5-2011

Exploitation of costimulatory SA-4-1BBL in the development of therapeutic cancer vaccines.

Abhishek K. Srivastava
University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd

EXPLOITATION OF COSTIMULATORY SA-4-1BBL IN THE DEVELOPMENT OF

THERAPEUTIC CANCER VACCINES

By

Abhishek K. Srivastava

M.S. Western Kentucky University, 2006

M.S. University of Louisville, 2008

A Dissertation

Submitted to the Faculty of the

Graduate School of the University of Louisville

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

University of Louisville

Louisville, Kentucky

May 2011

EXPLOITATION OF COSTIMULATORY SA-4-1BBL IN THE DEVELOPMENT OF

THERAPEUTIC CANCER VACCINES

By

Abhishek K. Srivastava
M.S. Western Kentucky University, 2006
M.S., University of Louisville, 2008

A Dissertation Approved on

March 31, 2011

by the following Dissertation Committee:

<div style="text-align:center">

_____
Haval Shirwan
Dissertation Director

_____
Esma Yolcu

_____
Jill Suttles

_____
Jun Yan

_____

</div>

DEDICATION

I dedicate my dissertation to my beautiful wife Shweta Srivastava and my parents Mr. Umesh Chandra Srivastava and Mrs. Indu Srivastava. Without their patience, understanding, support, and most of all love, the completion of this work would have not been possible. Above all, I would like to dedicate my dissertation to Almighty God who has given me strength and good health while completing my Doctoral dissertation.

ACKNOWLEDGMENTS

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This entire journey of my dissertation is an outcome of my Doctoral Research which was performed at University of Louisville, Kentucky, whereby I have been accompanied and supported by many people. I am very glad that I have now the opportunity to express my gratitude for all of them.

The first person I would like to thank is my advisor, Dr. Haval Shirwan, without whose guidance and encouragement my research and dissertation would not have been possible. I owe him lots of gratitude for having shown me this way of quality research and helping me finding my path of success. I am truly grateful to Dr. Esma Yolcu who has been an exceptional help and support pillar throughout this journey. Her expertise and guidance has immensely impacted my professional and personal life.

I am really thankful to my committee members Dr. Jill Suttles and Dr. Jun Yan in for their unconditional support and time. I would also like to thank Dr. James Lillard and Dr. Shailesh Singh for their scientific insights as my committee members for short-term. A special thanks to Department of Microbiology and Immunology faculty, staffs and students, ICT, IPIBS program for providing me excellent support either directly, or indirectly to pursue my Doctoral degree.

I am truly grateful to all my past and present lab members who have been incredibly helpful and supportive during this entire process. I am thankful to Rajesh,

ABSTRACT

EXPLOITATION OF COSTIMULATORY SA-4-1BBL IN THE DEVELOPMENT OF

THERAPEUTIC CANCER VACCINES

Abhishek K. Srivastava

March 31, 2011

Cancer accounts for nearly one-quarter of deaths in the United States, exceeded only by heart diseases. Despite the development of various strategies to treat cancer, it remains one of the most deadly diseases worldwide due to the limited effects of treatments available. The limited efficacy of these current treatment modalities, such as surgery, radiotherapy, and chemotherapy, are often due to their association with adverse side effects arising from lack of specificity for tumors, and most importantly their failure of eliminating residual and micro-metastatic tumors, which can lead to recurrences. Therefore, there is a dire need to develop tumor-specific therapies that not only eliminate primary tumors, but also micro-metastasis and prevent recurrences. In this regard, therapeutic cancer vaccines based on tumor-associated antigens (TAAs) has evolved as a promising approach due to their safety profile, ease of production, storage, transportation, administration to a broad patient population and most importantly establishment and/or maintenance of long-term immunological memory critical for the control of recurrences, a major cause of cancer death. However, despite theoretical promise, development of therapeutic cancer vaccines has been facing numerous set-backs mostly due to the weak immunogenicity of TAAs, tolerance to self-TAAs and various immune evasion

mechanisms employed by progressing tumors. Therefore, we hypothesized that use of natural costimulatory ligands of TNF family as adjuvant may overcome these limitations due to their effect on cells of innate, adaptive, and regulatory immunity without any sign of toxicity. The tumor necrosis factor receptor (TNFR)/TNF superfamily represents a crucial group of costimulatory receptor/ligands as most of the receptors of this family are inducibly expressed on various immune cells. 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 immunological memory. Among these family members, 4-1BB/4-1BBL signaling has recently been much appreciated as its signaling provides the essential survival signals, particularly in CD8$^+$ T cells. 4-1BB signaling into T cells allows CD8$^+$ T cell expansion, cytokine production, development of CTL effector function, and prevention of apoptotic cell death by up-regulating anti-apoptotic Bcl-xL and Bcl-2 molecules. As the aim of tumor immunotherapy is to generate long-lasting immune response, particularly CD8$^+$ T cell specific response, for the destruction of tumor cells, in this project, we focused on the utilization of 4-1BBL either alone or in combination with other immunomodulators, as a component of TAA-based subunit vaccines and tested its efficacy in preclinical mice tumor models.

First, we report that a single immunization with a therapeutic vaccine formulation containing novel form of soluble SA-4-1BBL, and survivin (SVN), a bona fide self antigen, resulted into the eradication of SVN-expressing 3LL tumors in 75% of mice in the absence of autoimmunity. The efficacy of vaccine was further improved to complete tumor eradication with an additional vaccination 6 days after the first vaccination. CD8$^+$

T cells and NK cells effector function was found to be critical for the efficacy of vaccine, but not the CD4$^+$ T cells.

Next, we tested the vaccine formulation containing combination of SA-4-1BBL and toll-like receptor 4 agonist monophosphoryl lipid A (MPL) with distinct mechanisms of action as a novel adjuvant system. A single immunization with both adjuvants and HPV E7 protein resulted in eradication of 100% of E7 expressing TC-1 tumors. Combined adjuvants had better therapeutic efficacy over the individual adjuvants, while SA-4-1BBL monotherapy outperformed MPL, 80% vs. 50%. Similarly, a single vaccination with SVN resulted in control/eradication of established 3LL pulmonary metastases that was further improved by a booster injection. The therapeutic efficacy of combined adjuvants as well as SA-4-1BBL as monotherapy was achieved in the absence of detectable toxicity and correlated with enhanced CD8$^+$ T cell function and increased intratumoral CD8$^+$ T effector/CD4$^+$FoxP3$^+$ T regulatory cell ratio. In marked contrast, vaccination with MPL as monotherapy resulted in an unfavorable intratumoral CD8$^+$ T effector/CD4$^+$FoxP3$^+$ T regulatory cell ratio that played a definitive role in vaccine efficacy. Depletion of T regulatory cells improved MPL efficacy to 100%, whereas elimination of CD8$^+$ T cells totally abrogated the efficacy of combined adjuvants.

In last, we report that combination of SA-4-1BBL and SA-OX40L, another member of TNF ligand family, was also able to eradicate TC-1 tumors in 100% of mice. This efficacy was mainly dependent on CD8$^+$ T cells as depletion of these cells completely abrogated the efficacy. Importantly, combination of these two ligands was also able to eradicate a 3-4 mm established tumors in 50% of mice.

Taken together, these data provide important mechanistic insight into the mode of action of SA-4-1BBL alone or in combination either MPL or SA-OX40L adjuvants and demonstrate its utility as a novel adjuvant system for the development of therapeutic TAA-based subunit cancer vaccines with significant clinical implications. These data also shed lights into the mode of action of MPL and SA-OX40L as a part of vaccine adjuvant systems and set the stage for their utilization in the development of new vaccine strategies.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Cancer is the one of the leading causes of death in the United States and worldwide. Despite growing efforts in the development of various strategies to treat cancer, it remains one of the most deadly diseases with the limited effects of treatments available. The limited efficacy of these current treatment modalities, such as surgery, radiotherapy, and chemotherapy, are often due to their association with adverse side effects arising from lack of specificity for tumors, and most importantly their failure of eliminating residual and micro-metastatic tumors, which can lead to recurrences (1). Therefore, there is a dire need to develop tumor-specific therapies that not only eliminate primary tumors, but also micro-metastasis and prevent recurrences. In this scenario, therapeutic cancer vaccines based on tumor associated antigens (TAAs) are preferred alternatives to conventional treatments primarily because of their safety profile and generation of long-term immunological memory critical for the control of recurrences, which are major cause of death from cancer.

The theory of cancer immune-surveillance suggests that the immune system has the ability to recognize precursors of cancer and, in most cases, mount an effective response to destroy these precursors before they become clinically apparent (2). This notion is supported by observations that mice lacking essential components of the innate or adaptive immune responses are susceptible to the development of spontaneous or

designed to enhance the ability of immune system can reduce the development of malignant disease in animal models. If immune-surveillance plays a key role in controlling the tumor progression, then one would expect the patients with pre-malignancy will mount vigorous immune responses. There are, indeed, scientific evidences indicating that this might be the case. Patients with monoclonal gammopathy, a pre-malignant disease, mount strong T cell responses against autologous pre-malignant B cells, whereas no responses are found in patients with malignant multiple myeloma (3). Additionally, breast cancer-associated proteins mucin-1 and HER2 specific CD8^{+} T cells from breast cancer patients are able to regress autologous human tumors that have been transplanted into immunodeficient non-obese diabetic mice (4). These studies suggest that, although tumors induce at least transient immune responses, cancer can still develop. This indicate that such cellular immune responses are either too low to prevent the tumor progression or evaded by tumor cells as its safety mechanism. Therefore, the development of therapeutic cancer vaccines capable of generating a robust tumor-specific immunity may serve as a promising tool for cancer treatment. However, despite demonstrated efficacy in test tubes and experimental animals, efforts to harness the immune system to fight cancer using cancer vaccines have been challenging for decades and have largely ended in failure. This may be due to the weak antigenic nature of self tumor-associated antigens (TAAs), both central and peripheral tolerogenic mechanisms, and various immune evasion mechanisms employed by the progressing tumors. These limitations can potentially be overcome by developing vaccine formulations including adjuvants that not only generate potent immune responses against TAAs with long-term immunological memory, but also overcome various immune evasion mechanisms.

2

Cancer Vaccines:

The development of successful vaccines against infectious diseases is one of medicine's most important accomplishments. Their influence on majority of human population is aptly expressed by vaccinologist Stanley Plotkin when he says: "The impact of vaccination on human health of the world's peoples is hard to exaggerate. With the exception of safe water, no other modality, not even antibiotics, has had such a major effect on mortality reduction and population growth" (5). The success of these vaccines primarily lies in their ability to induce long-lived antibody responses that protect against highly immunogenic bacteria and viruses. However, development of therapeutic vaccines against cancer faces a much greater challenges because their target often consist of tumor-associated self-antigens (TAAs), which are poorly immunogenic and often heterogeneously expressed by genetically unstable tumor cells that undergo mutations and frequently suppress immune responses at the cellular level in the tumor microenvironment. Therefore, despite several encouraging advances in the area of vaccinology, cancer is still a major public health problem worldwide, and requires new strategies for the development of potent vaccines to optimize patient outcomes.

In the last several years, numerous strategies such as cell-based vaccines, DNA- or RNA-based vaccines, protein/peptide vaccines, and vector-based vaccines, have been employed to overcome this weak immunogenic response against TAAs (6). The idea of using these first generation cancer vaccines is primarily based on their ability to activate antigen-presenting cells (APCs) and stimulate antigen-specific cytotoxic T lymphocyte (CTL)-mediated immune response. In this context, dendritic cells (DCs), the most effective APCs, have been targeted to enhance their ability to activate T cells. In the last

several years, a variety of strategies have been employed to "instruct" DCs to induce robust immune responses against tumor antigens, thus breaking immune tolerance. The main focus of these strategies revolve around the efficient antigen-loading that include, i) infecting DCs with viral, bacterial, or yeast vectors, ii) pulsing DCs with proteins or peptides, iii) loading DCs with whole tumor cells or lysates, and iv) transfecting DCs with DNA or RNA.

Viral, Bacterial and Yeast Vectors-based Vaccines:

The common vectors used in the development of therapeutic cancer vaccines include virus, bacterial and yeast vectors. These vectors are used to deliver recombinant genes such as genes expressing TAAs, costimulatory molecules, and cytokines into APCs. As a result, these recombinant vectors may induce increased immune response against the gene of interest that has been inserted into the vector. Poxvirus vectors (vaccinia virus prototype) are among the most utilized vectors for the development of vaccines. The advantage of using these vectors is that they replicate within the cytoplasm of infected cells, which is important for the safe use of this vector as recombinant vaccines, and no genetic sequence from the virus will be inserted into the host genome.

PSA-TRICOM, a poxvirus-based vaccine has been evaluated in 2 phase II clinical trials in patients with metastatic hormone-refractory prostate cancer (7). In the first trial, vaccinated patients had a greater 3-year overall survival and improved in median overall survival of 8.5 months when compared to the placebo controls. In the second phase II clinical trial at National Cancer Institute (NCI), patients vaccinated with PSA-TRICOM had improved median overall survival of 9.2 months. This study also suggested that Tregs play a significant role in the modulation of antitumor immunity. PANVAC-VF,

another poxvirus-based vaccine, was also tested in a phase III clinical trial in patients with advanced pancreatic cancer (8). The vaccine is also under evaluation in several other CEA- or MUC1-expressing carcinomas. A pilot study at NCI has shown the tolerance of PANVAC-VF in 25 patients with metastatic carcinomas. The vaccine generated an antigen specific T effector response in 3/8 patients measured by ELISPOT, tetramer, and intracellular IFN-γ staining (9).

In the last several years, a variety of different bacterial and yeast vectors have also been used for vaccine development. Among these, *E coli, Salmonella, Yersinia, Shigella, Listeria monocytogenes*, and *Sccharomyces cerevisiae* are the most investigated as vaccine vectors. The rationale for utilization of these microbes-based vectors as delivery vehicle for TAAs is primarily based on their ability to induce pro-inflammatory responses *via* interaction of pathogen-associated molecular patterns (PAMPs) with pattern-recognition receptors (PRRs) expressed on APCs. These interactions are the key controller of the activation of innate and adaptive immune responses (10). Most importantly, by the utilization of genetic engineering and efficient fermentation technologies, these vectors can be used for the production of large-scale, cost effective antitumor vaccines. Although, viral and bacterial based vaccines showed some advantages, these systems have various limitations, such as requiring mitotic cell division for transduction, low maintenance of transgene expression, and immune elimination of infected cells that limits gene expression *in vivo*. In addition, there are safety concerns arising from mutations in the transferred viral genome leading to virulent forms (11).

Protein or Peptide-based vaccines:

The use of proteins or peptides has long been an attractive approach to stimulate an effective immune response against variety of cancers. This strategy covers a broad spectrum of possibilities of utilizing single protein or combination of proteins (12), fusion proteins, peptides, and agonist peptides (13, 14). The major advantages of using these protein or epitope-based strategies are their ease of production, storage, transportation and low cost.

Recently, in the preclinical settings, we have demonstrated that both E7-peptide and E7 whole protein in combination with SA-4-1BBL, a costimulatory chimeric adjuvant based vaccine successfully eradicated the established TC-1 tumors in over 70% of mice (15, 16). Survivin (SVN) protein based vaccine has also shown a promising outcome in eradicating the tumors in both transplantable and pulmonary lung metastases tumor models. In the clinical settings, Oncophage, a HSP gp96 peptide-based vaccine has been tested in two phase III clinical trials against melanoma and renal cell carcinoma (RCC) (17, 18). Although, in the first phase III clinical trials in melanoma patients vaccine was able to provide survival benefits after 10 or more vaccinations, but in the second clinical trial in RCC patients, no difference in recurrence-free survival or overall survival was observed between patients receiving Oncophage versus no treatment. Stimuvax, a MUC-1 peptide-based vaccine which widely expressed on many tumors (lung, breast, prostate, colorectal), has also been evaluated in phase II B clinical trial in non-small cell lung cancer (NSCLC) (19) and showed positive signs of vaccine efficacy. Based on these data, Merck is currently conducting three large phase III clinical trials of Stimuvax.

Despite some promising clinical outcomes, use of specific proteins or peptides for the development of therapeutic vaccines clearly requires a careful selection of targeted TAAs, and their epitopes along with the ability to understand their structural and functional characteristics. Most importantly, this approach have certain drawbacks associated with it: i) weak immunogenicity ii) tumors can easily escape immune recognition through antigenic loss or mutation, iii) limited subset of patients due to HLA-restriction, and iv) poor ability to activate balanced $CD8^+$ and $CD4^+$ T cell response.

Whole tumor cells or tumor lysate-based vaccines:

Theoretically, whole tumor cells contain all relevant TAAs and, as such, have the potential to elicit an effective anti-tumor immune response when administered in conjunction with an appropriate immunomodulator. In principle, these vaccines can have more advantages over the single-target approaches as it not only can target both different and unknown antigens at the same time but also able to stimulate robust adaptive immune responses due to availability of both MHC class I and class II epitopes.

In the last two decades, several different vaccines derived from whole tumor cells or tumor lysate have been tested in both preclinical and clinic settings. In preclinical settings, vaccination with whole tumor cells was shown to stimulate the immune response through direct tumor-antigen presentation as well as prolonged release of TAAs that allow for sufficient tumor-antigen uptake by host APCs and subsequent activation of immune effector cells (20). Mice vaccinated with irradiated whole tumor cells in combination with an adjuvant, and subsequently challenged with non-irradiated tumor cells, were found to efficiently generate anti-tumor immune recall responses (21). In clinical settings, OncoVAX (Vaccinogen), an autologous irradiated tumor cells-based

7

vaccine has shown over 20% reduction in risk of colon cancer progression in patients compared to control groups. This efficacy was translated into improved overall survival of patients (22). Reniale, an autologous tumor cell lysate-based vaccine against RCC with adjuvant treatment showed greater benefits in patients with a higher risk. GVAX, another vaccine composed of two irradiated prostate cancer cell lines LNCaP and PC-3, has demonstrated some local immune responses in preclinical studies. Based on this encouraging preclinical study, it was further tested in clinical trials. Despite these encouraging immunological results, GVAX failed to meet the defined endpoint in two phase III clinical trials.

However, despite containing all relevant TAAs and thus avoiding the problem of identifying appropriate tumor antigens, the use of autologous tumor cells for cancer vaccines also faces major limitations (23). In addition to the weak immunogenic nature of unmodified autologous tumor cell-based vaccines, the lack of sufficient tumor mass for vaccine preparation due to early detection as well as the inability to surgically access for certain cancers represent other limitations. Although tumor cells may be cultured for an extended time period to generate enough cells for vaccine preparation, the inability of certain tumor cells to grow under culture conditions poses another problem. As an alternative, allogeneic tumor cells have been used as a source of TAAs, which ideally would be taken up by APCs and presented to autologous immune effector cells. Potentially, this would provide a limitless source of TAAs through well established cell lines. Although allogeneic tumor cell lines allow for standardized and large scale production of potential cancer vaccines, such vaccines were demonstrated to direct the

majority of the immune response towards the alloantigens, and not TAAs, due to dominant antigenic competition, leading to inefficient anti-tumor immune responses (23).

DNA and RNA-based vaccines:

DNA-based vaccines formulated to encode TAAs and/or immunostimulatory molecules to elicit an effective anti-tumor immune response have emerged as a safer and advantageous alternative to gene therapy in both preclinical and clinical settings. In preclinical studies, intradermal injection of mice transgenic for rat neu and HER-2/neu homologue with rat neu cDNA in combination with plasmids encoding costimulatory molecules CD80/CD86 and 4-1BBL resulted in the induction of both cellular and humoral immune responses (24). Recently, several phase I/II clinical trials have been conducted with DNA vaccines targeting variety of TAAs such as PSA, PAP, gp100, CEA, HSP65 against prostate cancer (25), melanoma (26), colorectal cancer (27), and head and neck carcinomas (28). In most of the cases, these trials showed very low immunogenicity of TAAs. In another phase I clinical trial, Melan-A/MART-1 DNA plasmid vaccine in patients with stage IV melanoma was well tolerated and induced an immune response. However, the vaccine did not induce regression of established disease (29). Despite pre-clinical and very low clinical success, the development of clinically successful naked DNA cancer vaccines faces major hurdles, such as the need for high doses of plasmid DNA, inefficient delivery of the gene of interest, low expression efficiency of the introduced gene, lack of vaccine efficacy, and finally safety concerns.

In recent years, mRNA-based cancer vaccines are also breaking the grounds and are being used as an attractive immunotherapeutic approach against cancers (30, 31). The advantage of utilizing this approach is primarily because of its safety as transfected

mRNA does not integrate into the host genome (32). The principle behind this approach is that mRNA-based vaccine containing mRNA-coding TAA is transfected into DCs and translated into proteins which further processed and loaded on MHC for its presentation, thus activating an antigen-specific T cell response (33). Several clinical studies have been conducted by employing mRNA-based vaccine approach against different cancers such as prostate (34), RCC (35), ovarian (36), lung, breast (37), neuroblastoma (38) and melanoma (39). A phase I clinical trial with PSA-mRNA-based vaccine was able to increase PSA-specific CTL response against metastatic prostate cancer (40). In another clinical trial, vaccine containing DCs transfecting with total RNA extracted from clear cell carcinoma against metastatic RCC showed significant increase in tumor specific $CD4^+$ and $CD8^+$ T cells with simultaneous decrease in Treg frequency (36).

Why vaccine fails:

As mentioned above, although all the vaccine approaches have demonstrated efficacy in various preclinical studies, they have been either ineffective or demonstrated limited efficacy in clinical settings. These strategies are mainly associated with lack of specificity, inefficient delivery, safety concerns, labor intensive, costly, and in some cases patient-customized which severely limits their broad clinical applications. Among all the concerns, one of the major hurdles for the clinical success may be due to the lack of tumor specific antigens (TSAs) and/or weak immunogenic nature of TAAs, usually recognized by the immune system as self-antigen. Theoretically, TSAs, mostly composed of mutant proteins, are the ideal target for cancer vaccines due to their specificity; however, the major drawback of targeting TSAs is the uniqueness of these mutations to each tumor, potentially leading to the development of patient customized

10

immunotherapy. On the other hand, TAAs are commonly expressed on the tumors with same histology and are shared among tumors of different origins. But one of the major limitations associated with TAAs are their weak immunogenic nature due to the tolerance of self -antigens. The next major obstacle for clinical success may include active immune evasion mechanisms employed by progressing tumors. These obstacles may be overcome by using potent adjuvants/immunomodulators that not only boost the existing immune responses to the tumor in patients, but also generate new immune responses and, most importantly, overcome the immune evasion mechanisms.

Why adjuvants are critical?

The term "adjuvant" was first proposed by Ramos in 1920s which originated from the latin *adjuvo* means "to help". Adjuvants, substances that traditionally added to vaccines are used to enhance the magnitude, breadth, quality and longevity of the innate system to increase or modify a subsequent adaptive immune response, with minimal toxicity. Addition of adjuvants in the vaccine may substantially reduce the amount of antigen and/or number of immunizations required for optimum immune response. That is why, in the last several decades, adjuvants have been known to be a critical determinant of the success or failure of a vaccine and Charlie Janeway famously described the nature of adjuvants as "the immunologists dirty little secret".

In the year between 1920s-1930s, it was found that aluminum salts can improve the immune response (41), thus Alum became the first vaccine adjuvant and until a year ago it has been the only approved adjuvant in USA for human vaccines. Adjuvants are necessary to boost the optimal immune response to weak antigens. Most importantly, adjuvants are important to overcome various tolerance mechanisms and facilitate the

11

induction of CTL response that can traffic to and lyse tumor cells. In this regard, the application of alum as adjuvant for cancer vaccines is limited (42) because alum-based vaccines primarily induce effective Th2 responses (43) with minimal efficacy in eliciting Th1 immunity (44), which is necessary for the eradication of tumors. In this context, the choice of adjuvants is critical. Adjuvants can affect the nature of the elicited immune responses, such as Th1 *vs.* Th2 responses, T cell *vs.* B cell responses, generation and maintenance of immunological memory, and reversal of the immunoregulatory mechanisms, such as Treg-mediated suppression and T cell anergy. As such, adjuvants that modulate innate, adaptive, and regulatory immunity in favor of the generation of effective anti-tumor immune responses may have the best efficacy. Of critical importance is the development of adjuvants with safety profiles in humans. Therefore, the development of effective therapeutic cancer vaccines for the clinic will require adjuvants/immunomodulators that can efficiently stimulate innate and adaptive immune responses as well as reverse/inhibit tumor-mediated immune suppressive mechanisms.

Adjuvants for therapeutic cancer vaccines:

In the last several years, despite the fact that adjuvants are the critical determinants of the success or failure of the vaccines, there has been great deal of pessimism regarding their use for the development of therapeutic cancer vaccines. This is mainly due to the lack of full characterization of mechanistic insight and precise knowledge of the constituents of many adjuvants until recently (45, 46). Some of the recently tested vaccine formulations, like viral vectors, are designed to express their own adjuvants while others, like peptide-based vaccines, do not and hence require co-administration of adjuvants for the induction of potent immune response.

12

For the several decades, the choice of adjuvants available for cancer vaccines has been very limited, mostly or in part due to toxicity concerns raising significant regulatory hurdles. In fact, only aluminum-salt based adjuvants have been used for human use in the United States for decades until recently when combination of aluminum hydroxide with monophosphoryl lipid A (MPLA) has been licensed. Approval of recombinant vaccines against HPV and HBV, albeit in preventing setting, has provided a great hope for the development of several adjuvants to augment the efficacy of the cancer vaccines. As oppose to adjuvants whose characterizations are poorly understood, a growing focus has been shifting towards the use of natural ligands or synthetic agonists for well-defined PRRs as adjuvants. Among all PRRs, toll-like receptors (TLRs) are the largest and most well characterized family of a diverse set of germ line-encoded receptors which recognize broad classes of conserved molecular structures common to groups of microorganisms (47-49).

Due to the critical role of TLR signaling on the innate, adaptive, and regulatory arms of the immune systems, TLR agonists have emerged as ideal adjuvants for cancer immunotherapy. Perhaps addition of various TLR agonists to vaccine formulations has been one of the most significant developments in the area of cancer vaccine. These agonists include TLR-3 (poly I:C), TLR-4 (MPL), TLR-5 (flagellin), TLR-7 (Aldara), TLR-7/8 (Resiquimod), and TLR-9 (CpG). Either alone or with various formulations, these TLR agonists have been shown to enhance the vaccine efficacy. In case of preclinical studies, these agonists have been demonstrated to generate anti-tumor immunity by enhancing innate immunity through the activation of DCs, NK cells, monocytes, and macrophages and induction of cytokines with both direct and indirect

anti-tumor activity. Engagement of TLRs on APCs, such as DCs, results in their maturation and migration to lymph nodes where they initiate adaptive immune responses and generate long-lasting memory against tumors. In case of clinical studies, MPLA has already been licensed in United States by FDA and used as a component of Melacine, Cervarix, a preventive HPV vaccine by GlaxoSmithKline Biological (GSK) (50), and is also a component of NSCLC vaccine in late-stage clinical trials. RC-529 (GSK, DynaVax), another synthetic TLR-4 agonist has been licensed for an HBV vaccine in Europe (51). CpG oligodeoxynucleotides (ODN) has also shown great promise as an adjuvant for TAA-based cancer vaccines (52).

Despite some promising clinical results, safety profile of these TLR agonists has been of the major hurdles which need to be addressed for the use of these agonists as a component of vaccine formulations. TLR agonists as components of vaccines have been found to be associated with severe toxicity, resulting from non-specific activation of lymphocytes and plausibly from signaling into non-immune cells (53-55). The limited effect of TLR-signaling on the induction of adaptive immunity, which is critical to the establishment of long-term immunological memory and prevention of tumor recurrences, have also been one of the major challenges for these agonists to be used as an adjuvant component of therapeutic cancer vaccines (56-58). Most importantly, TLR-signaling has been found to be involved in the generation of regulatory immunity which plays a critical role in immune evasion and allow tumors to counterbalance the anti-tumor immunity. For example, TLR-4 signaling has been found to expand Tregs *ex vivo* and induce IL-10 producing CD4$^+$ Tregs *in vivo* (54). Similarly, CpG, a TLR-9 agonist was involved in the conversion of CD4$^+$ T effector cells into Tregs *via* plasmacytoid DCs (59), and was

14

found to induce $CD19^+$ dendritic cells to acquire potent T cell suppressive functions through the production of indoleamine 2,3-dioxygenase (60). Based on above discussed outcomes, there is a dire need for the discovery and development of alternative adjuvants which not only has the potent immunomodulatory activities on cells of innate, adaptive, and regulatory immunity but also provides safety without significant toxicity at therapeutic doses.

Costimulatory 4-1BBL and OX40L as a platform for the development of therapeutic cancer vaccines:

An effective therapeutic cancer vaccine should aim to generate anti-tumor immunity by targeting T cells, APCs, and NK cells to eliminate the existing tumors and to promote immunological memory that prevents the recurrences. Most importantly, therapeutic cancer vaccine should also ideally prevent the generation and/or function of Tregs. In this context, costimulatory molecules of TNFR super-families play critical roles in modulating innate, adaptive, and regulatory immune responses. Several preclinical studies have demonstrated the effectiveness of agonists that express the extracellular functional domain of TNF ligands and cross-link their costimulatory receptors for effective signal transduction (61, 62).

In recent years, there have been growing interests in two costimulatory molecules, 4-1BB and OX40, which are shown to function primarily on $CD8^+$ and $CD4^+$ T cells, respectively. It is well known that signaling through 4-1BB and OX40 induces very similar responses although not identical. OX40 is expressed early after $CD4^+$ T cell activation and mediates the effector T cells differentiation (63). Similarly, stimulation of 4-1BB induces very identical effects on $CD8^+$ T cells (64). Nevertheless, signaling

15

through these two costimulatory pathways have shown to overlap in their effects on both CD4$^+$ and CD8$^+$ T cells (65, 66). Agonistic ligands to these costimulatory receptors have been, either alone or in combination, widely tested as effective adjuvants of therapeutic cancer vaccines in both preclinical and clinical settings (62, 67). OX40 ligation has been shown to induce T cell mediated antitumor responses against variety of tumors including sarcoma, colon, breast, and prostate cancer (62, 68, 69). On the other hand, 4-1BB ligation has shown effects on DC activation, survival, cytokines such as IL-6 and IL-12 which results into robust T cell stimulation (70, 71). Most importantly, 4-1BB ligation renders T effector cells resistant to suppression by Tregs (15) while OX40 directly inhibits the Tregs suppression (72). These agonistic antibodies induce robust amplification of T cell mediated immune responses, inhibit apoptotic cell death (73, 74) and stimulate long-lived T cell responses (75). In most of the studies, the anti-tumor responses generated by these agonists are associated with increased activity of CD8$^+$ and CD4$^+$ T effector functions. However, the biggest drawback with the use of agonistic antibodies for costimulatory molecules lies in their involvement in the induction of severe toxicity. These toxicities primarily arise from nonspecific, and systemic activation of lymphocytes (76, 77). These toxicities have further shown to be associated with cytokine-mediated disruption of lymphocyte trafficking, splenomegaly, multifocal hepatitis, and lyphodenopathy (78, 79). Additionally, immunization of agonistic anti-CD28 monoclonal Ab has found to be lethal due to systemic inflammatory responses in a phase I clinical trial (80). Therefore, utilization of agonistic Abs as a component of therapeutic vaccines requires a rigorous scrutiny due to its potential toxicity in humans and demands the

development of agonists that provide appropriate immune signal transduction in the absence of toxicity.

In this context, recently we have proposed the utilization of natural ligands as an alternative to agonistic Abs that may have both qualitative and quantitative differences in the generation of potent immune signal transduction with superior therapeutic efficacy in the absence of severe toxicity. Due to limitation of these ligands to be functional only as membrane bound proteins, not in soluble form, we have previously generated a chimeric 4-1BB ligand (SA-4-1BBL) by fusing its functional extracellular domain to a modified form of core streptavidin (SA) for potent signal transduction. Due to virtue of SA, this chimeric protein exists in stable tetramers and higher structures and allows this ligand for the generation of potent immunological activities in soluble forms. The activity of this natural SA-4-1BBL has already been established in our lab (81) and has been demonstrated to be superior to agonistic anti-4-1BB Ab (3H3) in terms of qualitative and quantitative differences (79). SA-4-1BBL in combination with both E7 peptide and protein (TAA)-based vaccines demonstrated potent efficacy by eradicating > 70% of E7-expressing TC-1 tumors (15, 16). Based on our previous observations, we sought to determine if SA-4-1BBL can be utilized as a successful vaccine adjuvant in other tumor model with different TAA and if efficacy of SA-4-1BBL based vaccine can further be improved by addition of another immunemodulator in the vaccine formulation. Therefore, the first major goal of this Ph.D. dissertation was to test the therapeutic efficacy of SA-4-1BBL in combination with survivin (SVN), a bona fide self-TAA in 3LL lung carcinoma model and determine the mechanistic insight of vaccine efficacy. The second goal was to determine the efficacy of SA-4-1BBL in combination with

another immunomodulator such as TLR-4 agonist MPLA and costimulatory SA-OX40L, due to their distinct mode of action, in different preclinical tumor mice models.

CHAPTER 2

SA-4-1BBL AS AN ADJUVANT COMPONENT OF A VACCINE CONTAINING RECOMBINANT SURVIVIN INDUCES POTENT ANTI-TUMOR EFFICACY IN A CD8$^+$ T CELL AND NK CELL DEPENDENT MANNER

Introduction

Cancer remains one of the deadliest diseases despite progress in the discovery of various strategies with the limited effects. The limited efficacy of these current treatment modalities are often due to their association with adverse side effects arising from lack of specificity for tumors, and most importantly their failure of eliminating residual and micrometastatic tumors, which can lead to recurrences (1). In this scenario, therapeutic vaccines based on tumor associated antigens (TAAs) are preferred alternatives to these conventional treatments for cancer primarily because of their safety profile and generation of long-term immunologic memory critical for the control of recurrences, which are the main cause of death from cancer. In order to succeed, vaccination against TAAs requires targeting the right TAA. The main properties of an ideal target TAA should be: i) over-expressed in tumors but little or undetectable in normal tissues, ii) essential for the survival of the tumors so that tumor immune escape can be avoided, and iii) expressed in many tumors so that vaccine could target variety of cancers. In this context, survivin (SVN) is known as one of the very few candidate TAAs that meet all these criteria (82).

SVN is a 16.5kD protein which is the smallest member of the inhibitor of apoptosis proteins (IAP) family. It is involved in the suppression of apoptotic cell death along with the regulation of cell division (82). SVN is expressed transiently in normal cells while constitutively expressed in most human cancers and its over-expression correlates with tumor progression and constitutes a poor patients survival (83, 84). It is now known that SVN can generate cellular immune response as several SVN-derived peptide epitopes that stimulate major histocompatability complex (MHC) class I restricted cytotoxic T lymphocytes (CTLs) have been identified in both cancer patients and in tumor-bearing mice (85-87). Since SVN-specific CTLs were able to target the tumor cells, it is suggested that potential tolerance to self-antigen can be effectively overcome and SVN-based immunotherapy can further be exploited in the clinic.

However, the development of successful therapeutic vaccines based on SVN has not been fully realized as they have failed to mount an optimal clinical effect in a broad patient population. The efficacy of such vaccines is curtailed by the weak antigenic nature of SVN due to its potential immune tolerance to dominant epitopes and various immune evasion mechanisms employed by the progressing tumors. To overcome these limitations, therapeutic vaccine formulations containing SVN requires the inclusion of potent adjuvants that not only generate robust immune responses with long-term immunological memory, but also overcome various immune evasion mechanisms. In this context, we recently hypothesized that costimulatory ligands, particularly 4-1BBL can be exploited as potential adjuvant of choice for the development of therapeutic cancer vaccines as it plays a crucial role in the generation and maintenance of $CD8^+$ T cell responses which is critical for the eradication of tumors (15, 16).

4-1BBL is known as one of the most potent costimulatory ligand of tumor necrosis factor (TNF) family due to its pleiotropic effects on variety of cells of innate (71), adaptive (88) and regulatory immunity (89). Inasmuch as 4-1BBL is a cell surface membranous protein and has no function in soluble form, recently we generated a chimeric protein, from hereafter called as SA-4-1BBL, by fusing the extracellular functional domain of 4-1BBL to a modified form of core streptavidin (SA). This soluble SA-4-1BBL has shown potent immune activity as an adjuvant component of TAA-based vaccines by targeting various cells of innate, such as DCs, NK cells, and adaptive, such as $CD4^+$ and $CD8^+$ T cells, in various preclinical tumor models (15, 16). Most importantly, SA-4-1BBL also modulates regulatory immunity by reversing tumor induced clonal anergy, renders T effector cells resistant to suppression by $CD4^+CD25^+FoxP3^+$ regulatory T cells, and inhibits the conversion of T effector cells into Tregs through the production of IFN-γ.

We hypothesized that SA-4-1BBL can be exploited for the development of therapeutic vaccines by targeting SVN self-TAA expressed by most of the tumors. Here we report that single vaccination with SVN+SA-4-1BBL based vaccine resulted in the significant eradication of SVN expressing 3LL tumor. The efficacy of vaccine was mainly dependent on $CD8^+$ T cells and NK cells effector function but not on the $CD4^+$ T cells. This efficacy was further improved to complete tumor eradication when an additional vaccination was administered 6 days after the first vaccination. This improved efficacy was correlated with increased level of IFN-γ and cytotoxicity. Additionally, SVN+SA-4-1BBL based vaccine also generate an effective humoral response without a sign of autoimmunity. These results provide the mechanistic insight of SVN+SA-4-1BBL

21

vaccine in 3LL tumor model and emphasize the critical role of CD8$^+$ T cells and NK cells in the vaccine efficacy. These observations also demonstrate the utility of additional vaccinations for the optimal efficacy of self-TAA-based vaccine strategy that can target variety of tumors and can successfully be translated into clinic.

Materials and Methods

Mice and cell lines

C57BL/6 and C57BL/6.SJL mice were purchased from The Jackson Laboratory, Taconic, or bred in our barrier animal facility at the University of Louisville. All animals were cared for in accordance with institutional and National Institutes of Health (NIH) guidelines. The 3LL, TC-1 and A20 cell lines were purchased from American Type Culture Collection (Manassas, VA).

Cloning, expression, and purification of recombinant SVN

Mouse-cDNA was subcloned into the pTWIN-1-6X-His bacterial expression vector (New England Biolabs) using Nde I and BamH I restriction sites. After transformation into C2566 competent *E. coli* cells (New England Biolabs), cultures were grown at 37 °C and induced using IPTG. Cells were harvested 3 h after induction, pelleted, and resuspended in 100 ml of lysis buffer (20mM Tris, pH 7.0, 500mM NaCl, 5mM imidazole, 5mM β-ME, 10μM ZnCl2). Cells were lysed by ultrasonication and inclusion bodies and insoluble material were pelleted at 10,000×g for 10 min. Pellet was washed three times by resuspending in lysis buffer containing 1% Triton X-100, rotating 30 min at room temperature, and repelleting at 35,000×g for 30 min. The final pellet was

resuspended in 100 ml lysis buffer containing 6M Guanidine-HCl and rotated at RT overnight to solubilize inclusion bodies. After, centrifugation at 35,000×g for 30 min at 4 °C to remove the insoluble material, supernatant collected for IMAC purification using Talon® cobalt resin according to the manufacturer's protocol (Clontech) with the exception of including 0.1% Triton X-114 to remove endotoxin. All purification steps were performed in the presence of 10µM ZnCl2 and 5mM β-ME to assist with proper folding and reduce oligomerization. Protein was dialyzed against PBS, concentrated using an Amicon Ultra MWCO 10,000 and sterile filtered using a 0.22µm filtration device. Protein concentration was measured using BCA and Bradford methods (Pierce). Construction, expression, purification, and characterization of SA-4-1BBL have been previously described (15). All the proteins had undetectable levels of endotoxin (SVN = 0.066 EU/µg protein, SA-4-1BBL=0.004 EU/µg protein).

Tumor models and immunizations

C57BL/6 mice were inoculated s.c with $1x10^5$ live 3LL cells into the right flank. For therapy, mice were vaccinated s.c. on day 6 post-tumor challenge with either SVN protein (50 µg) alone or with SA-4-1BBL (25 µg). For the two vaccination therapy, booster was administered either 3 days or 6 days or 9 days after first vaccination. Tumor size was measured with caliper twice a week. Tumor progression was determined by survival of mice which was defined when either tumor reached a size of 12 mm in diameter or when tumors ulcerated or mice showed signs of discomfort, at which point they were euthanized.

Intracellular cytokine assay

For intracellular cytokine staining, lymph node cells were resuspended in MLR

medium at 2×10^6/ml and stimulated with PMA (5 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) for 2 hrs in a 37 °C, 5% CO_2 incubator. GolgiPlug (1 µl/ml, BD PharMingen) was added to the activation mixture and cells were incubated for an additional 4 hrs. Cells were then stained with anti-CD44-APC and anti-CD8-APC-Cy7, fixed with 4% paraformaldehyde, and stained with anti-IFN-γ-PE-Cy7 or isotype controls. FACS Caliber or LSR-II (BD Bioscience) was used for FACS acquisition followed by data analysis using CellQuest (BD Biosciences), FlowJo (Tree Star), and Diva (BD Biosciences) software.

In vitro CTL and NK cells killing assays

For *in vitro* cytotoxicity assay, long-term tumor free animals from SVN alone and SVN+SA-4-1BBL groups were boosted and one week post vaccination, spleen was harvested and splenocytes were cultured with 10µg SVN protein per ml in complete MLR medium supplemented with 50 IU/ml of IL-2. Five days post culture; viable lymphocytes (effector cells) were recovered by centrifugation over a Ficoll gradient. Effector cells were then collected and tested for cytotoxic activity against [^{3}H]-thymidine labeled 3LL tumor target for CTL killing and YAC-1 tumor target for NK cell killing, respectively at various Effector/Target (E:T) for 4 h.

In vitro lymphocyte proliferation

Splenocytes from naive and long term boosted mice were cultured (2×10^5/well) with 5 µg/ml of soluble SVN protein for 5 days in complete MLR medium. Cultures were pulsed with [^{3}H]-thymidine for the last 16 hrs of the culture period, and harvested on a Tomtec Harvester 96 (Tomtec Inc., Hamden, CT) for quantification of incorporated radioactivity. Results were expressed as mean ± SD cpm of triplicate wells.

Depletion assay

A depleting Ab against mouse CD8$^+$ T cells (500ug/mouse, Clone 53.6.72, Bioexpress), NK1.1 (500ug/mouse, Clone PK 136, Bioexpress), and CD4$^+$ T cells (300ug/mouse, Clone GK1.5, Bioexpress) were used *via* intra peritoneal injection to deplete CD8$^+$ T cells, NK cells and CD4$^+$ T cells one day before vaccination. The depletion of these cells was complete as monitored at day 5 post treatment.

Detection of antibodies

Anti-SVN antibody was detected in mouse sera using enzyme-linked immunosorbent assay (ELISA). In brief 50 ng of SVN was coated on a 96-well plate by overnight incubation at 4 °C. Plate was washed and blocked followed by incubation with serum samples for 1.5 hrs at room temperature. Plate was washed and incubated with anti-mouse IgG conjugated with horseradish peroxidase (HRP) for 1 hr followed by incubation with substrate for 20 min. Reaction was stopped by sulfuric acid and measured on spectrophotometer at 490 nm.

For autoantibody detection against ssDNA, ELISA was performed. In brief, plate was coated with 1µg/well of heat-denatured calf thymus DNA (ssDNA, from Sigma). Plate was washed twice followed by blocking with buffer containing 5% BSA + 0.5% Tween 20 + 0.1% naïve B6 serum. Serum dilution was added to the plate followed by incubation at 4 ^{0}C for overnight. Plate was washed 3 times followed by addition of anti mouse IgG-HRP and absorbance was measured at 450nm.

Statistics

Statistical analyses were performed using the One Way Anova, and Kaplan-Mayer curves test using SPSS software. For each test, P values have been displayed and less than 0.05 and 0.001 were considered significant (*) and very significant (**), respectively.

Results

Expression and purification of recombinant SVN

We generated plasmid encoding the pTWIN-1 expression vector, which contains mouse SVN cDNA and 6X-His residues at C-terminus (Fig. 1A). The recombinant SVN was expressed in bacteria as inclusion bodies, denatured with Guanidine-HCl, and purified using IMAC taking advantage of a C-terminus 6X-His tag. The purified protein runs approximately as a 16.5 kDa band on SDS-PAGE (Fig. 1B top panel) and reacts with SVN antibody when analyzed on western blot (Fig. 1B bottom panel).

Next we tested if the 3LL cell line we are planning to use for this study expresses SVN. We looked at the expression of SVN in both cell lysate and supernatant from 3LL cell line along with other tumor cell lines like TC-1 and A20. As shown in Figure 2, cell lysate from all the cell line scored positive for SVN (Fig. 2A) while we did not see SVN expression in supernatant for either cell lines (Fig. 2B). These observations are consistent with SVN being a universal TAA.

Single vaccination with SVN+SA-4-1BBL eradicated 3LL tumor and this efficacy was primarily dependent on CD8^{+} T cells with partial involvement of NK cells

First we evaluated the therapeutic efficacy of SVN+SA-4-1BBL vaccine in 3LL

tumor model. C57BL/6 mice were challenged s.c. with 1×10^5 live 3LL cells and

vaccinated once with either SVN alone (50 µg) or with SA-4-1BBL (25 µg) six days

post-tumor challenge. Tumor growth and lifespan of the mice were followed for over a

90-day period. As shown in Figure. 3, SVN+SA-4-1BBL vaccine was able to eradicate

tumors in approximately 70% of mice while efficacy of SVN alone could only reach to <

20%. In contrast, all PBS control mice were expired within 60 days.

The therapeutic efficacy of the vaccine was associated with robust CD8$^+$ T cells, NK

cells effector and T cell proliferative responses

One of the major reasons for the failure of current tested SVN-based vaccines in

the clinic is due to their limited ability to mount optimal CD8$^+$ T cells effector response

on a broad patient population. The importance of T cell in the successful therapy can be

deduced from this fact that the strategies which augment T cell effector responses to

weak tumor antigens can only be translated into effective therapy (90). Therefore we

evaluated the impact of SVN+SA-4-1BBL vaccine on CD8$^+$ T cells effector response by

measuring the IFN-γ in the long-term tumor free animals one week after boosting with

SVN+SA-4-1BBL. As shown in Figure 4A, long-term mice subjected to SVN+SA-4-

1BBL vaccine had significantly enhanced number of CD8$^+$ T cells expressing IFN-γ as

compared to naïve mice, which translated into a robust SVN-specific CTL killing

response against 3LL targets (Fig. 4B).

As we (unpublished data) and others have already demonstrated that 3LL cells

acquire low level of MHC class I (91, 92) as a means to escape CTL and survive. As cells

missing or low expression of MHC class I molecules lose resistance to NK cells and

become susceptible to NK cell-mediated lysis (93, 94), we next tested the role of NK cells on vaccine efficacy. We performed the NK cell specific killing assay against YAC-1 targets and observed a significant higher killing at all E:T ratios in mice subjected to SVN+SA-4-1BBL as compared with naïve mice (Fig. 5A).

We also tested if long-term animals are able to generate T cell proliferation response after SVN exposure. To determine SVN-specific proliferation response, splenocytes from naive and long term boosted mice were cultured in the presence of soluble SVN protein for 5 days. As shown in Fig. 5B, long-term boosted mice generated significantly higher SVN protein-specific *in vitro* lymphoproliferative response as compared with naïve animals.

Taken together, these data suggest the requirement of both $CD8^+$ T cells and NK cells for the success of SVN+SA-4-1BBL based therapeutic effects.

$CD8^+$ T cells are critical while NK cells also play an important role in the efficacy of SVN+SA-4-1BBL vaccine

As SVN+SA-4-1BBL vaccine was able to generate potent $CD8^+$ T cells effector response with moderate NK cell killing response, which correlated with the therapeutic efficacy, we next depleted these cells to evaluate the direct involvement of these cells in the efficacy of vaccine. As shown in Figure 6, depletion of $CD8^+$ T cells completely abrogated the efficacy of the vaccine as all $CD8^+$ T cells depleted animals died before day 50 due to tumor burden or discomfort. NK cells were also found to be a key player in the efficacy of vaccine as depletion of NK1.1 attenuated the survival of the mice from 80% to 40%. We did not find any involvement of $CD4^+$ T cells as depletion of these cells did not affect the efficacy of the vaccine.

28

Additional vaccination improved the therapeutic efficacy of the vaccine and this efficacy was dependent on the timing of the vaccine

We next evaluated if additional vaccine will further improve the therapeutic efficacy of the vaccine. Therefore, we boosted the animals with SVN alone or SVN+SA-4-1BBL at various time points. As shown in Figure 7A, an additional booster of SVN+SA-4-1BBL on day 12 (6 days after first vaccination) improved the survival of the mice to 100% while SVN alone could improve the efficacy to only 25%. Interestingly, the timing of booster was critical to vaccine efficacy as booster on day 9 and day 15 could not improve the survival benefit over one vaccination (Fig. 7B). Although we observed attenuated therapeutic efficacy of vaccine on day 9 booster than single vaccination, it did not reach to a significant level.

Taken together, these data suggest that prime-boost regimen is required and timing of the boost is critical for the optimal SVN+SA-4-1BBL vaccine efficacy in 3LL model system.

Improved efficacy with additional vaccination was associated with $CD8^+$ T cells effector function, but not the NK cells

Our earlier data of *in vivo* depletion (Fig. 6) demonstrated the critical role of $CD8^+$ T cell in the therapeutic efficacy of the SVN+SA-4-1BBL vaccine. Therefore, we next evaluated the mechanistic basis of this therapeutic advantage of additional booster by measuring the $CD8^+$ T cell effector responses. As shown, this therapeutic advantage was strictly due to increased $CD8^+$ $IFN\text{-}\gamma^+$ T cell (Fig. 8A) and cytotoxic responses (Fig. 8B), while NK cell response was not changed when compared with one vaccination (Fig.

29

8C). Taken together, these data suggest that additional vaccination enhance antitumor immunity by increasing the CD8$^+$ T cell effector function but not the NK cell response.

SVN+SA-4-1BBL-based vaccine generated humoral response without any sign of autoimmunity

Recently, involvement of humoral immune response to tumor immunotherapy has been widely appreciated (95, 96), especially after the approval of Herceptin, an antibody-based vaccine, as a part of treatment regimen against Her2 expressing breast cancer. Therefore, we further investigated if SVN+SA-4-1BBL vaccine could also augment the humoral response. Sera from vaccinated mice were analyzed for titer to SVN-specific antibody. As shown in Figure 9A, SA-4-1BBL mediated costimulation triggered the humoral response when compared with SVN alone.

We also evaluated the effect of SVN+SA-4-1BBL vaccine on sign of autoimmunity as one of the major issues with the utility of self-antigens is the induction of autoimmunity which could be detrimental to the patients. Therefore, we tested the presence of autoantibody in the sera of vaccinated mice. As shown in Figure 9B, SVN-SA-4-1BBL vaccine did not generate antibody against ssDNA as compared to sera from lupus animals as a positive control.

Discussion

The efficacy of TAA-based therapeutic vaccine depends on the utility of immunogenic antigens and potent adjuvants which increase the breadth and intensity of the immune response of the vaccine. In the present study, we provide the evidence of

30

mechanistic role of the SVN+SA-4-1BBL vaccine and demonstrate that two vaccination of this novel tumor vaccine results in the 100% survival of mice against 3LL tumors. This level of efficacy, to our knowledge, has never been achieved before with SVN. The efficacy of vaccine is associated with CD8 and NK cell effector functions. Physical depletion of CD8, CD4, and NK cells suggests that $CD8^+$ T cells are the most critical player in the vaccine efficacy while NK cells also have an important contribution but not the $CD4^+$ T cells. Importantly, the efficacy of vaccine is dependent on the timing of the treatment. SVN+SA-4-1BBL also generate a potent humoral response without any sign of autoimmunity.

In recent years, TAA-based therapeutic vaccines have emerged as one of the most attractive approach for the treatment of cancers. In this context, SVN has been extensively used as an ideal TAA target for immunotherapeutic approaches due to its high levels of expression in most of the cancers with potential clinical applications. Over-expression of SVN correlates with tumor progression and constitutes a poor patients survival (83, 84). In fact the advantage of utilization of SVN for vaccine development is not only relying on its broad expression patterns on majority of tumors, but also on the fact that down-regulation or loss of its expression would hamper the tumor progression and/or enhance the tumor cell death. These virtues make SVN based immunotherapy an ideal approach to treat variety of cancers. However, the development of successful therapeutic vaccines based on SVN self-antigen has been very limited in terms of tumor eradication. In this regard, utilization of effective adjuvant as a part of vaccine formulation holds great importance for the vaccine success. Recently, we have demonstrated that costimulatory ligand SA-4-1BBL can be exploited as potential

adjuvant of choice for the development of therapeutic cancer vaccines against TC-1 tumors (15, 16). Consistent with our previous study, here we demonstrate that SVN in conjunction with SA-4-1BBL costimulatory adjuvant resulted into an impressive efficacy in the eradication of 3LL tumors in > 70% of mice while efficacy with SVN alone could reach to < 20%. The better efficacy of SVN+SA-4-1BBL was correlated with increased $CD8^+$ T cells IFN-γ response which resulted into the higher CTL and NK cell killing responses against 3LL and YAC-1 tumor cells, respectively.

Our results further show that an additional booster vaccination improves the efficacy of SVN+SA-4-1BBL vaccine from 70% to 100% in this model. Importantly, this improved efficacy of additional booster was correlated with increased $CD8^+$ IFN-γ^+ T cells response and CTL cytotoxicity. Physical depletion of CD8+ T cells completely abrogated the therapeutic efficacy directly confirmed the critical role of $CD8^+$ T cells in the vaccine efficacy in this model system. One of the major reasons for the failure of current tested SVN-based vaccines in the clinic is due to their limited ability to mount optimal $CD8^+$ T cells response on a broad patient population. The importance of T cell in the successful therapy can be deduced from this fact that the strategies which augment T cell responses to weak tumor antigens can only be translated into effective therapy (90). In this regard, SVN+SA-4-1BBL vaccine utilized in this study has shown great promise due to virtue of SA-4-1BBL as it targets $CD8^+$ T cell effector function which translates into an effective therapy (15). Role of $CD8^+$ T cells generated by SVN+SA-4-1BBL vaccine in our study is in the agreement with several other reports which suggest that T cells mounts a vigorous cytotoxic response against survivin both *in vitro* and *in vivo* (97, 98). Additionally, Xiang et al. (87) has also demonstrated that survivin-based DNA

vaccine induces potent antitumor response against Lewis Lung Carcinoma and efficacy of vaccine was dependent on antigen-specific CD8$^+$ T cell response which triggered tumor cell apoptosis, together with release of IFN-γ. SVN+SA-4-1BBL vaccination also enhanced the memory response evaluated by the increased SVN-specific T cell proliferation in the long-term animals suggested that vaccine has potential to check the recurrences.

We next observed that vaccine was also able to generate NK cell killing against YAC-1 target cells. Depletion of NK cells reduced the efficacy from > 70% to 40% further confirmed the involvement of NK cells in the efficacy of SVN+SA-4-1BBL vaccine in this model which was found to be additional mode of vaccine action. As we (unpublished data) and others have already demonstrated that 3LL cells acquire low level of MHC class I (91, 92) as a means to escape CTL and survive. Cells missing or low expression of MHC class I molecules lose resistance to NK cells and become susceptible to NK cell-mediated lysis (93, 94). This is consistent with the previous reports by several groups demonstrating the crucial role NK cells play in killing of class I negative tumors (99).

Next our results show that timing of the additional booster vaccination is critical for the optimal vaccine efficacy. We administered the additional booster vaccination at different time points after the first vaccination and to our surprise, we found out that additional booster given 6 days after first vaccination was only able to improve the efficacy of vaccine while booster on 3 days or 9 days after first vaccine didn't affect the vaccine efficacy. This suggests that timing of the booster is critical for the optimal efficacy. Surprisingly, booster on 3 days (early) after first vaccination relatively, although

not significantly, lower the therapeutic efficacy of the vaccine when compared to single vaccination. Recently, it has been reported that, depending on the timing of the treatment, anti-4-1BB Ab can either cause activation-induced cell death (AICD) or enhance immunity in viral setting (100). When given early after infection, anti-4-1BB was immunosuppressive and associated with depletion of virus-specific $CD8^+$ T cells while at the later time points; the $CD8^+$ T cell response was augmented.

One of the major concerns for self-TAA based immunotherapy is the potential autoimmune abnormalities. Next we demonstrate that SVN+SA-4-1BBL vaccine did not generate any sign of autoimmunity as we couldn't detect any autoantibody against ssDNA in the sera of the mice treated with vaccine. However, we detected higher SVN-specific antibodies (humoral response) in the mice subjected to SVN+SA-4-1BBL vaccine. Although generation of anti-SVN antibodies following therapeutic immunization is currently not being seen as one of the predominant aspects of potential antitumor response, existence of circulating antibodies against SVN has already been reported in variety of human cancers (101, 102). Therefore, presence of these SVN-specific antibodies in the cancer patients may predict as a means of host defense against tumors. In this perspective, generation of SVN-specific humoral response by our vaccine may be advantageous from the therapeutic point of view.

In conclusion, the vaccine strategy proposed in this study stand a chance of translating into the clinics as the operational approach employed in this study along with virtue of general applicability of our vaccine as a potent means of immunotherapy, provides a rational for the further development of future therapeutic strategy against cancer.

A

pTWIN-1   pTWIN-1

6X-His   mSVN

Nde I   Kpn I   BamH I

B

Marker Pre-binding Post-binding Wash 1 Wash 2 Elution 1 2 3 4 5

15K→

SDS page
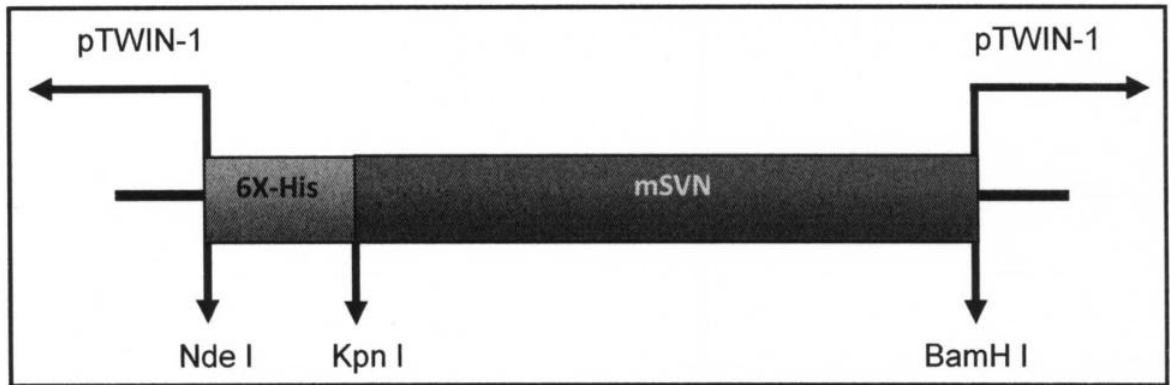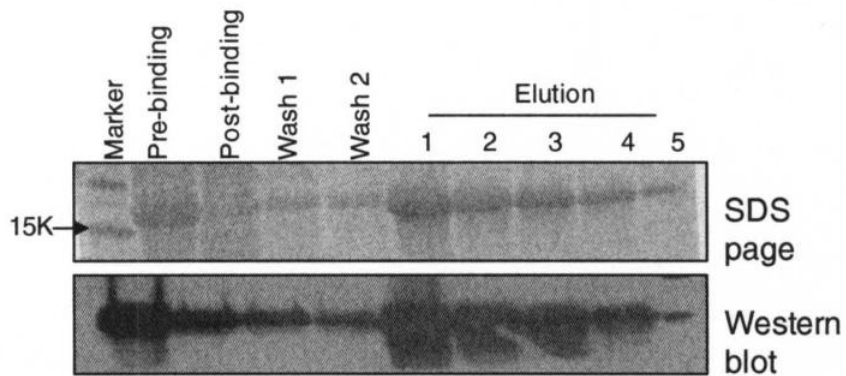
Western blot

Figure 1

Production of recombinant mouse-Survivin. (A) Schematic representation of pTWIN-1 expression vector with SVN, (B) Coomassie blue staining (top) and western blot (bottom) patterns of IMAC purified recombinant SVN.

A

SVN (+ control)
TC-1 cell line
A20 cell line
3LL cell line

Tumor cell lysate

B

SVN (+ control)
TC-1 cell line
3LL cell line
Serum only
SVN standard
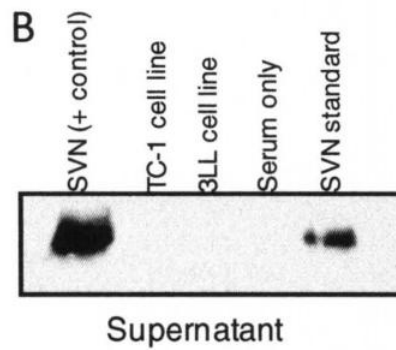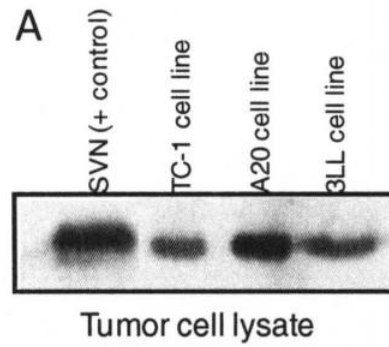
Supernatant

Figure 2

SVN expression on different tumor cell lines. Cell lysate (A) and culture supernatant (B) from TC-1, 3LL and A20 cell lines were prepared and analyzed using western blot. Expression of SVN was detected by anti-SVN Ab.
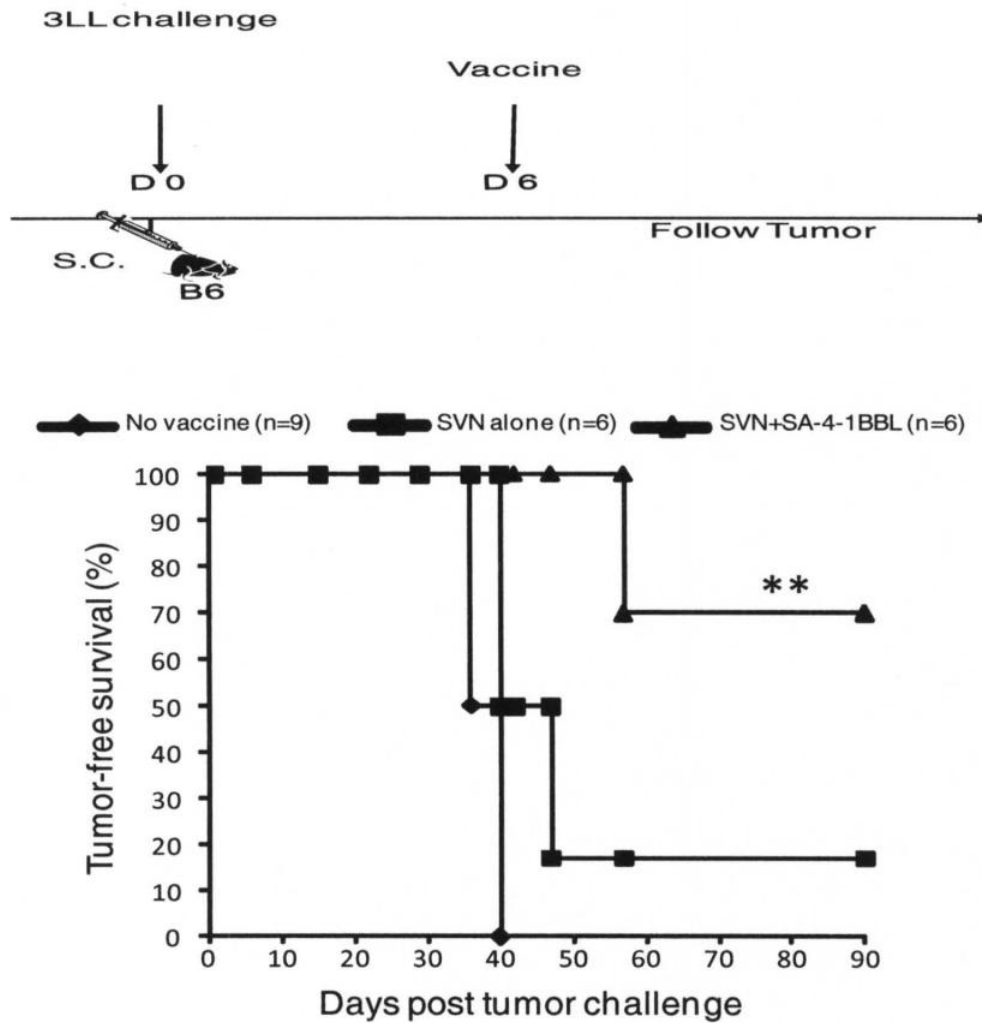
Figure 3

SVN+SA-4-1BBL vaccine eradicates SVN expressing 3LL tumors. C57BL/6 mice were challenged with live 1×10^5 3LL cells and vaccinated once s.c. on day 6 post-tumor challenge with either SVN (50 μg) alone or with SA-4-1BBL (25 μg). ** $P < 0.001$ for SVN+SA-4-1BBL *vs.* all other groups.
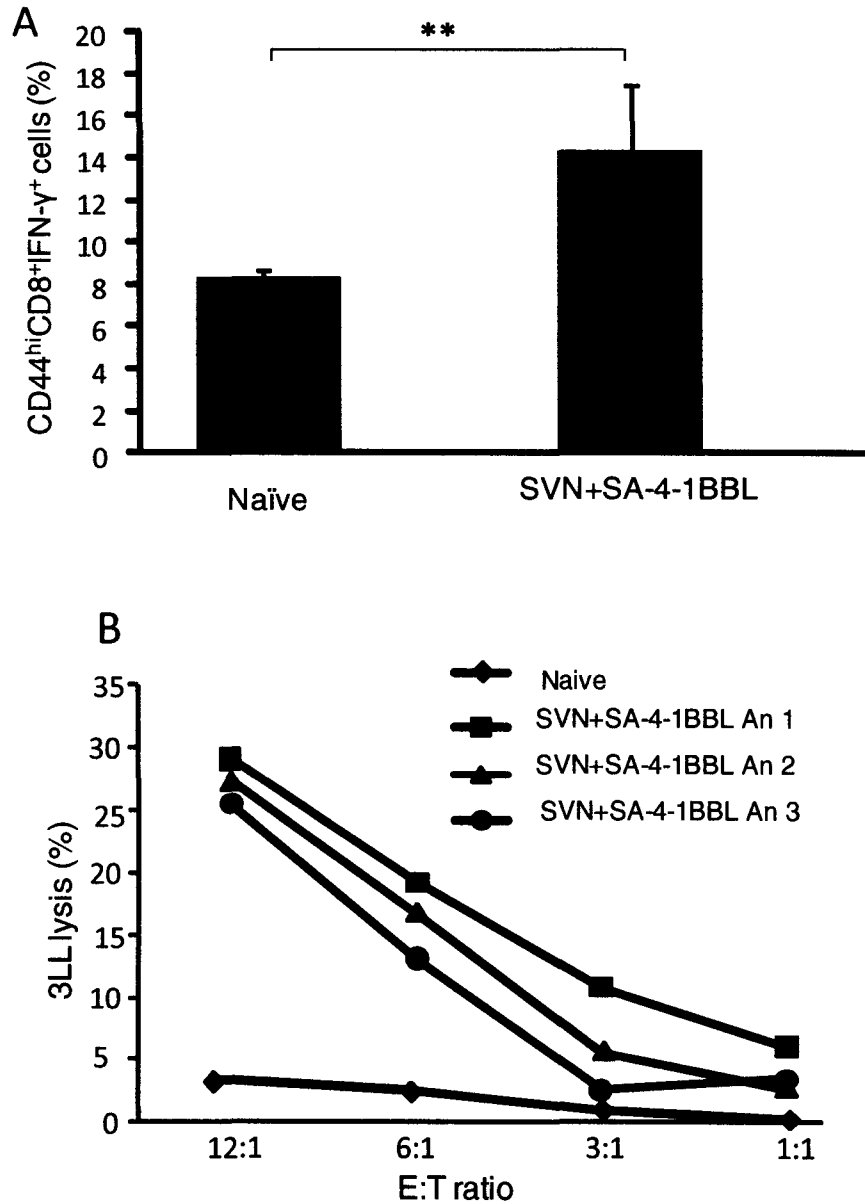
Figure 4

SVN+SA-4-1BBL based vaccine induces a strong antitumor $CD8^+$ T cell response. Long-term survived animals treated with SVN+SA-4-1BBL vaccine develop E7-specific $CD8^+$ T cell effector response after boosting as determined by (A) increased $CD8^+$ T cell intracellular IFN-γ^+ expression, (B) increased CTL killing. n = 3 or 4; ** $P < 0.001$ were calculated using student t-test. Data is representative of two independent experiments.
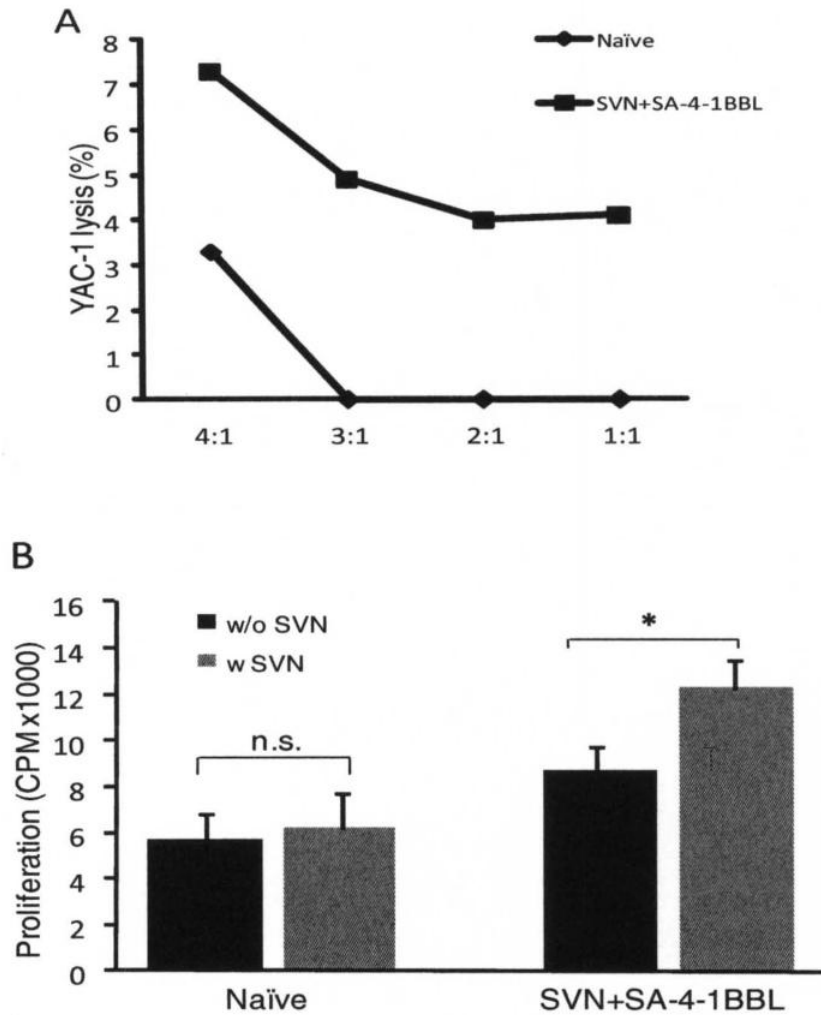
Figure 5

SVN+SA-4-1BBL based vaccine induces NK cell killing and T cell proliferation responses. Splenocytes from long-term survived animals boosted with SVN+SA-4-1BBL vaccine was cultured in the presence of IL-2 for 5 days to be used as effector cells and plated against YAC-1 target cells in various E:T ratios to determine the NK cell killing response (A). Splenocytes from above was also cultured with or without 5μg/ml SVN for 5 days to determine SVN-specific proliferation (B). (n =3 or 4), * P < 0.05 were calculated using student t-test, ns = not significant. Data is representative of two independent experiments.
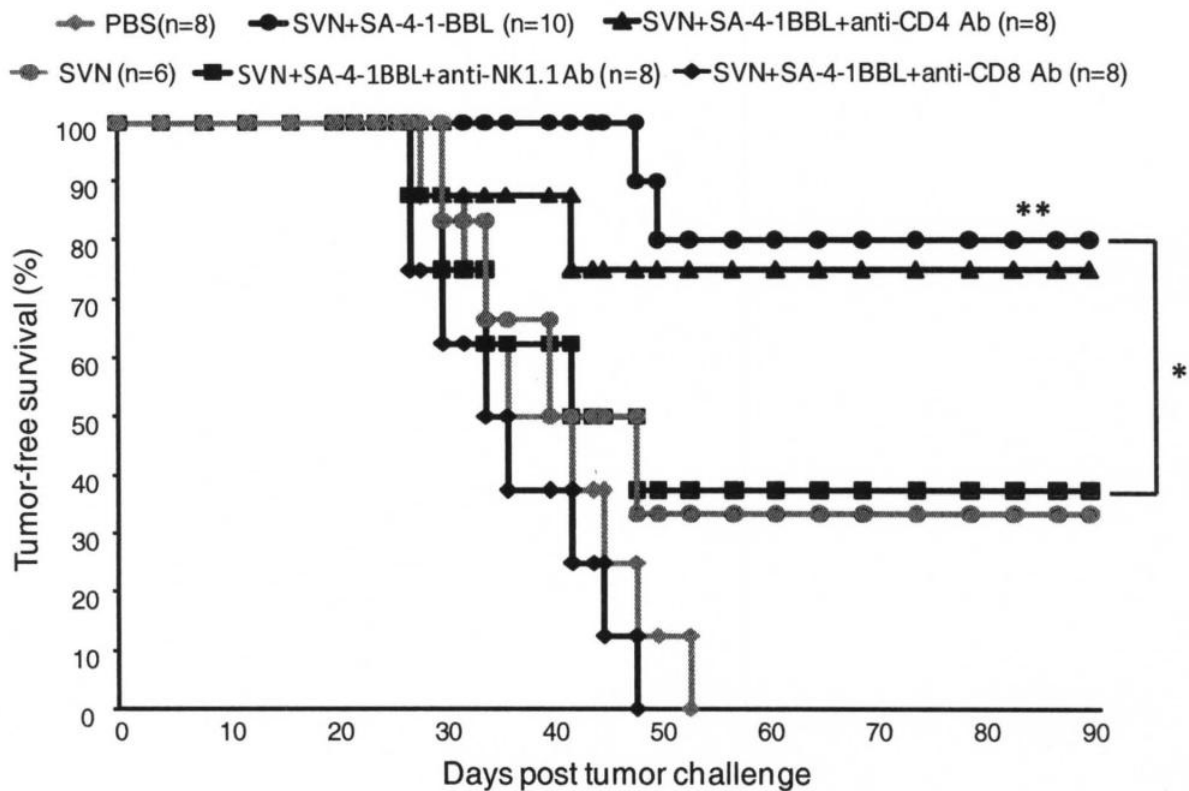
Figure 6

Efficacy of SVN+SA-4-1BBL based vaccine is mainly dependent on $CD8^+$ T cells followed by NK cells but not on $CD4^+$ T cells. The role of $CD8^+$ T cells and NK cells in the efficacy of vaccine was evaluated by depleting these cells with single i.p. injection of anti-CD8 (500 µg), anti-CD4 (300 µg) and anti-NK1.1 (500 µg) Abs/mouse one day before vaccination. The depletion of these cells was complete as monitored at day 5 post treatment. ** P < 0.001 for SVN+SA-4-1BBL *vs.* SVN+SA-4-1BBL+anti-CD8 Ab group while * P < 0.05 for SVN+SA-4-1BBL *vs.* SVN+SA-4-1BBL+anti-NK1.1 Ab group. (This experiment was performed in collaboration with Rajesh Sharma).
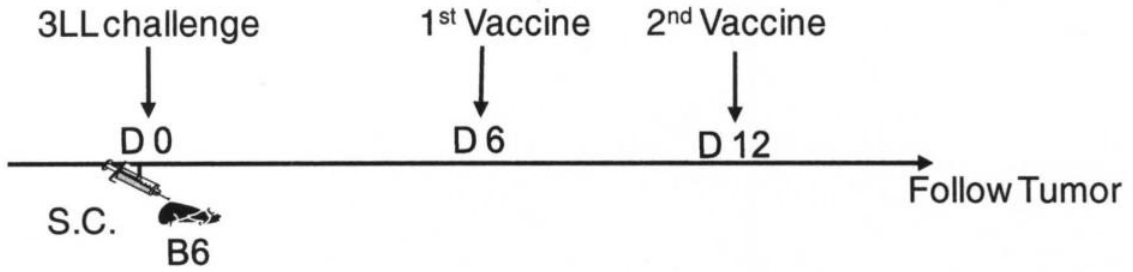
B
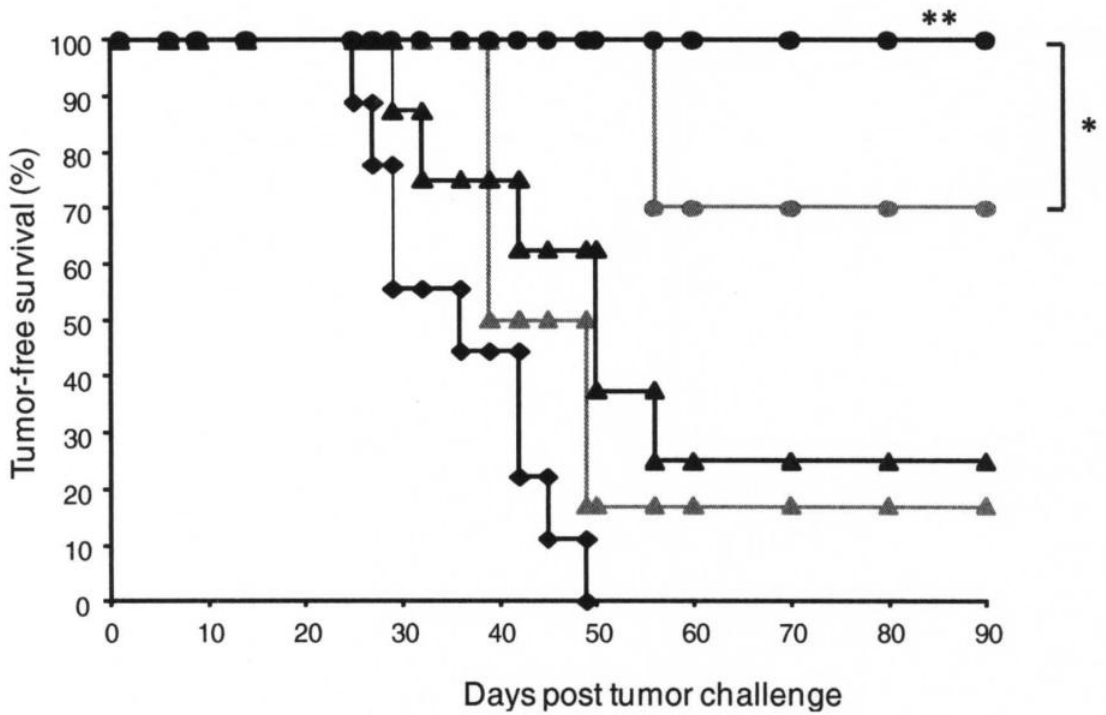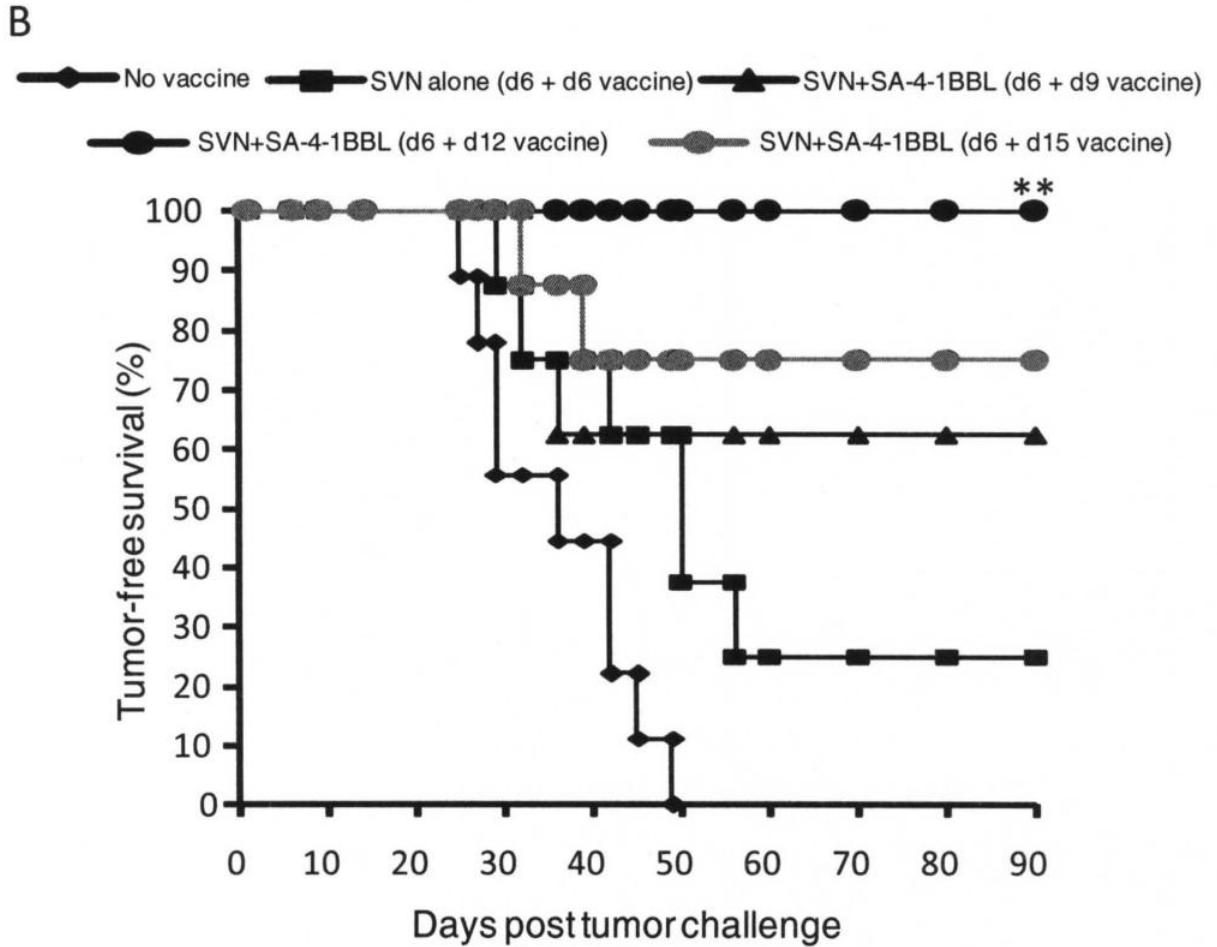
Figure 7

Additional vaccination of SVN+SA-4-1BBL completely eradicates the tumor and this improved efficacy is dependent on the timing of the booster. (A) C57BL/6 mice were challenged with live 1×10^5 3LL cells and vaccinated either once s.c. on day 6 or twice on day 6 and day 12 post-tumor challenge with either SVN (50 µg) alone or with SA-4-1BBL (25 µg) (B) booster was also administered either 3 days or 9 days after the first vaccination. ** $P < 0.001$ for SVN+SA-4-1BBL two vaccine *vs.* SVN alone and PBS controls while * $P < 0.05$ for SVN+SA-4-1BBL two vaccine *vs.* SVN+SA-4-1BBL one vaccine group. Data is pooled from two independent experiments.
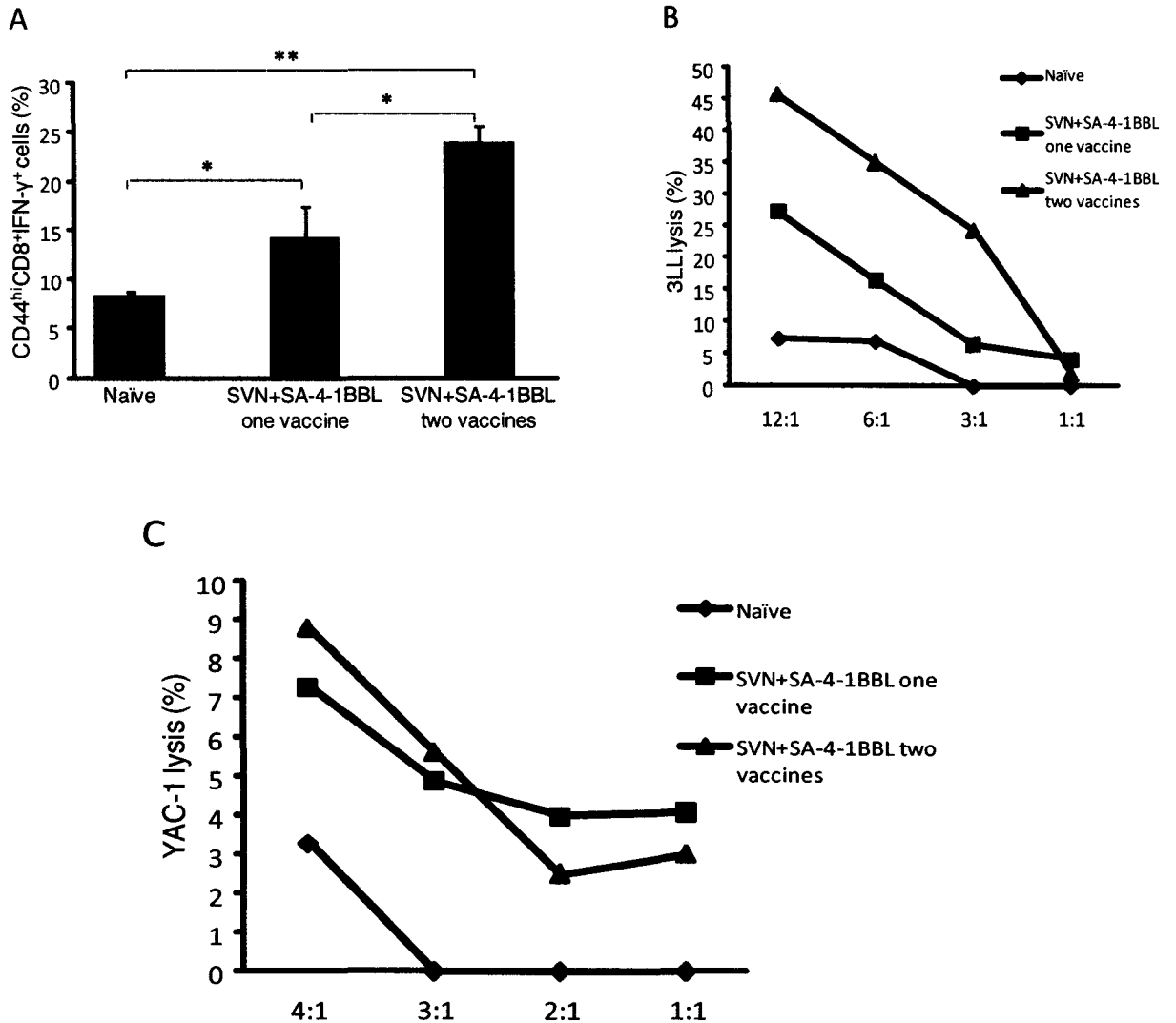
Figure 8

The increased therapeutic efficacy of booster vaccination is associated with improved $CD8^+$ T cells effector response but not the NK cells as demonstrated by (A) improved $CD8^+$ T cell intracellular $IFN-\gamma^+$ expression, (B) increased CTL killing, but (C) no difference in NK cell killing response. *P* values were calculated using student t-test.
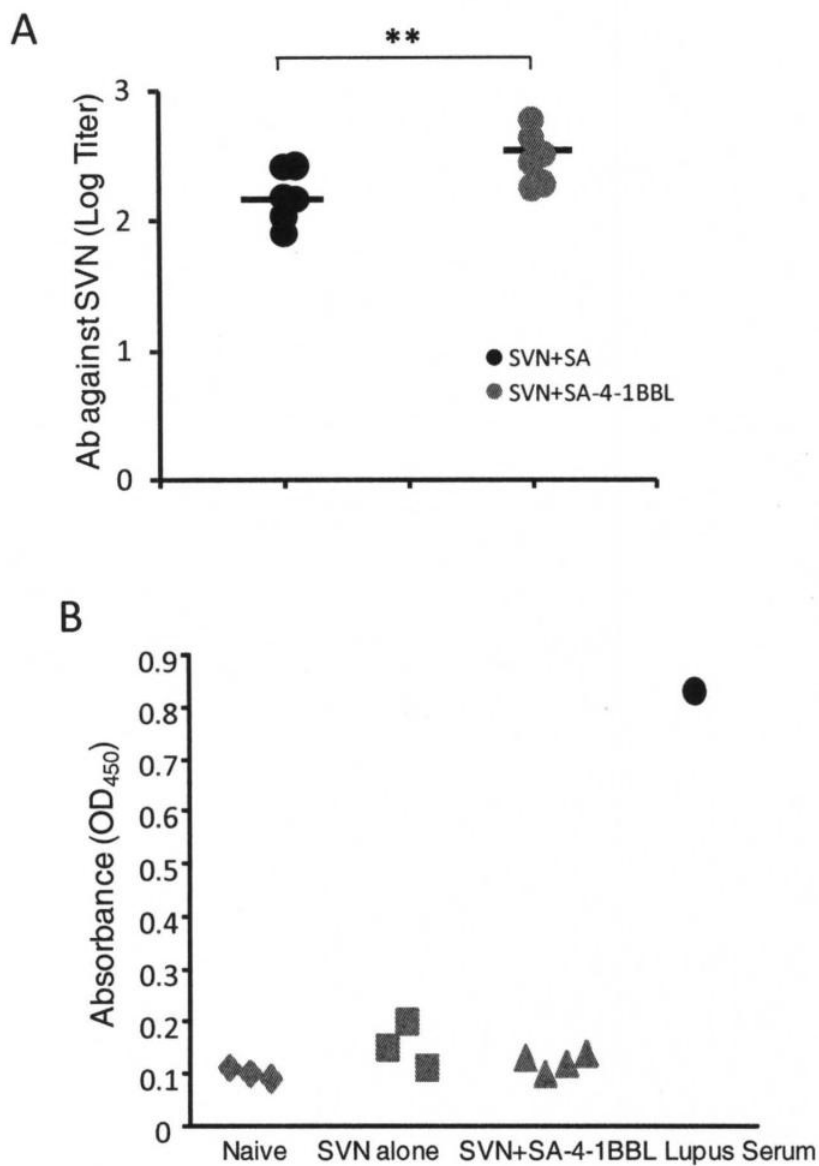
Figure 9

SVN+SA-4-1BBL based vaccine induced SVN-specific antibody without sign of autoimmunity. (A) Sera from naïve (n=6) and long-term tumor free animals (n=6) were tested for SVN-specific antibody using ELISA. (B) Sera from above animals were also tested for autoantibodies against ssDNA. Sera from pooled (n=3) lupus animals were used as positive control. *P* values were calculated using student t-test.

CHAPTER 3

SA-4-1BBL AND OX40L COMBINATION AS AN ADJUVANT SYSTEM SHOWS

ROBUST THERAPEUTIC EFFICACY IN ESTABLISHED TC-1 TUMORS

Introduction

One of the major advantages of utilization of vaccination strategy against cancers is its ability to elicit an immune response to primary tumors and maintain an immunological memory upon the reoccurrences. Given the most solid tumors usually express MHC class I, but not MHC class II, role of $CD8^+$ T cells in the eradication of tumors attracted more attention than $CD4^+$ T cells. However, it is critical to consider the role of $CD4^+$ T cells in the exertion of $CD8^+$ T cell independent antitumor response as well as the generation and persistence of memory CTL responses for designing the vaccine against tumors. $CD4^+$ T cells can induce antitumor immunity by recruiting and activating macrophages and eosinophils and this effect can be independent of $CD8^+$ T cells (103, 104). $CD4^+$ T cells generate IFN-γ response which has antitumor or antiangiogenic effects (105). In contrast, $CD4^+$ T cells also help enhancing the antitumor immunity of $CD8^+$ T cells (106, 107). These above observations indicate the importance of $CD4^+$ T cells in tumor immunotherapy and justify the design of vaccines that aimed at inducing a $CD4^+$ T cell response for effective therapy.

We started this study based on our previous observations with E7+SA-4-1BBL vaccine in TC-1 tumor (16) and SVN+SA-4-1BBL vaccine (Chapter 2) in 3LL tumor

which suggests the lack of CD4$^+$ T cells involvement in the vaccine efficacy. These findings could be interpreted as the inability of SA-4-1BBL-based vaccine to induce CD4$^+$ T cells response or that CD4$^+$ T cells do not play any role in the vaccine efficacy. We, therefore, addressed this issue by including an adjuvant with potent impact on CD4$^+$ T cells in the SA-4-1BBL based vaccine formulation. In this regard, costimulatory OX40L was the primary adjuvant of interest due to its critical role on the generation of potent CD4$^+$ T cell responses.

Recently, the costimulatory OX40, a member of tumor necrosis factor receptor (TNFR) superfamily has been extensively studied as a potent antitumor agent. OX40 has been shown to be expressed by CD4$^+$ T cells and CD8$^+$ T cells (67, 108). OX40 has been widely studied on CD4$^+$ T cells due to its high level expression after T cell activation. Signaling *via* OX40 has shown increased migration, increased cytokine production by effector cells, and increased numbers of CD4$^+$ memory T cells (108-110). Agonistic ligand to OX40 receptor has been widely tested as effective adjuvant of therapeutic cancer vaccines in both preclinical and clinical settings. Administration of OX40L/immunoglobulin fusion protein or OX40R mAb resulted in a significant prolongation of survival of tumor-bearing mice (62). In most of the studies, the antitumor responses generated by these agonists are associated with increased activity CD4 effector functions. Most importantly, OX40 signaling directly inhibits the Tregs suppression (72) makes it an attractive adjuvant for the development of therapeutic cancer vaccines. Therefore, we hypothesize that by providing CD4$^+$ T cells help and targeting Tregs via OX40L will further enhance the antitumor efficacy of SA-4-1BBL vaccine which primarily targets the CD8$^+$ T cells (Fig. 10).

In this study, we generated a novel form of OX40L by fusing the extracellular functional domain of this molecule to a modified form of core streptavidin (SA) to generate a chimeric molecule (SA-OX40L) that exists as tetramers and oligomers owing to the structural features of SA. We here in show that this chimeric SA-OX40L has preferential activity on CD4$^+$ T cells *in vitro* while has activity on both CD4$^+$ T cells and CD8$^+$ T cells *in vivo*.

Next, based on our previous observation of > 70% of therapeutic efficacy with SVN+SA-4-1BBL vaccine, we sought to improve this efficacy by combining SA-4-1BBL with SA-OX40L which may further overcome the immune tolerance. A single vaccination of SA-4-1BBL and SA-OX40L combination as a part of E7-based therapeutic vaccine resulted into eradication of E7-expressing TC-1 tumors in 100% mice. The efficacy of the combination vaccine is correlated with higher antigen specific CD8$^+$ T cell responses as compared to single agents. The combination vaccine was also able to eradicate 3-4 mm size TC-1 tumors in 50% of the mice while single agent has only 10% efficacy. Together, these data suggest that combination of SA-4-1BBL and SA-OX40L can be exploited to use as a new therapeutic vaccine strategy which has great promise to be successful in the clinic.

Material and methods

Mice and cell lines

C57BL/6 and C57BL/6.SJL mice were bred and maintained under specific pathogen-free conditions in our barrier animal facility at the University of Louisville. All

animals were cared for in accordance with institutional and NIH guidelines. TC-1 cell line was purchased from ATCC (Manassas, VA) and maintained as published (15, 111).

Antibodies and other reagents

Fluorochrome-conjugated anti-CD8-APC-Cy7, anti-CD62L-PE, anti-CD44-APC, anti-TNF-PE, anti-IFN-γ-PE-Cy7, and anti-IL-2-PerCp-Cy5.5, and isotype controls were purchased from BD Bioscience, eBioscience, and BioLegend. The HPV16 RAHYNIVTF E7 peptide (E7$_{49-57}$), SA-4-1BBL, E7, and SA proteins were reported previously (15, 111)

Construction and expression of chimeric SA-OX40L protein

The extracellular functional domain of OX40L molecule was synthesized at GenScript USA Inc. (Piscataway, NJ). The accurate sequence for OX40L was digested with *Eco*RI and *Xho*I and subcloned into pMT-Bip-V5-HisA expression vector containing a 6x-His Tag and core streptavidin (SA) sequence as previously published (81). The chimeric SA-OX40L was expressed using *Drosophila* expression system (DES; Invitrogen Life Technologies) followed by purification using IMAC Sepharose column, endotoxin testing, and quantification.

Receptor Binding Assay

Receptor binding assay was performed as previously published (81). In brief, splenocytes were stimulated with 5 µg/ml Con A (Sigma-Aldrich) in complete MLR media for 48 h. Naïve or Con A activated splenocytes were then incubated with SA-OX40L (200ng/10^6 cells) or molar equivalent of SA control protein on a shaker at 4 ^{0}C

48

for 30 min. Following incubation, cell were washed extensively, stained with CD4-APC, CD8-PercP and SA-FITC, and analyzed on flow.

T cell proliferation assay

Both $CD4^+$ and $CD8^+$ T cells were sorted from naïve C57BL/6 mice and cultured separately with 0.25 µg/ml anti-CD3 (BD Biosciences) and irradiated syngeneic splenocytes in the presence of various concentrations of SA-OX40L or SA control proteins. After four days, culture was pulsed with $[^3H]$thymidine during the last 16 h of the culture and harvested on a Tomtec Harvester 96 for analysis.

For *in vivo* proliferation assay, OT-I and OT-II (CD45.2) cells were labeled with 2.5 µM CFSE and $2x10^6$ OT-I and OT-II cells were transferred by i.v. into naïve C57BL/6-SJL (CD45.1). After 24h, mice were immunized s.c with OVA (3 µg) alone or with SA-4-1BBL (5 µg) or SA-OX40L (5 µg) or combination of both agents (5 µg+ 5 µg). After 3 days, peripheral LNs 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 flowcytometry.

Tumor models and vaccination

C57BL/6 mice were subcutaneously (s.c.) challenged with $1x10^5$ TC-1 cells into the right flank followed by single s.c. vaccination on day 6 post-tumor challenge with various vaccine formulations containing E7 protein (50 µg) alone as control or with SA-4-1BBL (25 µg), SA-OX40L (25 µg), or the combination of both adjuvants (25 µg/adjuvant). The diameters of tumors were measured with caliper and mice were euthanized when tumor reached a size of 12 mm in diameter, ulcerated, or mice showed

signs of discomfort. For depletion study, CD8$^+$ and CD4$^+$ T cells were depleted using Abs against CD8 (clone 53.6.72) and CD4 (clone GK 1.5) at 500 µg/mice via intra peritoneal (i.p.) injection one day before vaccination.

For therapy with established tumors, mice with ~3-4 mm TC-1tumors were vaccinated with above described formulations twice at 5 days interval. Tumors were followed for maximum of 100 days or until mice were euthanized due to tumor reached a size of 12 mm in diameter, ulcerated, or mice showed any sign of discomforts.

Flow cytometry

Spleens and/or tumor draining lymph nodes (TdLNs) were processed as described previously (16). For memory T cell typing, lymphocytes were stained with anti-CD8-APC-Cy7, anti-CD62L-FITC, and anti-CD44-APC Abs. For intracellular cytokine staining, lymphocytes (1x10^6 cells/mL) were stimulated either with 10 µg/mL E7$_{49-57}$ peptide or whole E7 protein for 2 hrs followed by incubation with GolgiPlug (1 µl/mL, BD PharMingen) overnight. Cells were first stained with anti-CD8-APC-Cy7, anti-CD4-Alexa700, and anti-CD44-APC, fixed with 4% paraformaldehyde followed by staining with anti-IFN-γ-PE-Cy7, anti-IL-2-Percp-Cy5.5, or isotype controls.

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

Generation of chimeric SA-OX40L with demonstrated activity in soluble form

Under physiological conditions, cross-linking of costimulatory receptors by cell membrane-bound ligands is critical for potent signal transduction and immune cell activation. Due to this prerequisite, most of the costimulatory ligands have attenuated

50

activity in the soluble form. Although agonistic Abs to these receptors is effective in delivering immunological signals, we chose to use natural ligands since signals generated by agonistic Abs is associated with severe toxicity (79). Based on our previously constructed chimeric SA-4-1BBL molecule which has shown potent activity in the soluble form in the absence of severe toxicity (15, 79, 81), we generated a chimeric SA-OX40L costimulatory molecule due to its critical role on both effector and regulatory arm of the immune system.

The extracellular domain of mouse OX40L, which is critical for binding and delivery of immunological signals via its receptor OX40, was cloned C-terminal to SA to ensure proper protein folding as OX40L is a type II membrane protein (Fig. 11A). This chimeric gene was then subcloned into pMT-Bip-V5-HisA expression vector and expressed using *Drosophila* expression system. As shown in Fig. 11B, under native condition SA-OX40L protein existed as tetramers and higher structures while under native condition it existed as monomers, owing to the structural characteristics of SA (Weber et al. Science, 1989). The chimeric SA-OX40L was able to bind OX40 receptor expressing on both activate $CD4^+$ and $CD8^+$ T cells while binding to naïve T cells was undetectably low (Fig. 11C).

Soluble SA-OX40L induce T cell proliferation

To evaluate the functional attributes of chimeric SA-OX40L *in vitro*, both sorted $CD4^+$ and $CD8^+$ T cells were stimulated with suboptimal anti-CD3 Ab in the presence of various concentrations of SA-OX40L. Costimulatory SA-OX40L was able to generate an effective proliferative response in $CD4^+$ T cells (Fig. 12A) but not in $CD8^+$ T cells (Fig.

51

12B). This response was both dose dependent and SA-OX40L specific as SA control protein fail to generate any response.

We next tested the functionality of SA-OX40L *in vivo* by utilizing OT-I and OT-II system. As consistent with *in vitro* study, SA-OX40L generated a vigorous proliferative response in $CD4^+$ OT-II cells. On the other hand, just oppose to *in vitro* study, SA-OX40L was able to generate $CD8^+$ OT-I proliferative response *in vivo* but it was not as robust as OT-II proliferation (Fig. 13).

Combination of SA-4-1BBL and SA-OX40L as the adjuvant component of E7 TAA-based vaccine completely eradicates the E7-expressing TC-1 tumors

As previously reported, a single vaccination with E7+SA-4-1BBL based vaccine was able to eradicate E7-expressing established TC-1 tumors in > 70% of mice and this efficacy was preferentially due to the involvement of $CD8^+$ T cell effector functions (16, 112). Keeping the aggressiveness of TC-1 tumors and utilization of single adjuvant in mind, this efficacy was turned out to be very impressive. Although, $CD8^+$ T cells are very critical for the elimination of tumors, involvement of $CD4^+$ T cells help cannot be ignored. Since 4-1BB signaling preferentially targets the $CD8^+$ T cells, probably this could be a possible reason our previously reported E7+SA-4-1BBL vaccine couldn't reach to its optimal potential. Therefore, we hypothesize that whether the therapeutic efficacy of this vaccine can further be improved by modifying the formulation to include SA-OX40L as the second adjuvant with primary effect on the $CD4^+$ T cells. A single s.c. vaccination with E7 protein mixed with SA-4-1BBL and SA-OX40L resulted in the eradication of TC-1 tumors in all mice for 90 day observation period (Fig. 14). In contrast, monotherapy with SA-4-1BBL and SA-OX40L resulted in eradication of tumor

in only > 70% and > 60% of mice, respectively. Mice that expired from tumor in the single agent groups, however, had slow kinetics of tumor progression as compared with both PBS and E7 protein control groups where all mice expired from the tumor burden around day 60 (Fig. 14). Taken together, these data demonstrate that combination of SA-4-1BBL and SA-OX40L formulation is effective in eradicating the TC-1 tumors than the individual agents.

The therapeutic efficacy of SA-4-1BBL and SA-OX40L combination is associated with the CD8$^+$ T cell effector and memory responses

CD8$^+$ T cell effector and memory responses are the key mechanisms immune system employ to eliminate the primary tumor and control the recurrences, respectively, in various tumor settings, including the TC-1 model (16, 113, 114). We, therefore, assessed the effect of combination vaccine on the CD8$^+$ T cell effector and long-term memory responses. Mice that had eradicated the tumor in response to various vaccine formulations were boosted s.c. with the same formulations and intracellular cytokine response of CD8$^+$ T cells to the dominant E7$_{49-57}$ epitope was tested one week later (15). In congruent with the therapeutic efficacy, combination of SA-4-1BBL and SA-OX40L generated a better antigen-specific cytokine response than single adjuvant therapy as assessed by CD8$^+$ T cell expressing IFN-γ single (Fig. 15A), and IFN-γ IL-2 double cytokines (Fig. 15B). There was no significant difference found between SA-4-1BBL and SA-OX40L monotherapy at cytokine levels. Additionally, combination vaccine formulation also generated the most effective CD8$^+$ T cell memory recall responses as compared to those including SA-4-1BBL and SA-OX40L as single agents (Fig. 15C). Most importantly, combination vaccine was also able to significantly reduce the

$CD4^+Foxp3^+$ Tregs in the spleen as compared to single agents (Fig. 15D). Combination vaccine was also able to generate better $CD4^+$ and $CD8^+$ T cell proliferation response *in vivo* as compared to single agents (Fig. 16). Collectively, these data demonstrate that combination of SA-4-1BBL and SA-OX40L generate potent $CD4^+$ and $CD8^+$ T cell proliferative and $CD8^+$ T cell effector and memory responses that correlate with the therapeutic efficacy of the vaccine against the TC-1 tumor.

$CD8^+$ T cells are the most critical player followed by $CD4^+$ T cells in the therapeutic efficacy of both SA-OX40L+SA-4-1BBL combination therapy as well as SA-OX40L monotherapy

Due to generation of $CD8^+$ and $CD4^+$ T cells responses by SA-4-1BBL and SA-OX40L, we used Abs against CD8 and CD4 molecules to deplete $CD8^+$ and $CD4^+$ T cells, respectively. Mice with TC-1 tumors were treated with depleting Abs one day before vaccination with E7 in conjunction with combination of SA-OX40L+SA-4-1BBL or SA-OX40L monotherapy. As shown in Fig. 17, depletion of $CD8^+$ T cells almost completely abrogated (100% *vs.* 16%) while depletion of $CD4^+$ T cells also significantly abrogated (100% *vs.* 40%) the therapeutic efficacy of SA-OX40L+SA-4-1BBL combination therapy. Depletion of $CD8^+$ T cells (66% *vs.* 20%) and $CD4^+$ T cells (66% *vs.* 33%) also significantly abrogated the efficacy of SA-OX40L monotherapy. Based on our previous data, depletion of $CD8^+$ T cells completely abrogated the efficacy of the SA-4-1BBL monotherapy while $CD4^+$ T cell depletion doesn't affect the efficacy (data not shown). Taken together, these data provide direct involvement of both $CD8^+$ and $CD4^+$ T cells in the therapeutic efficacy of combination as well as SA-OX40L mono therapies.

Combination of SA-4-1BBL and SA-OX40L vaccine formulation eradicates the established E7-expressing TC-1 tumors

Next we tested if combination vaccine formulation is able to eradicate more stringent established tumors. Mice with 3-4 mm size of TC-1 tumors were vaccinated twice on day 6 and day 12 with various vaccine formulations and followed for 100 days. As we expected, combination vaccine formulation was even able to completely eradicate 3-4 mm TC-1 tumors in 50% of mice (Fig. 18). In contrast, single agents were only able to eradicate tumors in > 10% of mice while all control mice died within 60 days.

Discussion

In this study, we demonstrated that the chimeric SA-OX40L had potent activity on both $CD4^+$ and $CD8^+$ T cells with preferential effects on $CD4^+$ T cells *in vivo*. Next, we demonstrated that addition of SA-OX40L in SA-4-1BBL vaccine formulation further improved the efficacy of the vaccine to 100% in TC-1 cervical cancer model. The efficacy of combination therapy was associated with robust primary $CD8^+$ T cell effector and long-term memory as well as $CD4^+$ T cell responses. Most importantly, the combination vaccine was also able to eradicate the more stringent 3-4 mm large TC-1 tumors in 50% of mice.

The choice of addition of OX40L, another member of costimulatory TNF ligand family, to SA-4-1BBL based vaccine was due to its potent effects on $CD4^+$, $CD8^+$ T and Tregs with preferential influence on $CD4^+$ T cells. As SA-4-1BBL primarily targets the $CD8^+$ T cells, attaining an additional $CD4^+$ T cells help is expected to improve the

therapeutic efficacy of the vaccine as $CD4^+$ T cell help is essential for generation of primary $CD8^+$ T cell response (115, 116). We chose to generate SA-OX40L instead of using already available agonistic anti-OX40 Ab due to qualitative and quantitative differences between natural ligand and agonistic Abs (79). As we expected, the generated chimeric SA-OX40L was able to induce potent $CD4^+$ and $CD8^+$ T cell proliferative response with preferential effect on $CD4^+$ T cells, which is consistent with previous studies with anti-OX40 Abs. Most importantly, combination of SA-4-1BBL and SA-OX40L was able to generate potent proliferative responses in both $CD4^+$ OT-II and $CD8^+$ OT-I cells *in vivo*. This efficacy further translated into a better therapeutic outcome against established TC-1 tumors.

The therapeutic outcome may be contributed by several mechanisms including synergy between 4-1BB and OX40 signaling. Although 4-1BB costimulation strongly targets the $CD8^+$ T cells while OX40 targets $CD4^+$ T cells, there is considerable overlap in their expression and functional mechanisms. Due to wide cellular distribution of these two receptors, it may increase the possibility of potential targets and therefore, enhance the chances of SA-4-1BBL and SA-OX40L to act on one or more cell types. It is likely that $CD8^+$ T cells are the common target for both the ligands as both the receptors are reported to be expressed on $CD8^+$ T cells following TCR signaling and enhance $CD8^+$ T cell response with the binding of agonistic mAbs, mostly *via* improved $CD8^+$ T cell effector function and their survival (67, 117). Therefore, dual costimulation through SA-4-1BBL and SA-OX40L could enhance effector function of $CD8^+$ T cells. Consistence with this notion, we observed an improved effector $CD8^+$ T cells response, as measured by IFN-γ and IL-2 double cytokines, in the mice subjected to SA-4-1BBL and SA-

OX40L combination therapy when compared to single agents. Most importantly, depletion of $CD8^+$ T cells in the combination therapy almost completely abrogated the vaccine efficacy suggested the critical role these cell population play in the combination therapy.

The effect of SA-OX40L on combination therapy, an alternative to its direct effect on $CD8^+$ T cells, could also be due to its influence on promoting $CD4^+$ T cells help. Although previous reports has shown the null effect of $CD4^+$ T cell help on the efficacy of anti-4-1BB and anti-OX40 mAbs combination therapy as depletion of $CD4^+$ T cells did not abrogate the efficacy (118), we observed the completely opposite result which suggests the involvement of $CD4^+$ T cell in vaccine efficacy. Physical depletion of $CD4^+$ T cells in our model system significantly compromised the efficacy if SA-OX40L monotherapy as well as combination of SA-4-1BBL and SA-OX40L combination therapy, suggesting the influence of SA-OX40L on $CD4^+$ T cells. This observation is in congruence with our initial functional assays where SA-OX40L shows preferential effects on $CD4^+$ T cells.

Finally, both 4-1BB and OX40 expression have been reported on Tregs cell and signaling *via* agonistic Abs and/or natural ligands to these receptors have been reported to abrogate the Treg-mediated immunosupression (81, 119). Inhibition of Treg-mediated suppression is one of the most critical phenomena required for therapeutic vaccine efficacy. Consistence with this notion, we also observed the lower Treg numbers in spleen of the mice subjected to combination therapy. Although the exact mechanistic basis of effect of SA-4-1BBL and SA-OX40L on the reduced number of Tregs observed in our model is unknown, it is possible that SA-OX40L may preferentially induce

apoptosis in Treg cells as previously reported (120) or SA-4-1BBL may block the conversion of Teff cells into induced Tregs as supported by our unpublished data demonstrating that SA-4-1BBL blocks tumor- and TGF-β-induced conversion of Teff cells into induced Treg cells through IFN-γ (Madireddi et al., manuscript submitted).

In conclusion, regardless of potential mechanisms, our data strongly supports the use of combination of SA-4-1BBL and OX40L as the immunomodulatory components of therapeutic vaccines against cancers with significant clinical potential.
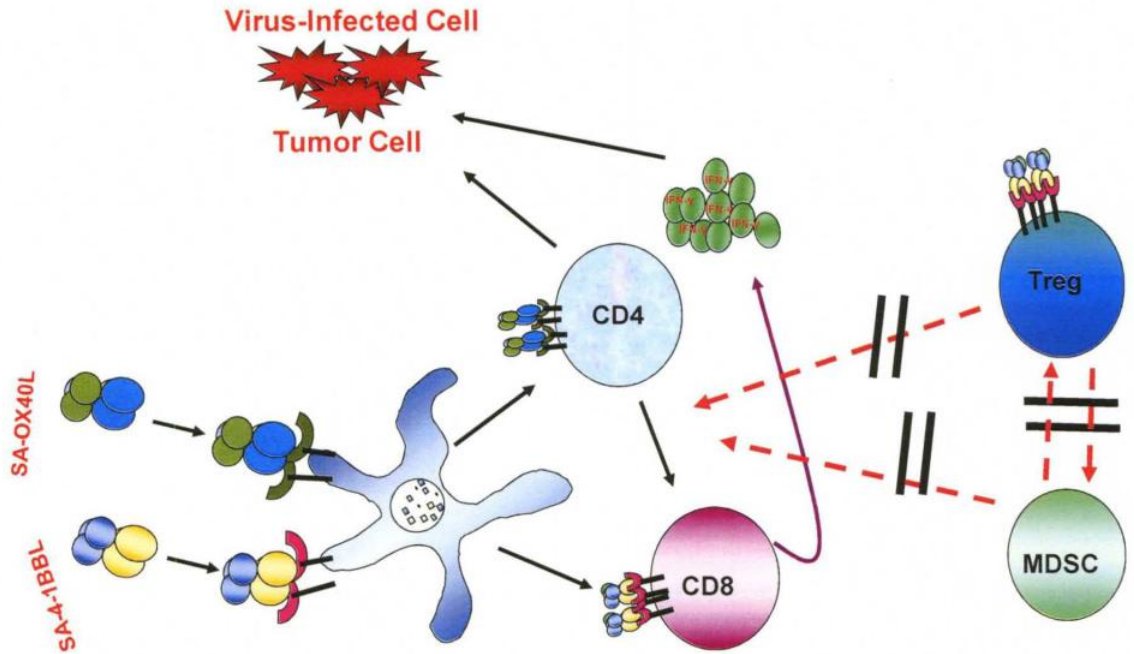
Figure 10

Hypothesis: Coordination between CD4$^+$ and CD8$^+$ T cells. SA-OX40L primarily targets CD4$^+$ T cells while SA-4-1BBL targets CD8$^+$ T cells. SA-OX40L-mediated CD4$^+$ T cells further become either effector cells or help SA-4-1BBL-mediated CD8$^+$ T cells effector response for robust anti-tumor immunity. Additionally, both SA-OX40L and SA-4-1BBL together reverse the Treg-mediated suppression either directly or indirectly, provide additional critical support to antitumor immunity.
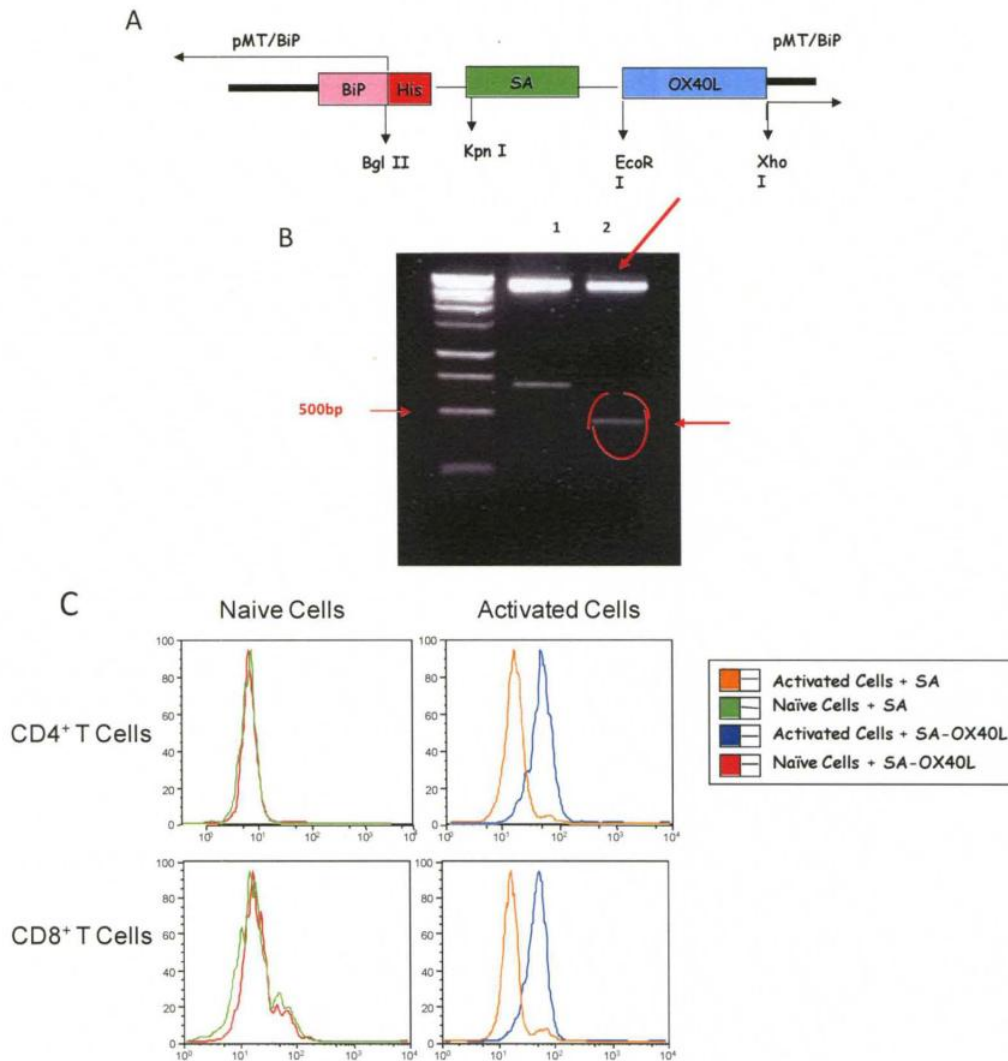
Figure 11

Construction and characterization of SA-OX40L protein. The extracellular domain of mouse OX40L was cloned C-terminal to core SA in the PMT/BiP/V5-HisA vector (A). After sub-cloning, right size of OX40L band (*lane 2*) was shown by gel electrophoresis (B). Binding of OX40L to OX40 receptor (C). Naive or ConA activated C57BL/6 splenocytes were incubated with SA-OX40L (200 ng/1x10^6 cells) or equimolar amount of SA protein and binding of SA-OX40L on CD4$^+$ (top panel) and CD8$^+$ T cells (bottom panel) was assessed by flow cytometry using anti-SA.

A

CD4 T cell proliferation

B

CD8 T cell proliferation
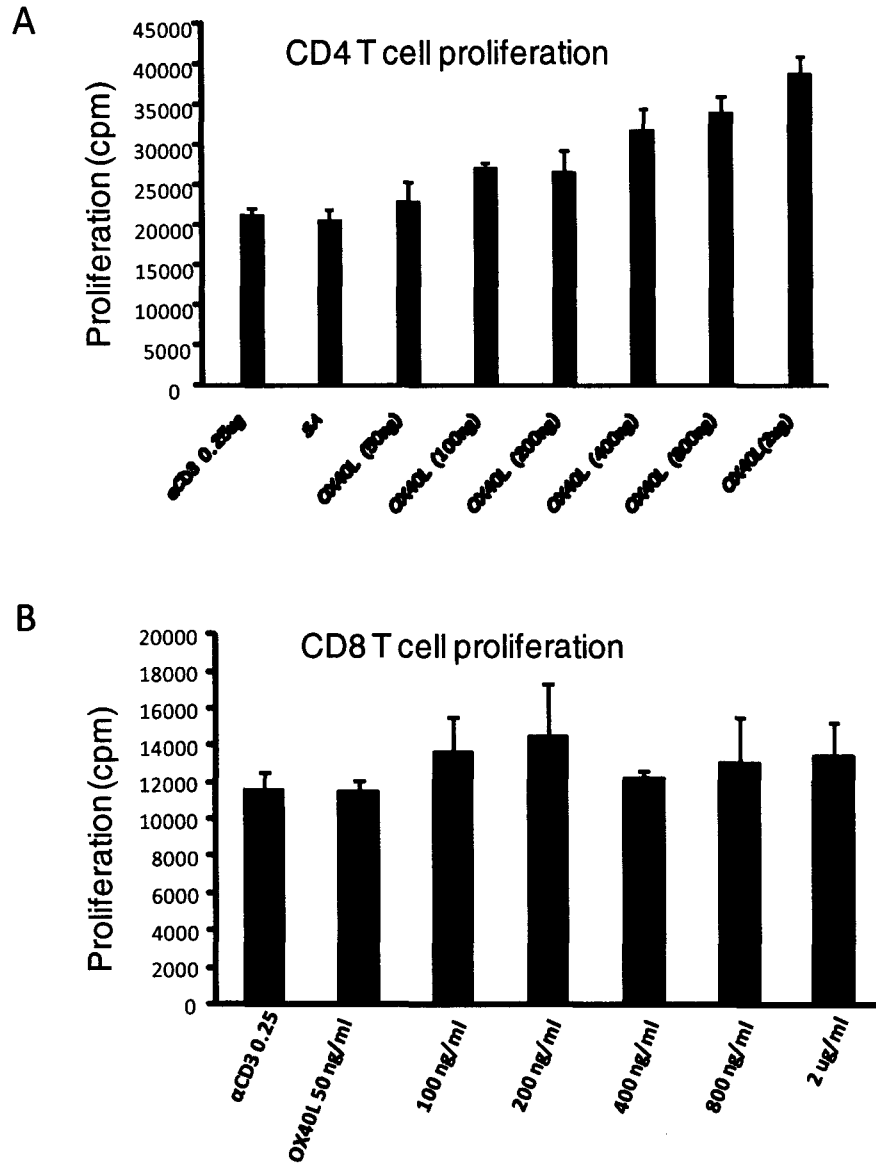
Figure 12

SA-OX40L induces CD4$^+$ T cell proliferation but not CD8$^+$ T cell *in vitro*. CD4$^+$ T cell

(A) and CD8$^+$ T cell (B) proliferation. Flow purified CD4$^+$ and CD8$^+$ T cells were

stimulated for 48h with suboptimal dose of anti-CD3 Ab with various doses of SA-

OX40L in the presence of irradiated syngeneic splenocytes. Cells were pulsed with [^{3}H]-

thymidine for the last 16 h of culture. Data are representative of minimum of three

independent experiments.
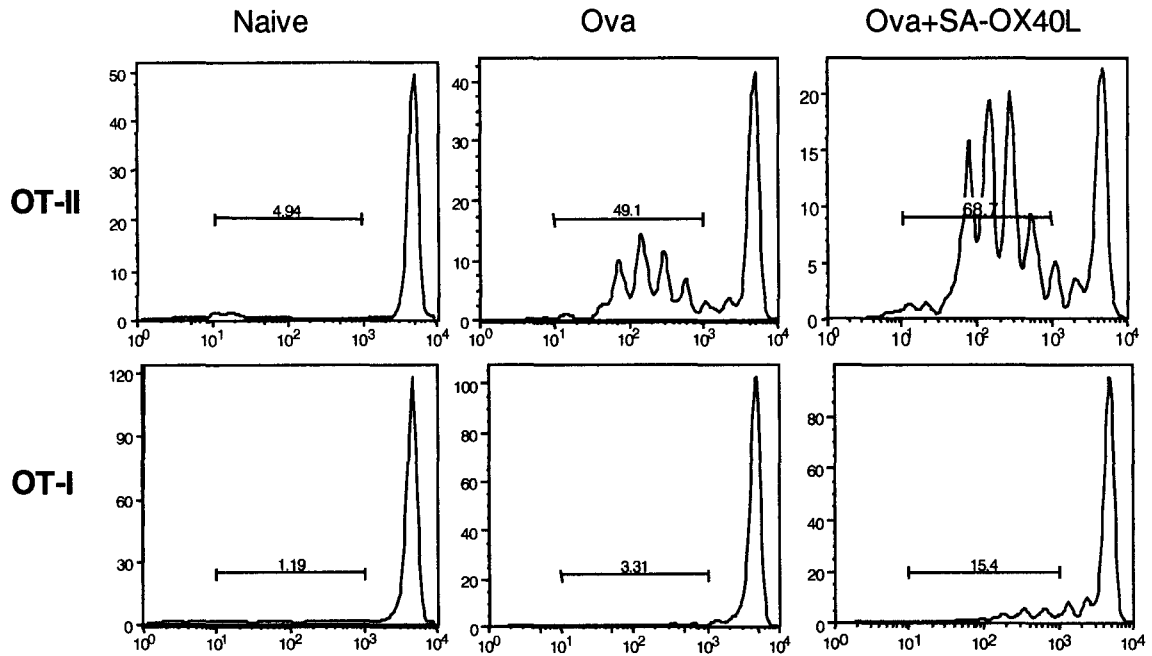
Figure 13

In vivo OT-I and OT-II cell proliferation in peripheral LNs. Two million OT-I and OT-II T cells (CD45.2$^+$) each were labeled with CFSE and injected (i.v.) into congenic C57BL/6.SJL (CD45.1$^+$) mice. Mice were vaccinated 24h later with either OVA (3 µg) alone or in combination with SA-OX40L (5 µg). Proliferation of both OT-II (top panel) and OT-I (bottom panel) was assessed using FACS caliber 3 days after vaccination by gaiting on CD4$^+$CD45.2$^+$ and CD8$^+$CD45.2$^+$ cells. Data are representative of minimum of three independent experiments.
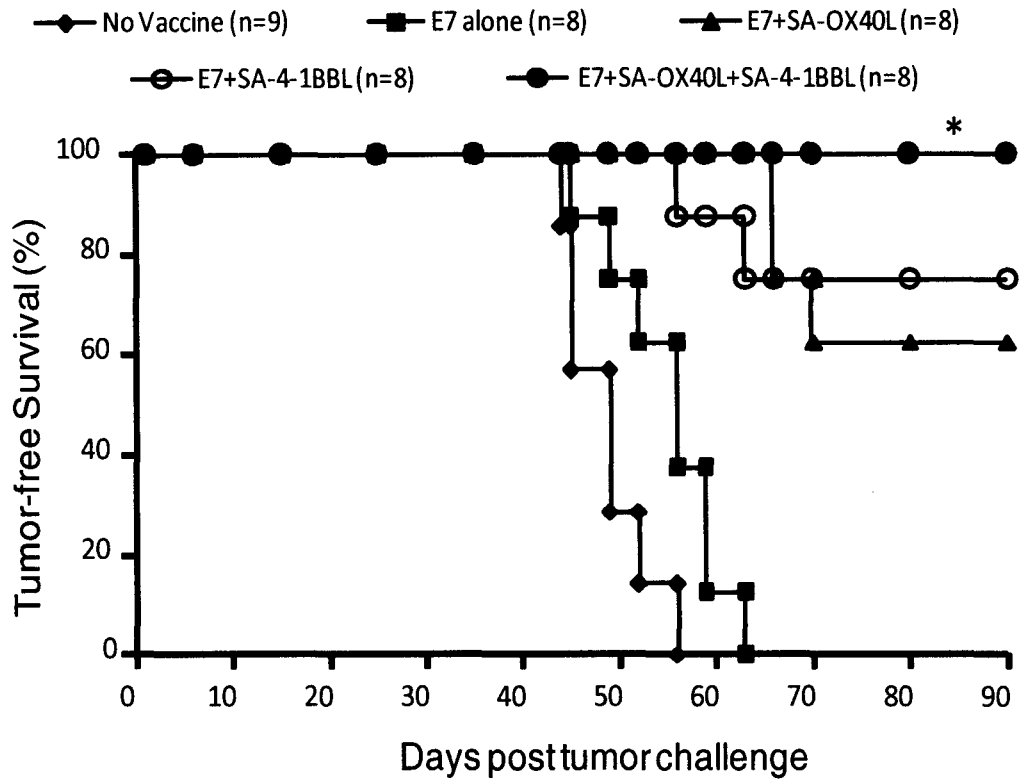
Figure 14

Combination of SA-4-1BBL and SA-OX40L completely eradicates the TC-1 tumors. C57BL/6 mice were challenged with 1×10^5 live TC-1 cells in the right flank and vaccinated s.c. on day 6 post tumor challenge with either E7 alone (50 µg) or with SA-4-1BBL (25 µg) or SA-OX40L (25 µg) or combination of both agents (25 µg + 25 µg). * *P* < 0.05 for combination therapy *vs.* all other groups.
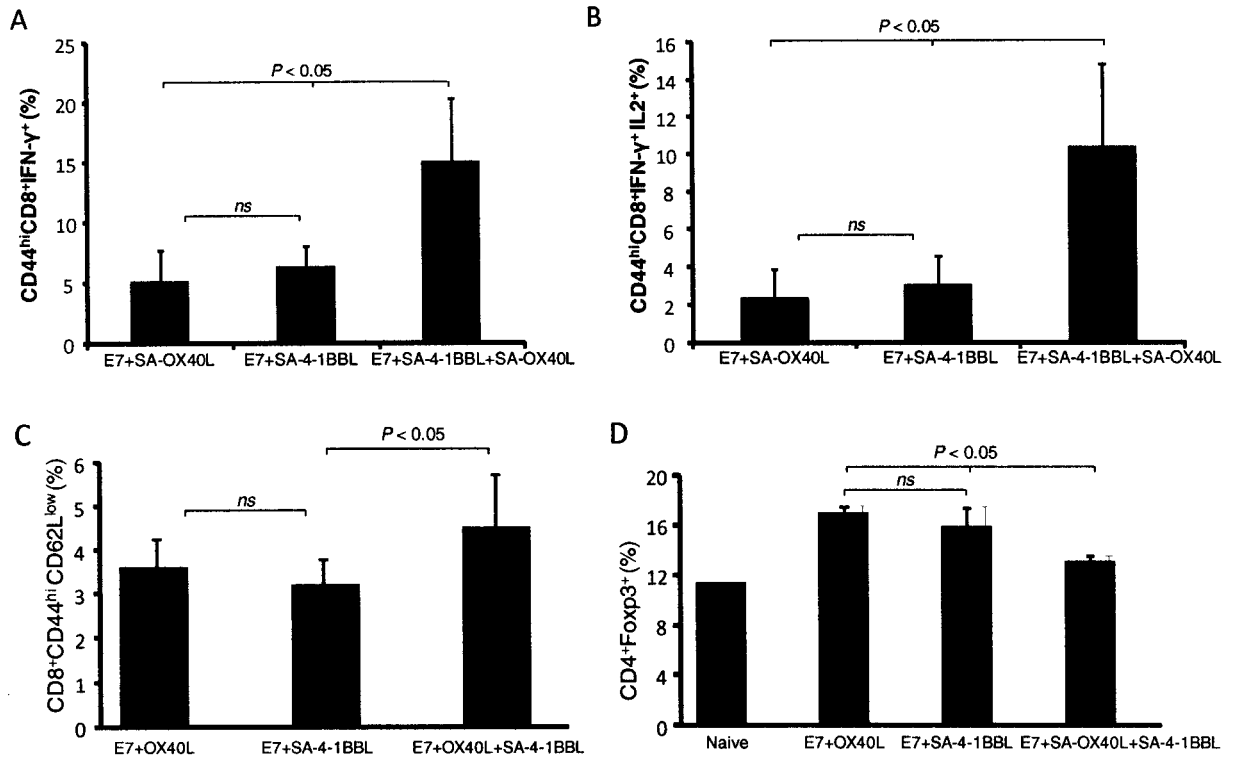
Figure 15

Vaccination with the SA-4-1BBL+SA-OX40L induces strong multi-functional cytokine and memory responses by $CD8^+$ T cells, and reduces Treg percentage that correlates with vaccine efficacy. Long-term (> 90 days) surviving mice were boosted with the same indicated vaccine formulations used for primary vaccination. 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-γIL-2 double cytokines (B). Splenocytes from the same groups were phenotyped to test the percentage of effector memory $CD44^{hi}CD62L^{low}CD8^+$ T cells (C) and $CD4^+Foxp3^+$ cells (D). Data for each panel are representative of two independent experiments that include 3-4 mice per group. P values were as shown and calculated using one way ANOVA and Tukey HSD test (ns = not significant).

64
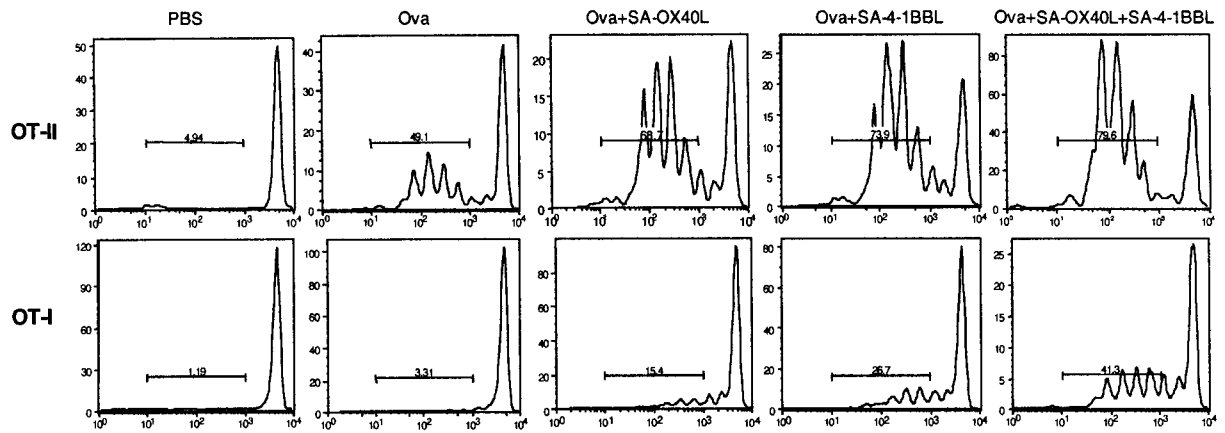
Figure 16

Combination of SA-4-1BBL and SA-OX40L generates better OT-I and OT-II cell proliferation *in vivo*. Two million OT-I and OT-II T cells (CD45.2$^+$) each were labeled with CFSE and injected (i.v.) into congenic C57BL/6.SJL (CD45.1$^+$) mice. Mice were vaccinated 24h later with either OVA (3 µg) alone or in combination with SA-OX40L (5 µg) or SA-4-1BBL (5 µg) or combination of both agents (5 µg + 5 µg). Proliferation of both OT-II (top panel) and OT-I (bottom panel) was assessed using FACS caliber 3 days after vaccination by gating on CD4$^+$CD45.2$^+$ and CD8$^+$CD45.2$^+$ cells. Data are representative of minimum of three independent experiments. This figure also contains partial data from figure 13.
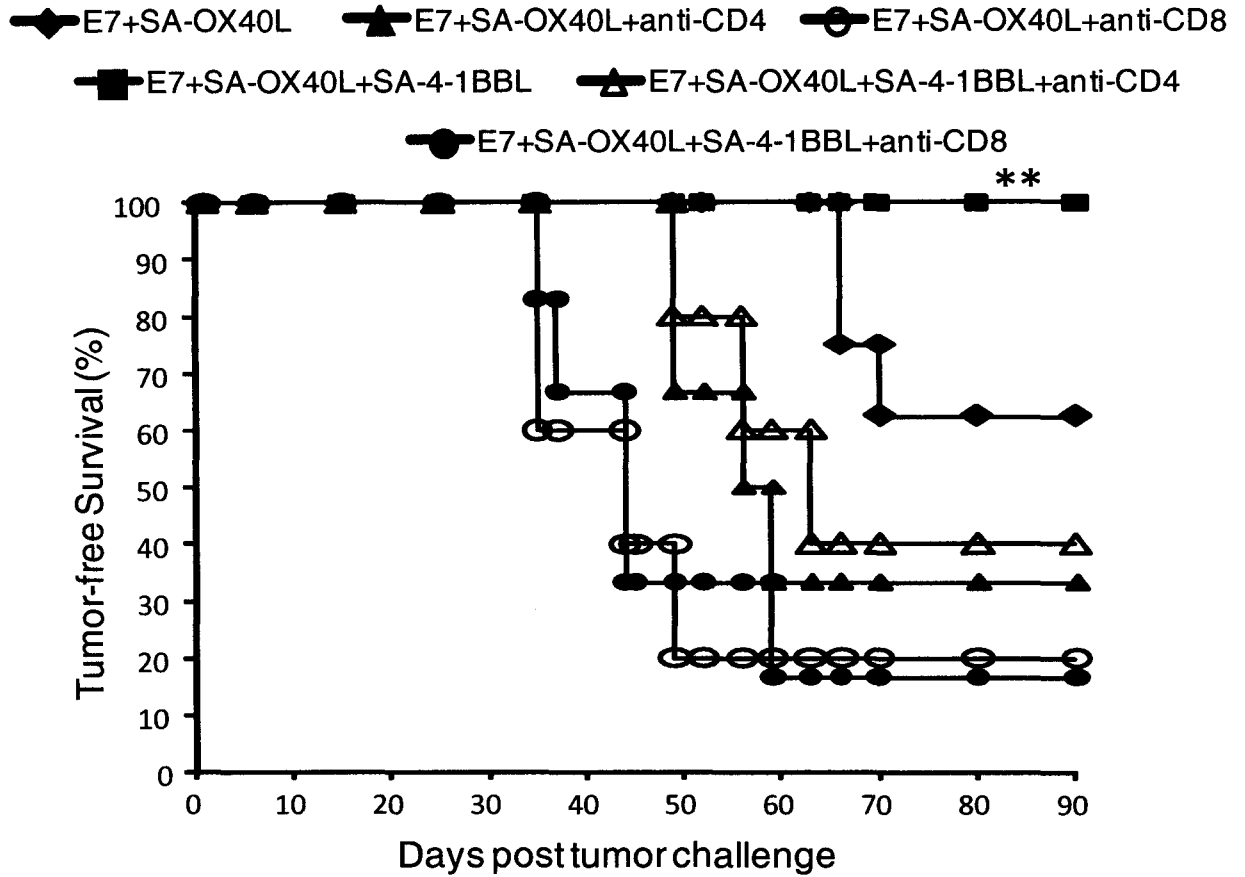
Figure 17

Therapeutic efficacy of SA-OX40L+SA-4-1BBL combination and SA-OX40L mono therapies requires both $CD8^+$ and $CD4^+$ T cells. $CD4^+$ and $CD8^+$ T cells were depleted using Abs against CD8 and CD4 molecules, respectively, one day before vaccination with E7 TAA and the indicated vaccine formulations using the TC-1 tumor model. ** P < 0.001 for E7+SA-OX40L+SA-4-1BBL vs. $CD4^+$ and $CD8^+$ T cells depleted groups.
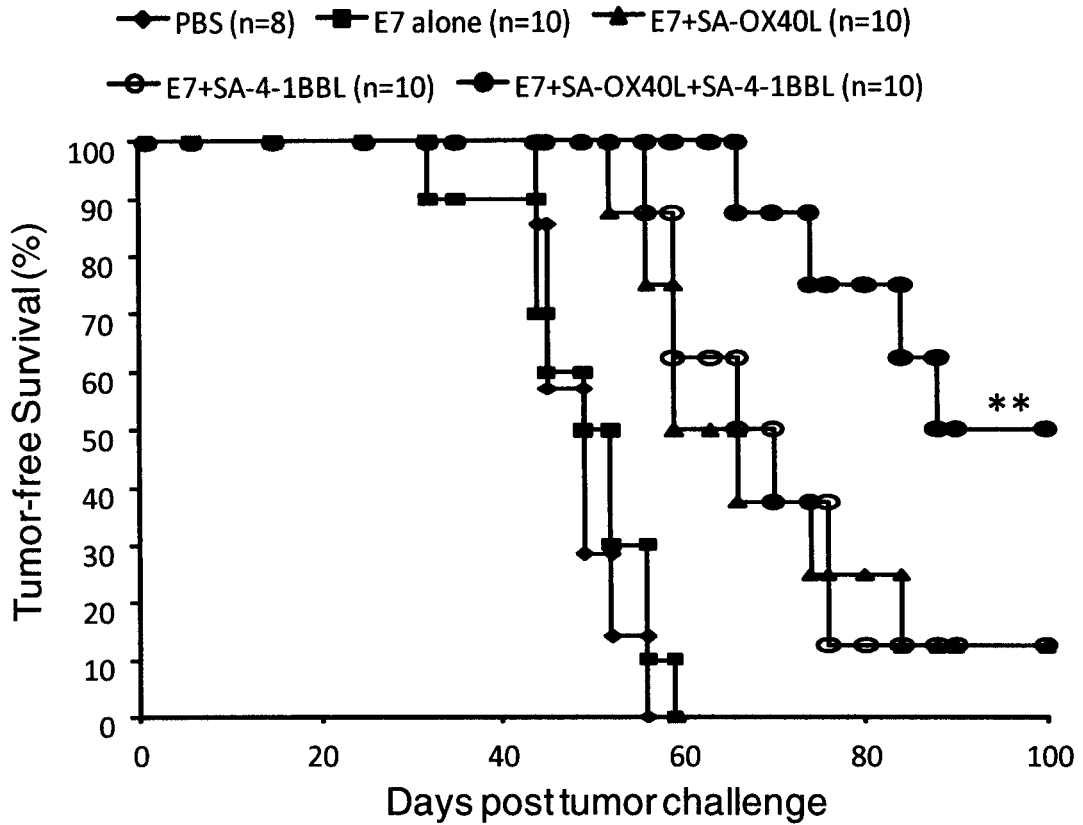
Figure 18

Combination of SA-4-1BBL and SA-OX40L has robust therapeutic efficacy in eradicating 3-4 mm established TC-1 tumors. C57BL/6 mice were challenged with 1×10^5 live TC-1 cells in the right flank. Once tumor size reaches 3-4 mm in size, mice were vaccinated s.c. with either E7 alone (50 μg) or with SA-4-1BBL (25 μg) or SA-OX40L (25 μg) or combination of both agents (25 μg + 25 μg) twice on day 5 and day 10, and followed for 100 days. ** $P < 0.001$ for combination therapy vs. all other groups.

CHAPTER 4

SA-4-1BBL AND MPL COMBINATION AS AN ADJUVANT SYSTEM SHOWS

ROBUST THERAPEUTIC EFFICACY BY INCREASING INTRATUMORAL

CD8$^+$ T EFFECTOR/CD4$^+$Foxp3$^+$ T REGULATORY CELL RATIO

Introduction

Therapeutic vaccines are preferred alternatives to conventional treatments for

cancer primarily because of their safety profile 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 (121, 122). These limitations can potentially be overcome by

developing vaccine formulations including adjuvants that not only generate potent

immune responses against TAAs with long-term immunological memory, but also

overcome various immune evasion mechanisms.

Recent advances in our understanding of the immune system, mechanistic basis of

immune activation, response, and establishment of long-term immunological memory,

and key molecules involved in regulating such responses have provided an unparalleled

68

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 (123, 124). 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 (125). However, the efficacy of MPL as the adjuvant component of therapeutic vaccines against cancer remains to be demonstrated. MPL primarily targets innate immunity, leading to the recruitment, activation, and maturation of antigen presenting cells (APCs), such as DCs that facilitate the generation of adaptive immune responses (126) (Fig. 19).

Unlike TLR pathway, costimulation directly targets adaptive immunity and is critical for the generation of primary as well as memory T and B cell responses (124), which led us to recently propose costimulatory ligands as potential adjuvants of choice for the development of therapeutic cancer vaccines (111). In particular, we focused on 4-1BBL as the costimulatory member of TNF family because of the critical role of this molecule in the generation and maintenance of $CD8^+$ T cell responses (127, 128) and the importance of $CD8^+$ T cells in eradication of tumors (129, 130). 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 (112). 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 that translate into robust therapeutic efficacy in various preclinical models (111, 112, 131, 132). 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 (111), and inhibiting the conversion of Teff cells into Treg cells through the production of IFN-γ (Madireddi et al., manuscript submitted). These combined effects results into better therapeutic efficacy in cancer setting as compared with two TLR agonists, CpG and MPL (111).

Given that MPL primarily targets APCs , such as DCs and macrophages, for the initiation of adaptive immunity (126) and 4-1BBL targets $CD8^+$ T cells for activation, acquisition of effector function, survival, and long-term memory (133-135), and the critical role of $CD8^+$ T cells for tumor eradication (129, 130), we hypothesized that an adjuvant system composed of both of these molecules may have potent therapeutic efficacy as the component of TAA-based vaccine formulations against cancer (Fig. 20). Consistent with this notion, a single vaccination with both adjuvants 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 intratumoral $CD8^+$ Teff/Treg cell ratio played a definitive role in the efficacy of the vaccine, which was confirmed by the depletion of CD8+ Teff and Treg cell populations. Taken together, these data demonstrate the utility combination of SA-4-1BBL and MPL together as a novel adjuvant system for the development of

therapeutic TAA-based subunit cancer vaccines which may have a significant clinical potential.

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 (Manassas, VA) and maintained as published (111).

Antibodies and other reagents

Fluorochrome-conjugated anti-CD8-APC-Cy7, anti-CD62L-PE, anti-CD44-APC, anti-TNF-PE, anti-IFN-γ-PE-Cy7, and anti-IL-2-PerCp-Cy5.5, and isotype controls were purchased from BD Bioscience, eBioscience, and BioLegend. MPL was purchased from InvivoGen (San Diego, CA). *The* HPV16 RAHYNIVTF E7 peptide (E7$_{49-57}$), SA-4-1BBL, E7 and mouse SVN proteins were reported previously (111).

Tumor models and vaccination

C57BL/6 mice were challenged subcutaneously (s.c.) with 1×10^5 live TC-1 cells into the right flank. For therapy, mice were vaccinated s.c. on day 6 post-tumor challenge with various vaccine formulations containing E7 protein (50 μg) alone as control or with SA-4-1BBL (25 μg), MPLA (25 μg), or the combination of both agents (25 μg/agent). The doses of E7, SA-4-1BBL, and MPL used in this study were based on our previously published studies (111). Mice were euthanized when tumor reached a size of 12 mm in

diameter, ulcerated, or mice showed signs of discomfort. $CD8^+$ and $CD4^+$ T cells were depleted using Abs against CD8 (clone 53.6.72) and CD4 (clone GK 1.5) at 500 µg/mice via intra peritoneal injection one day before vaccination.

For the pulmonary tumor model, 2×10^5 live 3LL cells were injected i.v. into the tail vein of mice. Mice were vaccinated s.c. either once on day 6 or twice on days 6 and 12 post-tumor challenge with various vaccine formulations containing SVN protein (50 µg) alone as control or with SA-4-1BBL (25 µg), MPL (25 µg), or the combination of both agents (25 µg/agent). Mice were euthanized 27 days post-tumor challenge for analysis of lung tumor burden as described (112, 136).

Flow cytometry and confocal microscopy

Spleens and/or tumor draining lymph nodes (TdLNs) were processed as described previously (111). For memory T cell typing, lymphocytes were stained with anti-CD8-APC-Cy7, anti-CD62L-FITC, and anti-CD44-APC Abs. For intracellular cytokine staining, lymphocytes (1×10^6 cells/mL) were stimulated either with 10 µg/mL $E7_{49-57}$ peptide for 2 hrs followed by incubation with GolgiPlug (1 µl/mL, BD PharMingen) overnight or with PMA (5 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) for 2 hrs followed by incubation with GolgiPlug (1 µl/ml) for an additional 4 hrs. 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 followed by acquisition and analysis as previously reported (112). Intratumoral $CD8^+$ T cells and $CD4^+Foxp3^+$ Treg cells were analyzed using confocal microscopy as described (112).

Analysis of autoantibody to ssDNA

A ssDNA ELISA was performed to assess the presence of auto-Abs in treated mice as described (137). Briefly, ninety six titer plates coated with 1 µg/well of heat-denatured calf thymus DNA (ssDNA, Sigma) were blocked with PBS containing 5% BSA + 0.5% Tween 20 + 0.1% naïve C57BL/6 serum. Serum dilutions were added to wells and incubated at 4°C overnight. Wells were washed 3 times, incubated with anti-mouse IgG-HRP, and absorbance was measured at 450 nm.

Results

Combined use of SA-4-1BBL and 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 established TC-1 tumors in > 70% of mice (112). 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 (126, 138). A single s.c. vaccination with E7 protein mixed with SA-4-1BBL and MPL resulted in the eradication of established TC-1 tumors in all mice, which remained tumor-free over an observation period of 90 days (Fig. 21A). In contrast, monotherapy with SA-4-1BBL and MPL resulted in eradication of tumor in only 80% and 50% of mice, respectively. Mice that expired from tumor in the single agent groups, however, had slow kinetics of tumor progression as compared with both PBS and E7 protein control groups where all mice expired from the tumor burden within 50 days (Fig. 21B). Taken together, these data demonstrate that SA-4-1BBL/MPL as an adjuvant system 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 the vaccine is associated with robust effects 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 (111, 129-131). We, therefore, assessed the CD8$^+$ T cell effector and long-term memory responses elicited by various vaccine formulations. Mice that had eradicated the tumor in response to various vaccine formulations were boosted s.c. 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 (129). Consistent with the therapeutic efficacy, vaccination with E7 protein and SA-4-1BBL/MPL generated a better antigen-specific cytokine response than single adjuvant therapy as assessed by CD8$^+$ T cell expressing IL-2, IFN-γ, and TNF-α triple cytokines (Fig. 22A-C). Consistent with the therapeutic responses, mice vaccinated with SA-4-1BBL formulation generated significantly ($P < 0.05$) better IFN-γ response than the MPL formulation (Fig. 22A). We also observed E7 TAA-specific TC-1 killing (Fig. 23A) responses in mice vaccinated with both adjuvants as compared with single adjuvants. Consistent with the therapeutic efficacy, SA-4-1BBL as monotherapy generated better killing responses than E7 antigen alone, whereas MPL failed to do so. Importantly, vaccine formulation with the SA-4-1BBL/MPL also generated the most effective CD8$^+$ T cell memory recall responses as compared to those including SA-4-1BBL and MPL as single agents (Fig. 23B). Collectively, these data demonstrate that SA-4-1BBL and MPL

adjuvants work together to generate potent $CD8^+$ T cell effector and memory responses that correlate with the therapeutic efficacy of the vaccine against the TC-1 tumor.

Vaccination with the SA-4-1BBL/MPL adjuvant system results in a favorable intratumoral $CD8^+$ Teff/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 (139, 140) and depletion of Treg cells results in better immune efficacy of therapeutic vaccines (141, 142). Therefore, we evaluated the effect of SA-4-1BBL/MPL adjuvant system on the status of intratumoral Treg and Teff cells. Mice bearing ~3-4 mm TC-1 tumor were vaccinated s.c. with various vaccine formulations. One week post-vaccination, tumors were harvested and analyzed for the presence of intratumoral $CD8^+$ T cells and $CD4^+FoxP3^+$ Treg cells using confocal microscopy. There was a significant reduction in the number of intratumoral Treg cells in mice vaccinated with either SA-4-1BBL as a single adjuvant or in combination with MPL when compared with PBS controls or E7 protein alone. (Fig. 24A top panel and B). Interestingly, vaccine formulation containing MPL as a single adjuvant 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 if a 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 (143). Vaccination with SA-4-1BBL/MPL had the most pronounced effect on the

number of intratumoral CD8$^+$ T cells infiltration followed by SA-4-1BBL, whereas MPL had a moderate effect that was similar to the E7 protein alone (Fig. 24 A bottom panel and 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. 24D). In marked contrast, MPL as a single adjuvant 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 in synergy to increase the intratumoral Teff/Treg cell ratio that correlates with the potent efficacy of this adjuvant system in eliminating established tumors.

CD8$^+$ T cells are critical to the therapeutic efficacy of SA-4-1BBL/MPL adjuvant system 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 vaccine therapeutic efficacy, we used Abs against CD8 and CD4 molecules to deplete CD8$^+$ Teff and Treg cells, respectively. Mice with established TC-1 tumors were treated with depleting Abs one day before vaccination with E7 protein admixed with SA-4-1BBL/MPL or MPL as monotherapy. As shown in Figure 25, depletion of CD8$^+$ T cells completely abrogated the therapeutic efficacy of SA-4-1BBL/MPL adjuvant system, while depletion of CD4$^+$ T cells, including Treg cells, improved the therapeutic efficacy of MPL from 50 to 100%. Taken together, these data provide direct evidence for the opposing roles of CD8$^+$ T and Treg cells in vaccine efficacy and point to the importance of Teff/Treg cell ratio as a predictor of vaccine efficacy/failure.

Vaccination with SA-4-1BBL/MPL adjuvant system and SVN eradicates established 3LL pulmonary metastatic tumors

The robust efficacy of vaccination with xenogeneic E7 TAA in combination with SA-4-1BBL/MPL in eliminating TC-1 tumors in all mice led us to test if this efficacy is translatable to SVN as a weak and potentially tolerant self-TAA using the 3LL pulmonary metastasis model. Mice were challenged i.v. with a lethal dose of live 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. As shown in Fig. 26A, vaccine formulation containing both adjuvants had the most therapeutic efficacy over single adjuvants in controlling tumor growth as demonstrated by both lung weight and presence of tumor nodules. Similar to the TC-1 model, vaccine formulation containing SA-4-1BBL had better efficacy in controlling tumor growth than MPLA, which had statistically significant ($P < 0.05$) 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 ($P < 0.05$) higher number of CD8$^+$ T cells expressing IFN-γ^+ as compared with PBS and SVN alone controls (Fig. 26B).

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. As shown in Figure 27, boosting with SA-4-1BBL/MPL resulted in complete eradication of lung tumor in all mice. Booster vaccination with single adjuvants was also effective in eradicating and/or controlling tumor burden that reached statistical significance ($P < 0.05$) as compared with PBS and SVN alone controls. 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 adjuvant system is achieved in the absence of detectable clinical toxicity and autoimmunity

Autoimmunity is a potential setback to effective self-TAA-based therapeutic vaccine formulations using potent adjuvants to induce immune responses to such antigens (144). 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 as well as 3LL 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, whereas the serum from mice with full blown lupus had high levels of such Abs (Fig. 28). Importantly, we did not detect signs of acute toxicity in vaccinated mice based on weight loss, unexpected mortality, gross anatomy, and macroscopic analysis of body organs, demonstrating the safety profile of this adjuvant system.

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. MPL collaborated with SA-4-1BBL as the adjuvant component of HPV E7 TAA-based vaccine to generate robust primary $CD8^+$ T cell effector and long-term memory responses that translated into improved therapeutic efficacy in the TC-1 cervical cancer mouse model. The therapeutic

efficacy of the adjuvant system was totally dependent on $CD8^+$ T cells and associated with a favorable intratumoral $CD8^+$ Teff/$CD4^+$Foxp3$^+$ Treg cell ratio. Importantly, the therapeutic efficacy of the adjuvant system was not limited to the xenogeneic E7 TAA, since a vaccine formulation containing SVN as a bona fide self-TAA was equally effective in eradicating/controlling tumors in the 3LL metastatic pulmonary cancer model.

The choice of SA-4-1BBL and MPL as the adjuvant system is due to their distinct mechanisms of action and targeting different immune cells for activation. 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 (126). 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 (133-135). Importantly, 4-1BB signaling appears to have the most effect on $CD8^+$ T cells, which are critical to the eradication of tumors as demonstrated in various preclinical settings, including the TC-1 model (111, 129-131). Therefore, MPL in the adjuvant system is expected to work with SA-4-1BBL for the activation of $CD8^+$ T cells through the activation of DCs and antigen cross-presentation (145), 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. This scheme is supported by our current findings that MPL collaborates with SA-4-1BBL in generating robust $CD8^+$ T cells primary and long-term memory responses that translate into effective therapy in two different established tumor models,

79

TC-1 cervical and 3LL pulmonary carcinoma, with two different antigens, HPV E7 xenogeneic and SVN bona fide self-TAA antigens. Consistent with this notion, depletion of CD8$^+$ T cells one day before vaccination completely abrogated the efficacy of the SA-4-1BBL/MPL adjuvant system in eradicating TC-1 tumors. In addition to its direct effect on CD8$^+$ T cells, SA-4-1BBL may also augment the effect of MPL on DCs by improving their antigen uptake and cross-presentation. This notion is supported by observations that a subpopulation of DCs constitutively express 4-1BB receptor (146, 147), and vaccination with SA-4-1BBL enhances their antigen uptake and cross-presentation (111, 112).

CD4$^+$CD25$^+$FoxP3$^+$ Treg cells play a critical role in immune evasion mechanisms employed by acute (148) as well as chronic infections (149, 150) and cancer (122, 141, 151), and as such serve as an important barrier for the efficacy of vaccines. Therefore, vaccine formulations that specifically control the number and/or function of Treg cells while enhancing the number of Teff cells may have desired therapeutic efficacy in settings of cancer and chronic infections. Consistent with this notion are studies demonstrating that the physical depletion of Treg cells or modulation of their regulatory function using Abs to various cell surface markers have protective and therapeutic effects against various tumors in preclinical models (151-155). Importantly, 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 (154). Consistent with preclinical studies, 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 (139-141). 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 adjuvant system. 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 (112). 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-based vaccine since their depletion one day before vaccination resulted in eradication of all tumors (Fig. 25). This finding, to our knowledge first, demonstrating that MPL efficacy is compromised by Treg cells is significant and provides an important mechanistic insight in improving the efficacy of this FDA approved adjuvant for the development of therapeutic cancer vaccines.

Although the primary targets of MPL are cells of innate immunity, a series of recent studies have demonstrated that this adjuvant may also directly targets cells of adaptive immunity. The expression of TLR-4 has been shown on CD4$^+$ T effector and Treg cells (156, 157). Importantly, stimulation via this receptor on CD4$^+$ Teff cells was shown to inhibit ERK1/2 signaling pathway, resulting in the inhibition of their function in an experimental colitis model (157). In marked contrast, stimulation of Treg cells with the TLR-4 agonist lipopolysaccharide resulted in their survival, expansion, and improved regulatory function *in vivo* (156), which may account for the unfavorable intratumoral CD8$^+$ Teff/Treg cell ratio seen in the MPL monotherapy group. Although the exact

mechanistic basis of the synergistic effect of SA-4-1BBL and MPL on the intratumoral CD8$^+$ T/Treg cell ratio observed in our model is unknown, i) SA-4-1BBL may preferentially induce apoptosis in Treg cells as reported for the agonists of OX-40 pathway (158), 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 unpublished data demonstrating that SA-4-1BBL blocks tumor- and TGF-β-induced conversion of Teff cells into induced Treg cells through IFN-γ (Madireddi et al., manuscript submitted). The increased expression of IFN-γ in response to SA-4-1BBL/MPL adjuvant system in the present study is further consistent with this notion. Importantly, although we observed enhanced E7 TAA-specific frequency of CD8$^+$ T cells expressing IFN-γ in the periphery of mice vaccinated with MPL as monotherapy, this effect did not result in increased number of CD8$^+$ T cells in the tumor, suggesting that these cells may not be trafficking into the tumor. In contrast, vaccination with SA-4-1BBL/MPL adjuvant system resulted in significantly higher numbers of CD8$^+$ Teff cells both in the periphery and within the tumor, suggesting that both adjuvants in combination may affect the trafficking/entry of CD8$^+$ Teff into the tumor and/or improve their survival.

Importantly, the therapeutic activity of SA-4-1BBL/MPL adjuvant system was achieved in the absence of detectable acute toxicity and chronic autoimmunity. 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 (132). The safety of MPL has already been demonstrated both in preclinical and clinical settings (125, 126, 138).

In conclusion, the studies presented in this communication demonstrate the robust efficacy of the SA-4-1BBL/MPL adjuvant system 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. Importantly, the therapeutic efficacy of the vaccines was observed in the absence of detectable acute toxicity or chronic autoimmunity. The better immune and therapeutic efficacy of SA-4-1BBL/MPL over MPL as monotherapy combined with MPL being a clinically approved adjuvant (125) emphasizes the importance of further developing this adjuvant system and assessing its efficacy as component of subunit therapeutic vaccines against cancer and chronic infections.
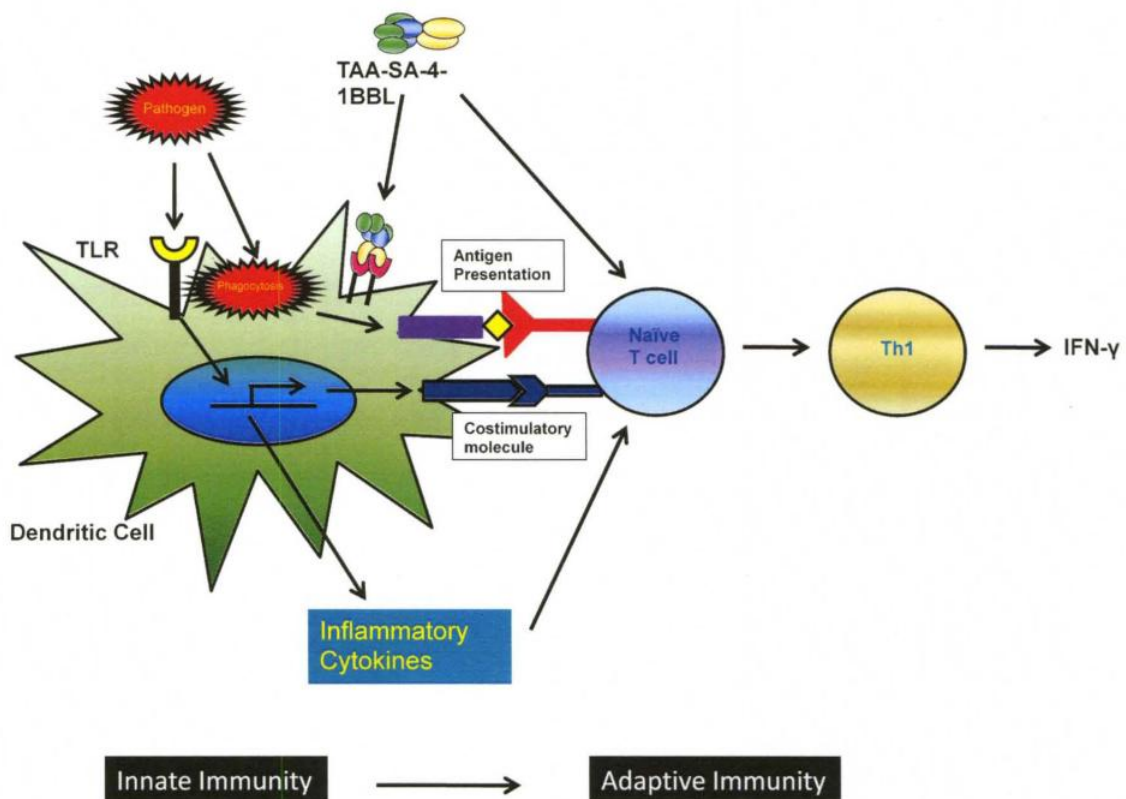
Figure 19

Targeting innate and adaptive immunity. Innate immune cells such as dendritic cell engulf pathogens and present pathogen-derived peptide antigens to naïve T cells. Additionally, TLRs recognize pathogen-derived components and induce expression of costimulatory molecules and inflammatory cytokines. On the other hand, costimulatory SA-4-1BBL also induce the dendritic cells activation and antigen cross- presentation. Phagocytosis-mediated antigen presentation, TLR-mediated expression of co-stimulatory molecules and inflammatory cytokines together with SA-4-1BBL-mediated dendritic cell activation and cross-presentation instruct development of antigen-specific adaptive immunity, especially Th1 cells.

activation and cross-presentation instruct development of antigen-specific adaptive immunity, especially Th1 cells.
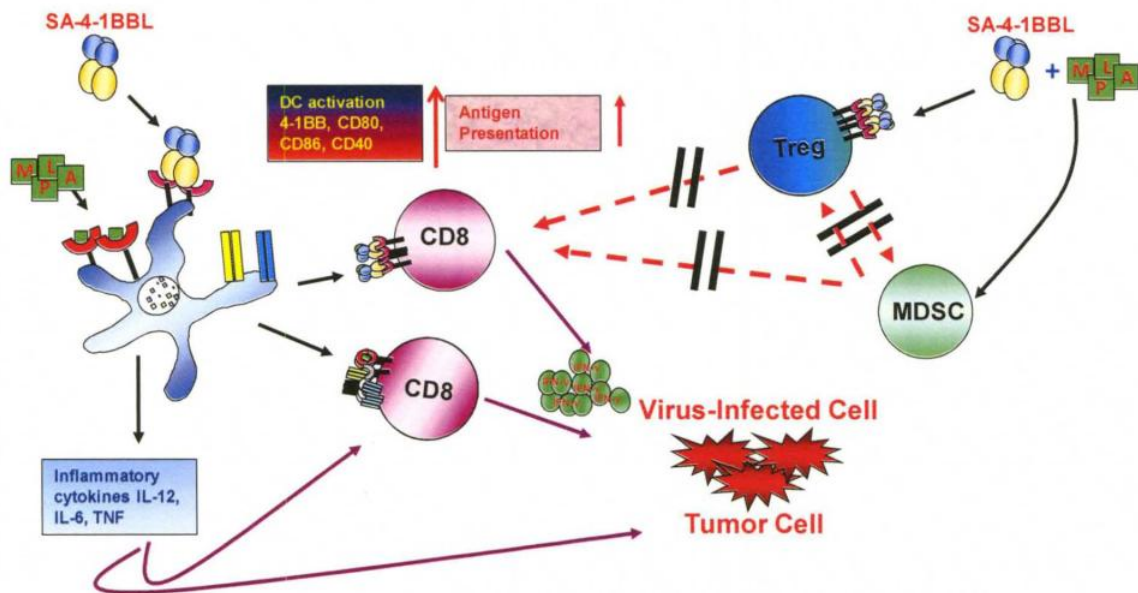
Figure 20

Hypothesis: Synergy between innate and adaptive immunity. MPL is recognized by TLR-4 expressed on dendritic cells and induce expression of costimulatory molecule 4-1BB and inflammatory cytokines such as IL-12, IL-6, and TNF. On the other hand, costimulatory SA-4-1BBL also activates the dendritic cells by inducing CD80, CD86, and CD40 expression and enhances antigen cross- presentation. MPL-mediated expression of 4-1BB and inflammatory cytokines together with SA-4-1BBL-mediated dendritic cell activation and cross-presentation instruct development of antigen-specific adaptive immunity synergistically to target tumor cells. Additionally, combination of SA-4-1BBL and MPL also targets the Tregs and MDSCs to avert their suppression on effector cells. Take together, combination therapy targets all arm of the immune system including innate, adaptive and regulatory.

A

B
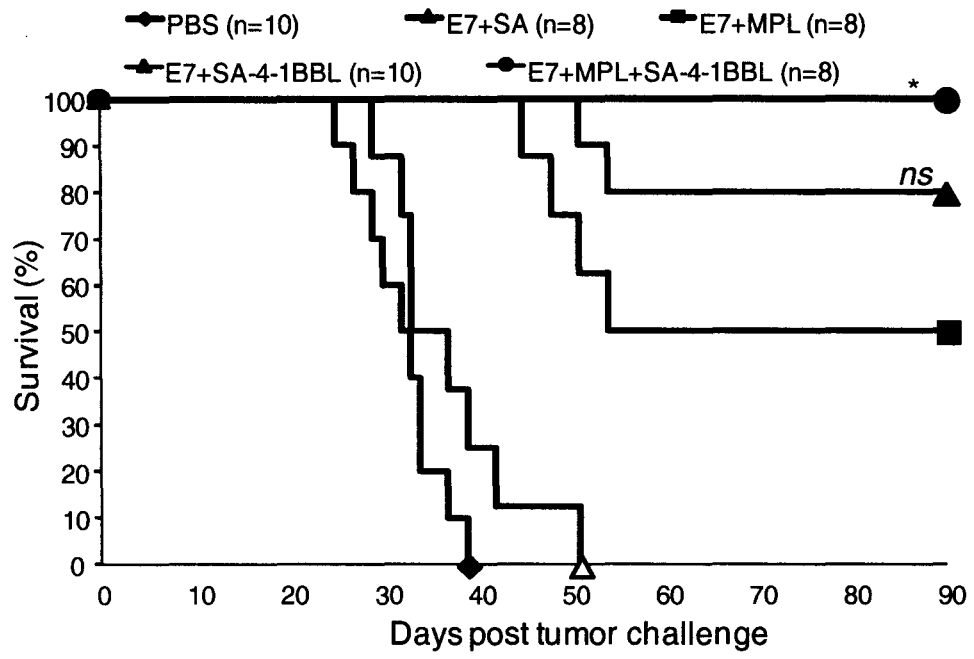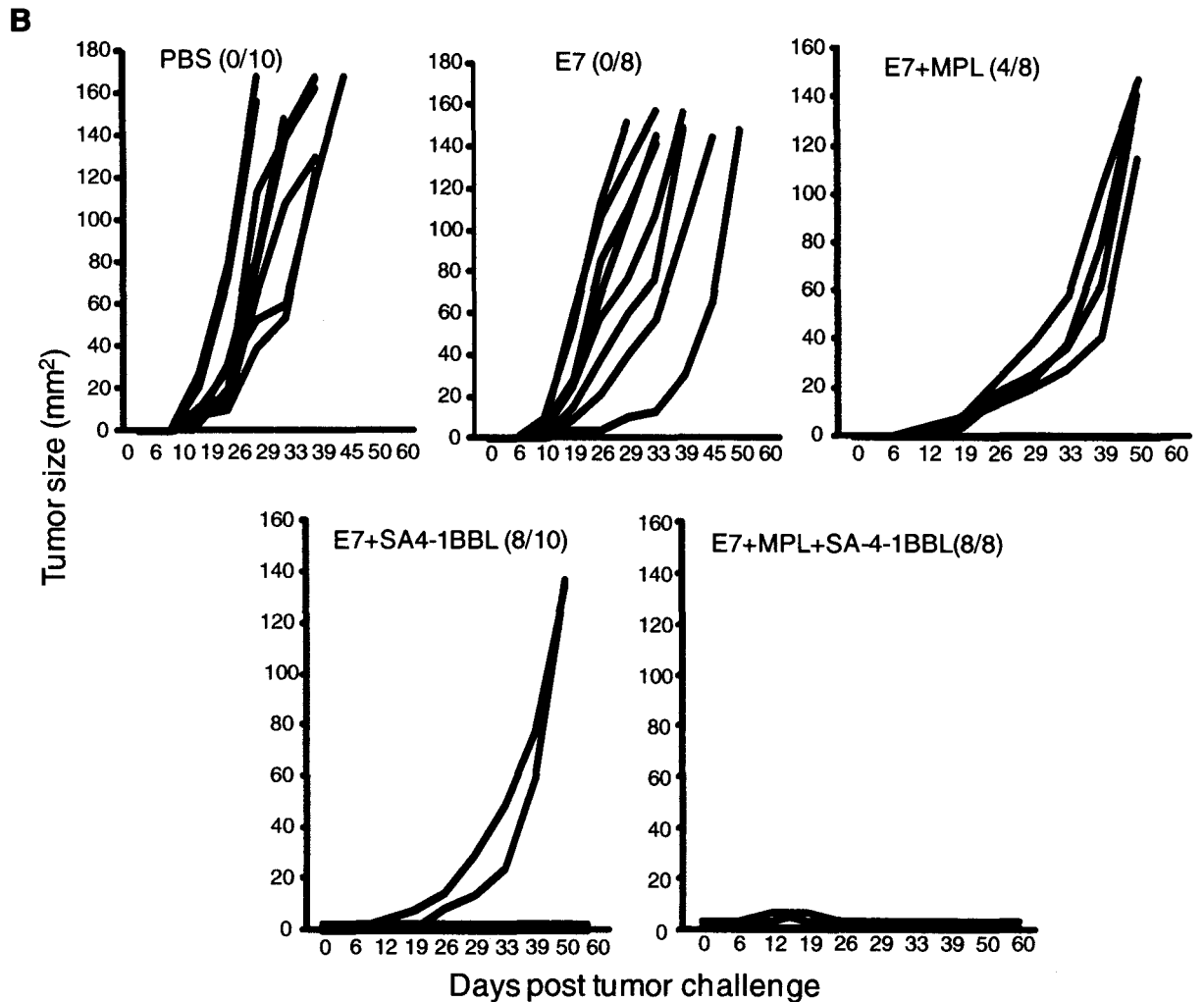
Figure 21

A single vaccination with the SA-4-1BBL/MPL adjuvant system results in the eradication

of established TC-1 tumor in all mice. A, C57BL/6 mice were challenged s.c. with 1×10^5

live TC-1 cells and left unvaccinated (PBS) or vaccinated once s.c. on day 6 post-tumor

challenge with E7 (50 μg) mixed with control SA protein (10 μg) or SA-4-1BBL (25 μg),

MPL (25 μg), or the combination of both agents (25 μg/agent). The log-rank test and

Kaplan-Meier method were used for analyses. *P < 0.05 as compared to all the other

groups, but SA-4-1BBL that was not significant (*ns*). B, Data from (A) are presented for

individual animals in each group.
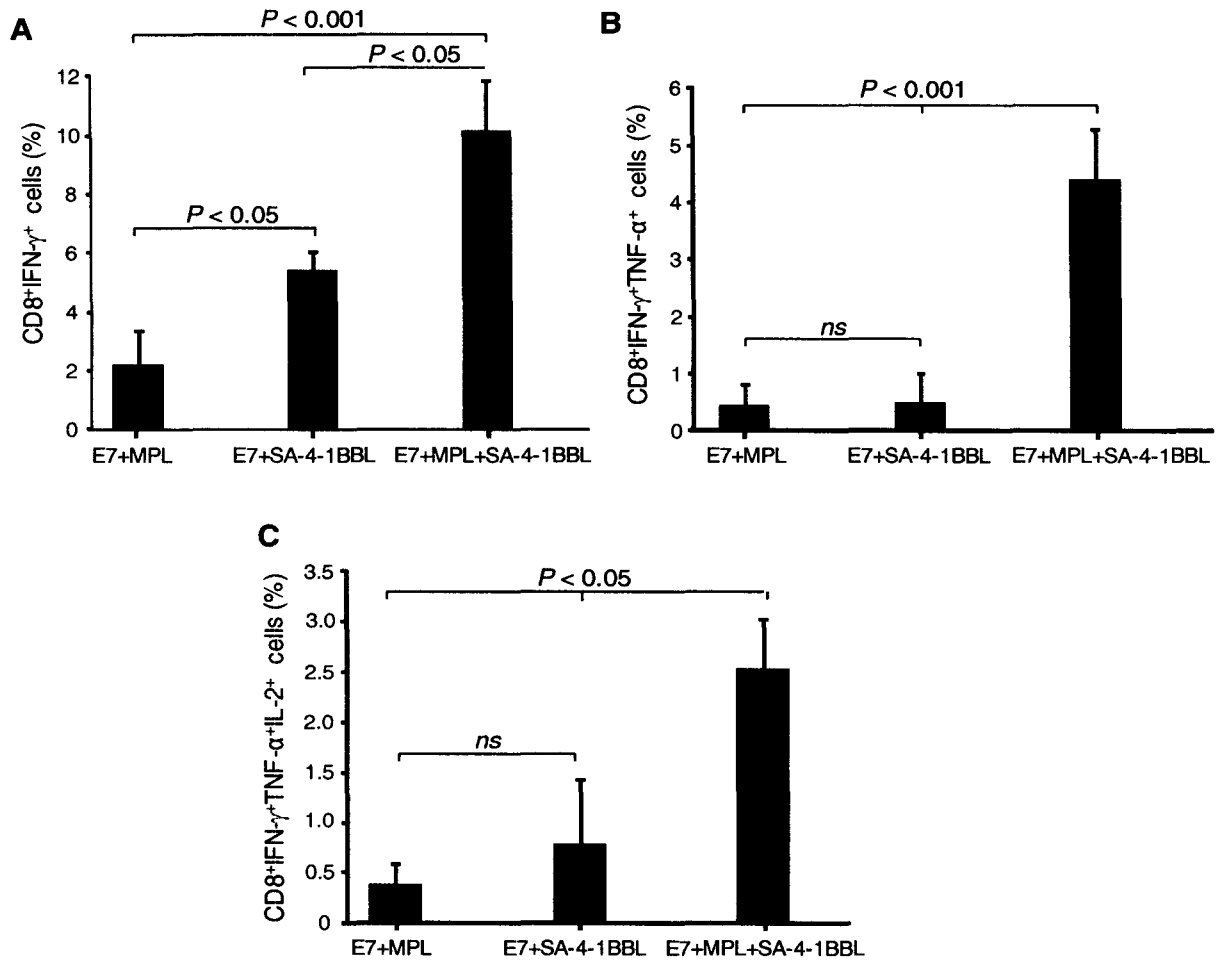
Figure 22

Vaccination with the SA-4-1BBL/MPL adjuvant system induces strong multi-functional cytokine responses by CD8$^+$ T cells that correlate with vaccine efficacy. Long-term (> 90 days) surviving mice were boosted with the same indicated vaccine formulations used for primary vaccination. 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. Data for each panel are representative of two independent experiments that include 3-4 mice per group. *P* values were as shown and calculated using one way ANOVA and Tukey HSD test (*ns* = not significant).
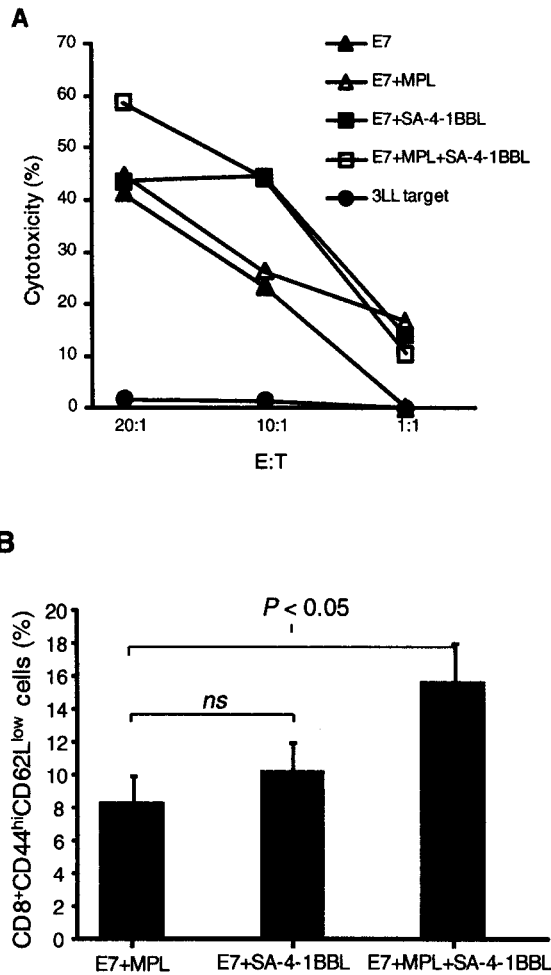
Figure 23

Vaccination with the SA-4-1BBL/MPL adjuvant system induces cytolytic and memory responses that correlate with vaccine efficacy. Long-term (> 90 days) surviving mice were boosted with the same indicated vaccine formulations used for primary vaccination. A, Splenocytes were stimulated with $E7_{49-57}$ peptide and IL-2 for 5 days and used as effectors against TC-1 tumors. 3LL tumor cells were used as irrelevant targets B, Splenocytes from the same groups were phenotyped to test the percentage of effector memory $CD44^{hi}CD62L^{low}CD8^{+}$ T cells. Data for each panel are representative of two independent experiments that include 3-4 mice per group. P values were as shown and calculated using one way ANOVA and Tukey HSD test (ns = not significant).

A

Tumor infiltrating CD4+Foxp3+ Treg cells

PBS   E7   E7+MPL   E7+SA-4-1BBL   E7+MPL+SA-4-1BBL
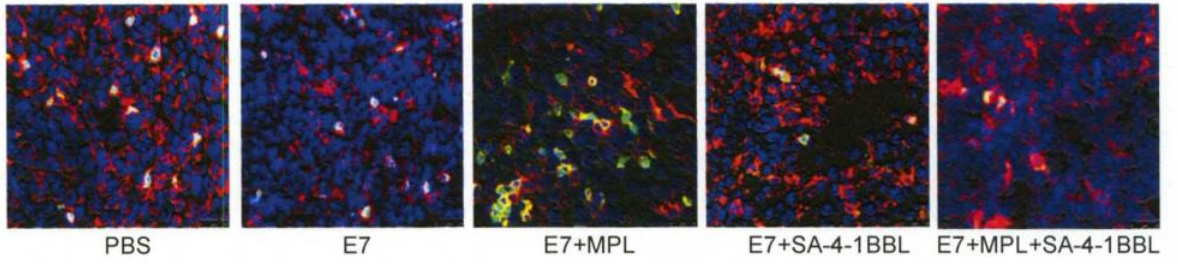
Tumor infiltrating CD8+ T cells

PBS   E7   E7+MPL   E7+SA-4-1BBL   E7+MPL+SA-4-1BBL
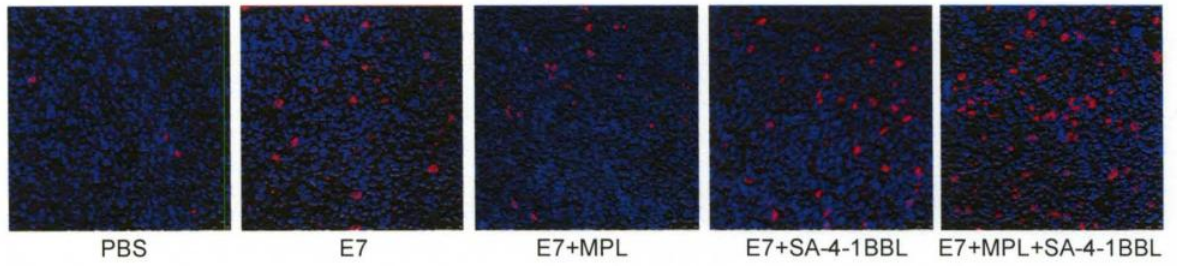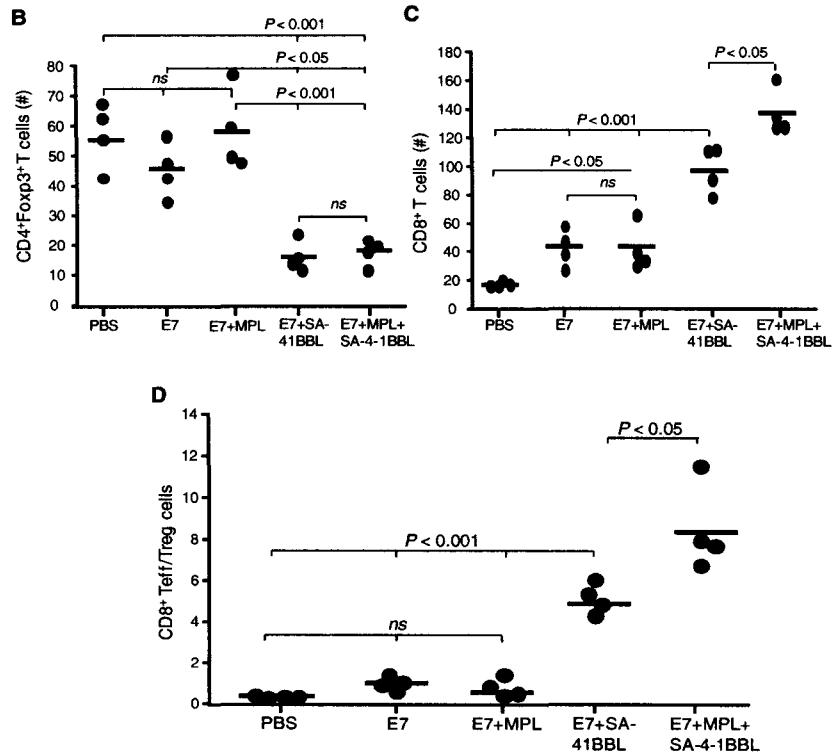
Figure 24

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 (~3-4 mm in diameter; $n = 4$ per group) 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 (top panel) stained with anti-CD4 Ab (red), anti-Foxp3 Ab (green), and Hoechst (blue), and $CD8^+$ T cells (bottom 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). P values were as shown and calculated using one way ANOVA and Tukey HSD test (ns = not significant).
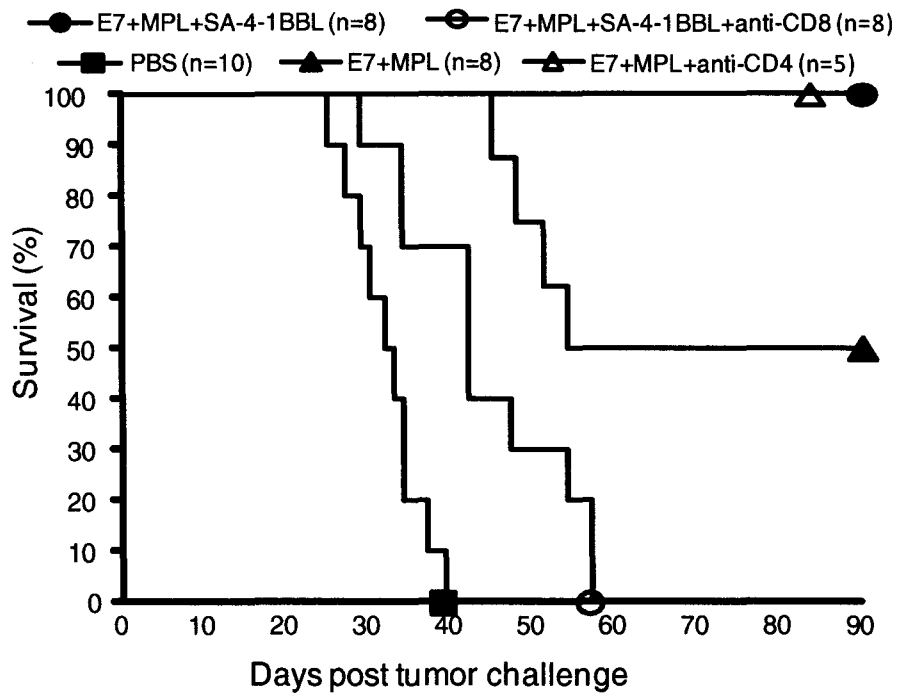
Figure 25

Therapeutic efficacy of SA-4-1BBL/MPL adjuvant system requires $CD8^+$ T cells while Treg cells compromise the efficacy of MPL monotherapy. $CD8^+$ T cells and Treg cells were depleted using Abs against CD8 and CD4 molecules, respectively, one day before vaccination with E7 TAA and the indicated adjuvant system using the TC-1 established tumor model. Data for PBS, E7+MPL, and E7+MPL+SA-4-1BBL groups were taken from Fig. 1.
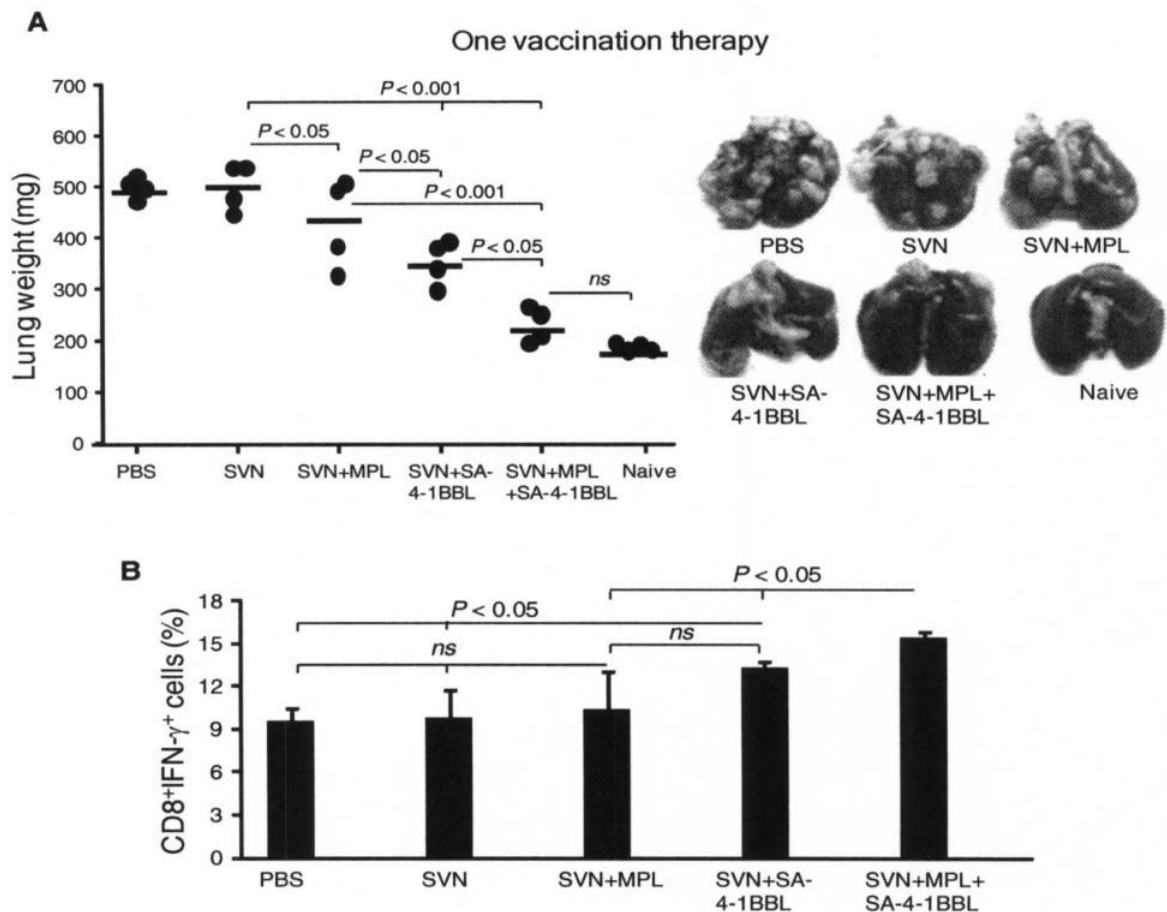
Figure 26

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 2×10^5 live 3LL cells by i.v. tail injection and vaccinated once s.c. on day 6 or twice on days 6 and 13 post-tumor challenge with SVN (50 µg) alone or antigen 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 splenocytes harvested from mice in (A).
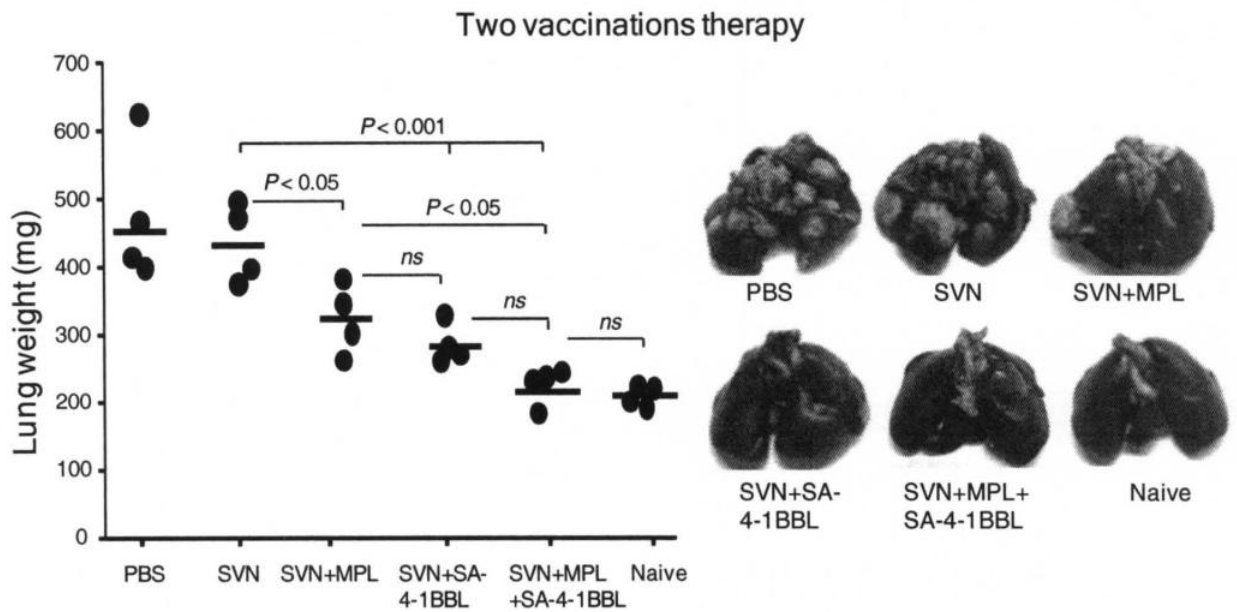
Figure 27

Additional vaccination with SA-4-1BBL/MPL adjuvant system almost completely eradicates the lung tumors. Lungs harvested from mice with two vaccinations are assessed as in (Fig. 14). *P* values were as shown and calculated using one-way ANOVA and Post Hoc LSD test (*ns* = not significant).
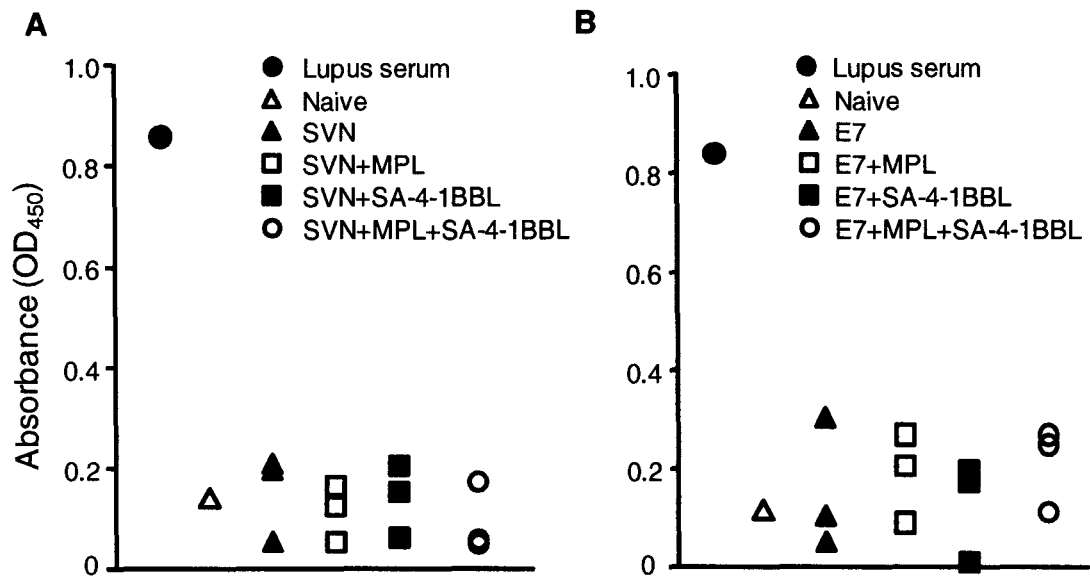
Figure 28

Vaccination with the SA-4-1BBL/MPL adjuvant system does not promote autoimmunity. Sera were harvested from mice challenged with 3LL tumor cells shown in Fig. 26 and TC-1 tumor cells in Fig. 21A at the experimental end points and tested for the presence of autoantibody against ssDNA in ELISA. Serum pooled from a minimum of 3 naïve and 3 lupus mice were used as negative and positive controls, respectively.

CHAPTER 5

CONCLUSIONS AND FUTURE PROSPECTIVES

The immune system has a potential to specifically recognize and eliminate cancer cells without damaging normal healthy cells. As a result, therapeutic vaccination strategy has been a promising approach to harness the immune system against disseminated cancers. Among all, therapeutic cancer vaccines based on TAA presents an attractive choice due to their safety profile, ease of production, storage, transportation, administration to a broad patient population and most importantly establishment and/or maintenance of long-term immunological memory critical for the control of recurrences. However, the effectiveness of these vaccines will not only depend on their ability to promote robust immune response but also overcome various immune evasion mechanisms employed by progressing tumors. In this context, utilization of immunomodulators which modulate the innate, adaptive, and regulatory immunity for controlling the tumor progression will have better chance to succeed in clinic. Therefore, utilization of SA-4-1BBL as a choice of adjuvant was a rational attempt due to its pleiotropic effects on cells of innate, adaptive and regulatory immunity. In this project, we first hypothesized that costimulatory SA-4-1BBL can be exploited to be a component of successful therapeutic cancer vaccines in preclinical tumor models. We show that a single vaccination of soluble form of SA-4-1BBL with SVN is able to eradicate SVN-expressing 3LL carcinoma in over 70% of mice without a sign of autoimmunity.

During the last several years, there has been better understanding about the suppressive mechanisms employed by progressing tumors that hampers the induction of robust immune responses. These understandings have revealed new insight into how suppressive cells such as Tregs and MDSCs could potentially be cleared or inhibited for better therapeutic efficacy. In this regard, combination of diverse agents (combination therapy) has shown a great potential against cancers due to their distinct mechanism of actions. For example, combination of chemotherapeutic agents have been shown to enhance cross-presentation leading to the augmentation of tumor-specific adaptive immune response. Similarly, combination of adjuvants such a as TLRs that target primarily innate immune response and stimulate Th1 cytokines promote the efficiency of T cell priming. Although an enormous efforts has been made in understanding the role of combination therapy on tumor-immune interaction at both molecular and cellular levels, clinical transitions is still in its infancy. But the development of effective cancer vaccines received a great boost after a vast understanding of DC as well as effector/Treg biology which clearly laid the ground for the development of improved clinical protocols. It is now believed that rather than quantity, it is important to generate high quality and high avidity polyclonal and poly-functional effector $CD8^+$ T cells which are able to reject tumors and long-lasting memory $CD8^+$ T cells which are able to control recurrences. Therefore, in this study, we attempted to utilize several combinatorial strategies that are usually presented in discordant manner which set a platform for the development of future therapeutic cancer vaccines. We here show that combination of SA-OX40L+SA-4-1BBL and SA-4-1BBL+MPL based therapeutic vaccine effectively enhanced poly-functional $CD8^+$ T effector cells. Combination SA-4-1BBL+MPL was also able to

increase intratumoral $CD8^+$ T effector/$CD4^+FoxP3^+$ T regulatory cell ratio. These all mechanistic outcomes eventually translated into a complete eradication of tumors in both TC-1 and 3LL tumor models. In last, this study also supports that future immunotherapeutic cancer research should address multifunctional nature of the disease using combination protocols.

The promising results from this overall Ph.D. study may provide a new avenue for cancer vaccine immunotherapy. However, despite the exciting improvements in the efficacy of various vaccine formulations studied in this study including tumor-free survival, there is still much to determine about the immunological mechanisms by which these results can be translated into the clinics. Additionally, it is also imperative to improve our understanding of CTL activation, NK activation, decreased Treg numbers and functionality, and impact of tumor escape.

In overall, these studies provide proof-of-principle data of the utilization SA-4-1BBL alone or in combination with other immunomodulators (adjuvant systems) which set a platform for clinical studies. Based on these studies, it is required to test the efficacy of these adjuvant systems in more clinically relevant spontaneous tumor models with the standard clinical practice. Eventually, testing these adjuvant systems in clinical trials will assess the therapeutic potential of these adjuvant systems, and if proven efficacious in therapeutic regimen, these adjuvant systems can be utilized against broad cancer types with well defined TAAs.

REFERENCES

(1) Kasimir-Bauer S, Mayer S, Bojko P, Borquez D, Neumann R, Seeber S. Survival of tumor cells in stem cell preparations and bone marrow of patients with high-risk or metastatic breast cancer after receiving dose-intensive or high-dose chemotherapy. Clin Cancer Res 2001;7:1582-9.

(2) Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest 2007;117:1137-46.

(3) Dhodapkar MV, Krasovsky J, Osman K, Geller MD. Vigorous premalignancy-specific effector T cell response in the bone marrow of patients with monoclonal gammopathy. J Exp Med 2003;198:1753-7.

(4) Beckhove P, Feuerer M, Dolenc M, Schuetz F, Choi C, Sommerfeldt N, et al. Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. J Clin Invest 2004;114:67-76.

(5) Plotkin SA. Of vaccination and infectious diseases in 2003. Adv Exp Med Biol 2004;549:1-4.

(6) Palena C, Abrams SI, Schlom J, Hodge JW. Cancer vaccines: preclinical studies and novel strategies. Adv Cancer Res 2006;95:115-45.

(7) Vergati M, Intrivici C, Huen NY, Schlom J, Tsang KY. Strategies for cancer vaccine development. J Biomed Biotechnol 2010;2010.

(8) Goldman B, DeFrancesco L. The cancer vaccine roller coaster. Nat Biotechnol 2009;27:129-39.

(9) Gulley JL, Arlen PM, Tsang KY, Yokokawa J, Palena C, Poole DJ, et al. Pilot study of vaccination with recombinant CEA-MUC-1-TRICOM poxviral-based vaccines in patients with metastatic carcinoma. Clin Cancer Res 2008;14:3060-9.

(10) Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 2007;7:179-90.

(11) Mancheno-Corvo P, Martin-Duque P. Viral gene therapy. Clin Transl Oncol 2006;8:858-67.

(12) Bolhassani A, Rafati S. Heat-shock proteins as powerful weapons in vaccine development. Expert Rev Vaccines 2008;7:1185-99.

(13) Cereda V, Poole DJ, Palena C, Das S, Bera TK, Remondo C, et al. New gene expressed in prostate: a potential target for T cell-mediated prostate cancer immunotherapy. Cancer Immunol Immunother 2010;59:63-71.

(14) Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, et al. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. J Exp Med 2004;200:297-306.

(15) Sharma RK, Elpek KG, Yolcu ES, Schabowsky RH, Zhao H, Bandura-Morgan L, et al. Costimulation as a platform for the development of vaccines: a peptide-based vaccine containing a novel form of 4-1BB ligand eradicates established tumors. Cancer Res 2009;69:4319-26.

(16) Sharma RK, Srivastava AK, Yolcu ES, MacLeod KJ, Schabowsky RH, Madireddi S, et al. SA-4-1BBL as the immunomodulatory component of a HPV-16 E7 protein based vaccine shows robust therapeutic efficacy in a mouse cervical cancer model. Vaccine 2010;28:5794-802.

(17) Testori A, Richards J, Whitman E, Mann GB, Lutzky J, Camacho L, et al. Phase III comparison of vitespen, an autologous tumor-derived heat shock protein gp96 peptide complex vaccine, with physician's choice of treatment for stage IV melanoma: the C-100-21 Study Group. J Clin Oncol 2008;26:955-62.

(18) Wood C, Srivastava P, Bukowski R, Lacombe L, Gorelov AI, Gorelov S, et al. An adjuvant autologous therapeutic vaccine (HSPPC-96; vitespen) versus observation alone for patients at high risk of recurrence after nephrectomy for renal cell carcinoma: a multicentre, open-label, randomised phase III trial. Lancet 2008;372:145-54.

(19) Butts C, Murray N, Maksymiuk A, Goss G, Marshall E, Soulieres D, et al. Randomized phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV non-small-cell lung cancer. J Clin Oncol 2005;23:6674-81.

(20) Ward S, Casey D, Labarthe MC, Whelan M, Dalgleish A, Pandha H, et al. Immunotherapeutic potential of whole tumour cells. Cancer Immunol Immunother 2002;51:351-7.

(21) Webster WS, Thompson RH, Harris KJ, Frigola X, Kuntz S, Inman BA, et al. Targeting molecular and cellular inhibitory mechanisms for improvement of antitumor memory responses reactivated by tumor cell vaccine. J Immunol 2007;179:2860-9.

(22) Uyl-de Groot CA, Vermorken JB, Hanna MG, Jr., Verboom P, Groot MT, Bonsel GJ, et al. Immunotherapy with autologous tumor cell-BCG vaccine in patients with colon cancer: a prospective study of medical and economic benefits. Vaccine 2005;23:2379-87.

(23) de Gruijl TD, van den Eertwegh AJ, Pinedo HM, Scheper RJ. Whole-cell cancer vaccination: from autologous to allogeneic tumor- and dendritic cell-based vaccines. Cancer Immunol Immunother 2008;57:1569-77.

(24) Disis ML, Scholler N, Dahlin A, Pullman J, Knutson KL, Hellstrom KE, et al. Plasmid-based vaccines encoding rat neu and immune stimulatory molecules can elicit rat neu-specific immunity. Mol Cancer Ther 2003;2:995-1002.

(25) Pavlenko M, Roos AK, Lundqvist A, Palmborg A, Miller AM, Ozenci V, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. Br J Cancer 2004;91:688-94.

(26) Yuan J, Ku GY, Gallardo HF, Orlandi F, Manukian G, Rasalan TS, et al. Safety and immunogenicity of a human and mouse gp100 DNA vaccine in a phase I trial of patients with melanoma. Cancer Immun 2009;9:5.

(27) Conry RM, Curiel DT, Strong TV, Moore SE, Allen KO, Barlow DL, et al. Safety and immunogenicity of a DNA vaccine encoding carcinoembryonic antigen and hepatitis B surface antigen in colorectal carcinoma patients. Clin Cancer Res 2002;8:2782-7.

(28) Michaluart P, Abdallah KA, Lima FD, Smith R, Moyses RA, Coelho V, et al. Phase I trial of DNA-hsp65 immunotherapy for advanced squamous cell carcinoma of the head and neck. Cancer Gene Ther 2008;15:676-84.

(29) Weber J, Boswell W, Smith J, Hersh E, Snively J, Diaz M, et al. Phase 1 trial of intranodal injection of a Melan-A/MART-1 DNA plasmid vaccine in patients with stage IV melanoma. J Immunother 2008;31:215-23.

(30) Kyte JA, Gaudernack G. Immuno-gene therapy of cancer with tumour-mRNA transfected dendritic cells. Cancer Immunol Immunother 2006;55:1432-42.

(31) Weide B, Garbe C, Rammensee HG, Pascolo S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. Immunol Lett 2008;115:33-42.

(32) Yamamoto A, Kormann M, Rosenecker J, Rudolph C. Current prospects for mRNA gene delivery. Eur J Pharm Biopharm 2009;71:484-9.

(33) Pascolo S. Messenger RNA-based vaccines. Expert Opin Biol Ther 2004;4:1285-94.

(34) Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, et al. Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer. J Immunol 2005;174:3798-807.

(35) Su Z, Dannull J, Heiser A, Yancey D, Pruitt S, Madden J, et al. Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res 2003;63:2127-33.

(36) Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005;115:3623-33.

(37) Morse MA, Nair SK, Mosca PJ, Hobeika AC, Clay TM, Deng Y, et al. Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest 2003;21:341-9.

(38) Caruso DA, Orme LM, Amor GM, Neale AM, Radcliff FJ, Downie P, et al. Results of a Phase I study utilizing monocyte-derived dendritic cells pulsed with tumor RNA in children with Stage 4 neuroblastoma. Cancer 2005;103:1280-91.

(39) Kyte JA, Mu L, Aamdal S, Kvalheim G, Dueland S, Hauser M, et al. Phase I/II trial of melanoma therapy with dendritic cells transfected with autologous tumor-mRNA. Cancer Gene Ther 2006;13:905-18.

(40) Heiser A, Coleman D, Dannull J, Yancey D, Maurice MA, Lallas CD, et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest 2002;109:409-17.

(41) Tagliabue A, Rappuoli R. Vaccine adjuvants: the dream becomes real. Hum Vaccin 2008;4:347-9.

(42) McKee AS, Munks MW, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 2007;27:687-90.

(43) Grun JL, Maurer PH. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. Cell Immunol 1989;121:134-45.

(44) Bomford R. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. I. Effect on the antibody response to bovine serum albumin and sheep red blood cells of Freund's incomplete and complete adjuvants, alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyl dipeptide and saponin. Clin Exp Immunol 1980;39:426-34.

(45) Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol 2009;9:287-93.

(46) Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. Nat Med 2005;11:S63-S68.

(47) Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783-801.

(48) Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197-216.

(49) Sansonetti PJ. The innate signaling of dangers and the dangers of innate signaling. Nat Immunol 2006;7:1237-42.

(50) Schwarz TF. Clinical update of the AS04-adjuvanted human papillomavirus-16/18 cervical cancer vaccine, Cervarix. Adv Ther 2009;26:983-98.

(51) Baldwin SL, Shaverdian N, Goto Y, Duthie MS, Raman VS, Evers T, et al. Enhanced humoral and Type 1 cellular immune responses with Fluzone adjuvanted with a synthetic TLR4 agonist formulated in an emulsion. Vaccine 2009;27:5956-63.

(52) Kim TG, Kim CH, Won EH, Bae SM, Ahn WS, Park JB, et al. CpG-ODN-stimulated dendritic cells act as a potent adjuvant for E7 protein delivery to induce antigen-specific antitumour immunity in a HPV 16 E7-associated animal tumour model. Immunology 2004;112:117-25.

(53) Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499-511.

(54) den Haan JM, Kraal G, Bevan MJ. Cutting edge: Lipopolysaccharide induces IL-10-producing regulatory CD4+ T cells that suppress the CD8+ T cell response. J Immunol 2007;178:5429-33.

(55) Krieg AM. Toll-free vaccines? Nat Biotechnol 2007;25:303-5.

(56) Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, et al. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 2006;314:1936-8.

(57) Ishii KJ, Akira S. Toll or toll-free adjuvant path toward the optimal vaccine development. J Clin Immunol 2007;27:363-71.

(58) Meyer-Bahlburg A, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. J Exp Med 2007;204:3095-101.

(59) Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu YJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. J Immunol 2004;173:4433-42.

(60) Mellor AL, Baban B, Chandler PR, Manlapat A, Kahler DJ, Munn DH. Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling. J Immunol 2005;175:5601-5.

(61) Melero I, Shuford WW, Newby SA, Aruffo A, Ledbetter JA, Hellstrom KE, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat Med 1997;3:682-5.

(62) Weinberg AD, Rivera MM, Prell R, Morris A, Ramstad T, Vetto JT, et al. Engagement of the OX-40 receptor in vivo enhances antitumor immunity. J Immunol 2000;164:2160-9.

(63) Weinberg AD, Vella AT, Croft M. OX-40: life beyond the effector T cell stage. Semin Immunol 1998;10:471-80.

(64) Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 2005;23:23-68.

(65) Cannons JL, Lau P, Ghumman B, DeBenedette MA, Yagita H, Okumura K, et al. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. J Immunol 2001;167:1313-24.

(66) De ST, Smith J, Baum P, Fanslow W, Butz E, Maliszewski C. Ox40 costimulation enhances the development of T cell responses induced by dendritic cells in vivo. J Immunol 2002;168:661-70.

(67) Taraban VY, Rowley TF, O'Brien L, Chan HT, Haswell LE, Green MH, et al. Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses. Eur J Immunol 2002;32:3617-27.

(68) Redmond WL, Gough MJ, Charbonneau B, Ratliff TL, Weinberg AD. Defects in the acquisition of CD8 T cell effector function after priming with tumor or soluble antigen can be overcome by the addition of an OX40 agonist. J Immunol 2007;179:7244-53.

(69) Ruby CE, Montler R, Zheng R, Shu S, Weinberg AD. IL-12 is required for anti-OX40-mediated CD4 T cell survival. J Immunol 2008;180:2140-8.

(70) Choi BK, Kim YH, Kwon PM, Lee SC, Kang SW, Kim MS, et al. 4-1BB functions as a survival factor in dendritic cells. J Immunol 2009;182:4107-15.

(71) Wilcox RA, Chapoval AI, Gorski KS, Otsuji M, Shin T, Flies DB, et al. Cutting edge: Expression of functional CD137 receptor by dendritic cells. J Immunol 2002;168:4262-7.

(72) Piconese S, Valzasina B, Colombo MP. OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. J Exp Med 2008;205:825-39.

(73) Laderach D, Movassagh M, Johnson A, Mittler RS, Galy A. 4-1BB co-stimulation enhances human CD8(+) T cell priming by augmenting the proliferation and survival of effector CD8(+) T cells. Int Immunol 2002;14:1155-67.

(74) Rogers PR, Song J, Gramaglia I, Killeen N, Croft M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity 2001;15:445-55.

(75) Bansal-Pakala P, Jember AG, Croft M. Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. Nat Med 2001;7:907-12.

(76) Ferlin WG, von der WT, Cottrez F, Ferrick DA, Coffman RL, Howard MC. The induction of a protective response in Leishmania major-infected BALB/c mice with anti-CD40 mAb. Eur J Immunol 1998;28:525-31.

(77) Hixon JA, Anver MR, Blazar BR, Panoskaltsis-Mortari A, Wiltrout RH, Murphy WJ. Administration of either anti-CD40 or interleukin-12 following lethal total body irradiation induces acute lethal toxicity affecting the gut. Biol Blood Marrow Transplant 2002;8:316-25.

(78) Niu L, Strahotin S, Hewes B, Zhang B, Zhang Y, Archer D, et al. Cytokine-mediated disruption of lymphocyte trafficking, hemopoiesis, and induction of lymphopenia, anemia, and thrombocytopenia in anti-CD137-treated mice. J Immunol 2007;178:4194-213.

(79) Schabowsky RH, Elpek KG, Madireddi S, Sharma RK, Yolcu ES, Bandura-Morgan L, et al. A novel form of 4-1BBL has better immunomodulatory activity than an agonistic anti-4-1BB Ab without Ab-associated severe toxicity. Vaccine 2009;28:512-22.

(80) Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med 2006;355:1018-28.

(81) Elpek KG, Yolcu ES, Franke DD, Lacelle C, Schabowsky RH, Shirwan H. Ex vivo expansion of CD4+CD25+FoxP3+ T regulatory cells based on synergy between IL-2 and 4-1BB signaling. J Immunol 2007;179:7295-304.

(82) Reed JC, Wilson DB. Cancer immunotherapy targeting survivin: commentary re: V. Pisarev et al., full-length dominant-negative survivin for cancer immunotherapy. Clin. Cancer Res., 9:6523-6533, 2003. Clin Cancer Res 2003;9:6310-5.

(83) Adida C, Haioun C, Gaulard P, Lepage E, Morel P, Briere J, et al. Prognostic significance of survivin expression in diffuse large B-cell lymphomas. Blood 2000;96:1921-5.

(84) Chakravarti A, Noll E, Black PM, Finkelstein DF, Finkelstein DM, Dyson NJ, et al. Quantitatively determined survivin expression levels are of prognostic value in human gliomas. J Clin Oncol 2002;20:1063-8.

(85) Casati C, Dalerba P, Rivoltini L, Gallino G, Deho P, Rini F, et al. The apoptosis inhibitor protein survivin induces tumor-specific CD8+ and CD4+ T cells in colorectal cancer patients. Cancer Res 2003;63:4507-15.

(86) Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, et al. Generation of survivin-specific CD8+ T effector cells by dendritic cells pulsed with protein or selected peptides. Cancer Res 2000;60:4845-9.

(87) Xiang R, Mizutani N, Luo Y, Chiodoni C, Zhou H, Mizutani M, et al. A DNA vaccine targeting survivin combines apoptosis with suppression of angiogenesis in lung tumor eradication. Cancer Res 2005;65:553-61.

(88) Saoulli K, Lee SY, Cannons JL, Yeh WC, Santana A, Goldstein MD, et al. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. J Exp Med 1998;187:1849-62.

(89) Robertson SJ, Messer RJ, Carmody AB, Mittler RS, Burlak C, Hasenkrug KJ. CD137 costimulation of CD8+ T cells confers resistance to suppression by virus-induced regulatory T cells. J Immunol 2008;180:5267-74.

(90) Rosenberg SA. Shedding light on immunotherapy for cancer. N Engl J Med 2004;350:1461-3.

(91) Juul-Madsen HR, Olsson L. Discrepancy between transcriptional products and cell surface expression of MHC class I antigens in metastatic and non-metastatic Lewis lung tumor cells. APMIS 1990;98:624-36.

(92) Sacchi A, Falcioni R, Tibursi G, Apollonj GC. MHC antigens expressed on 3LL metastatic variants: correlation with the expression of a TSP-180 protein. Adv Exp Med Biol 1988;233:141-50.

(93) Lanier LL. NK cell receptors. Annu Rev Immunol 1998;16:359-93.

(94) Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 2001;19:197-223.

(95) Nanni P, Nicoletti G, De GC, Landuzzi L, Di CE, Cavallo F, et al. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents

mammary carcinogenesis in HER-2/neu transgenic mice. J Exp Med 2001;194:1195-205.

(96) Nanni P, Landuzzi L, Nicoletti G, De GC, Rossi I, Croci S, et al. Immunoprevention of mammary carcinoma in HER-2/neu transgenic mice is IFN-gamma and B cell dependent. J Immunol 2004;173:2288-96.

(97) Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, thor SP. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer Res 2001;61:5964-8.

(98) Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 2003;101:1535-42.

(99) Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity 2003;19:561-9.

(100) Zhang B, Maris CH, Foell J, Whitmire J, Niu L, Song J, et al. Immune suppression or enhancement by CD137 T cell costimulation during acute viral infection is time dependent. J Clin Invest 2007;117:3029-41.

(101) Chang JT, Wong FH, Liao CT, Chen IH, Wang HM, Cheng AJ. Enzyme immunoassay for serum autoantibody to survivin and its findings in head-and-neck cancer patients. Clin Chem 2004;50:1261-4.

(102) Yagihashi A, Asanuma K, Nakamura M, Araya J, Mano Y, Torigoe T, et al. Detection of anti-survivin antibody in gastrointestinal cancer patients. Clin Chem 2001;47:1729-31.

(103) Corthay A, Skovseth DK, Lundin KU, Rosjo E, Omholt H, Hofgaard PO, et al. Primary antitumor immune response mediated by CD4+ T cells. Immunity 2005;22:371-83.

(104) Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med 1998;188:2357-68.

(105) Qin Z, Blankenstein T. CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity 2000;12:677-86.

(106) Huang H, Bi XG, Yuan JY, Xu SL, Guo XL, Xiang J. Combined CD4+ Th1 effect and lymphotactin transgene expression enhance CD8+ Tc1 tumor localization and therapy. Gene Ther 2005;12:999-1010.

(107) Marzo AL, Kinnear BF, Lake RA, Frelinger JJ, Collins EJ, Robinson BW, et al. Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. J Immunol 2000;165:6047-55.

(108) Maxwell JR, Weinberg A, Prell RA, Vella AT. Danger and OX40 receptor signaling synergize to enhance memory T cell survival by inhibiting peripheral deletion. J Immunol 2000;164:107-12.

(109) Evans DE, Prell RA, Thalhofer CJ, Hurwitz AA, Weinberg AD. Engagement of OX40 enhances antigen-specific CD4(+) T cell mobilization/memory development and humoral immunity: comparison of alphaOX-40 with alphaCTLA-4. J Immunol 2001;167:6804-11.

(110) Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, et al. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J Exp Med 1996;183:2185-95.

(111) Sharma RK, Elpek KG, Yolcu ES, Schabowsky R-H, Zhao H, Bandura-Morgan L, et al. Costimulation as a platform for the development of vaccines: a peptide-based vaccine containing a novel from of 4-1BBL eradicates established tumors. Cancer Res 2009;69:4319-26.

(112) Sharma RK, Schabowsky R-H, Srivastava A, Elpek KG, Madireddi S, Zhao H, et al. 4-1BB ligand as an effective multifunctional immunomodulator and antigen delivery vehicle for the development of therapeutic cancer vaccines. Cancer Res 2010;70:3945-54.

(113) Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996;56:21-6.

(114) Sharma RK, Yolcu ES, Elpek KG, Shirwan H. Tumor cells engineered to codisplay on their surface 4-1BBL and LIGHT costimulatory proteins as a novel vaccine approach for cancer immunotherapy. Cancer Gene Ther 2010;17:730-41.

(115) Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 1998;393:478-80.

(116) Ridge JP, Di RF, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 1998;393:474-8.

(117) Takahashi C, Mittler RS, Vella AT. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. J Immunol 1999;162:5037-40.

(118) Lee SJ, Myers L, Muralimohan G, Dai J, Qiao Y, Li Z, et al. 4-1BB and OX40 dual costimulation synergistically stimulate primary specific CD8 T cells for robust effector function. J Immunol 2004;173:3002-12.

(119) Zheng G, Wang B, Chen A. The 4-1BB costimulation augments the proliferation of CD4+CD25+ regulatory T cells. J Immunol 2004;173:2428-34.

(120) Gough MJ, Ruby CE, Redmond WL, Dhungel B, Brown A, Weinberg AD. OX40 agonist therapy enhances CD8 infiltration and decreases immune suppression in the tumor. Cancer Res 2008;68:5206-15.

(121) Dougan M, Dranoff G. Immune therapy for cancer. Annu Rev Immunol 2009;27:83-117.

(122) Schabowsky RH, Madireddi S, Sharma R, Yolcu ES, Shirwan H. Targeting $CD4^+CD25^+FoxP3^+$ regulatory T-cells for the augmentation of cancer immunotherapy. Curr Opin Investig Drugs 2007;8:1002-8.

(123) Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;11:373-84.

(124) Croft M. The role of TNF superfamily members in T-cell function and diseases. Nat Rev Immunol 2009;9:271-85.

(125) Romanowski B, de Borba PC, Naud PS, Roteli-Martins CM, De Carvalho NS, Teixeira JC, et al. Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. Lancet 2009;374:1975-85.

(126) Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 2009;183:6186-97.

(127) Myers L, Lee SW, Rossi RJ, Lefrancois L, Kwon BS, Mittler RS, et al. Combined CD137 (4-1BB) and adjuvant therapy generates a developing pool of peptide-specific CD8 memory T cells. Int Immunol 2006;18:325-33.

(128) Lee HW, Nam KO, Park SJ, Kwon BS. 4-1BB enhances CD8+ T cell expansion by regulating cell cycle progression through changes in expression of cyclins D and E and cyclin-dependent kinase inhibitor p27kip1. Eur J Immunol 2003;33:2133-41.

(129) Feltkamp MC, Smits HL, Vierboom MP, Minnaar RP, de Jongh BM, Drijfhout JW, et al. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol 1993;23:2242-9.

(130) Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996;56:21-6.

(131) Sharma RK, Yolcu ES, Elpek KG, Shirwan H. Tumor cells engineered to codisplay on their surface 4-1BBL and LIGHT costimulatory proteins as a novel vaccine approach for cancer immunotherapy. Cancer Gene Ther 2010;17:730-41.

(132) Schabowsky RH, Elpek KG, Sharma RK, Yolcu ES, Bandura-Morgan L, Mittler RS, et al. A novel form of 4-1BBL has better immunomodulatory activity than an agonistic anti-4-1BB Ab without Ab associated severe toxicity. Vaccine 2009;28:512-22.

(133) Bukczynski J, Wen T, Ellefsen K, Gauldie J, Watts TH. Costimulatory ligand 4-1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses. Proc Natl Acad Sci U S A 2004;101:1291-6.

(134) Cannons JL, Lau P, Ghumman B, DeBenedette MA, Yagita H, Okumura K, et al. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. J Immunol 2001;167:1313-24.

(135) Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 2005;23:23-68.

(136) Sorenson BS, Banton KL, Frykman NL, Leonard AS, Saltzman DA. Attenuated Salmonella typhimurium with interleukin 2 gene prevents the establishment of pulmonary metastases in a model of osteosarcoma. J Pediatr Surg 2008;43:1153-8.

(137) Cohen PL, Caricchio R, Abraham V, Camenisch TD, Jennette JC, Roubey RA, et al. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med 2002;196:135-40.

(138) Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 2007;316:1628-32.

(139) Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942-9.

(140) Gobert M, Treilleux I, Bendriss-Vermare N, Bachelot T, Goddard-Leon S, Arfi V, et al. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer Res 2009;69:2000-9.

(141) Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005;115:3623-33.

(142) Powell DJ, Jr., Felipe-Silva A, Merino MJ, Ahmadzadeh M, Allen T, Levy C, et al. Administration of a CD25-directed immunotoxin, LMB-2, to patients with metastatic melanoma induces a selective partial reduction in regulatory T cells in vivo. J Immunol 2007;179:4919-28.

(143) Quezada SA, Peggs KS, Curran MA, Allison JP. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. J Clin Invest 2006;116:1935-45.

(144) Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003;100:8372-7.

(145) Burgdorf S, Scholz C, Kautz A, Tampe R, Kurts C. Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat Immunol 2008;9:558-66.

(146) Wilcox RA, Chapoval AI, Gorski KS, Otsuji M, Shin T, Flies DB, et al. Cutting edge: Expression of functional CD137 receptor by dendritic cells. J Immunol 2002;168:4262-7.

(147) Zhang L, Wang Q, Wang X, Ding P, Song J, Ma C, et al. Anti-CD137 monoclonal antibody promotes the direct anti-tumor effect mediated by peripheral blood-derived human dendritic cells in vitro. Cell Mol Immunol 2004;1:71-6.

(148) Toka FN, Suvas S, Rouse BT. CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. J Virol 2004;78:13082-9.

(149) Chen X, Zhou B, Li M, Deng Q, Wu X, Le X, et al. CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease. Clin Immunol 2007;123:50-9.

(150) Xu D, Fu J, Jin L, Zhang H, Zhou C, Zou Z, et al. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol 2006;177:739-47.

(151) Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, et al. Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. J Exp Med 2005;201:779-91.

(152) Elpek KG, Lacelle C, Singh NP, Yolcu ES, Shirwan H. $CD4^+CD25^+$ T regulatory cells dominate multiple immune evasion mechanisms in early, but not late phases of tumor development in a B cell lymphoma model. J Immunol 2007;178:6840-8.

(153) Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, Ngiow SF, et al. Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res 2010;70:7788-99.

(154) Teng MW, Ngiow SF, von SB, McLaughlin N, Sparwasser T, Smyth MJ. Conditional regulatory T-cell depletion releases adaptive immunity preventing carcinogenesis and suppressing established tumor growth. Cancer Res 2010;70:7800-9.

(155) Zhou Q, Bucher C, Munger ME, Highfill SL, Tolar J, Munn DH, et al. Depletion of endogenous tumor-associated regulatory T cells improves the efficacy of adoptive cytotoxic T-cell immunotherapy in murine acute myeloid leukemia. Blood 2009;114:3793-802.

(156) Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med 2003;197:403-11.

(157) Gonzalez-Navajas JM, Fine S, Law J, Datta SK, Nguyen KP, Yu M, et al. TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice. J Clin Invest 2010;120:570-81.

(158) Gough MJ, Ruby CE, Redmond WL, Dhungel B, Brown A, Weinberg AD. OX40 agonist therapy enhances CD8 infiltration and decreases immune suppression in the tumor. Cancer Res 2008;68:5206-15.

112

CURRICULUM VITAE

Abhishek K. Srivastava

(Cell) 270-243-0362 (Work) 502-852-2065; abhishek.srivast@gmail.com; aksriv01@louisville.edu

Scientist with broad experience in the development of vaccine formulations, antibodies, cell therapies and their testing in preclinical animal models. Specialties in strategic analysis, micro planning, competitive intelligence, technology transfer, immunology, molecular biology, tissue and cell culture, recombinant protein production and imaging techniques.

PROFESSIONAL EXPERIENCE

Graduate Research Assistant, 2006-Present

Institute for Cellular Therapeutics, University of Louisville, KY

Develop and test therapeutic vaccine formulations against cancer and infectious diseases in preclinical mouse models. Generate novel adjuvant systems and define their mode of action for testing into clinical settings.

- Establish the mouse models of cervical, lung and breast cancer for vaccine testing
- Help in the development of vaccine formulations and animal model system for infectious disease such as influenza, tuberculosis
- Provide technical support and troubleshooting to internal and external collaborators
- Design and conduct experiments, analyze data, interpret results and draw conclusions
- Independently and in collaboration write protocols, abstracts, and manuscripts
- Maintain cell lines and Material Safety Data Sheet

Summer Research Intern, 2004-2005
Purdue University, West Lafayette, IN

Identify the toxic metal induced genes in heavy metal accumulating plants

- Develop the cDNA libraries of *Sesbania drummondii* plant in the presence of mercury and lead toxicity and identify the genes responsible for these metal accumulation
- Select the gene candidates for effective phytoremediation of lead toxicity

Graduate Teaching/Research Assistant, 2004-2006
Western Kentucky University, Bowling Green, KY

Identify the accumulation of lead, mercury and copper by *Sesbania drummondii*. As a result, USDA funded this project for further research.

- Establish the technology for testing the accumulation of toxic metals in plants
- Characterize the accumulation of toxic metals in rye grasses

Block Monitor/Project Monitor, 2002-2004
National Polio Surveillance Project (Govt. of India/WHO), Lucknow, India

Provide training, technical and logistic assistance to government counterparts as needed to promote timely reporting and investigation of acute flaccid paralysis (AFP) within block area.

- Plan, organize, and conduct meetings with government officials, professional organizations, hospital staff, non-government organizations, and others to promote an effective implementation of activities related to surveillance of AFP and polio eradication
- Maintain up-to-date data related to immunization activities and ensure analysis and interpretation of this data for improvement of immunization activities.

EDUCATION

Institution and Location	Department	Degree	Year
University of Louisville	Microbiology and Immunology	Ph.D.	2006 - March 2011
University of Louisville	Microbiology and Immunology	M.S.	2006 - 2008
Western Kentucky University	Biology	M.S.	2004 - 2006

AWARDS

- **State Scholarship Award** during Junior High School in India.

- **National Scholarship Award** during High School in India.
- **Graduate Assistantship** at Western Kentucky University (WKU).
- **Robert J. Wurster International Student Scholarship** at WKU. (2005)
- **First place for oral presentation** at Kentucky Academy of Science. (2005)
- **Second place for oral presentation** at ASA. (2006)
- **Travel Award** from American Society of Plant Biologist. (2006)
- **Outstanding Graduate Student Award of 2006** at WKU. (2006)
- **IPIBS Fellowship** from University of Louisville. (2006-2008)
- **Mario Escobar Young Investigator Award.** (2009)
- **Michael Tanner Memorial Award for Excellence in Graduate Student Sciences**
- **Condict Moore Student Research Second Place Award.** (2009)
- **AACR-Bristol-Myers Squibb Oncology Scholar-in-Training Award.** (2010)
- **Scholar-in-Training Award.** (2010)
- **Travel Award** by AMLI. (2010)
- Research on **"Development of Therapeutic Vaccine against Cancer"** was commended as one of the best research at Research Louisville. (2010)
- **Third Place Award** at JGBCC Annual Retreat. (2010)
- **Condict Moore Graduate Student Research First Place Award.** (2010)

RESEARCH GRANT SUPPORT

- Received **Summer Financial Support** from Biotechnology Center at WKU, USA. (2005)
- Received **Graduate Student Research Grant** at WKU, USA. (2006)
- Received **Graduate School Research Grant** from Commission on Diversity and Racial Equality (CODRE) for superlative research efforts at University of Louisville, USA. (2010)

PUBLICATIONS

1. **Srivastava AK** *et al.* Identification of lead-regulated genes by suppression subtractive hybridization in the heavy metal accumulator *Sesbania. drummondii.* **Planta** 225(6), 2007.
2. Sahi SV, Israr M, **Srivastava AK** *et al.* Accumulation, speciation and cellular localization of copper in *S. drummondii.* **Chemosphere** 67(11): 2257-2266, 2007.
3. **Srivastava AK** *et al.* Genes induced in response to mercury-ion exposure in heavy metal hyperaccumulator *S. drummondii.* **Environmental Science and Technology** 43(3), 2009.
4. Schabowsky RH, Sharma RK, Madireddi S, **Srivastava AK** *et al.* ProtEX technology for the generation of novel therapeutic cancer vaccines. **Experimental and Molecular Pathology** 86(3): 198-207, 2009.

5. Sharma RK, Schabowsky R-H, **Srivastava AK** *et al.* 4-1BB ligand as an effective multifunctional immunomodulator and antigen delivery vehicle for the development of therapeutic cancer vaccines. **Cancer Research** 70(10): 3945-54, 2010.

6. Sharma RK, **Srivastava AK** *et al.* SA-4-1BBL as the immunomodulatory component of a HPV-16 E7 protein based vaccine shows robust therapeutic efficacy in a mouse cervical cancer model. **Vaccine** 28(36): 5794-802, 2010.

7. Madireddi S, Schabowsky R-H, **Srivastava AK** *et al.* SA-4-1BBL costimulation inhibits conversion of naïve CD4+ T cells into CD4+CD25+FoxP3+ T regulatory cells by production of IFN-γ. **Communicated in Cancer Discovery.**

8. **Srivastava AK** *et al.* Costimulatory SA-4-1BBL and monophosphoryl lipid A as an adjuvant system shows robust therapeutic efficacy by increasing intratumoral $CD8^+$ T effector/ $CD4^+Foxp3^+$ T regulatory cell ratio. **Under review in Cancer Research.**

9. **Srivastava AK** *et al.* Survivin and 4-1BBL based soluble vaccine eradicates established lung tumors by activating an efficient CD8+ T cell and natural killer cell response. **Communicated.**

10. **Srivastava AK** *et al.* The role of Myeloid derived suppressor cells in 4-1BBL based cancer immunotherapy. **Manuscript ready for submission.**

11. **Srivastava AK** *et al.* Combination of costimulatory SA-4-1BBL and SA-OX40L provides advantageous therapeutic efficacy in the eradication of established tumors. **In preparation.**

PUBLISHED ABSTRACTS

1. **Srivastava AK** *et al.* Isolation and characterization of induced genes in response to lead in *S. drummondii*. Kentucky Academy of Science, 2005.

2. **Srivastava AK** *et al.* Callus culture of *S. drummondii*, a medicinally important plant. ASA Southern Branch Annual Meeting, San Antonio, TX, 2005.

3. **Srivastava AK** *et al.* Growth and copper accumulation in *S. drummondii* exposed to high concentrations of copper. ASA (Southern Branch), Orlando, FL, 2006.

4. **Srivastava AK** *et al.* Identification and characterization of lead-responsive genes in *S. drummondii* by suppression subtractive hybridization. American Society of Plant Biologist Meeting, Boston, MA, 2006.

5. **Srivastava AK** *et al.* Costimulation tunes anti-tumor immunity: A novel platform for the development of therapeutic vaccines. Research Louisville, KY, 2009.

6. Sharma RK, **Srivastava AK** *et al.* A novel vaccine approach for lung cancer immunotherapy. AMLI Annual Meeting, Boston, MA, 2009.

7. **Srivastava AK** *et al.* Costimulation tunes anti-tumor immunity: A novel platform for the development of therapeutic vaccines. JGBCC, Louisville, KY, 2009.

8. Sharma RK, Schabowsky R-H, **Srivastava AK** *et al.* SA-4-1BBL as an immune modulator and vehicle to deliver antigens into DCs for generation of robust antitumor therapeutic immune responses. JGBCC Annual Retreat, Louisville, KY, 2009.

9. **Srivastava AK** *et al.* Costimulation based immunotherapy: A novel approach to battle cancer. 2nd Graduate Student Symposium, Louisville, KY, 2010.

10. Sharma RK, **Srivastava AK** *et al.* Vaccination with survivin as a self TAA and SA-4-1BBL is effective in eradicating established lung carcinomas in $CD8^+$ T cell and NK cell dependent manner. AAI Annual Meeting, Baltimore, MD, 2010.
11. Sharma RK, **Srivastava AK** *et al.* SA-4-1BBL serves as an effective immunostimulatory component of HPV-16 E7 protein based therapeutic vaccine in a mouse model of cervical cancer. AAI Annual Meeting, Baltimore, MD, 2010.
12. Sharma RK, Schabowsky R-H, **Srivastava AK** *et al.* SA-4-1BBL costimulatory ligand as an immune modulator and effective vehicle to deliver antigens into DCs for the generation of robust therapeutic antitumor immune responses. AACR Meeting, Washington, DC. 2010.
13. Sharma RK, Schabowsky R-H, **Srivastava AK** *et al.* SA-4-1BBL as an immune modulator and vehicle to deliver antigens into dendritic cells for the generation of robust antitumor immune responses. AAI Annual Meeting, Baltimore, MD, 2010.
14. Sharma RK, **Srivastava AK** *et al.* 4-1BB ligand as a potent and safe adjuvant for the development of T cell based therapeutic cancer vaccines. AMLI, Orlando, FL, 2010.
15. **Srivastava AK** *et al.* Combination of costimulatory 4-1BBL and TLR agonist MPL triggers antitumor immunity by promoting favorable T effector/Treg cells ratio. JGBCC Annual Retreat, Louisville, KY, 2010.
16. **Srivastava AK** *et al.* Costimulatory SA-4-1BBL and monophosphoryl A as a novel adjuvant system for the development of cancer vaccines with robust therapeutic efficacy. Accepted for AACR Annual Meeting, Orlando, FL, 2011.

MENTORSHIP AND TRAINING

- Mentored and trained high school student "**Jenci Hawthorne**" for her science project
- Trained first year graduate students during lab rotation in Dr. Shirwan's laboratory.

COMPETENCIES/BEHAVIORS

- Excellent communicator, regularly presenting at local, regional, and national conferences
- Strong interpersonal and communication skills, technical writing
- Effective leadership and management skills, training and supervision of students and collaborators
- Ability to work in a team-oriented environment