the control of recurrences, which are the main cause of death from cancer. Therapeutic vaccines based on tumor-associated antigens (TAA) 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 immunologic 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 immunologic 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 human papilloma virus (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 streptavidin (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+ regulatory (Treg) cells (13), and inhibiting the conversion of Teff cells into Treg cells through the production of IFNγ (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/IFNγ 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 antigen presenting cells (APC), such as dendritic cells (DC) 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 (Louisville, KY). 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-IFNγ-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 Biosciences, eBioscience, Invitrogen, or BioLegend. MPL was purchased from InvivoGen. The HPV16 RAHYNIVTF E7 peptide (E749–57), 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 1 × 10^5 TC-1 cells and vaccinated subcutaneously on day 6 after tumor challenge. For established tumor study, mice with approximately 9 mm^2 established tumors were vaccinated twice at 10 days interval. For the pulmonary tumor model, 2 × 10^5 live 3LL cells were injected i.v. into the tail vein of mice. Mice were vaccinated subcutaneously once on day 6 after tumor challenge and euthanized 27 days after tumor challenge for analysis of lung tumor burden as described (10).

CD8+ and CD4+ T cells were depleted using antibodies against CD8 (clone 53.6.72) and CD4 (clone GK 1.5) at 500 μg/mouse via i.p. once 1 day before vaccination, whereas IFNγ blockade was performed by injecting the anti-IFNγ antibody (XMG1.2; 500 μg/mouse) 6 hours before tumor inoculation, followed by 3 more doses every 3 days after tumor challenge.

**Cytotoxicity assay**

Splenocytes were cultured with 10 μg of E749–57 peptide/mL in complete MLR medium supplemented with 50 IU/mL of IL2 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 (1 × 10^6 cells/mL) were stimulated with either 10 μg/mL of E749–57 peptide for 2 hours followed by overnight incubation with GolgiPlug (1 μL/mL, BD PharMingen) or stimulated with phorbol 12-myristate 13-acetate (PMA; 5 ng/mL, Sigma) and ionomycin (500 ng/mL, Sigma) for 2 hours followed by an additional 4-hour incubation with GolgiPlugg. Cells were first stained with anti-CD44-APC and anti-CD8-APC-Cy7, fixed with 4% paraformaldehyde, and then stained with anti-IFNγ-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 autoantibodies 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

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nitrigen (BUN), and creatinine (CREA) levels 18 hours after vaccination. Liver tissues were also collected from these mice, fixed in 3.7% formaldehyde, embedded in paraffin, and sliced and stained with hematoxylin and eosin for pathologic changes.

Statistical analysis
Statistical analyses were performed using the Student 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 (Supplementary Fig. S1A).

The therapeutic efficacy of the combined adjuvants was next tested in a more stringent and clinically relevant setting. Mice with approximately 9 mm² established tumors treated with a prime-boost vaccination 10 days apart. Combined adjuvant treatment resulted in complete eradication of established tumors in 75% of mice, whereas SA-4-1BBL and MPL monotherapies resulted in approximately 40% and 25% tumor eradication, respectively (Fig. 1B). Apart from inducing moderate therapeutic benefit, SA-4-1BBL monotherapy also slows the tumor growth compared with MPL monotherapy (Supplementary 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⁺ Teff 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⁺ Teff and long-term memory responses elicited by SA-4-1BBL/MPL. Mice that had eradicated the tumor in response to various vaccine formulations (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 E749–57 epitope (8). Consistent with the therapeutic efficacy, SA-4-1BBL/MPL generated a better antigen-specific IFNγ, IL2, and TNFα triple cytokine response than SA-4-1BBL and MPL monotherapies as assessed by percentage (Fig. 2A–C) and absolute number (Supplementary Fig. S2) of CD8⁺ T cells. In addition, SA-4-1BBL/MPL induced higher E7 TAA-specific

Figure 1. SA-4-1BBL/MPL adjuvant system eradicates established TC-1 tumors. A, C57BL/6 mice were challenged subcutaneously with 1 × 10⁵ TC-1 cells and left unvaccinated (PBS) or vaccinated once subcutaneously on day 6 after 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 approximately 9 mm² established tumors were vaccinated twice at 10 days interval. The log-rank test and Kaplan–Meier methods were used for analyses. In A, ***, P < 0.001 compared with all the other groups, except SA-4-1BBL. *, P ≤ 0.05 SA-4-1BBL while in B, *, P < 0.05 compared with SA-4-1BBL and MPL groups. Data are pooled from two independent experiments.
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 IFNγ and E7 TAA-specific CD8⁺ T-cell killing responses than MPL monotherapy (Fig. 2A and D). Importantly, SA-4-1BBL/MPL also generated the most effective CD8⁺ T-cell memory recall responses as compared with SA-4-1BBL and MPL monotherapies (Supplementary Fig. S3). Collectively, these data demonstrate the robust effect of SA-4-1BBL and MPL adjuvants in the generation of potent CD8⁺ Teff and memory responses that correlate with impressive therapeutic efficacy against TC-1 tumor.

Vaccination with the SA-4-1BBL/MPL adjuvant system induces strong antitumor CD8⁺ Teff 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-specific CD8⁺ T cells expressing intracellular IFNγ mono (A), IFNγ/TNFα double (B), and IFNγ/TNFα/IκBα 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 are expressed as a percentage of total CD8⁺ T cells (D). Splenocytes from mice from above groups were stimulated with E7 peptide and harvested 3 days later and assessed for E7 peptide-specific CD8⁺ T-cell killing responses as compared with PBS control, and indeed performed worse than E7 protein alone that appreciably, but not statistically 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 and 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 significantly, 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⁺ Teff 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 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 cells against cancer (26). Vaccination with SA-4-1BBL/MPL had the most pronounced effect on the number of intratumoral CD8⁺ T cells against cancer (26). Vaccination with SA-4-1BBL/MPL had the most pronounced effect on the number of intratumoral CD8⁺ T cells against cancer (26). Vaccination with SA-4-1BBL/MPL had the most pronounced effect on the number of intratumoral CD8⁺ T cells against cancer (26). 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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\(\gamma\) are critical to the therapeutic efficacy of SA-4-1BBL/MPL while Treg cells are detrimental to the efficacy of MPL monotherapy

To test whether 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 antibodies 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, whereas depletion of CD4\(^+\) T cells, including Treg cells, improved the therapeutic efficacy of MPL from 50% to 100%. Blockade of IFN\(\gamma\) using a neutralizing antibody significantly abrogated the therapeutic efficacy of SA-4-1BBL/MPL (20% tumor

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 (~9 mm\(^3\)) were vaccinated subcutaneously with E7 protein (50 \(\mu\)g) alone or with SA-4-1BBL (25 \(\mu\)g), MPL (25 \(\mu\)g), or a combination of both agents (25 \(\mu\)g/agent). One week after vaccination, tumors were harvested and stained for intratumoral CD8\(^+\) T cells and CD4\(^+\) \(\text{Foxp3}^+\) Treg cells followed by analysis using confocal microscopy. A, confocal pictures of tumor sections showing CD4\(^+\) \(\text{Foxp3}^+\) Treg cells (right) stained with anti-CD4 antibody (red), anti-Foxp3 antibody (green), and Hoechst (blue), and CD8\(^+\) T cells (left) stained with anti-CD8 antibody (red) and Hoechst (blue). Quantitative analysis of intratumoral CD4\(^+\) \(\text{Foxp3}^+\) 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 represent four animals per group (each dot represents single animal). *, \(P < 0.05\); **, \(P < 0.001\); ns > 0.05.
eradication vs. 100% without blocking (Fig. 4B). Taken together, these data establish a critical role for CD8+ T cells and IFNγ-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 whether this efficacy is translatable to SVN, a weak and potentially tolerant self-TAA, using the 3LL pulmonary metastasis model. Mice were challenged intravenously with a lethal dose of 3LL cells followed by subcutaneous 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 IFNγ+ 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 (Supplementary Fig. S4). Booster vaccination with SA-4-1BBL and MPL monotherapies also improved the rejection of tumor burden (Supplementary 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 antibodies against ssDNA as a sign of systemic autoimmunity. There was lack of significant amount of autoantibodies to ssDNA in all the groups tested compared with autoantibodies 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 draining lymph nodes (dLN) 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 with naïve mice. Pathologic analysis of liver tissue from SA-4-1BBL/MPL–vaccinated mice (Fig. 6D) or enumeration of various cell populations harvested.
from the dLN or spleen (Supplementary Table S1) did not show any significant changes as compared with 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 whether 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\(\gamma\) and associated with a favorable intratumoral CD8\(^+\) TefCD4\(^-\)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\(^+\)

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 \(2 \times 10^5\) 3LL cells by intravenous tail injection and vaccinated once subcutaneously on day 6 after tumor challenge with SVN (50 \(\mu\)g) alone or with SA-4-1BBL (25 \(\mu\)g), MPL (25 \(\mu\)g), or a combination of both agents (25 \(\mu\)g/agent). A, lungs were harvested 27 days after tumor challenge and assessed for tumor growth by weight and macroscopic presence of tumor nodules. B, intracellular IFN\(\gamma\) response of CD8\(^+\) T cells was assessed after PMA and ionomycin stimulation of lymphocytes harvested from mice in A. *, \(P \leq 0.05\); ***, \(P \leq 0.001\); nonsignificant, ns > 0.05.
T cells critical for antitumor efficacy (8, 9, 11, 13). Therefore, MPL in SA-4-1BBL/MPL combination is envisioned to enhance SA-4-1BBL costimulatory function on effector CD8⁺ T cells through the activation of DCs and antigen crosspresentation (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). Interestingly 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 (Supplementary Table S2). However, SA-4-1BBL may augment the effect of MPL on DCs in vivo by improving their antigen uptake, crosspresentation, 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 crosspresentation (10, 13). Furthermore, it has been demonstrated that monocyte/macrophage proliferation and survival was only achieved by 4-1BB signaling, but not lipopolysaccharide as a TLR-4 agonist (35).

One of the main outcomes of using SA-4-1BBL and MPL adjuvants together was the generation of potent CD8⁺ Teff 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 antitumor efficacy is perhaps due to its ability to produce polyfunctional effector cytokines, such as IFNγ, IL2, and TNFα, found in this study. It is well documented that polyfunctional Teff cells that simultaneously produce multiple effector cytokines are more effective in killing tumors than cells that secrete 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 IFNγ⁺ IL2⁺ TNFα⁺ 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 antitumor 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 IFNγ-
dependent 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 immunologic 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 dLN 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, nonspecific lymphoproliferation, altered lymphocyte trafficking, generalized lymphomegaly and splenomegaly, and hepatitis, all of which were observed with similar doses of an agonistic antibody 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 result 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.

**Disclosure of Potential Conflicts of Interest**

H. Shirwan and E.S. Yolcu are inventors on patents on SA-4-1BBL. No potential conflicts of interest were disclosed by the other authors.

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